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EUROPEAN JOURNAL OF GYNAECOLOGICAL ONCOLOGY (ISSN 0392-2936) publishes original peer reviewed works in the fields of female genital cancers and related subjects and also proceedings of gynecologic oncology society meetings all over the world. The Journal is covered by CURRENT CONTENTS, SCISEARCH, RESEARCH ALERT, INDEX MEDICUS, MEDLINE, EMBASE/Excerpta Medica, CURRENT ADVANCES IN CANCER RESEARCH, BIOSIS.
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Technetium-99m-sestamibi scintigraphy in gynecological cancer imaging

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Summary

A new diagnostic method, technetium-99m-sestamibi scintigraphy, and its potential use in gynecological oncology is described. The biochemical mechanisms of uptake and retention of technetium-99m-sestamibi in neoplastic cells are presented and the grounds for the potential use of the tracer in predicting the response to first-line chemotherapy in ovarian cancer patients are discussed. Based on the available literature data and on our own studies, the sensitivity and specificity of technetium-99m-sestamibi scintigraphy in ovarian cancer diagnosis are assessed, and the current place of this method among other functional imaging methods applied in gynecological oncology is discussed. Technetium-99m-sestamibi scintigraphy seems to provide an attractive alternative method to the expensive PET imaging, and can be easily performed in most hospitals. However, further studies in a larger series of patients are necessary before this method is widely applied.

Key words: 99mTc-sestamibi scintigraphy; Ovarian cancer; Chemotherapy.

Hexakis (2-methoxyisobutylisonitrile) technetium-99m (99mTc-sestamibi) is a radiopharmaceutical, originally developed for myocardial perfusion imaging, as its myocardial accumulation depends on blood flow and relates to mibi cation retention by mitochondria [1-3]. The potential use of 99mTc-sestamibi in oncology was first proposed in 1989 by Hassan et al. [4]. The authors showed an abnormal, elevated uptake of the marker in ten out of 13 patients with lung cancer, with a sensitivity of 77%. These results, along with the subsequent data on bone cancer published by Caner et al. [5], inspired a series of studies on 99mTc-sestamibi application in cancer diagnosis.

O’Tauma et al. [6] have shown 99mTc-sestamibi to be a highly specific agent for detection of metabolic activity in childhood brain tumors. An intense uptake of the tracer was presented in tumor-containing areas compared to uninvolved brain. These findings “opened the doors” to the use of 99mTc-sestamibi for diagnostic purposes in patients with central nervous system tumors. Further studies on 99mTc-sestamibi proved its value for imaging primary and metastatic tumors of the thyroid gland, brain, bronchus and breast [7-11]. The physical properties of 99mTc-sestamibi, i.e., gamma-ray energy and radiation dose, enables its usage with the conventional gamma radiation camera systems.

18Fluorodeoxyglucose (FDG) is a functional molecular imaging agent that detects the increased glucose metabolism of malignant tumors. FDG- position emission tomography (PET) imaging can reveal biochemical differences between normal and malignant tissues [12]. Hybrid PET-CT is a scanner that allows acquisition of spatially registered PET and CT data in one procedure, simultaneously providing anatomic and functional information and potentially improving both disease detection and its characterization.

Recently, PET-CT has emerged as a clinically important tool for evaluating patients with gynecologic malignancies, i.e., cervical, endometrial, and ovarian cancers [13-15], and the role of PET-CT in guiding therapeutic decisions is growing. PET-CT, with its functional imaging capabilities, offers the best available modality for detecting lymph nodes involved by cervical cancer [16]. The identification of metastatic lymph nodes with conventional CT and MRI is based on size, with short-axis diameter greater than 1 cm being a widely accepted criterion for the diagnosis of neoplastic involvement [17]. However, metastatic deposits in normal-sized lymph nodes and reactive lymph node enlargement do not allow us to reliably diagnose cancer infiltration either by CT or MRI, which can lead to false negatives and positives. Among contemporary imaging methods, functional imaging, besides morphological imaging, has emerged as a clinically important tool for diagnosing female genital tract diseases. There is a growing role of radioisotope-employing methods, especially in gynecological oncology. The development of specific new-generation tracers improves sensitivity and specificity of nuclear medicine techniques, which can be of a particular value when morphological imaging is ineffective or not conclusive.
Mechanisms of 99mTc-sestamibi cellular uptake

The 99mTc-sestamibi complex was described by Riche in the mid 1980s [19]. Stereochemical analysis has shown a stable complex with a central technetium atom (Tc-99m), surrounded by six mibi ligands (2-methoxyl isobutyl isonitrile), with surface methoxyl groups being the only functional groups.

The mechanisms of 99mTc-sestamibi uptake and retention in a cell have been extensively studied by Piwnica-Worms et al. [20, 21] and Kronauge et al. [22], who examined novel agents for myocardial perfusion imaging. The neutral 99mTc-sestamibi complex was found to accumulate not exclusively in myocardial cells, and was shown to be stable in vivo. Further studies revealed biophysical grounds of the 99mTc-sestamibi complex retention in a cell [23]. The fundamental myocellular uptake mechanism of 99mTc-sestamibi was demonstrated to involve passive distribution across plasma and mitochondrial membranes. In this respect, the 99mTc-sestamibi complex behaves like lipophilic cationic traces that diffuse across the cellular membrane into the cell in response to transmembrane potential. The large negative transmembrane potentials, especially in mitochondria, are responsible for tracer retention within the intracellular structures [21]. Tracer retention follows the Nernst equation [24]. Biochemical and cellular pharmacological studies with the use of electron probe X-ray microanalysis (EPXMA) and conventional electron microscopy confirmed the accumulation of the lipophilic complex at the inner mitochondrial membrane. However, the majority of the complex remains unbound within the mitochondrial matrix.

The main qualities of the 99mTc-sestamibi as a radiopharmaceutical are as follows:
- high specific activity of 10^8 Ci/mol,
- rapid distribution kinetics,
- low level of non-specific binding to plasma proteins,
- short half-life of approximately 6.02 h [25].

In a subsequent study Piwnica-Worms et al. [26, 27] discovered an additional factor influencing intracellular accumulation of 99mTc-sestamibi, a trans-membrane P-glycoprotein (Pgp).

Pgp is a 170kD protein, first described by Ling and Juliano [28, 29]. Increased expression of Pgp, encoded by the MDR1 gene, is associated with the development of multidrug resistance (MDR), thus it is linked to cancer treatment failure.

A classical experiment by Piwnica-Worms [26] has shown the potential use of the 99mTc-sestamibi complex to rapidly characterize Pgp expression in human tumors in vivo. Pgp was shown to actively transport the complex outside the cell. Studies on the cell lines with a different MDR1 gene expression confirmed this mechanism [30].

There is increasing interest in the use of 99mTc-sestamibi scintigraphy in oncology, both for diagnostic and predictive purposes. In our preliminary studies, we have examined the employment of 99mTc-sestamibi scintigraphy in patients with gynecological tumors. To date, most patients included in the study presented benign and malignant ovarian tumors, others were diagnosed with endometrial, uterine and vulvar carcinomas. Before scintigraphic imaging, all patients had been clinically examined, and abdominal and pelvic ultrasound scanning along with color Doppler scanning had been performed. At the beginning, we were afraid that physiological colon uptake of the tracer would make the imaging of gynecological lesions impossible, but scanning proved to be successful if performed within a few minutes after 99mTc-sestamibi injection. Physiological uptake of the tracer by the liver, gallbladder and small intestine is an obstacle in interpreting abdominal images, and further studies and experience are necessary to improve pre-operative diagnosis. Tracer uptake was assessed separately in pelvic and abdominal areas. Out of 70 patients with ovarian tumors 30 were diagnosed by histopathological methods with ovarian cancer as FIGO Stages IB to IV. 99mTc-sestamibi scintigraphy was characterized by 70% sensitivity at 70% specificity, lower to that of color Doppler ultrasound (sensitivity 93%, specificity 81%). However, when abdominal metastases were diagnosed, 99mTc-sestamibi scintigraphy was found more sensitive at a comparable specificity.

Piwnica-Worms et al. [27] in the studies on cancer cell lines have shown that 99m-Tc sestamibi is a substrate for Pgp and can be used for cellular Pgp detection [31-33]. 99m-Tc sestamibi accumulation was found to diminish with
the increase of MDR1 expression in breast cancer cell lines [34, 35]. Thus, 99m-Tc-sestamibi uptake may provide information about the Pgp status of the cells. These discoveries opened up extensive studies on 99m-Tc-sestamibi scintigraphy application for predicting response to chemotherapy in patients with breast and lung carcinomas, malignant melanoma, sarcomas and lymphomas [36-42]. The 99m-Tc sestaMIBI complex application in gynecological oncology was first examined in our pilot study [43] on 12 patients. We have demonstrated that the tracer accumulation assessment in patients undergoing chemotherapy may be useful for monitoring treatment response. We have also addressed the question of the value of 99m-Tc-sestamibi scintigraphy for monitoring the response to first-line chemotherapy in ovarian cancer patients. The 5-year survival rate for patients with clinically advanced ovarian cancer patients remains poor in spite of considerable efforts to improve the effects of treatment. The majority of cases are diagnosed at a late stage because there are no reliable screening techniques and early-stage ovarian cancer is generally asymptomatic. The essential ovarian cancer treatment is optimal surgery followed by chemotherapy. We have examined 25 patients with epithelial ovarian cancer FIGO Stages Ib to IV, following primary surgical treatment. All patients were scheduled for scintigraphy after surgery and after three and six courses of paclitaxel/platin chemotherapy, which is now the “gold standard” of the first-line chemical treatment of ovarian cancer patients. Following the sixth course of treatment, the response to treatment was assessed by physical examination, ultrasound and CT imaging, and CA 125 level measurement.

Before treatment, 18 patients (72%) presented 99m-Tc-sestamibi uptake. After treatment, 13 women had a complete clinical remission, ten patients presented disease progression, and the remaining two had a partial clinical remission. In patients with complete clinical remission, there was no uptake of the tracer, in patients with partial remission the tracer uptake remained unchanged, while in 80% of those with disease progression there was a high level of tracer accumulation.

We have found a strong correlation between the scintigraphic and the standard estimation of response to chemotherapy in ovarian carcinoma patients. Only one patient with FIGO Stage IIIc ovarian carcinoma and progression of disease after completion of chemotherapy, and with a high level of 99m-Tc-sestamibi uptake before treatment, presented no tracer uptake after treatment. This is in accordance with the results of Goldstein [44] and Bourhis et al. [45], who have shown low levels or the lack of P-glycoprotein expression in ovarian cancer at the time of diagnosis. The lack of false-positive results in our study points to the high specificity of 99m Tc sestaMIBI scintigraphy in estimating the response to treatment in patients with ovarian cancer. False-negatives were shown in two patients. Similar results were presented by Marshall et al. [46] and Dunnwald et al. [47] in breast cancer patients.

In summary, 99m-Tc-sestamibi scintigraphy in ovarian cancer patients seems to have a clinical value both for the preoperative diagnosis and for monitoring of treatment response. Further studies in a larger series of patients are necessary to confirm the utility of this method.

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Genetic polymorphisms, the metabolism of estrogens and breast cancer: a review

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Introduction

Breast cancer is the most common female cancer and the second cause of cancer death in women. Despite recent breakthroughs in the knowledge of the molecular pathology of the disease, such as the discovery of mutations in the genes BRCA1 and 2, p53 and PTEN, exposure to endogenous and exogenous estrogen throughout life cannot explain the heterogeneity of prognosis nor clinical features of patients. Recently, many gene polymorphisms in the metabolism of breast cancer have been described as possible neoplasm etiologic factors. This review is an attempt to summarize the current knowledge about these polymorphisms and to determine new target genes for diagnosis and treatment of the disease. Polymorphisms in the genes CYP17, CYP19, CYP1A1, CYP1A2, CYP1B1, UGT1A1, SULT1A1, 17-hydroxysteroid-dehydrogenase, COMT, GST, ESR1, and ESR2 are described.

Key words: Breast cancer; Metabolism of estrogens; Estrogen receptors; Polymorphisms; Metabolizing genes.

Risk factors for breast cancer

Today, the most important risk factors for breast cancer include high serum estrogen levels, many ovulation cycles due to early menarche (younger than 12), or late menopause (older than 55), hormonal replacement therapy after menopause, use of oral estrogen contraceptives for ten years or more, and obesity after menopause (BMI > 30.7 kg/m²) [4]. Obese women show higher serum estrogen levels due to greater estrogen biosynthesis and lower serum estrogen binding protein levels. However, they have longer menstrual cycles and, therefore, fewer ovulations, which may be protective before menopause.

Other risk factors are high bone and breast density (> 75% at mammography), as markers for high estrogenic action, nulliparity or first pregnancy older than 30 years, breast feeding for less than two years, BRCA 1 and 2 mutations and smoking habit [5]. Pregnancy and breast feeding are believed to induce the final ductal maturation, preventing malignant transformation.

The prognostic factors currently used are surgical staging, nuclear and histological grades, and the expression of ESR1 and HER2. However, a recent review [6] has highlighted the importance of many genes that are related to the estrogen metabolism pathway and disease progression (“70-gene-profile”), as possible markers for breast cancer outcome.

Estrogen metabolism and action

The estrogen synthesis pathway is summarized in Figure 1. Dehydroepiandrosterone is produced in the adrenal glands and converted to testosterone and androstenedione by enzymes of the complex CYP17. They are further metabolized to estrone and estradiol by the enzyme aromatase (member of the complex CYP19) in the ovaries, during the first half of the ovulatory cycle, but also in the breasts, adipose tissue, liver, and muscles. The extraglandular production of these hormones is of major importance after menopause, when breast estrogen levels can be from 10 to 50 times the serum concentration [7]. Locally, in many tissues, estrone and estradiol can be converted to estriol by enzymes of the complex CYP1A1. Estrone, estradiol and estriol are interchangeable due to the action of the enzyme 17-hydroxysteroid-dehydrogenase, and in the breast the action of estradiol predominates.

The primary metabolism of estrogens can follow two different pathways:

Summary

Breast cancer is the most common female cancer and the second cause of cancer death in women. Despite recent breakthroughs, much of the etiology of this disease is unknown and the most important risk factor, i.e., exposure to endogenous and exogenous estrogen throughout life cannot explain the heterogeneity of prognosis nor clinical features of patients. Recently, many gene polymorphisms in the metabolism of breast cancer have been described as possible neoplasm etiologic factors. This review is an attempt to summarize the current knowledge about these polymorphisms and to determine new target genes for diagnosis and treatment of the disease. Polymorphisms in the genes CYP17, CYP19, CYP1A1, CYP1A2, CYP1B1, UGT1A1, SULT1A1, 17-hydroxysteroid-dehydrogenase, COMT, GST, ESR1, and ESR2 are described.
1) **Conjugation** – Enzymes UDP-Glucuronosyltransferase 2 (UGT1A1) and Sulfotransferase-E1 or A1 (SULT1E1/SULT1A1) add the radicals glucuronil and sulfate, which are hydrosoluble to estrogens, and which are later excreted in the urine.

2) **Hydroxilation** – In the liver, breast and other tissues, enzymes related to the complex P450 promote the hydroxilation of estrogens, generating many compounds which will suffer secondary metabolism.

   A) 16-α-hydroxyestrogens (16-α-OH-E1/E2) are normally produced in small amounts by enzymes in the complex CYP1A1 [7] and later glucuronized by the enzyme UGT1A1. One study [8] showed lower urinary levels of this product among women in Finland when compared to women from Asia, who have a risk up to three times lower for breast cancer. This suggests a possible protective action of 16-α-OH-E1/E2.

   B) Catecholestrogens: 2-hydroxyestrogens and 4-hydroxyestrogens (2-OH-E1/E2 and 4-OH-E1/E2) – the main metabolites of estrogens [10] – are produced by the complexes CYP1A1, 1A2 and 3A4 (2-hydroxyestrogens) and CYP1B1 and 3A4 (4-hydroxyestrogens), and are methylated by the enzyme catechol-O-methyltransferase (COMT). The products from this reaction are conjugated to sulfate and glucuronil by the enzymes SULT1A1 and UGT1A1; 2-hydroxyestrogens are inactive and antiangiogenic whereas 4-hydroxyestrogens have an estrogenic action even greater than estradiol and, after glucuronization, can cause damage to DNA. Therefore, a reduction in the 2-hydroxyestrogen/4-hydroxyestrogen ratio is related to a higher breast cancer risk [9].

   C) Catecholestrogens: 2,3-hydroxyestrogens and 3,4-hydroxyestrogens (2,3-OH-E1/E2 and 3,4-OH-E1/E2) – the complexes CYP1A1, 1A2 and 3A4 can produce to a lesser degree, 2,3-hydroxyestrogens and the complexes CYP1B1 and 3A4, 3,4-hydroxyestrogens. These metabolites are oxidated by the complex P450, yielding quinones, which are later conjugated to glutathione by enzyme glutatino-S-transferase (GST). This process yields reactive oxygen intermediates which can damage DNA and the 3,4-hydroxyestrogens after this reaction can cause depurination [10].

   Estrogens have a proliferative action and are responsible for breast and endometrium maturation, and maintenance of bone density. They act on two receptors, ESR1 and ESR2 (α and β), translated on different sites: ESR1 in the long arm of chromosome 6 (6q25.1) and ESR2 in the long arm of chromosome 14 (14q22-24). Both are intracellular receptors, with two binding-sites for estrogens, and a binding-site for DNA. Structurally, they differ only in their binding site for estrogens, in which they show only 58% homology [11, 12]. After activation, they dimerized and migrate to the cellular nucleus, binding to regulatory regions of DNA and activating the expression of transcription factors. There is some evidence that their activation also stimulates the transcription of mitochondrial DNA and the production of cAMP [7], TGF- and EDGF [9].

   ESR1 is the most common in the whole body and responsible for the action of estrogens in the uterus, ovaries, endothelium and bones. ESR2 can be found in bones, cardiovascular epithelium, normal breasts, and ovaries [11].

   **Polymorphisms In Enzymes Related To Estrogen Metabolism And Their Role In Initiation And Progression Of Breast Cancer**
CYP17

A polymorphism is a genetic variant that appears in at least 1% of the population.

The polymorphism A2 or MSP1 of this enzyme consists of a substitution of thymine (T) to cytosine (C) in the position 743572 in the promoting region of the gene. It is found in 32% of Asians, 22% of Japanese, 14% of Caucasians and 13% of Afro-Americans [13] and enhances the enzyme action, yielding higher levels of estrogens and androgens during childbearing ages [14-16], but lower levels after menopause [17]; these associations are stronger for Hispanics [16]. These findings support the theory that polymorphism enhances breast cancer risk, but one study [18] has related the alteration to a higher production of 2-hydroxyestrogen, which is anti-neoplastic.

In fact, some groups have related the polymorphism to a higher breast cancer risk for women in childbearing ages [19-23] and after menopause [24]. Others [14, 25-32], however did not find any relation at all, but discovered that polymorphism enhances the risk of hormonal replacement therapy [29] and lack of breastfeeding [30]. Studies in India found a higher risk for cancer [20, 24], but opposite results were reported in China [25, 32].

CYP19

The number of TTTA repeats in the intron 4 of the gene is polymorphic. Ten repeats have a prevalence of 8.2% in China [25], 6.3% in Russia [19] and 8.7% in Australia [33]. The polymorphism is said to influence gene splicing and stimulate a higher conversion of androgens to estrogens. In fact, one study [17] attributed this alteration to estradiol levels up to 21% higher than normal.

Many studies found no correlation between this polymorphism and breast cancer in China [25], England [33, 34] and the United States [35], however, others found a strong correlation with breast cancer risk [18, 19, 26, 36], size of the tumor [36] and family history of breast cancer [26]. In the latter, the polymorphism seemed to influence the risk for patients’ daughters, regardless of the daughters’ polymorphism, suggesting a role of intrauterine exposure to estrogen in the genesis of breast cancer.

CYP1A1

Four polymorphisms are known in this gene and the m1 (or Msp 1, or CYP1A1*2A) has a prevalence of 4.2% among Afro-Americans [37], 3.5% in Africa [38] and 38.3% in China [39]. The substitution of T for C in the position 3801, a noncoding region of the gene, is related to higher levels of 2-hydroxyestrogens [40], which in theory, reduces the risk of breast cancer.

In fact, there is a lower incidence of the disease in Chinese women bearing the polymorphism [41], nevertheless the same does not happen in Taiwan [42]. There is a higher risk for Afro-Americans [37] and Africans [38], but no relation for Indians [43] or Caucasians. Just one group [44] found a higher risk for earlier and higher grade cancer in Caucasians carrying the alteration.

CYP1A2

The polymorphism CYP1A2*F, a substitution of cytosine to adenine in position 167 of the promoter-region, is present in 67.3% of Canadians [45] and 68% of Europeans [46]. It is related to lower enzymatic activity and a higher 16-α-hydroxyestrogen/catecholestrogen ratio, which was associated with a lower breast cancer risk in one study [47], but not in others [48, 49].

CYP1B1

This gene is highly polymorphic and the polymorphisms m1 and m2 are of major importance. The m1 polymorphism is a substitution of valine to leucine in position 432 of the enzyme and can be encountered in 25% of Afro-Americans, 67% of Caucasians and 83% of Chinese [50]. The alteration is related to a higher production of 4-hydroxyestrogens, reducing the 2-hydroxyestrogen/4-hydroxyestrogen ratio [40] and enhancing the risk of breast cancer.

