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Introduction

Over the past quarter of a century, several scientific developments have challenged traditional concepts in ovarian cancer. First, it was recognized that ovarian cancer is not a homogeneous disease, but rather a group of diseases—each with different morphology and biological behavior. Approximately 90% of ovarian cancers are carcinomas (malignant epithelial tumors) and, based on histopathology, immunohistochemistry, and molecular genetic analysis, at least five main types are currently distinguished: high-grade serous carcinoma (HGSC [70%]); endometrioid carcinoma (EC [10%]); clear-cell carcinoma (CCC [10%]); mucinous carcinoma (MC [3%]); and low-grade serous carcinoma (LGSC [<5%]) [1,2]. These tumor types (which account for 98% of ovarian carcinomas) can be reproducibly diagnosed by light microscopy and are inherently different diseases, as indicated by differences in epidemiologic and genetic risk factors; precursor lesions; patterns of spread; and molecular events during oncogenesis, response to chemotherapy, and prognosis [2,3]. Much less common are malignant germ cell tumors and potentially malignant sex cord-stromal tumors. The biomarker expression profile within a given histotype is consistent across stages. Ovarian cancers differ primarily based on histologic type.

In the era of personalized cancer medicine, reproducible histopathologic diagnosis of tumor cell type is a sine qua non for successful treatment. Different tumor histotypes respond differently to chemotherapy. The FIGO Committee on Gynecologic Oncology unanimously agreed that histologic type should be designated at staging.

The finding of high-grade serous tubal intraepithelial carcinoma (STIC), in patients with BRCA mutation undergoing risk-reducing salpingo-oophorectomy (RRSO) [4] also influenced the new FIGO staging. Although STIC is capable of metastasizing and, therefore, cannot be considered a true carcinoma in situ, compelling evidence for a tubal origin of BRCA-positive HGSC has accumulated over the past decade [5,6]. The relative proportion of HGSCs of ovarian and tubal derivation is unknown, mainly because tumor growth in advanced-stage cancers conceals the primary site. Even in cases involving BRCA mutation, evidence of a tubal origin of HGSCs is incomplete and a multicentric origin of these tumors cannot be excluded.

The process of the proposed changes to the staging of ovarian, fallopian tube, and primary peritoneal cancer started three years ago under the leadership of the Chair of the FIGO Committee on Gynecologic Oncology, Professor Lynette Denny. The proposal was sent to all relevant gynecologic oncology organizations and societies worldwide. The new staging was reached by consensus of those participating in the FIGO meeting held in Rome, Italy, on October 7, 2012 and approved two weeks later.
The following is the consensus agreement that resulted from these efforts and represents new criteria for staging of these gynecologic cancers.

Stage I: Tumor confined to ovaries or fallopian tube(s)

T1-N0-M0

IA: Tumor limited to 1 ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings

T1a-N0-M0

IB: Tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings

T1b-N0-M0

IC: Tumor limited to 1 or both ovaries or fallopian tubes, with any of the following:

IC1: Surgical spill

T1c1-N0-M0

IC2: Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface

T1c2-N0-M0

IC3: Malignant cells in the ascites or peritoneal washings

T1c3-N0-M0

Stage II: Tumor involves 1 or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer

T2-N0-M0

IIA: Extension and/or implants on uterus and/or fallopian tubes and/or ovaries

T2a-N0-M0

IIB: Extension to other pelvic intraperitoneal tissues

T2b-N0-M0

Stage III: Tumor involves 1 or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes

T1/T2-N1-M0

IIIA1: Positive retroperitoneal lymph nodes only (cytologically or histologically proven):

IIIA1(I) Metastasis up to 10 mm in greatest dimension

IIIA1(II) Metastasis more than 10 mm in greatest dimension

IIIA2: Microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes

T3a2-N0/N1-M0

IIIB: Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes

T3b-N0/N1-M0

IIIC: Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)

T3c-N0/N1-M0

Stage IV: Distant metastasis excluding peritoneal metastases

Stage IVA: Pleural effusion with positive cytology

Stage IVB: Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)

Any T, any N, M1

Main changes

The primary site (i.e. ovary, fallopian tube, or peritoneum) should be designated where possible. In some cases, it might not be possible to delineate the primary site clearly; such cases should be listed as “undesignated.” The histologic type should be recorded.

Stage I ovarian or fallopian tube cancer is confined to the ovaries or the fallopian tubes and peritoneal fluid/washings. Tumor rupture, surface involvement by tumor cells or presence of malignant cells in the ascites or peritoneal washings warrants a stage of IC. It is not possible to have stage I peritoneal cancer.

Stage II ovarian cancer comprises a small and heterogeneous group making up less than 10% of ovarian cancers. It is defined as extension or metastasis to extraovarian/extratubal pelvic organs and may include curable tumors that have directly extended to adjacent organs but have not yet metastasized, as well as tumors that have seeded the pelvic peritoneum by metastasis and, therefore, have a poor prognosis. The Committee felt that subdividing this small category further into IIB1 and IIB2 (i.e. microscopic and macroscopic pelvic peritoneal metastases) was not based on evidence/biology. All stage II disease is treated with adjuvant chemotherapy, so subclassification is not essential. Also, the old substage IIC (i.e. IIA or IIB but with tumor on
surface, capsule ruptured, or ascites or positive peritoneal washing) was considered redundant and eliminated.

Most ovarian cancers are HGSCs that usually present in stage III, with the vast majority (84%) stage IIIC [7]. These tumors characteristically spread along peritoneal surfaces involving both pelvic and abdominal peritoneum. Less than 10% of ovarian carcinomas extend beyond the pelvis with exclusively retroperitoneal lymph node involvement. Evidence in the literature indicates that these cases have a better prognosis than that of tumors with abdominal peritoneal involvement [8–14]. The new staging includes a revision of stage III patients and assignment to stage IIIA1 based on spread to the retroperitoneal lymph nodes without intraperitoneal dissemination. Stage IIIA1 is further subdivided into IIIA1(i) (metastasis ≤10 mm in greatest dimension) and IIIA1(ii) (metastasis >10 mm in greatest dimension), even if there are no retrospective data supporting quantification of the size of metastasis in IIIA1. Involvement of retroperitoneal lymph nodes must be proven cytologically or histologically.

**Stage IV** is defined as distant metastasis and includes patients with parenchymal liver/splenic metastases and extra-abdominal metastases; 12%–21% of patients present with stage IV disease [7]. Extension of tumor from omentum to spleen or liver (stage IIIC) should be differentiated from isolated parenchymal metastases (stage IVB).

**References**


Correlation of progression-free and post-progression survival with overall survival in phase III trials of first-line chemotherapy for advanced epithelial ovarian cancer

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Summary

Purpose of Investigation: The authors examined the relation between post-progression survival (PPS) and overall survival (OS) in phase III trials of first-line chemotherapy for advanced epithelial ovarian cancer. Materials and Methods: The authors partitioned OS into progression-free survival (PFS) and PPS and evaluated the relation between OS and either PFS or PPS. They also examined whether any association might be affected by the year of completion of trial enrollment. Results: The average PPS was longer in recent trials than in older trials (26.9 vs. 20.2 months, \( p = 0.0002 \)). For all trials, PPS was strongly associated with OS \( (r = 0.94) \), whereas PFS was more moderately but still strongly correlated with OS \( (r = 0.83) \). The average proportion of median OS accounted for by median PPS significantly increased from 54.1% in older trials to 60.3% in recent trials \( (p = 0.0001) \). Conclusion: The present findings indicate that, especially for recent trials, PPS is more highly associated than PFS with OS in first-line chemotherapy for advanced epithelial ovarian cancer.

Key words: Chemotherapy; Ovarian cancer; Overall survival; Progression-free survival; Phase III trial.

Introduction

Ovarian cancer is one of the most pernicious female cancers. Each year, approximately 255,000 females will be diagnosed with cancer of the ovaries and roughly 140,000 will die from the disease worldwide. The most common type of ovarian cancer is epithelial, which accounts for about 90% of all ovarian cancers. Epithelial ovarian cancers are frequently diagnosed at an advanced stage of the disease with a consequent poor prognosis. The response rate to chemotherapy is, however, highest among gynecologic cancers with many patients who undergo a combination of surgery and chemotherapy achieving complete remission.

Overall survival (OS) is the most objective parameter in selecting the best treatment regimen for cancer patients because it most accurately reflects a clear benefit to human beings. In recent clinical trials, however, substantial improvements in progression-free survival (PFS) do not necessarily imply a prolonged OS. This is because there are various effective therapies that can be administered after failure of first-line treatment, or after trial completion or withdrawal from a trial according to protocol, all of which make it difficult to demonstrate that gain in OS results from first line treatment alone rather than as a result of the sequential administration of later effective therapies.

The effect of therapies instituted after disease progression on survival in clinical trials is thus of interest. Since it has been shown that post-progression therapies influence OS, post-progression survival (PPS) has recently become of interest as a determinant of OS. However, little is known about PPS.

For this study, we divided OS of phase III randomized controlled trials for treatment-naïve patients with advanced epithelial ovarian cancer into PFS and PPS and assessed the association of each with OS.
Excluded trials and of large systematic reviews were searched. Trials that provided data for both OS and either PFS or time to progression (TTP) were included, whether or not these parameters were explicitly defined. Trials were excluded if they investigated only immunotherapy regimens or hormonal therapies.

Data abstraction and clinical endpoint
Primary endpoints were analyzed in detail, following the definitions used by the authors of each trial. When not specifically stated by the authors, the primary endpoint was taken to be that used for calculation of sample size. Two endpoints (PFS and TTP) based on tumor assessment were collectively referred to as PFS in this study, in line with the approach adopted in recent reports [1, 2]. Median OS and median PFS were extracted from all trials that provided data for each treatment group. Median PPS was defined as median OS minus median PFS for each trial. The following information was obtained from each report: year of trial publication, line of treatment, number of patients in each treatment arm, number of treatment arms in each trial/ type of agents, age of the patients, number of patients with serous cystadenofibroma, and number of patients with clear cell adenocarcinoma.

The aims of this analysis were: (i) to evaluate (quantify) the correlation of PFS and PPS with OS in phase III randomized controlled trials in advanced epithelial ovarian cancer, (ii) to evaluate if these correlations changed according to the period within which the trial was published (before or after December 1999).

Data analysis
The survival data (median OS, median PFS, median PPS, median PFS, and median OS) was summarized as the mean for all trial arms. The percentage of OS accounted for by PPS for each trial arm was calculated as: 100 – (100×median PFS/median OS). To assess the relation between median OS and either median PFS or median PPS, Spearman’s rank correlation coefficient was used. To account for differences in sample size and patient’s characteristics among trial arms, analyses were weighted by the number of patients in each arm.

In addition, all trials were divided into two groups on the basis of the year in which trial enrollment was completed. Given that the median year for completion of enrollment for the 24 analyzed trials [3–27] was December 1999, a division was made at year 1999 (older trials being June 1993 to December 1999 inclusive recent trials being January 2000 to August 2006 inclusive) in order to evaluate a possible change in PPS, and to assess whether the evaluated relations might be dependent on the year of completion of trial enrollment. Differences in the survival data between older and recent trials were determined by Students’s t-test the average survival data using Students’s t-test. All reported p-values correspond to two-sided tests, and those with p-values < 0.05 were considered statistically significant. Analyses were carried out with SAS for Windows release 9.3.

Results
Characteristics of the trials
A total of 2,379 potentially relevant publications were identified from the search. Of these, 2,335 studies were excluded for at least one of the following reasons: they examined other malignancies or combined modality treatments, they investigated only immunotherapy regimens or hormonal therapies; they examined other malignancies or combined modality treatments (e.g. radiotherapy); they were not randomized;
they were phase I or II trials; they were review articles, letters or commentaries; they represented subgroup analyses of other trials or they were the duplicates of similar retrieved studies; they contained no information about the year of completion of trial enrollment; they were phase III randomized controlled trials investigating second and sequential chemotherapies.

Review of the remaining publications yielded 24 trials that were considered to be highly relevant for the present study. The main characteristics of the 24 phase III trials included in the analysis are listed in Table 1. A total of 6,386 patients with advanced epithelial ovarian cancer were enrolled, with a median number of patients per study of 456.5 (range 42–4,312). The average median age of the patients was 59.0 years. Eleven trials used an endpoint based on tumor assessment (PFS or TTP) as the primary endpoint, whereas OS was assessed as the primary endpoint in ten trials. In the other three trials the primary endpoint was not specified.

Median OS, PFS, and PPS of all trials and subgroups based on year of completion of trial enrollment (older trials, up to December 1999; recent trials, January 2000 and later)

The survival data (all trials and trial arms according to the year in which trial enrollment was completed) are shown in Table 2. The median OS was 41.2 months, while the median PFS and PPS were 17.2 and 24.1 months, respectively, for all arms (n = 52). The median OS, PFS and PPS in older trials were 36.8, 16.6, and 20.2 months, respectively. The median OS, PFS, and PPS in recent trials were 44.5, 17.6 and 26.9 months, respectively. Although the average median PFS in older trials was the same as that in recent trials, the average median PPS was longer in the recent trials than in the older trials (26.9 and 20.2 months, respectively, \( p = 0.0002 \)). The average proportion of median OS accounted for by median PPS significantly increased from 54.1% in older trials to 60.3% in recent trials (\( p = 0.0001 \)).

### Relation between OS and either PFS or PPS

The relation between median OS and either median PFS or median PPS for all trials (52 arms, PPS/OS ratio: 57.7%) is shown in Figures 2 and 3, respectively. It was found that median PPS was more strongly associated with median OS (\( r = 0.94, p < 0.0001 \)) on the basis of Spearman’s correlation coefficient, whereas median PFS was more moderately but still strongly correlated with median OS (\( r = 0.83, p < 0.0001 \)).

The correlation between median OS and median PFS in recent trials (\( r = 0.79, p < 0.0001 \)) was similar to that in older trials (\( r = 0.84, p < 0.0001 \)). In addition, the slope of the two regression lines were found to be roughly parallel (\( p = 0.6187 \)). The association between median OS and median PPS in recent trials (\( r = 0.86, p < 0.0001 \)) was similar to that in older trials (\( r = 0.85, p < 0.0001 \)) with no difference in the slope of the regression lines (\( p = 0.8652 \)).

### Discussion

In the present study, median PPS was defined as median OS minus median PFS for each treatment arm of phase III randomized controlled trials for chemotherapy-naïve patients with advanced epithelial ovarian cancer, as previously described [1, 28]. The relation between median OS and either median PPS or median PFS by correlation analysis was also investigated revealing that median OS was more strongly associated with median PPS than with median PFS. Moreover, the average proportion of median OS accounted for by median PPS was found to be significantly increased in recent trials than in older trials. According to recent studies, chemotherapy sensitivity affects the overall survival greatly [29, 30]. Therefore the choice of drugs should be decided by whether the patient is chemotherapy-sensitive or not. Specifically, monotherapy has been selected for women who relapsed more than six months after completing initial chemotherapy [31, 32], whereas combination therapy with platinum-based therapy was selected for women who experienced relapse ≥ six months after therapy [33–35]. The recent prolongation of PPS is likely the result of the increasing number of active compounds, being adminis-

### Correlation of progression-free and post-progression survival with overall survival in phase III trials of first-line chemotherapy etc.

#### Table 2. — The survival data (all trials and trial arms according to the year in which trial enrollment was completed).

<table>
<thead>
<tr>
<th>Trial type</th>
<th>n</th>
<th>PFS (months)</th>
<th>P-value</th>
<th>PPS (months)</th>
<th>P-value</th>
<th>OS (months)</th>
<th>P-value</th>
<th>PPS/OS (%)</th>
<th>P-value</th>
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<tr>
<td>Overall</td>
<td>52</td>
<td>17.2</td>
<td></td>
<td>24.1</td>
<td></td>
<td>41.2</td>
<td></td>
<td>57.7</td>
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<tr>
<td>1st-line (up to December 1999)</td>
<td>29</td>
<td>16.6</td>
<td>0.3058</td>
<td>20.2</td>
<td>0.0002</td>
<td>36.8</td>
<td>0.0018</td>
<td>54.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>1st-line (December 1999 and later)</td>
<td>23</td>
<td>17.6</td>
<td></td>
<td>26.9</td>
<td></td>
<td>44.5</td>
<td></td>
<td>60.3</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. — Relation between median OS and median PFS for 52 arms of 24 phase III trials for advanced epithelial ovarian cancer. (A) All trials. (B) Older trials (trial enrollment finished between June 1993 and December 1999). (C) Recent trials (trial enrollment finished between January 2000 and August 2006). The area of each dot is proportional to the number of patients in each trial arm. The \( r \) values represent Spearman’s rank correlation coefficient.

Figure 3. — Relation between median OS and median PPS for 52 arms of 24 phase III trials for advanced epithelial ovarian cancer. (A) All trials. (B) Older trials (trial enrollment finished between June 1993 and December 1999). (C) Recent trials (trial enrollment finished between January 2000 and August 2006). The area of each dot is proportional to the number of patients in each trial arm. The \( r \) values represent Spearman’s rank correlation coefficient.
tered appropriately. Many factors other than more effective cures could be contributing to prolonged survival in trials of advanced epithelial ovarian cancer patients: inclusion criteria in modern trials are more stringent and treatment of ovarian cancer increasingly effective. Broglio and Berry [28] recently focused on PPS, which they termed survival post-progression (SPP) and defined as OS minus PFS, in a hypothetical clinical trial setting under the assumption that there was a treatment difference in PFS but not in PPS. As the median PPS increased, the probability of detecting a statistically significant difference in OS decreased substantially. For a trial with an observed \( p \) value for improvement in PFS of 0.001, there was > 90% probability for statistical significance of the difference in OS if the median PPS was two months, whereas this probability decreased to only 50% if the median PPS was six months. In the present study, for recent trials for advanced epithelial ovarian cancer, it was found that median PPS constituted more than half of median OS and that median PPS was > 27 months. Similar results were found by Hayashi et al. [2] in non-small-cell lung cancer (NSCLC) for which an increasing number of effective drugs are available. Hayashi et al. evaluated the relation between PPS and OS, and found that median PPS constituted more than half of median OS and that median PPS was > six months for NSCLC, which was a similar trend as the present study.

Evaluation of PFS as a surrogate endpoint for OS has often been conducted by quantifying the strength of the association between these endpoints at the individual level (referred to as individual-level surrogacy) and of that between the effects of treatment on these endpoints (trial-level surrogacy) [36–39]. The present examination of the correlation between PFS and OS was not an exercise in surrogate validation because of the lack of investigation into the correlation between the effects of chemotherapy on these endpoints. However, the present study has yielded the key finding that PPS, not PFS, is highly associated with OS.

The present study has several limitations. First, the analysis was based on abstracted data rather than on individual patient data. The use of individual patient data might have allowed a better characterization of the relation between OS and other endpoints based on tumor assessment, including PFS and TTP. However, if such an approach had been used, it would have restricted the analysis to a small number of trials and would have hindered its replication by independent researchers. Second, the results of this study potentially have several confounders because many heterogeneous trials have been included into the analysis. The results would be generally uninterpretable without appropriate adjustment for patient characteristics dependent on differences in predefined eligibility criteria for enrollment in the clinical trials. Third, the assessment of disease progression could be subject to measurement error and bias in individual patients, and the quality of measurement for endpoints based on tumor assessment can vary between centers and trials. Finally, following the example of a previous report for ovarian cancer, the two endpoints (PFS and TTP) based on tumor assessment are considered as the same parameter. PFS is defined as the time from patient random selection to tumor progression or death, whereas TTP is defined similarly but considers death as the time point when censoring occurs. TTP is the same as PFS if death does not occur during treatment. Given that death rarely occurs before disease progression in ovarian cancer, PFS could reasonably be considered the same as TTP for the analysis. Indeed, separate analysis of clinical trials that use PFS (46 arms) or TTP (six arms) revealed a consistent association between OS and PPS (data not shown). These data thus support this approach in which these two endpoints (PFS and TTP) are collectively referred to as PFS in the present analysis.

To the best of the present authors’ knowledge, this study is the first to analyze PPS in advanced epithelial ovarian cancer. The findings indicate that, especially for recent trials, PPS is strongly associated with OS for first-line chemotherapy in patients with advanced epithelial ovarian cancer. Therefore, OS remains an appropriate endpoint of clinical trials for chemotherapy-naive patients with advanced epithelial ovarian cancer. Given the great effect of PPS on OS, appropriate assessment of clinical course after disease progression (second-line and later) in each clinical trial will be required.

References

Prevention, diagnosis and treatment of cervical cancer precursor lesions at the Xingu Indigenous Park, Brazil

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Summary
Purpose: To describe the prevention, diagnosis, and treatment of cervical cancer precursor lesions at the Xingu Indigenous Park (PIX) from 2005 to 2006. Materials and Methods: Observational, transversal study. The research sample consisted of 503 sexually active women aged 12 years and older. The research was performed in three stages: screening, colposcopy, and surgical treatment by large loop excision of the transformation zone. Results: The cytopathological screening coverage was of 99.6%. The rate of cytologic atypia was 11.7%. Together, low-grade squamous intraepithelial lesions (LSILs) and high-grade squamous intraepithelial lesions (HSILs) were observed in 4.6% of the women. The cytological examination returned a sensitivity of 54%, specificity of 97%, a positive predictive value of 88%, and a negative predictive value of 83%. In the anatomopathological examinations of biopsies, the rate of HSILs was 30.2%. The sensitivity of the anatomopathological examination of biopsies was 72.2%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 44.4%. Conclusions: Viable strategies for preventing, diagnosing, and treating cervical cancer precursor lesions in women from the PIX include increasing annual coverage of cytopathological examinations, early detection of cervical intraepithelial lesions, and treatment and follow-up of detected cases.

Key words: Cervical intraepithelial neoplasia; Mass screening; Cervical neoplasm prevention; Indigenous health; South American Native Indians.

Introduction
Cervical cancer, the second-most common tumour of women worldwide, occurs primarily in developing countries, affecting the most vulnerable social groups. In Brazil, the expected number of new cases for 2012 is 17,540. In 2009, cervical cancer was the third-leading cause of cancer death in women, with 5,063 deaths [1]. Even in developed countries, it remains a public health concern. In the European Union, 34,000 new cases and over 16,000 cervical cancer-related deaths are reported each year [2].

The human papillomavirus (HPV) is considered cervical cancer’s main causal agent [3-5]. The prevalence of HPV infection in Latin American indigenous women is higher than in the rest of the female population [6-13]. This population is also exposed to cofactors that increase cervical cancer risk, including multiple sexual partners, sexually transmitted diseases (STDs), sexual activity at an early age, multiparity, and low socioeconomic status [14-18]. Low access to preventive screening for early detection and treatment of this cancer and its precursor lesions are other important cofactors for cervical cancer risk. The greater exposure of the Brazilian indigenous population to STDs is related to cultural issues of sexuality, the intensification of contact with the non-indigenous population, and the increased frequency and permanence of the indigenous population in urban areas. [19] The scarce access to information and prevention are also significant issues.

Information on the morbidities and mortality rate associated with cervical cancer in indigenous women is limited, and the data on cervico-vaginal cytopathological screening coverage are worrisome. Geographic isolation, the lack of infrastructure, and of specialised diagnostic support and the turnover of health professionals in indigenous areas are obstacles to the development of adequate screening programs. In addition, indigenous women, due to ignorance, shame, cultural beliefs or distrust, often refuse to undergo preventive screening.

According to official figures, the indigenous population of Brazil corresponds to 734,000 people in more than 200 different groups [20]. They are distributed throughout the country in different sociocultural contexts.

Cervical cancer is still barely noticeable in the setting of public health policy. A greater understanding of the epidemiology of this cancer in indigenous Brazilian women is needed. Thus, the aims of this study were to describe a strategy of prevention, diagnosis, and treatment of cervical cancer precursor lesions in women from the Xingu Indigenous Park (Parque Indígena do Xingu, PIX); to analyse their cytopathological examination coverage rate; to measure the prevalence of cytological atypia; and to determine...
the accuracy of cytopathological and anatomopathological examinations of colposcopically directed biopsies during 2005 to 2006.

**Materials and Methods**

**Design, population, and ethical aspects**

This was an observational, transversal, retrospective study using data obtained from documents related to preventing, diagnosing, and treating cervical cancer from 2005 to 2006. This research was conducted in the PIX, which is located in the state of Mato Grosso, Brazil, and encompasses an area of 26,420 km². This region is characterised by the biodiversity of the Amazon rainforest. Its total population in January 2006 was 2,299 inhabitants. The research sample consisted of 503 indigenous women aged 12 years and over with a history of current or previous sexual activity. The sample population belonged to seven ethnic groups – Kaiabi, Kamayurá, Yudja, Kisêdjê, Ikpeng, Trumai, and Wauja – distributed in 35 villages. The inclusion of women as young as 12 was due to their early onset of sexual activity and the cytological atypia previously observed in younger women [21].

This study was approved by the Research Ethics Committee of the Federal University of São Paulo (Universidade Federal de São Paulo, UNIFESP) and by the National Committee for Ethics in Research of the Ministry of Health, as this study focused on an indigenous population.

**Strategy**

UNIFESP has conducted a health program in the PIX designated the ‘Xingu Project’ since 1965 [22]. Until 2005, cervical cancer prevention in the PIX had characteristically low cytopathological screening coverage and low regional access to colposcopy and treatment. An increase in the incidence of precursor lesions, with the occurrence of five cervical cancer-related deaths, was observed from 2000 to 2005.

In light of this situation, beginning in 2005, a partnership was developed between the Xingu Project and the Nucleus of Prevention of Gynaecologic Diseases (Núcleo de Prevenção de Doenças Ginecológicas, NUPREV), Department of Gynaecology, UNIFESP. NUPREV is a specialised service in colposcopy and the treatment of cervical cancer precursor lesions. It is affiliated with the Brazilian Association of Pathology of the Lower Genital Tract and Colposcopy, which is affiliated with the International Federation for Cervical Pathology and Colposcopy, offering postgraduate courses with certification in colposcopy.

The data that were analysed were collected over three stages:

1. **Cervical cancer screening: October 2005**

To identify the population to be screened, individual medical records, which represent an unprecedented collection of demographic information in the Xingu Project, were used. Through these medical records, it was possible to identify the entire PIX population by name, age, gender and village.

Nurses from the Xingu Project with field work experience and training in cytopathological screening collection at NUPREV participated in this stage. Their training included both theoretical and practical collection of cervico-vaginal smears for one month. The nurses and indigenous health agents visited 35 villages in 30 days. The research team used powerboats to visit the tribes. Most villages had a rudimentary health clinic that offered basic care facilities. In the other villages, the authors improvised treatment areas in the homes of the community. In places with no gynaecological table, the cervico-vaginal smear was performed on stretchers.

As a light source, a flashlight fixed to the examiner’s forehead was used. Women experiencing their menstrual cycle and in late puerperium were excluded from the study and were evaluated later. Two women were not screened because they lived outside the PIX. All women agreed to undergo a gynaecological examination after talking about the importance of cancer prevention. The authors decided to initiate immediate treatment in cases of vulvo-vaginitis and cervicitis, according to the service protocol.

The gynaecological examination included inspection of the vulva, followed by the introduction of a disposable vaginal speculum to expose the cervix. Exfoliated cells from the vaginal fornix, ectocervix, and endocervix were collected. The material was deposited on a single histological slide, identified, and fixed with a spray solution. This material was placed in individual microscope slide boxes and then sent to the Cytopathology Laboratory of UNIFESP for processing and evaluation. The cytological classification followed the criteria of the Bethesda System 2001 [23].

2. **Colposcopy: February 2006**

The second stage of this work was to perform colposcopy on the women from the PIX, with the participation of a medical specialist from NUPREV and Xingu Project staff. Women with cytological atypia were subjected to colposcopy and biopsy when necessary. A portable colposcopic device (DF Vasconcelos) with 6X-40X magnifying lenses and blue and green filters was used. A gasoline-powered generator was used as a source of electricity. Women who had been selected for further examinations were transported by boat from the villages to one of three basic health service units with better infrastructures located in the PIX.

During the examination, the vulva and vaginal introitus were inspected by introducing a disposable speculum and exposing the cervix, followed by the application of 3% acetic acid to the cervix and vagina. Subsequently, Schiller’s iodine test was used to visualise changes in the cervix and the vaginal wall epithelium. After removal of the speculum, a 5% acetic acid solution was applied to evaluate the vulva, inguino-crural grooves, pubic mound, perineum and the perianal region. In the presence of abnormal colposcopy findings, a biopsy was performed with 4-5 mm Gaylor-Medina type forceps, followed by haemostasis with 50% ferric perchlorate.

A total of 58 women were examined, 43 of who underwent cervical biopsy. One woman was not examined because she lived outside the PIX. After the authors explained the procedure, no women refused to participate. To classify the colposcopic findings, the nomenclature of Barcelona 2002 was used [24]. The biopsy specimens were placed in a vial with 10% formalin solution and then sent to the Pathology Department of UNIFESP. The results of pathological examinations followed the classification of Richart 1990 [25].

3. **Surgery by large loop excision of the transformation zone (LLETZ): May 2006**

The third phase of this study consisted of surgical treatment by LLETZ, with the participation of three medical experts from NUPREV and the Xingu Project staff. A total of 20 surgical procedures were performed in the city of Canarana, Mato Grosso, where the women were transported by water, land or air, depending on their place of residence. The surgeries were performed outside the PIX because complications, such as excessive bleeding of the cervix after excision of the transformation zone by LLETZ, have been previously observed among those women. In this way, back-up medical staff members were available in case of emergency.
Table 1. — Distribution of atypical cytology and colposcopic findings in indigenous women from the PIX, Brazil, 2005–2006.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Cytologic atypia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASC-US</td>
<td>15</td>
<td>25.4</td>
</tr>
<tr>
<td>ASC-H</td>
<td>12</td>
<td>20.3</td>
</tr>
<tr>
<td>AGC</td>
<td>7</td>
<td>11.9</td>
</tr>
<tr>
<td>LSIL</td>
<td>15</td>
<td>25.4</td>
</tr>
<tr>
<td>HSIL</td>
<td>8</td>
<td>13.6</td>
</tr>
<tr>
<td>CIS</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>AIS</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Colposcopic findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>14</td>
<td>23.7</td>
</tr>
<tr>
<td>Abnormal</td>
<td>43</td>
<td>72.9</td>
</tr>
<tr>
<td>Unsatisfactory</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Not performed</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>100.0</td>
</tr>
</tbody>
</table>


Prior to the surgical procedure, colposcopy was performed to confirm and delineate the lesion. A dispersive electrode (neutral plate) was positioned under the patient. Infiltration injection of anaesthetic with vasoconstrictor was applied at three, six, nine, and 12 hours near the outer margins of the transformation zone. The electrosurgical generator was programmed to dispense cutting waves of 40 watts. The appropriate size of electrode loop was chosen depending on the extent of the injury, and a smoke evacuator with a biological filter was used for the surgeries. After cutting and removal of surgical specimens, the device was reprogrammed to dispense coagulation waves of 80 watts using a ball electrode to achieve haemostasis. Ferric perchlorate (50%) was applied to the cervix and the vagina. The surgical specimen was fixed in a polystyrene plate and placed in a vial containing 10% formaldehyde. The patients was counselled regarding postoperative care. The median of 25 years. The 12- to 29-year age group represented 61.4% of the sample, and women over 50 years amounted to 12.4%, resulting in a predominantly young population. A total of 59 women (11.7%) exhibited cytological atypia.

Table 1 shows that low-grade squamous intraepithelial lesions (LSILs) and high-grade squamous intraepithelial lesions (HSILs) represented 39.0% of the cases. Two cases (3.4%) had suspected carcinoma in situ and adenocarcinoma in situ, respectively. Among the women subjected to colposcopy, 43 (72.9%) had an abnormal-appearing exam, with predominant acetowhite epithelium in 35 cases (81.4%), and associated images in eight cases (18.6%). One colposcopic examination was considered unsatisfactory due to intense cervical inflammation. One woman did not undergo the test because she no longer resided in the PIX.

With respect to the age-group distribution of atypical cytology, 80.0% of LSIL cases occurred in the 12- to 29-year age group. In the 20- to 49-year age group, HSILs were more frequent (100%). Significant differences were observed in the analysed data (Table 2).

Table 3 shows the accuracy of the cytopathologic smear in relation to the HSIL results from the anatomopathological biopsy examination, which is considered the gold standard. The cytopathologic smear returned a sensitivity of 54%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a specificity of 97%, a positive predictive value of 80.0%, a sensitivity of p < 0.05.

Table 2. — Age-group distribution of atypical cytology in indigenous women from the PIX, Brazil, 2005–2006.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>ASC-US N</th>
<th>ASC-H N</th>
<th>AGC N</th>
<th>LSIL N</th>
<th>HSIL N</th>
<th>CIS N</th>
<th>AIS N</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 to 19</td>
<td>4</td>
<td>26.7</td>
<td>1</td>
<td>8.3</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>46.7</td>
</tr>
<tr>
<td>20 to 29</td>
<td>6</td>
<td>40.0</td>
<td>2</td>
<td>16.7</td>
<td>2</td>
<td>28.6</td>
<td>5</td>
<td>33.3</td>
</tr>
<tr>
<td>30 to 39</td>
<td>2</td>
<td>13.3</td>
<td>4</td>
<td>33.3</td>
<td>5</td>
<td>71.4</td>
<td>1</td>
<td>6.7</td>
</tr>
<tr>
<td>40 to 49</td>
<td>1</td>
<td>6.7</td>
<td>2</td>
<td>16.7</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>6.7</td>
</tr>
<tr>
<td>50 to 59</td>
<td>1</td>
<td>6.7</td>
<td>1</td>
<td>8.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>1</td>
<td>6.7</td>
<td>2</td>
<td>16.7</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>6.7</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>100.0</td>
<td>12</td>
<td>100.0</td>
<td>7</td>
<td>100.0</td>
<td>15</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Fisher’s exact test p = 0.028. ASC-US: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells, cannot exclude a high-grade lesion; AGC: atypia of glandular cells; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; CIS: carcinoma in situ; AIS: adenocarcinoma in situ.
The cytopathologic smear coverage reached 99.6% of sexually active women 12 years of age and above. The existence of demographic records, the definition of the target population, the performance of examinations in the villages, the involvement of indigenous health agents, and the efforts of the nursing staff responsible for the organisation of the study were decisive in achieving this high coverage. The scarce information regarding the collection of cytopathologic smears among indigenous women in Brazil shows that the coverage rates are worrisome, ranging from 5.1% to 23.5% [30]. These women face obstacles to accessing preventive screening, with the result that screening is not always available to them [18, 31]. The absence of organised programs for the prevention of cervical cancer in indigenous areas has also been observed.

The few studies on the indigenous population in Brazil report variations in the prevalence of cytologic atypia, from 4% to 22.3% [16-18, 30, 32-33]. This study identified a prevalence of 11.7%, which is higher than that found in the general Brazilian female population, which ranges from 1.1% to 7.8% [34-39]. In European countries, the rate of cytological abnormalities is approximately 3.7% [40]. The present findings suggest a greater rate of abnormal cytology among indigenous women compared to women in the non-indigenous population. Further studies are needed to confirm this hypothesis.

The presence of LSILs in women between the ages of 12 and 14 years reinforces the need to extend preventive actions to very young women. The early onset of sexual activity in indigenous women coexists with the freedom to exchange sexual partners. This population is vulnerable to STDs, especially HPV, because preventive measures, such as condom use, are not common. The inclusion of indigenous women in programs to control the rates of cervical cancer and STDs should be encouraged.

LSILs and HSILs in cytopathologic smears were more prevalent in the 20- to 49-year age group, similar to other...
Atypical squamous cells of undetermined significance (ASC-US) or atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H) were found in 5.4% of the cytopathological exams. There is no consensus on the optimal prevalence of ASCs; however, experts consider values below 5% acceptable [41]. The results of this study may be associated with intense inflammatory processes, especially in young women, and atrophy in those of older age. Women with ASC-US were treated for inflammation and were subsequently subjected to the new cervico-vaginal smear collection and colposcopy.

Regarding the colposcopic findings, similar data have been reported in the literature; acetowhite epithelium is the most frequent finding in abnormal colposcopies [42].

The sensitivity performance of cytologic smears in the PIX was within the parameters described in the literature, although higher rates have been reported [43, 44]. However, the predictive values were high, which suggests a reliable and extensible screening method that can be applied to other indigenous peoples of Brazil.

The accuracy of the anatomopathological examination of biopsies was significant, demonstrating that the cyto-collophistological correlation was instrumental in the detection of a greater number of cervical cancer precursor lesions.

In the years after this survey, the cervical cancer preventive actions in the PIX were maintained, with greater than 90% cytologic smear coverage. There was a reduction in the occurrence of cytologic atypia from 11.7% in 2005 to 6.0% in 2007. The results of the adopted strategies included increased coverage of cytologic smears, early detection of cervical intraepithelial lesions, assurance of treatment and follow-up of detected cases, and a reduction in the incidence of cervical cancer and its precursor lesions.

Health workers, especially nurses, play a crucial role in preventive actions and early detection of cervical cancer. These workers are the most involved with indigenous health care in Brazil and can play an important role in the control of this disease among indigenous peoples.

The absence of cervical cancer-related deaths after six years of prevention argues that the program developed in the PIX was effective and reduced both morbidity and mortality from this type of cancer. The participation of experts from UNIFESP was instrumental in the success of this work, which positively impacted the health of indigenous women in Brazil. It is the present authors’ belief that universities have an important role to play in indigenous health and that the knowledge gathered in this study can be applied to other contexts.

Cervical cancer and HPV infection are important public health issues that affect indigenous women, requiring intervention to control their incidence. Improving preventative measures and providing immunisation against HPV in this population are priorities.

It is necessary to expand the access of Brazilian indigenous women to preventive cervical cancer screening, providing diagnosis, treatment, and appropriate follow-up. Moreover, it is essential to promote public policies addressing sexual and reproductive health for this population, to improve the information infrastructure, and to develop more comprehensive epidemiological investigations that may reveal the true health status of the population.

Acknowledgements

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References


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CO₂ laser vaporization for the treatment of vaginal intraepithelial neoplasia: effectiveness and predictive factors for recurrence

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Summary
Objective: To evaluate the outcome of vaginal intraepithelial neoplasia (VaIN) treatment with CO₂ laser vaporization in terms of local recurrence and progression to vaginal carcinoma. Additionally, the authors investigated the predictive factors for first recurrence.

Materials and Methods: The medical records of all patients treated for VaIN with CO₂ laser vaporization at Sant’Anna Hospital in Turin (1995-2012), were retrospectively reviewed. A univariate logistic model was applied to evaluate selected clinical features as predictive factors for recurrence. A multivariate logistic regression analysis was then carried out including significant risk factors after univariate analysis (\(p<0.05\)).

Results: The analysis included 285 out of 302 patients. Seventy-one (25%) women relapsed; of these 24 VaIN 1 (22%), 37 VaIN 2 (27%), and ten VaIN 3 (26%). The median time to the first recurrence was 5.2 months (1.4−127.8) for VaIN 1, 6.6 months (1−85.2) for VaIN 2, and 3.6 months (1.2−62) for VaIN 3. Sixty-one out of 71 patients were retreated with CO₂ laser vaporization. At the last follow-up visit, 273 (96%) women were free from VaIN. No patients progressed to vaginal carcinoma. The multivariate model showed a higher risk of VaIN recurrence in the case of previous hysterectomy (HR 3.3, 95% CI 1.7−6.3, \(p<0.001\)) and concomitant H-SIL on the Pap smear (HR 1.9, 95% CI 1.2−3.1, \(p=0.008\)).

Conclusion: CO₂ laser vaporization is an effective low impact treatment for VaIN. Despite this, VaIN recur, in particular in cases of previous hysterectomy and concomitant H-SIL on the Pap smear. An intensive follow-up is proposed for women with a high risk of VaIN relapse.

Key words: CO₂ laser vaporization; Predictive factors; Relapses; Vaginal intraepithelial neoplasia.

Introduction
The vaginal intraepithelial neoplasia (VaIN) was first reported by Hummer in 1933 as “squamous cell atypia without stromal invasion” [1]. VaIN represents 0.4−1% [2, 3] of all intraepithelial neoplasias of the lower genital tract with an incidence of 0.2−0.3 new cases out of 100,000 women per year [3]. The incidence of VaIN is higher among patients aged 40 to 61 years [4], and recent data suggest an increasing incidence in patients aged 30-35 years [4, 5].

Human papillomavirus (HPV) plays a critical role in the development of anogenital neoplasia and has been found in 93.6−98.8% [6, 7] of VaIN cases. Other risk factors for VaIN are a history of hysterectomy [8−10], diagnosis of cervical intraepithelial neoplasia (CIN) or vulvar intraepithelial neoplasia (VIN) [6−9], previous pelvic radiotherapy [11], immunosuppression [12], and smoking [13].

VaIN most commonly affects the upper third of the vagina [3, 4] and lesions are often multifocal [2, 10, 14]. Since most cases are characterized by asymptomatic disease, the diagnosis is often driven accidentally by a Pap smear [4, 7] or during a colposcopy performed for other reasons. The biopsy of all colposcopic abnormalities is, however, mandatory to confirm the presence of VaIN and to map its extension.

The increasing incidence of VaIN, especially in young women, the frequent relapses of VaIN after treatment (10−42%) [5], and the desire to maintain sexual function have prompted gynecologists to identify new ways of treating this disease, in order to balance radical treatment and the incidence of complications. For these reasons, treatment has changed from upper or total vaginectomy along with hysterectomy to a more conservative and tailored procedure, related to the clinical, morphological and topographical features of the lesion [16]. As far as the excisional procedures are concerned, options include wide local excision (WLE) with a combination of sharp and gauze dissection, a loop electrosurgical excision procedure (LEEP), cold knife excision, and CO₂ laser excision. In addition, we must also consider ablative treatments (electrosurgery, cryotherapy, CO₂ laser vaporization and cavitational ultrasonic surgical aspiration [CUSA]), medical therapies (with 5-fluorouracil (5-FU), imiquimod or trichloroacetic acid), and brachytherapy.
Materials and Methods

A retrospective review of the medical records of all patients treated for VaIN with CO2 laser vaporization at the Colposcopy and Laser Surgery unit at Sant’Anna Hospital of Turin, between 1995 and 2012 was performed. All patients included in the analysis had a histological confirmation of VaIN (after cytology and colposcopy examination) and at least three months of follow-up.

Patient data were collected from colposcopic registers and hospital records. For each woman, the authors have reported the age and parity at first diagnosis, the result of the colposcopic examination, and state of immunosuppression. Additionally, the authors attempted to investigate the predictive factors for the first recurrence of VaIN.

The extent of the vaginal lesion was determined by a colposcopic examination after application of 3% acetic acid and Lugol iodine to the vaginal mucosa. The authors used a satinated metal speculum to prevent laser reflection, linked to a suction system and iodine to the vaginal mucosa. The authors used a satinized metal speculum to prevent laser reflection, linked to a suction system and CO2 laser vaporization can be considered an excisional and ablative technique. It is easy to repeat if needed, with few side effects, high precision, and a minimal impact on psychological and sexual function [2, 17, 18]. Hoffman et al. [19] described this technique, reporting a mean operative time of 42 minutes, with five ml of blood loss and an 8% complication rate. Benedet et al. [20] analyzed some biopsies from patients with VaIN and found epithelial involvement from 0.1 mm to 1.4 mm in thickness; epithelial destruction to a depth of 1.5 mm should therefore be sufficient to destroy the VaIN-containing epithelium without damaging the normal surrounding structures. Surgical specimens, however, are not available after CO2 laser vaporization, the equipment is very expensive, and a long training period is needed for the operator [21].

The aim of this study was to review a large cohort of patients with VaIN treated with CO2 laser vaporization in the present institution and to evaluate the outcome of this treatment in terms of local recurrence and the rate of progression to vaginal carcinoma. Additionally, the authors considered prognostically significant when the p-value was < 0.05. All statistical analyses were carried out using SPSS version 17.

This study was approved by the local ethical committee. Data processing, the formulation of any epidemiological analysis, and the prognostic correlation between biological and clinical data was strictly anonymous.

Table 1. — Patient features.

<table>
<thead>
<tr>
<th>Median age (years)</th>
<th>38 (14-76)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade of VaIN</td>
<td></td>
</tr>
<tr>
<td>VaIN 1</td>
<td>110 (38.6%)</td>
</tr>
<tr>
<td>VaIN 2</td>
<td>136 (47.7%)</td>
</tr>
<tr>
<td>VaIN 3</td>
<td>39 (13.7%)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
</tr>
<tr>
<td>Nulliparous</td>
<td>102 (35.8%)</td>
</tr>
<tr>
<td>1 child</td>
<td>41 (14.4%)</td>
</tr>
<tr>
<td>≥ 2 children</td>
<td>36 (12.6%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>106 (37.2%)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Right vaginal wall</td>
<td>54 (18.9%)</td>
</tr>
<tr>
<td>Left vaginal wall</td>
<td>22 (7.7%)</td>
</tr>
<tr>
<td>Ant-post vaginal wall</td>
<td>11 (3.9%)</td>
</tr>
<tr>
<td>Fornix</td>
<td>72 (25.3%)</td>
</tr>
<tr>
<td>Vaginal vault</td>
<td>5 (1.8%)</td>
</tr>
<tr>
<td>Multiple</td>
<td>121 (42.4%)</td>
</tr>
<tr>
<td>Colposcopy</td>
<td></td>
</tr>
<tr>
<td>Aceto-white epithelium</td>
<td>238 (83.5%)</td>
</tr>
<tr>
<td>Punctuation</td>
<td>6 (2.1%)</td>
</tr>
<tr>
<td>Mosaic</td>
<td>2 (0.7%)</td>
</tr>
<tr>
<td>Condylomatosis</td>
<td>9 (3.2%)</td>
</tr>
<tr>
<td>Multiple</td>
<td>23 (8.2%)</td>
</tr>
<tr>
<td>Various</td>
<td>5 (1.8%)</td>
</tr>
<tr>
<td>CIN association</td>
<td></td>
</tr>
<tr>
<td>CIN 1</td>
<td>33 (11.6%)</td>
</tr>
<tr>
<td>CIN 2</td>
<td>36 (12.6%)</td>
</tr>
<tr>
<td>CIN 3</td>
<td>14 (4.9%)</td>
</tr>
<tr>
<td>Total</td>
<td>83 (29.1%)</td>
</tr>
<tr>
<td>VIN association</td>
<td></td>
</tr>
<tr>
<td>VIN 1</td>
<td>2 (0.7%)</td>
</tr>
<tr>
<td>VIN 2</td>
<td>4 (1.4%)</td>
</tr>
<tr>
<td>VIN 3</td>
<td>3 (1.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>9 (3.2%)</td>
</tr>
<tr>
<td>HPV cytopathic effect</td>
<td>38 (13.3%)</td>
</tr>
<tr>
<td>Pap smear performed</td>
<td></td>
</tr>
<tr>
<td>at vaIN diagnosis</td>
<td></td>
</tr>
<tr>
<td>ASC-US</td>
<td>12 (4.2%)</td>
</tr>
<tr>
<td>ASC-H</td>
<td>1 (0.4%)</td>
</tr>
<tr>
<td>L-SIL</td>
<td>151 (53%)</td>
</tr>
<tr>
<td>H-SIL</td>
<td>83 (22.1%)</td>
</tr>
<tr>
<td>Negative</td>
<td>26 (9.1%)</td>
</tr>
<tr>
<td>Inadequate</td>
<td>26 (9.1%)</td>
</tr>
<tr>
<td>Immunosuppression*</td>
<td></td>
</tr>
<tr>
<td>Previous</td>
<td></td>
</tr>
<tr>
<td>Benign disease</td>
<td>9 (3.2%)</td>
</tr>
<tr>
<td>Malign disease</td>
<td>6 (2.1%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>3 (1.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>18 (6.3%)</td>
</tr>
</tbody>
</table>

*One patient had a selective immunoglobulin A (IgA) deficiency (SIGAD), one patient had acute myeloid leukemia (AML) treated with chemotherapy drugs, six patients underwent immunosuppressive therapy for autoimmune diseases, and two patients underwent immunosuppressive therapy to avoid rejection after organ transplantation. VaIN: vaginal intraepithelial lesion; Ant: anterior; post: posterior; CIN: cervical intraepithelial lesion; VIN: vulvar intraepithelial lesion; HPV: human papillomavirus; ASCUS: atypical squamous cells of undetermined significance; ASC-H: atypical squamous cells-cannot exclude high-grade squamous intraepithelial lesion; L-SIL: low grade squamous intraepithelial lesion; H-SIL: high grade squamous intraepithelial lesion.
**Results**

From 1995 to 2012, 302 women were diagnosed with VaIN in the present unit. Among them, 285 women were included in the study according to the inclusion criteria (11 patients were excluded because of a follow-up shorter than three months, VaIN regressed spontaneously in four patients, one woman was treated with LEEP, and another patient was treated with topical 5-FU followed by radiotherapy). Patient features are summarized in Table 1. The median age at VaIN diagnosis was 38 years (14–76). Most cases of VaIN were low or intermediate grade: 110 (38.6%) VaIN 1, 136 (47.7%) VaIN 2, with only 39 (13.7%) cases of VaIN 3. The median age for women with VaIN 1 was 35 years (14–64), 38 years (22–76) for VaIN 2, and 43 years (22–70) for VaIN 3. This difference, however, was not statistically significant ($p = 0.10$).

Association with CIN was found in 83 (29.1%) women; among them, 16 had a previous diagnosis of CIN, while 67 had diagnosis of CIN concomitant to VaIN diagnosis. Association with VIN was found in nine women (3.2%), while an association with both CIN and VIN was confirmed in only one woman (0.4%), who had concomitant VaIN 2, CIN 2, and VIN 3.

Eighteen women (6.3%) had a history of hysterectomy. The median time between uterine surgery and VaIN laser treatment was 219 months (3.1–365.7); in particular, women who underwent hysterectomy for benign disease developed VaIN after a median time of 135 months (4.6–365.7), while in women who underwent hysterectomy for malignant disease, the median time to VaIN was reduced to 62 months (3.1–280.4) ($p = 0.76$). Median follow-up time was 58 months (3–204).

Among the 285 women affected by VaIN, 71 (25%) relapsed; in particular, 24 (22%) VaIN 1, 37 (27%) VaIN 2, and ten (26%) VaIN 3 recurred. Eighteen (75%) cases of VaIN 1, 22 (59%) VaIN 2, and three (30%) VaIN 3 relapsed with the same grade of previous lesion; six (25%) VaIN 1 and no VaIN 2 relapsed with a superior grade; 15 (40.5%) VaIN 2 and seven (70%) VaIN 3 relapsed with an inferior grade compared to the initial lesion. The median time to the first recur-

---

**Table 2. — Median time to first recurrence of VaIN and concordance of VaIN grade at diagnosis and VaIN grade at first recurrence.**

<table>
<thead>
<tr>
<th>Grade of lesion at first diagnosis</th>
<th>Median time at first recurrence (months)</th>
<th>Grade of lesion at first relapse</th>
<th>Concordant</th>
<th>Superior</th>
<th>Inferior</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>VaIN 1</td>
<td>5.2 (1.4–127.8)</td>
<td>18</td>
<td>6</td>
<td>\</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>VaIN 2</td>
<td>6.6 (1–85.2)</td>
<td>22</td>
<td>\</td>
<td>15</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>VaIN 3</td>
<td>3.6 (1.2–62)</td>
<td>3</td>
<td>\</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.0 (1–127.8)</td>
<td>43</td>
<td>6</td>
<td>22</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

VaIN: vaginal intraepithelial lesion.

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Figure 1. — VaIN relapses in the present series and their treatments. Multiple relapses of VaIN occurred in this series, but they were successfully retreated.

* According to the original VaIN grade: 24 (22%) VaIN 1, 37 (27%) VaIN 2 and 10 (26%) VaIN 3. ** According to the original VaIN grade: 6 (5.5%) VaIN 1, 8 (5.9%) VaIN 2 and 4 (10.3%) VaIN 3. *** According to the original VaIN grade: 2 (1.8%) VaIN 1, 1 (0.7%) VaIN 2 and 1 (2.6%) VaIN 3. **** According to the original VaIN grade: 1 (0.9%) VaIN 1 and 1 (0.7%) VaIN 2. ***** According to the original VaIN grade: 1 (0.7%) VaIN 2. VaIN: vaginal intraepithelial neoplasia; Pt: patients; 5-FU: 5-fluouracil.
The multivariate model confirmed a higher risk of VaIN recurrence only in cases of previous hysterectomy (HR 3.3, 95% CI 1.7–6.3, p < 0.001) and concomitant H-SIL on the Pap smear (HR 1.9, 95% CI 1.2–3.1, p = 0.008).

Discussion

Patient and lesion features drive treatment in patients affected by VaIN [16]. In particular, the topography of VaIN, the size, the presence of an erosion area, grading, multifocality, the association with CIN and VIN, and the opportunity to evaluate by colposcopy the extension of the lesion are the main factors to be taken into account [21]. In the case of a single lesion not situated on the vaginal vault and completely visualized during colposcopy, the best treatment is CO2 laser vaporization, with few complications and the possibility of repeated treatment [2]. Yalcin et al. [22] reported no intraoperative or early post-operative complications in 24 women treated with CO2 laser vaporization; only one case of stricture of the upper vagina after multiple rounds of laser treatment was observed. In the case of a single lesion situated on the vaginal vault or not completely visualized during the colposcopy, laser surgery has demonstrated a higher failure rate, therefore an excisional technique or topical 5-FU application are the best choices [23]. 5-FU is useful in the case of multiple lesions and in the case of lesions which are difficult to reach and after radiotherapy [14].

The age of the patient, co-morbidities, the desire to preserve sexual function, and previous vaginal therapies have to be taken into account in the choice of VaIN therapy. In sexually active young women, minimally invasive and repeatable techniques, like CO2 laser vaporization or topical application of 5-FU, would be the best first choice. In the case of persistence or multiple recurrence of VaIN, more aggressive techniques such excisional therapy should be considered. For older women, however, the literature suggests direct excisional therapy or radiotherapy [21].

In the present authors’ experience, CO2 laser vaporization has proven to be an effective technique: 75% of women were free from VaIN after the first application and 93% after the second treatment. This study confirms data from a previous study [24] that reported a global effectiveness of 94.4% for CO2 laser vaporization in 36 patients treated for VaIN (all grades). Among the present patients, none progressed to invasive vaginal carcinoma.

In the present series, VaIN 3 (which progresses more frequently to vaginal carcinoma [2, 19]) was less frequent than VaIN 1 and 2, and the patients were closely followed up. Moreover, most vaginal cancers occur in elderly women, with the peak incidence during the sixth and seventh decades (older than the mean age of this series), and less than 10% of these tumors are diagnosed in patients less than 40 years of age [26]. These reasons seem to explain the lack of progression to invasive vaginal cancer in the present study. One out of four of the
present patients relapsed; this is consistent with the literature showing a global recurrence rate of 20-40% [17, 18, 21].

The median age of women with VaIN in the present study (38 years, range 14-76) was lower compared to the literature (range 40-61 years) [4, 27], and the authors suggest a trend of a higher risk of VaIN recurrence in women aged ≥ 38 (HR 1.7, 95% CI 1.0–2.8, p = 0.05).

Most authors suggest that patients with low grade lesions should be followed up because most of these lesions regress spontaneously [14]; however, other authors affirm that this practice is not safe, because of the frequent association of these lesions with high risk HPV infection [28]. Patients with high grade VaIN must always be treated [1, 14, 29] because a high risk of progression to invasive vaginal cancer has been observed (20% without treatment within three years [2] and 8-11.5% after treatment [14, 19]). In the present unit, the authors treated low grade lesions persisting for at least two rounds and high grade lesions at the first diagnosis.

In contrast to the results of a study by Hoffman et al. [19] (recurrence rate of 42% for high grade VaIN), more recent studies have supported the use of CO2 laser vaporization for the treatment of high grade VaIN, reporting a long term relapse rate of 20-30%, especially for multifocal lesions [17, 18]. These data are consistent with the present study, with a recurrence rate for VaIN 2-3 of 26.9%.

The median time for the first recurrence after treatment of VaIN 3 was 3.6 months vs. 5.2 months after VaIN 1 (Table 2): this difference was not statistically significant (HR 1.1, 95% CI 0.5–2.2, p = 0.8). Most of VaIN 1 (75%) and VaIN 2 (59%) cases relapsed with the same grade of the initial lesion; most VaIN 3 (70%) cases relapsed with an inferior grade compared to the previous lesion, and no VaIN progressed to invasive vaginal carcinoma. Therefore, the present authors observed early but indolent relapses after CO2 laser therapy; hence women should be clearly informed and offered a careful follow-up.

When the lesion is localized on the vaginal vault scar or hidden in the recess of its angles, it is difficult to reach with CO2 laser vaporization or other ablative techniques; therefore, excisional treatment is suggested [21, 30]. As shown in Table 1, the present authors treated five lesions localized on the vaginal vault: they were single, completely visualized during colposcopy, and not within the vaginal vault scar. By stratifying the present patients according to the different site of the lesion, a statistically significant difference in the risk of recurrence was not found (p = 0.9), nor was stratifying the patients according to lesions in the upper third of the vagina versus other sites, as analyzed by Dodge et al. [5]. Comparing VaIN on the vaginal vault post hysterectomy versus other sites, the present authors noted that lesions on the vaginal vault had a higher risk of recurrence (HR: 4.2, 95% CI 1.5–11.8, p = 0.006), confirming that CO2 laser vaporization on the vault is not an effective treatment.

Regarding the association of VaIN with cervical or VIN, the literature reports a VaIN-CIN association in 37-65% of patients [4, 5], a VaIN-VIN association in 10% of patients [10], and a CIN-VIN-VaIN association in 9% of patients [5]. In the present study, the association of VaIN with CIN and VIN was lower (29.1% and 3.2% of patients, respectively) and only one patient (0.4%) had concomitant CIN-VIN-VaIN. The presence of intraepithelial lesions in at least two different sites of the lower genital tract (vagina, cervix, vulva, and anus) is called lower genital tract syndrome. While this syndrome is considered an important risk factor for the development of VaIN relapses [5, 15], the presence of concomitant single CIN or VIN is not considered a significant risk factor for VaIN recurrence [5, 15, 22]. The present study reports an association with CIN in 20.5% of VaIN relapses and an association with VIN in 44.4%, confirming that single CIN or VIN association is not a statistically significant factor for VaIN recurrence (CIN: HR 1.4, 95% CI 0.8–2.3, p=0.3; VIN: HR 2.1, 95% CI 0.8–5.6, p = 0.2).

Cervical cytology performed differently if compared to cervical histology, with H-SIL as a statistically significant factor for VaIN relapse. Out of 83 patients with VaIN and concomitant H-SIL on the Pap smear, 29 (34.9%) developed a VaIN relapse, versus 41 (21.6%) relapses among 190 patients with other Pap smear results (ASCUS, ASC-H, L-SIL, and negative) (HR 1.7, 95% CI 1.1–2.8, p = 0.03). H-SIL on the Pap smear may have arisen directly from high grade VaIN in vaginal cells collected by the Pap smear, without the involvement of any cervical cells.

Finally, the present authors examined the role of previous hysterectomy. As is known, this is an important risk factor for the development of VaIN, especially in the case of hysterectomy performed for CIN or cervical carcinoma with a rate of VaIN diagnosis of 4-55% [9]. For this reason, some authors suggest these patients should have cytology and colposcopy included in their follow-up [15]. The data regarding VaIN after hysterectomy for benign disease are very contrasting [8]. In the present study, 18/285 patients (6.3%) with VaIN had a history of hysterectomy, and 11 of them (61.1%) relapsed (HR 3.4, 95% CI 1.8–6.5, p < 0.001). The present numbers were too small to stratify the data in terms of the indication for the previous hysterectomy. However, the multivariate model confirmed the role of two negative predictive factors for the recurrence of VaIN: previous hysterectomy (HR 3.3, CI 1.7–6.3, p < 0.001) and concomitant H-SIL on the Pap smear (HR 1.9, 95% CI 1.2–3.1, p = 0.008).

Some limitations of the present study include its retrospective and monocentric nature and the lack of some data regarding the demographic features of women (i.e. parity). Moreover, the authors analyzed HPV co-infection only by the observation of cytopathic effects and not by an HPV DNA test.
The strengths of the present study include the large number of patients with VaIN treated with a standardized technique by the same experts (R.V., L.P. and G.M.) and a long median time of follow-up, i.e., 58 months.

Figure 2 provides a suggestion for the scheduled follow-up for women with a high risk of VaIN relapse. A colposcopy should be performed 30 days after treatment, followed by scheduled visits (including colposcopy, cytology, and eventually histology) after three months, then every six months, and then annual visits. Based on the present data, recurrences could occur after a long period of time (127.8 months), therefore the present authors consider it worthwhile to continue follow-up until ten years after treatment.

The present data suggest that CO₂ laser vaporization can be used successfully as a low impact treatment for high and low grade VaIN completely visualized by colposcopy. Careful counseling regarding frequent VaIN relapses and long-term follow-up are needed for these patients because VaIN often recurs, especially in women with a history of hysterectomy or concomitant H-SIL on the Pap smear. The first recurrence could occur quite early, and multiple sequential relapses could also occur, but most of them can be successfully retreated with laser.

References


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Overexpression of PI3K-p110α in the progression of uterine cervical neoplasia and its correlation with pAkt and DJ-1

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1 Department of Pathology and 2 Department of Obstetrics and Gynecology, Eulji Medical Center, Eulji University School of Medicine, Seoul (Korea)

Summary

Objective: To investigate the expression of PI3K-p110α, pAkt, PTEN, the signaling molecules from PI3K/Akt signaling pathway, DJ-1, an oncprotein and HSP90α, a molecular chaperone, and their correlation in uterine cervical neoplasia, in order to elucidate their role in cervical carcinogenesis. Materials and Methods: Using immunohistochemistry, the authors analyzed the expression of PI3K-p110α, pAkt, PTEN, DJ-1 and HSP90α, and their correlation in ten normal tissues, cervical intraepithelial neoplasia (CIN) including 30 CIN1 and 31 CIN3, and 33 cases of invasive squamous cell carcinoma (SCC). Results: The expression of all proteins significantly increased in CIN3 compared to CIN1, and only the expression of PI3K-p110α significantly increased in invasive SCC compared to CIN3. There was a significant positive correlation between the expression of PI3K-p110α and DJ-1, as well as PI3K-p110α and pAkt in CIN3 and invasive SCC. Conclusion: Overexpression of PI3K-p110α is associated with progression of uterine cervical neoplasia, and the expression of pAkt and DJ-1 is positively correlated with PI3K-p110α expression in this process.

Key words: PI3K-p110α; DJ-1; pAkt; Cervical intraepithelial neoplasia; Squamous cell carcinoma.

Introduction

Development of uterine cervical carcinoma is associated with multiple molecular events, in addition to human papillomavirus (HPV) infection [1-3]. Frequent genetic aberration on chromosome 3q has been observed in cervical dysplasia and carcinoma by comparative genomic hybridization [4]. The area of gain has been refined at 3q26.3, encoding the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K-p110α) and an oncogene in this region is called PIK3CA [3]. PI3K phosphorylates the membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP2) to form phosphatidylinositol 3,4,5-triphosphate (PIP3), which triggers phosphorylation of Akt [5]. pAkt, a phosphorylated and active form of Akt, affects diverse cellular processes including survival, proliferation, protein synthesis, and glucose metabolism [6]. On the other hand, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) negatively regulates PI3K activity by dephosphorylation PIP3 [7]. Loss of PTEN or overexpression of pAkt can result in the development of many types of tumors [7, 8].

DJ-1 has been characterized as an oncogenic product that transforms NIH3T3 cells and has a stronger cooperative transforming activity with H-Ras [9]. DJ-1 acts as a negative regulator of PTEN and increases cell survival by hyperphosphorylation of Akt in mammalian cells [10]. Overexpression of DJ-1 has been shown to predict a poor prognosis in pancreatic [11] and esophageal cancers [12]. Heat shock protein 90 (HSP90) is a molecular chaperone that maintains the stability and activity of oncogenic client proteins including ERBB2, Akt, steroid hormone receptors, mutant p53, HIF-1alpha, and hTERT [13]. Thus, inhibition of HSP90 provides simultaneous repression of multiple signaling pathways that have been involved in the development of malignancy [13]. HSP90 has two isoforms, HSP90α and HSP90β, and HSP90α is stress-inducible and overexpressed in many cancer cells [14].

There has been no report concerning the expression of DJ-1, HSP90α, PI3K-p110α, pAkt, and PTEN and their correlation in cervical neoplasia, although each molecule has been examined individually [8, 15-17]. In this study, the authors analyzed the expression of these markers in normal cervix, cervical intraepithelial neoplasia (CIN), and invasive squamous cell carcinoma (SCC), and their association during the progress of cervical neoplasia.

Materials and Methods

The present authors retrieved 30 (27 punch biopsies, two conizations, and one hysterectomy) cases of CIN grade 1 (CIN1), 31 (23 conizations and eight hysterectomies) cases of CIN grade 3 (CIN3), and 33 (9 conizations and 24 hysterectomies) cases of invasive SCC from the files of the Department of Pathology, Eulji Medical Center, Eulji University School of Medicine, Seoul, Korea, between 2000 and 2004. HPV DNA data of patients were not available and the authors used p16INK4a immunostaining as an ancillary test for a marker of HPV infected CIN [18]. Ten cases
of histologically normal and p16INK4a negative cervical mucosa from hysterectomy specimen were included. CIN2 cases were excluded because of its poor reproducibility of histopathologic diagnosis. The stage of 24 invasive SCC patients underwent hysterectomy was classified into nine cases of Stage Ia, ten Stage Ib, and five Stage Ila-IIlb according to the international Federation of Gynecology and Obstetrics (FIGO) staging system. After reviewing all hematoxylin and eosin-stained slides from each case, a representative block of each lesion was selected. Tissue microarray (TMA) was constructed using a manual microarray instrument. The tissue cores (two mm in diameter) from the targeted areas were taken from the paraffin blocks and arranged in a new recipient block for TMA.

**Immunohistochemistry**

The authors performed immunohistochemical staining using an autostainer. Four micron tissue sections were cut from TMA blocks and positioned on poly-L-lysine coated slides. After deparaffinization and rehydration, antigen retrieval was performed using citrate buffer (pH 6.0) at 121°C for ten minutes. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for five minutes, and the sections were incubated with specific antibodies against DJ-1 (1:1,000), HSP90α (1:10,000), pAkt (1:500), PI3K-p110α (1:500), PTEN (1:250), and p16INK4a (p16INK4a kit). Color was developed using diaminobenzidine, and the sections were counterstained with hematoxylin. Positive and negative controls for DJ-1, HSP90α, pAkt, PI3K-p110α, and PTEN were optimized as previously described [19]. Diffuse cytoplasmic staining of pAkt, PI3K-p110α, and PTEN were considered positive. In the case of DJ-1 and HSP90α, either cytoplasmic or nuclear staining was considered positive. Immunohistochemical staining results were evaluated independently by two pathologists (S.K. Choi and H. Lee). When the interpretation between two observers was different, final decision was reached by re-evaluating the slides at a conference microscope. The immunoreactivity was determined semiquantitatively by assessing the percentage of stained cells and the intensity of staining. The percentage of positive cells was divided into three groups as staining in < 5%, 5-50%, and > 50% of the neoplastic cells. The staining intensity was rated as negative, weak and strong. The overall staining score was classified as: 0, no expression (less than 5% staining with any intensity); 1, low expression (5-50% staining with any intensity); 2, moderate expression (50-80% staining with strong intensity); and 3, high expression (over 80% staining with strong intensity).
Overexpression of PI3K-p110α in the progression of uterine cervical neoplasia and its correlation with pAkt and DJ-1

Statistical analysis

Comparative analysis of immunoexpression between CIN1, CIN3, and invasive SCC was performed using Chi square test and Fisher’s exact test. Correlation between markers was analyzed by Spearman correlation test in CIN3 and invasive SCC. CIN1 was excluded in correlation test between markers because PI3K-p110α and pAkt were not expressed in CIN1. Statistical significance was set at \( p < 0.05 \). All analysis was performed using the SPSS ver. 14.0.

Results

Staining pattern of DJ-1, HSP90α, PI3K-p110α, pAkt, and PTEN in the normal cervix, CIN1, CIN3, and invasive SCC is shown in Figure 1. In normal cervical squamous epithelia, HSP90α and PTEN expression was observed in basal layer, whereas the expression of DJ-1, PI3K-p110α, and pAkt was negative. HSP90α staining was strong nuclear and cytoplasmic, and PTEN staining was weakly cytoplasmic in basal layer.

Table 1 summarizes the staining score of markers according to the disease severity. The expression of all markers was significantly upregulated in CIN3 compared to CIN1. However, comparing CIN3 to SCC, only the expression of PI3K-p110α significantly increased in SCC. DJ-1 was not stained in koilocytes and only weakly expressed in nuclei of the cells of basal layer in 20% of CIN1 cases. Diffuse cytoplasmic and nuclear DJ-1 expression was observed in 90.3% of CIN3 (low expression, 61.3% and high expression, 29.0%) and 100% of SCC cases (low expression, 51.5% and high expression, 48.5%). HSP90α was expressed in koilocytes as well as basal layer cells of all CIN1 cases (low expression, 93.3% and high expression, 6.7%). HSP90α staining was diffusely cytoplasmic and nuclear in 87.1% of CIN3 (low expression, 54.8% and high expression, 32.3%) and 93.9% of SCC cases (low expression, 72.7% and high expression, 21.2%). PI3K-p110α staining was negative in all CIN1, but mostly weakly cytoplasmic in 67.7% of CIN3 (low expression, 45.2%) and 93.9% of SCC cases (low expression, 87.8%). pAkt staining was also negative in all CIN1, but weakly cytoplasmic in 45.2% of CIN3 and 45.5% of SCC, and strong only in 3% of SCC cases. PTEN expression showed weak cytoplasmic staining pattern in 3.3% of CIN1, 48.4% of CIN3, and 42.4% of SCC cases.

The authors identified positive correlations in the expression of PI3K-p110α and DJ-1 (Spearman’s correlation coefficient (cc), 0.266; \( p = 0.043 \)) and PI3K-p110α and pAkt (Spearman’s correlation coefficient (cc), 0.288; \( p = 0.021 \)) in CIN3 and SCC (Table 2).

Table 1. — DJ-1, HSP90α, PI3K-p110α, pAkt, and PTEN staining score in CIN1, CIN3, and SCC.

<table>
<thead>
<tr>
<th>Score</th>
<th>CIN1</th>
<th>CIN3</th>
<th>SCC</th>
<th>( P_{1-3} )</th>
<th>( P_{3-S} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N %</td>
<td>N %</td>
<td>N %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DJ-1</td>
<td></td>
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</tr>
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<td>16  48.5</td>
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</tbody>
</table>

CIN1: cervical intraepithelial neoplasia grade 1; CIN3: cervical intraepithelial neoplasia grade 3; SCC: squamous cell carcinoma; N: number. \( P_{1-3} \) reflects \( p \)-value between CIN1 and CIN3; \( P_{3-S} \), \( p \)-value between CIN3 and SCC. Variables found to be significant (\( p < 0.05 \)) are shown in bold.

Table 2. — Spearman correlation between markers in CIN3 and SCC.

<table>
<thead>
<tr>
<th></th>
<th>HSP90α</th>
<th>PI3K-p110α</th>
<th>pAkt</th>
<th>PTEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ-1</td>
<td>0.055 (( p = 0.667 ))</td>
<td><strong>0.266 (( p = 0.033 ))</strong></td>
<td>0.144 (( p = 0.257 ))</td>
<td>0.232 (( p = 0.066 ))</td>
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<tr>
<td>HSP90α</td>
<td>-0.135 (( p = 0.286 ))</td>
<td>-0.023 (( p = 0.858 ))</td>
<td>-0.076 (( p = 0.549 ))</td>
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<tr>
<td>PI3K-p110α</td>
<td><strong>0.288 (( p = 0.021 ))</strong></td>
<td></td>
<td></td>
<td>0.210 (( p = 0.097 ))</td>
</tr>
<tr>
<td>pAkt</td>
<td></td>
<td></td>
<td></td>
<td>0.226 (( p = 0.073 ))</td>
</tr>
</tbody>
</table>

CIN3: cervical intraepithelial neoplasia grade 3; SCC: squamous cell carcinoma. Variables found to be significant (\( p < 0.05 \)) are shown in bold.
Discussion

In this study, the authors found a significantly increased expression of PI3K-p110α, pAkt, DJ-1, HSP90α, and PTEN in CIN3 compared to CIN1. The staining of PI3K-p110α was completely negative in CIN1 and normal cervical tissue, whereas PI3K-p110α was overexpressed in CIN3 (67.7%) and SCC (93.9%), corresponding to previous studies [3, 20]. Amplification of PIK3CA gene copy number was shown in CIN3 and SCC specimens by quantitative real-time polymerase chain reaction [3]. Zhang et al. showed overexpression of PI3K at the protein and mRNA level in cervical cancer tissues compared with non-neoplastic tissues and higher PI3K expression in HeLa cells than in immortal human keratinocyte HaCaT cells [20]. There were studies representing gain of chromosome 3q in HPV-infected tumor cells that would indicate activation of PI3K [1, 4], but there is no study on the expression of PI3K in koilocyte. In the present study, PI3K-p110α was not expressed in koilocyte and it remains to be seen whether genetic alteration of PI3K-p110α is associated with morphologic change of HPV-infected cells. Recently, mutation of PI3KCA exon 9 and exon 20 was found in 23% of cervical cancer patients and FIGO Stage I/II patients with PIK3CA mutant tumors showed worse overall survival [21]. Schwarz et al. showed mutational activation of PI3KCA in cervical cancer was associated with incomplete metabolic response, resulting in poor response to chemoradiotherapy [15]. LY294002, PI3K inhibitor inhibited the proliferation of cells and the expression of pAkt and phospho-mTOR, providing a possible therapeutic strategy of PI3K inhibitor for cervical cancer [20]. As mutational status of PI3KCA is associated with overall survival in cervical cancer patients and response rate of the patients treated with PI3K/Akt/mTOR inhibitor [22], it would be required to evaluate the tumoral PIK3CA mutational status.

Positive correlation between pAkt and PI3K-p110α in this study corresponds to previous results [3], but no significant difference in expression rate of pAkt in CIN3 (45.2%) and SCC (48.5%) was found.

DJ-1 promotes cell survival through modulating PI3K/Akt pathway [10]. In esophageal SCC patients, significant correlation is present between DJ-1 and pAkt expression, and a high level of nuclear DJ-1 is significantly associated with high distant metastatic potential and poorer survival [12]. Higher expression of DJ-1 has been associated with higher pathologic stage in esophageal and glottic SCC [12, 23], and urothelial carcinoma [19]. The present authors previously showed that DJ-1 expression was significantly higher in invasive urothelial carcinoma (T1-T3) compared to non-invasive urothelial carcinoma (Ta) of bladder [19]. DJ-1 is characterized as one of the molecular markers indicative of cervical cancer progression by proteomic analysis by Arnouk et al. demonstrating significant increase of DJ-1 in SCC compared to normal tissue [17]. In the present study, DJ-1 was significantly overexpressed in CIN3 (90.3%) compared to weak expression in basal layer of CIN1 (20%) with negative staining in koilocytes, supporting the assumption that molecular change of DJ-1 occur during latency period of HPV infection [17]. Although the authors showed no significant difference of DJ-1 expression between CIN3 and SCC, the result of high expression rate of DJ-1 in CIN3 (90.3%) and SCC (100%), and positive correlation between DJ-1 and PI3K-p110α suggests the involvement of DJ-1 in progression of cervical cancer through PI3K/Akt signaling pathway.

Contrary to other proteins, HSP90α was abundantly expressed in nuclei and cytoplasm of most cells through CIN to SCC tissues, including koilocytes in this study. As heat shock proteins (HSPs) are known to interact with p53, upregulation of HSP90 may be induced by destabilization of p53 by oncogenic HPV E6 expression under negative feedback control [16, 24]. Castle et al. demonstrated that expression of HSP40, HSP60, and HSP70 increased in proportion to the severity of CIN, while expression of HSP90 was negligible in normal cervix and CIN, but the reason for lack of HSP90 was not explained [16]. HSP90α expression in cervical neoplastic lesion may reflect its involvement in whole process from early HPV infection to invasive cancer. However, the present authors did not have data of HPV DNA testing and therefore further study including HPV genomic organization data and assessing their association with HSP90α would be needed [25]. It is well known that Akt is one of client proteins of HSP90, and HSP90 inhibitors reduce Akt expression in gynecologic cancer cells [26], however, any correlation between expression of HSP90α and pAkt was not observed in our results.

PTEN staining result in the present study was conflicting compared to previous data showing that loss of PTEN expression was associated with tumor progression in the cervical epithelium [8], and pelvic lymph node metastasis in early-stage cervical cancer [27]. However, direct mutation of PTEN gene has been generally absent in cervical cancer [28]. In addition, a study showing evenly distributed PTEN immunostaining in normal cervical mucosa and tumor tissue suggests little impact of PTEN on tumor suppression [3]. Combined analysis of PTEN mutation would be needed to define the role of PTEN in cervical carcinogenesis.

In summary, this study is the first attempt to represent simultaneous assessment of PI3K-p110α, pAkt, DJ-1, HSP90α, and PTEN in CIN and SCC of uterine cervix. The present results suggest that the overexpression of PI3K-p110α is associated with stepwise progression from CIN to SCC, and pAkt and DJ-1 may be positively correlated with PI3K-p110α, irrespective of PTEN loss in PI3K/Akt signaling pathway. Further studies including the mutational analysis of these molecules and their association with HPV infection will provide better understanding of their role in cervical carcinogenesis.
References


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Anti-Hsp20 antibody concentrations inversely correlated with tumor progression in ovarian cancer

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Summary

Purpose: To investigate the serum concentrations of anti-heat shock protein 20 (anti-Hsp20) antibodies in women with ovarian cancer at different clinical stages, and the relationship between these concentrations and tumor progression. Materials and Methods: Blood samples were obtained from 72 patients undergoing surgery for ovarian cancer, 21 women with ovarian carcinoid, and 42 healthy women. Anti-Hsp20 antibody concentrations were determined by enzyme-linked immunosorbent assay. Results: Mean anti-Hsp20 antibody concentrations were significantly lower in patients with ovarian cancer than in the control group. The anti-Hsp20 antibody concentrations were negatively correlated with ovarian cancer malignancy. Conclusions: The present findings suggest that anti-Hsp20 antibodies may play a protective role against ovarian cancer progression, and that anti-Hsp20 antibodies may be a new index for the early diagnosis and treatment of ovarian cancer.

Key words: Heat shock protein; Hsp20; Ovarian cancer.

Introduction

Heat shock proteins (HSPs) are a subset of molecular chaperones best known for their rapid and abundant induction following stress. HSPs, classified by their molecular weight, are highly expressed in many malignant tumors—including ovarian cancer—and most seem to play roles in many aspects of tumor progression and response to therapy, probably due to their antiapoptotic properties [1, 2]. Hsp27 typically functions as a chaperone, but previous studies indicate that it also plays fundamental roles in maintaining intracellular redox potential and stabilizing the cytoskeleton [3, 4]. High Hsp27 expression induced by chronic cellular stress may lead to apoptosis suppression, thus facilitating malignant transformation [5].

The present authors’ previous studies of HSP20 revealed its antiapoptotic effect on cardiomyocytes [6, 7]. They also found that Hsp20 expression levels decrease with tumor progression in ovarian cancer patients, suggesting that HSP20 could have a suppressive effect on ovarian cancer progression [8]. In the present study, the authors investigated the relationship between the serum concentration of anti-Hsp20 antibodies in women with ovarian cancer and the progression of ovarian cancer malignancy.

Materials and Methods

Subjects

The study subjects included 145 randomly selected women who were hospitalized for a suspected ovarian tumor between April 2011 and May 2013 and who intended to undergo surgical intervention. Another group of 42 healthy women of similar age were recruited as a control group. All participants were ethnic Han Chinese, and lived in the area of the Shandong Province in the middle-eastern region of China. Table 1 presents the demographics and clinical characteristics of the study population. The Ethics Committee of Jinan Third People’s Hospital approved the research protocol, and written informed consent was obtained from each patient and control participant.

Women with ovarian cancer were excluded if they had received hormone therapy or chemotherapy, or if their condition occurred in combination with other malignancies. After screening, the ovarian cancer group included 72 patients: six (18%) International Federation of Gynecology and Obstetrics (FIGO) Stage I cases, ten (29%) FIGO Stage II cases, 12 (35%) FIGO Stage III cases, and six (18%) FIGO Stage IV cases. The histological types were as follows: serous papillary carcinoma (n = 50), mucinous carcinoma (n = 10), clear cell carcinoma (n = 4), endometrioid carcinoma (n = 6), endometrioid carcinoma (n = 4), and mixed cystadenocarcinoma (n = 3). Twenty-one women were diagnosed with benign ovarian carcinoid, with the different histological types including serous cystadenoma (n = 6), mucinous cystadenoma (n = 3), mixed cystadenoma (n = 3), and simple ovarian cyst (n = 6).

Surgical specimens

Ovarian tissues for histological type analysis were obtained from patients by surgical resection at the Department of Obstet-
rics and Gynecology, Jinan Third People’s Hospital. Resected tissue was divided such that part was examined by intraoperative frozen section analysis and the rest was snap-frozen in liquid nitrogen and stored at −80°C.

Serum samples
Patient blood samples were collected from the median cubital vein at 8:00 am after fasting. Blood was collected in a clotting tube. Within four hours of collection, clotted blood was centrifuged at 2000 × g for ten minutes, and then serum was aliquoted and stored at −80°C until assay.

Enzyme-linked immunosorbent assay (ELISA)
In the serum samples, the concentration of IgG antibodies against Hsp20 was determined by ELISA. Human recombinant Hsp20 from Prospec was used as an antigen. The ELISA plates were coated with the antigen solution at a concentration of two mg/ml in 50 mmol/L of carbonate buffer at pH 9.6. The tested serum samples were diluted 400-fold using 0.5% bovine serum albumin in phosphate-buffered saline with Tween-20. Horseradish peroxidase-conjugated goat anti-human IgG was used to detect the bound IgG antibodies. Tetramethylbenzidine solution was used as the enzymatic reaction substrate. The Power Wave XS plate reader was used to read the absorbance at 450 nm (reference wavelength, 630 nm), and the results were calculated using KC Junior software.

Calibration was performed using pooled sera obtained from 50 healthy blood donors. Dilution of 1:400 was considered as 100 arbitrary units (AU/ml). The optimal sera dilution was chosen experimentally; the 1:400 dilution produced an optimal sample absorbance to background absorbance ratio for most of the studied samples. The calibration curve consisted of six standards with concentrations ranging from 0 to 400 AU/ml. The coefficient of the intra-assay variation was 8%. Determinations were performed during a single series. The sensitivity was about 1 AU. The obtained results were presented using basic parameters of descriptive statistics.

Statistical analysis
Data are expressed as mean ± standard deviation (SD). Differences were analyzed for significance by one-way repeated-measures ANOVA, with further analyses performed using the Newman–Keuls test for multiple comparisons between treatment groups. The results were considered significant at p < 0.05. Analyses were performed using GraphPad Prism version 4.0 for Windows.

Results
Comparison of anti-Hsp20 antibodies among different groups
Figure 1 shows the results of ELISA for anti-Hsp20 expression in serum from patients with ovarian cancer Stages I, II, III, and IV, from ovarian carcinoid patients, and from control subjects with normal ovaries. Serum anti-Hsp20 concentrations were significantly reduced in malignant cases compared to in healthy controls, as well as in malignant cases compared to in benign cases (p < 0.05 for both). Concentrations of anti-Hsp20 antibodies also significantly differed between ovarian cancer stages I and II (p < 0.05), with a trend towards an inverse correlation between cancer stage and anti-Hsp20 antibody expression in tumor tissues (Figure 1).

Discussion
The present results showed that the concentrations of anti-Hsp20 antibodies were lower in ovarian tumor tissue than in normal tissue, with a trend toward anti-Hsp20 antibody concentration decreasing in correlation with increasing ovarian cancer progression tissues. These findings are
Anti-Hsp20 antibody concentrations inversely correlated with tumor progression in ovarian cancer

Consistent with those of the authors’ previous study, which demonstrated that HSP levels in tumor tissues were attenuated in association with ovarian cancer progression, such that HSP expression was inversely related to the grade of malignancy. To the authors’ knowledge, this is the first report of a significant association between concentrations of anti-Hsp20 antibodies and ovarian cancer progression.

Olejek et al. previously reported that the mean concentration of anti-Hsp27 antibodies was significantly higher in a group of patients with ovarian carcinoma than in the control group. Their analysis of the association between anti-Hsp27 antibodies and the stage of clinical progression revealed higher concentrations of anti-Hsp27 antibodies in less advanced ovarian carcinoma specimens [8]. Thus, it seems that anti-Hsp20 antibodies and anti-Hsp27 antibodies have different responses to tumor malignancy. Further studies are warranted to investigate the underlying mechanisms behind these associations.

Conclusion

The present results strongly suggest that anti-Hsp20 antibody concentration decreases with tumor progression in ovarian cancer patients, thus indicating a possible suppressive effect of anti-Hsp20 antibodies on ovarian cancer progression. The authors are currently conducting studies to investigate the underlying mechanism of this effect, as well as to optimize the detection of anti-Hsp20 antibodies in serum for the early identification of ovarian cancer.

References


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Pathological factors associated with omental metastases in endometrial cancer


Department of Obstetrics and Gynecology, School of Medicine, Keio University, Tokyo (Japan)

Summary

Purpose of investigation: This study aimed to assess the role of omentectomy in the surgical therapy of endometrial cancer. Materials and Methods: A retrospective study was performed in 98 patients who were pathologically diagnosed with endometrial cancer and had initially undergone surgical therapy at the present institution. This study analyzed the relationship between omental metastasis and clinicopathological factors. Results: Omental metastasis was detected in nine patients (9%). On univariate analysis, significant number of omental metastatic lesions were detected in few cases by positive peritoneal cytology, adnexal metastasis, gross dissemination, and lymphovascular space involvement. On multivariate analysis, adnexal metastasis were a significant risk factor. The sensitivity of the special histological type and the specificity of the macroscopic peritoneal dissemination and adnexal metastasis were all high. Conclusion: Omentectomy plays a significant role in determining the exact surgical staging in cases with non-endometrioid cancer, adnexal metastasis, and macroscopic peritoneal dissemination.

Key words: Endometrial cancer; Omentectomy; Omental metastasis; Pathological factor; Uterine cancer.

Introduction

Endometrial cancer is a malignant tumor, the incidence of which is increasing in Japan because of the growth in the population of nulliparous females, attributed to delayed marriages and lifestyle changes. The initial management of endometrial cancer includes surgery, and the standard surgical procedure is hysterectomy with bilateral salpingo-oophorectomy [1] and retroperitoneal lymphadenectomy/biopsy (pelvic and paraaortic lymph nodes). The present authors frequently change surgical procedures depending on the histological type of the tumor or the presence of extrauterine metastasis or myometrial invasion.

Risk factors for recurrence are deep myometrial invasion [2], endometrioid adenocarcinoma grade 3 (G3) [2, 3], non-endometrioid adenocarcinoma [4, 5], lymphovascular space invasion (LVSI) [4, 6], adnexal metastasis [7], cervical involvement [7, 8], lymph node metastasis, and distant metastasis; of these, the latter is a particularly important factor in recurrence and poor prognosis.

Accurate risk assessment is important for adequate postoperative therapy and the estimation of prognosis. Lymph node metastasis is often observed in patients with endometrial cancer, and biopsy or lymphadenectomy of regional lymph nodes is a reliable diagnostic indicator of extrauterine metastasis. Endometrial cancer spreads throughout the uterine tube, accompanied by organized peritoneal dissemination, the assessment of which is also important, depending on the histological type.

Peritoneal dissemination is diagnosed on the basis of macroscopic observations and peritoneal biopsy. The omentum is a lymphatic tissue comprised of many vessels and microvessels, and omentectomy may be useful in the evaluation of peritoneal dissemination.