This risk was not statistically significant in many studies [51-55], but a meta-analysis found a higher risk for Caucasians [56] and women in Turkey with a BMI higher than 24 kg/m² [57]. However, no relation was found for the Chinese [56, 58] and even a reduction in risk was reported for Africans [56]. The risk seems to be directly proportional to age [56], but for cancers diagnosed after menopause, the polymorphism is related to a higher expression of ESR1 [51, 55] and better disease-free survival [59].

The m2, a substitution of asparagine for serine in the position 453, has a prevalence of 17.4% in Caucasians [51], 3.4% in Afro-Americans [51] and 29% in Europeans [54]. It enhances the enzymatic activity, reduces the levels of 2-hydroxyestrogens and 16-α-hydroxyestrogens, and rises the levels of 4-hydroxyestrogens [18], specially after menopause [55], which theoretically enhances breast cancer risk. In fact, one study [45] found a higher risk with family transmission, especially for women with a BMI higher than 27 kg/m², but it was not confirmed by other studies [51, 54, 55].

UGT1A1

The UGT1A1*28 is one extra TA repeat in the TATA-box in the promoting region of the gene and happens in 13% of the Chinese population [60]. In theory, it reduces enzymatic activity, lowers the estrogen inactivation ratio and enhances estrogen serum and tissue concentrations. In fact, carriers of the polymorphism have higher breast densities [61].

Two studies attributed the alteration to a higher risk, one in women with a family history of breast cancer [62], the other in young Chinese women without any family history, low BMI and late menarche [60], suggesting that in this population the alteration is a risk factor independent of estrogen levels. The polymorphism has also been associated with more aggressive tumors, a higher probability of being larger than 2 cm at diagnosis [62], and lower expression of estrogen receptors [63].
ESR1. The polymorphism PvuII is located in intron 1 and confirmed in a recent study conducted in Brazil [80].

Higher breast cancer risk [44, 79]. This, however, was not deletion [78] and two others attributed the alteration to a mutation in breast cancer tissues of women carrying the polymorphism to a higher risk of lymph node metastasis [36, 64].

17-Hydroxysteroid-Dehydrogenase

The polymorphism B1 is a substitution of adenine to guanine in position 1954 (exon 6), generating a substitution of serine to glycine in position 312. Some studies attributed the polymorphism to a higher conversion of estrone to estradiol and a higher risk of breast cancer [70]. Afterwards, the risk was confirmed for obese women after menopause [32] and young women with normal BMI [22].

COMT

The polymorphism COMT-L is present in 27.4% of North-Americans [58], 25% of Caucasians [71] and 5.2% of Chinese [31]. A substitution of A to G, and the resulting substitution of valine for methionine in position 158, results in an enzymatic activity four times less and, consequently higher levels of 2-hydroxyestrogens [17, 18].

There are many studies assessing breast cancer risk regarding this alteration: one group [72] found a reduced risk, probably due to the anti-proliferative action of 2-hydroxyestrogens, while others attributed the polymorphism to a higher risk [31], both for fertile women [31, 72] and women after menopause [71], and some found no relation at all for women in China [58, 73], Europe [74, 75], Turkey [57] and North-America [76]. One study encountered a risk not related to age or hormone levels [74] and others found a risk influenced by BMI [72] and smoking habit [77].

In 2005, a meta-analysis [58] concluded that there was no relation between this polymorphism and breast cancer, but most of the eligible studies were conducted in Chinese women who have a lower risk of breast cancer.

GST

The polymorphism GSTM1 0/0 (null) inactivates the enzyme, allowing quinone conjugates to accumulate and damage the DNA. One study found a higher rate of somatic mutations in breast cancer tissues of women carrying the deletion [78] and two others attributed the alteration to a higher breast cancer risk [44, 79]. This, however, was not confirmed in a recent study conducted in Brazil [80].

ESR1

Four polymorphisms have been extensively studied in ESR1. The polymorphism PvuII is located in intron 1 and corresponds to the alteration c454-397C-T. It has been found in 35% of black women, 13% of Caucasians and 16% of Hispanics [81]. It is related to a higher estrogenic action, responsible for higher breast and bone density after menopause, higher serum cholesterol, earlier menarche [82] and menopause [83] and better response to hormonal replacement therapy [84].

Two groups [85, 86] found no association between the polymorphism and breast cancer, but others found a higher risk for ductal cancer for all ages [87], especially after menopause [83]. The risk seems to be directly proportional to BMI [83], to number of ovulation cycles and to serum estrogen binding protein levels [12]. One group has attributed the alteration to a lower probability of expressing progesterone receptors [86], but no influence in the expression of estrogen receptors [88].

The polymorphism Xbal, in intron 1, consists of the alteration c454-351A-G and occurs in 34% of the general population [83]. It is related to a better response to estrogens, later onset of menopause, and higher breast density after hormonal replacement therapy [89].

There is a direct relation between the number of polymorphic alleles and risk for ductal cancer [83], but this relation is statistically significant only for women older than 45 years and after menopause [87]. The risk was confirmed for the Chinese [87] and Korean [85] populations, but not for women in Norway [90], Hispanics or Caucasians [91]. One study related the polymorphism to a higher expression of ERS1 in the tumor cells [86].

Allele 1 in codon 325 is the alteration CCC-CCG in exon 4 and has a prevalence in the general population of 55.4% [92]. The polymorphism leads to endogenous activation of the receptor [93], and is related to more aggressive ESR1-negative tumors [94]. What is more, it influences the action of ESR1 over the expression of e-cadherin, an adhesion molecule which regulates cell proliferation [95]. Two groups detected a higher breast cancer risk in women bearing this polymorphism, one analyzing the general population [96], while the other studied women with a family story of breast cancer [97]. One study related this SNP to a higher risk of lymph node metastasis [92], not confirmed by later studies [87, 96].

The multiple repetitions of GT in intron 1 of the gene ESR1 have been poorly analyzed. One group associated longer repetitions to higher breast cancer risks [98], and others related the alteration to higher mortalities among tumors expressing ESR1 [12, 84, 98]. Eighteen repetitions were associated with the highest mortality rates. All three studies are based on the Shanghai Breast Study database and found an interaction of this polymorphism and the polymorphisms PvuII e Xbal in breast cancer risk.

ESR2

During carcinogenesis breast tissue expresses higher levels of ESR1 and loses up to 60% of its ESR2 expression [99]. This led to the theory that ESR2 may control the mitogenic action of ESR1 [100].
The effect of polymorphisms in this gene on breast cancer is poorly understood. In China, two alterations have been described: C(14206)T and C(33390)G, in intron 5 and exon 7, respectively. In a case-control study with 1,134 cases and 1,235 controls [12], the polymorphism C(33390)G was strongly associated to breast cancer, with an odds ratio of 2.5, which could be as high as 4 for women with high estrogen and low estrogen-binding protein levels. The alteration C(14206)T has been associated to a higher incidence of benign fibroadenoma.

Two groups have recently evaluated the association of the number of CA repeats in the gene and breast cancer risk in Caucasians. One of them found out that shorter repetitions are an independent risk factor [101], while the other found a significant risk only when the repeats were associated with other alterations in ESR1 and the androgen receptor [102].

**Conclusion**

The evaluation of gene polymorphisms must be cautious. The heterogeneous distribution in populations make it very difficult in study comparisons carried out in different countries. Furthermore, the change of function promoted by the alteration is much more minimal and, if the impact of a polymorphism seems huge, the presence of confounding factors should be considered. Analyzing risk factors for neoplasms is also difficult. The disease is multifactorial and many of these factors are unknown and vary in different populations.

For this reason, there are polymorphisms that, in theory, are related to higher breast cancer risk, but their prevalence in the population is so high that they are unlikely to be independent risk factors. For example, the polymorphism m1 of the gene CYP1B1 has been associated to a higher breast cancer risk in Caucasians by two meta-analyses [56, 57], and can be found in up to 65% of this population [50]. There are also polymorphisms related to a higher risk in some populations, but not in others, like the same CYP1B1, which was not related to breast cancer in the Chinese population, despite being present in 83% of this population [56, 58]. This difference is certainly due to other factors affecting the gene-gene and environment interaction.

However, there is enough evidence to conclude that polymorphisms affecting the metabolism and action of estrogens play important roles in breast cancer. Based on the data presented in this article, we suggest the following associations of polymorphisms and breast cancer, which must be confirmed by proper studies:

- **Probably associated with breast cancer:** UGT1A1*28, SULT1A1 Arg213His.
- **Association only for some populations:** CYP17A2, CYP1A1m1, CYP1B1m1, ESR1 XbaI.
- **Probably not associated with breast cancer:** COMT-L.
- **Insufficient data:** CYP19(TTTA)n, 17β-Hydroxysteroid-dehydrogenase-B1, CYP1A2*F, CYP1B1m2, STM1 (null), ESR1 325 (CCC-CCG), ESR 1 PvuII, ESR1 (GT)n, ESR2 C(33390)G, ESR2 (CA)n.

When analyzing the prevalence of polymorphisms in different populations, it becomes clear that the polymorphism SULT1A1 Arg213His, probably associated with breast cancer, is less common in the Chinese, who have a reduced breast cancer risk, and the ESR1 PvuII, also related to a higher risk, is more common in black women, who have higher incidences of the disease. These findings do not fully explain the different incidence of the disease in these two groups, but suggest that it may be related to gene polymorphisms.

A more careful analysis of the polymorphisms presented here and the discovery of new ones may detect new factors influencing breast cancer risk and prognosis, with an important impact on the diagnosis and treatment of the disease.

**Acknowledgments**

We would like to thank Anderson Luis do Nascimento and the gynecologist Ismael Dale Cotrim Guerreiro da Silva for their assistance.

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Peri- and intratumoral T and B lymphocytic infiltration in breast cancer

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**Summary**

**Purpose:** To investigate peritumoral and intratumoral infiltrates in surgical specimens obtained from patients with invasive breast cancer, and of relating these to tumor size. **Methods:** Twenty-six surgical specimens obtained from patients diagnosed with breast cancer underwent immunohistochemical preparation and CD3, CD8, CD20 and CD68 labeling. The positive cells were counted in the tissue samples and correlated with the tumor size determined by imaging methods (TIA $\leq$ 2 or TIB $> 2$ cm). **Results:** There was a significant reduction in intratumoral B lymphocytes (CD20$^+$), although this reduction could only be observed in TIA. In relation to peritumoral T lymphocytes (CD3$^+$), there was a significant reduction in TIB, in comparison with TIA. Peritumoral and intratumoral CD3$^+$ and CD68$^+$ presence in completely opposite ways in both sizes of tumors. **Conclusion:** Peritumoral and intratumoral infiltrates of T and B lymphocytes are different and depend on tumor size.

**Key words:** Infiltration cells; Lymphocytes; Macrophages; Neoplasia; Immunohistochemistry.

**Introduction**

Since the first research on tumors and the immune system, studies have attempted to correlate cell infiltrates with the immune response to tumors in several organs \cite{1, 2}, including in breast cancer cases \cite{2, 3}. It was thus hoped to gain a better understanding of the specific mechanisms through which the immune system may respond to tumors. Through such understanding, it might at some time in the future become possible to interfere with the pathogenesis of tumor cells and induce their eradication. One of the most interesting findings regarding lymphocytic infiltrates in peritumoral tissue and peripheral blood is the increase in the CD4$^+$/CD8$^+$ ratio in patients with breast cancer \cite{1, 2}. Breast cancer patients present suppressed neutrophil migration response upon chemotherapy, and this is probably due to the circulation of factors involved in mechanisms that inhibit neutrophil migration \cite{4}. There has been speculation that these cells may have an antitumor function, but this has not yet been confirmed \cite{5}.

Another question that needs further study is whether inflammation and lymphocytic infiltration occur in order to favor the antitumor response to breast cancer, or not. Tumors with markers indicating a worse prognosis have been shown to exhibit increased quantities of lymphocytic infiltrates, and of natural killer (NK) cells in particular \cite{6}. A study on precursors by Underwood concluded that lymphocytic infiltration is a sign of favorable prognosis \cite{7}. Corroborating this hypothesis, Ogmundsdóttir \textit{et al.} showed that lymphocytes may stimulate the epithelium of breast cancer, and that this stimulation is strongly correlated with the expression of class I MHC molecules (major histocompatibility complex) molecules by the tumor cells \cite{8}. In contrast, there are other studies demonstrating that leukocytic inflammation and infiltration are associated with a worse prognosis \cite{9, 10}. Stewart and Tsai \cite{11}, in a wide-ranging review of 35 independent studies, found that in 23 of these a high degree of lymphocytic infiltration in tumors was correlated with worse prognosis for the patients.

The role of immune response in patients with breast cancer is still a source of controversy with regard to its relationship with prognostic factors and its relevance, to the point that the response may alter the way in which the therapy is conducted. Another point that should be considered is tumor size versus cellular type (intra- and peritumoral). Independent of their size, different factors and/or different concentrations could be secreted that might induce or inhibit the selective migration of different intra- and peritumoral cell types. Thus, the present study had the aims of studying peritumoral and intratumoral infiltrates of T and B lymphocytes and macrophages in biopsies obtained from patients with invasive breast cancer, and of relating these to tumor size.

**Materials and Methods**

**Patients**

Twenty-six surgical specimens from female patients (26 patients in total) with an anatomopathological diagnosis of invasive breast cancer were analyzed. The patients were attended by the mastology team of the Teaching Hospital of the Federal University of Triângulo Mineiro.
Patient ages ranged from 26 to 81 years (x = 50.6 ± 11.3), parity from one to ten deliveries (x = 3 ± 2.2), age at the time of menarche from ten to 16 years (x = 13.1 ± 1.5) and age at the time of first pregnancy from 16 to 44 years (x = 24 ± 6.8). All 26 patients had children and 23 had breastfed, with length of breastfeeding ranging from two to 120 months (x = 24.4). Age at the time of menopause (n = 13) ranged from 40 to 55 years (x = 47.2 ± 4.7).

The predominant histological type was invasive ductal carcinoma, which accounted for 21 (80.8%) of the 26 cases studied. There were also two cases of invasive lobular carcinoma, one of tubular-lobular carcinoma, one of inflammatory carcinoma, and one of mixed ductal and adenosquamous carcinoma.

Tumor size

The average diameter of the tumor ranged from 2.0 to 7.0 cm. Most of the tumors were in clinical Stage IIB (n = 18), and there were also three cases in each of Stages I and IIIA, and one case in each of Stages IIA and IIIB.

Patients were assessed at the time of diagnosis using imaging methods (mammography and ultrasound). Longitudinal and transverse diameters were obtained and the greatest diameter was considered for the staging. Clinical staging of the disease was based on the TNM system, as updated by the Union Internationale Contre le Cancer (UICC) [12] and the American Joint Committee on Cancer (AJCC) [13]. Patients with Stage IV were excluded from the study. Mammography and ultrasound (US) were used for tumor measurement, because the clinical measurement method involves normal breast tissue and, if peritumoral edema were present, this could overestimate the size. Histopathologic size was not used because some patients were submitted to neoadjuvant chemotherapy. Image tumor sizes (T) were divided for statistical purposes in tumors less than or equal to 2 cm (TIA, n = 17) or tumors larger than 2 and < 6 cm (TIB, n = 9).

Anatomopathological analysis

The products from the surgical specimens utilized for diagnosing the neoplasia were fixed in 10% formol. All the specimens contained representative samples of the tumor. There were also two cases of invasive lobular carcinoma, one of tubular-lobular carcinoma, one of inflammatory carcinoma, and one of mixed ductal and adenosquamous carcinoma.

Immunohistochemistry and cell counting technique

The slides were deparaffinized using xylol and immediately hydrated in pure alcohol and subsequently in water. They were immersed in phosphate-buffered saline (PBS) solution (pH 6) and left in 3% oxygenated water for 15 min. Next, they were placed in a steaming pan for 30 min with a citrate buffer and then cooled, rinsed, washed and left in PBS solution for 10 min. The slides
were then incubated for 20 hours with the primary antibodies: CD3 (polyclonal, subclass IgG1/Kappa, dilution 1:800; Dako), CD8 (clone 1A5, subclass IgG1/Kappa, dilution 1:50, Dako), CD20 (clone L26, subclass IgG1/Kappa, dilution 1:600, Dako) and CD68 (clone KP1, subclass IgG1/Kappa, dilution 1:1000; Dako). All these were diluted in 1% PBS-BSA (phosphate-buffered saline solution containing 1% bovine serum albumin). Afterwards, they were then washed twice with PBS and incubated with the secondary antibody (antirabbit or antimouse biotinylated antibodies) for 30 min. They were again washed and then incubated with peroxidase-conjugated streptavidin (Dako LSAB 2 peroxidase kit) for 30 min. Finally, the slides were washed and developed using diaminobenzidine solution for 5 min and mounted for analysis, using Entellan. The same technique was applied to lymphoid tissue (amygdala), as a control.

Following these immunohistochemical procedures, the distribution patterns and morphological details of the cells were analyzed and compared, and carefully recorded for each specimen. The scoring standard adopted by Georgiannos et al. [14] was utilized for counting the lymphoid cells. Briefly, the slides were categorized according to the proportion of stained cells: 0 = none; 1 = rare cells; 2 = moderate number of stained cells; 3 = abundance of stained cells (Figure 1a-c). Initially, the cells were observed at low magnification (10x), to obtain a general impression of the cell distribution, and in particular the maximum count. Following this, they were examined in detail (magnification: 40x) to obtain the final counts. These procedures were done by means of analyzing the peritumoral or intratumoral CD20.

![Figure 2. Distribution of different cell types according to the intensity of peritumoral and intratumoral infiltrates. The intensity of immune cell infiltrates in 26 biopsies obtained from patients with breast cancer was evaluated by immunohistochemistry and determined in accordance with the protocol devised by Georgiannos et al. The different cell types studied were: T lymphocytes (CD3) cytotoxic lymphocytes (CD8) B lymphocytes (CD20) and macrophages (CD68). The results were expressed as infiltrate intensity ratios (high/low), according to cell localization: peritumoral (white) or intratumoral (black). * p = 0.03 (Fisher test), comparing peritumoral and intratumoral CD20.](image-url)
breast tissue around the neoplasia. Infiltrations of lymphoid cells in regions distant from the neoplastic focus were not taken into consideration.

Statistical Analysis

Analysis of the slides was performed on the final result obtained following concordance between three observers. The slides were categorized for analysis according to the proportion of stained cells: low infiltration when the proportion was 0 and 1 (respectively, none and rare stain cells), and high infiltration when the proportion was 2 or 3 (respectively, moderate and abundance of stain cells). The results are presented as the ratio of the numbers of high cases/low cases (2-3 stain cells/0-1 stain cells). The Fisher test was used for statistical analysis (GraphPad Prism version 3.00-GraphPad Inc., San Diego, CA). Results were considered to be statistically significant when p ≤ 0.05.

Results

Figure 2 shows the intensities of infiltrated T lymphocytes – CD3 (A), cytotoxic lymphocytes – CD8 (B), B lymphocytes – CD20+ (C) and lymphocytes and macrophages – CD68 (D), according to localization in relation to the tumor (peritumoral or intratumoral). Infiltrated CD3, CD8, CD20 were less stained, and CD68

Figure 3. Distribution of different cell types between peritumoral and intratumoral localization, according to tumor size. The intensity of immune cell infiltrates in 26 biopsies obtained from patients with breast cancer was evaluated by immunohistochemistry and determined in accordance with the protocol devised by Georgiannos et al. Tumor size was determined by imaging methods (TI) and grouped as less than or equal to 2 cm (TIA, n = 17) or larger than 2 cm (TIB, n=9). The different cell types studied were: T lymphocyte (CD3 – A), cytotoxic lymphocyte (CD8 – B), B lymphocyte (CD20 – C) and macrophage (CD68 – D). The results were expressed as infiltrate intensity ratios (high/low), according to cellular localization: peritumoral (■) or intratumoral (●). * p = 0.0127 (Fisher test), comparing peritumoral CD3 in TIA and TIB tumors. * p = 0.0024 (Fisher test), comparing CD20 in TIA tumors between peritumoral and intratumoral localization.
was similar. There was a statistically significant difference between the peritumoral and intratumoral intensities of B lymphocytes.

Figure 3 presents the intensities of the labeled immune cells according to two parameters: localization of the infiltrate (peritumoral or intratumoral) and tumor size (TIA or TIB). It can be seen that the results shown in Figure 2 are attributed to tumor size. A significant difference was observed for peritumoral T lymphocytes, since TIA presented a reduction in comparison to TIB. Another significant difference was seen in relation to the CD20 marker for tumors sized less than or equal to 2 cm: peritumoral infiltrates presented a greater number of cells than did intratumoral infiltrates. There were lower staining counts of intratumoral CD8 lymphocytes in both TIA and TIB, and lower staining of intratumoral CD68 in TIB.