According to the National Comprehensive Cancer Network (NCCN) guidelines [9], omentectomy is recommended in cases with increased CA-125 levels and following histological types: serous adenocarcinoma, clear cell adenocarcinoma, and carcinosarcoma. Furthermore, it is recommended in cases with suspicious extrauterine lesions according to magnetic resonance imaging (MRI) and computed tomography (CT). However, indications for omentectomy remain controversial [10].

In this report, the authors discuss pathological features in relation to confirming the role of omentectomy in the surgical therapy of endometrial cancer.

Materials and Methods

Subjects included 98 patients who were pathologically diagnosed with endometrial cancer at the present institution and had initially undergone surgical therapy between 2007 and 2011 [including hysterectomy, bilateral salpingo-oophorectomy (BSO), and omentectomy]. Clinicopathological factors of these cases were retrospectively analyzed. At the present institution, endometrial biopsies or curettage were performed in cases with a definitive diagnosis of endometrial cancer. Moreover, pelvic MRIs, thoracoabdominal CTs, and hysteroscopies were performed.
to evaluate the clinical stage; appropriate surgical procedures were then selected, including lymphadenectomy.

Hysterectomy encompasses simple hysterectomy, modified radical hysterectomy, and radical hysterectomy; the choice of procedure is determined on the basis of cervical stromal invasion and myometrial invasion. BSO was performed in all cases, whereas pelvic lymphadenectomy was performed in all cases except those with Stage IA and endometrioid adenocarcinoma G1 without myometrial invasion. Pelvic lymphadenectomy was performed in following cases: pelvic lymph node metastasis, adnexal metastasis, myometrial invasion that involved at least one-half thickness, endometrioid adenocarcinoma G3, and non-endometrioid adenocarcinoma, including serous adenocarcinoma, clear cell adenocarcinoma, and carcinosarcoma. Omentectomy was performed in all cases where para-aortic lymphadenectomy was performed. Partial omentectomy was performed in principle, but subtotal omentectomy was performed in cases where macroscopic peritoneal dissemination was detected. In some cases with severe complications, retroperitoneal lymphadenectomy was not performed.

Intraoperative pathological diagnosis was achieved by the pathologist in most cases after evaluating the histological type, myometrial invasion, and distant metastasis; this determination assisted in determining the surgical technique to be employed for lymphadenectomy. Intraoperative peritoneal cytology was not performed at this institute.

Risk factors for recurrence were assessed on the basis of the pathological diagnosis. Postoperative multidrug adjuvant chemotherapy included platinum drugs, which was administered for three to six cycles in all cases with risk factors. The follow-up period was ten to 15 years following initial therapy, and follow-up examinations to check for recurrence included gynecological examinations, vaginal cytology, thoracoabdominal CTs, and the identification of tumor markers.

This study analyzed following pathological factors: histological type, peritoneal cytology, adnexal metastasis, myometrial invasion, LVSI, cervical stromal invasion, parametrial invasion, regional lymph node metastasis, and peritoneal dissemination.

This study was conducted with the approval from the ethics committee of the School of Medicine, Keio University (approval number: 20120243).

**Statistical analysis**

The SPSS software (version 20) was used for statistical analysis, which was performed using Fisher’s exact test and Student’s t-test. P values < 0.05 were considered statistically significant. Kaplan–Meier curves were used to evaluate the progression-free survival and were compared using standard log-rank tests.

**Results**

**Patient characteristics**

Patients’ median age was 59 years (range: 34–77 years). Omentectomy was performed in all cases. Complications developing because of omentectomy were not clearly determined in any case. Pelvic and para-aortic lymphadenectomy were performed in most cases, but retroperitoneal lymphadenectomy were not performed in 7% cases. Patient characteristics and clinicopathological features of all 98 cases are presented in Table 1.

Sixty-six patients were diagnosed with endometrioid adenocarcinoma (19, 24, and 23 with grades 1, 2, and 3, respectively), 16 with serous adenocarcinoma, three with clear cell adenocarcinoma, ten with carcinosarcoma, and three with undifferentiated carcinoma. Omental metastasis was detected in 1.5% cases with endometrioid adenocarcinoma, 31.2% with serous adenocarcinoma, 33.3% with clear cell adenocarcinoma, and 20.0% with carcinosarcoma.

**Table 1. — Patients’ characteristics.**

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<th>Peritoneal cytology</th>
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<th>Cervical involvement</th>
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<td>71±109.3</td>
<td>31.0±55.7</td>
<td>822.0±556.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>24</td>
<td>4</td>
<td>20</td>
<td>NS</td>
<td></td>
<td></td>
<td>24</td>
<td>4</td>
<td>71±109.3</td>
<td>31.0±55.7</td>
<td>822.0±556.0</td>
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<td></td>
<td>–</td>
<td>74</td>
<td>5</td>
<td>69</td>
<td>NS</td>
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<td></td>
<td>74</td>
<td>5</td>
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<td>31.0±55.7</td>
<td>822.0±556.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>65</td>
<td>7</td>
<td>58</td>
<td>NS</td>
<td></td>
<td></td>
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<td>7</td>
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<td>31.0±55.7</td>
<td>822.0±556.0</td>
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<tr>
<td></td>
<td>–</td>
<td>33</td>
<td>2</td>
<td>31</td>
<td>NS</td>
<td></td>
<td></td>
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<td>2</td>
<td>71±109.3</td>
<td>31.0±55.7</td>
<td>822.0±556.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>NS</td>
<td></td>
<td></td>
<td>4</td>
<td>3</td>
<td>71±109.3</td>
<td>31.0±55.7</td>
<td>822.0±556.0</td>
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<tr>
<td></td>
<td>–</td>
<td>94</td>
<td>6</td>
<td>88</td>
<td>NS</td>
<td></td>
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<td>6</td>
<td>71±109.3</td>
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<td>822.0±556.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>33</td>
<td>1</td>
<td>32</td>
<td>NS</td>
<td></td>
<td></td>
<td>33</td>
<td>1</td>
<td>71±109.3</td>
<td>31.0±55.7</td>
<td>822.0±556.0</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>65</td>
<td>8</td>
<td>57</td>
<td>NS</td>
<td></td>
<td></td>
<td>65</td>
<td>8</td>
<td>71±109.3</td>
<td>31.0±55.7</td>
<td>822.0±556.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td>7</td>
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<td>71±109.3</td>
<td>31.0±55.7</td>
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</tr>
<tr>
<td></td>
<td>–</td>
<td>91</td>
<td>2</td>
<td>89</td>
<td>NS</td>
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<td>2</td>
<td>71±109.3</td>
<td>31.0±55.7</td>
<td>822.0±556.0</td>
</tr>
</tbody>
</table>

* endometrioid adenocarcinoma; ** lymphovascular space involvement; *** progression-free survival.
The following pathological factors revealed significant correlation with omental metastasis: positive peritoneal cytology, adnexal metastasis, LVSI, peritoneal dissemination, CA125, and CA19-9 levels. Patients without omental metastasis had a significantly better prognosis. The median follow-up period was 25 months (range: 2–60 months).

Table 2. — Clinicopathological factors of the nine cases with omental metastasis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Suspicious omental metastasis</th>
<th>pTN</th>
<th>Histological type</th>
<th>MI</th>
<th>AM</th>
<th>LM</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56</td>
<td>–</td>
<td>pT1AN0</td>
<td>Serous</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>–</td>
<td>pT1BN1</td>
<td>Serous</td>
<td>&gt;1/2</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>+</td>
<td>pT3BN0</td>
<td>Serous</td>
<td>&gt;1/2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>+</td>
<td>pT3AN0</td>
<td>Serous</td>
<td>&gt;1/2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>+</td>
<td>pT3AN0</td>
<td>Serous</td>
<td>&gt;1/2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>–</td>
<td>pT2N0</td>
<td>Clear</td>
<td>&gt;1/2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>–</td>
<td>pT3AN0</td>
<td>Carcinosarcoma</td>
<td>&gt;1/2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>+</td>
<td>pT3AN0</td>
<td>Carcinosarcoma</td>
<td>&lt;1/2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
<td>–</td>
<td>pT3AN0</td>
<td>EM G1</td>
<td>&gt;1/2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>


Table 3. — Univariate and multivariate analysis of pathological factors.

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis (p value)</th>
<th>Multivariate analysis (p value)</th>
<th>Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-EM histological type (VS EM)</td>
<td>0.429</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Positive peritoneal cytology (VS negative)</td>
<td>&lt;0.001</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Adnexal metastasis (VS negative)</td>
<td>0.001</td>
<td>0.003</td>
<td>8.864 (2.062-38.108)</td>
</tr>
<tr>
<td>Myometrial invasion &gt; 1/2 (VS &lt;1/2)</td>
<td>0.052</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Cervical involvement (VS negative)</td>
<td>0.144</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis (VS negative)</td>
<td>0.524</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Peritoneal dissemination by inspection (VS negative)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. — Sensitivity and specificity of pathological factors.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-EM histological type (VS EM)</td>
<td>88.9%</td>
<td>47.2%</td>
</tr>
<tr>
<td>Adnexal metastasis (VS negative)</td>
<td>66.7%</td>
<td>87.7%</td>
</tr>
<tr>
<td>Myometrial invasion &gt; 1/2 (VS &lt;1/2)</td>
<td>66.7%</td>
<td>30.0%</td>
</tr>
<tr>
<td>Cervical involvement (VS negative)</td>
<td>44.4%</td>
<td>77.5%</td>
</tr>
<tr>
<td>Lymph node metastasis (VS negative)</td>
<td>22.2%</td>
<td>64.0%</td>
</tr>
<tr>
<td>Peritoneal dissemination by inspection (VS negative)</td>
<td>77.8%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

The following pathological factors revealed significant correlation with omental metastasis: positive peritoneal cytology, adnexal metastasis, LVSI, peritoneal dissemination, CA125, and CA19-9 levels. Patients without omental metastasis had a significantly better prognosis. The median follow-up period was 25 months (range: 2–60 months).

Analysis of pathological factors in omental metastasis cases

Pathological factors involved in omental metastasis cases are presented in Table 2. Omental metastasis was detected in nine of 98 cases (9%), including one case with endometrioid adenocarcinoma, five with serous adenocarcinomas, one with clear cell adenocarcinoma, and two with carcinosarcomas.

In seven of these nine cases, the macroscopic peritoneal dissemination was detected during surgery, along with four other cases wherein peritoneal dissemination had already been detected before surgery by either CT or MRI. Microscopic omental metastasis were detected in two cases: one with serous adenocarcinoma and another with carcinosarcoma; both these cases were subsequently diagnosed with serous adenocarcinoma with preoperative endometrial biopsy. In both cases, tumor markers were found at levels lower than cut-off values, with no omental metastasis identified by either CT or MRI, and with no omental involvement detected by intraoperative inspection and palpation. In all cases, peritoneal cytology was positive, and no metastasis was detected in other organs.

Analysis of predictive factors of omental metastasis

According to univariate analyses, the hazard of omental metastasis were significantly higher in the groups of positive peritoneal cytology, adnexal metastasis, and gross peritoneal dissemination (Table 3). Histological type, myometrial invasion, and adnexal metastasis were diagnosed by aforementioned intraoperative pathological diagnosis. Among these pathological factors, multivariate analysis revealed that the presence of adnexal metastasis was a significant risk factor (Table 3). Sensitivity and specificity are presented in Table 4.
sion were higher. The specificity of the macroscopic peritoneal dissemination and adnexal metastasis were higher.

Prognosis

Figure 1 represents Kaplan–Meier curves for the progression-free survival of cases with and without omental metastasis. Except for one case with a short follow-up period (seven months), all cases with omental metastasis suffered relapse and revealed a significantly poorer prognosis ($p < 0.001$).

Discussion

The significance of omentectomy remains controversial in endometrial cancer. According to the NCCN guidelines, omentectomy is considered essential in cases with increased CA125 levels and in non-endometrioid histological type of cancers (serous adenocarcinoma, clear cell adenocarcinoma, and carcinosarcoma). It has been reported that the incidence of complications did not increase because of omentectomy, although partial omentectomy under the transverse colon occupied approximately 12–20 minutes of surgical time [11].

Omental metastasis was detected in 9% endometrial cancer cases in this study, which does not comply with results of past studies (3%–8%) [10, 12, 13]. The cases with omental metastasis were classified as Stage IVB according to the FIGO 2008 staging. In cases classified as Stage IVB, prognosis is assumed to be poor; one study reported a median overall survival of 24 months for such cases [14]. Although the therapeutic significance of omentectomy is thus unclear based on previous studies. With regards to the poor prognosis, confirmation of the presence of omental metastasis in clinical Stage I is very important.

There was a prospective analysis for the investigation of omental metastasis in clinical Stage I [13] and a retrospective study on the limited histological type of endometrioid adenocarcinoma [11]. These reports demonstrated that there remains the possibility that some omental metastasis could not be detected in those studies although omental metastasis existed.

Previous studies discussed predictive factors for omental metastasis. Chen et al. [13] reported that following pathological factors revealed significant correlation with omental metastasis: serous adenocarcinoma, adnexal metastasis, peritoneal dissemination around the Douglas’ pouch, lymph node metastasis, and endometrioid adenocarcinoma G3. Dilek et al. [12] reported that adnexal metastasis, lymph node metastasis, and deeper myometrial invasion were correlated, and Fujiwara et al. [10] and Metindir et al. [11] reported that positive peritoneal cytology revealed significant correlation with omental metastasis. Other previous reports had discussed these predictive factors, but the significance of this correlation does require further research. In the present study, following pathological factors did not contradict findings of previous studies: non-endometrioid histological type, macroscopic peritoneal dissemination, and adnexal metastasis.

In the present study, sensitivity was higher in cases with a non-endometrioid histological type, specificity was higher in cases with macroscopic peritoneal dissemination and adnexal metastasis, and all nine cases with omental metastasis involved one of the three pathological factors. If we consider these three factors in preoperative and intraoperative stages, omentectomy should be performed in order not to miss omental metastasis.

In the present study, omental metastases were detected in about 1/3 cases with serous adenocarcinoma, and peritoneal dissemination was also often detected in cases with serous adenocarcinoma. Slomovitz et al. [15] reported that extraterine spread was detected in 38% of 32 serous adenocarcinoma cases without myometrial invasion (endometrial intraepithelial carcinoma, EIC). On the basis of these findings, omentectomy should be performed in cases with presumed serous adenocarcinoma following preoperative endometrial biopsy.

In Case 1 from the present study, no peritoneal dissemination was suspected on preoperative CT, MRI, and intraoperative observation (Table 2). Moreover, no myometrial invasion, LVSI, adnexal metastasis, or lymph node metastasis was detected on postoperative pathological diagnosis, although microscopic omental metastasis was detected. In this case, the indication for omentectomy was based on the presence of non-endometrioid histological type—serous adenocarcinoma. If omentectomy had not been performed in this case, this patient would have been classified as Stage IA (i.e., early cancer). In our study, all cases had positive peritoneal cytology findings; however, no previous studies
have discussed the determination of surgical procedure based on rapid intraoperative peritoneal cytology. Further studies on the accuracy of rapid intraoperative peritoneal cytology and the relationship between cytology and determination of surgical procedure are needed.

Conclusion

During the surgical therapy for endometrial cancer, omentectomy was shown to be beneficial in the exact surgical staging in cases with non-endometrioid histological type cancers, adnexal metastasis, and macroscopic peritoneal dissemination.

Acknowledgements

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References


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The opinion of gynecologists on the management of early-stage, high-grade endometrioid endometrial cancer

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Summary

Purpose of investigation: There is no consensus on the management of Stage I endometrioid endometrial cancer (EEC) with grade 3 histology. This study evaluates the opinion of gynecologists in The Netherlands on the management of Stage I grade 3 EEC. Materials and Methods: Members of the Dutch Gynecologic Oncology Working Group were requested to complete a digital questionnaire on the management of Stage I, grade 3 EEC. Actual treatment of patients with Stage I, grade 3 EEC was assessed by analysis of PALGA, the Dutch Pathology Registry. Results: Most gynecologists prefer routine lymphadenectomy or complete staging (62.3%), while these were actually performed in 27.3% of the cases. Gynecologic oncologists are more likely to perform a lymphadenectomy than general gynecologists. There was a wide variation of clinical practice. Conclusion: The results of this study underline the need for additional research into management of Stage I, grade 3 EEC as well as the need for conclusive guidelines.

Key words: Endometrioid histology; Endometrial cancer; Grade 3; Questionnaire; Lymphadenectomy; Guidelines.

Introduction

Cancer of the uterine corpus is the most common gynecologic malignancy in the Western world, with incidence and mortality rates of respectively, 1,913 and 484 in The Netherlands in 2011 [1]. Most patients present with endometrioid endometrial cancer (EEC) at an early stage, and have a favorable prognosis. Standard treatment of clinical Stage I EEC consists of hysterectomy and bilateral salpingo-oophorectomy. Based on the PORTEC trials, in The Netherlands, vaginal brachytherapy (VBT) is recommended when two out of three risk factors (age ≥ 60 years, myometrial invasion (MI) ≥ 50%, and grade 3 histology) are present, and external beam radiotherapy (EBRT) is recommended when all three are present [2-4].

The Dutch guidelines on the treatment of endometrial cancer were revised in 2010 on several aspects as shown in Table 1 [4]. Most important were recommendations to consider more extensive diagnostics in high-risk patients, a shift from pelvic and para-aortic lymphadenectomy to lymph node sampling, and the consideration of chemotherapy in high-risk patients. However, in the revised guideline, the management of patients with clinical Stage I, grade 3 EEC is still left to the clinicians’ opinion.

The primary aim of this study was to analyze the opinion of gynecologists in The Netherlands on the management of clinical Stage I, grade 3 EEC and to assess differences between gynecologists with different levels of oncologic training. Furthermore, these opinions were compared to the recommendations of the revised guidelines and the actual treatment of patients with Stage I, grade 3 EEC in The Netherlands.

Materials and Methods

Gynecologic cancer care in The Netherlands is organized by regional hospitals around tertiary referral centers. Gynecologic oncologists are trained as fellows and work in referral centers. Patients with endometrial cancer can be treated by both general gynecologists and gynecologic oncologists and are referred on indication. The Dutch Gynecologic Working Group is a division of the Dutch Society of Obstetrics and Gynecology and both gynecologic oncologists and general gynecologists can become a member.

A digital questionnaire was spread among all (around 200) members of the Dutch Gynecologic Oncology Working Group. Questions are related to the following, fictive case: a 55-year-old patient is diagnosed with poorly differentiated (grade 3), clinical Stage I, endometrioid endometrial cancer by either pipelle or dilatation and curettage. Medical history does not obstruct surgery. Questions (shown in Table 2) ranged from the diagnostic work-up to the primary and adjuvant therapy. After every section, there was space for comments (questions 6, 16, and 19).

To assess actual practice of performing lymphadenectomies, the Dutch nationwide network and registry of histo- and cytology (PALGA) was consulted [5]. All patients diagnosed pre-operatively with grade 3 EEC between July 2009 and June 2010 were selected. In the pathology report, it was mentioned
whether lymph nodes were received postoperatively and what their origin was. Only completely documented cases were eligible for inclusion. As clinical FIGO Stage is not registered in this database, this search contained patients suspected of all stages EEC.

IBM SPSS 20 was used for statistical analysis. The difference in answers between gynecologic oncologists and general gynecologists was calculated using the Pearson’s chi square, the Fisher’s exact test, and bivariate regression analysis. Results were considered significant at a \( p \) value ≤ 0.05. Further analysis of the data was observational.

**Results**

A total of 61 out of 200 gynecologists returned the questionnaire, leading to a response rate of 30%. All answers are shown in Table 2. Half of the responders (n=31) were gynecologic oncologists. There was no consensus among responders concerning the need for abdominal CT-scan and CA-125 measurement. With respect to the primary treatment, 37.7% would omit routine lymphadenectomy (n=21), or recommend sampling of suspicious lymph nodes only (n=2). The majority of the responders, 62.3% (n=38) was in favor of more extensive surgical staging. The age of the patient did not influence the opinion on initial management for most responders. In case of patients with grade 3 histology and MI ≥ 50%, external beam radiotherapy is advised. Chemotherapy is not advised.

**Table 1. — Summary of the old and the new Dutch guideline for Stage I endometrial cancer.**

<table>
<thead>
<tr>
<th>Diagnostics</th>
<th>Old</th>
<th>New [4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>History taking, examination, transvaginal ultrasound, and chest radiography.</td>
<td>History taking, examination, transvaginal ultrasound, and chest radiography.</td>
<td>CA-125 when extra-uterine disease is suspected.</td>
</tr>
<tr>
<td>CA-125 when extra-uterine disease is suspected.</td>
<td>CA-125 when extra-uterine disease is suspected.</td>
<td>CT-scan or MRI can be considered in patients with grade 3 histology.</td>
</tr>
<tr>
<td>No CT-scan or MRI.</td>
<td>No CT-scan or MRI.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Surgery</th>
<th>Old</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hysterectomy and bilateral salpingo-oophorectomy.</td>
<td>Hysterectomy and bilateral salpingo-oophorectomy.</td>
<td></td>
</tr>
<tr>
<td>A lymphadenectomy might be considered in patients with grade 3 histology.</td>
<td>Sampling of suspicious nodes.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adjuvant Therapy</th>
<th>Old</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>When ≥ 2 risk factors* are present, vaginal brachytherapy is advised. If there is both grade 3 histology and MI ≥ 50%, external beam radiotherapy is advised.</td>
<td>When ≥ 2 risk factors* are present, vaginal brachytherapy is advised.</td>
<td></td>
</tr>
</tbody>
</table>

| * Risk factors: age ≥ 60 years, grade 3 histology and myometrial invasion (MI) ≥ 50%. |

Results

It was commented that an abdominal CT-scan could be helpful in diagnosing metastases (n=6) and nodal disease (n=5). Nine responders recommended abdominal CT only in case of an elevated CA-125 or in combination with CA-125 measurement. Three responders preferred MRI for assessing myometrial invasion. There were no comments concerning primary surgery. Three responders found it impossible to decide on adjuvant treatment without knowing the extent of myometrial invasion.

Seventy patients retrieved by the PALGA search were treated for grade 3 EEC between July 2009 and June 2010, and the database contained complete information on primary surgical treatment in 55 (79%). In total, 14 patients (25.5%) underwent a lymphadenectomy. Nine (16.4%) underwent a pelvic lymphadenectomy and five (9.1%) both a pelvic and para-aortal lymphadenectomy. Positive nodes were found in three of these 14 cases (23%).

**Discussion**

This study shows that there is no agreement in The Netherlands concerning the diagnostic work-up and primary and adjuvant treatment for patients with Stage I, grade 3 EEC, where the guideline is not strictly directive. Compared to general gynecologists, gynecologic oncologists are much more likely to perform a systematic lymphadenectomy in this group of patients.

Literature concerning the diagnostic work-up of clinical Stage I, grade 3 EEC is inconclusive and the guideline therefore states that CA-125 measurement and CT or MRI may be considered [6]. This is represented in the questionnaire, with no agreement concerning necessity of a CT-scan and CA-125 measurement.

Interestingly, the guideline recommends sampling of suspicious lymph nodes, whereas the majority of gynecologists feel that a lymphadenectomy should be performed. This was significantly more among gynecologic oncologists compared to general gynecologists, which might be explained by the fact that the former have had more surgical training and are more confident to perform a lymphadenectomy. Several large studies have presented conflicting data on the role of routine lymphadenectomy in clinical Stage I EEC [7-9]. This controversy led to the revised Dutch guideline against routine lymphadenectomy, in contrast with the American guideline, which recommends complete surgical staging when grade 3 histology is present [10].
Table 2. — Questionnaire and responses.

1. What is your background?
   - Resident: 3 (4.9%)
   - Gynecologist: 27 (44.3%)
   - Gynecologic oncologist: 31 (50.8%)

2. How many years are you registered?
   - 16 (sd 8.4)

3. What would be the primary surgical treatment?
   - TAH/VH + BSO: 21 (34.4%)
   - TAH/VH + BSO + lymph node sampling: 2 (3.3%)
   - TAH/VH + BSO + pelvic lymphadenectomy: 12 (19.7%)
   - TAH/VH + BSO + pelvic and para-aortal lymphadenectomy: 23 (37.7%)
   - Total surgical staging: 3 (4.9%)

4. If the patient was 65 years old, would this change the surgical treatment?
   - No: 59 (96.7%)
   - Yes: 2 (3.3%)

5. If so, what would the primary treatment be?
   - TAH/VH + BSO: 12 (34.3%)
   - TAH/VH + BSO + pelvic lymphadenectomy: 2 (100%)
   - Total surgical staging: 11 (31.4%)

6. Would you make an abdominal CT-scan?
   - No: 26 (42.6%)
   - Yes: 35 (57.4%)

7. If so, would it change your policy?
   - No: 23 (65.7%)
   - Yes: 12 (34.3%)
   - Unknown: 1 (33.3%)

8. Would you measure CA-125?
   - No: 25 (41%)
   - Yes: 35 (57.4%)
   - Unknown: 1 (1.6%)

9. If so, would it change your policy?
   - No: 1 (6%)
   - Yes: 2 (100%)

10. Would you make an abdominal CT-scan?
    - No: 57 (93.4%)
    - Yes: 3 (4.9%)
    - Unknown: 1 (1.6%)

11. Would you measure CA-125?
    - No: 1 (33.3%)
    - Yes: 2 (66.7%)
    - Not applicable: 58

12. If so, would it change your policy?
    - No: 1 (100%)
    - Yes: 1 (33.3%)
    - Not applicable: 58

13. Would you measure CA-125?
    - No: 2 (100%)
    - Yes: 1 (33.3%)
    - Not applicable: 58

14. If so, would it change your policy?
    - No: 2 (100%)
    - Yes: 1 (33.3%)
    - Not applicable: 58

15. If there was no lymphadenectomy performed, what should the adjuvant treatment be?
    - No adjuvant therapy: -
    - Radiotherapy according to the PORTEC 1-2 trials, if applicable participation in the PORTEC 3 trial: 56 (91.8%)
    - Radiotherapy according to the PORTEC 1-2 trials and chemotherapy: 2 (3.3%)
    - Chemotherapy: -
    - Unknown: 3 (4.9%)

16. If a lymphadenectomy was conducted, all nodes were negative and there was no sign of extra-uterine disease, what should the adjuvant treatment be?
    - No adjuvant treatment: 27 (44.3%)
    - Radiotherapy according to the PORTEC 1-2 trials, if applicable participation in the PORTEC 3 trial: 33 (54.1%)
    - Unknown: 1 (1.6%)
The Dutch guideline is based, among others, on the PORTEC I and II trials concerning adjuvant therapy [2, 3]. Nevertheless, none of the responders felt that observation alone was safe enough for patients who did not undergo a lymphadenectomy, which illustrates the hesitation of gynecologists to follow the guideline by omitting a routine lymphadenectomy. A problem with the consideration of adjuvant therapy in the questionnaire (questions 17 and 18, Table 2) is the fact that the extent of myometrial invasion, required in deciding on adjuvant treatment, is not given.

When looking at the actual treatment at patients with grade 3 EEC, there was a large discrepancy between the opinion on routine lymphadenectomy and the actual amount of lymphadenectomies performed (25.5%). The total number of patients treated for grade 3 EEC as retrieved from the PALGA-registry should be considered a random sample, as it is known that not all cases are labeled systematically in this system and are therefore missed during a search. It has to be noted that the clinical stage is not recorded in the PALGA-registry.

The major strength of this study is that it compared gynecologists’ opinions, the guideline, and actual treatment. Results demonstrated that variation is low among the points of consensus, and variation was large among the points of doubt in the management of Stage I, grade 3 EEC. Moreover, it showed that while gynecologists tend to follow the guidelines on routine lymphadenectomy, it is not the optimal treatment in their opinion. Many studies have looked at either endometrioid endometrial cancer with good prognosis or non-endometrioid endometrial cancer with poor prognosis, leading to comprehensive guidelines. For Stage I, grade 3 EEC, however, the guidelines remain inconclusive. In a time of centralization and standardization of care, the finding that gynecologists tend to comply with the guidelines will hopefully lead to more research into Stage I, grade 3 EEC and subsequently to comprehensive guidelines.

It is known from literature that the response rates of questionnaires among physicians, and especially gynecologists, is very poor, even when an incentive is used [11]. The major limitation of this study was the low response rate. However, while the authors did not have the funding to provide an incentive, their response rate is comparable to that of a questionnaire with an incentive. Another weakness of this study may be the fact that the myometrial invasion was not given in the case description, as pointed out by several responders. Despite these points, the authors feel that the current data support the sense of urgency to find consensus on the management of Stage I, grade 3 EEC.

Conclusion

In conclusion, the current study illustrates that gynecologists in The Netherlands adhere well to the guidelines concerning the management of clinical Stage I, grade 3 EEC. However, with respect to the lymphadenectomy there is a discrepancy between: 1) the opinion and the guideline, 2) the opinion of gynecologic oncologists and general gynecologists, and 3) the opinion and the actual performed lymphadenectomy in grade 3 EEC. Moreover, there is little agreement on decisions left to the discretion of the gynecologist. Although the actual treatment is in line with the guideline, these data show the need for agreement on the management of patients with clinical Stage I grade 3 EEC.

References

Serum human epididymis protein 4 can be a useful tumor marker in the differential diagnosis of adnexal masses during pregnancy: a pilot study

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Summary

Purpose: The purpose of this study was to evaluate serum concentrations of human epididymis protein 4 (HE4) and cancer antigen 125 (CA 125) in healthy women and their pregnant counterparts to determine the influence of pregnancy on these biomarkers. Materials and Methods: Serum concentrations of CA 125 and HE4 were measured in 27 healthy non-pregnant women and 26 healthy pregnant women in the first and second trimesters. Results: Higher concentration of CA 125 was found in pregnant than in non-pregnant women (p = 0.002). There was no difference in CA 125 concentrations between first and second trimesters (p = 0.13). Serum HE4 concentration was not different in pregnant group compared to non-pregnant women (p = 0.510). Likewise, no difference was found in HE4 levels between the trimesters (p = 0.485). There was a positive correlation between increasing parity and CA 125 (p = 0.023), but not HE4 (p = 1.0). Conclusion: HE4 serum biomarker is unaffected by pregnancy status and may be useful for evaluation of doubtful pelvic masses in pregnancy. Contrarily, increased serum levels of CA 125 could yield increased number of false-positive results.

Key words: Cancer antigen 125; Human epididymis protein 4; Tumor markers in pregnancy.

Introduction

Advances in the use of ultrasound for assessing pregnancy have led to an increase in the detection of adnexal masses in gravid women [1]. As the adnexal masses are detected at asymptomatic stage, the outcomes of these lesions have improved. Predictably, symptomatic or persistent masses into late gestation are usually associated with a higher rate of complications (torsion 1-22%, rupture 0-9%, obstruction of labor 2-17%) [2-4], and malignancy [3, 5] than those observed asymptotically in early gestation [6, 7]. Management strategies for ovarian masses in pregnancy have not been well defined. Ultrasound assessment of masses can help to determine the risk of malignancy and guide the surgical management. In doubtful pelvic masses, it is necessary to utilize an approved biomarker for better characterization of the masses, but alterations in the levels of tumor biomarkers in pregnancy can render them useless in this period. Cancer antigen 125 (CA 125) was proposed as a serum biomarker for ovarian cancer in 1983 and approved for routine management of this disease. However, a major problem with CA 125 is its low diagnostic specificity. High concentrations found in benign gynecological conditions, particularly in premenopausal patients such as ovarian cysts, myomas, endometriosis, and non-gynecological conditions including effusions, liver or renal disease and also malignant diseases [8, 9]. Likewise, concentration of CA 125 alters during menstrual phase and pregnancy [10, 11]. Human epididymis protein 4 (HE4) has been proposed as a novel tumor marker to increase the diagnostic specificity of early stage ovarian cancer. United States Food and Drug Agency (FDA) approved the clinical use of HE4 in the monitoring of epithelial ovarian cancer in 2009. The aim of this study was to evaluate serum concentrations of HE4 and CA 125 in healthy patients and in their pregnant counterparts to determine the influence of pregnancy on the concentrations of these serum biomarkers and the reliability of HE4 as a tumor marker in pregnant population.

Materials and Methods

The authors enrolled 30 healthy pregnant women who admitted for antenatal follow up at the present department as a study group and 30 age-matched healthy non-pregnant women as a control group. Written informed consent was obtained from all participants and institutional ethics committee approved the study. All participants were subjected to an ultrasonographic examination to rule out the presence of any adnexal masses. Serum samples were obtained twice from the pregnant group: once at admission in the first trimester and once in the second trimester. Serum concentrations of CA 125 and HE4 were measured using commercial kits (Beckman Coulter, Fullerton, CA, USA and Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. All statistical analyses were performed using SPSS software (version 17.0). The results were expressed as mean ± standard deviation. The differences between the groups were evaluated using the unpaired Student’s t-test. Correlation analysis was performed using the Pearson correlation coefficient. The level of significance was set at p < 0.05.
trimester and again at the second trimester follow-up. Samples were obtained once from the control group when they attended the gynecology department for an annual check-up. Exclusion criteria were multifetal pregnancy and the existence of any systemic, gynecological, or non-gynecological disease that could elevate the serum CA 125 concentrations. Two women in the pregnant study group were excluded due to miscarriage, and two were excluded because they were lost to follow up in the second trimester. Three participants in the control group were excluded due to hemolyzed blood samples. The final study comprised 26 healthy pregnant women in the study group and 27 healthy nonpregnant women in the control group.

The blood samples were obtained by venous puncture in the present hospital, centrifuged, and stored at -80°C until assayed. The serum levels of CA 125 and HE4 were determined with a chemiluminescent enzyme immunoassay and an ELISA immunoassay, respectively. This solid-phase noncompetitive immunoassay was based on a direct sandwich technique using two mouse monoclonal antibodies (2H5 and 3D8) directed against two epitopes in the C-WFDC domain of HE4.

Data are reported herein as the median-min-max. All statistical analyses were performed using non-parametric tests (Wilcoxon’s signed rank test, Mann–Whitney U-test). Correlations were evaluated with Spearman’s rank correlation coefficients. Analyses were performed using SPSS software, version 9.0 for Windows (SPSS). The level of statistical significance was set at $p < 0.05$.

## Results

This study included 79 serum samples from a study group of 26 healthy pregnant women with a median age of 28 years (18–37) and a control group of 27 healthy nonpregnant women with a median age of 26.5 years (18–39) ($p = 0.873$). The median parity in the study and the control group was 1.0 and 1.5, respectively ($p = 0.011$) (Table 1).

The results of the CA 125 measurements in the study and control groups are shown in Table 2. Higher serum concentration of CA 125 was found in the pregnant women than in the controls, while the levels were not statistically different between the first and second trimesters ($p = 0.13$).

The results of the HE4 measurements in the study and control groups are shown in Table 3. No statistical significant difference in the concentration of HE4 between the first and second trimesters ($p = 0.485$).
ence was observed in sera HE4 levels between pregnant and non-pregnant groups ($p = 0.510$). Likewise, there was no statistically significant difference in HE4 levels between the first and second trimester of pregnancy ($p = 0.485$). Figure 1 displays a scatterplot of the serum CA 125 and HE4 levels for all subjects.

The serum concentrations of CA 125 and HE4 were compared with parity in all groups. A statistically significant elevation in the CA 125 concentration was found with increasing parity ($R = 0.31$, $p = 0.023$), but there was no relationship between the levels of HE4 and parity ($R = 0.00$, $p = 1.0$).

Discussion

The management strategy of ovarian masses in pregnancy is an unresolved issue among obstetricians. Although some propose elective removal in the second trimester, others argue that a conservative approach results in spontaneous resolution of most masses, which might provoke unnecessary surgery. Tumor markers, such as CA 125, have a restricted role in the discrimination of benign versus malignant lesions due to increased levels in pregnant sera [6, 11]. This rise in the levels of CA 125 begins 30–40 days after the last menstrual period, peaks between 35–60 days, and starts to decrease by the end of the first trimester [12]. The present results confirmed the elevation in serum CA 125 concentrations in pregnancy, but the authors found no difference between the first and second trimester. Another limitation of using CA 125 as a biomarker in the discrimination of adnexal masses in pregnancy is that up to 20% of ovarian cancers and almost 50% of early-stage disease do not express this antigen [8, 13, 14]. Surgical findings support that, the majority of ovarian cancers during pregnancy are diagnosed as Stage 1, with the disease confined to the ovaries [7, 15]. Therefore, it is necessary to combine CA 125 with novel markers that can provide better diagnostic efficiency.

Schummer et al. established that the HE4 gene, also known as WDFC2, is primarily overexpressed in patients with ovarian carcinomas [16]. This finding was later confirmed by gene-expression profiling studies [17, 18]. Furthermore, HE4 has a relatively subtype-specific expression pattern primarily restricted to the serous and endometrioid subclasses of epithelial ovarian carcinomas [19, 20]. Nonetheless, high or moderate HE4 expression can also be detected in adenocarcinomas of the lung, breast, transitional cell, endometrial and pancreatic carcinomas, but ovarian serous carcinomas have the highest expression [21]. Hellstrom et al. concluded that HE4 was less frequently positive in benign gynecological disease and may be more beneficial than CA125 [22]. Further studies showed that HE4 had the highest sensitivity (83%) as a single marker for ovarian cancer detection in patients with pelvic masses, particularly in those with early-stage disease [23]. Most serum HE4 studies have suggested that the sensitivity and the specificity of HE4 in gynecological diseases are better than those of CA 125 and that both tumor markers are complementary [9, 24]. Moore et al. proposed that the concentration of HE4 is lower in pregnant women compared with their premenopausal counterparts and that this was attributed to increased renal clearance in pregnancy [25]. They also reported that levels of HE4 did not change related with different trimesters of pregnancy. Consistent with their reports, a comparison of HE4 in the first and second trimesters revealed no statistical difference in the current study. This study also showed no significant difference in the serum concentration of HE4 in pregnancy, but elevated levels of CA125 when compared with nonpregnant controls. According to the literature, the elevation in CA125 in pregnancy occurs predominantly during the first trimester, probably because of its role in early fetal development [26, 27]. However, several studies also reported that this elevation persists throughout pregnancy [11, 28].

The present results revealed a positive correlation between increasing parity and CA 125 ($R = 0.31$, $p = 0.023$) but not HE4. There are several studies indicating elevated, decreased, or unaltered concentrations of CA 125 with increasing parity [29, 30]. One limitation of the present study was that the results are based on a small number of study subjects and controls. Large multicentric trials should be advocated to confirm these results. The findings of the current study suggest that HE4 is a credible marker, which does not fluctuate in pregnancy, and that it may be useful for the evaluation of ovarian cysts and doubtful pelvic masses in pregnancy. Contrarily, increased CA125 serum concentrations could yield an increased number of false-positive results.

References


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Prevalence and predictors of abnormal Papanicolaou smears in HIV-infected women

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Summary
Purpose of investigation: To characterize the risk factors for abnormal cervical cytology among women with human immunodeficiency virus (HIV), and to determine the relationship between antiretroviral therapy (ART) and cytology results. Materials and Methods: Retrospective study of clinical data of 115 HIV-infected women between January 2008 and December 2011. Analysis of cervical smears history, as well as, epidemiologic, medical, and sexual factors, administration of ART, CD4 cells count, and HIV viral load were performed. Results: Mean age was 35.9 ± 6.5 years. Average time of HIV infection was 10.5 ± 4.5 years. HPV infection prevalence was 37.4%, the majority was high-risk. An abnormal Papanicolaou smear was found in 43.5%. Atypical squamous cell of undetermined significance (ASC-US) was reported in 7.8%, low-grade squamous intraepithelial lesions (LSIL) in 32.2%, and high-grade squamous intraepithelial lesions (HSIL) in 3.5%. HPV infection was the only statistical predictor of abnormal cytology (p < 0.001; OR = 0.042). ART, CD4 cells count, and HIV viral load did not correlate to regression of abnormal cytology. Conclusion: These women should be followed-up according to current cervical cancer screening guidelines, independently of the therapy, CD4 cells count, and HIV viral load.

Key words: HIV-infected women; Cytology; Human papillomavirus (HPV); Predictors.

Introduction
Invasive cervical cancer and its precursors are the most significant gynecologic manifestations of human immunodeficiency virus (HIV) infection. Innumerous reports [1-4] suggest that HIV-infected women have higher incidence of squamous intraepithelial lesions (SIL) than the general population, with an increased risk of developing invasive cervical neoplasia. The contribution of immunosuppression and other risk factors to the increased incidence of SIL in HIV-infected women remains controversial. Antiretroviral therapy (ART), especially highly active antiretroviral therapy (HAART), undoubtedly lowered the incidence of innumerous opportunistic diseases related to HIV infection, such as Kaposi’s sarcoma and non-Hodgkin lymphoma. However, its impact on the incidence of SIL and cervical cancer has not been yet clarified [5-8].

The objectives of this study were to evaluate the prevalence of human papillomavirus (HPV) infection and characterize possible risk factors for abnormal cervical cytology among women with HIV infection, and to determine the potential relationship between antiretroviral therapy and cytology results.

Materials and Methods
The present study was approved by the Ethical Committee of the Coimbra Hospital and University Center, a tertiary care hospital with a Cervical-Vulvar Unit. The authors conducted a retrospective analysis of HIV-infected women in their Outpatient Clinic. These patients were commonly referred to the unit by the Infectious Disease Specialist who followed them. The authors analyzed the data of 131 patients (16 were excluded because of incomplete data), between January 2008 and December 2011, including cervical smear history as well as epidemiologic (age, years of HIV infection, race, smoking habits, drug abuse), medical (mode of transmission of HIV, other infections like hepatitis B or C), and sexual factors (age at first intercourse, number of sexual partners, history of prostitution, and HPV infection), administration of ART, CD4 cells count, and HIV viral load.

In the present clinical protocol, all HIV-infected women were advised to have a Papanicolaou smear taken every year. All smears were taken by gynecologists and ThinPrep technology was used systematically. They were processed in the pathology department and classified based on the Bethesda System guidelines. According to the present protocol, colposcopy was offered to all women with cervical cell abnormalities. If indicated, lesions were further evaluated by biopsy. Definitive treatment was provided as indicated.

In order to analyze progression or regression of the cytological abnormalities, the authors considered two consecutive Papanicolaou smears with a time interval not inferior to one year. For this study they analyzed 83 patients who met the criteria. Regression was considered when the second cytology (C2; follow-up) had lower-grade abnormalities than the first (C1; baseline); no change, when both had the same classification; progression, when the second cytology had higher-grade abnormalities than the first.

Women were asked if they were taking ART and also to specify the medication. CD4 cells count and HIV viral load were taken into consideration, if done two months earlier, or after the clinical examination and Papanicolaou smear.
Table 1. — Sociodemographic characteristics and HIV progression markers for abnormal cytological results.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Frequency (%)</th>
<th>NILM</th>
<th>Abnormal</th>
<th>p value</th>
</tr>
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<tr>
<td>Race</td>
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<td>40 (42.1)</td>
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<td>8 (44.4)</td>
<td>10 (55.6)</td>
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<tr>
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<td>2 (100)</td>
<td>0</td>
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<td>Age of first intercourse</td>
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<td></td>
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<td>12-15 years</td>
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<td>17 (60.7)</td>
<td>11 (39.3)</td>
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<td>16-18 years</td>
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<td>29 (51.8)</td>
<td>27 (48.2)</td>
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<td>&gt; 18 years</td>
<td>31 (27)</td>
<td>19 (61.3)</td>
<td>12 (38.7)</td>
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<td>Number of sexual partners</td>
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<td>18 (66.7)</td>
<td>9 (33.3)</td>
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<td>2-5</td>
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<td>30 (52.6)</td>
<td>27 (47.4)</td>
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<td>12 (45.2)</td>
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<td></td>
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<td>35 (43.2)</td>
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<td>5 (83.3)</td>
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<td>1 (20)</td>
<td></td>
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<td>12 (52.2)</td>
<td>11 (47.8)</td>
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<tr>
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<td>56 (56.6)</td>
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<td>1 (33.3)</td>
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<td>4 (57.1)</td>
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<td>2 (40)</td>
<td>3 (60)</td>
<td></td>
</tr>
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<td>No</td>
<td>110 (95.7)</td>
<td>63 (57.3)</td>
<td>47 (42.7)</td>
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<td>22 (19.1)</td>
<td>10 (45.5)</td>
<td>12 (54.5)</td>
<td></td>
</tr>
<tr>
<td>No</td>
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<td>55 (59.1)</td>
<td>9 (40.9)</td>
<td></td>
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<td>Abnormal</td>
<td></td>
</tr>
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<td>22 (19.1)</td>
<td>11 (50)</td>
<td>11 (50)</td>
<td></td>
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<tr>
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<td>93 (80.9)</td>
<td>54 (58.1)</td>
<td>39 (41.9)</td>
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</tr>
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<td>Abnormal</td>
<td>&lt;0.001</td>
</tr>
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<td>36 (46.8)</td>
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<td>38 (33)</td>
<td>24 (63.2)</td>
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<td>Abnormal</td>
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<td>4 (80)</td>
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<td>18 (58.1)</td>
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<td>&gt;350</td>
<td>79 (68.7)</td>
<td>46 (58.2)</td>
<td>33 (41.8)</td>
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</table>

Statistical analysis was performed using SPSS v.20.0. Univariate analysis was used to determine which variables were associated with abnormal cytology. Chi-square tests were used to test categorical variables. Variables at least marginally associated with abnormal cytology (p < 0.15) in univariate analyses were further investigated in multivariate logistic regression models. Analyzing the statistical significance of the predictors in the regression models, the reference value of p < 0.05 was adopted. With a sample of 115 patients and considering an α level of 0.05, the power of the study to detect a medium W effect size of 0.30 was 78% for contingency tables with three degrees of freedom, 83% for contingency tables with two degrees of freedom, and 90% for contingency tables with one degree of freedom.Odds ratio (OR) and 95% confidence intervals (CI) were also estimated.

Results

A total of 115 HIV-infected patients were analyzed. Table 1 shows epidemiologic data, risk factors, and correlates them to different cytological results. In this population, 43.5% had abnormal Papanicolaou smears. An atypical squamous cell of undetermined significance (ASC-US) was reported in 7.8%, low-grade squamous intraepithelial lesions (LSIL) in 32.2% and high-grade squamous intraepithelial lesions (HSIL) in 3.5%. Negative cytological results for intraepithelial lesions (NILM) were found in 56.5%. The prevalence of HPV infection was 37.4%.

Mean age of the patient population was 36.98 ± 6.52 years. Mean time of infection by HIV was 10.48 ± 4.47 years. There was no significant difference in age, race, number of sexual partners, mode of HIV transmission, history of prostitution, smoking habits, and the cytological results. Other infections and drug abuse were marginally associated with abnormal cytology (p = 0.08 and 0.13, respectively). Only HPV infection could be considered a statistical predictor of abnormal cytology (p < 0.001, OR = 0.042) and a risk factor. Women with a combination of three risk factors – other infections, HPV infection, and drug abuse – were 35% more likely to have abnormal cytology.

Table 2 shows the changes in cervical cytology status in consecutive Papanicolaou smears. Considering NILM, 26.1% had progression but the majority (73.9%) was stable. On the other hand, the majority of LSIL did not change and persisted. The only two cases of HSIL showed regression, because the second cytology was performed after treatment (the two biopsies revealed cervical intraepithelial neoplasia (CIN) 3 and a conization were carried out).
Prevalence and predictors of abnormal Papanicolaou smears in HIV-infected women

Tables 3, 4, and 5 show the association between ART, CD4 cells count and HIV viral load, and changes in cervical cytological status, respectively. About 44.8% of women that were not on ART at baseline began treatment. The majority of women were under ART, had CD4 cells count superior to 200/mm³ and almost negative viral load. The present authors found no differences in regression or progression of the cytological results when the three conditions were analyzed in isolation or combined (Table 6).

Discussion

The prevalence of abnormal Pap smears in HIV infected women was 43.5% in the present study, which is similar to many others described in the literature [2-4]. The prevalence of HPV infection in this study (37.4%) was not as high as others have described, mainly Heard et al. [9] who found a prevalence of HPV infection in European women with HIV infection near 49.5% (46.3-52.8%). However, the prevalence of HPV infection in the present study was clearly associated with abnormal cytological results and could be considered a statistical predictor of disease and a risk factor, which is in agreement with other reports in the literature [2, 3].

The present study showed, in agreement with others [2, 3], that in addition to HPV and HIV infection, no other characteristics usually associated with abnormal Papanicolaou smears abnormalities, such as early intercourse, smoking habits, number of sexual partners, and sexually transmitted infection, were significant.

The prevalence of HSIL was 3.5% which corresponds to only two cases. After the abnormal Pap smear and a colposcopy suggesting a high-grade lesion, a biopsy was performed and the histology revealed CIN 3. A conization was offered in both cases and the histology revealed a CIN 3 with free margins. Follow-up with Papanicolaou smear and colposcopic examination showed regression of the cytological abnormalities. No case of cervical cancer was found in the present study. The low incidence of high-grade cytological abnormalities and absence of cervical cancer could be explained by a highly cohort of women with great compliance to cervical cancer screening and intervention.

A synergistic action of HIV-induced immunosuppression and HPV infection may actually favor the onset of precancerous cervical lesions. HAART regimens have dramatically improved HIV prognosis. However, while systemic benefits are unquestionable, the beneficial actions that it may exhibit locally, on the cervix, are still unclear [6].

Table 3. — Antiretroviral therapy and changes in cervical cytological status.

<table>
<thead>
<tr>
<th>Cytological result</th>
<th>Antiretroviral therapy</th>
<th>Comparison between C1 and C2</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
<td>C2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regression</td>
<td>No change</td>
<td>Progression</td>
</tr>
<tr>
<td>Abnormal</td>
<td>On ART</td>
<td>10 (38.5)</td>
<td>15 (57.7)</td>
</tr>
<tr>
<td></td>
<td>Off ART</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Normal</td>
<td>On ART</td>
<td>1 (20)</td>
<td>4 (80)</td>
</tr>
<tr>
<td></td>
<td>Off ART</td>
<td>3 (60)</td>
<td>2 (40)</td>
</tr>
</tbody>
</table>

Legend:

C1 and C2: first and second cytology;
time interval between C1 and C2 not inferior to one year;
regression: C2 < C1; no change: C1 = C2; progression: C2 > C1.

Table 4. — HIV viral load and changes in cervical cytological status.

<table>
<thead>
<tr>
<th>HIV viral load (number of copies/mL)</th>
<th>Comparison between C1 and C2</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regression</td>
<td>No change</td>
</tr>
<tr>
<td>C1 &lt;50</td>
<td>14 (63.6)</td>
<td>26 (54.2)</td>
</tr>
<tr>
<td>50-10³</td>
<td>4 (18.2)</td>
<td>13 (27.1)</td>
</tr>
<tr>
<td>10³-10⁶</td>
<td>4 (18.2)</td>
<td>9 (18.7)</td>
</tr>
<tr>
<td>C2 &lt;50</td>
<td>17 (77.3)</td>
<td>34 (70.8)</td>
</tr>
<tr>
<td>50-10³</td>
<td>3 (13.6)</td>
<td>7 (14.6)</td>
</tr>
<tr>
<td>10³-10⁶</td>
<td>2 (9.1)</td>
<td>7 (14.6)</td>
</tr>
</tbody>
</table>

Legend:

C1 and C2: first and second cytology;
time interval between C1 and C2 not inferior to one year;
regression: C2 < C1; no change: C1 = C2; progression: C2 > C1.

Table 5. — HIV viral load and changes in cervical cytological status.

<table>
<thead>
<tr>
<th>CD4 cells count (cells/mm³)</th>
<th>Comparison between C1 and C2</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regression</td>
<td>No change</td>
</tr>
<tr>
<td>C1 &lt;200</td>
<td>1 (4.5)</td>
<td>3 (6.3)</td>
</tr>
<tr>
<td>200-350</td>
<td>7 (31.8)</td>
<td>11 (22.9)</td>
</tr>
<tr>
<td>&gt;350</td>
<td>14 (63.6)</td>
<td>34 (70.8)</td>
</tr>
<tr>
<td>C2 &lt;200</td>
<td>1 (4.5)</td>
<td>3 (6.3)</td>
</tr>
<tr>
<td>200-350</td>
<td>3 (13.6)</td>
<td>5 (10.4)</td>
</tr>
<tr>
<td>&gt;350</td>
<td>18 (81.8)</td>
<td>40 (83.3)</td>
</tr>
</tbody>
</table>

Legend:

C1 and C2: first and second cytology;
time interval between C1 and C2 not inferior to one year;
regression: C2 < C1; no change: C1 = C2; progression: C2 > C1.

Table 6. — Adjusted odds ratio and significance of antiretroviral therapy, CD4 cells count and HIV viral load in regression or progression of the cytological results.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>p value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiretroviral therapy</td>
<td>0.999</td>
<td>1.009</td>
</tr>
<tr>
<td>CD4 cells count</td>
<td>1.169</td>
<td>1.268</td>
</tr>
<tr>
<td>HIV viral load</td>
<td>0.464</td>
<td>0.025</td>
</tr>
<tr>
<td>Antiretroviral therapy * CD4 cells count * HIV viral load</td>
<td>0.999</td>
<td></td>
</tr>
</tbody>
</table>

Legend:

Cox & Snell Nagelkerke R² = 0.09 R² = 0.14
cent studies [7- 8, 10-11] have indicate that HAART may show a beneficial effect on cervical lesions but this evidence is still under debate. In the present study, the majority of women were under retroviral therapy but not necessarily HAART.

Although the most used therapy to treat HIV infection is HAART, a proportion of the present study population was receiving an older combination of antiretroviral drugs because of their long history of disease. Moreover, most of the present patient population had CD4 cells count superior to 200/mm³ and negative viral load despite the antiretroviral therapy. Furthermore, the authors found no differences in regression or progression of the cytological results when analyzed for antiretroviral therapy, CD4 cells count, and HIV viral load. In agreement with Soncini et al. [12], the actions of HIV infection and antiretroviral therapy on the cervix are not clear. On the other hand, some publications clearly showed that antiretroviral therapy, such as HAART, induces high levels of CD4 cells count and negative HIV viral load influencing the regression of abnormal cytological results [13, 14], and even decreasing HPV prevalence [15].

The present study had several limitations. It was a retrospective analysis of clinical data of patients followed in the Outpatient Clinic with no control group. The present sample size (115 HIV positive patients) was too limited to draw meaningful conclusions.

Conclusion

It is clear that HIV-infected women have a higher risk of developing cervical neoplasia. After the advent of HAART, several studies were performed to increase our knowledge on the effect of this therapy on abnormal cytological results and cervical lesions. The present study and similar studies reinforce that HPV is a predictor and risk factor for cervical cellular abnormalities in HIV-infected women. Hence, HIV infected women under HAART should be followed-up according to current cervical cancer screening guidelines, independently of the therapy, CD4 cells count, and HIV viral load.

References


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Expression and significance of LRIG3 in human cervical squamous cell carcinoma

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Summary
Objective: To explore the effect of LRIG3 in the development of cervical squamous cell carcinoma. Materials and Methods: LRIG3 and epidermal growth factor receptor (EGFR) expressions were detected by immunohistochemistry. Western blot method was used to detect LRIG3 and EGFR protein expression at different time points. MTT, flow cytometry, and transwell chamber were adopted to examine the proliferation, apoptosis, and strength of invasion of Hela229 cell respectively. Results: During the process of normal cervix deteriorating into CIN and CSCC, LRIG3 expression gradually decreased, while EGFR increased. After the transfection of LRIG3 ASODN into cells, the amount of LRIG3 expression and the cell apoptosis rate both decreased gradually, EGFR protein expression and cell proliferation rate both increased (p < 0.05), other groups remained unchanged (p > 0.05). The cell migration ability in LRIG3 ASODN transfection group was stronger than that of other groups (p < 0.05). Conclusion: LRIG3 plays an important role in Hela229 cell proliferation, apoptosis, and invasiveness.

Key words: LRIG3; Cervical squamous carcinoma; Immunohistochemical method; EGFR.

Introduction
Cervical cancer is one of the most common gynecologic malignant cancers in female reproductive system with a mortality rate ranking third in the female cancer deaths. Globally, there are 370,000 new cases of this disease every year [1]. Epidermal growth factor receptor (EGRF) is a key factor in the genesis and development of cancers with a vital role in cell proliferation, apoptosis, and metastasis. Researchers also found that expression of the EGRF is often increased in cervical dysplasia and carcinoma, and the human papillomavirus (HPV) oncoproteins stimulate cell growth via the EGFR pathway [2], and changes in activity is one of the important factors leading to carcinogenesis, development and cancer metastasis [3].

LRIGs are a type of group of newly discovered genes, including three members: LRIG1, LRIG2, and LRIG3, among which LRIG1, considered to be a cancer suppressor gene, can play its negative regulation role in tumors of epithelial origin through negative feedback regulation growth factor signal [4, 5]. Given that LRIG3 is rich in leucine repeated sequences extracellularly, and its structural composition is also similar to that of LRIG1, therefore, it is believed that its functions are probably similar to that of LRIG1. There are few reports on the study of LRIG3, hence in this paper, the immunohistochemistry method was used to examine LRIG3 and EGFR protein expressions in cervical squamous cell carcinoma (CSCC), cervical intraepithelial neoplasia (CIN), and normal cervical epithelium (NCE) to explore the relationship between LRIG3 and CSCC. By using the antisense nucleotide technology, LRIG3 expressions in Hela229 cell of CSCC was inhibited, thus detecting its effect on EGFR expressions and cell proliferation and invasiveness, further exploring the role of LRIG3 in the genesis and development of CSCC. All the achievements in this paper will provide theoretical basis for molecular targeted therapy in CSCC.

Materials and Methods
Research objects
Cervical mucosa tissues of CSCC or suspected patients who were diagnosed in the First Affiliated Hospital of Zhengzhou University from January 2009 to November 2011, were collected, including tissues of 45 patients with CSCC, tissues of 20 patients with CIN, and tissues of 30 patients with NCE, who were diagnosed as the normal cervix or chronic cervicitis by operation biopsy. Specimens, fixed in 10% formalin and dehydrated conventionally, were cut into slices after paraffin embedding.

No immunosuppressive therapy, radiotherapy, and chemotherapy were given in patients before operation, all CSCC, HE sliced by the hospital pathologist, were confirmed such.

Patients’ general conditions: all patients had complete clinical and pathological data, and had signed informed consent with patients before employing their medical records. The CSCC group aged 31-73 years, with the average age 44.6±15.8 years, among which there were 21 cases with an age less than 45 and 24 cases equal or more than 45 years. With regards to tumor diameter, there...
were 29 cases with that more than four cm and 16 cases less than four cm; there were 26 patients with tumor interstitial infiltration depth \( \geq 1/2 \) of the muscle layer, and 19 patients \( < 1/2 \) of the muscle layer. According to the clinical staging criteria designated by the International Federation of Gynecology and Obstetrics (FIGO, 2000), there were 18 cases in the first stage, 17 cases in the second stage, and ten cases in the third or fourth stage. Meanwhile, there were 19 cases with lymph node metastasis and 26 cases without. CIN group aged 29-68 years with an average age of 41.5±13.6 years, among which there were 12 cases in the I-II grade and eight cases in the III grade. Influences of weight, age of the first pregnancy, pregnant times, menstrual cycle, and other factors were excluded. The ages of NCE group were 32-74 years with an average age of 48.9±11.4 years.

**Detection of LRIG3 and EGFR expressions in CSCC, CTN, and NCE groups by immunohistochemistry**

Immunohistochemical staining procedures were conducted following the manufacturer’s instructions of SP series kit.

Determination of the results: immunohistochemical positive result assumed a brown-yellow staining. LRIG3 staining area mainly concentrated in the cell membrane or in the cytoplasm, while EGFR was located in the cell membrane and/or cytoplasm. Two pathologists, who were completely unaware of any clinical and pathological data of the samples, made the decision about the results according to the uniform scoring standard and the double-blind method. A two-level scoring method was adopted to determine the samples with positive expression. The concrete scoring method was according to the percentage of the number of positive cells accounting for the number of total cells: 0 points, when the proportion of positive cells <1%; 1 point, 2%~25%; 2 points, 26%~50%; 3 points, 51%~75%; 4 points, >75%. Staining intensity scoring: 0 points, no staining of samples; 1 point, weak staining; 2 points, moderate staining; 3 points, strong staining. The total score was a multiplying result of scores of the above two methods. Samples were defined as four categories according to the scores: 0-1, negative (-); 2-4, weak positive (+); 5-8, positive (++); 9-12, and strong positive (+++).

**Detection of LRIG3 and EGFR expressions in Hela229 cell with ASODN technology**

The secondary structure of LRIG3 was stimulated by adopting RNA structure3.5. Aimed at LRIG3 mRNA, a LRIG3 ASODN sequence, a SODN sequence, and a MSODN sequence were designed, and the three sequences were all under phosphorothioate modification. The three designed LRIG3 ODN sequences were listed as follows: LRIG3 ASODN, 5’-GGTCTCCAATTCATTGTGTG-3’, LRIG3 SODN, 5’-ACACAAUGAAUUGAGACC-3’, and LRIG3 MSODN, 5’-TTAGCTTTGCTGGATGTCAG-3’. Hela229 cells to be transfected were divided into blank control group, MSODN group, SODN group, and ASODN group (experimental group), then LRIG3 ASODN, SODN, and MSODN with 250 \( \mu g/ml \) concentration were transfected into Hela229 cells, which were cultured in the six orifice plate until 24, 48, and 72 hours, meantime setting up positive control group (cells added into apoptosis agent) and negative control group (cells untreated).

1. Respectively, LRIG3 ASODN, MSODN, SODN with 250 \( \mu g/ml \) concentration were transfected into Hela229 cells, which were cultured in the six orifice plate until 24, 48, and 72 hours, meantime setting up positive control group (cells added into apoptosis agent) and negative control group (cells untreated).

2. Cells in logarithmic growth phase were shifted into 96 orifice plate. Once fully covered in the plate, the ODN was transfected into cells. Meantime, negative control and blank control were set up, each group with three multiple holes. After reaching the already setting time (24, 48, and 72 hours), each hole was added MTT for testing, OD value was evaluated under the 492 nm wavelength. Calculation of cell growth rate: cell growth rate (%) = \([A(A-\text{experimental group})-A(\text{blank group})]/(A_0-A(\text{blank group}))\times100\%\), and \(A_0\) was OD492 value in experimental group at the beginning of this experiment.

**Detection of cell apoptosis by Annexin-V-FITC/PI double staining method**

Detection of cell apoptosis was done in accordance with AnnexinV-FITC/PI kit instruction and the specific steps were as follows:

1. The secondary structure of LRIG3 was stimulated by adopting RNA structure3.5. Aimed at LRIG3 mRNA, a LRIG3 ASODN sequence, a SODN sequence, and a MSODN sequence were designed, and the three sequences were all under phosphorothioate modification. The three designed LRIG3 ODN sequences were listed as follows: LRIG3 ASODN, 5’-GGTCTCCAATTCATTGTGTG-3’, LRIG3 SODN, 5’-ACACAAUGAAUUGAGACC-3’, and LRIG3 MSODN, 5’-TTAGCTTTGCTGGATGTCAG-3’. Hela229 cells to be transfected were divided into blank control group, MSODN group, SODN group, and ASODN group (experimental group), then LRIG3 ASODN, SODN, and MSODN with 250 \( \mu g/ml \) concentration were transfected into Hela229 cells, which were cultured in the six orifice plate until 24, 48, and 72 hours, meantime setting up positive control group (cells added into apoptosis agent) and negative control group (cells untreated).

**Testing of cell invasion ability with the transwell chamber method**

Testing of cell invasion ability with the transwell chamber method was done, respectively, into Hela229 cells, which were cultured in the six orifice plate until 24, 48, and 72 hours, meantime setting up positive control group (cells added into apoptosis agent) and negative control group (cells untreated).

**Testing of cell apoptosis by Annexin-V-FITC/PI double staining method**

Detection of cell apoptosis was done in accordance with AnnexinV-FITC/PI kit instruction and the specific steps were as follows:

1. Respectively, LRIG3 ASODN, MSODN, SODN with 250 \( \mu g/ml \) concentration were transfected into Hela229 cells, which were cultured in the six orifice plate until 24, 48, and 72 hours, meantime setting up positive control group (cells added into apoptosis agent) and negative control group (cells untreated).

2. Then, cells were collected and washed. 1~5×10^6 cells were taken out to be resuspended and centrifugated at 100 rpm for five minutes. The supernatant was abandoned and 500 μl binding buffer was used to suspend cells.

3. The cell suspension was added into one μl Annexin-V-FITC and five μl propidium iodide, after which incubated at room temperature away from light for 15 minutes after mixed.

4. Cell cycle was detected by the flow cytometer with the excitation light wavelength 488 nm and the emission light wavelength more than 630 nm; 10,000 cells were tested and the operation had to be finished within 30 minutes.

5. Simultaneously, PI and Annexin V-FITC were used for the single staining of Hela229 cells, which was taken as the benchmark.

Cell Quest software was adopted to analyze the results and calculate the proportion of cell apoptosis. Determination of the results included that as follows: cell necrosis area (PI+, Annexin V-, located in the upper left region), terminal cell apoptosis area (PI-, Annexin V+, located in the upper right region), living cell area (PI-, Annexin V-, located in the lower left region), and early cell apoptosis area (PI-, Annexin V+, located in the upper right region).

**Testing of cell invasion ability with the transwell chamber method**

1. The cells were cultured in serum-free medium, hungry for 12 to 24 hours. After trypsin digestion of cells in different groups, RPMI1640 medium without fetal bovine serum was made into cell suspension. Then the cell concentration was adjusted to 5×10^6/ml after cell counting.

2. The cryopreserved Matrigel was melted at 4°C, and the pre-cooling RPMI1640 medium without serum was diluted to one mg/ml.

3. After two hours’ ultraviolet irradiation of transwell chamber, it was put into 24 orifice plate with 100 μl Matrigel added into each orifice, and then cultivated for four hours in the incubator containing 5% CO₂ at 37°C.

4. 600μl RPMI1640 medium containing 10% fetal bovine serum was added to the lower layer of the chamber, while 100 μl cell suspension liquids in different groups was added to the upper layer of the chamber. The chamber was placed in the incubator containing 5% CO₂ and was cultivated for 24 hours at 37°C.

5. The medium was removed from the upper layer of the chamber, and membrane was taken out. The redundant cells on Matrigel matrix were gently wiped off with cotton swabs. Then after washed by PBS buffer for one time, 70% methanol was used to fix the membrane for 45 minutes and stained by hematoxylin for five minutes.
6. Cells which migrated to membrane were observed under the microscope, and three multiple plates were set up for different types of cells, repeated for three times. The total number of cells in five high power fields for each plate was recorded. With the average value as the number of transmembrane cells was used to evaluate the invasiveness of cells.

Data statistics and analysis
Statistical analysis of the whole data was conducted adopting SPSS16.0 software package. All statistical measurement data are shown in the form of average number ± the standard deviation. Chi-squared test was used for the comparison of samples. And t-test or ANOVA was adopted for comparison among groups. When \( p < 0.05 \), the difference was statistically significant.

Results

Immunohistochemical analysis of LRIG3 and EGFR in each group
LRIG3 and EGFR protein expressions in NCE tissues, CIN tissues and CSCC tissues were examined by immunohistochemical method. Figures 1 and 2 show the expressions of LRIG3 and EGFR in the three groups of tissues. Then statistical analysis of the LRIG3 and EGFR expressions are shown in Tables 1 and 2. It indicated that positive expression of LRIG3 and EGFR in different cervical tissues reached the extremely significant level. During the process of normal cervix deteriorating to CIN and CSCC, the amount of LRIG3 expression gradually decreased, while that of EGFR was on the contrary (as seen in Figures 1 and 2).

LRIG3 and EGFR expressions in Hela229 cells at different time points after transfection
The oligonucleotides were transfected into Hela229 cells in vitro. At the time point of 24, 48, and 72 hours after transfection, the amount of LRIG3 and EGFR protein was respectively semi-quantitatively evaluated. Results shown in Tables 3 and 4 indicate that after the LRIG3 ASODN was transfected into cells, the amount of LRIG3 protein gradually decreased, while that of EGFR protein gradually increased (\( p < 0.05 \)). Compared with other groups, the difference of LRIG3 and EGFR expressions was significant (\( p < 0.05 \) or \( p < 0.01 \)). In summary, the operation of ASODN transfected into cancer cells could reduce the LRIG3 protein level significantly and improve EGFR protein expression level.

Effect of LRIG3 antisense nucleotide on Hela229 cells proliferation and apoptosis
The cell proliferation rate in ASODN group was significantly higher than that of the other groups at 24 hours after LRIG3 ASODN transfection into Hela229 cells. As the extension of LRIG3 expression suppression time, the cell pro-
liferation rate gradually increased ($p < 0.05$), while that in MSODN group and SODN group had no obvious changes ($p > 0.05$) (Table 5).

After transfection of oligonucleotide into Hela229 cells in different groups, the cell apoptosis rate in ASODN group was significantly lower than that of the other groups ($p < 0.01$). With the extension of transfection time, the cell apoptosis rate in ASODN group kept a ever-decreasing trend, the amount of apoptosis at the time of 48 and 72 hours after transfection were both lower than that at 24 hours ($p < 0.05$) (Figure 3). The above results suggested that through anti-sense oligonucleotide transfection, the Hela229 cell proliferation rate can be obviously improved and cell apoptosis rate can be decreased.

<table>
<thead>
<tr>
<th>Group</th>
<th>Negative</th>
<th>Weak</th>
<th>Positive</th>
<th>Strong positive</th>
<th>Positive rate (%)</th>
<th>$\chi^2$ value (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSCC group</td>
<td>29</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>35.36</td>
<td></td>
</tr>
<tr>
<td>CIN group</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>1</td>
<td>70.00</td>
<td>32.637 (&lt;0.01)</td>
</tr>
<tr>
<td>NCE group</td>
<td>0</td>
<td>9</td>
<td>17</td>
<td>4</td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. — Effect of oligonucleotide transfection on LRIG3 protein expression in Hela229 cells.

<table>
<thead>
<tr>
<th>Time of transfection</th>
<th>ASODN group</th>
<th>SODN group</th>
<th>MSODN group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>0.144±0.010</td>
<td>0.233±0.015</td>
<td>0.223±0.021</td>
<td>0.247±0.029</td>
</tr>
<tr>
<td>48 hours</td>
<td>0.077±0.004</td>
<td>0.218±0.032</td>
<td>0.221±0.014</td>
<td>0.267±0.017</td>
</tr>
<tr>
<td>72 hours</td>
<td>0.057±0.004</td>
<td>0.227±0.027</td>
<td>0.230±0.023</td>
<td>0.241±0.026</td>
</tr>
</tbody>
</table>

Note: *: compared with the expression at 24 hours after transfection, $p < 0.05$; ◊: compared with the expression 48 hours after transfection, $p < 0.05$; ▼: compared with ASODN group at the same time point, $p < 0.05$.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of trans membrane cells</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASODN group</td>
<td>246±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SODN group</td>
<td>168±10◊</td>
<td>54.240</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MSODN group</td>
<td>177±8◊</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>176±6◊</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: ◊: compared with ASODN group, $p < 0.05$.

Result of Transwell invasion experiment

Hela229 cells, with a 72-hour 250 μg/ml LRIG3 ASODN transfection, were used in transwell invasion experiment. The results showed that compared with other groups, the number of trans-membrane cells in transfection ASODN group had a significant increase and the difference had statistical significance ($p < 0.05$), while the number of trans-membrane cells in other groups had no obvious difference ($p > 0.05$) (as seen in Table 6 and Figure 4). It can be seen that Hela229 cells transfected by ASODN had a stronger migration, easier for migration.
Discussion

This study gives a detection of LRIG3 and EGFR expressions in CSCC, CIN tissues, and NCE tissues by immunohistochemical method. The results indicated during the process of normal cervix deteriorating to CSCC, that LRIG3 expression gradually decreased in cervical tissues, while EGFR expression gradually increased. It indicates that LRIG3 may play its biological function through negative feedback to EGFR. In vitro experiment, LRIG3 expression level was regulated by adopting antisense nucleotide technology. It was found that after the LRIG3 expression was inhibited, the EGFR protein expression level was improved simultaneously. With the extension of LRIG3 expression suppression time, EGFR expression level also shows an ever-increasing trend. In terms of CSCC Hela229 cell proliferation and apoptosis, the growth rate of cells with suppressed LRIG3 expression rises, the apoptosis rate decreases, and the migration ability becomes stronger than that of other groups ($p < 0.05$). These results show that after the suppression of LRIG3 gene expression, Hela229 cell proliferation ability and invasion ability has significantly enhanced, along with the rise of EGFR expression level. Therefore, it is considered that LRIG3 shows a negative feedback regulation to EGFR expression, LRIG3 may be able to suppress the genesis and development of cervical squamous cell carcinoma or have a certain reversal function to the aggressive development of CSCC.

Currently, researches on LRIGs abnormal expression mostly focus on LRIG1. Compared with the normal tissues, decreases [6, 7] of LRIG1 protein and mRNA were found in prostate cancer cells, renal cancer cells, colon cancer cells, and so on, which indicates a close relationship between LRIG1 and the genesis and development of tumors. Few studies on the relationship between LRIG1 and tumor have been reported so far. However there is certain similarity between LRIG3 and LRIG1 in terms of protein structure, and they may be similar in function. Moreover, the located region of LRIG3 gene on human chromosome was the disease-prone parts of human tumor gene deletion. Therefore, a preliminary inference can be conducted that LRIG3 may have close relationship with the genesis of some certain human tumor. Muller et al. conducted a study of human cervical adenocarcinoma, which indicated that high staining intensity of LRIG1 and high fraction of LRIG3-positive cells were significantly associated with patient survival, and positive correlations were found between LRIG1 and LRIG3 staining intensity and HPV status [8]. In the study of astrocyte tumor, it was found LRIG3 has close relationship with tumor proliferation index, tumor grading and patients’ survival rate, and it is an independent prognostic factor [9]. Cai et al. [10] designed shRNA targeting LRIG3 by employing RNA interference technology. As the result of shRNA transfected into GL15 cells, it was found that the decrease of LRIG3 expression, suppression of cell proliferation and increase
of cell apoptosis appeared simultaneously. So it was inferred that LRIG3 suppress tumor perhaps by regulating EGFR signal system. In this experiment, LRIG3 shows a low expression in CSCC, while EGFR shows high expression. There is a close relationship between EGFR/LRIG3 and the genesis of CSCC. Meanwhile it also demonstrates when LRIG3 expression is inhibited, EGFR expression rises. That is to say, LRIG3 presents negative feedback regulation of EGFR expression with tumor cell proliferation and invasion abilities are enhanced. All of these facts indicate gene LRIG3 can inhibit cell proliferation ability raised by EGFR. The anticancer role of LRIG3 may come into play through EGFR signal pathway. The negative feedback regulation of EGFR by LRIG3 can be completed through the following ways [11, 12]: (1) decrease of receptor sensitivity caused by too much phosphorylation; (2) internalization of ligand-receptor complexes; (3) inhibition of intracellular and transmembrane protein; (4) proteolysis of some special signal transduction proteins.

Though the mechanism of negative feedback has not been very clear, it is undoubted that LRIG3 produces effects on the genesis and development of tumor by acting on EGFR signal pathway. Therefore, research work in the next step can be focused on the specific ways of LRIG3 regulation on EGFR expression or the study of regulatory path.

In conclusion, analysis of combining LRIG3 with EGFR may provide a new reference standard for the diagnosis of CSCC and a new approach to cervical cancer molecular targeted treatment.

Acknowledgements

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References


Introduction

Pregnancy-associated breast cancer (PABC) is defined as breast cancer diagnosed during pregnancy or within one year after delivery [1]. PABC is a rare situation, but represents a real challenge, with a prevalence of 0.2% to 3.8% of all breast cancers (7.3% of breast cancers in patients younger than 40 years) and an incidence of one in 3,000 to one in 10,000 pregnancies [2, 3]. However, since more women are becoming pregnant later due to cultural and social developments, its diagnosis is likely to increase in clinical practice [4, 5].

The prognosis for breast cancer has been considered to be worsened by the coexistence of pregnancy. To date, significant controversy still exists regarding the pathological tumor features and prognosis of patients diagnosed with pregnancy-associated breast cancer (PABC). The aim of the present study was to analyze the different prognostic factors and outcome in PABC subset versus a non-PABC control group matched for age and year of diagnosis.

Materials and Methods: A total of 56 PABC cases were diagnosed from 1990 to 2008, for whom 73 non-PABC patients were identified. Pathological characteristics, immunohistochemical features, and differences in overall and disease-free survival were compared between both groups. Results: Compared to non-PABC controls, PABC patients presented more advanced disease (31% vs 13%, \( p = 0.024 \)) and greater lymph node involvement (53% vs 34%, \( p = 0.034 \)). Pathological and tumor features tended to present poorer prognostic factors in the PABC subset. Survival was poorer in the PABC patients (five-year DFS 68% in PABC vs 86% in non-PABC, \( p = 0.12 \)). However, analysing survival adjusted for stage and age, the authors did not find significant differences between both groups. Conclusions: PABC patients tended to be diagnosed in advanced breast disease and presented tumors with adverse pathological prognostic factors. While the authors found a poorer outcome in PABC group, no significant differences were observed with stage-matched analysis. The present results may suggest that the poorer prognosis observed within PABC women could not be due to pregnancy itself, but with a delay in diagnosis and tumor subtype pathological features.

Key words: Breast cancer; Pregnancy; Prognostic factors; Outcome; Pathological features.
Materials and Methods

The authors recorded the experiences of PABC over years at Quirón Dexeus University Hospital, Barcelona. From 1990 to 2008, 56 patients were diagnosed with PABC and matched 1:1.5 to non-PABC controls by age and date at diagnosis (73 controls). All patients were identified from the authors’ prospective Breast Cancer Database.

The PABC criteria included only patients diagnosed with invasive breast cancer during or within one year after delivery. All patients were pathologically diagnosed at the present center. Before 2007 the diagnosis was performed by fine-needle aspiration and beyond 2007 by core biopsy. All cases were subsequently pathologically confirmed in the surgical specimens.

Since 2002 sentinel lymph node biopsy was introduced as a part of the axillary staging protocol, therefore patients treated later did not undergo an axillary lymphadenectomy if the sentinel node was negative.

Clinical and pathological data such as hormone receptors status, Her2 Neu, histological tumor grade, histological tumor type, lymph node involvement, tumor size were retrieved from medical and pathological records.

Histological and immunohistochemical study

Histological grade was performed according to the Elston and Ellis modification of the Scarff-Bloom-Richardson grading system [18]. Hormone receptors were analyzed by immunohistochemistry. Determination of estrogen receptors (clone 6F11), progesterone receptors (clone 16), and Her2 Neu (clone CB11) was performed by IHC using, in all cases, the manufacturer’s pre-diluted antibody. A tumor considered positive for estrogen or progesterone receptors was defined as having 10% or more of stained tumor cells nuclei. Positive Her2 was considered as overexpression 3 + or 2 + if FISH technique was positive. Ki67 was not reported in the study since it had not been routinely recorded at that time.

Statistical analysis

The description of quantitative variables was performed using median, mean, range (minimum and maximum), and standard deviation. The qualitative variables were presented by means of the description of proportions. Quantitative variables were compared with Wilcoxon Mann-Whitney or Student’s t-test, and categorical variables were analyzed with either the Pearson chi-square or Fisher’s exact test. Survival was estimated using Kaplan-Meier curves. A Cox regression model was performed for the stage-adjusted survival subanalysis. All statistical analysis were performed using the SPSS Statistics 20.0 program. All tests were two-sided and the significance level was set at 0.05.

Results

A total of 56 PABC patients were diagnosed between 1990-2008, for whom 73 controls non-PABC were identified. In the present center the PABC rate was 1/1230 pregnancies and 1/76 of all breast cancer cases (1.31%).

Patient characteristics are shown in Table 1. The mean age of PABC and non-PABC group was 35.4 ± 4.7 (27-50) years and 37.3 ± 6.6 (27-52), respectively (age-matched). The PABC diagnosis was made during postpartum term in 34 cases (62%), and during pregnancy in 22 patients (38%): six cases in the first trimester (12%), four cases in the second trimester (8%), and nine in the third trimester (18%).

### Table 1. — Clinical patients’ characteristics.

<table>
<thead>
<tr>
<th></th>
<th>PABC cases (%)</th>
<th>Non-PABC cases (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cases</td>
<td>56 (100)</td>
<td>73 (100)</td>
<td>Match</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.39 (27-50)</td>
<td>37.31 (27-52)</td>
<td>Match</td>
</tr>
<tr>
<td>Year of diagnosis</td>
<td>1990-2008</td>
<td>1990-2008</td>
<td>Match</td>
</tr>
<tr>
<td>Term in diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st trimester</td>
<td>6 (12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd trimester</td>
<td>4 (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>9 (18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puerperium</td>
<td>34 (62)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laterality</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB</td>
<td>48.2 (27)</td>
<td>53.4 (39)</td>
<td>0.62</td>
</tr>
<tr>
<td>LB</td>
<td>51.8 (29)</td>
<td>46.6 (34)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. — Pathological and tumor features in PABC and non-PABC groups.

<table>
<thead>
<tr>
<th></th>
<th>PABC cases (%)</th>
<th>Non-PABC cases (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (2cm or less)</td>
<td>22 (44)</td>
<td>32 (52)</td>
<td>0.03</td>
</tr>
<tr>
<td>T2 (&gt;2 -5cm)</td>
<td>14 (28)</td>
<td>25 (40)</td>
<td></td>
</tr>
<tr>
<td>T3 (&gt;5cm)</td>
<td>7 (14)</td>
<td>4 (6)</td>
<td></td>
</tr>
<tr>
<td>T4 (skin/muscle)</td>
<td>7 (14)</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (I+II)</td>
<td>33 (69)</td>
<td>52 (87)</td>
<td>0.024</td>
</tr>
<tr>
<td>Advanced (III+IV)</td>
<td>15 (31)</td>
<td>8 (13)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>8</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>38 (76)</td>
<td>52 (74)</td>
<td>0.58</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>6 (12)</td>
<td>6 (9)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>6 (12)</td>
<td>12 (17)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Histological tumor grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>8 (24)</td>
<td>17 (45)</td>
<td>0.17</td>
</tr>
<tr>
<td>Grade II</td>
<td>16 (49)</td>
<td>15 (39)</td>
<td></td>
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<tr>
<td>Grade III</td>
<td>9 (27)</td>
<td>6 (16)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>23</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td>28 (53)</td>
<td>24 (34)</td>
<td>0.03</td>
</tr>
<tr>
<td>Estrogen receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26 (67)</td>
<td>42 (78)</td>
<td>0.23</td>
</tr>
<tr>
<td>Negative</td>
<td>13 (33)</td>
<td>12 (22)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>17</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Progesterone receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20 (51)</td>
<td>41 (76)</td>
<td>0.01</td>
</tr>
<tr>
<td>Negative</td>
<td>19 (49)</td>
<td>13 (24)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>17</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Her2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (35)</td>
<td>9 (31)</td>
<td>0.78</td>
</tr>
<tr>
<td>Negative</td>
<td>19 (65)</td>
<td>20 (69)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>27</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Tumor subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR+/Her2-</td>
<td>14 (48)</td>
<td>19 (66)</td>
<td>0.17</td>
</tr>
<tr>
<td>Her2+</td>
<td>10 (35)</td>
<td>9 (31)</td>
<td></td>
</tr>
<tr>
<td>Triple -</td>
<td>5 (17)</td>
<td>1 (3)</td>
<td></td>
</tr>
</tbody>
</table>

NA: not assigned.
Pathological and tumor features are summarized in Table 2. PABC patients presented more advanced tumor stage at diagnosis compared to non-PABC (31% advanced disease in PABC group vs 13% non-PABC group, \( p = 0.024 \)). Larger tumors were found within PABC patients (40% T2 stage in PABC vs 28% non-PABC (\( p = 0.03 \). In the present series, lymph node involvement was found in 28 PABC patients (53%) versus 24 non-PABC patients (34%) (\( p = 0.034 \)). No relevant differences were found according tumor histology or laterality between both groups. The majority was diagnosed with invasive ductal carcinoma in both groups, 52 PABC cases (74%) and 38 (76%) in control group (\( p = 0.5 \)).

Regarding immunohistochemical analysis, adverse prognostic factors were found within PABC patients compared to non-PABC group: negative-ER (33% vs 22%; \( p = 0.23 \)), negative-PR (49% vs 24%; \( p = 0.014 \)) and positive-Her2 (35% vs 31%; \( p = 0.78 \)).

The most common tumor subtype in PABC patients was positive-HR/negative-Her2 (48%), but in comparison to non-PABC group, triple negative tumors were found in a high rate (17% vs 3%, \( p = 0.17 \)).

At a median follow-up of 3.6 years (1-14) for PABC subset and 8.8 years (1-31) for the control group, four patients (7.4%) had a locoregional recurrence, five (14%) cases metastasized, and seven (13%) died in PABC group, related to 5 (6.9), 7 (16) and 0 in non-PABC group (Table 3).

Survival in the PABC subset tended to be lower (Figure 1). The five-year DFS was 64% in PABC patients and 86% in non-PABC patients (\( p = 0.12 \)) and five-year OS was 74% and 100%, respectively (\( p = 0.21 \)). However, DFS analysis matching stage did not find significant differences between both groups (Figure 2).

**Discussion**

The overall PABC rate in the present series between 1990-2008 was 1/1230 pregnancies and 1.31% of all breast cancers, higher than the rate described in literature [2]. This is likely due to a delay in pregnancy among patients at our center, with a mean parity age of 34 years.

Classically, PABC has been considered an aggressive disease with a poor outcome, mainly, due to the independent effect of pregnancy. This belief has led it to be considered as an intractable situation in which surgery was not effective [19, 20]. However, in recent years some published data have suggested a poor prognosis related to the delay in diagnosis and the young age of PABC patients [17, 21, 22].

The current study represents a large series in a single center evaluating the pathological features in PABC group compared with non-PABC patients. The authors found that in the PABC group patients were diagnosed with an advanced TNM stage and presented greater nodal involvement compared to non-PABC patients (31% vs 13%, \( p < 0.05 \); 52% vs 34%, \( p < 0.05 \); respectively). These findings corroborate the observa-
tions by Middleton et al. who found a more advanced disease among PABC patients [23]. This may reflect a delay in diagnosis and treatment due to childbirth. The physiological changes induced by gestation make physical examination more challenging. This difficulty must be added to the lack of attention to breast symptoms (breast pain, nipple discharge, increased mammary density, mastitis) by the obstetrician who can consider them as normal changes in the pregnant breast. All these factors could result in a more advanced PABC diagnosis.

Regarding prognostic factors, the present series showed a high rate of Grade III histological tumor, negative hormone receptors, and positive Her2. These results are in agreement with those previously published where the PABC group had adverse prognostic factors [5, 23-25]. Although the most common tumor subtype within PABC patients was positive-HR/negative-Her2, the present authors found a high rate of triple negative tumors (17% vs 3%, p = 0.17). This could suggest that the poor outcome is not directly related to pregnancy but to tumor subtypes and its pathological characteristics. Such findings may be attributed to the young age of PABC patients who are more likely to develop breast tumors with such features [23, 26]. However, the present authors’ matching process allowed them to correct the impact of age, and they found even a greater percentage of adverse prognostic factors in PABC subset.

In the prognosis and outcome analysis, PABC patients had greater mortality (p = 0.02) and poorer survival (five-year DFS 64% vs 86%), but the adjustment for age and stage showed no considerable differences between both groups. If pregnancy itself is the main cause of the poorer prognosis within women with PABC, PABC patients would be expected to have more recurrences and greater mortality compared to non-PABC patients with the same stage at the time of diagnosis.

From the present study the authors may conclude that PABC patients are diagnosed with advanced breast cancer. The poorer prognosis observed within PABC women may not be associated with pregnancy itself, but with tumor subtype pathological features and a delay in diagnosis related to advanced disease, as the matched-stage analysis found no major differences in survival between both groups.