Discussion

Infiltration by immune cells is a common feature in many human tumors, and it has been suggested that the degree of infiltration is a measure of the host immune response. Several studies have attempted to correlate the infiltrate with the prognosis of breast cancer. Another question is the difference in peri- or intratumoral lymphocyte infiltration. It is an interesting point that immune cells sometimes migrate to the peritumoral site, but the infiltration is different in the intratumoral site. Is it possible that some cytokines or mediators produced by neoplastic cells could inhibit the migration of some types of cells, and thus tumors that have this evasion mechanism have a poor prognosis? Menard et al. analyzed 1,919 cases of primary ductal and lobular infiltrating breast carcinomas from women with long-term follow-up and showed that 16-17% of the tumors presented infiltrate that was independent of the patient’s age at diagnosis. However, they were unable to find any correlation between the infiltrate and the prognosis [3].

Analysis of the infiltrate in our study showed frequent presence of the four cell types studied. T CD3+ lymphocytes were in most cases present in higher infiltrates, while T CD8+ lymphocytes were found in lower infiltration. These data are in agreement with a previous study [1]. In a study on 60 cases of malignant breast neoplasia, Georgiannos et al. [14] did not find any cases of category 0 for CD3+. The CD3+ lymphocytes were present in high infiltration in all TIB cases, while CD8+ in these cases was more frequently found in lower infiltration. T CD8+ lymphocytes were present at lower concentrations when the patients presented tumors > 2 cm. As CD8+ T lymphocytes were also present with CD3+, we could deduce that the cells stained by CD3 antibody and not by CD8 could be CD4+ T lymphocytes.

Campbell et al. [15] investigated the profile of intracellular T cell cytokines in the peripheral blood of patients with breast cancer. They found a significant reduction in the percentages of CD4+ and CD8+ cells that were producing IL-2, IFN-gamma, TNF-alpha and IL-4, in comparison with the control group of healthy individuals.

Patients with breast cancer have been found to present lower absolute numbers of lymphocytes in the peripheral blood [16]. In contrast, higher numbers of suppressive Treg CD4+ CD25+ lymphocytes are present in the peripheral blood and tumoral microenvironment [17, 18].

In the present study, it was also observed that T CD3+ and CD8+ lymphocytes had an important participation in the tumoral microenvironment. Although there was a large presence of infiltrate containing these lymphocytes, in relation to the presence of others, it was ineffective in eradicating the primary tumor. The presence of T CD3+ lymphocytes in the infiltrate was more frequent in tumors of more than 2 cm in diameter. Nonetheless, some studies still question whether the infiltrate is related to favorable evolution of the disease [9, 10]. However, the present data suggest that the bigger the tumor is, the greater the immune response is, as represented by T CD3+ lymphocytes. This therefore suggests that larger infiltrations by intratumoral T lymphocytes could be related to less favorable prognosis.

B lymphocytes, which in the present study were labeled with CD20+ antibodies, have been frequent findings in peritumoral infiltrates [19-22]. Nevertheless, our data showed that this occurred only in TIA, while in TIB there was increased intratumoral CD20. There was no relationship between the levels of B lymphocytes and clinical stage, which has also been found in other studies [22], or with the other variables studied. B lymphocytes produce antibodies that bond to various antigens. When they are present in intratumoral infiltrates, they produce antitumor antibodies and inhibit the growth of autologous tumor cells, although this response is not limited to specific tumor antigens [23]. However, the majority of these antibodies are against auto-antigens and not just against tumor antigens [20].

Another cell type studied was macrophages. Interestingly, these cells presented an inversion that was dependent on tumor size: TIA had more high intratumoral infiltrate than peritumoral infiltrate, while TIB had more peritumoral macrophages than intratumoral macrophages. The presence of macrophages was the opposite of what was observed for T lymphocytes (CD3+). These data are similar to what is presented in the literature. Ben-Hur et al. [24] studied 17 cases of invasive breast carcinoma and, in evaluating the CD68+ macrophages, found few cells in different areas of the tumor. There is uncertainty regarding the function of the presence of macrophage infiltrates, but an association between poor prognosis and severe macrophage infiltrate in breast cancer cases has been found in several studies [24-26].

Studies on animal models have demonstrated that infiltrating macrophages in the tumor induce apoptosis in T CD8+ cells by means of a mechanism that requires cell contact and mediation by tumor necrosis factor (TNF) and nitric oxide [27]. High levels of macrophages in focal areas of the tumor are associated with increased vascular density, and the groups of macrophages are found in avascular areas of the tumor, which is associated with worse prognosis [28]. Aggressive tumors rapidly increase
their vascular supply in some areas, while leaving other areas in prolonged hypoxia which subsequently leads to necrosis. This might attract macrophages to the interior of the tumor, to contribute to the process of angiogenesis [29]. Disease-free survival and overall survival are worse when there are large quantities of macrophages [29-31]. As the evolution of long-term cases becomes available, we may be able to verify the prognosis for such patients.

Conclusion

Taken together, peri- and intratumoral infiltration may contribute to tumor regression or progression. Another interesting finding that deserves further study is the infiltration of T lymphocytes and macrophages. However, these findings are very complex and also require further studies. In the present study our aim was to demonstrate these findings are very complex and also require further studies. In the present study our aim was to demonstrate that tumor size might be related to peritumoral and intratumoral immune cell infiltrates in breast cancer cases. In conclusion, our results suggest that peritumoral and intratumoral infiltrates of T and B lymphocytes are different and depend on tumor size.

Acknowledgements

We thank FAPEMIG, FINEP and CNPq for funding.

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Self-sampling for human papillomavirus (HPV) testing as cervical cancer screening option. Experience from the LAMS Study

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Summary

Purpose: To compare Hybrid Capture II (HC2) in detecting high-risk (HR) HPV in patient-collected vaginal samples with those obtained using gynaecologist collected samples. Methods: Patients were submitted to Pap smears, visual inspection with acetic acid (VIA) and HC2 for hr-HPV. Results: A total of 1,081 HC2 tests for HR-HPV were performed: 770 (71.2%) samples were collected by a physician and 311 (28.8%) were self-collected by the patients. In detecting any cervical lesion, the sensitivity of HC2 collected by a physician was higher (92.86%) than that (37.5%) in the self-sampling group. Negative predictive value (NPV) was high for both, 99.69% and 93.75%, respectively. Using the CIN2 cutoff, performance of HC2 was significantly improved: 92.9% and 62.5%, respectively. HC2 specificity for any cervical lesion and for CIN2 or higher were close to 90% in both groups. Conclusions: Self-sampled HPV testing is a powerful option to increase the detection of cervical lesions in women segregated from prevention programs.

Key words: Hybrid capture; HPV; Cervical cancer; Liquid based cytology; Self-sampling.

Introduction

Human papillomavirus (HPV) is the most widespread sexually transmitted disease (STD), with an estimated global prevalence of 10.4% among women with normal cytology, although substantial differences are encountered in different regions [1]. Relatively few high-risk HPV types (HR-HPV), most notably HPV16 and HPV18, are associated with more than 99% of all cervical carcinomas [2].

Effective prevention of cervical cancer with organized cytology-based screening programs necessitates well-trained professionals with different skills. Until now, such programs have only been implemented in some highly developed countries [3], and on the global scale, the vast majority of women diagnosed as having cervical cancer have never participated in organized cytological screening [4]. Since the demonstration of HR-HPV types as the necessary cause of cervical cancer [5], and recognition that cervical cytology suffers from low sensitivity, the use of HPV testing by Hybrid Capture II (HC2) was approved by the United States Food and Drug Administration (FDA) in 2003 to be used concomitantly with cytology or alone [6]. In view of these facts, HR-HPV testing has been a part of new strategies for the screening of HPV induced-lesions in the US [7].

A variety of self-sampling devices have been introduced for collection of vaginal samples for HC2 testing. These systems have been tested in several studies, and shown to be a potentially viable screening option for women outside the regular programs of screening [8-11]. Indeed, the sensitivity of such self-collected vaginal samples for HPV testing has varied from 66.1% to 90% [3, 8-11]. Based on this experience, self-sampling for HPV testing seems a promising first-line option in cervical cancer screening, particularly in settings where cervical cytology is not readily available or insufficient in quantity to ensure wide enough coverage of the whole female population. In this setting, only women testing positive for hr-HPV should be referred for additional examinations [3]. Additionally, a high level of concordance between self-collected samples and physician sampling have been experienced. Restricting the results in HR-HPV, the concordance remains high but in contrast, low-risk HPV is more frequently identified in self-collected samples [11].

Revised manuscript accepted for publication October 1, 2007
Interestingly, women generally found the self-sampling option more suitable than the test performed by clinicians, but they were not confident that the test had been done properly [12]. Important demographic differences were also reported, e.g., married women having more positive attitudes towards self-sampling than single women, and Asian women having more negative attitudes than women in other ethnic groups [12]. Adolescents and young adult women seemed to prefer clinician to self-testing, largely because of concerns about self-collection accuracy [13]. These observations are essential issues to be considered by the authorities who want to plan self-sampling HPV testing as an alternative tool for primary screening, because a remarkably high prevalence of hr-HPV (three to six times higher than the expected prevalence in women of comparable age) can occur; apparently, these results closely depend on to the skill of the population analyzed [13, 14].

In spite of encouraging data, there are several divergent results regarding the agreement between clinician- and self-collected vaginal samples for HPV, and the sensitivity value of HPV clinician testing and self-testing to detect cervical lesion [14]. Most of the disagreements discussed above may be largely related to differences in recruitment and data collection procedures, study populations, analytic methods and outcome measures [14].

In our ongoing multi-center study in Latin America, a cohort of over 12,000 women have been examined using eight different diagnostic tests as potential screening tools in low-resource settings. The main objective of this study was to compare the results of HC2 assay (for hr-HPV) in two types of samples: i) patient-collected vaginal samples, and ii) samples collected by gynecologists.

Materials and Methods

The enrolled cohort is part of the Latin American Screening (LAMS) study, a prospective multicenter cohort study that tested optional cervical cancer screening methods and assessed the natural history of HPV infections and CIN in four clinical centers in Brazil (Leonor Mendes de Barros Hospital, HLMB; Hospital de Clínicas de Porto Alegre and State University of Campinas) and Argentina (First Chair, Gynecology Hospital of Clinics). The study design and the baseline data of the LAMS study have been detailed recently [15].

The present analysis comprises the HLMB cohort only. In this cohort, patients were screened for cervical cancer with Pap smears, visual inspection with acetic acid (VIA) and HC2 for high risk HPV (HR-HPV).

HC2 was collected by a physician or by a self-sampling method and inclusion of the patient in either group was randomly performed. The self-collected sample by the patient was performed after preliminary oriented-instruction by a well-trained nurse. General characteristics of the patients were reported.

Women testing positive for any of the tests were referred for colposcopy, and cervical biopsies were performed if necessary.

All patients gave their written consent to participate in the study, which was approved by the local Ethics Committee.

Histological specimens

All cases referred for colposcopy and biopsies were taken according to clinical evaluation. The cases were primarily classified according to WHO’s 1994 classification [16] and, afterwards revised according WHO’s 2003 classification [17].

Hybrid Capture Assay

The HC2 protocol was performed according to the instructions of the manufacturer (Digene Co., Gaithersburg, MD, USA). In estimation of the viral load, samples with relative light units (RLU) > 20 were considered to harbor a high viral load and, those with 5-19.9 were intermediate, and those with 1-4.99 were low [18]. Only HR-HPV was tested (carcinogenic types included: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) [19].

Statistical methods

In statistical analyses, two different statistical softwares were used: SPSS for Windows (Version 11.5) and STATA/SE 8.2. The performance indicators (sensitivity, SE, specificity, SP, negative predictive value NPV, and positive predictive value, PPV) for conventional Pap tests and liquid based cytology (LBC) were calculated from the 2 x 2 contingency tables, using colposcopic biopsies as the gold standard. The chi-square test was used to analyze correlations between categorical data, with Pearson’s correlation and Fisher’s exact test, and calculating OR and their 95% CI where appropriate. In all statistical analyses p < 0.05 was regarded as significant.

Results

A total of 1,081 tests of HC2 for hr-HPV were performed: 770 (71.2%) samples were collected by a physician and 311 (28.8%) were self-collected by the patients.

Table 1 shows the principal characteristics of the patients regarding age, years of education, age at first intercourse, number of pregnancies, number of deliveries or caesarean sections, number of abortions (prenatal births), number of partners since first intercourse and during the previous 12 months, and the number of Pap tests during the lifetime. Interestingly, the values of both groups were quite similar. Mean and median age were around 37 years. The other parameters revealed that the women in our case series have comparable cultural attitudes.

Table 2 depicts the race distribution which revealed a significant difference among women in both groups (sampled by a physician or self-collected samples). High-risk HPV infection in white women was more prevalent in comparison to the results observed in black and mixed (p = 0.0001). No other variable was significantly different in either group, including contraception methods, which demonstrates the homogeneity of the women’s history regarding contraceptive usage, and previous history of sexually transmitted disease.

Table 3 exhibits the results of hr-HPV Pap smear examination, HC2 tests and VIA correlated to the method of sample collection. All parameters were more significantly positive in the self-sampling group than in material sampled by a physician. The differences were particularly interesting in cases with any cytological abnormality (p = 0.008) and in positive VIA (p = 0.0001).
Table 1. — Quantitative history variables of the patients tested for hr-HPV by the two sampling methods for HC2 assay.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HC2 sampling by physician</th>
<th>HC2 self-sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± Std. deviation</td>
<td>Mean ± Std. deviation</td>
</tr>
<tr>
<td>Age</td>
<td>37.55 ± 9.73</td>
<td>36.97 ± 9.97</td>
</tr>
<tr>
<td>Years of education</td>
<td>7.1 ± 3.5</td>
<td>7.5 ± 3.5</td>
</tr>
<tr>
<td>Age at first sexual intercourse</td>
<td>18.5 ± 3.8</td>
<td>18.9 ± 4.5</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>2.8 ± 2.2</td>
<td>2.5 ± 1.9</td>
</tr>
<tr>
<td>No. of deliveries</td>
<td>1.7 ± 1.9</td>
<td>1.4 ± 1.6</td>
</tr>
<tr>
<td>No. of cesarean sections</td>
<td>0.6 ± 1.0</td>
<td>0.7 ± 1.0</td>
</tr>
<tr>
<td>No. of aboritions/prenatal births</td>
<td>0.5 ± 0.9</td>
<td>0.4 ± 0.8</td>
</tr>
<tr>
<td>No. of partners since the first intercourse</td>
<td>2.5 ± 3.3</td>
<td>2.4 ± 2.1</td>
</tr>
<tr>
<td>No. of partners during the past 12 months</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>No. of life-time Pap smears</td>
<td>6.5 ± 4.8</td>
<td>6.6 ± 5.2</td>
</tr>
</tbody>
</table>

* Mann-Whitney U-test; HC2, Hybrid capture II; HR-HPV, high-risk human papillomavirus.

Table 2. — Race, contraception, STD and smoking history of patients tested for HR-HPV by the two sampling methods for HC2 assay.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HC2 sampling by physician</th>
<th>HC2 self-sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± Std. deviation</td>
<td>Mean ± Std. deviation</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>511 (66.5%)</td>
<td>194 (62.4%)</td>
</tr>
<tr>
<td>Black</td>
<td>71 (9.2%)</td>
<td>53 (17%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>164 (21.4%)</td>
<td>50 (16.1%)</td>
</tr>
<tr>
<td>Other</td>
<td>22 (66.5%)</td>
<td>14 (4.5%)</td>
</tr>
<tr>
<td>Contraception</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>216 (28.1%)</td>
<td>90 (28.9%)</td>
</tr>
<tr>
<td>Hormonal</td>
<td>193 (25.1%)</td>
<td>76 (24.4%)</td>
</tr>
<tr>
<td>Condom</td>
<td>109 (14.2%)</td>
<td>55 (17.7%)</td>
</tr>
<tr>
<td>IUD</td>
<td>67 (8.7%)</td>
<td>26 (8.4%)</td>
</tr>
<tr>
<td>Tubal sterilization</td>
<td>142 (18.5%)</td>
<td>50 (17.8%)</td>
</tr>
<tr>
<td>Other</td>
<td>42 (5.5%)</td>
<td>14 (5.2%)</td>
</tr>
<tr>
<td>History of STD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>55 (7.2%)</td>
<td>18 (5.8%)</td>
</tr>
<tr>
<td>Partners</td>
<td>68 (8.8%)</td>
<td>22 (7.1%)</td>
</tr>
<tr>
<td>Previous pap smear</td>
<td>718 (93.4%)</td>
<td>297 (95.5%)</td>
</tr>
<tr>
<td>Smoking (current or past)</td>
<td>279 (36.3%)</td>
<td>108 (34.7%)</td>
</tr>
</tbody>
</table>

* Pearson’s chi-square; ** Pearson’s chi-square with continuity correction; HC2, Hybrid capture II; HR-HPV, high-risk human papillomavirus; IUD, intrauterine contraceptive device; STD, sexually transmitted disease.

Table 3. — Results of Pap smear, HC2 for HR-HPV and VIA in the two groups of sampling.

<table>
<thead>
<tr>
<th>Exam</th>
<th>HC2 sampling by physician</th>
<th>HC2 self-sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>Pap</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASCUS or higher</td>
<td>72/770 (9.4%)</td>
<td>47/311 (15.1%)</td>
</tr>
<tr>
<td>HSIL or higher</td>
<td>10/770 (1.3%)</td>
<td>12/311 (3.9%)</td>
</tr>
<tr>
<td>HC2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>108/770 (14%)</td>
<td>63/311 (20.3%)</td>
</tr>
<tr>
<td>VIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>89/770 (11.6%)</td>
<td>64/311 (20.6%)</td>
</tr>
</tbody>
</table>

* Pearson chi-square; HC2, Hybrid capture II; HR-HPV, high-risk human papillomavirus; VIA, Visual inspection with acetic acid.

Discussion

HPVs infect epithelial cells and cause a variety of lesions including warts to cervical neoplasia and cancer. High-risk HPV DNA is found in almost all cervical cancers (> 99.7%), with HPV16 being the most prevalent type in both low-grade disease and cervical neoplasia [20]. Identifying HR-HPV in women with cervical cancer is critical to understanding the pathogenesis of cervical cancer [21]. Presently, the identification of HR-HPV has been determinedly advocated by epidemiologists who are clearly identifying the correlation between HR-HPV infection and cervical high-grade lesions [1, 7, 11, 22, 23].

The complexity of cytologic-based screening and the necessity of resources, infrastructure, professional expertise, together with the need for repeated and well-controlled screenings at regular intervals, make cervical cytologic screening very difficult to be efficiently implemented in poor countries [22]. Additionally, the accuracy and reproducibility of the Pap test is far from acceptable as the primary screening option in low resource settings [24]. Recognition of HR-HPV DNA is also important to improve the identification of cervical lesions alone or associated with cytological examination [25, 26]. Recently, we studied HC2 as an optional tool for primary screening, and the results clearly demonstrated this tendency due to the superior correlation of positive hr-HPV testing with biopsy-proven high-grade lesions when compared with cytology, conventional or liquid-based preparations [27]. Importantly, the HC2 option seems to be more cost-effective than cytology and its use is encouraged for low resource countries [7, 28-31], and it is more accurate for women aged 30 years or more [7, 29]. HC2
hr-HPV also has an important predictive impact for both negative and positive results. Oncogenic HPV infections comprise a significant risk factor for incident cervical abnormalities [32-35]. Remarkably, among older women where HPV may be added to general screening, the estimated absolute risk of high grade lesions in HC2-positive women is believed to be superior to 20% within ten years which indicates that even a single positive HPV test in cytologically negative women is substantially predictive of high-grade CIN; this fact supports the use of HC2 testing to stratify women into different risk categories [36].

In this context, self-sampling screening could be an important option to select cervical lesions in women out of the regular health system programs in poor regions of developing countries. There are several data that robustly demonstrated this potential [4, 8-13], including in Brazil [3]. Complementary, the performance of HC2 for HR-HPV in self-collected material and those collected by a physician was slightly different. In the physician material for HC2 analysis. Even with a performance slightly inferior to those obtained by a physician, the ability to collect optimal samples by women was clearly ratified in that HC2 can be performed elsewhere with confidence to collect optimal samples by women was clearly ratified in that HC2 can be performed elsewhere with confidence.

Noteworthy conclusions can be assessed with our results. Self-sampling is a reliable tool for women to collect material for HC2 analysis. Even with a performance slightly inferior to those obtained by a physician, the ability to collect optimal samples by women was clearly ratified in that HC2 can be performed elsewhere with confidence. These observations are similar to those reported by Holland and co-workers in Brazil [3].

Importantly, HC2 for HR-HPV showed a consistent and superior performance when compared with other screening options, including cytology, as we have already observed previously [27]. Additionally, HC2 showed high sensitivities to detect CIN2 or higher lesions (92.86%) which support the high clinical sensitivity of HC2 tests to
identify high-grade lesions. Moreover, the NPV was almost 100% for CIN2 or higher (99.85%) which evidently demonstrates that HR-HPV testing negative with HC2 in self-sampling material is a safe and reliable resource for population screening. Importantly, the specificity for high grade lesions was superior by almost 90% implicating an additional gain for the self-sampling option. Our results found comparable values in the literature which strongly support the reproducibility of HC2 for HR-HPV collected by the self-sampling method [38, 39].

Conclusion

Self-sampling screening in remote areas of developing countries should be seriously considered as a powerful tool to reduce the prevalence of cervical cancer and its high-grade precursors, and to cooperate with the efforts to decrease mortality [3]. Obviously, these assumptions must be further measured in screening programs to test the efficiency in a large population.