References


Effectiveness of third-line chemotherapy in recurrent ovarian cancer patients


Department of Obstetrics and Gynecology, School of Medicine, Keio University, Tokyo (Japan)

Summary

Objective: Despite recent advances in the treatment of recurrent ovarian cancer, little evidence exists describing the benefit of third-line chemotherapy. The present authors previously reported that the treatment-free interval (TFI) after second-line chemotherapy may predict a survival benefit of third-line chemotherapy, however the length of TFI was uncertain due to limited cases. In this study, the authors evaluated the length of TFI, which is correlated with the effectiveness of third-line chemotherapy and a prognostic factor of third-line chemotherapy. Materials and Methods: The authors reviewed the medical records of 85 women with recurrent ovarian cancer who received third-line chemotherapy after a paclitaxel/carboplatin (PC) regimen as first-line chemotherapy. Results: The response rate [complete response (CR) + partial response (PR)] and clinical benefit rate [(CBR): CR + PR + stable disease (SD)] during the TFI after second-line chemotherapy for 0–3 months, 3–6 months, and 6–12 months and ≥12 months were 9.8%, 0%, 0%, 43.8% and 15.7%, 50%, 66.7%, and 93.8%, respectively. The median overall survival (OS) from the onset of third-line chemotherapy was longer for TFI ≥3 months than for TFI 0–3 months (795 days vs. 281 days, p < 0.001). Finally, according to univariate (HR = 0.256; p < 0.001) and multivariate (HR = 0.264; p < 0.001) analyses, TFI was the independent significant prognostic factor for OS. Conclusions: TFI less than three months after second-line chemotherapy may predict little survival benefit of third-line chemotherapy.

Key words: Epithelial ovarian cancer; Recurrent ovarian cancer; Third-line chemotherapy.

Introduction

At present, there are no effective screening methods for epithelial ovarian cancer, ultimately resulting in the vast majority of patients being identified during advanced stages. International Federation of Obstetricians and Gynecologists (FIGO) Stage III or IV patients usually relapse in more than 70% patients, indicating difficulties in achieving complete cure. Ovarian cancer is the ninth most common disease in women and the fifth leading cause of cancer death [1]. Thus, the goal of primary treatment is to cure, and on recurrence, life extension or palliation of cancer-related symptoms is the objective for better quality of life (QoL). There is concern regarding whether treatment of relapsed patients is beneficial or whether multiple chemotherapies threaten QoL. The present authors previously reported that the treatment-free interval (TFI) after second-line chemotherapy may predict a survival benefit of third-line chemotherapy [2]; however, the length of TFI was uncertain because of limited patients. In this study, the authors performed retrospective analysis of 85 patients of recurrent ovarian cancer who received paclitaxel/carboplatin (PC) as first-line therapy, particularly evaluating the patients who benefited from third-line chemotherapy.

Materials and Methods

Patients

The authors retrospectively reviewed the medical records of women with recurrent ovarian cancer who received third-line chemotherapy. Eighty-five patients who started to receive third-line chemotherapy between July 1997 and December 2009 were included. Among them, 40 patients were also included in the present authors’ previous study [2]. All patients underwent initial debulking surgery. They received primary chemotherapy consisting of PC regimen and second-line chemotherapy after the first relapse. The patients who underwent surgery at relapse were excluded. All patients were followed up at the Department of Obstetrics and Gynecology, Keio University Hospital. Treatment decisions regarding third-line chemotherapy were usually made by the attending clinician. Data were collected on age, FIGO staging, histologic type, extent and outcome of surgery, prior chemotherapeutic treatments and disease responses, intervals between primary, secondary, and tertiary treatments, and overall survival (OS) after receiving the third-line drug.

Definition of sensitivity of primary chemotherapy

“Refractory,” “resistant,” and “sensitive” in first recurrence were defined as follows: refractory, progression, partial remission, or stable disease at the time of primary chemotherapy, resistant, complete remission, and relapse less than six months after primary chemotherapy, and sensitive, complete remission, and relapse six months or more after discontinuing primary chemotherapy. TFI before third-line chemotherapy was defined as the interval between the last day of second-line chemotherapy and the first day of third-line chemotherapy.
Evaluating response of third-line chemotherapy

Response was based on two-dimensional measurements of the lesions based on computed tomographic images. A complete response (CR) was defined as no evidence of disease on imaging studies, with normalization of the serum CA125 level. Partial response (PR) was defined as >50% decrease in tumor size. Progressive disease (PD) was defined as >25% increase in tumor size or the appearance of a new lesion. Stable disease (SD) was defined as neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. The CA125 response criteria were not used. OS was defined as the interval from the first day of administration of the third-line drug to the day of death or the last day of observation.

Statistical analysis

Patients were categorized by age (< median vs. ≥ median), debulking status (complete vs. incomplete), platinum sensitivity (sensitive vs. refractory/resistant), histology (serous vs. non-serous), FIGO staging (Stage I, II vs. Stage III, IV), and TFI (0–3 months vs. ≥ three months). Factors influencing OS were analyzed by Cox’s proportional hazard test and the log-rank test. After investigation of multicollinearity of these factors, multivariate Cox’s proportional hazard test was applied. P-value <0.05 was considered statistically significant. Statistical calculations were performed using SPSS Statistics software, version 20 for Windows.

Results

Patients

Median age at the onset of third-line chemotherapy was 56 years (range: 32–76). Clinical stage and histology were as follows: Clinical Stage (I: 6; II: 3; III: 52; IV: 24); histology (serous: 47; clear cell: 17; endometrioid: 11; mucinous: three; undifferentiated: three; others: four). In the first recurrence, 40 patients were platinum-sensitive and 45 were platinum-resistant. Thirty-two patients received a platinum/taxane regimen, 18 received cisplatin + irinotecan, six received cisplatin + doxorubicin + cyclophosphamide, three received other combination therapies, 15 received irinotecan, five received taxane monotherapy, five received pegylated liposomal doxorubicin (PLD), and one received carboplatin as second-line chemotherapy.

The clinical background of the third-line settings are shown in Table 1. TFI from the last day of second-line chemotherapy to the first day of third-line chemotherapy was 0–3 months in 51 patients, 3–6 months in six patients, 6–12 months in 12 patients, and ≥ 12 months in 16 patients. Forty-five patients received single-agent chemotherapy (taxane: 21; irinotecan/topotecan: seven; liposomal doxorubicin: nine; other: eight) and 40 received combination chemotherapy (platinum/taxane: 30; cisplatin + irinotecan: three; cisplatin + doxorubicin + cyclophosphamide: four; other: three) as third-line chemotherapy.

Relationships between TFI and RR or CBR of third-line chemotherapy

To evaluate the length of TFI, which can predict the benefit of third-line chemotherapy, the authors initially investigated RR and CBR of TFI 0–3 months, 3–6 months, 6–12 months, and ≥ 12 months, respectively (Table 2). RR was 0–3 months in 9.8%, 3–6 months in 0.0%, 6–12 months in 0.0%, and ≥ 12 months in 43.8% patients. CBR was 0–3 months in 15.7%, 3–6 months in 50%, 6–12 months in 66.7%, and ≥ 12 months in 93.8% patients.
TFI less or more than three months can predict survival after third-line chemotherapy

Next, the authors evaluated the relevance of survival and each TFI. The median OS for all patients was 330 days (range, 24–3,335). Fifty-three patients died, 20 were alive with disease, and 12 were alive with no evidence of disease on the last day of August 2011. The median OS was 281 days in TFI 0–3 months, 612 days in TFI 3–6 months, 788 days in TFI 6–12 months, and 1110 days in TFI ≥ 12 months (Table 2). Based on these findings and the fact that CBR rather than RR associates with the effect of third-line chemotherapy, the authors divided all patients into two groups (TFI: 0–3 months, ≥ 3 months) and compared OS between the two groups (Figure 1). The median OS in the TFI 0–3 months group was significantly shorter than that in the TFI ≥ three months group (281 days vs. 795 days, p < 0.001).

Table 3. — Univariate and multivariate analyses of the effect of various prognostic factors on OS.

<table>
<thead>
<tr>
<th>Hazard Ratio (95% CI)</th>
<th>p-value</th>
<th>Hazard Ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (&lt; median vs. ≥ median)</td>
<td>1.046 (0.609-1.795)</td>
<td>0.871</td>
<td>1.535 (0.838-2.812)</td>
</tr>
<tr>
<td>Debulking status (complete vs. incomplete)</td>
<td>1.096 (0.609-1.971)</td>
<td>0.761</td>
<td>1.719 (0.801-3.690)</td>
</tr>
<tr>
<td>Platinum sensitivity (sensitive vs. refractory/resistant)</td>
<td>2.847 (1.604-5.052)</td>
<td>&lt;0.001</td>
<td>1.892 (0.977-3.666)</td>
</tr>
<tr>
<td>Histology (serous vs. non-serous)</td>
<td>1.147 (0.666-1.976)</td>
<td>0.620</td>
<td>1.270 (0.641-2.518)</td>
</tr>
<tr>
<td>FIGO staging (Stage I, II vs. Stage III, IV)</td>
<td>0.579 (0.260-1.288)</td>
<td>0.180</td>
<td>0.358 (0.127-1.008)</td>
</tr>
<tr>
<td>TFI (0-3 months vs. ≥ three months)</td>
<td>0.256 (0.139-0.470)</td>
<td>&lt;0.001</td>
<td>0.264 (0.133-0.527)</td>
</tr>
</tbody>
</table>

TFI three months is the independent prognostic factor of third-line chemotherapy benefit

The authors next analyzed the prognostic significance of multiple factors such as age, debulking status, platinum sensitivity, histology, FIGO staging, and TFI. Among then, TFI is the single independent factor for determining the benefit of third-line chemotherapy by univariate (HR = 0.256, p < 0.001) and multivariate analysis (HR = 0.264, p < 0.001), although platinum sensitivity of the first relapse may also serve as a prognostic factor with a greater number of patients (univariate p < 0.001, multivariate p = 0.059) (Table 3).

Discussion

For almost two decades, the paclitaxel/platinum regimen has been the standard chemotherapy for ovarian cancer [3]. Despite improvement in the prognosis of ovarian cancer, it is still difficult to achieve complete remission, and effective therapy is needed. There are several reports describing the benefits of second-line chemotherapy; however, the clinical evidences regarding advantages of third-line chemotherapy are few.

In epithelial ovarian cancer, “platinum sensitivity” is the well-recognized clinical factor [4]. On second-line setting of platinum-sensitive relapse, platinum-based combination chemotherapy is recommended. In addition to the traditional PC treatment, carboplatin in combination with PLD [5] or gemcitabine [6] is a favorable regimen. On the other hand, regarding platinum-resistant relapse, single usage of PLD, topotecan, paclitaxel, or gemcitabine have shown similar activity, and six randomized trials failed to show superiority in outcomes for combination vs. single agent [7].

In the third-line setting, the concept of a platinum-free interval is not clear. However, in clinical situations, the decision to give third- or fourth-line chemotherapy to patients is often contemplated. In this study, platinum sensitivity of the first relapse was shown to help predict the efficacy of third-line chemotherapy, but this result was not significant. Furthermore, debulking status of operation, histology, or FIGO staging did not predict the efficacy of third-line chemotherapy. These data indicate that in cancers that are beyond second relapse, tumor characteristics...
are more aggressive and can withstand chemotherapeutic damage. Hanker et al. reported optimal tumor debulking and platinum sensitivity of first-line chemotherapy as independent prognostic factors for PFS up to the third relapse, and maximum of three lines of subsequent relapse treatment seem to be beneficial [8]. Griffiths et al. reported that treatment efficacy declined rapidly with successive lines of therapy after platinum resistance and suggested that disease progression on two consecutive lines of therapy should be used as a guide to discontinue chemotherapy [9]. Hoskins et al. reported that an interval between two consecutive relapses measuring less than six months was a proposed marker for discontinuing further chemotherapy [10]. The present data indicates that populations with TFI < three months show little response to third-line therapy, which is mostly consistent with prior reports.

The present authors previously reported that TFI from second-line chemotherapy is the predictive marker of third-line chemotherapy; however, they were unable to determine the specific time window among the TFI three to six month group because of limited patients [2]. In this present study, the authors evaluated more patients with recurrent ovarian cancer who received the CP regimen in the first-line setting. According to RR and CBR of each TFI, CBR is thought to be the better indicator of third-line chemotherapy compared with RR, with RR of TFI <12 months being very low and difficult to evaluate. Overall, the authors determined TFI of three months to be a prognostic indicator of third-line chemotherapy. In the clinical situation, third-line chemotherapy for TFI < three months may have little-to-no clinical benefit or may actually threaten QoL.

Acknowledgements

The authors thank Keiko Abe and Tomomi Noda for their secretarial work.

References

Clear cell endometrial cancer: a CTF multicentre Italian study

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Summary

Endometrial clear cell carcinoma (CCC) is a rare entity and only accounts for 1-6% of all endometrial cancers. CCC is considered an aggressive subtype of endometrial cancer with worse prognosis compared with type I cancer and more frequent relapses at distant and extrapelvic sites. These characteristics require specific treatment modalities, but rarity of the disease does not allow to identify evidence based indications for therapies. Objective of the present study is to analyse a series of cases treated in a multicentre Italian setting. Materials and Methods: Sixty-five endometrial CCC were treated in the period 1990-2010 in the participating institutions. Slides of the pathological specimens were reviewed by a single pathologist of each institution and debatable cases were collegially reviewed. Clinical records were collected by a common database. Demographic, surgical pathological, and follow-up data were registered. Results: All patients received primary surgery. Stage of disease according FIGO 2009 was as follow: 1a: 16.9%, 1b: 35.4%, 2: 9.2%, 3a: 9.2%, 3b: 3.1%, 3c: 16.9%, 4a: 3.1%, and 4b: 6.1%. Adjuvant post-operative treatment was adopted in 53.8% of cases. A relapse was detected in 29.2% of cases with a majority of extrapelvic sites (68.4%). Five-year survival rate was significantly related to stage of disease with an excellent prognosis for Stage Ia e Ib disease with a complete staging. In these cases adjuvant treatment does not show significant improvement of survival. Relapsed cases show a response rate to treatment in 26% of cases (predominantly chemotherapy). Conclusion: CCC requires extensive surgical staging. Stage I disease completely staged does not require adjuvant therapy. More advanced stages require adjuvant chemotherapy.

Key words: Clear cell endometrial cancer; Post-treatment relapses; Clinical outcome.

Introduction

Uterine cancer is the most common gynaecological malignancy in Western countries. It is estimated that in the United States 47,130 new diagnosis were reported in 2012 [1]. In Italy 7,465 new cases were registered in 2011 by the Consortium of the Tumour Registry (AIRTUM) [2]. The vast majority of uterine cancers originate from the endometrium and only a limited number, about 8%, are sarcomas. Endometrial cancers include endometrioid cancer (Type 1) and non-endometrioid cancer (Type 2). Type 2 endometrial cancers include serous papillary carcinomas (UPSC) and clear cell carcinomas (CCC), accounting respectively 7-10% [3, 4] and 1-6% of all endometrial carcinomas [4-6].

Clear cell tumours, like UPSC, are an aggressive subtype of endometrial carcinomas with a tendency to relapse outside the pelvis. Both CCC and UPSC develop more frequently in post-menopausal women but are not associated with estrogen use, obesity, and are more common in black women [7].

Survival of women with CCC is generally worse than those with low-grade but similar to high-grade endometrioid tumors [8]. CCC is more likely to present with extraterine spread compared to low-grade endometrioid histology [9, 10].

Due to its rarity, it has been difficult to study CCC in controlled clinical trials, making the development of evidence-based management challenging [5].

The aims of the present study are to investigate patterns of care, treatment failures, and survival rates in patients treated for endometrial CCC in a multicentre retrospective Italian study.

Materials and Methods

Clinical records of endometrial CCC patients were treated in the period 1990-2010 in five Italian institutions (Obstetrics and Gynecology, Institute University of Brescia, Pisa, Turin, European Institute of Oncology-Milan, and Venice Mestre Hospital) where reviewed. Significant information concerning demographic, clinical, surgical, pathological, and data derived from follow-up programs where collected in a common database. Sixty-five cases of CCC were collected after a pathological revision of histological material that had been performed by a single pathologist in the local institution and subsequent collegial revision of the slides for questionable cases. All patients received the treatment in the participating institutions and surgery was the primary treatment in all cases.

The patients were restaged retrospectively according to International Federation of Gynaecology and Obstetrics (FIGO) classification 2009. Post-operative treatment was given according to local protocols and was established on the basis of pathological
findings, age, and general conditions. All patients were followed-up until they died or until December 2011. The medium follow-up was 133 months (range 6-216 months).

Statistical methods
The SAS statistical package (release 8.2) was used for computations. The time from surgery to death or last observation was defined as overall survival. The cumulative probability of survival was estimated by the products-limit method. The long-rank R test was used to compare the homogeneity of survival functions across strata defined by categories of prognostic variables.

Results
Patients characteristics at presentation are summarized in Table 1. All the patients received primary surgery; 62 patients by laparotomy (95.3%) and three by laparoscopy (4.6%).

Type of hysterectomy was: standard Type I (Piver and Rutledge) in 48 cases (73.85%) and Type II in 17 cases (26.15%). Additional surgical procedures to hysterectomy are listed in Table 2. Among the 55 cases in which the histotype was available for revision, both in biopsy and definitive surgical specimen, an agreement was registered in 63.6% of cases. Lymph-node metastases were observed in seven out of 51 cases submitted to lymphadenectomy (13.7%).

Tumor stage according to FIGO classification 2009 was as follows: Stage 1a: 11 (16.9%), Stage 1b: 23 (35.4), Stage 2: six (9.2%), Stage 3a: six (9.2%), Stage 3b: two (3.1%), Stage 3c: 11 (16.9%), Stage 4a: two (3.1%), and Stage 4b: four (6.1%). Residual tumour after surgery was described in six patients (9.2%). Adjuvant postoperative treatment was adapted in 35 patients (53.8%).

Adjuvant treatment was radiotherapy (external beam ± brachytherapy) in seven out of 35 patients (20.0%), chemotherapy in 16 patients (45.7%), and radio-chemotherapy in 12 patients (35.6%). Chemotherapy regimens consisted in combination of carboplatin-taxol in 14 patients, taxol-epirubicin-platinum in ten patients, and single carboplatin in four patients.

A relapse was detected during the follow up in 19 patients (29.2%). Characteristics of recurrences are detailed in Table 3. The majority of recurrences were extra-pelvic (13 out of 19: 68.4%) at peritoneal, distant or multiple sites. In 47.3% of cases the relapse was clinically detectable but only in 28.3% were symptomatic.

The five-year survival rate was significantly related to the disease stage. Early stages (Ia and Ib) had an excellent prognosis compared with all other stages (Figure 1). Among patients with Stage I and II with complete surgery, the survival rate did not show a significant difference according to different types of adjuvant treatment and no treatment at all (Figure 2).

Time of relapses showed that the events occurred mainly during the 36th month following primary treatment (Figure 3). Therapy at recurrence (19 cases) was radiotherapy in five (26.3%) cases, chemotherapy in ten (52.6%) cases, radiochemotherapy in three (15.7%) cases, and hormonal therapy in one case (5.2%).
Response rate in relapsed cases after radiological assessment was: complete response in four cases (21.0%), partial response in one (5.3%) case, stable disease in one (5.3%) case, and progression of the disease in 12 cases (63.1%).

Discussion

Endometrial CCC is a rare entity and only accounts for 1-6% of all endometrial cancer (11-13). The present case series confirmed that CCC is rarely associated with diabetes, hypertension, and use of hormonal replacement therapy as usually described for common endometrial cancer [3, 4, 7]. Preoperative biopsies are reliable to detect the definitive histotype in the majority of cases (35 out of 55 evaluable cases in this series: 63.6%) which reflect data referred by other studies [14].

Comprehensive surgical staging is advocated in endometrial CCC as subclinical extrauterine spread is frequent [15]. In the present series, 52.3% of patients were confirmed to have a Stage I disease after intensive surgical staging. Among patients with more advanced disease (25 cases with Stages III and IV), 19 (76%) had a complete resection of the disease.

Based on the limited available evidence and the present experience, it may be concluded that optimal cytoreduction of metastatic disease appears to be feasible and of benefit in patients with this disease [5].

The impact of clear cell histology in comparison to other high-risk endometrial cancer histologies such as UPSC is controversial [9, 7, 16].

The present series confirmed that Stage I CCC after comprehensive surgical staging had an excellent prognosis significantly better of what described in the present authors’ recent report on UPSC treated in the same period in their institutions [17]; this is in agreement with the series described by Thomas et al. (15) in which patients with true Stage I disease after intensive surgical staging and without adjuvant treatment showed no hematologic, lymphatic or peritoneal failures at median follow up of 44 months. Since UPSC and CCC are thought to have poorer survival than type I endometrial cancer, presumably due to early metastatic occurrence, it is important to evaluate this lesions in light of those who are intensively surgically staged [9]. This consideration has an important role in the evaluation of debatable results of adjuvant therapy in Stage I disease and in general in CCC [5, 9, 18, 19].

All the studies reported in literature are small, retrospective, with heterogeneity of patients including various types of high-risk endometrial cancer and treated with various modalities of radiation therapy. The present results confirm the indication of various studies [5, 9, 15] that adjuvant pelvic radiotherapy does not appear to have a beneficial effect either in Stage I correctly staged CCC, due to the good prognosis of this disease, nor in high-risk apparent Stage I disease due to the sites of relapse which account for 68.4% of extrapelvic diseases. These assess-
ments induced to consider chemotherapy as post-surgical adjuvant treatment in CCC.

Small and retrospective studies have suggested a potential role of adjuvant administration of platinum-based chemotherapy in this setting [20-23]. All these studies included a heterogeneous group of patients with endometrial cancer including CC type at various stages of disease or at relapse. Based on the available data, the combination of carboplatin and paclitaxel ± doxorubicin appears to have efficacy in the treatment of women with advanced endometrial CCC and in relapsed patients and should be considered at present the schedule of reference.

In conclusion: endometrial CCC has a worse prognosis compared to type I histology due to the high percentage of extrauterine occult diffusion at presentation. This implies the need of intensive surgical staging aiming to identify correctly Stage I disease, which does not require adjuvant treatment; on the other hand, more advanced disease requires adjuvant treatment due to the high percentage of recurrent disease also in extrapelvic sites. This implies the need of administering combination chemotherapy including carboplatin and paclitaxel.

References

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Upregulation of microRNA-224 sensitizes human cervical cells SiHa to paclitaxel

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Summary

Purpose: The purpose of this study was to identify the drug resistant role of miR-224 expression in cervical cancer. Materials and Methods: The expression of miR-224 pre- and post-paclitaxel treatment was determined by using stem-loop real-time reverse transcription polymerase chain reaction (RT-PCR). The authors exogenously upregulated miR-224 expression in SiHa cells using miRIDIAN miR-224 mimic transfection and observed its impact on paclitaxel sensitivity using Cytotoxicity assays. Results: MiR-224 was significantly downregulated with fold values at 2.130435 and 4.26087 under five and ten nM paclitaxel treatments, respectively. MiR-224 expression is markedly increased in SiHa cells after transfected with miRIDIAN miR-224 mimic. Exogenous miR-224 facilitates paclitaxel sensitivity in cervical cancer cells. The IC50 value was decreased in SiHa with overexpression of miR-224 compared with miRNA-negative control (p < 0.0001). Conclusion: The results suggests that miR-224 might serve as a predictor for paclitaxel response or a therapeutic target in cervical cancer therapy.

Key words: miR-224; Chemo-resistance; Cervical cancer; Paclitaxel.

Introduction

Chemotherapy resistance is one of the key causal factors in cancer death and has baffled scientists and oncologists worldwide for a long time [1-2]. For cervical cancer (CC), the third most common gynecologic cancer in global women [3], chemotherapy is still an adjuvant treatment for the complement of surgery or radiation due to its insensitivity to anticancer drugs [4-5]. The molecular genetic basis of chemoresistance is complex and involves multiple processes, including drug transport and metabolism, DNA repair, and apoptosis [6]. Currently, the factors associated with chemoresistance in cervical cancer remain poorly understood.

MicroRNAs are small, single-stranded, noncoding RNAs composed of 19 to 25 nucleotides (~22 nt), which have been proven to be a key regulator in gene expression and modulated up to one-third of all genes [7-10]. Recently, an increasing number of evidence demonstrates that miRNAs may play an integral role in modulating chemosensitivity [11, 12]. For instance, upregulation of miR-138 and downregulation of miR-27a generally increased cisplatin sensitivity in advanced bladder cancer [13]; downregulation of miR-21 increased the paclitaxel sensitivity in glioblastoma cells [14]. Thus, exploration of the role of a specific miRNA and its mechanism in cancer chemoresistance may assist in discovering new approaches to reverse chemoresistance of cancers.

Given the importance role of miRNAs in modulating chemosensitivity, the present authors’ previous study identified a characteristic miRNA expression profile that was associated with paclitaxel chemosensitivity in cervical squamous cell carcinoma [15]. Among 21 differentially expressed miRNAs, miR-224 was found to be the most obviously downregulated miRNAs after paclitaxel chemotherapy in cervical cancer. Accumulating studies have found that miR-224 is dysregulated in various human malignancies and can potentially affect many cancer-related cellular processes, including transcription, cell differentiation, cell death, growth, and cell proliferation [16-20]. However, little is known about the role of miR-224 in drug resistance of CC. The aim of this study was to investigate the drug resistant role of miR-224 expression in CC.

Materials and Methods

Cell Culture

The HPV 16-positive human cervical carcinoma cell line SiHa was obtained from the American Type Culture Collection (ATCC) and was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO2.

Chemotherapy inducement

Paclitaxel was used at a final concentration of five and ten nM, respectively. The cancer cell line SiHa cells were seeded 3×105 per well in six-well plates and incubated overnight, and then
Table 1. — The primers used in this study.

<table>
<thead>
<tr>
<th>miRNA reverse transcription primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-224</td>
<td>5'-AGCGGTTGCTAGTGGCAACTGGATTTCCGAGGT-3'</td>
<td>5'-GTGCAGGGTGCCGAGGT-3'</td>
</tr>
<tr>
<td>U6snRNA</td>
<td>5'-CTCGCTTCAGCAGCA-3'</td>
<td>5'-AACGCTTCACGAAATTTGCCGT-3'</td>
</tr>
</tbody>
</table>

Gene forward primer reverse primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
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</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-224</td>
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<td>U6snRNA</td>
<td>5'-CTCGCTTCAGCAGCA-3'</td>
<td>5'-AACGCTTCACGAAATTTGCCGT-3'</td>
</tr>
</tbody>
</table>

Abbreviation: miRNA = microRNA.

treated with paclitaxel for 72 hours. After 72 hours of induction, cells were harvested for further experimentation.

**Transfection**

miRIDIAN miR-224 mimic (miR-224) and miRIDIAN microRNA mimic negative control 1 (negative control) were purchased. For transient transfection, SiHa cells were seeded in plates at 60% confluency overnight, then transfected with miR-224 mimic to over-express the miR-224 level using DharmaFECT 1 reagent at a final concentration of 100 nM in accordance with the manufacturer’s instructions. After overnight incubation, the culture medium was replaced with fresh Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum before further study. The expression level of miR-224 in the transfected cells at 72 hours post-transfection was directly confirmed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The relative level of miR-224 in transfected cells was examined by qRT-PCR. Mock-transfected cells served as blank control and negative control mimic was transfected as negative control.

**RNA extraction and real-time RT–PCR**

Total RNAs containing miRNAs were extracted from the harvested cells using one ml TRIZOL reagent following manufacturer’s instructions. The quantity and concentration of RNA were spectrophotometrically assessed by measuring absorbance at A260/280. For miRNA qRT-PCR, stem-loop RT-PCR was performed. Briefly, the RNAs were reverse-transcribed to cDNA with a miR-224-specific stem-loop-like RT primer following the manufacturer’s protocol. For miRNA quantification, each reverse transcription reaction consisted of 0.5 ug of total RNA, mixed with 2.5 ul of 5×RT buffer containing dNTPs, 0.2 ul of 10 umol/l stem-loop RT primer, 0.2 ul RNase inhibitor protein, and 0.5 ul reverse transcriptase in a final volume of 10 ul, and then incubated at 70°C for 10 minutes, 0°C for three minutes, 42°C for 60 minutes and at 70°C for 15 minutes. Real-time PCR was performed with an Applied Biosystems 7900HT Fast Real-time PCR system using the SYBR Premix Ex Taq (perfect real time). PCR volume was 20 ul, containing 1 ul reverse transcript product. Cycling conditions were 1 cycle of 95°C for 30 seconds and 40 cycles of 95°C for five seconds and 60°C for 30 seconds. PCR was performed in triplicate. Real-time PCR was performed using SYBR Premix Ex Taq. The U6 snRNA was used as endogenous control for miRNA. The sequences of the primers are given in Table 1. The ΔCt method was used to determine relative quantization of miRNA expression in samples, and the fold change was determined as 2-ΔΔCt.

**Cytotoxicity assays**

Cells were triplicately seeded in flat-bottomed 96-well plates at a density of 6×103 per well in 100 ul culture medium and allowed to adhere overnight. Then the cells were transfected with miR-224 mimic or negative control mimic. After 24 hours incubation, the culture medium was replaced with fresh medium and supplemented with various paclitaxel doses (0, 5, 10, 20, 50, and 100 nM). After three days, replaced the medium with 100 ul fresh medium, 10 ul Cell Counting Kit-8 (CCK8; 1:10) was added to each well according to manufacturer’s instructions. After two hours in culture at 37°C, the cell viability was determined by measuring the absorbance at 450 nm using a 550 Bio-Rad plate-reader. Once the absorbance at 450 nm was recorded, the inhibitory concentration of 50% of cells (IC50) was calculated. The assays were conducted in triplicate and repeated at least three times.

**Statistical analysis**

The experiments were repeated at least three times. Results are expressed as mean ± SD. An independent Student’s t-test or an ANOVA was used to compare continuous variables. P < 0.05 was considered as statistically significant. All analyses were performed using SPSS 16.0 software (SPSS).

**Results**

**Paclitaxel decreases miR-224 expression in cervical cancer cells.**

The SiHa cells were cultured with different amounts of paclitaxel (0, 5, and 10 nM) for 72 hours. Then the authors observed the expression of miR-224 pre- and post-paclitaxel treatment by using stem-loop real-time RT-PCR. The result showed that miR-224 was significantly downregulated with fold values at 2.130435 and 4.26087 under 5 and 10 nM paclitaxel treatments, respectively (Figure 1). This finding may suggest that paclitaxel induces a decrease in miR-224 expression in a clear dose-dependent manner.

**miR-224 expression is markedly increased in SiHa cells after transfected with miRIDIAN miR-224 mimic.**

MiRNA mimic or negative control was transfected into the human cervical carcinoma cell line SiHa cells and the expression of miR-224 was detected in 72 hours post-transfection. The result showed a significant upregulation of miR-224 expression in SiHa cells transfected with miR-224 mimic compared with the negative control. miR-224 was overexpressed in SiHa cells with an increased 1605.742-fold (p < 0.0001) compared with the negative control (Figure 2).
Upregulation of microRNA-224 sensitizes human cervical cells SiHa to paclitaxel

Exogenous miR-224 facilitates paclitaxel sensitivity in cervical cancer cells.

To confirm the involvement of miR-224 in regulating paclitaxel sensitivity in cervical cancer cells, the authors exogenously upregulated miR-224 expression using miRIDIAN miR-224 mimic and observed its impact on paclitaxel sensitivity using CCK-8. Furthermore, the drug sensitivity testing was determined with CCK-8 assay at 72 hours with different paclitaxel doses (0, 5, 10, 20, 50, and 100 nM). As shown in Figure 3, paclitaxel sensitivities were significantly increased after forced overexpression of miR-224 in SiHa cells compared with miRNA-negative controls. The IC50 value was decreased in SiHa with overexpression of miR-224 (15.72 ± 1.887 nM) compared with miRNA-negative control (66.09 ± 5.966 nM) (p < 0.0001). The present findings suggested that miR-224 positively modulates the sensitivity to paclitaxel in cervical cancer cells.

Discussion

Chemotherapy is one of the major widely used treatment methods in cancer, while many kinds of cancer are still refractory to chemotherapy [21, 22]. Deep understanding of drug resistance mechanisms is needed to improve the chemotherapy response. Nowadays, drug resistance is considered as a multifactorial phenomenon involving several major mechanisms, such as increased repair of DNA damage, reduced apoptosis, altered metabolism of drugs, and increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cells [23-26]. The activation of drug resistance

Figure 1. — The downregulated expression of miR-224 is paclitaxel dependence. The value of miR-224 was 0.98, 0.46, and 0.23 respectively, under 0, 5 and 10 nM paclitaxel treatment. MiR-224 was significantly decreased with the increase of paclitaxel (p = 0.03).

Figure 2. — miR-224 expression is markedly increased in SiHa cells after transfected. Real-time PCR validated the expression of miR-224 was increased by 1605.742-fold compared with the negative control (p < 0.0001).

Figure 3. — The overexpression of miR-224 can promote the paclitaxel sensitivity in cervical cancer SiHa cells. The expression of miR-224 was upregulated by using miRIDIAN miR-224 mimic and its influence on paclitaxel sensitivity in SiHa cells at 72 hours was observed using CCK-8. Paclitaxel sensitivity was significantly increased after enforced overexpression of miR-224 in SiHa cells compared with negative control.
mechanisms can occur at the genetic level through gene amplification, the transcriptional level through epigenetic modifications, or the proteomic level through mutation or aberrant expression [27]. Recently, the evidence of the roles of microRNAs in determining drug sensitivity/resistance has been emerging.

MiR-224 was originally identified and ends mapped by cloning from Weri cells in human [28]. Its sequence maps to chromosome X [29]. Accumulating studies have found that miR-224 may act as a potential oncogenic miRNA. For instance, miR-224 expression is frequently upregulated in hepatocellular carcinoma [16], colorectal cancer [17], medullo-blastoma [30], thyroid cancer [31], pancreatic ductal adenocarcinoma [32], prostate cancer [33], and renal cancer [34]. However, there are also some opposite findings, such as the downregulated expression of miR-224 in ovarian cancer and oral carcinoma [35-36]. This discrepancy may be related to different actions of the same miRNA in different kinds of cancer. In cervical cancer, the present authors’ previous study and Rao et al.’s study all identified miR-224 to be significantly upregulated by 2.7-3.2 fold in cervical cancer tissues compared with cervical normal tissues [15, 37]. Furthermore, miR-224 upregulation was associated with aggressive progression and poor prognosis in cervical cancer [38]. Until now, there has been no study of the association between miR-224 expression and chemotherapy sensitivity in cervical cancer.

By comparing the miRNA microarray profiles, the present authors previously found that 21 miRNAs (including miR-224) were differentially expressed between self-paired pre- and post-chemotherapy cancer tissues. Then 5 (miR-375, miR-424, miR-181b, miR-224, and miR-27a) were selected and validated in paclitaxel-treated cervical cancer cell lines (Caski and SiHa), of which miR-224 was the most significantly downregulated with fold values range at 1.91–2.05 and 6.3–7.89 under 5 and 10 nM paclitaxel treatment, significantly downregulated with fold values range at 1.91–2.05 and 6.3–7.89 under 5 and 10 nM paclitaxel treatment, respectively. Here, the authors further observed the association between miR-224 expression and paclitaxel treatment in cervical cancer SiHa cell line and found that miR-224 expression was downregulated in SiHa cells following paclitaxel treatment in vitro in a clear dose-dependent manner. Moreover, they demonstrated that exogenous miR-224 facilitated paclitaxel sensitivity in cervical cancer cells in vitro and paclitaxel sensitivities were significantly increased after forced overexpression of miR-224 in SiHa cells compared with miRNA-negative controls. Thus, the present findings suggest that paclitaxel may induce an acquired drug resistance in cervical cancer cells. The authors demonstrated in this study that the upregulation of miR-224 expression by a miR-224 mimic contributed to sensitizing human cervical cancer SiHa cells to the anticancer drug paclitaxel. In the clinic, the development of secondary drug resistance probably downregulated miR-224 expression in cervical cancer cells and enforced overexpression of miR-224 may be able to reverse paclitaxel resistance in cervical cancer.

In summary, the authors find for the first time, to their knowledge, that paclitaxel downregulates miR-224 expression and overexpressed miR-224 inversely reduces chemo-resistance in cervical cancer in vitro. The present study highlights a potential role of miR-224 in the development of drug resistance in cervical cancer and suggests that miR-224 might serve as a predictor for paclitaxel response or a therapeutic target in cervical cancer therapy.

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References


Tracking of cervical cancer in 7,519 patients: a study of the prevalence of altered cytologies

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Summary

Purpose: To evaluate the outcome and adherence of 535 patients with cytological changes. Materials and Methods: Study of 7519 smear tests harvested in 2007. Results: Of the 7,519 (100%) patients analyzed, 6,964 (92.6%) had cytology negative for intraepithelial lesion or malignancy, 535 (7.1%) abnormalities in epithelial cells, and 20 (0.3%) were unsatisfactory. Of these 535 (100%) patients, 511 (95.5%) were referred to the outpatient clinic and colposcopic exam submitted and 24 (4.5%) did not return to the clinic. The group participated in the ambulatory visits, 302 (59.1%) underwent colposcopy-guided biopsy, and the remaining 209 (40.9%) examinations were negative. Conclusion: The cytological examination remains the method of choice for cervical cancer screening. It includes low costs, is effective, and well-accepted. Early diagnosis minimises the cost of research. Universities have an important role in the training of health professionals and also in the development of research.

Key words: Uterine cervical neoplasm; Biopsy; Cytopathology; Screening.

Introduction

In the 1940s the tracking of cervical cancer began, following a publication by Papanicolaou and Traut [1] about the cytological coloration technique which allowed the identification of malignant neoplasias using a vaginal smear.

Globally, cervical cancer [2, 3] represents a public health problem, with an incidence twice as high in less developed countries, while it is also the third most common form of cancer among women. According to estimates [2, 3] there will be 530,000 new cases of cancer in 2012, and it will be responsible for the death of 275,000 women.

The beginning of sexual activity at a precocious age, the greater number of sexual partners, and multiparity are considered the preponderant risk factors [2] for cervical cancer. There is epidemiological evidence that indicates that it is a sexually transmitted disease (STD) caused by the human papillomavirus (HPV) and is an important risk factor in cervical carcinogenesis. Meisels and Morín [4]; Zür Hausen et al. [5], and Pater and Pater [6] have confirmed the presence of HPV DNA in the genome of carcinomatous cells. Zür Hausen [7] observed the presence of HPV in more than 90% of squamous cervical carcinomas. In this manner, the relationship between HPV and cervical cancer defined in the above research, as well as in others, can be confirmed.

Epidemiological studies [8, 9] have demonstrated that HPV is responsible for the progression of cervical intraepithelial neoplasias (CIN) with a high level, which can occur up to two years after the primary infection.

Cervical cancer [10] has a long clinical evolution, allowing its detection in an incipient phase. Since the 1960s the practice of tracking has led to a satisfactory reduction in the incidence of mortality in the majority of developed countries.

Papanicolaou’s exam is part of this strategy [2] since it has a low cost, is simple to carry out and administrate, can be applied to the whole population, has high sensitivity, and is very specific, as well as allowing the precocious treatment of cancer and its precursory lesions. It is therefore necessary to guarantee the organization, the integrity and the quality of the program, as well as the follow-up of patients.

Stimulated by the Viva Women Program (the National Program for the Control of Cervical and Breast Cancer), created in 1996 in Brazil [11], the control of cervical cancer was consolidated as a priority in the National Policy for Oncological Care (INCA, 2005), in the Pact for Health (Brasil, 2006), and the Plan for Strengthening the Network for the Prevention, Diagnosis, and Treatment of Cancer in 2011. Despite all these government actions, it is the second most frequent tumor [2] in the female population and the fourth cause of death for women due to cancer in Brazil. In 2012, 17,540 cases of cervical cancer are expected [2], with an estimated risk of 17 cases in every 100,000 women and 4,800 fatal victims per year. The initial age group at risk [2] is 20 - 29 years old and the risk increases until it reaches its peak between 50 and 60 years.
The International Union Against Cancer (UICC) concluded in 2006 [3-12] that in the population tracking there were errors in sampling, in the technique of preparing and processing slides, in the control of laboratory quality, in monitoring, in the interpretation of the smear, and in the preparation and writing of reports. Most responsible for false-negative rates were the precarious collection of samples (60% and interpretation errors 40%).

The importance of cytological tracking in the detection of precancerous and invasive lesions has served as a stimulus to evaluate the number of patients with altered cytological exams and lacking outpatient care after the preventive exam.

The main objective of the study was to evaluate the result of 535 cytological changed examinations and specifically, the forwarding and follow-up of patients with altered cytology results and how patients adhere to outpatient control.

Materials and Methods

A transversal study with a retrospective analysis of 7,519 exams of patients who are users of the Sistema Único de Saúde (SUS – Single Health System) in the outpatient clinics of the Department of Gynecology, Escola Paulista de Medicina (EPM) and Universidade Federal de São Paulo (UNIFESP), who during their consultations had material collected for the Papanicolaou exam, in the period between January and December 2007. Included were all patients who were not menstruating and who had not previously done an endovaginal ultrasound, used vaginal cream, and/or had sexual relations in the previous 72 hours.

Cytopathological exam

The exams were carried out by medical residents, post-graduate students, attending doctors, and nursing professionals. The material collected was sent to the Cytopathological Laboratory of the EPM/UNIFESP Department of Gynecology. The collection of the cytopathological exam used the triple technique (collects ectocervical and endocervical, vaginal).

Cytopathological tracking

The cytological readings were interpreted according to the Bethesda System Terminology (BST 2001 - Table 1). Patients with altered cytologies were directed to the Gynecological Diseases Prevention Group (Núcleo de Prevenção de Doenças Ginecológicas – NUPREV) in the Department of Gynecology (EPM/UNIFESP), for diagnostic confirmation using colposcopy exams, biopsies, and anatomopathology exams, and afterwards received the suitable treatment.

Data collection

The results of the cytopathological exams were obtained from the Information System of the Cytopathology Laboratory, Department of Gynecology (EPM/UNIFESP), catalogued in accordance with the entry date of the exam. After this, the development of the patients with abnormalities in epithelial cells was analyzed, with data from January to September 2008 being collected to check anatomo-pathological reports and the scheduling of further outpatient consultations.

Clinical conduct

To analyze the data, the protocol suggested by the National Institute of Cancer (Nacional do Câncer), INCA-2012 [11], was used to follow up the patients who had done the Papanicolaou exam, covering the behavior expected in accordance with the result of the cytopathological exam. Those with references to material that was unsatisfactory for oncological evaluation had to be immediately repeated. Altered cytologies due to low grade intraepithelial lesions (LSIL), epithelial cell abnormalities of undetermined significance (ASC-US), epithelial cell abnormalities cannot exclude - HSIL (ASC-H), and atypical glands had to be treated within, if there was a specific and repeated agent, in a maximum of six months. If cellular alterations persisted, it was important to continue the investigation. In regard to results with high level lesions, epidermoid carcinomas, and invasive adenocarcinomas, investigation with colposcopy and biopsy was indicated.

Ethics committee

This project was submitted to and approved by the Research Ethics Committee of Universidade Federal de São Paulo/Hospital São Paulo.

Results

The research included the tracking of cervical cancer using the Papanicolaou exam in patients between 11 and 89 years of age, with an average age of 45. No patients were excluded after the collection of material.

Of the 7,519 (100%) patients analyzed, 6,964 (92.6%) had negative cytologies for intraepithelial lesions or malignity, 535 (7.1%) had abnormalities in epithelial cells, and 20 (0.3%) of the samples were unsatisfactory. The 20 patients with unsatisfactory exams were recalled twice and 14 continued to have unsatisfactory cytology.

Table 1 shows the results of the cytopathological exams of the 535 (100%) patients who had abnormalities in epithelial cells. Of this total, 173 (32.3%) had atypical cells with an indeterminate meaning, 269 (50.3%) were LSIL, 66 (12.3%) were HSIL, 24 (4.5%) were spinocellular carcinoma, and three (0.6%) were adenocarcinoma. Also in relation to these 535 (100%), 24 (4.5%) did not return to the outpatient clinic, 511 (95.5%) returned and were directed to the specialized service.

Of the group of 511 (100%) patients who returned, 302 (59.1%) underwent a colposcopy, directed biopsy, and anatomo-pathology, with the treatment carried out according to the biopsy result. For the other 209 (40.9%), the col-

<table>
<thead>
<tr>
<th>Cytological results</th>
<th>No. of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atypias with undetermined significance</td>
<td>173 (32.3%)</td>
</tr>
<tr>
<td>LSIL</td>
<td>269 (50.3%)</td>
</tr>
<tr>
<td>HSIL</td>
<td>66 (12.3%)</td>
</tr>
<tr>
<td>Spinocellular carcinoma</td>
<td>24 (4.5%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>3 (0.6%)</td>
</tr>
<tr>
<td>Total cases</td>
<td>535 (100%)</td>
</tr>
</tbody>
</table>
poscopy exam was negative and no biopsy carried out, as there was expectant behavior.

Of the 302 (100%) who were biopsied, 76 (25.2%) had atypias with an undetermined significance and the other 226 (74.8%) had intraepithelial lesions of a low or high degree, carcinoma, and adenocarcinoma.

Table 2 shows the cyto-histological correlation of the 76 patients with atypias with an undetermined significance.

<table>
<thead>
<tr>
<th>Biopsy result</th>
<th>Cytologies with atypias with an undetermined significance N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic cervicitis</td>
<td>45 (59.2%)</td>
</tr>
<tr>
<td>LSIL</td>
<td>27 (35.5%)</td>
</tr>
<tr>
<td>HSIL</td>
<td>1 (1.3%)</td>
</tr>
<tr>
<td>Spinocellular carcinoma</td>
<td>1 (1.3%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>2 (2.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>76 (100%)</td>
</tr>
</tbody>
</table>

Table 3 shows the cyto-histological correlation of the 226 patients who received LSIL, HSIL, CEC, and adenocarcinoma cytological results.

<table>
<thead>
<tr>
<th>Biopsy N (%)</th>
<th>LSIL N (%)</th>
<th>HSIL N (%)</th>
<th>CEC N (%)</th>
<th>Adenocarcinoma N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic cervicitis</td>
<td>50 (25.7%)</td>
<td>8 (25.7%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>58 (25.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSIL</td>
<td>86 (25.7%)</td>
<td>10 (25.7%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>96 (25.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSIL</td>
<td>11 (4.9%)</td>
<td>39 (17.3%)</td>
<td>8 (3.6%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>58 (25.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinocellular carcinoma</td>
<td>0 (0%)</td>
<td>1 (0.4%)</td>
<td>10 (4.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>11 (4.9%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (1.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>3 (1.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>147 (65%)</td>
<td>58 (25.7%)</td>
<td>18 (8.0%)</td>
<td>3 (1.3%)</td>
</tr>
<tr>
<td>226 (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

lesions, and carcinomas, who were not biopsied during the colposcopy.

Of the 535 patients with altered cytology, 511 (95.5%) continued treatment in a specialized outpatient clinic. Only 24 (4.5%) did not return to the clinic. Various researches were carried out in the records of these 24 (100%) patients to describe outpatient absenteeism. It was found that 17 (70.8%) used the hospital register (HR) of other patients and seven (29.2%) had not made appointments for socio-economic reasons.

Table 5 shows the result of the cytological exams of the patients who did not return to the clinic. Of these, the most frequent were the 17 (70.8%) who had LSIL cytological results, followed by five (20.8%) with atypias with an undetermined significance, one (4.2%) with HSIL, and one (4.2%) with CEC.

Discussion

The high level of prevalence and mortality of cervical cancer [2] found in developing countries causes serious difficulties in public health. Frequent sick leave and hospitalizations prevent patients from exercising their social role in the family and at work. For this reason, educational measures [13], which can explain the natural history of the disease, show the risk factors, and stimulate patient participation in regular exams, are objective attitudes involving the community, with the intention of reducing morbimortality rate.

Medeiros et al. [14] observed that among 760,501 women who did the Papanicolaou exam, only 6.4% had altered cytologies. Rama et al. [15], in a transversal study with 5,477 women, found that 6.4% of cytologies had ab-
normal results. Corroborating this data, 7,519 patients were tracked and 535 (7.1%) were identified with epithelial cells.

It is a fact that the slow evolution of this cancer allows diagnosis in the intraepithelial phase, in asymptomatic women, where treatment is low cost [16]. In this research Ostor [17] managed to evaluate the regression, persistence, and progression of intraepithelial lesions. He concluded that there was regression in 60% of low grade intraepithelial neoplasia cases, with persistence of 30% and progression in about 10% to high grade intraepithelial neoplasia and 1% to invasive carcinoma. Consequently, delays in tracking can lead to progression in the lesion, meaning more invasive treatment is necessary. Hutchinson et al. [18] estimated that around 25% of cases of cervical cancer occur in patients who are regularly examined at least once every three years.

According to the Ministry of Health [19], around 40% of patients who do cytological exams (Papanicolaou) do not return to the clinic to check the result. Often they are the target of tracking. In this study it was found that of the 535 (100%) with altered oncotic cytology, only 24 (4.5%) did not return to the clinic during the period of the research. Tracking of cervical cancer [19] in developed countries improved after criteria were established to control and call patients.

In the study by Thuler et al. [20], it was found that of the 10,505,773 exams included in the Cervical Cancer Information System (Sistema de Informação do Câncer do Colo do Útero - SISCOLO) in 2002, 1.66% (144,415 exams) were considered unsatisfactory. These type of exam results cause disturbances for patients and increase the cost of prevention programs. Generally they are mainly associated with problems in the collection and setting of samples. In this study of the 7,519 (100%) patients submitted to cervical cancer tracking, only 20 (0.3%) had unsatisfactory exams.

Triple collection is part of the routine of cervical cancer tracking in the UNIFESP Department of Gynecology. The use of a single slide and triple collection minimizes the cost of the exam, facilitates the reading of slides, as well as increasing the operational capacity of the laboratory, without compromising the final result.

Using SISCOLO, Thuler et al. [20] managed to evaluate the monitoring of 1,028 laboratories which carried out Papanicolaou exams and showed the importance of this data for the improvement of cervical cancer tracking programs in Brazil.

Conclusion

Of the 7,519 patients who did a cytological exam, 535 had altered results and were sent to a specialized clinic for further investigations. Out of these 535 (100%) patients, 511 (95.5%) patients were sent to a specialized clinic for a colposcopy, and 24 (4.5%) did not return to the clinic. Finally, of the group who went to the specialized clinic, 302 (59.1%) had a biopsy directed by the colposcopy, while the exam was negative for the other 209 (40.9%). Consequently they had expectant behavior. Patients who did not obtain a cyto-histological correlation were investigated afterwards.

The cytological exam is considered the oldest and best established prevention program. It continues to be the method of choice for tracking cervical cancer. It has a low cost, is efficient, and well-accepted. However, it is a vulnerable to errors in collection and the preparation of slides, and has subjectivity in reading. Precocious diagnosis and treatment minimizes the cost of the entire investigatory process. In a general manner, universities have a very important role in the training of specialist doctors, health professions, and also in the preparation of research projects.

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[11] Programa Nacional de Controle do Câncer do Colo do Útero. Available at: http://www2.inca.gov.br/wps/wcm/connect/9ab3788046a6903a40f0d189676b0/pdf_pncc_coloutero.pdf?MOD=AJPERES&CACHEID=9ab”378804a6903a40f0d189676b0

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An evaluation comparing Californium$^{252}$ neutron brachytherapy with neoadjuvant intra-arterial embolism chemotherapy assisted surgery effect for treating advanced cervical carcinoma patients

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$^1$ Department of Gynecology and Obstetrics, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou
$^2$ Department of Radiation Oncology, Wu Jing Zong Dai Hospital of Guangdong province, Guangzhou (China)

Summary

Purpose of investigation: To compare the therapeutic and side effects of using Californium$^{252}$ ($^{252}$ Cf) neutron brachytherapy with neoadjuvant intra-arterial embolism chemotherapy in combination with surgery for treating Stage Ib2-IIb cervical cancers (CCs). Materials and Methods: Thirty-two Stage Ib2-IIb CC patients were enrolled and randomly divided into two groups from January 2007 to April 2010 in the present Hospital. Prior to surgery within four weeks, a total of 17 cases were treated with $^{252}$Cf neutron brachytherapy (700-800 cGy doses at point A) once a week (Group A), and 15 cases were treated by neoadjuvant intra-arterial embolism chemotherapy using a combination of bleomycin, carboplatin, and cyclophosphamide twice (Group B). The clinical symptoms and signs, side effects, and relapse condition follow up until July 2013 were compared between the two groups for the perioperation. Results: Reductions in tumor mass and CR+PR were not significantly different between the groups before the surgery ($p > 0.05$). Abdominal pain and pelvic adhesions were significantly more severe in Group B ($p < 0.05$). There were no significant differences in surgical time, blood loss or the other side effects between Groups A and B ($p > 0.05$). The percentage of pelvic tumor recurrences in Group A was lower than that of the patients in Group B (11.8% vs 20.0%) although with no significant difference at present. No distant metastasis has been found in both two groups. Conclusion: Except for less abdominal pain and pelvic adhesions, $^{252}$Cf neutron brachytherapy has perioperative effects similar to those of neoadjuvant intra-arterial embolism chemotherapy.

Key words: Advanced cervical cancer; Neoadjuvant intra-arterial embolism chemotherapy; Californium$^{252}$ neutron brachytherapy.

Introduction

Cervical cancer (CC) is the second most common cancer in women worldwide and is a leading cause of cancer-related deaths in women in underdeveloped countries. CC is one of the most common cancers affecting a woman’s reproductive organs. In China, epidemiological research in the central and western regions has shown that CC is becoming a major health concern for women in the countryside. Nearly 100,000 cases of CC are diagnosed annually, with approximately 20,000 deaths in 2001 alone in China [1-2].

Surgery for CC may be an option in the early stages when the tumor is confined, while radiotherapy, chemotherapy or radiochemotherapy is used in the late stages of CC. No significant increases in curative rates have been observed from photon radiotherapy, despite important progress in radiotherapy techniques and quality assurance. Among the reasons for this lack of effect is the varying radiosensitivity of different tumor subpopulations. Treatment with Californium$^{252}$ ($^{252}$Cf), as a source of gamma/neutron radiation in brachytherapy, provides new treatment modalities to overcome this limitation [3-5]. However, its effects when combined with surgery need to be reevaluated. In addition, neoadjuvant chemotherapy combined with surgery for CC has recently become an important treatment for advanced CC, especially Stage Ib disease [6-7]. Intra-arterial embolism chemotherapy based on cisplatin or carboplatin has become the most common form of neoadjuvant chemotherapy. The primary purpose of this study was to compare the perioperative therapeutic effects of $^{252}$Cf neutron brachytherapy and neoadjuvant intra-arterial embolism chemotherapy when they are used with surgery to treat advanced Stage Ib-IIb CC. The relapse sites and survival were also analyzed.

Materials and Methods

Patients

From January 2007 to April 2010, a randomized study of 64 women with bulky or locally advanced Stage Ib–IIb CC was performed at the Department of Obstetrics and Gynecology of the First Affiliated Hospital of Sun Yat-sen University. The disease stage was determined according to the FIGO classification sys-
a week continuously over four weeks in a specialized intracavitary therapy room of the outpatient department at Wu Jing Zong Dui Hospital of Guangdong Province. The intracavitary 252Cf neutron component was administered in one uterovaginal application using a disposable cervical tube. Fifteen cases (Group B) were treated with two continuous courses of neoadjuvant intra-arterial embolism chemotherapy (consisting of a combination of 45 mg bleomycin, 0.35/m2 carboplatin, and 0.6/m2 cyclophosphamide) that were administered every three weeks. The examination, therapy, and short-term monitoring were performed by the same team of physicians (gynecologists and radiation oncologists).

The effects and side effects were evaluated following chemotherapy or radiotherapy prior to surgery. Ultrasound and MRI examinations were performed before and after the administration of chemotherapy or radiotherapy to evaluate the change in tumor size. A radical hysterectomy with pelvic lymphadenectomy was performed three weeks after chemotherapy or radiotherapy in all the patients. The surgical effects and relapse condition were also compared between the two groups.

Therapeutic effect criteria

The therapeutic effects were judged by the changes in tumor size before and after the chemotherapy or radiotherapy. The following criteria of the Union for International Cancer Control (UICC) were used: complete remission (CR) consisted of a tumor that completely disappeared macroscopically and no new lesions; partial remission (PR) consisted of a reduction in tumor size of ≥ 50% and no new lesions; stable disease (SD) consisted of a reduction in tumor size of ≤ 50% and no new lesions; and progressive disease (PD) consisted of no reduction in tumor size or the appearance of new lesions. CR and PR were considered to indicate effective treatment, and SD and PD were considered to indicate ineffective treatment. The patients who died for reasons other than cervical carcinoma and had no evidence of disease at the most recent checkup were included when calculating the overall survival rate.

Statistical analysis

A statistical comparison of the treatment results between the groups was performed with SPSS Version 13.0 using the Kaplan-Meier method for survival analysis and the log-rank test and Mann-Whitney nonparametric tests for side effects rates. The t-test and χ2 test in SPSS 13.0 were also used. A p value <0.05 was considered significant.

Results

Comparison of therapeutic effect between the two groups

Group A was treated with four courses of 252Cf neutron brachytherapy, and Group B was treated with two courses of neoadjuvant intra-arterial embolism chemotherapy at the same intervals. The tumor sizes in the two groups before

| Table 1. — Distribution of the two patient groups by stage, grade, and histopathology. |
|---------------------------------|------|-----------------|-----|-----|-----|
| Group A | n | Squamous cell carcinoma | G1 | G2 | G3 |
| Ib2 | 12 | 12 | 0 | 1 | 1 | 10 |
| Ia | 3 | 2 | 1 | 0 | 1 | 1 |
| IIb | 2 | 2 | 0 | 2 | 0 | 0 |
| Total | 17 | 16 | 1 | 3 | 2 | 11 |
| Group B | n | Squamous cell carcinoma | G1 | G2 | G3 |
| Ib2 | 10 | 9 | 1 | 0 | 2 | 6 |
| Ia | 4 | 3 | 1 | 0 | 1 | 2 |
| IIb | 1 | 0 | 1 | 1 | 0 | 0 |
| Total | 15 | 12 | 3 | 1 | 3 | 8 |

An evaluation comparing Californium\textsuperscript{252} neutron brachytherapy with neoadjuvant intra-arterial embolism chemotherapy assisted etc.

and after chemotherapy and radiotherapy were comparable, with no significant difference between the two groups ($p > 0.05$). The tumor sizes were significantly reduced after chemotherapy or radiotherapy in each group ($p < 0.05$). The tumor sizes before and after chemotherapy and radiotherapy are shown in Table 2.

According to the UICC therapeutic effect criteria, before surgery, CR occurred in three cases, PR in 11 cases, and PD in one case for Group A. The efficacy rate (CR+PR) of Group A was 82.3%. The tumor disappeared completely with presenting an erosion in three cases. The pathology of one PD case was adenocarcinoma. In Group B, CR occurred in three cases, PR in nine cases, and SD in three cases. The efficacy rate (CR+PR) was 80.0%. The tumor disappeared completely, presenting an erosion, in three cases. Vaginal hemorrhage occurred in two cases and was immediately controlled through neoadjuvant intra-arterial embolism chemotherapy. The pathology of one SD case was adenocarcinoma. The efficacy rates were not significantly different between the two groups (Table 3).

Side effects

Usual side effects such as abdominal pain, symptoms of gastrointestinal, fever, vaginal bleeding, renal dysfunction, and bone inhibition were compared between the two groups, and routine blood tests and hepatic-renal function were monitored in the two groups after chemotherapy or radiotherapy. The curative effects and complications after therapy are shown in Table 4.

The side effects rates for abdominal pain in Group A were significant lower than those in Group B. There was almost no recent toxicity reaction in Group A, except for one case of hematuria caused by contamination from vaginal bleeding. The hemoglobin level did not change after radiotherapy even in two cases with moderate anemia (Hbs of 64g/l and 80 g/l). There was no significant hepatic or renal dysfunction in each group.

Comparison of the following surgery

All patients had radical hysterectomies with adnexectomy and pelvic lymphadenectomy. The blood loss ranged from 400 to 1,500 ml and from 200 to 1,500 ml with averages of 673 ml and 450 ml in Groups A and B, respectively ($p > 0.05$). Group A had a slightly longer mean surgical time (288 minutes) than Group B (276 minutes; $p > 0.05$). During the procedures, no pelvic adhesions were found in Group A, but ten cases of different degrees of tissue necrosis and pelvic adhesions were noted in Group B. Of these ten cases, three were severe adhesions manifested by bilateral adhesion of the ureters, iliac ar-

Table 2. — Comparison of cervical tumor diameter (in mm) before and after chemotherapy and radiation between the two groups.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before therapy</td>
<td>48.8 ± 5.7</td>
<td>50.3 ± 10.9</td>
<td>0.156</td>
</tr>
<tr>
<td>After therapy</td>
<td>18.6 ± 4.7</td>
<td>20.7 ± 6.6</td>
<td>0.257</td>
</tr>
</tbody>
</table>

Tumor diameter: means of the maximal diameter, as measured by ultrasound examination and MRI.

Table 3. — Comparison of the late efficacy rates between the two groups before surgery.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>17.6% (3/17)</td>
<td>20.0% (3/15)</td>
<td>0.911</td>
</tr>
<tr>
<td>PR</td>
<td>64.7% (11/17)</td>
<td>60.0% (9/15)</td>
<td>0.823</td>
</tr>
<tr>
<td>SD</td>
<td>11.8% (2/17)</td>
<td>20.0% (3/15)</td>
<td>0.710</td>
</tr>
<tr>
<td>PD</td>
<td>5.9% (1/17)</td>
<td>0.0% (0/15)</td>
<td>0.794</td>
</tr>
<tr>
<td>CR + PR</td>
<td>82.3 (14/17)</td>
<td>80.0% (12/15)</td>
<td>0.911</td>
</tr>
</tbody>
</table>

Table 4. — Comparison of side effects and complications between the two groups before surgery.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Abdominal pain</th>
<th>Vomit</th>
<th>Fever</th>
<th>Vaginal bleeding</th>
<th>Renal dysfunction</th>
<th>Bone inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Group B</td>
<td>15</td>
<td>10</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>p value</td>
<td>0.001</td>
<td>0.350</td>
<td>0.202</td>
<td>0.737</td>
<td>0.794</td>
<td>0.189</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. — Comparison of operative conditions between the two groups.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean blood loss (ml)</td>
<td>673 ± 26</td>
<td>450 ± 15</td>
<td>0.238</td>
</tr>
<tr>
<td>Mean surgical time (minutes)</td>
<td>288 ± 38</td>
<td>276 ± 20</td>
<td>0.317</td>
</tr>
<tr>
<td>Pelvic adhesions</td>
<td>0/17</td>
<td>10/15</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 6. — Relapse patterns in patients with cervical carcinoma after treatment in the two groups.

<table>
<thead>
<tr>
<th></th>
<th>Stage IB2</th>
<th>Stage IIA</th>
<th>Stage IIB</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Recurrence in the pelvis</td>
<td>0/17</td>
<td>1/17</td>
<td>1/17</td>
</tr>
<tr>
<td></td>
<td>Distant metastasis</td>
<td>0/17</td>
<td>0/17</td>
<td>0/17</td>
</tr>
<tr>
<td>Group B</td>
<td>Recurrence in the pelvis</td>
<td>0/15</td>
<td>2/15</td>
<td>1/15</td>
</tr>
<tr>
<td></td>
<td>Distant metastasis</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
</tr>
</tbody>
</table>

The percentage of recurrence in the pelvis in Group A was lower than that of the patients in Group B (11.8% vs 20.0%) although with no significant difference.
tery, and surrounding tissue. Most cases of tissue necrosis around the ureters were formulated at the ureteral tunnel with adhesions that led to surgical difficulties. The pelvic adhesion rate of Group B was significantly higher than that of Group A ($p < 0.001$) (Table 5).

Follow-up of relapse and survival

Throughout the study period from January 2007 to July 2013, two cases in Stage Ib2 in Group A were lost to follow-up during 2011, and the remaining 15 patients in Group A were alive at the end of the study. All the patients in Group B were alive. The relapse patterns in both treatment groups are shown in Table 6.

In the patients treated with $^{252}$Cf pre-surgery, the percentage of pelvic tumor recurrences was 11.8% (2/17), which was lower than that of the patients treated with neoadjuvant intra-arterial embolism chemotherapy (20.0%, $p = 0.87$, $p > 0.05$) although with no significant difference at present. All the relapses were characterized as new lesions or vaginal residuals in the pelvis without distant metastasis.

Discussion

The therapeutic outcome and survival of patients with bulky or locally advanced CC remains poor because of the large amount of bleeding during surgery, surgical difficulties, and tumor residuals, which lead to pelvic lymph node metastasis. To reduce the tumor volume, decrease tumor cell activity, prevent dissemination, and improve the surgical resection possibility and survival rate, a comprehensive therapeutic regimen of combined surgery, radiotherapy, and chemotherapy has been developed according to FIGO 2003 guidelines [9]. Intra-arterial chemotherapy may increase the tumor’s exposure to high drug concentrations in the local pelvis, which can be four to 22 times that of intravenous chemotherapy, while decreasing systemic drug delivery to tissues. Combined with embolization, intra-arterial chemotherapy may prolong the response time of the chemotherapy, simultaneously increase ischemia, and necrosis of the tumor tissues themselves, and can also effectively control large vaginal hemorrhages [10-11]. All of the above considerations have identified to increase the surgical possibilities and success rates. In recent years, the efficacy rate and CR rate of platinum-combination chemotherapy have been >80% and 9% to 18%, respectively, in cases of advanced CC [12]. In the present study, intra-arterial embolism chemotherapy combined with cisplatin, bleomycin, and cyclophosphamide prior to surgery was given to patients with advanced Stage Ib2-Ib bulky and locally CC. The efficacy rates were the same (80%), but CR rate increased to 20%; these values are similar to other previously reported chemotherapy regimens.

The discovery of $^{244}$Cf as a neutron/gamma radiation source in 1950, followed by the discovery of its radionuclide $^{252}$Cf in 1956, which opened up the possibility of high linear energy transfer rays (high-LET) brachytherapy in tumor treatment. In early studies showed that, depending on the method of administration, the inclusion of $^{252}$Cf in tumor brachytherapy often produced better results than conventional brachytherapy, thereby opening the prospect of improved therapeutic outcomes [13, 14]. These investigations on $^{252}$Cf demonstrated that during high-LET irradiation, the section of the cell survival curve that benefits from the repair of sublethal damage is practically nonexistent. The lethal effects caused by the single-hit, irreparable damage of the neutron component become prominent. During $^{252}$Cf tumor therapy, therefore, there is no need for prolonged irradiation to achieve the desired biological effect [15]. The ability of X-rays and $\gamma$-rays to kill cancer cells decreases because of increased cellular hypoxia and decreased radiation sensitivity in locally advanced cervical carcinoma, which causes cancer cell residual and recurrence of the tumor due to hypoxia. From the mechanism and clinical trials, $^{252}$Cf neutron rays allow for more rapid regression of tumor tissues, a higher local control rate, and less recurrence due to their ability to kill hypoxic and aerobic cells. A decreased reliance on the cell cycle for the further biological effects of high-LET radiation leads to a lower oxygen enhancement rate (OER) [16], inhibition of sublethal and potentially lethal cell damage repair [17], and a minimal dependence of radiation sensitivity on the cell cycle. A previous study has reported that three courses of $^{252}$Cf neutron brachytherapy were effective in 72% of patients with locally advanced Stage Ib2 CC. This study concluded that $^{252}$Cf is more effective for ray-resistant tumors by comparing 117 cases of $^{252}$Cf brachytherapy to 110 cases of $\gamma$-ray brachytherapy. More than 90% of the radiation reached the target organs [18]. In the present study with advanced CC, the therapeutic efficacy rate of $^{252}$Cf brachytherapy was found to be 82.4%, which is similar to that of intra-arterial embolism chemotherapy. Although the mechanisms of $^{252}$Cf brachytherapy are completely different from those of intra-arterial embolism chemotherapy, the clinical therapeutic effects at the same time intervals were similar to each other.

According to this research, the incidence of abdominal pain was significantly higher in patients treated with intra-arterial embolism chemotherapy, which may have been caused by local tissue ischemia. Although pain control was often needed in some cases, there was obvious improvement by the following day. The high incidence of fever was found in the intra-arterial embolism chemotherapy group, which may have been related to the invasive nature of the procedure and the absorption of embolic agents. It should be noted in this study, however, that $^{252}$Cf brachytherapy allows the direct interaction of neutrons with the cells of the tumor population, thus minimizing...
the postradiation damage to healthy tissues [19]. The present authors found that $^{252}$Cf neutron brachytherapy caused no obvious abdominal pain and fever even bone marrow inhibition; as a result, the patients could be treated as outpatients, and even patients with the moderately anemic tolerated the therapy.

In subsequent surgeries, the incidence of pelvic adhesions was significantly higher in the intra-arterial embolism chemotherapy group, perhaps because the blocked vessels in the tumor led to tumor tissue ischemia and necrosis, hyperemia, and adhesions caused by the subsequent collateral circulation. However, in this study, the pelvic adhesions did not significantly increase the blood loss or surgical time, and no risk of injury around organs was found. By contrast, fewer pelvic adhesions were found in the continuous surgeries following $^{252}$Cf brachytherapy.

No deaths occurred in either the $^{252}$Cf neutron brachytherapy or the neoadjuvant intra-arterial embolism chemotherapy groups, perhaps because of the limited follow-up times, which currently range from 34 to 73 months. Further research is needed to compare the survival rates.

$^{252}$Cf brachytherapy improves surgical therapeutic effects similar to those of intra-arterial embolism chemotherapy in bulky or local cervical carcinomas while causing fewer pelvic adhesions and less discomfort, such as abdominal pain.

Acknowledgements

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Diagnostic accuracy of 1.5 Tesla breast magnetic resonance imaging in the pre-operative assessment of axillary lymph nodes

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Summary
The purpose of this study was to test the accuracy of 1.5 Tesla magnetic resonance imaging (1.5T MRI) in the preoperative evaluation of axillary lymph nodes in patients with invasive breast cancer. The authors retrospectively analyzed 26 patients with invasive breast cancer who had undergone sentinel lymph node biopsy (SLNB) and/or axillary lymph node dissection (ALND). All patients had been submitted to preoperative contrast enhanced breast 1.5T MRI. On the basis of lymph nodes morphological and dynamic characteristics, lymph nodes were classified as “negative” (short axis < 5 mm), “borderline” (short axis > 5 mm, absence of a hilum) or “positive” (short axis > 5 mm, absence of a hilum and also other suspicious features). The authors compared 1.5T MRI results with the outcome of histological analysis performed according to the TNM criteria; sensitivity (SE), specificity (SP), positive predictive value (PPV), and negative predictive value (NPV) of 1.5T MRI were evaluated. Considering only the lymph nodes “positive”, 1.5 T MRI showed: SE 37.8%, SP 99.3%, FP 0.7%, PPV 92.5%, and NPV 88.1%. However, considering also “borderline”, 1.5T MRI achieved: SE 75.7%, SP 99.3%, FP 0.7%, PPV 96.1%, and NPV was 95%. Contrast enhanced breast 1.5T MRI is not yet a valid alternative to histological analysis but it is a valid tool for a preoperative study of the topography of axillary lymph nodes and has the potential to become a routine method for evaluating the metastatic lymph nodes before submission to ALND.

Key words: Breast cancer; Axillary lymph nodes; Magnetic resonance imaging.

Introduction
Axillary lymph node status is one of the most important prognostic factors in patients with breast cancer [1-5]. For an accurate staging and prognosis, axillary lymph node dissection (ALND) with removal of at least ten lymph nodes is currently recommended [6]. However, this procedure may lead to side effects such as lymphedema, seroma, numbness of the arm, infection, pain, and damage to the motor nerve resulting in decreased mobility and impaired quality of life [6-18]. Screening and early detection of breast cancer, while the disease may still not have spread through the lymphatic system, have led to the development of sentinel lymph node biopsy (SLNB) to reduce morbidity linked to ALND [2, 19]. In patients with clinically negative axilla and early stage breast cancer, SLNB has thus become a standard procedure as it permits selective ALND in patients with sentinel lymph node metastases [20].

The purpose of this study was to test the diagnostic accuracy of 1.5 Tesla magnetic resonance imaging (1.5T MRI) in the preoperative evaluation of axillary lymph nodes in patients with invasive breast cancer.
micrometastasis (pNlmi): > 200 metastatic cells or one metastasis measuring 0.2 to two mm in greatest dimension [21,22].

Lymph nodes negative for malignancy, lymph nodes with ITC, and lymph nodes with micrometastases were classified as “negative” according to the meta-analysis published by Bilimoria et al., who concluded that these types of lymph nodes have no influence on the choice of treatment [24].

All MRI examinations were performed using a 1.5T magnet and a dedicated four-channel breast coil with the patient in the prone position. Image acquisition was carried out in accordance with the international guidelines issued by the European Society of Breast Imaging [25]. After localizer sequences in three orthogonal planes, the following sequences were acquired:

- axial T2-weighted short-time inversion recovery (TIRM) sequences (repetition time (TR)/echo time (TE) 6000/140 ms, field of view (FOV) = 340 x 340, slice thickness: four mm, matrix 320 x 190, NEX 2.00);
- axial T1-weighted fast spin echo (FSE) sequences (TR/TE 467/9.9 ms, FOV = 380 x 380, slice thickness: four mm, matrix 320 x 224, NEX 1.00);
- axial T1-weighted FLASH 3D Dynamic (FL 3D DYN) sequences in the axial plane, before and five times after contrast administration (TR/TE 5.8/2.8 ms, FOV 380 x 380, slice thickness: two mm, matrix 320 x 320, NEX 1.00).

Contrast medium was gadobenate-dimeglumine administered in a concentration of 0.2 mmol/kg injected through a 20 G intravenous cannula at the rate of two ml/sec using an automatic injector, followed by infusion of 20 ml saline solution at the same speed.

- Sagittal fat-saturated T1-weighted FSE sequence (TR/TE 467/9.9 ms, FOV = 112 x 320, slice thickness: five mm, matrix 320 x 224, NEX 1.00);
- Image post-processing included temporal subtraction (contrast-enhanced minus unenhanced image) of dynamic studies with fat saturation. MR images were interpreted retrospectively by two radiologists in consensus using the picture archiving and communication system which allows 3D maximum intensity projections (MIP) of the dynamic sequences and manual selection of the window. Both radiologists were familiar with the patient’s clinical history and had reviewed all previous mammograms and ultrasound images.

A predictive model was hypothesized according to the parameters for metastatic lymph node involvement reported in the literature [26], and lymph nodes were classified as “negative”, “borderline” or “positive”:

Depending on the length of the short axis, the lymph nodes were divided into two categories: lymph nodes with a short axis < five mm and lymph nodes with a short axis ≥ five mm. Lymph nodes with a short axis ≥ five mm were interpreted as “negative”, i.e. not invaded by macrometastases. Lymph nodes with a short axis ≥ five mm were studied for the following parameters:

A) Presence or absence of a discernible hilum.
B) Other morphological or dynamic changes such as shape (round or oval), margins (regular or irregular), cortex (homogeneous if C-shaped and thickness < three mm and inhomogeneous if not C-shaped and thickness ≥ three mm), perifocal edema (presence or absence of hyperintense signal from the tissue around the lymph node on T2-weighted images), symmetry (presence or absence of difference between lymph node distribution in the axilla near the affected breast and the contralateral axilla), and enhancement (presence or absence of contrast uptake in the peripheral portion of the lymph node on T1-weighted sequences after injection of contrast agent).

Lymph nodes with a short axis ≥ five mm and absence of a hilum but no other morphological or dynamic changes were classified as “borderline” [23].

Lymph nodes with a short axis ≥ five mm, absence of a discernible hilum and other suspicious features, such as round shape, irregular margins, not C-shaped and cortical thickening, perifocal edema, asymmetry in lymph node distribution between the axilla near the affected breast and the contralateral axilla and/or ring enhancement, were classified as “positive”.

In order to assess the accuracy of the method, 1.5T MRI results were compared with the outcome of histological analysis after ALND and/or SLNB, and sensitivity (SE), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) were analyzed.

Results

Of the 66 pre-surgical breast MRI examinations which were reviewed, 17 were excluded as no suspicious abnormality was visible on the dynamic images, 15 were excluded because the patients were lost to the present institution, six were excluded because it was not possible to assess axillary lymph nodes on the images, as MRI had been performed to study the primary tumor and not the axilla, and two were excluded because the images presented motion artifacts.

A total of 26 patients met the selection criteria and were included in this retrospective study. Mean age of the patients was 56 years (range 40-65); 17 patients (65.4%) were postmenopausal and nine patients (34.6%) were premenopausal.

Histopathological analysis of the surgical specimens showed that 14 patients had carcinoma Stage T1, nine had carcinoma Stage T2, and three had carcinoma Stage T3. Histological analysis furthermore showed that 20 patients had invasive ductal carcinoma, two had invasive lobular carcinoma, and four had mixed ductal-lobular carcinoma. With regards to hormone receptor status, 11 patients had Luminal A tumor, eight had Luminal B tumor, four had triple-negative tumor, and the remaining three patients had Her2 overexpression tumor. Tumors were graded as follows: four patients had grade 1 tumors, ten had grade 2 tumors, and 12 had grade 3 tumors. Mean tumor size was 23 mm in diameter (range 7-80). According to the TNM staging system, 20 patients were staged N1 and six were staged N2.

The total number of removed lymph nodes was 372 of which 66 proved to be metastatic at histological analysis while 306 were non-metastatic. MRI identified 52 affected lymph nodes of which 27 were classified as “borderline” and 25 as “positive” (Figure 1).

MRI evidenced round shape in 21 cases (40.3%), irregular lymph node margins in 14 cases (26.9%), not C-shaped and cortical thickening in 20 cases (38.4%), and ring enhancement in 16 cases (30.7%). There was asymmetry in lymph node distribution between the axilla near the affected breast and the contralateral axilla in four cases (15.4%). No lymph nodes presented perifocal edema.
Sixteen lymph nodes (30.7%) classified as “negative” at MRI according to the described criteria were identified as metastatic at histological examination, while two lymph nodes classified as “positive” at MRI were classified as “negative” at histological analysis.

Considering as metastatic only the lymph nodes classified as “positive” and comparing the result of breast MRI with histological outcome after ALND and/or SLNB, 1.5 T MRI proved to have a SE of 37.8% and a SP of 99.3%. False positive rate was 0.7%, PPV 92.5%, and NPV 88.1%.

However, considering as metastatic lymph nodes also those classified as “borderline”, 1.5T MRI achieved a SE of 75.7% and SP of 99.3%. False positive rate was 0.7%, PPV was close to 96.1%, and NPV was 95%.

Comparison between lymph node features detected at 1.5T MRI and histological outcome revealed that the following MRI findings occurred at a greater frequency in metastatic lymph nodes: no discernible hilum (80.7% of metastatic lymph nodes), round shape (40.3% of metastatic lymph nodes), and cortical thickening (38.4% of metastatic lymph nodes).

Discussion

In patients with breast cancer, the surgeon currently has to perform a careful preoperative clinical evaluation of the axilla in order to plan SLNB or ALND, as ALND and to a lesser extent SLNB, may lead to sequelae, particularly lymphedema and functional neurological deficit [27-29]. However, clinical examination has a low SP in the preoperative evaluation, and histological analysis confirms malignancy of palpable lymph nodes in less than 50% of cases.

Non-invasive diagnostic methods have improved in recent years, and MRI may become the imaging technique of choice in the evaluation of axillary lymph nodes. This technique has several advantages: it is operator-independent and it allows reevaluation over time as it is not a real-time examination like US imaging. MRI furthermore provides visualization of the axilla on several levels.

The aim of this study was to test the accuracy of 1.5T MRI in the preoperative assessment of axillary lymph nodes using a predictive model for MRI evaluation of axillary lymph nodes in breast cancer. In order to do this, morphological and dynamic criteria were established. On the basis of these parameters, a predictive model was hypo-
esized, and the detected lymph nodes were classified as “negative”, “borderline” or “positive”.

When only the “positive” lymph nodes were considered as metastatic, SE was very low as only 37.8% proved to be metastasis positive at histologic examination, while 0.5% were false positive. Considering as metastatic lymph nodes also those classified as “borderline”, MRI achieved higher values including a SE of 75.7%. Also, PPV and NPV were higher when “borderline” lymph nodes were classified as metastatic (92.5% vs 96.1% and 88.1% vs 95%, respectively).

1.5 T MRI thus achieved a high SP in the detection of metastatic lymph nodes (99.3% both when “positive” only and when “positive “ and “borderline” were considered as metastatic). SP is perhaps the most important parameter for evaluating an imaging technique, as the main objective is not to reduce the risk of overtreatment but to avoid undertreatment.

SE achieved in this study was in agreement with the values reported in the literature [30, 31]. The present results show that lymph nodes with a short axis measuring ≥ five mm, no discernible hilum, and at least one additional suspicious finding (most frequently round shape and cortical thickening) has a high probability of being metastatic. This suggests that lymph nodes classified as “borderline” on the basis of the predictive model proposed in this study should be considered as metastatic.

Today, treatment of patients with breast cancer and metastatic lymph nodes has greatly improved. The Z0011 study confirmed the usefulness of conservative surgery in selected patients with early stage breast cancer and a maximum of two macrometastatic lymph nodes [26]. The authors furthermore stated that ALND could be omitted without compromising overall survival in these patients. However, ALND is currently recommended in all patients who do not meet the selection criteria reported in the Z0011 study, i.e., mastectomized patients, patients with a single metastatic sentinel lymph node, undergoing neoadjuvant chemotherapy, and patients undergoing partial breast irradiation.

The outcome of the present study shows that 1.5 MRI can support management decisions in patients in whom ALDN is currently recommended. A limitation of this study is that lymph node assessment was not possible in six patients due to partial visualization of the axilla; however future investigation focused on the axillary will solve this problem. In two patients the images showed motion artifacts caused by breathing movements affecting the uptake of contrast agent.

Further studies are required to confirm the predictive value of 1.5T MRI in the preoperative evaluation of axillary lymph nodes using an appropriate evaluation model. However, the improved quality of images obtained using 3 Tesla (3T) MRI and diffusion-weighted imaging (DWI) may further refine this technique. The greater spatial and contrast resolution of 3T MRI can provide a more accurate evaluation of lymph nodes, and DWI sequences can add information about the microscopic cellular environment and tumor cellularity [32].

Conclusions

Despite the accuracy of the method, contrast enhanced breast 1.5 T MRI is not yet a sufficiently valid alternative to histological analysis. The SP of 1.5T MRI is high, and only 3.8% of lymph nodes classified as “negative” according to the described criteria were identified as metastatic lymph nodes at histological examination. However, the low SE negatively affects the value of this method as a decision making tool before ALND.

Nevertheless, MRI is a valid method for studying the topography of axillary lymph nodes, and it has the potential to become a routine tool for evaluating the axilla in patients with metastatic sentinel lymph nodes before submission to ALND [25].

Future studies using 3T MRI and DWI sequences may provide a greater diagnostic accuracy than the accuracy reported in this paper. Detailed information on the status of axillary lymph nodes before surgery can help a multidisciplinary team to improve the treatment of breast cancer patients.

References

Calcitriol does not significantly enhance the efficacy of radiation of human cervical tumors in mice

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Summary

Objective: Calcitriol can enhance the sensitivity of cancer cells to radiation in vitro. The authors aimed to investigate the potential synergistic effect of calcitriol and radiation in a xenograft mouse model of human cervical cancer. Materials and Methods: Tumor-bearing mice were fed with vehicle arachis oil or 2.5 μg/kg calcitriol daily for 15 consecutive days. Some mice received ten Gy radiation on day 7 post treatment. Tumor growth was monitored, and the tumor tissues were examined by histology and electron microscopy. Results: Treatment with either calcitriol or radiation significantly inhibited the growth of implanted cervical cancers (p < 0.05 vs. control) and increased the number of dead tumor cells in the tumor sections. However, there was no significant difference in the tumor weights between the mice with radiation alone and both radiation and calcitriol treatment. Conclusion: Calcitriol had anti-tumoral activity, but failed to enhance the efficacy of radiation in human cervical cancers.

Key words: Calcitriol; Cervical tumor; Radiation response.

Introduction

Cervical cancer is one of the more common malignancies and a leading cause of cancer-related morbidity and mortality in women worldwide. Cervical cancer is estimated to affect 529,800 women annually [1]. Currently, patients with localized advanced cervical cancer are usually treated with external beam radiotherapy (EBRT), concomitant chemotherapy, and brachytherapy (BT) [2]. These therapeutic strategies have improved significantly in the local control of tumor progression and the survival of patients with cervical cancer [3]. The prognosis of patients with advanced recurrent cervical cancer depends mainly on treatment and on the site and extent of recurrence [4, 5]. However, the currently used medicines for chemotherapy, such as platinum-based chemoradiation regimens, usually cause severe side-effects and are not well tolerated in some patients [3]. Therefore, the discovery of new safer medicines for the treatment of patients with advanced cervical cancer will be of great significance.

A previous study has revealed an inverse association between vitamin D intake and cervical neoplasia risk in Japanese women [6]. Calcitriol, 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3), the biologically active metabolite of vitamin D, has been shown to regulate the growth of various types of cancer cells [7, 8]. Moreover, accumulating evidence indicates that calcitriol inhibits the growth of several cancer cells, including breast cancer cells [9]. Calcitriol can induce cell cycle arrest and apoptosis, and inhibit tumor cell invasion, metastasis, and angiogenesis [10]. Treatment with calcitriol increases the sensitivity to ionizing radiation in breast and prostate cancer cells [11, 12]. However, little is known about the impact of treatment with calcitriol on the sensitivity to radiation in human cervical tumors.

In this present study, the authors investigated the effects of calcitriol or combined radiation therapy on the growth of implanted human cervical tumors in mice. Surprisingly, they did not find that treatment with calcitriol enhanced the sensitivity of human cervical tumors to radiotherapy in vivo. They discussed the potential reasons for the failure.

Materials and Methods

Cell culture

Human cervical carcinoma HeLa cells were provided by the Experimental Center of the Second Affiliated Hospital of Harbin Medical University, Harbin, China. The cells were maintained in high glucose Dulbecco’s Modified Eagle’s medium (DMEM) supplemented by 10% fetal bovine serum at 37°C in a 5% CO2-humidified incubator.

Establishment of tumor-bearing nude mice model

Female BALB/c nude mice at four to five weeks of age and weighing 20-22 grams were obtained from a Shanghai Animal Laboratory in China. The animals were housed under a specific pathogen-free facility and had free access to food and water. Every effort was made to minimize the numbers and suffering of animals used in the experiments. The experimental protocols were approved by the Animal Ethical Committee of the Second Affiliated Hospital of Harbin Medical University, and the work was undertaken within which and conformed to the provisions of the Declaration of Helsinki.

*First co-authors and contributed equally to this work.

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HeLa cells at logarithmic growth phase were harvested, and the cells (10⁷ cells in 0.1 ml of PBS per mouse) were implanted subcutaneously into the lateral thigh of the posterior limb of individual mice. After tumor cell inoculation, the development of solid tumors in mice was monitored. Two weeks after incubation, the tumors reached an appropriate five mm in one dimension, and the animals were used for the following experiments.