Acknowledgments

This study is a part of the ongoing LAMS (Latin America Screening) study, entitled: IMPROVING HEALTH SYSTEMS TOWARDS EQUALITY-BASED CONTROL OF CERVICAL CANCER IN LATIN AMERICA. Comparing Pap smear cytology, aided visual inspection, cervicography and human papillomavirus (HPV) testing as optional screening tools in Brazil and Argentina, and supported by the INCO-DEV Programme of the European Commission (Project #ICA4-CT-2001-10013). The kind cooperation of all professionals of all institutions involved in this project is gratefully acknowledged. The authors also express their special thanks to DIGENE Corporation for providing the Hybrid Capture Kits at our disposal.

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Association study of vascular endothelial growth factor gene polymorphisms in endometrial carcinomas in a Japanese population

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Introduction

Endometrial carcinoma is one of the most common gynecologic malignancies and the age-standardized incidence rate is rising in Japan, possibly due to changing lifestyle and dietary patterns. Prolonged unopposed estrogen stimulation such as the use of tamoxifen, late menopause, and obesity have been identified as major risk factors for endometrial carcinomas. Recent studies show a relationship between the hereditary syndrome such as the Lynch syndrome and endometrial carcinomas, and demonstrate that a genetic factor is another risk factor of endometrial carcinomas [1, 2].

Angiogenesis is an important step in the development of cancer and is necessary for primary tumor growth, invasiveness, and metastasis. Increased tumor vascularization and expression of angiogenic factors are associated with advanced tumor stage and poor prognosis in various cancers. Vascular endothelial growth factor (VEGF), the most potent endothelial cell mitogen, has been shown to play a critical role in tumor angiogenesis [3]. VEGF binds to two kinds of VEGF receptor tyrosine kinases on endothelial cells and activates intracellular signal transduction pathways, leading to the promotion of angiogenesis and vascular permeability [3].

VEGF has been shown to be up-regulated at mRNA and protein levels in a variety of carcinomas [6-12]. Elevated serum or intratumoral VEGF levels were associated with advanced stage disease and poor prognosis for several cancers, including breast [3], renal [4, 5], lung [6], endometrial [7, 8], and prostate carcinomas [9].

Serum levels of VEGF were significantly increased with the advanced FIGO stage of endometrial carcinomas [7]. Yokoyama et al. demonstrated that VEGF is a useful biomarker to predict myometrial invasion and lymph node metastasis in endometrial carcinoma [8]. These results suggest that VEGF may contribute to the pathogenesis of endometrial carcinomas.

Several studies have recently demonstrated positive associations between VEGF gene polymorphisms and several diseases. Among more than 30 single nucleotide polymorphisms (SNPs) located on the VEGF gene, three SNPs such as -460 C/T in the promoter region, +405 G/C, and +936 C/T polymorphisms were examined in 105 endometrial carcinomas and 179 controls using PCR-RFLP analysis. An association of these polymorphisms with three-year disease-free survival was evaluated using the Kaplan-Meier method. Results: No significant differences in the allele frequencies and genotype distributions of VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms were observed between endometrial carcinoma patients and controls. There were no significant differences in the frequencies of haplotype -460 T/+405 C between patients and controls. Furthermore, VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms were not associated with three-year disease-free survival of endometrial carcinoma patients. Conclusions: Although limited by sample size, our study did not demonstrate any evidence that VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms are associated with an increased risk of endometrial carcinomas in Japanese women.

Key words: Endometrial carcinoma; Gene polymorphism; Polymerase chain reaction; Restriction fragment length polymorphism; Vascular endothelial growth factor.

Summary

Objective: Vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a critical role in angiogenesis of endometrial carcinomas. Several studies have demonstrated positive associations between VEGF gene polymorphisms and several carcinomas. In this study we investigated whether VEGF gene polymorphisms are associated with endometrial carcinomas in a Japanese population. Methods: The allele frequencies and genotype distributions of VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms were examined in 105 endometrial carcinomas and 179 controls using PCR-RFLP analysis. An association of these polymorphisms with three-year disease-free survival was evaluated using the Kaplan-Meier method. Results: No significant differences in the allele frequencies and genotype distributions of VEGF -460 C/T (p = 0.54, 0.90), +405 G/C (p = 0.31, 0.17), and +936 C/T polymorphisms (p = 0.46, 0.24) were observed between endometrial carcinoma patients and controls. There were no significant differences in the frequencies of haplotype -460 T/+405 C between patients and controls. Furthermore, VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms were not associated with three-year disease-free survival of endometrial carcinoma patients. Conclusions: Although limited by sample size, our study did not demonstrate any evidence that VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms are associated with an increased risk of endometrial carcinomas in Japanese women.

Key words: Endometrial carcinoma; Gene polymorphism; Polymerase chain reaction; Restriction fragment length polymorphism; Vascular endothelial growth factor.
cancers [3, 10, 11, 14, 15]. The frequency of the VEGF -460 T/T genotype was shown to be significantly increased in patients with oral and prostate cancers compared to controls [10, 11], and that of the VEGF +405 C allele was significantly increased in prostate cancer with high histological grade [15]. The frequency of the VEGF +936 T allele was significantly lower in breast cancer patients compared to controls [3, 14].

However, it remains unknown whether VEGF gene polymorphisms are associated with endometrial carcinomas. In this study we investigated the possible associations between the VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms and endometrial carcinomas in a Japanese population by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

Materials and Methods

Subjects

The design of this study was approved by the Medical Ethics Review Committee of Kobe University Graduate School of Medicine. Written informed consent was obtained from all women involved in this study. The patient group consisted of 105 unrelated Japanese women with surgically confirmed endometrial carcinoma based on operative records and histological findings. All the patients had surgically confirmed Stage I to IV diseases according to the Federation of Gynecology and Obstetrics (FIGO) classification [16]. The age of patients with endometrial carcinoma ranged from 36 to 80 yrs with the mean age of 57.1 ± 11.0 yrs. Patients were excluded from the study if the operative records were unavailable or if there was any doubt about the diagnosis. Women who were non-Japanese (ethnicity/race) were excluded. Age-matched controls with a mean age of 56.6 ± 10.9 yrs consisted of 179 healthy Japanese women who had participated in a routine cancer detection program for gynecologic cancers at our hospital. They had no history or suggestive clinical evidence of endometrial pathology (Table 1). Patients were followed-up for cancer recurrence and mortality. Of the total 105 patients, 50 (47.6%) patients could be followed-up via in-person contact. Among them, four patients have died to date, and the remaining 46 participants are still living.

Genotyping

Genomic DNA was extracted from EDTA anticoagulated whole blood using the Wizard DNA Purification Kit (Promega, Madison, WI, USA). The –460, +405, and +936 polymorphisms in the VEGF gene were determined by using PCR-RFLP analysis. Genotyping for VEGF -460 C/T polymorphism was performed by the forward primer 5’-TACGTGCGAGGCTA-3’ and the reverse primer 5’-TACGTGCGAGGCCCTGA-3’, followed by digestion with the restriction enzyme BstUI. Genotyping for VEGF +405 G/C polymorphism was performed by the forward primer 5’-AATTATTTTTGCTTGC-3’ and the reverse primer 5’-TACGTGCGGAGGGCCTGA-3’, followed by digestion with the restriction enzyme BglII. Genotyping for VEGF +936 C/T polymorphism was performed by the forward primer 5’-AAGGAAAGAGACTCTGGCCG-3’ and the reverse primer 5’-TATGTGGGTTGTTGTTGTC-TACAGG-3’, followed by digestion with the restriction enzyme NlaIII.

The conditions for the genotyping were as follows: PCR in a 20 μl reaction mixture containing 20 ng of genomic DNA, 10 pmol of each primer, 250 μM of dNTPs and 1.0 unit of Taq gold DNA polymerase. The concentration of MgCl₂, varied between the PCR reactions for the different polymorphisms with 1.5 mM for VEGF -460 C/T polymorphism and VEGF +405 G/C polymorphism, and 2.0 mM for VEGF +936 C/T polymorphism. The PCR was conducted with ABI 9700 thermocycler (PE Applied Biosystem, Foster City, CA, USA) by using the following thermal profiles: an initial denaturing cycle of 94°C for 1 min, 32 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and a final cycle of 72°C for 5 min for VEGF -460 C/T polymorphism; an initial denaturing cycle of 96°C for 12 min, 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and a final cycle of 72°C for 5 min for VEGF +405 G/C polymorphism; an initial denaturing cycle of 96°C for 12 min, 35 cycles of denaturing at 94°C for 40 s, annealing at 64°C for 1 min, and extension at 72°C for 40 s, and a final cycle of 72°C for 5 min for VEGF +936 C/T polymorphism. Digestions with the appropriate restriction enzyme were performed according to the manufacturer’s instructions (New England Biolabs, Beverly, MA, USA) at 60°C for 24 h in the case of VEGF -460 C/T polymorphism, 65°C for 24 h in the case of VEGF +405 G/C polymorphism, and 37°C for 24 h in the case of VEGF +936 C/T polymorphism. DNA fragments were subjected to electrophoresis in a 2% agarose gel for the VEGF +405 G/C and +936 C/T and a 4% gel for the VEGF -460 C/T. Gel was stained with ethidium bromide (0.1 μg/ml) and visualized by ultraviolet illumination.

Statistical analysis

Genotype distributions were examined for the significant departure from the Hardy-Weinberg equilibrium by a goodness-of-fit (chi-square) test. Chi square analysis was used to examine differences in the proportions of genotype of three polymorphisms between patients and controls. Fisher’s exact test was applied when appropriate. Odds Ratios (OR) and 95% confidence intervals (CIs) were used to compare categorical variables; p < 0.05 was considered statistically significant.

Cases were divided into two subgroups consisting of women with Stage I-II and Stage III-IV disease, endometrioid and non-endometrioid cancers, and the allele frequencies and genotype distributions of the VEGF polymorphisms in the subgroups were analyzed separately. Haplotype frequencies and standardized disequilibrium coefficient (D’) were evaluated using the program Haploview (available at http://www.broad.mit.edu/mpg/haploview/tutorial/php); p < 0.05 was considered significant.

Three-year disease-free survival rates were evaluated using the Kaplan-Meier method and the differences in survival across different subgroups were determined using the log-rank test. Survival curves were compared to the log-rank test and the differences in survival rates were determined using the log-rank test.
different genotypes were assessed using the log rank test. The end point for disease-free survival was cancer recurrence/metastasis or death related to endometrial carcinoma. The disease-free survival period was calculated as the time from initial diagnosis to the end points of the study, censoring at the date of last contact.

Results

Genotyping of the VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms was successful in all 105 cases and 179 controls. The breakdown for stages of endometrial carcinoma are shown in Table 1.

The genotype distributions were all in Hardy-Weinberg equilibrium in both cases and controls. The genotype distributions and allele frequencies of the VEGF -460 C/T, +405 G/C, and +936 C/T polymorphisms in endometrial carcinoma patients and controls are shown in Tables 2, 3, and 4. The allele frequencies among control individuals with these polymorphisms were comparable to those of controls in other published studies using individuals from the Japanese population [19, 23]. The -460 allele frequencies of our controls were significantly different from those reported in UK (p = 0.0000005) [17] and Indian populations (p = 0.0000005) [18]. Of the VEGF +405 G/C polymorphism, the allele frequency in Indian controls is significantly different from those of Korean (p = 0.0001) [12] and Japanese (p = 0.0000002) [19]. There were no significant differences in the genotype distributions and allele frequencies of the VEGF-460 C/T, +405 G/C, and +936 C/T polymorphisms between endometrial carcinoma patients and the controls. Furthermore, the stratification by histologic types and staging failed to identify statistically significant differences between endometrial carcinoma patients and the controls (Tables 2, 3, and 4).

Table 2. — Distribution of VEGF -460 C/T polymorphism in endometrial carcinomas and controls.

<table>
<thead>
<tr>
<th>Disease</th>
<th>VEGF -460 genotype (%)</th>
<th>p value</th>
<th>T</th>
<th>C</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrial carcinomas (n = 105)</td>
<td>54 (51.4)</td>
<td>42 (40.0)</td>
<td>9 (8.6)</td>
<td>p = 0.54*</td>
<td>150 (71.4)</td>
</tr>
<tr>
<td>Histological types</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>endometrioid (n = 89)</td>
<td>46 (51.7)</td>
<td>36 (40.5)</td>
<td>7 (7.8)</td>
<td>p = 0.67*</td>
<td>128 (71.9)</td>
</tr>
<tr>
<td>non-endometrioid (n = 16)</td>
<td>8 (50.0)</td>
<td>6 (37.5)</td>
<td>2 (12.5)</td>
<td>p = 0.57*</td>
<td>22 (68.8)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II (n = 85)</td>
<td>46 (54.1)</td>
<td>31 (36.5)</td>
<td>8 (9.4)</td>
<td>p = 0.29*</td>
<td>123 (72.4)</td>
</tr>
<tr>
<td>III-IV (n = 20)</td>
<td>8 (40.0)</td>
<td>11 (55.0)</td>
<td>5 (5.0)</td>
<td>p = 0.74*</td>
<td>27 (67.5)</td>
</tr>
</tbody>
</table>

versus controls.

Table 3. — Distribution of VEGF +405 G/C polymorphism in endometrial carcinomas and controls.

<table>
<thead>
<tr>
<th>Disease</th>
<th>VEGF +405 genotype (%)</th>
<th>p value</th>
<th>G</th>
<th>C</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrial carcinomas (n = 105)</td>
<td>25 (23.8)</td>
<td>52 (49.5)</td>
<td>28 (26.7)</td>
<td>p = 0.31*</td>
<td>102 (48.6)</td>
</tr>
<tr>
<td>Histological types</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>endometrioid (n = 89)</td>
<td>20 (22.5)</td>
<td>43 (48.3)</td>
<td>26 (29.2)</td>
<td>p = 0.22*</td>
<td>83 (46.6)</td>
</tr>
<tr>
<td>non-endometrioid (n = 16)</td>
<td>5 (31.2)</td>
<td>9 (56.3)</td>
<td>2 (12.5)</td>
<td>p = 0.53*</td>
<td>19 (59.4)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II (n = 85)</td>
<td>20 (23.5)</td>
<td>44 (51.8)</td>
<td>21 (24.7)</td>
<td>p = 0.32*</td>
<td>84 (49.4)</td>
</tr>
<tr>
<td>III-IV (n = 20)</td>
<td>5 (25.0)</td>
<td>8 (40.0)</td>
<td>7 (35.0)</td>
<td>p = 0.51*</td>
<td>22 (55.0)</td>
</tr>
</tbody>
</table>

versus controls.

Table 4. — Distribution of VEGF +936 C/T polymorphism in endometrial carcinomas and controls.

<table>
<thead>
<tr>
<th>Disease</th>
<th>VEGF +936 genotype (%)</th>
<th>p value</th>
<th>C</th>
<th>T</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrial carcinomas (n = 105)</td>
<td>59 (56.2)</td>
<td>39 (37.1)</td>
<td>7 (6.70)</td>
<td>p = 0.46*</td>
<td>157 (74.8)</td>
</tr>
<tr>
<td>Histological types</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>endometrioid (n = 89)</td>
<td>51 (57.3)</td>
<td>32 (36.0)</td>
<td>6 (6.70)</td>
<td>p = 0.60*</td>
<td>134 (75.3)</td>
</tr>
<tr>
<td>non-endometrioid (n = 16)</td>
<td>8 (50.0)</td>
<td>7 (43.8)</td>
<td>1 (6.20)</td>
<td>p = 0.54*</td>
<td>23 (71.9)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II (n = 85)</td>
<td>47 (55.3)</td>
<td>31 (36.5)</td>
<td>7 (8.20)</td>
<td>p = 0.39*</td>
<td>125 (73.5)</td>
</tr>
<tr>
<td>III-IV (n = 20)</td>
<td>12 (60.0)</td>
<td>8 (40.0)</td>
<td>0 (0.00)</td>
<td>p = 0.44*</td>
<td>32 (80.0)</td>
</tr>
</tbody>
</table>

versus controls.
polymorphism, the allele frequency in our Japanese con-
exposure to environmental factors. In the VEGF -460 C/T the prevalence of polymorphism in the population and
tion because disease susceptibility is dependent on both
previous data and ours might be due to the ethnic varia-
tion of various cancers. The difference in the results between
may be a risk factor for the development and progression
increased compared to the controls in the same popula-
tion and the disease-free survival of this cancer.

Discussion

In this study we investigated the possible associations
between endometrial carcinomas and VEGF -460 C/T,
+405 G/C and +936 C/T polymorphisms in a Japanese
population. We could not find any associations between
these polymorphisms and endometrial carcinomas. We
performed separate analyses on histological types and
stratification by the FIGO stage, which failed to show any
significant differences. In addition, we evaluated the
associations of these polymorphisms with three-year
disease-free survival in the patients, but could not find
any associations between these polymorphisms and
disease-free survival. To the best of our knowledge, this
study appears to be the first report to demonstrate no
associations between endometrial carcinomas and VEGF
polymorphisms and disease-free survival in a Japanese
population.

Ku et al. investigated the possible association of VEGF
-460 C/T polymorphism and oral cancer by PCR-RFLP
analysis in a Taiwanese population, and reported that
VEGF -460 T/T genotype frequency in patients was sig-
ificantly increased compared to the controls [11]. Chen
et al. also reported that VEGF 640 T/T genotype fre-
quency in patients with prostate cancer was significantly
increased compared to the controls in the same popula-
tion [10]. These studies suggest that VEGF -460 T allele
may be a risk factor for the development and progression
of various cancers. The difference in the results between
previous data and ours might be due to the ethnic vari-
tion because disease susceptibility is dependent on both
the prevalence of polymorphism in the population and
exposure to environmental factors. In the VEGF -460 C/T
polymorphism, the allele frequency in our Japanese con-
trols is similar with that of the Korean population, but is
significantly different from that of Indian (p < 0.01) and
UK populations (p < 0.01). Therefore, our results may
not be directly applicable to other populations including
Indian and UK populations.

Several studies have demonstrated positive associations
between VEGF +405 G/C polymorphism and diseases
such as breast cancer [20], showing that the frequency of
VEGF +405 C allele was increased in patients compared to
controls. Awata et al. showed that fasting serum VEGF
levels were higher in a normal Japanese population with
VEGF +405 C/C genotype [21], suggesting that VEGF
+405 G/C polymorphism is associated with VEGF syn-
thesis. Taken together, VEGF +405 C allele may be
involved in the pathogenesis of several diseases, although
it still remains unclear how polymorphisms in the
untranslated region of the VEGF gene influence its
protein production [18, 21, 22]. In contrast, no associ-
ations have been noted between VEGF +405 G/C poly-
orphism and diseases such as preeclampsia [13]. Our
results coincide with the results of Han et al., Seo et al.,
and Papazoglou et al., which could not find a positive
association between this polymorphism and diseases.

There are several reports which have shown positive
associations between VEGF +936 C/T polymorphism
who and the progression or aggressiveness of tumors.
Kripl et al. reported that the carriers of VEGF +936 T
allele were at a decreased risk for breast cancer and that
carriers of +936 T allele showed significantly lower
VEGF plasma levels [14]. On the other hand, no associ-
ations were reported between VEGF +936 C/T polymor-
phism and diseases such as renal cell carcinoma in a
Japanese population [23] and breast cancer in Polish and
German populations [20]. We could not find any associ-
ations between endometrial carcinomas and this poly-
morphism in the population studied.

Lu et al. investigated the possible associations between
these three polymorphisms and breast cancer patients in
a Chinese population [3]. They found strong linkage dis-
equilibrium between the VEGF -460 C/T and +405 G/C
polymorphisms (D' = 0.94) and an association of the
VEGF -460 C and +405 G haplotype with poorer survival
of breast cancer patients, but failed to identify an associ-
ation of VEGF +936 C/T polymorphism with overall sur-
vival or disease-free survival [3]. In this study, we found
a similar strong linkage disequilibrium between the -460
T and +405 C alleles (D' = 0.91), but the haplotype anal-
ysis did not reveal a positive association between endome-
trial carcinoma patients and controls. We also evaluated
the association of VEGF -460 C/T, +405 G/C and +936
C/T polymorphisms with the three-year disease-free sur-
vival of endometrial carcinoma patients, but could not
find any clear associations between these polymorphisms
and the disease-free survival of this cancer.

In conclusion, this study appears to be the first descrip-
tion to demonstrate no associations between endometrial
cancer and the VEGF -460 C/T, +405 G/C, and +936 C/T
polymorphisms, suggesting that these two polymor-
phisms are unlikely to be involved in the pathogenesis of

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Table 5. — Haplotype frequencies of VEGF -460 C/T and
+405 G/C polymorphism in endometrial carcinomas and
controls.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Haplotype frequencies (%)</th>
<th>Odd Ratios</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>T C</td>
<td>49.6</td>
<td>44.4</td>
<td>1</td>
</tr>
<tr>
<td>T G</td>
<td>21.4</td>
<td>26.6</td>
<td>0.70</td>
</tr>
<tr>
<td>C G</td>
<td>27.2</td>
<td>27.9</td>
<td>0.88</td>
</tr>
<tr>
<td>C C</td>
<td>1.8</td>
<td>1.1</td>
<td>1.52</td>
</tr>
</tbody>
</table>

*Observed haplotype frequencies were estimated by the Expectation-Maximiza-
tion method using a Haploview program.