**Experimental assignment and treatments**

The tumor-bearing nude mice were randomized and fed with 0.2 ml of arachis oil or with 0.2 ml of arachis oil containing 2.5 μg/kg of calcitriol by gavage daily for 15 consecutive days. Some mice from each group received ten Gy radiation using a 6MV X-ray at a focus-surface distance of 100 cm and a dose rate of two Gy/min once at day 7 post calcitriol treatment. The growth of implanted tumors in the vehicle alone control, radiation, calcitriol alone, and combined calcitriol and radiation groups (n=5 per group) of mice were monitored every five days up to 15 days post-initial treatment using a vernier caliper in a blinded manner. The tumor volume (V) was calculated by the following formula:

\[ V = \frac{1}{2} \times a \times b^2 \]

(a was the length and b was the width of tumor). At the end of the experiment, the mice were sacrificed and their tumors were dissected and weighed. The tumor growth inhibition rate was calculated using the following formula:

\[ \text{Tumor growth inhibition} \% = \left( \frac{\text{Tumor weight in control group} - \text{Tumor weight in treatment group}}{\text{Tumor weight in control group}} \right) \times 100\% . \]

**Histological examination**

The dissected tumor tissues were fixed in 4% paraformaldehyde (PFA), dehydrated with a graded ethanol series, and embedded in paraffin. The tumor tissue sections (five μm) were deparaffinized and rehydrated. Subsequently, the sections were stained with haematoxylin and eosin (HE) and examined under a light microscope. Images were captured by Image-pro plus software.

**Transmission electron microscope (TEM) analysis**

For TEM analysis, tumor tissues were fixed with 2.5% glutaraldehyde and post-fixed with 1% OsO₄. After dehydration through a graded ethanol series and acetone, the tumor tissues were embedded in Epon812 for ultra thin sectioning. The ultra thin sections were then stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope.

**Statistical analysis**

Data shown are representative photoimages or expressed as the mean ± standard deviation (SD). The difference among the groups of mice was analyzed by analysis of variance (ANOVA) using SPSS 10.0 software. A p value < 0.05 was considered statistically significant.

**Results**

To determine the effect of treatment with calcitriol on the sensitivity of human cervical cancer to radiation, BALB/c nude mice were inoculated with human cervical cancer HeLa cells to induce solid tumors. When the mice developed tumors, they were randomized and fed with vehicle or calcitriol and some of mice were subjected to radiation. All of the animals survived 15 days after initial treatment. The tumor-bearing control mice, but not the mice, which received radiation or calcitriol, showed decreased appetite, decreased activity, and weight loss at five days post-treatment. As shown the Figure 1, calcitriol, radiation alone, or combined therapy significantly mitigated the loss of body weight in animals on day 10 and day 15 post-treatment. Treatment with either calcitriol or radiation alone significantly inhibited the growth of implanted human cervical tumors (Figure 2A). However, treatment with both calcitriol and radiation did not significantly enhance the inhibition of tumor growth in mice (p > 0.05 vs. the radiation alone). Similarly, treatment with either calcitriol or radiation alone significantly reduced the weight of the dissected tumors, but both treatments had no obvious synergistic effect on reducing the size of tumors in the mice (Figure 2B).

The authors next characterized the morphology of tumor tissues from different groups of mice by histological examination. While typical tumor structure and morphology, proliferative fibrosis, and inflammatory infiltrates were observed in the tumors from the controls mice, there were obviously less infiltrates and fibroblast proliferation as well as many dead tumor cells in the tumors from both the mice receiving radiation and calcitriol (Figure 3). There was no obvious difference in the tumor morphology from the mice that had been treated with radiation alone or combined with calcitriol. Further TEM analysis revealed that nuclear condensation, chromatin margination, and membrane damage were observed in the tumors from the mice that had been treated with radiation or combined with calcitriol, but not from the control or calcitriol alone-treated mice (Figure 4).
Calcitriol does not significantly enhance the efficacy of radiation of human cervical tumors in mice.

Figure 2. — Treatment with either calcitriol or radiation inhibits the growth of implanted cervical cancer in mice. Following treatment with calcitriol, the growth of implanted tumors was monitored at the indicated time points up to 15 days post-treatment. At the end of the experiment, the tumors in individual mice were dissected and weighed. Data are expressed as the mean ± SD of the tumor sizes or weights in different groups of mice (n=5 per group). (A) The growth of implanted tumors; (B) the tumor weights. *p < 0.05 vs. the control mice treated with vehicle alone.

Figure 3. — Histological analysis of tumors. At the end of the experiment, the tumor tissues from individual mice were dissected out and subjected to histological examination by HE staining. Data shown are representative photoimages of the implanted cervical tumors from individual groups of mice (n=5 per group). Scale bar: 100 µM; The black arrows: inflammatory infiltrates; The white arrows: dead tumor cells.

Figure 4. — TEM analysis of tumors. The tumor tissues from individual mice were dissected and subjected to TEM analysis. Data shown are representative photoimages of the implanted cervical tumors from individual groups of mice (n=5 per group). Scale bar: 500 nm; The black arrows: nuclear condensation; the white arrows: membrane damage.
Together, these data suggested that treatment with calcitriol inhibited the growth of human cervical tumors in mice, but failed to enhance the sensitivity of human cervical cancer to radiation.

Discussion

In this present study, the authors examined the potential role of calcitriol in radiation responses in cervical tumor-bearing nude mice. Their results indicated that, although radiation, calcitriol alone, or combined therapy significantly inhibited the tumor growth in vivo, there was no synergistic effect of combined therapy on inhibiting the growth of human cervical cancer in mice.

Previous studies have shown that calcitriol has anti-proliferative, anti-inflammatory, pro-differentiation, and pro-apoptotic activities in many human cancers [7, 10, 13, 14]. Evidently, treatment with calcitriol inhibits the growth and promotes apoptosis of breast cancer cells via induction of reactive oxygen species (ROS) [9]. Treatment with calcitriol also induces the apoptosis of ovarian cancer cells through the down-regulation of telomerase [15] by modulating MiR-498 expression. Consistent with these observations, the present findings indicated that treatment with calcitriol greatly suppressed the growth of human cervical tumor in nude mice. It is possible that calcitriol may also induce human cervical cancer cell apoptosis in vivo. Indeed, the authors obviously observed increased dead cells and apoptotic features in the tumor section from the calcitriol–treated mice. Although the presence of vitamin D receptor (VDR) in cervical tumors remains controversial, increased levels of VDR expression were detected in cervix carcinomas, as compared with that in normal corresponding tissues [16]. Furthermore, increased levels of VDR were also detected in the cervical cancer tissues from human patients, although there was no statistically significant correlation between the levels of VDR expression in the cancers, anti-Ki-67 or anti-p53 staining, and histopathological data (tumor stage, lymph node status, grading, histological tumor type) [17]. On the contrary, there VDR expression was detected in another study of one specimen [18]. The present authors are interested in further investigating the mechanisms underlying the action of calcitriol in inhibiting the growth of human cervical cancer, including measuring the VDR expression.

Radiotherapy represents an effective treatment modality for patients with cervical cancer [2]. However, many patients with advanced cervical cancer respond poorly with tumor progression and recurrence [4, 19]. Adjuvant chemotherapeutic agent may enhance radio-sensitization of cervical tumor cells by direct toxicity against tumor cells or by inhibiting radiotherapy-related repair in the tumor [20, 21]. Although cisplatin-based chemotherapy increases the sensitivity of cervical cancer to radiotherapy, this therapeutic strategy does not prolong the survival of patients with cervical cancer due to severe side-effects [22]. Previous studies have shown that treatment with calcitriol enhances the sensitivity of different types of human cancers to radiotherapy [7, 10, 13, 14]. However, the present authors found that treatment with calcitriol failed to enhance the sensitivity of human cervical cancer to radiation in mice. These data suggest that different types of human cancer may have various responses to calcitriol treatment. They are interested in further investigating the mechanisms underlying the failure of calcitriol treatment in radiotherapy for human cervical cancer.

Conclusion

In summary, the present study indicated that treatment with calcitriol and radiation alone significantly inhibited the growth of human cervical cancer in mice. However, treatment with calcitriol failed to enhance the sensitivity of human cervical cancer to radiotherapy in mice.

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References

Calcitriol does not significantly enhance the efficacy of radiation of human cervical tumors in mice.


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Diagnostic performances of CA125, HE4, and ROMA index in ovarian cancer

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Summary

Purpose of investigation: HE4 (human epididymis protein 4) is suggested to be used as a potential new biomarker to identify ovarian malignancies from benign adnexal masses. The aim of this study was to evaluate HE4, in comparison with CA125 and Risk of Ovarian Malignancy Algorithm (ROMA) index in benign gynecological diseases and ovarian cancer, and additionally to determine the reference range for HE4 in healthy Turkish women. Materials and Methods: CA125 and HE4 serum levels were determined in 96 patients with benign gynecological diseases, 47 patients with ovarian cancer and 106 healthy women using a specific analyzer. CA125 and HE4 cut-offs were 35 U/ml and 70 pmol/L, respectively. Results: HE4 had significantly higher concentrations in ovarian cancer than benign gynecologic disorders (p < 0.005). Tumor marker sensitivity in ovarian cancer was 78% for HE4, 63% for CA125, and 88% for ROMA index at 95% specificity. A significantly higher area under the Receiver operator characteristic (ROC) curve was obtained with HE4 and ROMA index than CA125 in the differential diagnosis of benign gynecological diseases versus ovarian cancer (0.929, 0.955, and 0.781, respectively). Reference limits for serum HE4 in healthy Turkish women was determined as 28.9 - 62.4 pmol/L for premenopausal and 23.7 - 152.4 pmol/L for postmenopausal women. Conclusions: In the diagnosis of ovarian cancer, HE4 had higher sensitivity, as a single tumor marker. The sensitivity of HE4 and ROMA index in postmenopausal women was higher than premenopausal women for detecting ovarian cancer.

Key words: CA125; HE4; ROMA index; Ovarian cancer.

Introduction

Ovarian cancer is the most frequent cause of death from gynecological cancer and second most commonly diagnosed gynecologic malignancy in Europe and United States. This cancer affects mainly women in postmenopausal state with a peak at 55-65 years. Patient’s medical history, clinical examination, imaging data, and tumor marker profile is used to differentiate malignant ovarian masses from benign disorders. Since ovarian cancer is asymptomatic at early stages and no effective screening approach is available, 80-90% of ovarian cancer patients are diagnosed with advanced stage disease and five-year survival rates are less than 30%; that is why it has historically been called the “silent killer” [1-4]. Tumor markers can be used for screening, early detection of cancer, diagnostic confirmation, prognosis, monitoring disease, response to therapy, and detection of recurrence. An ideal tumor marker should be highly specific to a certain type of cancer and highly sensitive in order to avoid false positive results, and it should also be inexpensive for screening the target population [5].

CA125 (carbohydrate antigen 125) is a high molecular weight glycoprotein that is present in the fallopian tube epithelium, endocervix, endometrium, peritoneum, pleura, and pericardium [6]. CA125 is the most widely used tumor marker in ovarian cancer but has low sensitivity for detection of cancer in the early stages; only 20-30% of patients are diagnosed at an early stage whereas 80-90% of patients are diagnosed at Stages III–IV. Additionally, 20% of ovarian cancers do not express CA125 antigen [7,8]. The lack of specificity of CA125 is due to its elevated levels in many benign gynecologic diseases (endometriosis, myomas, ovarian cysts), non-gynecological malignities (pancreatic, breast, bladder, liver, lung), and physiological conditions such as pregnancy and menstruation [9,10]. Therefore, a new tumor biomarker or combination of biomarkers with high sensitivity is needed for screening, early detection of ovarian cancer, and evaluation of response to treatment.

HE4 (human epididymis protein 4) is a novel tumor marker approved by the United States FDA for diagnosis and monitoring of progressive disease in patients with epithelial ovarian cancer. HE4 is a 25kD whey acidic protein with a four disulfide core that is predominantly expressed in epithelial cells of the epididymis, respiratory epithelium of proximal airways, and the normal female reproductive tract [11]. Recently, it was shown that dual marker combination of HE4 and CA 125 increased the sensitivity and specificity of either marker alone for the detection of ovarian and endometrial cancer [12-14].
The aim of this study was to assess the clinical value of preoperative HE4 in comparison with CA125 and ROMA (Risk of Ovarian Malignancy Algorithm) index in patients with benign gynecological diseases and ovarian cancer. In this study, the authors also evaluated HE4 levels in healthy Turkish women at different ages.

Materials and Methods

In this study, 96 benign gynecologic disorders and 47 ovarian cancer were evaluated for the diagnostic sensitivity of CA125, HE4, and ROMA index. Additionally, 106 healthy Turkish women, aged between 20-80 years, were analyzed to find a reference range for serum HE4 levels. The study was approved by the Local Ethical Committee of Hacettepe University, Faculty of Medicine.

All blood samples were obtained preoperatively and collected in ten-ml serum separator tubes (SST). Within four hours of collection, the blood samples were centrifuged at 1,500 g for ten minutes, the serum was separated, and stored at –80°C. CA125 and HE4 were analyzed in parallel using a specific system. The assay for HE4 and CA125 are both two-step chemiluminescence microparticle immunoassays (CMIA). In the first step, serum samples were incubated OC 125 coated or anti-HE4 coated paramagnetic microparticles. OC 125 antigen or HE4 antigen present in the serum bind to the OC 125 coated or anti-HE4 coated microparticles. In the second step, acridinium-labeled conjugate was added following washing step in the CA125 assay or HE4 assay. Chemiluminescent reaction was measured by relative light units which directly reflects CA125 or HE4 concentrations in the serum samples. The upper limits of normality was 35 U/ml for CA125 and 70 pmol/L for HE4.

The ROMA index is a predective probability algorithm that incorporates CA 125, HE4 and menopausal status to distinguish benign from malignant adnexal masses, and has been approved by the United States FDA [15]. In this study, ROMA index was calculated using logistic regression analysis according to Moore RG et al. [12]: For premenopausal women, Predictive index (PI) = -12.0 + 2.38 xLN(HE4) + 0.0626 xLN(CA125) For postmenopausal women, Predictive index (PI) = -12.0 + 2.38 xLN(HE4) + 0.0626 xLN(CA125)

The suggested cut-off value for ROMA index is 13.1% in premenopausal women and 27.7% in postmenopausal women. All surgical pathologic samples were examined by a gynecologic pathologist following the surgery, and each diagnosis was reviewed and classified as either benign or malignant.

Statistical Analysis

All the data were analyzed with SPSS statistical software (version 19.0). Tumor marker values between groups were compared using Kruskal-Wallis test. The level of statistical significance was set at \( p < 0.05 \). Cross-validation analysis was performed to obtain the sensitivities at set specificities of 80%, 90%, and 95%. Receiver operator characteristic (ROC) curves were constructed and the area under the curve (AUC) was compared between each tumor marker and marker combination using a non-parametric method. Reference limits for HE4 in healthy Turkish women was calculated by parametric method.

Results

Among the ovarian cancer patients, there were 14 premenopausal and 33 postmenopausal women. The benign patient population consisted of 83 premenopausal women and 13 postmenopausal women. In the benign group there were 11 adenoma, 21 endometriosis, 30 ovarian cysts, 28 leiomyomas, and six mucinous cyst adenoma. The mean age was 42 ± 10 for benign gynecologic diseases (premenopausal: 40 ± 9, postmenopausal: 55 ± 6), and 56 ± 14 for ovarian cancer (premenopausal: 41 ± 10, postmenopausal: 62 ± 10). Additionally, 106 healthy Turkish women (59 premenopausal, 47 postmenopausal), aged between 20-80 years, were evaluated to find a reference range for HE4.

The mean, median, minimum and maximum values of CA125, HE4, and ROMA index are given in Table 1. Comparison of the postmenopausal benign group to the premenopausal benign group did not show a statistical difference between the median levels of the CA125, HE4, and ROMA index \( (p > 0.05, \text{respectively}) \). CA125 levels in ovarian cancer were significantly higher than benign gynecologic diseases in postmenopausal women \( (p < 0.05) \), but not in premenopausal women \( (p > 0.05) \). Significantly higher HE4 levels and ROMA index was observed in ovarian cancer compared to benign gynecologic diseases both in postmenopausal and premenopausal women \( (p < 0.05) \), but a significant difference was not present in either HE4 levels or ROMA index between pre and postmenopausal ovarian cancer patients \( (p > 0.05) \). The utility of CA125, HE4, and ROMA index in discriminating benign gynecological disorders from ovarian cancer is given in Figure 1 by boxplot graphics.

The ROC curve analysis for evaluating the utility of HE4, CA125, and ROMA index in the diagnosis of ovarian cancer versus benign gynecologic diseases are shown in Figure 2. For the differential diagnosis of ovarian can-

Table 1. — CA125, HE4 and ROMA index values in benign gynecologic diseases and ovarian cancer:

<table>
<thead>
<tr>
<th>Disease Type</th>
<th>CA125 (U/ml)</th>
<th>HE4 (pmol/l)</th>
<th>ROMA index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>14.6</td>
<td>209.7</td>
<td>10.3</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>14.6</td>
<td>209.7</td>
<td>10.3</td>
</tr>
<tr>
<td>Total</td>
<td>14.6</td>
<td>209.7</td>
<td>10.3</td>
</tr>
<tr>
<td>Ovarian Ca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>176.2</td>
<td>600.7</td>
<td>263.3</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>176.2</td>
<td>600.7</td>
<td>263.3</td>
</tr>
<tr>
<td>Total</td>
<td>176.2</td>
<td>600.7</td>
<td>263.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CA125 (U/ml)</th>
<th>Median</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>12.1</td>
<td>5.1</td>
<td>52.9</td>
</tr>
<tr>
<td>Ovarian Ca</td>
<td>12.1</td>
<td>5.1</td>
<td>52.9</td>
</tr>
<tr>
<td>Total</td>
<td>12.1</td>
<td>5.1</td>
<td>52.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HE4 (pmol/l)</th>
<th>Median</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>49.9</td>
<td>29.9</td>
<td>92.9</td>
</tr>
<tr>
<td>Ovarian Ca</td>
<td>49.9</td>
<td>29.9</td>
<td>92.9</td>
</tr>
<tr>
<td>Total</td>
<td>49.9</td>
<td>29.9</td>
<td>92.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ROMA index</th>
<th>Median</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>9.5</td>
<td>3.8</td>
<td>17.6</td>
</tr>
<tr>
<td>Ovarian Ca</td>
<td>9.5</td>
<td>3.8</td>
<td>17.6</td>
</tr>
<tr>
<td>Total</td>
<td>9.5</td>
<td>3.8</td>
<td>17.6</td>
</tr>
</tbody>
</table>
Diagnostic performances of CA125, HE4, and ROMA index in ovarian cancer

Figure 1. — Box-plot graphics of CA125, HE4, and ROMA index for the discrimination of benign diseases versus ovarian cancer.

Figure 2. — ROC curves for CA125, HE4, and ROMA index in premenopausal (A), postmenopausal (B), and overall (C) ovarian cancer.
Z.G. Dikmen, A. Colak, P. Dogan, S. Tuncer, F. Akbiyik

In this study, the authors also examined serum HE4 levels of healthy Turkish women on the basis of age and menopausal status to refine normal ranges for this novel biomarker. Samples were collected from 106 healthy women aged between 20 and 80 years. For serum HE4 levels, the minimum value was 18.4 pmol/L, and the maximum value was 232 pmol/L. The authors observed that HE4 levels increased by age, especially after age 60 as seen in Figure 3. Median serum HE4 levels was 43 pmol/L for premenopausal women and 52.3 pmol/L for postmenopausal women; a significant difference was observed in the median HE4 levels. For Turkish women, serum HE4 normal range was determined as 28.9 - 62.4 pmol/L for premenopausal period and 23.7 - 152.4 pmol/L for postmenopausal period at 5 - 95 % confidence interval. The cut-off points are found as 65.4 U/ml for CA125, 85.4 pmol/L for HE4, and 17.85% for ROMA index in Turkish women.

**Discussion**

The aim of this study was to evaluate the diagnostic performances of HE4 and ROMA index compared to CA125 in Turkish women. CA125 is the present gold standard for the differential diagnosis of pelvic masses. Unfortunately, low diagnostic sensitivity and specificity is the most important problem with CA125 both in pre- and postmenopausal women. Several novel tumor markers such as soluble mesothelin-related peptide (SMRP), HE4, CA72-4, activin, inhibin, osteopontin, OVX1, M-CSF, LPA, prostasin, kallikrein, epidermal growth factor (EGFR), and ERBB2 (Her2) have been evaluated in patients with ovarian cancer. HE4 had the highest sensitivity as a single marker and dual marker combination of CA125 and HE4 had a greater sensitivity than either marker alone [16, 17]. Currently, the combination of multiple markers and algorithms is promising for ovarian cancer.

<table>
<thead>
<tr>
<th>Table 2. — The AUC values and the sensitivities of CA125, HE4, and ROMA index in ovarian cancer.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROC AUC (95% CI)</td>
</tr>
<tr>
<td>CA125 Premenopausal 0.752 (0.63-0.81)</td>
</tr>
<tr>
<td>Postmenopausal 0.876 (0.73-0.95)</td>
</tr>
<tr>
<td>Overall 0.781 (0.69-0.84)</td>
</tr>
<tr>
<td>HE4 Premenopausal 0.897 (0.75-0.91)</td>
</tr>
<tr>
<td>Postmenopausal 0.937 (0.82-0.98)</td>
</tr>
<tr>
<td>Overall 0.929 (0.87-0.96)</td>
</tr>
<tr>
<td>ROMA Premenopausal 0.905 (0.81-0.94)</td>
</tr>
<tr>
<td>Postmenopausal 0.986 (0.88-0.99)</td>
</tr>
<tr>
<td>Overall 0.955 (0.90-0.98)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3. — The sensitivity (%), specificity (%), PPV (%), and NPV (%) values in ovarian cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity Specificity PPV NPV</td>
</tr>
<tr>
<td>% % % %</td>
</tr>
<tr>
<td>CA125 Premenopausal 64.3</td>
</tr>
<tr>
<td>Postmenopausal 60.6</td>
</tr>
<tr>
<td>Overall 61.7</td>
</tr>
<tr>
<td>HE4 Premenopausal 78.5</td>
</tr>
<tr>
<td>Postmenopausal 84.8</td>
</tr>
<tr>
<td>Overall 91.4</td>
</tr>
<tr>
<td>ROMA index Premenopausal 83.3</td>
</tr>
<tr>
<td>Postmenopausal 96.3</td>
</tr>
<tr>
<td>Overall 89.7</td>
</tr>
</tbody>
</table>

PPV = positive predictive value; NPV = negative predictive value.
Jacobs et al. [18] developed the Risk of Malignancy Index (RMI) algorithm for predicting the risk of malignancy in women with a pelvic mass which employs pelvic ultrasonography (USG), CA125 and menopausal status. The following equation was used to calculate RMI: USG x Menopause x CA125. Unfortunately, USG imaging data can show variability between users and centers, which is the weakness of RMI algorithm. Recently, Moore et al. [12] developed the ROMA index, dual marker combination of CA125 and HE4 together with the menopausal status, to classify pre- and postmenopausal women into high and low risk groups. When RMI and ROMA index were compared for ovarian cancer patients, the ROMA index (94.3%) achieved a significantly higher sensitivity than RMI (84.6%) at 75% specificity [19].

Ovarian cancers usually occur in postmenopausal state with a peak between 55-65 years. Recent studies have shown that ROMA algorithm and HE4 were better than CA125 significantly in postmenopausal women in the distinction of ovarian cancer from benign diseases but not in premenopausal women [20-22]. These findings go along with the present results as the authors observed the highest AUC (0.955) and higher sensitivities at 80%, 90%, and 95% specificities for ROMA index in postmenopausal ovarian cancer. They have also shown that both HE4 and ROMA index were significantly higher than CA125 alone in postmenopausal ovarian cancer (p < 0.05). The present results indicate that HE4 has the highest PPV and specificity in the differential diagnosis of benign diseases versus ovarian cancer.

Moore et al. [17] found that HE4 levels were elevated only in 12% patients relative to CA125 levels which were elevated in 26% patients with benign gynecological disease. In the present study, false positivity rate was 10% for HE4, 26% for CA125, and 32% for ROMA index in patients with benign gynecological disease, indicating that HE4 has a greater specificity for benign disease than CA125 alone. Huhtinen et al. [13] conclude that measuring HE4 in addition to CA125 provides a more accurate tool for the differential diagnosis of ovarian cancer and endometriosis.

Some recent studies [17, 21, 23] have reported that HE4 has higher benefits for the early stage of ovarian cancer. When the present authors analyzed cancer patients diagnosed at Stage I and II (n = 26), CA125 was high only in eight patients (30%), whereas HE4 was elevated in 17 patients (61%), and ROMA index was positive in 13 patients (50%). The present results also support that HE4 alone had a significantly higher sensitivity than CA125 in Stage I disease and can be used for the early detection of ovarian cancer. The addition of CA125 to HE4 for the calculation of ROMA index decreased the sensitivity in patients with Stage I disease. Therefore, for the early detection of ovarian cancer, it has been suggested that the best algorithm is to classify patients at high risk with high HE4 levels and to determine the risk by ROMA index for patients with normal HE4 and high CA125 levels [24].

Anastasi et al. [25] suggested that HE4 levels are significantly higher in ovulatory phase than follicular phase of the menstrual cycle, but with no difference after 35 years of age. In contrast, Hallamaa et al. [26] have shown that menstrual cycle has no significant effect on HE4 levels, therefore HE4 measurement can be carried out at any phase of menstrual cycle and during hormonal medication such as use of contraceptives, extending the benefits for its use.

Compared with CA125, HE4 is inversely influenced by age; CA125 is higher in healthy premenopausal women, whereas HE4 tends to be higher in postmenopausal women [22]. Moore et al. [27] have shown that elevations in HE4 levels emerged with age after 40 years; the upper 95% percentile for HE4 was 89 pmol/L for premenopausal women, 128 pmol/l for postmenopausal women, and 115 pmol/L for all women. The median HE4 levels for premenopausal women (46.6 pmol/L) were significantly lower than postmenopausal women (57.6 pmol/L). Bolstad et al. [28] have shown that HE4 levels in Nordic population increase with age; 9% higher at 40 years, 20% at 50 years, 37% at 60 years, 63% at 70 years, and 101% at 80 years compared to 20 years. Therefore, any algorithm for detection of cancer involving HE4 should include age of the patients. In healthy Turkish women, the present authors observed that HE4 levels increase by age, especially after menopause. Normal reference limit was determined as 28.9 - 62.4 pmol/L for premenopausal women and 23.7 - 152.4 pmol/L for postmenopausal women. In healthy Chinese women (age ranged from 21 to 81 years), the upper limits of the 95% percentile intervals were 82.62 pmol/L for HE4, 30.91 U/ml for CA125, and 19.27 for ROMA by electrochemiluminescence immunooassay (ECLIA) [29].

Recent studies [30, 31] have assessed the prognostic value of preoperative serum HE4 levels and reported high HE4 levels is a marker of ovarian cancer aggressiveness and poor prognosis. It has been shown that urine HE4 levels can also be used for the diagnosis of both early and late stages of ovarian cancer and follow-up of the therapy [32].

Conclusion

In this study, the authors observed that HE4 has very good accuracy for the differential diagnosis of benign diseases and ovarian cancer, therefore serum HE4 measurement can be used as a preoperative test for predicting benign or malign nature of pelvic masses. The authors can comment that serum HE4 levels improve the specificity of laboratory identification of ovarian cancer; high serum HE4 with high CA125 may suggest the presence of gynecologic malignancy whereas high serum CA125 levels without elevated HE4 would direct towards benign gynecologic diseases.
References


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Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women and the most lethal gynecologic malignancy in the world [1]. Early symptoms of EOC patients are generally minor and easily overlooked. At initial diagnosis, most of the women suffer from advanced stage disease. Although improvement of the quality of cytoreductive surgery as well as the development of novel drugs and new chemotherapy regimens for EOC have been made, the prognosis of patients with advanced stage EOC remains poor. The five-year overall survival (OS) is approximately 20% for Stage III disease and 5% for Stage IV disease [2]. Previous studies have demonstrated diverse genetic alterations in EOC [3-7], but the highly complex molecular mechanisms underlying EOC carcinogenesis and progression remain obscure. Therefore, it is necessary to search novel markers for EOC, which can accurately identify biological characteristics of tumors, improve therapeutic strategies, and predict clinical outcome.

MicroRNAs (miRNAs) are single-stranded, small non-coding RNAs with 18–25 nucleotides in length [8]. They can negatively regulate gene expression through base-pairing to the 3’ untranslated region (3’UTR) of target messenger RNA (mRNA), resulting in translation inhibition or mRNA degradation [9, 10]. It is now clear that miRNAs play key roles in almost all biological processes, including differentiation, development, gene regulation, cell proliferation and apoptosis, and the development of various diseases, such as cancer. They function as tumor suppressors or oncogenes according to the roles of their target genes. Gene expression profiling studies have revealed that miRNA expression may be an excellent biomarker for cancer diagnosis and prognosis estimation [11, 12]. In terms of EOC, abnormal expression of several miRNAs such as miR-21 [13], miR-125b [13], miR-34a [14], and miR-15 [15] have been reported. Peng et al. showed decreased miR-100 expression in EOC and its correlation with advanced clinical stage, higher serum CA125 level, lymph node metastasis, and shorter OS [16]. Additionally, miR-100 could affect the growth of EOC cells by post-transcriptionally regulating polo-like kinase 1 (PLK1) expression. By performing miRNA expression profiling analysis, Hu et al. found that three miR-200 miRNAs (miR-200a, miR-200b and miR-429) in the miR-200b–429 cluster were significantly associated with cancer recurrence and OS [17]. Furthermore, overexpression of this miR-200 cluster could inhibit ovarian cancer cell migration. These findings suggest that miRNAs act not only as diagnostic and prognostic markers, but also as potential therapeutic targets of EOC.

Summary

Objective: MicroRNA-451 has been proved to be downregulated in many human malignancies and correlated with tumor progression. However, its expression and clinical significance in epithelial ovarian cancer (EOC) is still unclear. The aim of this study was to explore the effects of miR-451 in EOC tumorigenesis and development. Materials and Methods: The expression levels of miR-451 were quantified by qRT-PCR in 115 EOC and 34 normal ovarian tissues, and correlated with clinicopathological factors and prognosis. MTT, flow cytometric assay, and transwell invasion assay were used to test the proliferation, apoptosis, and invasion of SKOV-3 EOC cells transfected with miR-451 mimics or negative control (NC) RNA-oligonucleotides. Results: MiR-451 expression was significantly downregulated in EOC compared with normal ovarian tissues. Low level of miR-451 was associated with advanced FIGO stage \( p = 0.005 \), higher serum CA125 expression level \( p = 0.005 \), and lymph node metastasis \( p = 0.002 \). Multivariate Cox regression analysis identified decreased miR-451 expression as an independent factor predicting poor prognosis for EOC patients. In addition, transfection of miR-451 mimics in SKOV-3 was able to reduce cell proliferation, promote cell apoptosis, and inhibit cell invasion. Conclusions: miR-451 may act not only as a novel diagnostic and prognostic marker, but also as a potential target for molecular therapy of EOC.

Key words: MicroRNA-451; Epithelial ovarian cancer; Prognosis; Proliferation; Apoptosis; Invasion.
One of the tumor suppressive miRNAs is miR-451. miR-451 expression is increased in gastric cancer [18], breast cancer [19], lung cancer [20], glioma [21], and head and neck squamous cell carcinoma [22]. miR-451 has been reported to be correlated with chemosensitivity of MCF-7 breast cancer cells to doxorubicin (DOX) and A549 nonsmall cell lung cancer (NSCLC) cells to cisplatin [19, 23]. Recent studies have demonstrated that miR-451 may modulate the process of tumorigenesis and the behavior of cancer cells by suppressing a series of oncopgenes. However, expression of miR-451 in EOC and a link between miR-451 and clinicopathological tumor features have not been clearly understood.

In this study, the authors examined the expression of miR-451 in EOC and normal ovarian tissues. Then, the clinicopathological and prognostic significance of miR-451 expression in human EOC were statistically analyzed. Furthermore, the effects of miR-451 on EOC cell line SKOV-3 proliferation, apoptosis, and invasion were investigated.

Materials and Methods

Patients and tissue samples

A total of 149 fresh surgical tissue samples (115 EOC and 34 normal ovarian tissues) were collected at the Department of Obstetrics and Gynecology, The First Affiliated Hospital of Shantou University Medical College between 2005 and 2007. Normal ovarian tissues were obtained from tumor-free participants during surgery for other gynecological diseases. For example, they underwent surgery for a total hysterectomy, bilateral salpingo-oophorectomy, and pelvic and para-aortic lymphadenectomies. The ethical committees of the present hospital approved this study, and informed consent was obtained from all patients. No patient had undergone chemotherapy or radiotherapy before surgery. All tissue samples were flash frozen and stored at -80°C in liquid nitrogen until processed. Clinical tumor stage was classified according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. All EOC patients had been followed up from the time of surgical intervention to 2012. OS was measured up to the date of death due to any cause or, for living patients, the date of last follow-up.

Cell culture

Human ovarian cancer cell line SKOV-3 was obtained from the Beijing Institute for Cancer Research (Beijing, China). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in humidified air at 37°C with 5% CO₂.

RNA extraction and quantitative real-time PCR

Total RNA was isolated using a reagent according to the manufacturer’s instructions. RNA quality and quantity were assessed using a bioanalyzer system. Reverse transcription reaction was carried out starting from 10 ng of total RNA using the looped primers. Real-time PCR was performed using the standard Taqman MicroRNA assays protocol on ABI7500 real-time PCR detection system. U6 small nuclear RNA was used as an internal control. The RT primers were 5'-ACACTCCAGCTGGAACCGTTACATTACT-3' and reverse, 5'-CTGGTGTCGGTGGAACCCGAA-3'. U6 sense, 5'-CTGGTCTGCGCACA-3' and reverse, 5'-AACGCTTCAGAATTGGT-3'. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Each sample was measured in triplicate, and the relative amount of miR-451 to U6 was calculated using the equation 2^-ΔΔCt, where DCT = (CTmiR-451 - CTU6).

miR-451 transfection

MiR-451 mimics and negative control (NC) RNA oligonucleotides were utilized. The day before transfection, SKOV-3 cells were seeded in antibiotic free medium. Transfection was carried out using lipofectamine in accordance with the manufacturer’s procedure. The level of miR-451 mimics expression in SKOV-3 cells was assayed by real-time PCR.

Cell proliferation assay

The proliferation capacity of SKOV-3 cells transfected with miR-451 mimics or NC was evaluated with an MTT assay, which was performed following standard procedure in 96-well plates. In brief, cells were seeded at a density of 2,000 cells per well containing 100 ul of culture medium and cultured overnight. Twenty ul of five mg/ml MTX (dimethyl thiazolyl diphenyl tetrazolium) reagent was added to each well and cells were further incubated for four hours at 37°C. Then the medium was removed, and 100 ul of DMSO (dimethyl sulfoxide) was added to each well to dissolve the formazan. Cell proliferation was assessed daily for four consecutive days. The absorbance of the samples was measured with a spectrophotometer reader at 490 nm.

Detection of apoptosis by flow cytometry

Apoptosis was detected by flow cytometric analysis as previously reported [24]. Briefly, the cells were washed and resuspended at a concentration of 1×10⁶ cells/ml. Then, the cells were stained with Annexin V and propidium iodide (PI), using the Annexin V apoptosis detection kit. After incubation at room temperature in the dark for 15 minutes, the cells were immediately analyzed with a flowcytometer.

Cell invasion assay

Cell invasion assay was performed using 24-well transwell chambers (eight μm) and the upper chambers were first covered with one mg/ml Matrigel. After transfection, 2×10⁵ SKOV-3 cells resuspended in 100 ul serum-free medium were seeded into the upper chambers. 0.5 ml of 10% FBS-RMPI-1640 was added to the lower chambers. Following a 24-hour incubation, non-invaded cells on the top of the membrane were removed with a cotton swab, and the invaded cells were fixed with 95% ethanol and stained with 0.1% crystal violet. The number of invaded cells was determined by counting five random fields on each membrane.

Statistics

Statistical analyses were performed with SPSS software (version 16.0). Data were expressed as mean ± standard deviation (SD). The differences between groups were analyzed using the Student’s t-test or chi-square test. Survival probabilities were described by Kaplan–Meier curves and compared using the log-rank test. Cox regression (Proportional hazard model) was adopted to assess the independence of different prognostic factors. All tests were two-tailed, and the significance level was set at p < 0.05.
Expression and prognostic significance of microRNA-451 in human epithelial ovarian cancer

Results

miR-451 expression in EOC tissues and its association with clinicopathological characteristics

The expression levels of miR-451 in EOC and normal ovarian tissues were detected by qRT-PCR and normalized to U6 small nuclear RNA. For EOC tissues, the mean level of miR-451 expression was 3.5 (range, 1.4 - 9.2). For the normal ovarian tissues, the mean level of miR-451 expression was 8.7 (range, 2.9 - 17.8). The level of miR-451 was significantly lower in EOC tissues than in normal ovarian tissues ($p < 0.001$; Figure 1).

Next, the clinicopathological significance of miR-451 expression in human EOC was analyzed. The authors divided the patients into two groups according to their miR-451 expression levels using its median as a cutoff: high miR-451 expression group (n = 58) and low miR-451 expression group (n = 57). As shown in Table 1, downregulation of miR-451 was closely correlated with advanced FIGO stage ($p = 0.005$), higher serum CA125 expression level ($p = 0.005$), and lymph node metastasis ($p = 0.002$). However, there was no significant correlation between miR-451 expression and other clinicopathological variables including age, histological type, histological grade, and ascites.

Table 1. — Association of miR-451 expression with clinicopathological variables of EOC patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low miR-451 expression (n, %)</th>
<th>High miR-451 expression (n, %)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 50</td>
<td>38 (53.5%)</td>
<td>33 (46.5%)</td>
<td>0.339</td>
</tr>
<tr>
<td>&lt; 50</td>
<td>19 (43.2%)</td>
<td>25 (56.8%)</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>18 (56.2%)</td>
<td>14 (43.8%)</td>
<td>0.256</td>
</tr>
<tr>
<td>Mucinous</td>
<td>23 (48.9%)</td>
<td>24 (51.1%)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>16 (44.4%)</td>
<td>20 (55.6%)</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>27 (49.1%)</td>
<td>28 (50.9%)</td>
<td>0.323</td>
</tr>
<tr>
<td>G2</td>
<td>15 (53.6%)</td>
<td>13 (46.4%)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>15 (46.9%)</td>
<td>17 (53.1%)</td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>18 (34.0%)</td>
<td>35 (66.0%)</td>
<td>0.005</td>
</tr>
<tr>
<td>III/IV</td>
<td>39 (62.9%)</td>
<td>23 (37.1%)</td>
<td></td>
</tr>
<tr>
<td>Serum CA125 level (U/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5.0 x 10^5</td>
<td>20 (35.7%)</td>
<td>36 (62.3%)</td>
<td>0.005</td>
</tr>
<tr>
<td>≥ 5.0 x 10^5</td>
<td>37 (62.7%)</td>
<td>22 (37.3%)</td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>24 (44.4%)</td>
<td>30 (55.6%)</td>
<td>0.352</td>
</tr>
<tr>
<td>Yes</td>
<td>33 (56.5%)</td>
<td>28 (43.5%)</td>
<td></td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>20 (31.7%)</td>
<td>43 (68.3%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Yes</td>
<td>37 (71.2%)</td>
<td>15 (28.8%)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. — miR-451 expression in 115 EOC and 34 normal ovarian tissues detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Figure 2. — Kaplan–Meier curves for overall survival time of patients with EOC, divided according to miR-451 expression levels.
The authors further evaluated whether miR-451 expression had prognostic potential for OS of EOC patients. The Kaplan-Meier curve is shown in Figure 2. They found patients with low miR-451 expression were more likely to have a shorter OS ($p < 0.001$), when compared to patients with high miR-451 expression. Aside from miR-451 expression ($p < 0.001$, RR = 3.82), univariate Cox proportional hazard regression analysis revealed that histological grade ($p = 0.008$, RR = 2.45), FIGO stage ($p = 0.006$, RR = 3.12), and lymph node status ($p = 0.012$, RR = 2.16) were also predictive factors for prognosis. Multivariate Cox proportional hazard regression analysis confirmed that low-level miR-451 expression ($p = 0.008$, RR = 2.32) was an unfavorable prognostic factor independent of other clinicopathological factors, including FIGO stage ($p = 0.003$, RR = 1.79) and lymph node metastasis ($p = 0.016$, RR = 3.04; Table 2).

Table 2. — Univariate and multivariate analysis of prognostic variables by Cox regression analysis.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>RR P-value</td>
<td>RR P-value</td>
</tr>
<tr>
<td>(≥ 50 / &lt;50)</td>
<td>1.43 0.218</td>
<td>1.55 0.095</td>
</tr>
<tr>
<td>Histological type</td>
<td>0.96 0.146</td>
<td>2.46 0.272</td>
</tr>
<tr>
<td>(Serous/non-serious)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td>2.45 0.008</td>
<td>2.11 0.078</td>
</tr>
<tr>
<td>(G1/G2+G3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td>3.12 0.006</td>
<td>1.79 0.003</td>
</tr>
<tr>
<td>(III + IV / I + II)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascites (yes/no)</td>
<td>0.87 0.452</td>
<td>1.52 0.072</td>
</tr>
<tr>
<td>Serum CA 125</td>
<td>1.35 0.139</td>
<td>2.02 0.214</td>
</tr>
<tr>
<td>(≥ 5.0 $\times$ 10^5 / &lt;5.0 $\times$ 10^5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node involvement</td>
<td>2.16 0.012</td>
<td>3.04 0.016</td>
</tr>
<tr>
<td>(yes/no)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expression of miR-451</td>
<td>3.82 &lt;0.001</td>
<td>2.32 0.008</td>
</tr>
<tr>
<td>(low/high)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. — Effects of miR-451 transfection on the proliferation, apoptosis, and invasion of EOC cell line SKOV-3. (A) miR-451 expression after treatment with miR-451 mimics or negative control in SKOV-3 cells assessed by quantitative RT-PCR. (B) MTT assay demonstrated reduced cell proliferation in SKOV-3 cells after transfection of miR-451 mimics. (C) transfection of miR-451 mimics promoted cell apoptosis in SKOV-3 cells. (D) miR-451 suppressed SKOV-3 cell invasion in vitro. The Matrigel invasion assay showed that the number of invaded cells was significantly lower in the miR-451-transfected cells than in the NC-transfected cells.
Effects of miR-451 expression on proliferation, apoptosis, and invasion of SKOV-3 Cells

The downregulation of miR-451 in EOC tissues prompted the authors to investigate its biological functions in carcinogenesis. SKOV-3 cells were transfected with miR-451 mimics or NC. As shown in Figure 3A, the expression level of miR-451 in miR-451 mimics transfected cells was significantly higher compared with NC transfected cells \((p < 0.001)\). MTT assay demonstrated that transfection of miR-451 mimics reduced cell proliferation of SKOV-3 cells (Figure 3B; \(p < 0.01\)). The authors also observed promoted cell apoptosis in miR-451 mimics transfected cells (Figure 3C; \(p < 0.05\)). Furthermore, transwell invasion assay showed a significant decrease in invaded cell numbers after miR-451 transfection (Figure 3D, \(p < 0.01\)). These results indicate that miR-451 is involved in the negative regulation of EOC cell growth and invasion in vitro.

Discussion

Much work has been performed in an attempt to identify markers with diagnostic and prognostic implications for human EOC. Recently, many studies have shown that aberrant miRNAs are associated with tumorigenesis and progression in various human cancer types; however, their potential roles in EOC remain largely uncharacterized. In the current study, the authors firstly observed that miR-451 was downregulated in EOC tissues compared with normal ovarian tissues. Then, the downregulation of miR-451 in EOC tissues was significantly correlated with advanced FIGO stage, higher serum CA125 expression level, and lymph node metastasis. Furthermore, patients with low miR-451 expression showed poorer survival than those with high miR-451 expression. A multivariate analysis with the Cox proportional hazards confirmed that the status of miR-451 expression was an independent predictor of OS in EOC. Finally, transfection of miR-451 mimics in EOC cell line SKOV-3 was able to reduce cell proliferation, promote cell apoptosis, and inhibit cell invasion in vitro. To the authors’ knowledge, this is the first report regarding the involvement of miR-451 in EOC.