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control subjects, respectively (Table 5). No significant
difference was noted in the frequencies of haplotype -460
T/+405 C between cases and controls.

The possible associations between the genotypes in three
VEGF polymorphisms and three-year disease-free survival were evaluated in 55 patients using the Kaplan
Meier method. During the study period, seven patients
recurred. The Kaplan Meier survival curves showed no
associations between VEGF -460 C/T, +405 G/C, and
+936 C/T polymorphisms and disease-free survival in
endometrial carcinoma, respectively (p = 0.58, p = 0.14,
and p = 0.69).

In this study we investigated the possible associations
between endometrial carcinomas and VEGF -460 C/T,
+405 G/C and +936 C/T polymorphisms in a Japanese
population. We could not find any associations between
these polymorphisms and endometrial carcinomas. We
performed separate analyses on histological types and
stratification by the FIGO stage, which failed to show any
significant differences. In addition, we evaluated the
associations of these polymorphisms with three-year
disease-free survival of the patients, but could not find
any associations between these polymorphisms and
disease-free survival. To the best of our knowledge, this
study appears to be the first report to demonstrate no
associations between endometrial carcinomas and VEGF
polymorphisms and disease-free survival in a Japanese
population.
Association study of vascular endothelial growth factor gene polymorphisms in endometrial carcinomas in a Japanese population

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endometrial cancer in a Japanese population. Although this model is biologically plausible, we recognize that our conclusions are based on relatively small numbers and will require verification from additional independent studies because the sample sizes are not sufficient to conclude the differences as non-significant (the power level of < 80%).

Acknowledgements
This study was supported by a Grant-in-Aid for Scientific Research 16024212 from the Japanese Ministry of Education, Science and Culture.

References
HPV in men

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Summary

Purpose: To collect information about HPV in men and the (possible) correlation with HPV infection in women. Methods: Review of the literature. Results: An overview of HPV-related penile and anal malignancies in men and the risk factors of acquiring HPV. Conclusion: In men HPV is also partially responsible for anogenital malignancies. Although the prevalence of HPV-related malignancies in men is much lower than in women, it is useful to gain more knowledge. Especially knowing if men are really the HPV reservoir and transmitters for women can make a difference in deciding whether men should also be screened for HPV and if they are good candidates for vaccination.

Key words: HPV; Male; Penile lesions; Anal lesions; Screening.

Introduction

Human papilloma virus (HPV) infection is a sexually transmitted disease (STD) that mostly follows a self-limiting transient course for both sexes. Persisting infection causes clinical lesions, depending on the subtype of HPV. Low-risk types (6, 11) cause benign but bothersome lesions such as anogenital warts. High-risk types (16, 18, 31, 35, 45, 51, 52, 56, 58, 66) are associated with high-grade dysplasia and anogenital cancers. HPV infection is a necessary cofactor in causing cervical cancer, with the types 16 and 18 being responsible for more than 70% of the cervical cancers, killing 250,000 women a year worldwide. Because of this, research has mainly focused on women and screening methods, and treatments for women are well developed. Because of the low prevalence of HPV-related disease in men, the nature of the infection in men is less understood and a male screening/treatment organogram does not exist. More knowledge on HPV in men would be useful for women because men are believed to be the carriers and transmitters of the virus to women.

Overview

Men and HPV - clinical consequences

Anogenital HPV infection is found mostly in young men, with a peak incidence in their second and third decade. The incidence is higher in homosexual men [1, 2]. The incubating period varies from three weeks to nine months after sexual intercourse with an infected partner [3]. Of men who test negative for HPV at baseline, up to 13.8% screen positive during follow-up [4]. The risk of persisting HPV infection is associated with the detection of more than one HPV type at baseline screening [4]. Most common low-risk type is HPV 6; most common high-risk type is HPV 16. Penile cancer is mostly of squamous epithelial origin, differentiated in situ carcinomas (Bowen disease and erythroplasia of Queyrat) and invasive carcinomas (squamous cell carcinoma (SCC) and verrucous carcinoma). HPV subtypes are found in patients with Bowen disease [5], erythroplasia of Queyrat [6], bowenoid papulosis [7, 8] and penile lichen sclerosis [9]. Bowenoid papulosis remains mostly a benign condition and will only rarely evolve to an invasive cancer. It is mostly found in young, circumcised men with a rather active sexual life [10]. Erythroplasia of Queyrat (10 to 30%) [11] can progress to invasive SCC. Ulceration of the original lesions is mostly at the time suggestive of malign evolution. Both are observed in elderly uncircumcised white men [11, 12]. Penile lichen sclerosis is a chronic inflammatory disorder of unknown origin that may lead to meatal stenosis or phimosis [9]. Non-healing wounds are the first sign of malignancy – a process that can take up to 34 years. The incidence of penile cancer is 1/100,000 with penile squamous cell carcinoma (SCC) representing 95%. High-risk HPVs are detected with polymerase chain reaction (PCR) in 17% to 82% of the cases. HPV 6 and 11 are mostly associated with verrucous carcinoma; invasive growth is mostly associated with HPV 16, 18 and 54 [7]. The prevalence of HPV in patients with anal intraepithelial neoplasia is up to 85% [13].

Men and HPV – risk factors

HPV-infection: The degree to which HPV infection plays a necessary causative role in men remains a matter of debate. Men with a history of anogenital warts have a 5- to 6-fold risk of penile SCC [14]. Some cases of penile SCC and many of verrucous carcinoma are negative for HPV-DNA testing. In the latter, when HPV is found it is mostly low-risk HPV 6 and 11, showing that other cofactors must be involved [15]. Probably there are two pathways involved in penile carcinogenesis. One with sexual activity as a route for oncogenic HPV-transmission and the other as a result of synergistic effects of cofactors unrelated to HPV-infection in the patient or the patient’s partner [15]. Patients with lichen sclerosis have a significantly higher rate of oncogenic high-risk HPV than a control group (17.4 vs 8.7%) [16]. When lichen sclerosis is associated with low-risk HPV infection, the development to cancer is probably unrelated to the HPV infection.

HPV transmission: Sexual transmission is the predominant mode of HPV acquisition. In men having anal receptive intercourse, the virus acts in the same way as in the cervix with the dentate line in the rectum being similar to the squamous column.
HPV in men

Discussion

Regular screening programs are important to prevent HPV-related cancers, since HPV infection precedes the development of cancer by several years. The prevalence of malignant consequences of HPV infection in women and men in Western countries is rather low: 10/100,000 for cervical cancer, 1/100,000 for penile cancer and 1.5/100,000 for anal cancer. In the third world where there is a lack of good sexual education, standard screening programs and good medical care, the incidences are higher, except for anal cancer; respectively 44.1/100,000, 4.4/100,000, and 0.7/100,000 [34]. The best anatomical sites for HPV sampling in men are the glans, corona, prepuce and the shaft of the penis. The prepuce is probably the best single site for HPV detection. Screening and treatment of female patients is clear. Many governments are looking into if and how to include the vaccination in the routine vaccination program. HPV-vaccine is especially important for the third world; the highest reduction in mortality rate is to be expected from prevention, since screening and treatment are suboptimal. Should men be screened and vaccinated? At this time there is no current indication for testing men. First of all, HPV infection is very common, but finding HPV infection does not equal an increased risk of disease or cancer in these men or in their sexual partners. Furthermore, there is no standard test available and in addition, there is no therapy for eradicating the HPV infection. One considers that men will profit from the “herd”-effect, if all women should be vaccinated. The future will tell if maybe a subpopulation of men should be screened: men with promiscuous sexual behavior (sex workers), men with anal receptive intercourse, and male partners of women with CIN.

Conclusion

Since the last review in 2004 [35] research has focused more on men and HPV. The natural history of HPV in men is still mainly unknown. Although at this time there is a consensus that general screening of men is useless, especially because there is no real treatment, the number of reports on this subject is increasing.

References

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Stromal cells play a role in cervical cancer progression mediated by MMP-2 protein

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Summary

Metalloproteinases, especially metalloproteinase-2 (MMP-2), are known for their role in the degradation of the extracellular matrix. Nevertheless, a thorough understanding of MMP-2 expression in neoplastic lesions of the uterine cervix has yet to be accomplished. This study aimed to analyze the MMP-2 expression in cervical intraepithelial neoplasia III (CIN3) and in cervical squamous cell carcinoma, in tumor cells and adjacent stromal cells. MMP-2 expression was assessed by an immunohistochemical technique. MMP-2 expression was greater in the stromal cells of invasive carcinomas than in CIN3 (p < 0.0001). MMP-2 expression in stromal cells correlates with the clinical stage, gradually increasing as the tumor progresses (p = 0.04). This study corroborates that stromal cells play an important role in tumor invasion and progression, mediated by the progressive enhancement of MMP-2 expression from CIN3 to advanced invasive tumor. The intense MMP-2 expression most probably is associated with poor tumor prognosis.

Key words: Matrix metalloproteinase 2; Cervix cancer; Cervical intraepithelial neoplasia; Stromal cells.

Introduction

Cervical carcinoma is the second most common neoplasia in women worldwide, corresponding annually to 16% of all cases of tumors in women [1]. Over the last ten years, many studies have demonstrated the unequivocal association between the human papilloma virus (HPV) and cervical carcinoma. The histological appearance of CIN3 is characterized by both cellular atypia and structural disorganization restricted to the squamous epithelium and limited by the basement membrane. For the occurrence of both invasion and tumor metastasis, degradation of the basement membrane and the extracellular matrix (ECM) must occur, since these structures act as a physical barrier to cell migration [3]. Such events suggest that during the progression of CIN, local phenomena occur that would result in the formation of genetically-modified cell clones capable of producing substances that would permit degradation of the basement membrane and initiate evolvement to invasive carcinoma [4].

ECM degradation is mediated by families of extracellular proteinases including serine proteinases, cysteine proteinases and matrix metalloproteinases (MMPs), which play a key role in the evolution of human malignant neoplasias through an increase in proteolysis mediated by these proteins [3, 5, 6]. The MMP family is composed of 20 zinc- and calcium-dependent proteolytic enzymes, subdivided into collagenases, gelatinases, elastases, stromelysins and MMPs according to the specificity of the substrate and the homology of domains [5, 7]. MMPs may be produced by malignant epithelium and by adjacent stroma, suggesting an important role of cell-to-cell interaction [8]. ECM degradation by MMPs would facilitate tumor invasion and progression of the cancer [5]. MMP-2 is a gelatinase and its potential to degrade type IV collagen in the ECM has been shown to be of great importance in facilitating stromal and vascular invasion by tumor cells [9]. In vitro studies have identified the cell-mediated MMP-2 activation mechanism [10].

Studies on several tissues such as those from the colon, pancreas, prostate, bladder and breast [11-15] have shown an increase in MMP-2 expression in human tumor cells and its relationship with an increase in metastases resulting from its ability to remodel ECM and degrade the basement membrane. Some studies on the uterine cervix [16, 17] have described MMP-2 expression in CIN lesions and in invasive carcinoma. Nevertheless, the significance of MMP-2 expression in cervical neoplastic lesions and its relationship with the processes of invasion and metastasis still remain to be established.

Therefore, the aim of this study was to evaluate variations in MMP-2 expression in tumor cells and in the cells of adjacent stroma, comparing cases of CIN3 with cases of invasive squamous cell carcinoma of the cervix. It was hoped that findings would contribute towards improving the current understanding of the role of MMP-2 protein in the invasive process of squamous cell carcinoma of the cervix. From a clinical viewpoint, it is also interesting to consider whether MMP-2 expression plays a role as a possible prognostic marker of invasion and metastasis.
Materials and Methods

Samples

Following a review of clinical records, two groups were selected consisting of 45 women with a diagnosis of CIN3 and 45 with a diagnosis of invasive squamous carcinoma, all cases diagnosed by biopsy. Selection of cases for this study was carried out sequentially from January 2004 until the present among women receiving care at the Oncology Department of the Unicamp. The biopsy samples were reviewed for confirmation of diagnosis and those women with no other associated histological diagnosis and whose paraffin blocks contained sufficient material for immunohistochemical assays were included in this study.

Immunohistochemical analysis

Ninety blocks of paraffin-embedded material were cut into 5-μm thick sections using a microtome. Sections were deparaffinized in xylol and gradually rehydrated in an ethanol series. Following gradual hydration, nonspecific sites were blocked with a 10% hydrogen peroxide solution, after which antigen recovery was performed by immersion in a pH 8.9 buffer for 30 min at a temperature of 95°C. Next, the slides were incubated with primary monoclonal antibodies (matrix metalloproteinase 2, clone 17B11, code NCL-MMP2-507, Novocastra Laboratories, Newcastle, UK) in a chamber at 37°C for 30 min, and then incubated overnight. On the following day, the material was washed with PBS, and the LSAB peroxidase kit (LSAB/HRP, code K0690-1, Dako, Carpinteria, CA, USA) was used to detect the antigen-antibody reaction. After washing, the immunocomplex containing peroxidase was detected using 3,3-diaminobenzidine-HCL chromogen followed by hematoxylin counterstaining. For each reaction, a positive control was also evaluated, as recommended by the manufacturer. Negative controls were always evaluated and consisted of the same case as the positive control with the exclusion of the primary antibody in the reaction.

For each case, “hot-spot” reaction areas were selected and five photographs were taken that included the areas of epithelial-stromal transition. Photographs were taken at high magnification (400x) using a digital camera (Nikon, Coolpix, model 995). Images were transferred to a computer and analyzed using an imaging analyzer software package (ImageJ, 2000).

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The software program used for the statistical analysis of MMP-2 was EPI-INFO. The association between the categorical variables was analyzed using Fisher’s exact test and the magnitude of the association was calculated using the odds ratios (OR) and their respective 95% confidence intervals (CI).
Results

Immunohistochemical reaction for MMP-2 was observed in the cytoplasm of tumor and stromal cells in the two study groups (Figures 1A-1B). Staining was also observed in some cases in the endothelial and glandular cells, although these were excluded from the analyses.

MMP-2 was found to be intensely positive in the stroma (score > 5) in 2% (1/45) of cases of CIN3 and in 40% (18/45) of cases of invasive carcinoma. With respect to the tumor cells, intense MMP-2 staining was found in 18% of cases (8/45) and in 24% (11/45) of cases, respectively, of CIN3 and invasive carcinoma. Expression of MMP-2 was greater in the stromal cells of cases of invasive carcinoma compared to cases of CIN3, and this difference was statistically significant (p < 0.0001). However, no statistically significant difference was found in MMP-2 expression in tumor cells between cases of CIN3 and invasive carcinoma (p = 0.9423) (Table 1). With respect to CIN3, MMP-2 expression was significantly greater in epithelial tumor cells compared to stromal cells (p = 0.05), whereas in the cases of invasive carcinoma no statistically significant difference was found in MMP-2 expression between epithelial tumor cells and stromal cells (p = 0.08) (Table 2).

Table 1. — Comparison of MMP-2 protein expression (score) between the cases of CIN3 and invasive squamous carcinoma for stromal and tumor cells.

<table>
<thead>
<tr>
<th>Score</th>
<th>CIN 3</th>
<th>Stromal cells</th>
<th>CIN 3</th>
<th>Tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Invasive</td>
<td>CI 95%</td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>10</td>
<td>6</td>
<td>Reference</td>
<td>8</td>
</tr>
<tr>
<td>3-4</td>
<td>34</td>
<td>21</td>
<td>1.03 (0.33-3.25)</td>
<td>29</td>
</tr>
<tr>
<td>5-6</td>
<td>1</td>
<td>18</td>
<td>30.00 (3.15-285.71)</td>
<td>8</td>
</tr>
<tr>
<td>p*</td>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
<td>0.1524</td>
</tr>
</tbody>
</table>

* Fisher's exact test (score 1-3, score 3-4, score 5-6).

Table 2. — Comparison of MMP-2 protein expression (score) between stromal and tumor cells in CIN3 and invasive squamous carcinoma.

<table>
<thead>
<tr>
<th>Score</th>
<th>CIN 3</th>
<th>Stromal cells</th>
<th>CIN 3</th>
<th>Tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Invasive</td>
<td>CI 95%</td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>10</td>
<td>8</td>
<td>Reference</td>
<td>6</td>
</tr>
<tr>
<td>3-4</td>
<td>34</td>
<td>29</td>
<td>1.07 (0.37-3.06)</td>
<td>21</td>
</tr>
<tr>
<td>5-6</td>
<td>1</td>
<td>8</td>
<td>10.00 (1.03-97.5)</td>
<td>18</td>
</tr>
<tr>
<td>p*</td>
<td>0.05</td>
<td></td>
<td></td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Fisher’s exact test (score 1-3, score 3-4, score 5-6).

In accordance with the FIGO classification criteria for staging, 18 tumors were classified as Stages I-II, 26 as Stages III-IV and one case could not be classified due to the early death of the patient. A greater frequency of MMP-2 positivity with a score > 3 was found in the stromal cells in Stages III-IV compared to Stages I-II, and this difference was statistically significant (p = 0.04). No association was found between MMP-2 expression in tumor cells and staging (Table 3).

Discussion

Up to the present time, we have been able to evaluate MMP-2 expression in 45 cases of CIN3 and in 45 cases of invasive squamous cell carcinoma of the cervix, using immunohistochemistry to compare the localization of protein expression in both the neoplastic epithelial cells and in the adjacent stromal cells. Our results suggest that MMP-2 expression in stromal cells is greater in cases of invasive carcinoma compared to cases of CIN3, and that it is also greater in more advanced stages of invasive carcinoma. Intense MMP-2 expression was more frequent in epithelial tumor cells than in stromal cells in cases of CIN3. In cases of invasive carcinoma, expression of MMP-2 was similar in both stromal and tumor cells.

Our results are in agreement with those of Davidson et al. [18], who studied a smaller number of cases and reported higher MMP-2 positivity in stromal cells adjacent to invasive carcinoma compared to cases of CIN. In the same study, no differences were found in MMP-2 expression in tumor cells between cases of CIN and invasive carcinoma. In addition, the authors quantified mRNA for MMP-2 in stromal and tumor cells, observing a greater amount of mRNA in tumor cells compared to stromal cells. These investigators hypothesized that tumor cells would be responsible for synthesizing MMP-2, while stroma cells would activate it, which would justify the intense stromal staining for MMP-2.

The importance of stromal cells in defining the role of MMP-2 has also been described in cases of non-small cell lung cancer and rectal cancer [19, 20]. Other studies have suggested that tumor cells may be responsible for inducing the production of proteolytic enzymes in neighboring stromal cells [16, 21, 22]. A study carried out by Sun et al. suggests that the presence of ECM metalloprotease inducers (EMMPRIN) in tumor cells promotes latent MMP-2 production by fibroblasts [21]. The findings published by Sier et al. complement these observations, reporting that after being produced by the fibroblasts, MMP-2 would be activated on the surface of tumor cells in the tumor-stromal interface [22]. Irrespective of the localization of MMP-2 expression and activation, the above-mentioned investigators agree that the increase in MMP-2 expression is associated with a poorer prognosis in patients due to greater aggressivity of the tumor [19-22].

The presence of MMP-2 does not necessarily imply that there will be proteolytic activity, since the enzyme may be in its latent or active form, which cannot be dif-
fermented by immunohistochemistry. It is known that MT1-MMP (MMP-14) and TIMP-2 are necessary to activate MMP-2 [6]. Furthermore, in its active form, it may be inhibited by the presence of TIMP and RECK (reversion-inducing cysteine-rich protein with Kazal motifs) [23]. Thus, there are many possible control mechanisms in tissues, triggered by these proteins that try to reduce the process of degradation of the ECM that would lead to tumor invasion.

In an attempt to understand how the organism loses its ability to self-regulate for this protein, Smola-Hess et al. [23] recently demonstrated that the E7 protein from HPV type 16 was associated with an increase in MT1-MMP (MMP-14) expression in keratinocytes and consequently with an increase in MMP-2 activation. On the other hand, the E7 protein derived from HPV 1 was not able to induce MT1-MMP expression. This result may explain the participation of high-risk HPV types in the imbalance of MMP-2 protein found in tumor invasion.

Conclusion

This study corroborates that stromal cells play an important role in tumor invasion and progression, mediated by the progressive enhancement of MMP-2 expression from CIN 3 to early invasive tumor, and from this stage to advanced invasive carcinoma. Intense MMP-2 expression most probably is associated with poor tumor prognosis.

References


Cost of screening and treatment of cervical dyskaryosis in Germany

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Summary

Human papillomavirus (HPV) infection is the principal cause of cervical cancer. Clinical trials with HPV vaccines have shown high efficacy against HPV-induced precancerous cervical lesions. Before implementing a vaccination programme, up-to-date data on cervical dyskaryosis, incidence and annual treatment costs are needed. We assessed resource use and costs for 12 months following diagnosis for women with abnormal Pap smears in Germany based on a sample of 138 women who had received abnormal results on Pap smears taken during March and April of 2004. Most women had a Pap IIID (57%) vs Pap III (20%) or Pap IV (23%). Women with a Pap IV consulted their gynaecologist more frequently than those with a Pap III or Pap IIID (5.6 visits vs 4.2 and 4.6 visits, respectively). Only 9% of patients underwent colposcopy plus biopsy; this may be due to the lack of histological assessment by colposcopy and biopsy done currently in Germany. More women in the Pap IV group had a cold knife conisation, compared with those in the Pap IIID group, (84% vs 27%) hysterectomy (22% vs 4%) and laser coagulation (12.5% vs 4%). Median treatment duration was shorter for women with a Pap III than for those with Pap IIID and IV (3 vs 5 months, respectively). Overall, 28.3% of the women were hospitalised (median 5; range 1-33 days). The estimated average annual cost per patient was €1,055, €943 and €3,174 for Pap III, IIID and IV, respectively. The cost of managing precancerous cervical lesions in Germany was shown to be high.