Previous research has revealed dysregulated miR-451 expression in many human malignancies, and its functions as a tumor suppressor by targeting a number of oncogenic genes. For example, decreased miRNA-451 expression was confirmed in NSCLC tissues and correlated with advanced clinical stage, lymph node metastasis, and poor prognosis [25]. Upregulation of miR-451 significantly reduced the in vitro proliferation and enhanced apoptosis of NSCLC cell line SPC-A1 by targeting ras-related protein 14 (RAB14), and suppressed the development of tumors in nude mice. Tian et al. reported the anti-tumor function of miR-451 in glioma through targeting calcium binding protein 39 gene (CAB39) and inhibiting the PI3K/AKT pathway [21]. Bandres et al. identified a novel oncogene macrophage migration inhibitory factor (MIF) as a direct target of miR-451, and revealed the predictive effects of miR-451 on radiosensitivity and prognosis in gastric cancer patients [18]. In terms of the association between miR-451 and chemosensitivity, Bian et al.’s study indicated that ectopic overexpression of miR-451 could sensitize A549 NSCLC cells to cisplatin by increasing cisplatin-induced apoptosis [23]. Kovalchuk et al. reported that the enforced increase of miR-451 levels in MCF-7 breast cancer cells would downregulate expression of multidrug resistance 1 (MDR1) and increase sensitivity of the MCF-7 cells to DOX [19]. Taken together, miR-451 could not only be useful as a marker but also serve as a target for the development of novel therapeutic strategies to overcome tumor growth and drug resistance.

The mechanism by which miR-451 expression affects carcinogenesis, cancer progression and drug resistance is complex. Some useful targets have been identified during the past few years; however, there is no ‘one-to-one’ connection between miRNAs and target mRNAs. An average miRNA can have more than 100 targets [26]. Conversely, several miRNAs can converge on a single transcript target [27]. Thus, the potential regulatory circuitry afforded by miR-451 may be enormous, and its direct functional targets in EOC are not completely clear. Additionally, the current study was limited by its retrospective nature which led to the present results being considered exploratory, and the sample size was relatively smaller. Further prospective analysis containing a large number of tumor samples is worth performing.

In conclusion, the present findings suggested that miR-451 downregulation was closely correlated with tumor aggressive progression and poor prognosis of human EOC. Ectopic expression of miR-451 inhibited cell growth and invasion in vitro. Therefore, miRNA-451 may be a potential novel target for gene therapy of EOC.

References


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Effects of cyclopamine on the biological characteristics of human breast cancer MCF-7 cell line and its mechanism

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Department of Breast Surgery, the First Affiliated Hospital of Liaoning Medical University, Jinzhou (China)

Summary

Purpose: To observe the effects of cyclopamine on the biological characteristics of human breast cancer MCF-7 cell line and explore its mechanism. Materials and Methods: After human breast cancer MCF-7 cells were treated with different-concentration cyclopamine for different periods, MTT assay was used to detect the inhibitory effect of cyclopamine on MCF-7 cell proliferation, flow cytometry was used to determine the distribution of MCF-7 cell cycle and the effect of cyclopamine on MCF-7 apoptosis, and Western blot was used to measure the protein levels of cyclins D1 and p21 in MCF-7 cells. Results: In certain range, MCF-7 cell proliferation was inhibited by cyclopamine in a dose- and time-dependent manner, and the optimal inhibiting concentration was ten μmol/L and the optimal action time at 48 hours. With the time prolongation of cyclopamine action, the cells in G0/G1 phase were significantly increased, but the cells in S phase were significantly decreased (compared with blank control group, all \( p < 0.05 \)). With the time prolongation of cyclopamine action, apoptosis rate of MCF-7 cells was also significantly increased (compared with blank control group, all \( p < 0.05 \)). The level of cyclin D1 of MCF-7 cells was decreased, but cyclin p21 was increased (compared with blank control group, all \( p < 0.05 \)). Conclusion: Cyclopamine inhibits MCF-7 cell proliferation via arresting MCF-7 cell transformation from G1 phase to S phase. This may be associated with the expressions of Hedgehog (Hh) signaling pathway-related cyclins.

Key words: Breast cancer; MCF-7; Cyclopamine; Cyclin.

Introduction

Breast cancer is a type of common malignancy and its formation is associated with the abnormal activation of Hedgehog (Hh) signaling pathway [1]. In this study, the authors observed the effects of cyclopamine, a Hh signaling pathway-specific inhibitor, on human breast cancer MCF-7 cells and explored the possible mechanism in order to provide a theoretical basis for clinical application of cyclopamine in the treatment of breast cancer.

Materials and Methods

All study methods were approved by ethics committee of the First Affiliated Hospital, Liaoning Medical University.

Cell culture

MCF-7 cells were incubated in RPMI640 medium containing 10% of fresh fetal calf serum, 100 U/ml of penicillin and 100 U/ml streptomycin at 37°C at an atmosphere of 5% CO2. Medium was changed every two or three days and a passage was performed every four to seven days.

MTT assay

MCF-7 cells (1×10^4/ml) in logarithmic growth phase were inoculated in a 96-well plate (each well with 100 μl of MCF-7 cells). When cells were adherent, the final concentrations (1, 2, 5, 10, and 15 μmol/L) of cyclopamine were respectively added into each well (each well with 100 μl of cyclopamine and each group with five wells) followed by incubation for 24, 48, and 72 hours, respectively. MTT (0.5 mg/ml, 100 μl) was added. Four hours later, 150 μl of DMSO was added with shaking. About 15 minutes later, the absorbance of each well was determined at 490 nm with EL-LISA. Control group and blank control group were also set. The inhibition rate was calculated according to the following formula:

\[
inhibition\ rate = \frac{1 - absorbance\ in\ experiment\ group}{absorbance\ in\ control\ group} \times 100\%.
\]

Flow cytometry

MCF-7 cells (1×10^6/ml) were collected after treated with ten μmol/L of cyclopamine for 24 and 48 hours respectively, and then partial cells underwent PI single staining for analysis of cell cycle and other cells underwent AnnexitinV-FITC/PI double staining for analysis of apoptosis.

Western blot

MCF-7 cells were inoculated in a six-well plate at 1.5×10^6/well overnight, and then treated with ten μmol/L of cyclopamine for 24 and 48 hours, respectively. MCF-7 cells underwent clearing on ice, degeneration in water bath, semi-dry transmembrane followed by addition of mouse anti-human monoclonal antibody of cyclins D1 and p21. The membrane was placed in alkaline phosphatase-labeled secondary antibody, and then visualized with luminous liquid prepared according to the manufacturer’s instructions.

Statistical analysis

Statistical treatment was performed with SPSS16.0 software. Measurement data were expressed as (±s). Analysis of variance was used in experiment data and \( q \) test was used in the comparisons between groups. Statistical significance was established at \( p < 0.05 \).
Results

Inhibitory effects of cyclopamine on MCF-7 cell proliferation

As shown in Figure 1 and Table 1, with the increases in the concentration and action time of cyclopamine, the inhibitory effects of cyclopamine on MCF-7 cell proliferation were not always increased, and the optimal inhibiting concentration was ten μmol/L and the optimal action time was 48 hours. There were significant differences in inhibition rate between different-concentration groups at the same time point and between different time points at the same concentration (\( p < 0.05 \)). The concentrations were irrelevant to action time without interaction (\( p > 0.05 \)).

Flow cytometry

With the time prolongation of cyclopamine action, MCF-7 cells in G\(_0\)/G\(_1\) phase were increased, but MCF-7 cells in S phase were decreased (Figure 2). Compared with blank control group, more MCF-7 cells were arrested in G\(_1\) phase in cyclopamine groups, suggesting that cyclopamine action was associated with MCF-7 cell transformation from G\(_1\) phase to S phase, namely that the inhibition point of cyclopamine was G\(_1\)/S. With the time prolongation of cyclopamine action, MCF-7 cell apoptosis was increased. Compared blank control group, apoptosis rates in 24- and 48-hour-treated groups were significantly increased (all \( p < 0.05 \)) (Figure 3).

Western blot

Effects of cyclopamine on the expressions of cyclins D\(_1\) and p21 are shown in Figure 4. Compared with blank control group, cyclin D\(_1\) expression was significantly decreased in cyclopamine groups (\( p < 0.05 \)). Cyclin D\(_1\) expression in 24-hour-treated group was (34.30 ± 0.79) % of cyclin D\(_1\) expression in blank control group and in
48-hour-treated group was (21.46 ± 1.23) %. Compared with blank control group, cyclin p21 expression was significantly increased in cyclopamine groups (P<0.05). Cyclin p21 expression in 24-hour-treated group was (1.24 ± 0.02) times cyclin p21 expression in blank control group, and in 48-hour-treated group was (1.46 ± 0.02).

**Discussion**

**Relationship between Hh signaling pathway and tumor cells**

Hh gene is associated with Patched (Ptch) and smoothened (Smo) on the cell membrane. In the absence of Hh, Ptch, and Smo form a compound, inhibiting Smo activity. When Hh combines with Ptch, Ptch cannot inhibit Smo, hence Smo activity is released and the expressions of Smo signaling pathway-related downstream genes are up-regulated. Therefore, Hh signaling pathway-related molecular mutations can cause a variety of developmental defects or tumor [2, 3].

**Relationship between cyclopamine and Hh signaling pathway**

Cyclopamine, steroid alkaloids, and a kind of Hh signaling pathway-specific inhibitor, is extracted from the genus Veratrum. Cyclopamine can inhibit Hh signaling pathway activity [4]. It is reported that cyclopamine can inhibit tumor growth caused by the excessive activation of Hh signaling pathway and induce apoptosis [5].

**Relationship between cyclins and Hh signaling pathway**

The aberrant activation of Hh signaling pathway is related to tumorigenesis, and target genes or downstream molecules of Hh signaling pathway such as n2Myc, Egf, cyclin D, cyclin E, cyclin B, and BMP are involved in tumor cell proliferation and invasion [6]. Cyclin D1, an important member of G cyclin family, is regarded as an oncogene. Cyclin D1 can activate CDK4 and CDK6 in G1 phase and accelerate the transformation from G1 phase to S phase [7]. p21, a cell cycle-regulatory factor, can inhibit cyclin D1 activity and
lead to cell cycle arrest, interfering with cell proliferation and playing a negative regulatory role in cell proliferation [8].

Present study

In this study, after human breast cancer MCF-7 cells were treated with cyclopamine, MTT, flow cytometry, and Western blot were performed. This study suggests that cyclopamine can inhibit MCF-7 cell proliferation and induce MCF-7 cell apoptosis via downregulation of cyclin D1 and up-regulation of cyclin p21. Whether there are other mechanisms regarding cyclopamine-induced breast cancer cell apoptosis remain to be further studied.

References

Growing teratoma syndrome of the ovary presenting with liver metastasis: report of a case

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6 Pierre-et-Marie Curie University (Paris VI), Paris (France)

Summary
Growing teratoma syndrome (GTS) is a rare condition among patients with non-seminomatous germ cell tumors who present with enlarging metastatic masses during appropriate systemic chemotherapy in the context of normalized serum markers. This is an infrequent event in the progression of testicular tumors, and is even less common in the case of ovarian germ cell tumors. The pathogenesis of GTS is not completely understood and diagnosis can only be made with certainty after complete pathologic examination. Although histologically benign, GTS may present an enveloping growth with aggressive local expansion, which can be related to substantial morbidity and mortality. Surgery is the only recommended treatment and early recognition of this syndrome is essential as it offers hope for curative resection and avoids the use of ineffective chemotherapy. The authors present a brief review of the literature, along with the case report of a 37-year-old woman presenting GTS with liver involvement who was successfully treated by debulking surgery followed by major liver resection. This report demonstrates that complete surgical resection results in excellent disease control. More importantly, it highlights that clinicians need to be aware of the possible development of GTS when monitoring their patients with non-seminomatous germ cell tumors. These patients require coordinated care between oncologist, gynecologists, and general surgeons to obtain the best possible outcomes.

Keys words: Ovary; Neoplasms, Germ cell and embryonal; Teratoma; Liver; Treatment outcome.

Introduction
The growing teratoma syndrome (GTS) was first described in 1982 by Logothetis et al. [1]. It is defined as an enlarging retroperitoneal or other metastatic mass that consists of mature teratoma and is detected during or after systemic chemotherapy for non-seminomatous germ cell tumors. This is an infrequent event in the progression of testicular tumors, with reported incidence ranging from 1.9% to 7.6% [2]. It is even less common in the case of germ cell tumors of the ovary. Early recognition of this syndrome is essential as it offers hope for curative resection and avoids the use of ineffective chemotherapy. The authors report the case of GTS of the ovary associated with liver invasion that was treated by radical debulking surgery and extended right hepatectomy. Ten months after surgery the patient was disease-free.

Case Report
A 37-year-old multiparous woman who originally underwent a left salpingo-oophorectomy on December 2011 at another hospital for a left ovarian cyst whose final histology showed an immature grade III teratoma according to the FIGO (International Federation of Gynecology and Obstetrics) classification. Although recommended, no complementary treatment was proposed to the patient in her original institution. Four months later, she presented with a palpable mass and a pelvic recurrence of the disease was diagnosed. Laboratory findings included an elevated α-fetoprotein (AFP) of 2,431 ng/ml, and normal levels of carcino embryonic antigen (CEA) and human chorionic gonadotropin (HCG). She was then addressed to the Department of Gynaecological Surgery of the Pitié-Salpêtrière Hospital where she underwent a first debulking of the tumor including partial abdominal wall resection, left salpingectomy, and several peritoneal lesions resection. Histology showed a mature teratoma with a 10% immature teratoma component. After surgery she received four cycles of bleomycin, etoposide, and cisplatin (BEP) at three-week intervals. Tumor markers AFP and HCG were within normal limits after the second cycle of chemotherapy.

On September 2012, during regular follow up, a multi-detector computed-tomography (MDCT) scan revealed three hepatic lesions (Figure 1), metastatic pelvic lymph nodes, right colon soft tissue mass, and multiple pelvic masses with invasion of the right ureter. She was scheduled for surgery and complete debulking of the tumor including total abdominal hysterectomy, right salpingo-
oophorectomy, bilateral pelvic para-aortic lymphadenectomy, two segmental small bowel resections, right nephrectomy, rectosigmoid resection, partial splenectomy, and omentectomy was performed. Histology revealed a mature teratoma without any immature component.

Before liver resection, a MDCT-volumetry was performed and showed a small left liver remnant. A right portal vein embolization was then performed to induce liver hypertrophy. Six weeks later an extended right hepatectomy was performed (Figure 2). Final histopathology revealed a five-cm mature teratoma comprising cartilage, ciliated respiratory-type epithelium, enteric epithelium and neurogenic tissue with a supporting stroma of undifferentiated mesenchymal spindle cells without any immature component confirming the diagnosis of GTS of ovary. The patient received no further treatment and regular follow up including thoraco-abdominal-pelvic CT and tumor markers did not reveal any sign of recurrence within ten months follow-up.

Discussion

Although GTS was first named in 1982, the benign transformation or evolution of germ cell carcinomas after chemotherapy was first noted in the early 1970 [3, 4]. Actually, the earliest report was published in 1969 and described five patients who presented with primary testicular neoplasms of varying histologies, including seminoma and immature teratoma, whose metastatic sites consisted of well-differentiated teratomatous elements [5]. Immature ovarian teratoma represents less than 1% of all ovarian tumors. It is usually seen in women of the first two decades and contains immature neural tissues, the amount of which determines the grade of the tumor [1].

GTS is a rare complication of these malignant tumors. The hallmark feature of GTS is the normalization of tumor markers [AFP, HCG, lactate dehydrogenase]. Indeed, in cases where the tumor markers are not entirely in the normal range, it is imperative to exclude any non-malignant etiology (i.e., elevated AFP from liver dysfunction, elevated HCG from marijuana use or from elevated luteinizing hormone) [4,6].

Three criteria have been used to precisely describe this rare entity: (1) clinical or radiologic enlargement of metastases during or after chemotherapy, (2) normalization of previously elevated serum tumor markers (AFP or HCG), and (3) metastases consisting of pure mature teratoma without malignant cells on histologic examination. [7, 8] The present case reported here presents all three criteria, with an enlargement of peritoneal lesions seen just after the end of chemotherapy associated with the complete normalization of serum tumor markers.

Some authors distinguish GTS from chemotherapeutic retroconversion (CR) [2], which was first defined in 1977 by DiSaia et al. [9] in the context of immature ovarian teratoma. CR is a chemotherapy mediated transformation of a metastatic immature teratoma into mature teratoma. They hypothesized that there are two possible mechanisms for this process: chemotherapy either promotes the conversion of immature teratomatous tissue into mature tissue or chemotherapy destroys only the immature component, leaving the mature tissue behind [9].

Djordjevic et al. [2] pointed out that CR meets only two of the three criteria for GTS. In GTS, not only must the mature teratoma nodules have undergone CR, but they also must have the ability to grow, whereas in CR the nodules do not increase in size. This is a key difference between these two phenomena; it speaks to the proliferative ability of the GTS cells despite being terminally differentiated.
Growing teratoma syndrome of the ovary presenting with liver metastasis: report of a case

GTS of the ovary can present from the age of five to 38 years, with a mean age of 20 years [2]. The primary tumor was either a pure immature teratoma or a mixed germ cell tumor of the ovary [2]. GTS nodules usually appear within the first two years from the start of chemotherapy, but at least two cases have been reported where the first GTS nodules presented at five and 11 years [2, 8, 10]. Some investigators have suggested several characteristics that could predict the subsequent development of GTS in testicular or ovarian germ cell tumors. It includes the presence of mature teratomatous elements in the primary tumor, no reduction in tumor size after chemotherapy, incomplete resection of the primary tumor, FIGO stage III disease with peritoneal involvement, and the presence of predominantly immature neuroectodermal components in the primary tumor [8, 11].

Although GTS lesions are histologically benign, their enveloping growth and aggressive local expansion can cause substantial morbidity and mortality. It is imperative to perform an adequate and total resection because GTS recurrence is impressive, with reported rates of 72-83% in patients with partial resections versus 0-4% in those who undergo complete resections [6,11], moreover patients in whom surgery is delayed can develop inoperable disease. The present case illustrates well this “aggressive behavior”. After three months from the first debulking surgery and during chemotherapy that successfully placed tumor markers AFP and HCG values within normal limits there were hepatic, abdominal, and pelvic growths, which required an extensive debulking surgery.

Also malignant transformation of mature ovarian teratomas is a well-known phenomenon and malignancies of all three embryologic lineages can develop [2]. Usually patients who present with secondary malignancies arising from mature teratomas of the ovary are at least 15 years older than the average patient with mature cystic teratoma, and have tumors of a larger size [12, 13]. One can thus extrapolate that secondary malignancies may develop in masses of GTS of the ovary, and especially in those that have been left in the patient for many years. The chances of degeneration of mature teratoma into undifferentiated tumors or even carcinomas have been reported to be up to 3% of cases [2, 14].

Imaging is not a foolproof means of discriminating between malignant germ cell tumors and GTS. However, some features on imaging studies such as well-circumscribed lesions, onset, or an increased number of cystic changes with elements of fat, punctuate, curvilinear calcifications, or an increase in density of the masses are commonly associated with the presence of GTS [15-18]. The role of [18F]-Fluorodeoxyglucose positron emission tomography has not been established but it may help in identifying GTS lesions that usually present negative uptake [19].

Unlike testicular tumors where distant metastases are common, the overwhelming majority of ovarian GTS nodules are confined to the pelvis, abdomen, or the retroperitoneum [2, 7, 8] with, to the authors’ knowledge, only two reported cases of an ovarian GTS, where the GTS nodules were seen elsewhere (in the neck lymph nodes and in the lung) [16, 20]. As GTS nodules consist of mature tissue, they lack the ability to metastasize or to invade surrounding tissues [2].

Cases describing hepatic GTS lesions in the literature show that such metastasis are located at the capsule. They are characterized by a thick fibrous capsule that does not infiltrate the liver parenchyma; therefore, it gives a misleading impression of metastasis [2, 10, 20]. In the present case, although the lesion was located in the middle of segment VIII, it surprisingly did not invade liver parenchyma. Radical surgical excision is the only way to provide complete cure for these patients and in experimented centers extended liver resection or even liver transplantation have been successfully employed [21].

There are few reported cases in the testicular or ovarian GTS literature showing any benefit of post-resection chemotherapy or radiation therapy [1, 9, 18]. Results are usually disappointing but significant clinical improvement as well as stability of a partially resected mass have been reported with the use of interferon, or the humanized monoclonal antibody bevacizumab [22-24]. Surgery is the only way to achieve complete disease control but these medical therapies may play a role in reducing the size and alleviating surgical dissections [4].

Conclusion

This report demonstrates that complete resection is the only way to improve survival and controlling the disease. More importantly, it highlights that clinicians need to be aware of the possible development of GTS when monitoring their patients with immature ovarian tumors. These patients require coordinated care between oncologist, gynecologists and surgeons to obtain the best possible outcomes. Immature teratomas usually present very good response with chemotherapy and recurrence is not a common event, on the other hand, the mature component may recur and long-term follow up is warranted. Surgical excision is usually technically challenging in this cases. However, it should not constitute an obstacle for the surgeon because only complete resection improves survival.

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MS assembly of data analysis, searched the database, selected the articles and drafted the manuscript; GGP and JPL carried out the surgery (), examined the patient for various check-ups, were crucial in the revision process of the reviewed manuscript and helped to check the quality of written English; SV examined the patient for various check-ups, monitored chemotherapy and helped with an
References


Guide wire surgery in breast cancer and why to avoid scissors

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Summary

Case: A 58-year old woman presented with microcalcifications in her left breast. A biopsy showed a low-grade ductal carcinoma in situ. A tumorectomy was performed using a harpoon-shaped guide wire to remove the entire lesion. No additional therapy was given. Six months later, follow-up mammography revealed that the distal end of the guide wire was still present in the left breast.

Conclusion: When performing a tumorectomy using a guide wire, the completeness of the wire should be checked during surgery. Additionally cutting of the wire can be prevented by using a scalpel instead of scissors during surgery.

Key words: Guide wire; Breast cancer; Breast sparing surgery; Cancer; Multidisciplinary; Breast clinic.

Introduction

The preoperative guide wire localization by ultrasound or mammography is the method of choice for guiding the surgical resection in non-palpable breast cancers [1]. In this way only the tumor with a minimal but sufficient amount of normal breast tumor (one cm) is removed. This approach will allow a good cosmetic outcome in well selected case for breast conservative surgery. During surgery, care should be taken not to cut the wire. After removal of the tumor, the specimen should be oriented and imaging should be performed. The preoperative imaging of the breast should be compared with the per-operative imaging of the specimen in order to check whether the tumor is removed with a sufficient amount of normal breast tissue and the wire is complete. Current case report is written to highlight the importance of performing imaging of the removed specimen.

Case Report

A 58-year-old female presented with micro-calcifications in her left breast on a screening-mammography. Her medical history was unremarkable and she never had used hormonal substitution therapy. There was no family history of breast cancer. Clinical examination was within normal limits.

An extra diagnostic mammography of the left breast showed a cluster of microcalcifications in the superolateral quadrant. This lesion was classified as BIRADS-Iva (Figure 1). A mammotome-biopsy revealed a low-grade ductal carcinoma in situ (DCIS) with microcalcifications.

A tumorectomy of the left breast was performed, after marking the lesion with a harpoon-shaped guide wire (Figure 2). The guide wire was excised together with the tumor and an X-ray of this specimen was taken. All microcalcifications were present, but it was unclear if the guide wire was complete. Unfortunately no additional mammography of the left breast was performed at this time to determine if the distal end of the guide wire was still present in the breast or not.

A pathologic assessment of the biopsy material confirmed the earlier diagnosis of fibrocystic lesions with a small focus of atypical ductal hyperplasia. No additional therapy was recommended. During the six months follow-up appointment in the Breast Clinic, a mammography was performed. The mammography was within normal limits (BIRADS-score I), no microcalcifications were seen, but the tip of the guide wire was still present in the breast (Figure 3). The patient was discussed during the multidisciplinary meeting and based on the fact that the previous surgery did not show DCIS and the core biopsy did, it was recommended to remove the distal end of the guide wire together with the surrounding tissue in order to exclude persistent DCIS. The definitive pathology report did not show any signs of atypia or DCIS.

Discussion

In the present case, the guide wire for marking the non-palpable breast lesion was accidentally cut and lead to confusion in the follow-up. When surgery is performed for a non-palpable lesion, the completeness of excision as well as the guide wire should be checked by taking a X-ray of the specimen. If there is a discrepancy between the biopsies and the guide wire is not completely removed, a re-excision should be done.

The complication was the result of an excision performed by scissors. If an excision is done by a scalpel the wire cannot be cut. Likewise electrocoagulation will not cut the wire, but this method has as disadvantage that it influences the pathological assessment of the margins and the they can influence the analysis for estrogen and progesterone receptors. Furthermore the fume of electrocoagulation may cause cancer among the people who are standing around the operating table, like: nurses, anaesthesiologists, and surgeons if they inhale it.
Ideally a tumorectomy for breast cancer should be performed with a knife. The tumor-free margin should be one cm. To avoid discussion about the margin, one should not use electrocoagulation. Furthermore electrocoagulation can have a false negative interpretation of hormone receptors in the specimen. After the skin incision is made, one should place a Kocher above and below the presumed lesion. Then in one movement the lesion should be excised with the knife according to the orientation of the guide wire in the breast. A careful preoperative preparation together with a dedicated radiologist who places the guide wire is essential for a successful operation. Taking time to prepare oneself as a surgeon together with the multidisciplinary team for an operation is essential to have success. Checking the specimen during surgery is must. This is not only to know if one has completely excised the lesion which one wants to remove, but also to know that one did not leave any thing behind.

In conclusion, during breast cancer surgery for a non-palpable lesion, one should not only check for the excised lesion, but also for completeness of the guide wire by an X-ray in order to avoid confusion.

References


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Introduction

Women who have a BRCA1 mutation have a 39% to 46% risk of developing ovarian cancer by the age of 70, while women with a BRCA2 mutation carry a 10% to 27% risk by the age of 70 [1]. Bilateral salpingo-ovariectomy (BSO) is suggested to BRCA mutation carriers between the ages of 35 to 40, or when childbearing is complete [2]. Consequently, a minimally invasive approach to perform BSO is advisable.

Case Report

A 43-year old woman, 2 para, referred to our department with a BRCA1 mutation diagnosis and previous a bilateral mastectomy for breast cancer. BSO was prescribed and a SPAL approach and the use of a new diode laser for cutting and coagulation was suggested. After complete information the patient consented to surgical treatment. X-Cone trocar was placed through a 20-mm umbilical incision and pneumoperitoneum was achieved (Figure 1). Intra-abdominal visualization was obtained with introduction of a five-mm 30 degree laparoscope [3]. A five-mm sheath bending/grasping forceps was inserted [4]. To perform adhesiolysis and bilateral salpingo-oophorectomy, a Dual wave lengths laser system was used to generate a 980-+1470 nm laser through a diode semiconductor (Figures 2-3). A 1,000 µm fiber with an a-traumatic conic tip at the distal end was inserted in a dedicated laparoscopic sheath. A light guide allows to address the laser through optical fibers on the target tissue with an extreme precision. After coagulation of ovarian ar-

Summary

Herein the authors report the first case of prophylactic bilateral salpingo-ovariectomy (BSO) in single port access laparoscopy (SPAL) with use of diode laser in a patient with BRCA1 mutation. As fimbria could be considered the site of origin for many serous carcinomas in BRCA mutation carriers, many studies are carried out to evaluate the possibility of preventing ovarian carcinoma with BSO. SPAL is a development of endoscopic surgery which further reduces invasiveness of surgical procedures. Diode laser presents a recognized precision for tissue cutting and coagulation and its use could be highly advantageous in SPAL surgery and in particular in such situations avoiding fallopian tube histology distortion and consequently improve the prognosis of BRCA carriers.

Key words: Diode laser; Ovarian carcinoma; Prophylactic salpingo-ovariectomy; Single port access laparoscopy; SPAL.
teries with a bipolar forceps they were sectioned with the laser. Adnexae were removed separately with an endobag.

At the end of the laparoscopic procedure to possibly prevent or decrease the occurrence of post-surgical adhesions 500 cc of warm lactated Ringer’s solution was instilled in the pelvis [5]. Suture of the fascia was performed to prevent hernia formation (Vycril 0, single stitches). Closure of the cutaneous wound and reconstruction of the umbilical shape was performed with absorbable single stitches.

Results

No intraoperative and postoperative complications were observed. Pain score measured immediately after surgery in the recovery unit was 3. The postoperative pain scores after six, 24, and 48 hours were 3, 2, and 1, respectively. The patient received a painkiller only six hours after surgery. Patient was discharged the day after surgery and fully recovered in one week. Pathology evaluation of adnexae did not show any subclinical disease. At one-month follow-up the patient cosmetic satisfaction was assessed using the Body Image Questionnaire (BIQ) that showed the maximum scores.

Discussion

A new paradigm for the pathogenesis of ovarian cancer based on a dualistic model and the recognition that the majority of “ovarian” carcinomas originate outside the ovary, assist in organizing this complex group of neoplasms and facilitates the development of new and novel approaches to prevention, screening, and treatment. In particular, type II ovarian carcinomas are composed of tumors that are aggressive, present in advanced stage, and develop from intraepithelial carcinomas in the fallopian tube [1]. BRCA carriers have a very high risk of developing such carcinoma during their life [1]. Consequently a BSO is suggested at 40 years of age or when child-bearing has been completed. Nowadays the role of minimally invasive surgery as multiport laparoscopy in oncology is growing [6, 7]. Moreover, single-port access (SPAL) laparoscopic surgery has been recently introduced into the field of minimally invasive surgery and implemented in gynecologic procedures [4, 5]. The main advantage of SPAL surgery is the excellent cosmetic outcome, less postoperative pain, and a faster recovery [8-11]. Recently, an exponential growth in the use of diode lasers has been observed in almost every area of pure and applied sciences. The currently available 980+1470 Nm diode laser may have certain advantages. A diode is an electronic laser consisting of two semiconductor materials with the size of a grain of sand. This technology makes it possible for this laser to be the smallest available. A microprocessor-controlled system regulates the flow of electrical current transmitted from the base unit to the surgical site by a solid quartz-core, fiber-optic cable. The heated tip can then be used to incise, excise, and coagulate tissue while a zone (0.3-0.6 mm) of thermo-coagulation provides excellent hemostasis of vessels up to two mm in diameter. In contrast to the CO2 laser, the diode laser is more often used in contact mode with minimal tissue penetration of only 0.3-0.6 mm. A visible light beam is combined with the invisible laser beam. Its use in gynecology has been recently described for salpingectomy with an excellent surgical outcome and no complication [12].
In SPAL, diode laser could be very useful as it consents to cut and coagulate without changing instruments with extreme precision, causes minimal thermal damage, is surgically time-saving, and improves the possible advantages of this approach. In BRCA carriers, SPAL BSO with the use of diode laser could be the best minimally invasive surgical approach. Another possible advantage of diode laser could be the avoidance of any thermal distortion of the fallopian tube consenting an early detection of microscopic serous carcinoma developing in the fimbria.

References


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Squamous cell carcinoma of Bartholin gland coexistent with human papillomavirus

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Summary

Squamous cell carcinoma of the Bartholin’s glands is a very rare form of vulvar neoplasm. This case presents a 46-year-old female diagnosed with advanced primary squamous cell cancer of the Bartholin glands that has proven positive for human papillomavirus (HPV). The patient was treated with a wide excision of the tumor, an ipsilateral lymphadenectomy, and an adjuvant chemotherapy and irradiation. After two years of follow-up the patient remains in remission.

Key words: Squamous cell cancer; Bartholin’s gland; Human papillomavirus.

Introduction

Primary carcinomas of the Bartholin’s gland account for 5% of all vulvar cancers and 0.25% of all gynecological malignancies [1]. Squamous cell carcinoma account for approximately 40% of Bartholin gland carcinomas. High risk human papillomavirus (HPV) is investigated as possible cause. Although HPV infection itself does not indicate viral involvement in carcinogenesis, it still points out the possible role of HPV infection as an early step in carcinogenesis, similar to the vulvar one, marking HPV 16 and 18 as most significant ones [2,3]. Carcinoma of the Bartholin gland usually manifests as an enlargement of the gland area and may appear to be a Bartholin cyst. The average age of women with this tumor is 50 years, with most between 40 and 70 years. Bartholin gland tumors area typically solid, deeply infiltrative, and occupy the site of the gland, occasionally obscuring its presence. They range from one to seven cm in diameter [4]. Their clinical presentations include arising tumour mass, pain, pruritus, bleeding, discharge, or their combination. In as many of 50% of patients, there is an initial misdiagnosis of Bartholin’s gland cyst or abscess, resulting in diagnostic and therapeutic delay [5].

Case Report

In this report the authors present a case of 46-year-old female, with two childbirths in her medical history. Patient underwent conization because of a CIN III 20 years prior, and hysterectomy without adnexectomy two years prior due to uterine myoma. Patient is also being treated for insulin-dependent diabetes mellitus. Patient was admitted to the present Department complaining of a painless mass in the Bartholin gland area. Further inspection showed that the greater labia were mildly bulging, the skin color was regular, and that there was no sign of Bartholin gland abscess. By palpation the authors had ruled out the presence of a cyst in the gland’s secretory duct; however, a solid, painless six cm mass was discovered. This tumour mass was attached to deeper structures and immobile. Palpation of the inguinal areas had also revealed enlarged and fixated lymph nodes, with no exulceration on the surfaces. After clinical examination, the existence of a tumor in the pelvis and distant metastasis was ruled out, and radical excision and ipsilateral lymphadenectomy were carried out. The tumor was solid, six cm wide, and showed invasive growth into the surrounding adipose tissue in a deep and irregular infiltration manner. The suspicion of a primary malignant tumor of the Bartholin gland was confirmed by a histopathological diagnosis. It was a moderately differentiated squamous cell carcinoma (Figure 1). Qualitative immunohistochemistry analyses of the histological preparations were carried out on p16 (CINtec), which was diffusely positive in all layers of the epithelium (Figure 2), and on the proliferation marker Ki67, which also showed increased expression (Figure 3). Of the twelve inguinofemoral lymph nodes removed, eight were infiltrated by the tumor, and two had their capsules destroyed. After surgical treatment, adjuvant chemoradiation was applied (cisplatin + RT 45 Gy). Irradiation was applied to the areas of the tumor site, the vulva, the inguinofemoral region, and the pelvis. Two years after treatment, the patient is showing no signs of disease.

Discussion

The differential diagnosis for a Bartholin gland tumor most commonly includes cysts and abscesses, which occur in 2% of women, and other vulvovaginal disorders, such as vulvar carcinoma, acrochordons, hidradenomas, other dermatoses, and condyoma acuminata. The Bartholin gland is composed of columnar epithelium and the ducts are lined by stratified
squamous epithelium, which changes to transitional cell epithelium toward the terminal ducts. According to Pinn et al., squamous cell carcinoma is the only lesion of the Bartholin gland linked to HPV [6]. The criteria that have to be met for the diagnosis of a primary carcinoma of the Bartholin gland, according to Chamlian’s and Taylor [7], are:

1. Transition between normal gland and tumor
2. The tumor involves the area of Bartholin’s gland, is histologically compatible with Bartholin origin, and there is no evidence of a primary tumor elsewhere [5].

The criteria for the diagnosis of a primary carcinoma of the Bartholin gland have been met in this case.

Squamous cell carcinoma account for approximately 40% of Bartholin gland carcinomas, the same as adenocarcinomas. Other types include adenoid cystic carcinoma (15%), transitional cell carcinoma (less than 5%), adenosquamous carcinoma (less than 5%), and poorly differentiated adenocarcinomas [4].

The questions arising from this are: Why is there a squamous cell carcinoma in the gland? What is the pathophysiology of this process? What is the origin of a tumor of this cytological type?

Bartholin’s gland carcinomas can be squamous if they originate near the orifice of the duct, papillary if they arise from the transitional epithelium of the duct, or adenocarcinomas if they arise from the gland itself [8].

The research by Felix et al. has shown that squamous cell carcinomas and adenocarcinomas of the Bartholin’s gland are antigenically similar to one another, but are distinct from the normal squamous epithelium of the vulva and the ducts and acinus of Bartholin’s gland, and are similar to the epithelium of the transition zone, which is similar to the transition zone of the uterine cervix [9].

The association between squamous cell carcinomas of the genital tract and HPV has been well established [10].

Research done by Felix et al. has shown that six of seven squamous carcinomas of Bartholin’s gland contained the HPV DNA. Both the squamous cell and adenocarcinoma seem to be arising from the same cell type, and it could be said that, as in the cervix, both originate in the transitional zone, and are associated with HPV infections [9].

In order to determine the connection between the squamous cell carcinoma and the transforming HPV infection, CINtec p16 immunohistochemistry was used as a way to indirectly determine oncogenic activity of high-risk HPVs. Overexpression of the p16 biomarker is the direct consequence of loss of control of the cell cycle, caused by HPV oncoproteins. As a result of the loss of cell cycle control, accelerated proliferation occurs, which manifests as overexpression of Ki-67 [11].
Immunohistochemistry with p16 has shown, in the present preparation (Figure 2), overexpression and diffuse positivity in all layers of the epithelium, along with a heightened Ki-67 expression (Figure 3), all of which are simultaneous signs of a transforming infection, progression, and proliferation. It is interesting to note that the patient was treated much earlier with conization due to CIN 3 of the cervix. This is often found in other authors’ reports on primary carcinoma of the Bartholin gland as a sign of a general infection of the genital tract with HPV, and speaks in favor of the statement that HPV is the cause of the primary squamous cell carcinoma of the Bartholin gland. These results also open a new topic: is there a real possibility for prevention of primary squamous cell carcinoma of the Bartholin gland (along with cervical cancer) by using HPV vaccination?

References


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Paraneoplastic neurological syndromes (PNS) caused by occult breast cancer and metastatic carcinoma of the lymph node

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Summary
Paraneoplastic neurological syndromes (PNS) are immune-mediated, subacute, and progressive syndromes caused by remote effects of malignant tumours rather than the direct infiltration of tumours. The most common maladies related to PNS are small cell lung cancer, breast and ovarian cancer, and Hodgkin’s lymphoma. Diagnoses of PNS frequently precede tumour diagnoses because the primary tumour is often occult. It is difficult for clinicians to recognise PNS, because there are various neurological symptoms and signs in the patient but few abnormal results of the examinations. The examination of paraneoplastic panels (cerebrospinal fluid (CSF) and serum) is useful in the diagnosis of PNS, but the false negatives should be considered. Due to the severe neurological morbidity and mortality caused by PNS, early diagnoses are important to allow for time to treat the underlying tumour and to obtain functional improvement. It is worth noting that regular re-examination and follow-up are crucial for reducing the rates of misdiagnosis and missed diagnosis of PNS.

Key words: Paraneoplastic neurological syndromes; Occult breast cancer; Metastatic carcinoma of the lymph node.

Introduction
Generally speaking, metastases to the central nervous system are the most common reasons for neurological disorders in cancer patients. However, in some cases, this is not the case at all. Paraneoplastic neurological syndromes (PNS), which have been reported over sixty years, are immune-mediated remote effects of malignant tumours involving the nervous system and are related to various anti-neuronal antibodies. In some patients with breast cancer, it is easy to diagnose PNS based on their clinical manifestations and a positive paraneoplastic antibody test. In comparison, it is very difficult to diagnose PNS for a few patients with nothing but persistent unexplained neurological deficit, especially with a negative paraneoplastic antibody test. There is no doubt that recognition of this syndrome is very important, therefore the authors report a case of occult breast cancer detected by a whole-body positron-emission tomography and an immunohistochemical technique in metastatic axillar lymph nodes. The pathogenesis of paraneoplastic syndrome is also discussed in this case.

Case Report
In April, 2013, a 63-year-old woman presented with a 20-day history of headache, nocturnal fever (37.5°C), xerostomia, palpitations, lower extremity weakness, and gait ataxia. She gradually became wheelchair-dependent and was admitted to a hospital for lumbar puncture. Multiple cerebrospinal fluid (CSF) analyses, including paraneoplastic and myasthenia gravis panels (CSF and serum), were nondiagnostic. Routine blood tests were normal with the exception of increased HbA1c and LDL cholesterol. Her headache and fever subsided ten days later, but she began to suffer from double vision (the left eye was limited in adduction and abduction, and the right eye was limited in abduction), dizziness, masticatory atonia, and a limited ability to open her mouth. She was transferred to the present institution and gradually became dysphagic over the subsequent 15 days. Multiple magnetic resonance imaging scans of her brain and spine revealed nothing but mild lacunar infarction and cervical spondylosis, both of which were unlikely to cause symptoms detailed above but could have explained the bilateral Hoffman signs, Babinski signs, and tendon hyperreflexia upon neurological examination. The motor powers in both lower and upper extremities were 5-/5, she denied experiencing sensory disturbances, and an EMG was unremarkable. There were so many neurological symptoms and signs in the patient but few abnormal results of the examinations, which imply that PNS was still the most likely diagnosis. Other most likely differential diagnoses include motor neuron disease and myasthenia gravis. However, results of EMG fail to support any of these three diagnoses. Multiple CSF analyses exclude the diagnosis of myasthenia gravis and PNS. Multiple tumour markers (glycogen antigen, non-small cell lung cancer, and glial enolase) were abnormally elevated, and the paraneoplastic panel was re-examined. The second examination revealed an anti-Ri antibody-positive finding, which commonly indicates small cell lung cancer (SCLC) or breast cancer. A mammogram showed no clear lesions in either breast. A chest CT revealed no diagno-
sis with the exception of right axillary lymphadenectomy. Furthermore, a whole-body positron-emission tomography (PET) confirmed the hypermetabolic activity of the right axillary lymph nodes and provided no evidence of any primary lesion in the breasts or other organs (Figure 1). The patient underwent an axillary node biopsy, and histopathology showed moderately differentiated adenocarcinoma that was consistent with a mammary origin (Figure 2). The patient was diagnosed with PNS due to occult breast cancer and metastatic carcinoma of the lymph node. After taking exemestane (30 mg/day) for three months, she underwent a mastectomy of the right breast with axillary clearance and died of pulmonary embolism.

Discussion

PNS are immune-mediated, subacute, and progressive syndromes caused by remote effects of malignant tumours rather than the direct infiltration of tumours, but the pathogeneses are not well understood [1]. The main targets of the immune responses are the central, peripheral, or autonomic nervous system, but these responses also attack the muscle, skin, and endocrine system [2]. The present patient exhibited characteristics of ataxia and multiple cranial nerve palsies. The most common maladies related to PNS are SCLC, breast and ovarian cancer, and Hodgkin’s lymphoma [3]. Diagnoses of PNS frequently precede tumour diagnoses because the primary tumour is often occult [4]. It is difficult for clinicians to recognise PNS. Due to the severe neurological morbidity and mortality caused by PNS, early diagnoses are important to allow time to treat the underlying tumour and to obtain functional improvement. When patients develop weakness, ataxia, sensory/motor disturbances, autonomic nervous disorders, or psychiatric disorders, and these symptoms are all persistent, severe, and difficult to explain based on the findings of routine medical examinations, PNS should be involved in the differential diagnosis. Routine tumour markers and paraneoplastic antibodies should be identified in the serum or CSF. If necessary, the test results should be re-examined to prevent false negatives. Notably, FDG-PET plays an important role in the diagnosis of PNS [5]. However, in 20% of patients, the primary tumours are too occult to be identified even by autopsy [6], which demonstrates that regular re-examination and follow-up are crucial for reducing the rates of misdiagnosis and missed diagnosis of PNS.

Conclusions

PNS are immune-mediated, subacute, and progressive syndromes caused by remote effects of malignant tumours rather than the direct infiltration of tumours, and it is difficult for clinicians to recognise PNS. The examination of paraneoplastic panels (CSF and serum) and multiple tumour markers is useful for the diagnosis of PNS. Early diagnoses are important for the treatment of the underlying tumour and to obtain functional improvement. Regular re-examination and follow-up are also crucial for reducing the rates of misdiagnosis and missed diagnosis of PNS.

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Paraneoplastic neurological syndromes (PNS) caused by occult breast cancer and metastatic carcinoma of the lymph node

References


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