Key words: Human papillomavirus; Cervical cancer; Cervical cancer screening; Cervical dyskaryosis; Retrospective study; Resource use; Treatment cost.

Introduction

Human papillomavirus (HPV) infection is the primary cause of cervical cancer [1, 2]. Approximately 200 HPV types have been identified, and 35 of these have been shown to infect the genital tract epithelium [3]. They are classified into high-risk and low-risk HPV types, based on their oncogenic potential [4]. High-risk types 16 and 18 are associated with an estimated 70% of high-grade cervical lesions and cervical cancer, while other high risk types, e.g. 45, 31, 33, 52 and 58, account for only 19.6% of these conditions [5]. Low risk HPV types are associated with anogenital condylomata (i.e., genital warts) and mild dyskaryosis. HPV types 6 and 11 are associated with 90% of genital warts and also with low-grade cervical lesions [6, 7].

HPV infection is responsible for the development of the precursor lesions leading to cervical cancer. Cervical screening programmes enable the early detection and treatment of these precursor lesions or cervical dyskaryosis. Cytological screening using the conventional Papanicolaou tests (Pap smears) is currently the recommended method for cervical cancer screening in most countries. In Germany, cytologically-identified lesions are classified according to the modified Munich Cytological Classification, a variation of the most common Pap reporting convention (Table 1). In the Munich system, both Pap I and Pap II classifications correspond to a normal Pap smear, and the Pap IIw or Pap IIk classification normally requires additional follow-up. The Pap III to IVb classifications correspond to the precancerous stages of cervical cancer, and matches with LSIL (low-grade squamous intraepithelial lesion - Pap IIID) up to HSIL, possible neoplasia (high-grade squamous intraepithelial lesion - Pap IVb) according to the Bethesda classification.

After abnormal Pap smear results further procedures, including colposcopy and biopsy, may be performed to determine the CIN (cervical intraepithelial neoplasia) grade of the lesion. CIN lesions are treated by a variety of techniques including laser coagulation, loop excision or cold knife conisation.

Information regarding the actual costs related to cervical cancer screening exists in some European countries, such as France and the UK, but no such information is available for Germany. As healthcare systems are not uniform across Europe and healthcare pathways differ widely between countries, costs related to cervical cancer screening are likely to be different. Hence, it is necessary to collect and analyse country-specific data on the cost burden of HPN-related diseases. The aim of this study was to collect data on healthcare resource use and costs for the management of women with cervical dyskaryosis (Pap III, IIID and IV) in Germany.
Methods

Gynaecologists were selected using the ACNielsen regions database [8]. This database divides Germany into eight regions and defines the number of physicians to be recruited from each region to obtain a representative geographical distribution of gynaecologists. Between March and April 2005 a total of 50 gynaecologists were recruited for the study. Each gynaecologist was asked to provide data for the first three patients aged over 21 years, consulted during this period, who had been diagnosed with Pap III, IIID or IV in March or April 2004, and were living in Germany.

For each patient, information on socio-demographic data, clinical data (medical history, diagnosis, and outcome), healthcare resources used (specialists visits, diagnostics, medications, interventions, adverse events, hospitalisations) and work days lost were collected. Healthcare resources used and work days lost were recorded from the date of diagnosis (March/April 2004) for a follow-up period of up to one year from diagnosis. At each visit the Pap stage was reevaluated and if the Pap stage had regressed to a stage other than Pap III, IIID or IV, the resource use data were not collected for that particular visit.

The costs per patient were calculated by combining the healthcare resources used with the associated unit costs. Units costs were based on national sources [9, 10] and are expressed in euros (€) for the year 2005 (Table 2). For procedures (leep excision, laser coagulation, cold knife conisation, curettage, biopsy and haemostasis), office-based unit costs and hospital-based unit costs were applied. These latter include hospitalisation costs, as well as costs for laboratory tests. Hospitalisation costs were based on the German diagnostic related groups (DRG) system, which incorporates the mean length of stay [11]. Since colposcopy is currently not reimbursed, its cost was not considered in this cost analysis.

We estimated the cost of productivity loss by multiplying the mean number of work days lost per patient by the Gross Domestic Product (GDP) per person per working day. In 2004, the GDP per person per working day for Germany was € 119 [12]. In the estimation of the costs due to productivity loss, it was assumed that patients for whom data were missing had not taken sick leave. For the healthcare payer perspective, only direct costs related to treatment and management of women with Pap III, IIID and IV were analysed, whereas for the societal perspective direct and indirect costs were taken into account. The total cost of detection and treatment of precancerous cervical lesions for the German population in 2005 was estimated by multiplying the mean screening cost per patient by the number of women screened by Pap stage per year.

Statistics

Demographic, clinical, resource use and cost data were analysed using SAS® version 8 (SAS Institute INC). As the distribution of resource use and cost data was expected to be skewed, 95% confidence intervals (95% CI) were obtained using non-parametric bootstrapping techniques [13]. To identify patient characteristics that had a significant impact on overall costs, we used a multivariable model based on analysis of the variance (ANOVA) of the overall costs taking into account different patient characteristics as cofactors. The F-statistic was used to assess the overall significance of the model. The significance of each co-factor of the model was assessed by a t-test.

Results

Seventy-four gynaecologists were asked to participate in this study; 67 agreed and 50 gynaecologists provided patient data. Most gynaecologists were office-based (80%) and worked in private practice (68%). These gynaecologists were a representative sample in terms of the geographical distribution of gynaecologists in Germany except for the region of Schleswig-Holstein, which was under-represented; gynaecologists in this region represent 16% of all gynaecologists in Germany, whereas for the study they represented only 10% of those in the study.

Data for 138 patients out of the 152 patients initially included were analysed. Data for 14 patients were not analysed because either the protocol was not respected or the treatment duration was > 12 months. The mean age of the patients included in the study was 39 ± 11 years. Most patients were married (or living with their partner) (61%), had at least a high school degree (52%), were employed full-time (52%), and had at least one pregnancy (65%). Twenty-eight percent of the patients had never smoked before, 9% were ex-smokers and 49.2% were current smokers. For 61% of the patients (n = 84) infor-
mation on new sexual partners in the last 12 months was available. The median number of new sexual partners < 12 months was one; about 20% of the women had no new sexual partner < 12 months, while 7.2% had two or three < 12 months. Lastly, most women used hormonal contraceptives (38%) followed by intrauterine devices (13%); 30% said they did not use contraceptives.

Of the 138 patients analysed, 27 patients (20%) had Pap III diagnosis, 79 (57%) had a Pap IIID diagnosis, and 32 (23%) had Pap IV diagnosis. The overall mean treatment duration was 5.1 ± 3.7 months: for Pap III, 4.4 ± 3.8 months; for Pap IIID 5.5 ± 3.1 months and for Pap IV 4.9 ± 3.7 months. The mean number of consultations was 4.7 ± 1.9. This was higher for the patients with Pap IV (5.6 ± 1.4) than for patients with Pap IIID (4.6 ± 2.0) and those with Pap III (4.2 ± 2.0).

Only a minority of the patients (9%) underwent colposcopy with biopsy, which was not frequently performed as a diagnostic procedure (Table 3). More women with Pap IV (59%) underwent curettage compared with those with Pap III (19%) or Pap IIID (14%). During the study period, 47% of all patients underwent surgery (Table 4). Women with Pap IV underwent surgery more frequently than those with a Pap III or a Pap IIID: 96.9% compared with 26% and 34%, respectively. Cold knife conisation was the most frequent surgical intervention (Table 4). Adverse events related to the treatment of cervical lesions were reported by eight patients (four in the Pap IIID group and four in the Pap IV group): pelvic pain, (1 patient), severe bleeding (3 patients), infection (1 patient) and three patients had other adverse events (unspecified). Two of these patients had additional visits to the gynaecologist due to adverse events.

Overall, 39 (28.3%) women were hospitalised for a median duration of five days (range 1-33 days). More patients in the Pap IV group were hospitalised: 19 (59%) compared with six (22%) and 14 (18%) in the Pap III and IIID groups, respectively.

The mean duration of hospitalisation was 5.5, 5.0 and 3.5 days for the Pap III, Pap IIID and Pap IV groups, respectively.

Information on sick leave was available for only 93 patients of whom 47 (51%) took sick leave. The median duration of leave was 12 days (range 2-64 days), with 22 of the 26 women in the Pap IV group having sick leave compared with six out of 17 and 19 out of 50 in the Pap III and IIID groups, respectively.

The average direct costs per patient and the costs related to gynaecologist visits were significantly higher for patients in the Pap IV group compared with those in the Pap III group and Pap IIID patients (Table 5). Although curettage was infrequently used, it accounted for more of the costs than did colposcopy; it is mainly performed in hospital and is therefore more costly. The mean costs for medical interventions were statistically significantly higher in the Pap IV group than in the other groups due to the more frequent use of cold knife conisations and hysterectomies in patients in the Pap IV group (Table 4).

The mean indirect costs per patient due to sick leave were significantly higher in the Pap IV group compared with the Pap III and IIID groups (Table 5). The contribution of indirect costs to total costs was similar in all three Pap groups (40-45%); these costs were €442, €430 and €1,293 in the Pap III, IIID and IV groups, respectively. It was assumed that patients for whom data were missing had not taken sick leave. From the societal perspective, the mean total costs per patient were significantly higher for women in the Pap IV group than those for women in the Pap III and IIID groups (Table 5).

The effect of patient characteristics on the costs was estimated using a multivariable analysis using data available from 118 patients. The model included Pap stage, age, smoking habits and pregnancy status but the type of setting was not included since the majority of patients were from office-based, private practices. The Pap type had a significant effect on total costs (p < 0.0001), whereas age group, smoking habits and pregnancy status did not.

### Table 3. — Frequency of the use of procedures. Values are reported as number of women (percentages).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>All patients (n = 138)</th>
<th>Pap III group (n = 27)</th>
<th>Pap IIID (n = 79)</th>
<th>Pap IV group (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colposcopy</td>
<td>106 (77%)</td>
<td>24 (89%)</td>
<td>58 (73%)</td>
<td>24 (75%)</td>
</tr>
<tr>
<td>HPV-DNA</td>
<td>41 (30%)</td>
<td>5 (19%)</td>
<td>30 (38%)</td>
<td>6 (19%)</td>
</tr>
<tr>
<td>Curettage</td>
<td>35 (25%)</td>
<td>5 (19%)</td>
<td>11 (14%)</td>
<td>19 (59%)</td>
</tr>
<tr>
<td>Biopsy</td>
<td>13 (9%)</td>
<td>1 (4%)</td>
<td>6 (8%)</td>
<td>6 (19%)</td>
</tr>
</tbody>
</table>

### Table 4. — Frequency and type of surgical interventions. Values are reported as number of women (percentages).

<table>
<thead>
<tr>
<th>Intervention</th>
<th>All patients (n = 138)</th>
<th>Pap III group (n = 27)</th>
<th>Pap IIID (n = 79)</th>
<th>Pap IV group (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold knife conisation</td>
<td>54 (29%)</td>
<td>6 (22%)</td>
<td>21 (27%)</td>
<td>27 (84%)</td>
</tr>
<tr>
<td>Hysterectomy</td>
<td>12 (9%)</td>
<td>1 (4%)</td>
<td>4 (5%)</td>
<td>7 (22%)</td>
</tr>
<tr>
<td>Laser coagulation</td>
<td>7 (5%)</td>
<td>0</td>
<td>3 (4%)</td>
<td>4 (13%)</td>
</tr>
<tr>
<td>Leep excision</td>
<td>5 (4%)</td>
<td>2 (7%)</td>
<td>1 (1%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Cryotherapy</td>
<td>1 (1%)</td>
<td>0</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>Haemostosis</td>
<td>2 (2%)</td>
<td>0</td>
<td>1 (1%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>No intervention</td>
<td>73 (53%)</td>
<td>20 (74%)</td>
<td>52 (66%)</td>
<td>1 (3%)</td>
</tr>
</tbody>
</table>
It was assumed that women with a Pap I and II undergo 1.06 Pap smears each year (unit cost *).

Previously reported distribution of Pap types in Germany [18-20].

<table>
<thead>
<tr>
<th>Pap Stage</th>
<th>Number of women screened</th>
<th>Mean cost/ woman (€)</th>
<th>Total cost for German population (€)</th>
<th>Mean cost/ woman (€)</th>
<th>Total cost for German population (€)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pap I &amp; II</td>
<td>16,241,538</td>
<td>26.28**</td>
<td>426,872,556</td>
<td>26.27</td>
<td>426,872,556</td>
</tr>
<tr>
<td>Pap III</td>
<td>32,941</td>
<td>612.74</td>
<td>20,184,268</td>
<td>1,054.97</td>
<td>34,751,867</td>
</tr>
<tr>
<td>Pap IID</td>
<td>172,940</td>
<td>513.22</td>
<td>88,756,267</td>
<td>943.39</td>
<td>163,149,867</td>
</tr>
<tr>
<td>Pap IV</td>
<td>22,235</td>
<td>1,881.04</td>
<td>41,824,927</td>
<td>3,172.75</td>
<td>70,568,109</td>
</tr>
<tr>
<td>Total</td>
<td>16,470,478</td>
<td>577,592,856</td>
<td>695,297,361</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4 Previously reported distribution of Pap types in Germany [18-20].

** It was assumed that women with a Pap I and II undergo 1.06 Pap smears each year (unit cost € 24.79). This number has been calculated based on the fact that there are 18 million Pap smears performed annually [22], and that our study reported a mean number of Pap smears of 2.7, 3.6 and 3.1 respectively for Pap III, Pap IID and Pap IV.

Discussion

Our results show that most women enrolled in the study had a Pap IID (57%) vs Pap III (20%) or Pap IV (n = 23%) diagnosis. Patients in the Pap IV group consulted a gynaecologist more often than those in the Pap III or Pap IID groups (5.6 visits compared with 4.2 and 4.6 visits, respectively). The most common intervention was colposcopy (77%). Patient management, including treatment, was initiated on the basis of Pap-smear-detected cytological abnormalities, although diagnostics such as biopsy and colposcopy are recommended [14, 15]. Cold knife conisation was the most often prescribed surgical procedure, regardless of Pap stage. The median treatment duration was five months for women in the Pap IID and IV groups compared with three months for those in the Pap III group. Overall, 28.3% of the women were hospitalised for a median of five days (range 1-33 days). The estimated mean annual cost (direct and indirect) per patient associated with the screening and treatment of women with Pap III, IID and IV results were € 1,055, € 943 and € 3,174, respectively.

These data can be used to estimate the total annual cost for screening and treatment of pre-cancerous lesions in the German population. Assuming that 50% of women aged between 20 and 84 years old have a Pap smear annually, the number of women screened annually in 2005 was estimated to be 16,470,478 [16, 17]. Using the previously reported distribution of Pap types in Germany, the number of women diagnosed with a Pap I, II, III or IV can be calculated (Table 6) [18-20]. Assuming that women with Pap I or II have on average 1.06 Pap smears annually (Table 6) the mean cost per women with Pap I or II is € 26.28. Thus, the annual costs associated with the screening and management of all Pap stages can be estimated at € 578 million from the healthcare provider perspective, and € 695 million from the societal perspective. Total costs for Pap III, IID, IID and IV represent 26% (€ 150.8 million) and 39% (€ 268.5 million) of these costs, respectively.

This estimate for the healthcare provider is higher than that recently reported in the UK of £ 138.5 million (about € 206.4 million, with £1 = € 1.49, exchange rate 23 February 2007). This difference can be explained partly by the different screening programmes in these two countries. In Germany, women covered by statutory health insurance and aged over 20 years are eligible for a yearly consultation with their gynaecologist, which includes a Pap test every year, whereas in the UK national guidelines recommend screening women aged 25 to 49 years every three years and women aged 50 to 64 years every five years [21]. In Germany it is estimated that there are 18 million Pap tests performed every year [22], whereas in the UK it was estimated that in 2003 there were 4.8 million tests, which is nearly four times fewer [23]. In France, where screening is opportunistic, as in Germany, it was estimated that 6,111,787 Pap tests were performed in 2004 giving an estimated uptake of 27% in women between 20 and 69 years old [24]. The costs associated with the detection and treatment of cervical dyskaryosis in 2004 in France was estimated at € 174.2 million from the healthcare payer’s perspective and € 336 million from the societal perspective [25]. This estimate is lower than we report here for Germany. The difference in costs can mainly be attributed to the number of Pap smears performed annually in each country: 6,111,787 in France versus 18,000,000 in Germany (almost three times more). This difference is directly related to the difference in the current screening intervals for women with normal Pap smears in both countries: recommendation for annual screening in Germany versus every three years in France. However attributing the whole cost for the annual visit to the costs of Pap smear screening does not reflect the reality since in Germany women have an annual consultation with their gynaecologist which includes a physical examination and health advice. Women are advised about colposcopy, mammography and other examinations during this consultation with their gynaecologists. Despite this consideration, irrespective of the fee being totally or partially attributed to Pap smear screening, the German screening costs still exceed that of France and the UK.

The study has several limitations. There were few patients in the Pap III and IV groups giving a skewed distribution of the patients over the three Pap stages. Moreover, as we only evaluated the resource use and costs of Pap III, Pap IID and Pap IV stages, and not for Pap I and II, the average resource use and costs for patients who changed to another stage may have been underestimated. However, if we assume that change to another stage would mainly involve changing to Pap II, the impact on the average costs would be limited as the costs for this stage are low. Curettage was often performed, and cold knife conisation was the preferred type of treatment. The high cost of managing pre-cancerous cervical lesions may be related to histological assessment by cold knife conisation instead of colposcopy with biopsy. This specific management of cytological smears in Germany differs from corresponding algorithms in neighbouring countries.

It is difficult to say whether the treatment of the 138 patients in this study reflects that which German women with abnormal Pap smears would undergo; however it is a necessary first step in evaluating the costs in Germany. Despite these limitations, this is the first study in Germany to report on how women with abnormal Pap smears are managed. Our results show that the majority
of women with abnormal Pap smears are treated without histological confirmation of the lesions in Germany. This practice is in contrast with the UK and the US and could be partly due to the lack of national guidelines for the management of abnormal Pap smears in Germany, a lack of colposcopy clinics, and the fact that colposcopy is not a reimbursed health care procedure in Germany.

The results from recent clinical trials of prophylactic immunisation of young women with a quadrivalent HPV L1 virus-like particle vaccine (types 6, 11 16 and 18) show an efficacy of 100% (95% CI: 55.3% to 100%) in preventing type-specific HPV-associated disease and 95.8% (95% CI: 83.8% to 99.5%) in the reduction of the combined incidence of HPV infection and disease [26]. Thus, although this vaccine will not protect against all HPV infections, it has been shown to prevent 98% (95.89% CI: 86% to 100%) of HPV-16/18 related CIN grades 2, 3 and adenocarcinoma in situ in uninfected women. This same study, which followed the women for an average of three years post-vaccination, also reported 44% (95% CI: 26% to 58%) efficacy in preventing high-grade cervical lesions in women who were infected prior to vaccination. This reduction of lesions will dramatically reduce the costs associated with the treatment of HPV-related cervical disease [27].

In conclusion, the total cost of detecting and treating atypical Pap smears in Germany is high, estimated at €268.5 million per year from the societal perspective. The introduction of a HPV vaccine preventing cervical dyskaryosis, in combination with the cervical cancer screening programme, will result in a significant reduction of the burden of disease.

Acknowledgements

We thank the patients, the primary care staff and the gynaecologists who participated in the study.

We also thank Laurence Serradell for her contribution to the study (set-up and conduct), Nathalie Largeron, Sara Jow, Nina Latham and Katharina Buesch for help finalising the study reports and reviewing of the paper, Catherine Mary for drafting the manuscript and Margaret Haugh and Virginia Powers for help with editing and reviewing the paper.

Funding for this study was provided by SP MSD, Europe.

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Immunoexpression of HER family, neuregulin, MAPK and AKT in invasive ductal carcinomas of the breast

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Summary

Background: The purpose of this study was to investigate the frequency of expression of the erbB/HER family of growth factor receptors, their ligand neuregulin-α (NRGα) and the most important pathways activated by HER receptors that are mitogen-activated protein kinase (MAPK) and serine/threonine kinase (AKT) in invasive ductal carcinomas of the breast, not otherwise specified (IDC-NOS).

Methods: 59 of the IDC-NOS of the breast were studied for ER, PR, EGFR, c-erbB-2, c-erbB-3, c-erbB-4, neuregulin Ab-3, phospho-AKT, and phospho-p44/42 MAP kinase using the streptavidin-biotin horseradish method.

Results: Of the 59 tumours, 44 (75%) were ER+, 37 (63%) PR+, four (7%) EGFR+, seven (12%) c-erbB-2+, seven (12%) c-erbB-3+ and 14 (24%) c-erbB-4+ α

Strong cytoplasmic and/or nuclear immunoexpression was revealed in 17 (29%) cases for NRGα, 13 (22%) cases for p-AKT, and nuclear immunoexpression with p-MAPK was found in 17 (29%) cases.

Conclusion: The results suggest that high-grade breast carcinomas are not only associated with ER/PR- negativity, but seem to be activated by receptor tyrosine kinase growth factors.

Key words: Breast Carcinoma; HER family; Immunohistochemistry.

Introduction

The HER family of receptor tyrosine kinases (RTKs) consists of four receptors: Epidermal growth factor receptor (EGFR) (also called HER-1 or erbB-1), HER-2 (erbB-2), HER-3 (erbB-3), and HER-4 (erbB-4). The family is associated with extensive receptor-receptor interactions and diversity of ligands. HER ligands can be divided into three groups: The first group includes EGF, amphiregulin (AR), and transforming growth factor α (TGFα) that bind specifically to HER-1. The second group consists of betacellulin (BTC), heparin binding EGF, and epiregulin, which exhibit dual specificity for HER-1 and HER-4. The third group, composed of the neuregulins (also known as neu differentiation factors or heregulins), bind to HER-3 and HER-4 or only HER-4 [1]. Multiple erbB-receptor homo- or heterodimers trigger intracellular signalling leading to specific cellular responses, e.g., stimulation or inhibition of proliferation [2]. The EGFR family of RTKs and ligands play an important role in the pathogenesis of breast cancer (BCa) [3-5].

c-erbB-2 is the best studied member of the type 1 growth factor receptor (T1GFR) family and its amplification occurs in 15-25% of BCa cases [6]. This oncogene activates the phospho-inositol-3-kinase (PI-3K) pathway that inhibits apoptosis. The survival signal is also normally coupled to the MAPK pathway. Increased Her-2 expression in cancer enhances and prolongs signalling from both the PI-3K and MAPK pathways [1].

The main purpose of this study was to determine the immunoexpression of the following: erbB/Her family of growth factor receptors and their ligand neuregulin-α (NRGα), and the most important pathways activated by HER, MAPK and the serine/threonine kinase AKT in BCa [invasive ductal carcinomas of the breast, not otherwise specified (IDC-NOS)]. A further aim was to investigate coexpressions and correlations with the well-known histopathological prognostic parameters including tumour stage, grade, lymph node status, oestrogen receptor (ER), progesterone receptor (PR) status and clinical outcome.

Materials and Methods

The study included 59 cases of IDC-NOS (mean age 59 years) from modified radical mastectomies or lumpectomies from patients whose complete clinical records and follow-up information were available from Marmara University Hospital, Turkey during the period 1998-2004. All surgical material was fixed in 4% formalin and embedded in paraffin. The tumours were classified according to the pTNM system (sixth edition) and were graded according to Elston & Ellis [7]. The study was approved by the Ethics Committee of the Marmara University Hospital.

Immunohistochemistry

The primary antibodies used were summarised in Table 1. Immunohistochemistry was performed using the streptavidin-biotin immunoperoxidase method (UltraVision Detection System Anti-Polyvalent, HRP; Fremont, CA). Four-micrometer sections were cut on to Menzel SuperFrost® Plus glass slides. The slides were dewaxed overnight in an incubator at 37°C.
Deparaffinized in three changes of xylene and rehydrated in two changes of 95% ethanol. Sections were covered with 3% H2O2 in methanol for 20 min to block endogenous peroxidase activity of the tissue, followed by a washing procedure with distilled water. Antigen retrieval for ER, PR, c-erbB-2, c-erbB-3, c-erbB-4, NRG, phospho-p44/42 MAP kinase and phospho-AKT antibodies was performed by incubating the slides in a microwave oven (160 W) with 0.01 M citrate buffer (pH 6.0) for 15 min, followed by cooling for 20 min. at room temperature. The slides were incubated with Protease XXV at 37°C for 20 min for EGFR. The slides were washed twice with tris-buffered saline (TBS), and then blocked with normal serum for 5 min. Slides were then placed in a humid chamber and incubated for 30 min with the primary antibody as indicated in Table 1. After two rinses in TBS, the slides were incubated with biotin-conjugated secondary antibody for 10 min at room temperature. The slides were rinsed again and treated with horseradish peroxidase-conjugated streptavidin for 10 min at room temperature. Tissue staining was visualized with a DAB substrate chromogen solution. Slides were counterstained with hematoxylin, dehydrated, and mounted.

Table 1 — Primary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>SP1; Lab Vision</td>
<td>1/400</td>
<td>RT 30 min</td>
</tr>
<tr>
<td>PR</td>
<td>SP2; Lab Vision</td>
<td>1/400</td>
<td>RT 30 min</td>
</tr>
<tr>
<td>EGFR</td>
<td>111,6; Lab Vision</td>
<td>RTU</td>
<td>ON at 4°C</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td>e2-4001+3BS;</td>
<td>RTU</td>
<td>RT 30 min</td>
</tr>
<tr>
<td>c-erbB-3</td>
<td>HER-3; Lab Vision</td>
<td>RTU</td>
<td>RT 60 min</td>
</tr>
<tr>
<td>c-erbB-4</td>
<td>HER-4; Lab Vision</td>
<td>RTU</td>
<td>RT 60 min</td>
</tr>
<tr>
<td>Neuregulin Ab-3</td>
<td>NDF/GGF/Neuregulin;</td>
<td>RTU</td>
<td>1/150 RT 30 min</td>
</tr>
<tr>
<td>Phospho-p44/42</td>
<td>Thr 202/Tyr 204;</td>
<td>Cell Signalling</td>
<td>1/100 RT 60 min</td>
</tr>
<tr>
<td>Map Kinase</td>
<td>Cell Signalling</td>
<td>1/50</td>
<td>ON at 4°C</td>
</tr>
<tr>
<td>Phospho-AKT</td>
<td>Ser 473;</td>
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</table>

Abbreviations: RTU: ready to use, RT: room temperature, ON: overnight

Positive and negative control slides were included in each staining series. No significant staining was observed in the negative controls using serum replacing the primary antibody. The results of the immunohistochemistry procedures were assessed by two pathologists independently and the cases were discussed for resolution where discrepancies occurred.

Evaluation of immunostaining

ER and PR: Staining was scored as positive using a cut-off value of >10% of the tumour cell nuclei.

EGFR, c-erbB-2, c-erbB-3 and c-erbB-4: no staining or weak incomplete membrane staining in any proportion of tumour cells was scored as 0 or 1+, complete membrane staining that was either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells was scored as 2+, complete intense uniform membrane staining of >30% of invasive tumour cells was scored as 3+ and regarded as positive for HER family proteins [8].

EGFR staining was weak or moderately positive on cytoplasmic membranes of myoepithelial cells of all benign ducts whenever they were found around the tumours in the same sections.

p-AKT and NRGα: The same criteria as specified for the HER family above were used. Strong cytoplasmic and/or nuclear staining of >30% of the tumour cells compared to weak staining of the normal breast tissue as a positive control was evaluated as positive immunostaining for p-AKT and NRGα [9]. Cytoplasmic staining of ductal cells with NRGα in all benign ducts whenever they were found around tumours in the same sections was also noted.
Table 3A — Results of statistical analysis

<table>
<thead>
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<td>6</td>
<td>6</td>
<td>9</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>9</td>
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<td>9</td>
<td>9</td>
<td>9</td>
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<td>6</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>$p$ value</td>
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<td>0.06</td>
<td>0.011</td>
<td>0.005</td>
<td>0.067</td>
<td>0.0001</td>
<td>0.017</td>
<td>0.004</td>
<td>0.012</td>
<td>0.064</td>
<td>0.045</td>
<td>0.062</td>
<td>0.0001</td>
<td>0.03</td>
<td>0.063</td>
<td>0.064</td>
<td>0.029</td>
<td>0.059</td>
<td></td>
</tr>
</tbody>
</table>

Results

Among the 59 IDC-NOS cases studied, 75% (44/59) were ER positive and 63% (37/59) were PR positive (Table 2). There was a statistically significant correlation between tumour stage and lymph node status, and between ER and PR status ($p < 0.05$). With regard to clinical follow-up, 73% (43/59) of the patients were well with no evidence of disease, 8.5% (5/59) had metastatic disease and 18.6% (11/59) died of breast carcinoma. There was a tendency of correlation between ER negativity and poor clinical outcome ($p = 0.064$) (Table 3).
Figure 1A — Obvious circumferential distribution of EGFR.
Figure 1B — Complete intense uniform membrane staining of c-erb-2.
Figure 1C — Strong membranous staining with c-erb-3.
Figure 1D — Obvious circumferential staining of c-erb-4.
Figure 1E — Cytoplasmic and/or nuclear expression with NRGα
Figure 1F — Strong nuclear staining with p-AKT
Figure 1G — Strong nuclear staining with p-MAPK.
expression and PR immunoexpression (p < 0.05) (Table 3). There was no significant correlation with ER, EGFR, NRGα, p-MAPK or p-AKT immunoexpression.

**Phospho-AKT**

p-AKT protein expression was assessed in 22% (13/59) of the cases (Table 2) (Figure 1G). A tendency for a positive correlation was found with lymph node status (p = 0.067) and poor clinical outcome (p = 0.059) (Table 3).

**Coexpression profile of the proteins**

Five (9%) of the cases demonstrated coexpression with c-erbB-3+c-erbB-4, p-MAPK+p-AKT, NRG+ p-MAPK while four (7%) of the cases were positive for both c-erbB-2 and NRGα. There were only three (5%) cases coexpressing NRGα+p-MAPK+p-AKT. A statistically significant correlation was revealed between c-erbB-2 and c-erbB-3 overexpression and between c-erbB-3 and c-erbB-4 (p < 0.05). There was no statistical significance but there was a tendency of positive correlation of coexpression between c-erbB-2 and c-erbB-4 (p = 0.062) and between c-erbB-2 and EGFR (p = 0.064). Tumour high grade was found to be correlated with c-erbB-2 overexpression (p < 0.05) but we could not find any significant correlation between coexpression profiles and histopathological prognostic parameters such as tumour stage, lymph node or hormonal status, and clinical outcome of the patients.

**Discussion**

c-erbB-2 is a member of the T1GFR family and its amplification occurs in 15-25% of BCa, predicting a poor prognosis [6,10,11]. Although c-erbB-2 is the best-studied member of the T1GFR family, the other family members and their coexpression profile and correlations with NRGα, p-MAPK and p-AKT and with the well-known histopathological prognostic parameters such as tumour stage, grade, lymph node status, ER, PR status, and clinical outcome in IDC-NOS of the breast are not well established.

**EGFR:** EGFR occurs in 15-36% of BCas [12,13,14]. EGFR has been demonstrated in breast cancer cell growth and its overexpression was found to be indicative of poor prognosis. No relationship between EGFR expression and steroid receptor status was observed [15]. It has also been demonstrated that erbB1 overexpression in malignancies besides tumour proliferation results in increased tumour cell motility in vivo together with enhanced intravasation and metastasis and ErbB3-dependent motility and intravasation in BCa metastasis [16]. EGFR expression may have prognostic significance in patients with locally advanced BCa who are treated with anthracycline chemotherapy [17]. In our study group, EGFR overexpression was found in four cases (7%). Our rate of frequency appears to be low compared to other reports (15%-36%). This discrepancy could be due to the small number of patients (59 cases) studied and/or the antibodies that were used for the evaluation of the immunohistochemistry or the study group that the results were based on. It is well known that some histological types of BCa such as basal-like carcinomas, metaplastic carcinomas and squamous carcinomas of the breast have a high rate of EGFR overexpression [18-20]. Our study group consisted entirely of IDC (NOS) and the majority of the cases (91.5%) were Stage I or II. Three of the four EGFR positive cases had lymph node metastasis and two had metastatic disease. A statistically significant association with ER negativity and EGFR overexpression (p < 0.05) and a borderline positive correlation with EGFR and c-erbB-2 overexpression (p = 0.064) were revealed.

- Triple-negative BCa (ER-negative, PR-negative, and HER2-negative) is a high risk breast cancer that lacks the benefit of any specific therapy that targets these proteins. Those tumors have positive expression of basal cytokeratins (basal phenotype), P-cadherin, p53, and EGFR [21]. Among our cases there were only five cases that were triple negative for immunohistochemistry and while 60% died of BCa, the only case which demonstrated EGFR overexpression had a good clinical outcome with no evidence of disease.

**HER4:** There are reports supporting the hypothesis that HER1-3 is associated with driving tumour proliferation, whereas HER4 is involved in a non-proliferative or even a protective role [22]. Low nuclear grade, low proliferation rate and presence of HER4 expression in ductal carcinoma in situ (DCIS) were found to be independent predictors of nonrecurrence. HER4 expression has been suggested to identify women who could avoid radiotherapy after breast-conserving surgery for DCIS [23]. We also studied two DCIS cases that were not included in the study group and both cases were only positive for c-erbB-4; c-erbB-4 was found to antagonise the c-erbB-2 effect during the clinical course of BCa and its expression was associated with a more favourable outcome. It is therefore suggested that clarifying the status of c-erbB-4 expression could be significant to achieve the best results with immunotherapy against the c-erbB-2 receptor [14]. Our clinical follow-up results of the patients also demonstrate a statistically significant correlation between c-erbB-4 overexpression and good clinical outcome (p < 0.05).

**TGF family member coexpression:** TGF family member coexpression (HER1, HER2, HER3 but not HER4) was found to have a negative synergistic effect on patient outcome, independent of tumour size or lymph node status [24]. ErbB3 functions as an indispensable ErbB2 dimerisation partner and is required for proliferation of ErbB2-overexpressing tumour cells [25]. Strong cytoplasmic c-erbB-3 immunoexpression has been found to be significantly correlated with local recurrence in BCa but there were no significant associations with survival, Union International Contre Cancer (UICC) criteria, age, menopausal status, ER status, histological grade, c-erbB-2 status or the presence of vascular invasion [26]. On the other hand, combined ErbB-2 and ErbB-3 expression has been found to be associated with nodal involvement and reduced overall survival [13]. The strongest overall corre-
Immunoexpression of HER family, neuregulin, MAPK and AKT in invasive ductal carcinomas of the breast

Strong p-ERK staining in tumour cells was observed in 11 out of 245 (4.5%) BCa [33]. We revealed cytoplasmic and nuclear NRG1α immunoexpression in 29% of IDC cases and weak/moderate staining in the cytoplasm of the ductal cells of normal breast tissue whenever they were found around the tumour in the same sections. We could not find any statistically significant correlation between NRG1α immunoexpression and the other parameters that were studied. Cytoplasmic staining of NRG1 appears to be a marker for ductal cells of the breast.

MAPK: Strong p-ERK staining in tumour cells was associated with early stages, negative nodal status and long recurrence-free survival [34]. Nuclear ERK2 expression was found to be an independent prognostic factor of shortened overall survival of patients, while cytoplasmic ERK2 had an independent, favourable effect on both disease-free and overall survival [35]. In this study we have also found a statistically significant correlation between nuclear p-MAPK overexpression and PR positivity (p < 0.05). We could not find any statistically significant correlation between p-MAPK overexpression and the clinical outcome of the patients. This lack of association could be due to the small number of cases studied. Although we could not find any statistically significant correlation between p-AKT, NRG1α and p-MAPK, immunoexpressing tumour cells were similar. If nuclear staining occurs with p-AKT or NRG1α it is most commonly revealed in the ductal carcinoma cells which are at the outer section (basally located) of the tumour group and this is also a common finding for p-MAPK.

AKT: Activation of AKT and its prognostic value in BCa have been reported [36,37]. The AKT pathway was found to be activated in early breast cancer during the in situ stages [38]. Our two DCIS cases also demonstrated p-AKT overexpression (unreported data). p-AKT has been found to be significantly associated with c-erbB-2 overexpression and with a poor prognosis [39]. The results of our study support the notion that p-AKT overexpression has a borderline positive correlation with lymph node metastasis (p = 0.067) and poor clinical outcome (p = 0.059). Five of the seven cases who had strong p-AKT, immunoexpression either had metastatic disease or died as a result of BCa.

Conclusion: This study was entirely based on IDC-NOS and majority of the cases were tumour Stage I or II. The results indicate that high-grade histomorphology of BCas is not only associated with ER-/PR-negativity, but seems to be activated by receptor tyrosine kinase growth factors such as EGFR and c-erbB-2 overexpression. While c-erbB-4 overexpression may predict a good clinical outcome for patients, p-AKT expression seems to predict a poor clinical outcome. The expression and coexpression profile of receptor thyrosine kinase growth factors are usually not characterised in the pathological diagnosis. However information about tumourigenesis in diagnostic pathology reports targeting multiple erbB-receptors may provide an exceptional strategy for an effective cancer therapy.

Acknowledgement

This study has been supported by the Marmara University Research Foundation with project numbers: SAG-TUS-290906-0205 and SAG-YYT-200407-0068.

References


Differential gene expression analysis of ovarian cancer in a population isolate

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Summary

Gene expression products represent candidate biomarkers with the potential for early screening and therapy of patients with ovarian serous carcinoma. The present study, using patients that originate from the population isolate of South Tyrol, Italy, substantiates the feasibility of differential gene expression analysis in a genetically isolated population for the identification of potential markers of ovarian cancer. Gene expression profiles of fresh-frozen ovarian serous papillary carcinoma samples were analyzed and compared to normal ovarian control tissues using oligonucleotide microarrays complementary to 14,500 human genes. Supervised analysis of gene expression profiling data identified 225 genes that are down-regulated and 635 that are up-regulated in malignant compared to normal ovarian tissues. Class-prediction analysis identified 40 differentially expressed genes for further investigation as potential markers of ovarian cancer, including 20 novel candidates. Our findings provide a glimpse into the potential of population isolate genomics in oncological research.

Key words: Gene expression analysis; Microarray; Ovarian cancer; Molecular marker; Population isolate.

Introduction

Ovarian carcinoma is the most lethal gynaecological malignancy in North American and Western European women. Contributing to the poor prognosis are the preponderance of late-stage disease at diagnosis, the frequent development of drug resistance, and the lack of reliable markers for diagnosis, prognosis, and therapy [1, 2]. Ovarian carcinoma is not a single disease, but a group of heterogeneous neoplasms which derive mainly from the ovarian surface epithelium. The most common histological subtypes are: serous, mucinous, endometrioid, clear cell, transitional cell, and undifferentiated [3]. Previous studies have identified genes related to ovarian carcinoma, including p53, c-myc, c-erb-B2, and K-ras, but none of them can reliably be employed as diagnostic or prognostic markers [4]. The only validated molecular marker in ovarian cancer is CA125, a large glycoprotein of unknown function, which is expressed in over 80% of ovarian cancers [5]. Even though changes in CA125 levels correlate to the clinical course of the disease and can be used to predict tumor progression and response to therapy, it has serious limitations as a diagnostic or prognostic tool [6]. The identification of potential tumor markers is urgently needed for a better understanding of the underlying biochemical mechanisms and regulatory pathways involved in ovarian tumorigenesis. The key technology for the study of the vast amount of genetic data is the DNA microarray, which has established itself as an indispensable research tool for biological and medical research. The main benefit of DNA microarrays is that they allow the investigation of differential gene expression of several thousands of genes within two independent samples in a comprehensive manner. The comparison of the expression and mutation profiles obtained from tumor cells and healthy cells enables us to gain new insights into the complexities of cancer without detailed previous knowledge [7, 8].

In the present investigation we have compared the transcriptomes of ovarian epithelial tumors and adjacent healthy tissue from South Tyrolean cancer patients. The province of South Tyrol in Northern Italy is a cultural-linguistic island with a genetically relatively homogeneous population and a highly developed health system [9]. Owing to reduction in genetic heterogeneity, isolated population groups are considered highly valuable for studying disease genes and mutations, and their interaction with environmental and clinical factors [10]. Previous studies have shown that novel cancer-related genes can be successfully identified through the analysis of isolated populations [10, 11]. Here, we report the identification of 860 genes that are differentially expressed in ovarian tumor samples compared to normal ovarian tissue using the Affymetrix GeneChip® microarray technology. Our specific focus has been on 40 genes which we iden-
tified as classifiers of tumorous versus normal tissue through class prediction and hierarchical clustering. This study suggests that the analysis of differential gene expression of ovarian cancer can be successfully applied in a population isolate such as South Tyrol to expand our knowledge of the underlying biology of cancer.

Materials and Methods

Sample collection

For this study, we established a competence network across four regional hospitals of South Tyrol (Bolzano, Merano, Brunico, and Bressanone) and developed a full study protocol which was then approved by the local ethics committee of the Autonomous Province of Bolzano. Each study participant signed an informed consent. For each case in this study, an epithelial serous ovarian cancer sample and adjacent normal tissue from the same subject were collected. The sample type, the tumor histology, the percentage of tumorous cells and the percentage of necrotic cells in the tumor sample are indicated in Table 1. Normal ovarian tissue samples 6_N, 7_N and 8_N that have been included in this study as controls were obtained from a different source through an EC-approved collaboration with a clinical department.

Gene expression profiling

Total RNA was extracted using TRIzol® reagent (InVitrogen) and purified using the RNeasy® Mini Kit (QIAGEN). RNA integrity was assessed using the Agilent® 2100 bioanalyzer and the RNA Nano LabChip® Kit (Agilent Technologies). For cRNA probe preparation, 8 μg of total RNA was linearly amplified using the One-Cycle Target Labeling Assay according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA); 15 μg of fragmented cRNA were hybridised on GeneChip® Human Genome U133A array (Affymetrix®) after quality checking on GeneChip Test3 array (Affymetrix). Standard Affymetrix procedures were applied for quality assessment.

Microarray data analysis

Data handling was mainly done using the Bioconductor Affy package [12]. Probe set intensities were computed using the GCRMA method and loess normalization. Probe sets that did not show broad interquantile intensity ranges within the experimental samples were filtered out by applying the Interquantile (IQR) filtering procedure (IQR ≤ 0.5). The filtered data led to 8404 grade A probesets. Genes differentially expressed in tumor versus normal samples were identified using Significance Analysis of Microarray (SAM 2.1) software [13] (minimal fold change = 2; false discovery rate < 1). Multiclass classification was performed by Prediction Analysis of Microarrays (PAM) [14] on the 4055 probe sets that passed the IQR filter and which were expressed in all samples (intensity > 100 in non-log scale). To perform hierarchical clustering of the selected probe lists, Euclidean distance and linkage methods were respectively used as distance and linkage methods within Spotfire 8.1 software.

Gene functional annotations

The differentially expressed genes were annotated using the on-line tool “Database for Annotation, Visualization and Integrated Discovery” (DAVID). DAVID functional annotations are derived primarily from Entrez Gene at the National Centre for Biotechnology Information (NCBI).

Results

Within the competence network established across four South Tyrolean regional hospitals we developed an efficient workflow system for sample collection, tissue transport in RNAlater solution, and pathological analysis of the samples (Figure 1).

Differential expression analysis using a two-class unpaired data test (SAM) was performed on the 8404 probe sets that passed the IQR filter, resulting in a small number (149) of probe sets that were down-regulated in the tumor samples compared to normal ovarian tissue. To investigate if these results were due to non homogeneity among the samples, hierarchical clustering was performed on the selected probe sets list. Three of the normal ovarian samples (1_N, 2_N and 4_N) clustered with the tumor samples instead of the other normal ovarian tissues, probably due to the presence of tumor
### Table 2. — Forty genes identified as potential class predictors. References to studies which have previously identified the gene as being differentially regulated in ovarian cancer are indicated in the last column.

<table>
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<tr>
<th>PAM rank</th>
<th>Gene</th>
<th>Gene title</th>
<th>SAM fold change</th>
<th>SAM Ontology</th>
<th>OMIN</th>
<th>Ref</th>
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<td>1</td>
<td>KLF4</td>
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<td></td>
</tr>
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<td>6.5696</td>
<td>down transcription factor activity</td>
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</tr>
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<tr>
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<tr>
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<td>Tenascin XB</td>
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<tr>
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<td>3.43818</td>
<td>down chromosome organization and biogenesis</td>
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<td>47</td>
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</table>

Discussion

Over the last six years a number of studies on expression profiling of ovarian cancers have been published, but so far no reliable prognostic marker has been found and the molecular pathways involved in the initiation and progression of ovarian cancer are still poorly understood [15]. Cancer is a highly polymorphic disease resulting from the accumulation of genetic and epigenetic aberrations in interaction with environmental and clinical factors [16]. The importance of isolated populations for revealing the genetic etiology of common diseases, including cancer, has been highlighted in recent years [17, 18]. The advantages of studies on isolated populations are the more uniform genetic and environmental
backgrounds which allow a more efficient approach to the research of cancer. South Tyrol, the northern-most province of Italy, represents a very good location for the study of several diseases, in many fields of genetic medicine [19, 20].

In the present study we have analyzed five epithelial serous ovarian cancer tissues and the corresponding normal ovarian tissue from well characterized South Tyrolean cancer patients by microarray analysis. It has been shown that the genetic background of unrelated individuals causes variance in tissue gene expression levels [21]. Gene-expression profiling in individuals within a genetic isolate such as South Tyrol can somewhat reduce such inter-sample variations, thus allowing the detection of significant pathology-related changes in gene expression with fewer samples. In our sample-set we identified 225 genes that are down-regulated and 635 that are up-regulated in tumors compared to normal ovarian tissue. Through class prediction and hierarchical clustering we identified 40 genes which may be related to molecular events involved in the genesis and development of ovarian cancer. These genes included common oncogenes and tumor suppressor genes with known roles in carcinogenesis, as well as genes with no known role in cancer. At least 20 genes have already been reported in previous studies of ovarian cancerogenesis, including CD24 (small cell lung carcinoma cluster 4 antigen) [22-25], ITM2A (integral membrane protein 2A) [24, 26, 27], keratin 18 [4, 5, 24], TCEAL4 [28], NR2F2 [29], decorin [27, 30], and KLF2 [31]. CD24 is known to be upregulated in hematological malignancies and different types of solid tumors, including ovarian cancer [5, 24]. Recent
Differential gene expression analysis of ovarian cancer in a population isolate

Altogether, 50% of the 40 genes identified in our analysis have been described previously in studies of ovarian cancer, providing some validity to our study. To our knowledge, altered expression in ovarian cancer of the remaining 20 genes is first reported here, although some, such as KLF4 [32], AKAP12 [38], NDRG2 [39, 40] and ADAMTS1 [41], have been linked to other types of cancer such as KLF4 [32], AKAP12 [38], NDRG2 [39, 40] and ADAMTS1 [41]. These findings make decorin an interesting candidate for cancer therapy.

Figure 4. — Gene expression levels of five genes involved in the genesis and development of solid tumors in paired (tumor vs normal) ovarian tissues. Values are reported as mRNA expression levels, i.e. normalized fluorescent intensity. Each bar represents the average of single sample intensity values per population. Lines indicate standard deviations while * indicates whether the difference is significant between the two groups at p < 0.05 (Student's t-test).

In conclusion, our statistical analysis highlights 40 genes with differential expression in ovarian serous papilloma cancer when compared to normal tissue. We have discussed the roles in the development of cancer of several genes which might provide further insights into the etiology of ovarian cancer and aid its clinical management.
Acknowledgments
The authors are grateful to the study participants and the hospi-
tals of Bolzano, Merano, Brunico, and Bressanone (Depart-
ment of Gynecology) for their participation and collaboration
in this research project. We thank Prof. C. Wiedermann for
helpful comments on the manuscript. The study was supported
by the Ministry of Health of the Autonomous Province of
Bolzano and the South Tyrolean Sparkasse Foundation.

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cDNA array for the study and diagnosis of epithelial ovarian
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microisolates in South Tyrol (MICROS): study design and epi-
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Study of p53 codon 72 polymorphism in patients with breast cancer

Gynecology Department, Federal University of São Paulo, SP (Brazil)

Summary
Breast cancer is a common disease in Western societies, with an incidence of 46.31/100,000 women/year in Brazil. The tumor suppressor gene TP53 is one of the most studied genes regarding the presence of mutations. Indeed, 50% of all tumors are known to exhibit changes in the TP53 nucleotide sequence due to carcinogenic processes. As to the presence of polymorphism, the TP53 gene is polymorphic at the nucleotide residue 347 (codon 72).

In the current study, we examine if this polymorphism is associated with the clinicopathological parameters of breast cancer patients in a Brazilian population. One hundred and thirteen patients with breast cancer were included. The polymorphic region of the TP53 gene was PCR-amplified from genomic DNA obtained from buccal cells. Specific primers for the Pro and Arg allele were used.

Correlations of polymorphism with age, staging, nuclear grade, lymph node status, estrogen receptor status and lymphatic and/or blood vessel invasion were evaluated. Statistical analysis was performed using the Fisher’s exact test. The frequency of p53 Arg/Arg was 37% and of the heterozygous allele Arg/Pro it was 39%.

There was no correlation between polymorphism and clinicopathological parameters. According to our results, the TP53 polymorphism, at the 347 residue, is not associated with any clinicopathological findings of patients with breast cancer.

Key words: p53 polymorphism; Breast cancer; Prognosis.

Introduction
Breast cancer is a highly prevalent disease in Western populations, with higher incidence in North America and Western Europe [1]. In Brazil, it is estimated there were approximately 49 new cases per 100,000 women in 2005 [2]. Since this is such a prevalent and severe disease among women, advances in studies that make an attempt to detect new genes related to breast cancer, or even its interaction with environmental factors involved in mammary carcinogenesis, could result in improvement in prevention and treatment of this neoplasm.

Genetic factors are responsible for only 5% of cases, 85% of cases being sporadic and approximately 10% familial [1]. Studies on inherited susceptibility to breast cancer suggested that the predominant susceptibility genes are transmitted in an autosomal dominant mode [1]. Polymorphisms are among other genetic factors of lower penetrance that are responsible for familial cases.

This genetic variation is responsible for the remaining familial risks not attributed to high penetrance genes. Polymorphisms in genes that codify enzymes, receptors or other proteins which act in metabolic pathways and are potentially relevant in breast cancer, could influence the function of these proteins and create differences among individuals in their metabolic activity. It is not known if polymorphism per se produces a modest risk or if interaction with other carcinogenic factors is needed.

The TP53 gene is associated with breast cancer when mutated. This is a tumor suppressor gene located in the short arm of chromosome 17 and composed of only 11 exons. It codifies a 393-amino acid nuclear phosphoprotein called p53, with 53 kDa [3, 4]. It acts as the cell molecular guardian, monitoring the genome integrity through the control of the cell cycle, repair of DNA and apoptosis [5, 6]. The TP53 mutations are considered the most common genetic alterations in human cancer and are present in approximately 50% of all cancers [7-9]. In breast cancer, TP53 is mutated in 20-30% of tumors [10].

The prevalence of TP53 mutations in the germ lineage of women with breast cancer and aged under 40 years has been estimated in approximately 1% [1].

Polymorphism is defined as a variation sequence in a gene that occurs in more than 1% of alleles [1]. Polymorphism in TP53 is considered a risk factor for malignant diseases including breast cancer [7, 11], due to the crucial relation of this gene in genome maintenance.

Only codon 72 polymorphism seems to be related to breast cancer [12]. The polymorphic TP53 in codon 72 of the protein it codifies means it has variants in the same p53 protein, which do not characterize a mutation. In the amino acid structure, guanine or cytosine in residual nucleotide 347 results in an arginine (CGC) or proline (CCC) codon [11] for the amino acid.

The p53 Pro 72 is different from p53 Arg72 and this is reflected by different electrophoretic mobility. The Arg72 migrates faster in agarose gel [11, 13]. Tumors of patients with Pro 72 are smaller and grow less [11, 13]. The p53 Pro presents higher transcriptional activity than p53 Arg, which could be related to stronger affinity for transcript-
tional factors TAFII32 and TAFII70 [5]. On the other hand, p53 Arg seems to induce apoptosis faster than p53 Pro in vitro [5, 14].

There are studies in the literature that consider the Arg/Arg genotype in codon 72 as a risk factor for breast cancer when compared to a control population. This is related to the fact that the prevalence of Arg/Arg 72 is 20% in the general population and 62% in breast cancer patients [11].

Several authors studied this association, but the results are still inconclusive [11, 15]. Polymorphism in p53 codon 72 varies according to the ethnic group and to geographic latitude [5, 11, 16]. The frequency of Arg 72 genotype increases with latitude, whereas that of Pro 72 decreases with latitude. There is also an association of this polymorphism with larynx, colorectal, cervical, vesical and lung cancers.

In breast cancer patients a higher number of mutations were found in homozygous individuals for Arg; and even in those heterozygous - Arg/Pro - the frequency of mutations located in the arginine allele is higher. Therefore, the p53 Arg 72 polymorphism is already considered a risk factor in certain populations and could also be a prognostic factor. Its presence could be related to a higher number of lymph nodes affected and higher histological grade, among other factors.

It would be relevant to conduct this investigation in the Brazilian population. The objective of this study was to verify the association between the TP53 codon 72 polymorphism and clinical-pathological parameters of breast cancer patients, in order to verify the eventual prognostic value of this polymorphism.

Materials and Methods

This study was conducted in the Discipline of Mastology, Department of Gynecology at the Universidade Federal de Sao Paulo (UNIFESP), and patients who underwent surgery for breast cancer in the period from 1999 to 2004 were enrolled. The patients were chosen when they were admitted for postoperative follow-up visits at outpatient clinics.

The research project was approved by the Institution Research Ethics Committee. After signing an informed consent, all patients were submitted to buccal cell collection using a cytobrush. According to the protocol, data were gathered from patients’ medical charts, such as age at onset of disease, past history, staging, recurrences and metastases. Further, data on tumor immunochemistry and histopathological sections were obtained from the postoperative pathological record.

DNA extraction and genotyping: Cytological samples obtained were preserved at -80°C until further genomic DNA extraction. DNA was extracted according to the Kit GFX® protocol (Amersham-Pharmacia) for buccal cells. Analysis of the amount of DNA obtained in these extractions was made by spectrophotometry with 260 nm wave length (Spectronic model Genesys 5). The p53 genotyping was performed according to Brenna et al. Briefly, for the arginine allele reactions, 200 ng of the genomic DNA were used in a final reaction volume of 25 μl containing: 10 pmol/μl of each primer (sense 5’- GCC AGA GGC TGC TCC CCC CC-3’; anti-sense 5’- CGT GCA AGT CAC AGA CTF-3’). Electrophoresis was conducted in a 2% agarose gel containing ethidium bromide staining and the patterns obtained in this reaction have been described elsewhere [17].

Statistical analysis was made by Fisher’s exact test and the differences between allele status and clinicopathological parameters were considered significant when p < 0.05.

Results

One hundred and thirteen patients participated in the study. In the genetic analysis of the TP53 codon 72 polymorphism in this Brazilian population we obtained 61 (61/113) homozygous cases for arginine (Arg/Arg), 48 heterozygous cases (Arg/Pro) and four homozygous cases for proline (Pro/Pro).

The age varied from 25 to 84 years, with a mean of 53.88 years and median of 56 years. Among all patients, 87.6% (99/113) were aged 40 years or older, and they presented a similar distribution in both heterozygous (Arg/Pro) (87.5%) and homozygous Arg/Arg (86.2%), and Pro/Pro (100%) cases. There was a family history of breast cancer in 8.8% of the cases. Among homozygous arg/arg patients, 11.5% presented a positive family history, whereas only 6.2% of the heterozygous cases expressed this variable (Table 1).

The patients were classified as early stage (64.6%) and advanced stage (35.4%) (Table 1). Approximately 69% of the breast cancer patients presented an advanced stage (35.4%) (Table 1). Approximately 69%

![Table 1. — Correlation between P53 codon 72 polymorphism and clinical-pathological parameters.](image-url)
presented positive hormone receptors, and the percentage of negative hormone receptors was higher in homozygous (32.8%) than in heterozygous patients (27.1%). In the analysis of histopathological parameters, there was angiolympathic invasion in 49.2% of homozygous cases compared to only 41.7% of heterozygous cases (p = 0.406). The analysis of nuclear grade showed the cases with low nuclear grades (nuclear grade 1) corresponded to 18.3%, while 81.7% were high nuclear grade (nuclear grade 2 and 3).

In the analysis of polymorphisms there was similarity regarding the presence of high-grade tumors in both homozygous (82.1%) and heterozygous cases (82.2%) (Table 1). Out of 68 patients with nodal involvement, 65.8% presented capsular invasion among the homozygous and 60.7% among the heterozygous cases (Table 1). Only 6.2% of the women had local recurrence during the follow-up which varied from six months to five years.

The development of metastasis during follow-up occurred in 14.2% of patients, with similar percentages in both homozygous and heterozygous cases (Table 1). Among six cases of deaths, five were homozygous for arginine.

**Discussion**

The TP53 gene is polymorphic at position 72, containing a residue of proline or arginine in this position. Two decades ago it was initially studied as a change in the p53 mobility in SDS-polyacrylamide gel [18].

This alteration in mobility suggested that change in the amino acid could modify the protein structure and function, and produce functionally distinct proteins. This leads to the hypothesis that there could be individual variations in apoptosis induced by p53 due to natural population variations at the codon 72 of the p53 protein and therefore several authors have examined arginine homozygosity as a possible risk factor for skin and HPV-associated cervical tumorigenesis [11, 15, 19]. This occurs because despite Arg 72 pro-apoptotic activity, it seems to be more susceptible to a HPV 18 association and, consequently, to degradation of p53 function.

Papadakis et al. [11] found a higher prevalence (62%) of Arg/Arg genotype among breast cancer patients compared to the control group with no disease (20%). In our population of patients we also observed a higher incidence of Arg/Arg genotype (54%).

In Papadakis et al.’s control group, 67% of cases were heterozygous (Arg/Pro) and only 20% homozygous (Arg/Arg). This shows that even though a case-control study was not conducted, our sample is very similar to the genotypic situation of breast cancer patients in Greece. Only four of our cases were homozygous for proline (Pro/Pro) compared to 21% of Papadakis et al.’s cases, thus corroborating the hypothesis that the frequency of p53 codon-72 genotypes varies according to the ethnic group and to geographic location.

The pro72 allele is selected for individuals who live in high levels of ultra-violet radiation (low latitudes), since this polymorphism characteristically varies according to latitude. The lower the latitude, the more Pro 72 and the higher, the more Arg 72. The latitude of Sao Paulo is 23 degrees, which leads to a higher frequency of Arg and lower frequency of Pro/Pro, as shown in the study. In Bologna, 6.35% of breast cancer were Pro/Pro, close to our incidence (3.5%); 43.3% were heterozygous (Arg/Pro) and 47.8% homozygous for arginine [20].

In our study initial stages were predominant, but there was a higher percentage of homozygous for arginine in advanced stages, similar to the results obtained by Papadakis et al., in which only seven cases were Stage III and six of these were homozygous for Arg/Arg.

As for hormone receptors, 69% of our cases were positive for hormone receptors, like in Papadakis et al.’s study mentioned above [11]. However, our homozygous cases presented a higher number of negative hormone receptors, in disagreement with their analysis in which positive and negative hormone receptors are equally distributed among heterozygous and homozygous cases for arginine. As in the Greek study, our patients also had nuclear grade 2 and 3.

The attempt to find differences between the clinical-pathological characteristics of patients is based on studies reporting that variants in codon 72 polymorphism are not biochemically equivalent; they differ in the capability of linking to the transcriptional machinery and in apoptosis modulating a variety of experimental systems [18, 20]. Therefore, individuals presenting these variants could behave differently during manifestation of the condition, elucidating a variant that would modulate higher susceptibility to aggressive disease.

Bonafé et al. [20] found that the presence of arginine in one of the codon 72 alleles was associated with lower overall survival (OS) and disease-free survival (DFS), regardless of other established prognostic factors such as nodal involvement. This study also demonstrated that patients who maintained arginine in tumor tissue usually had positive estrogen receptors (p = 0.06).

Hence, those authors concluded that the presence of arginine in the polymorphism variant at the moment of diagnosis does not mean a poorer prognosis, but interferes in long-term OS and DFS. Like in our cases, this study did not show statistical significance between the clinical-pathological factors analyzed (nuclear grade, tumor size, nodal involvement, age, hormone receptor status and Ki67) as compared to variants of p53 codon 72. However, it shows similarity in expression of receptors.

In a study conducted on individuals with transitional cell carcinoma in the urinary tract [21], it was observed that patients with mutating tumors containing Arg in tumor tissue had larger lesions. There was no statistically significant correlation between the other parameters, such as age, tumor grade and gender. This makes us consider that the presence of arginine homozygosity, which could be correlated with poorer prognostic factors in breast cancer, would be observed if we had selected only patients presenting a mutant p53; later, a comparative study of prognostic factors and variants will be performed.
Arginine allele preferential retention in neoplastic tissue has been described in many carcinomas, such as head and neck, vulvar, esophageal, urinary and lung. It is speculated that this polymorphism may affect the mutation functions of p53, resulting in growth advantage for tumors in which the mutation resides in the arginine allele. The reason is when the mutation occurs at this site it is capable of inactivating p73. Yamamoto et al. [22] demonstrated p73 apoptotic activity, an increase in mitotic activity and a decrease of apoptosis in patients with blocked p73 activity. In that study, patients with a functional deficit of this protein had higher tumor proliferation and higher number of metastases, suggesting that p73 would be a prognostic factor. Despite all efforts and the increasing number of studies, the biopathological meaning of these variants is not yet clear.

Polymorphism acts as an intragenic modifier of mutant p53 behavior and has an effect in p53 biological activity. Currently, the meaning of p53 codon 72 polymorphism is still obscure in terms of cancer biopathology and epidemiology. Further studies using a spectrum of different carcinoma tissue samples are required to understand the association of polymorphism and human carcinogenesis. In conclusion according to our findings, different alleles of p53 codon 72 polymorphism have not shown any association with breast cancer clinical-pathological factors.

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Paget’s disease of the vulva. A ten-year experience

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Summary

Background: Paget’s disease of the vulva is a rare neoplasmatic lesion which mainly affects postmenopausal women. Method: We conducted a retrospective study during the period January 1996 till December 2005 in which 11 cases of Paget’s disease were detected. The clinical manifestations, management, specific pathological features, treatment and prognosis of each patient are presented. Conclusion: Surgical treatment is the current standard and long-term follow-up is required.

Key words: Paget’s disease of the vulva; Diagnosis; Management; Prognosis.

Introduction

In 1874 Sir James Paget described mammary Paget’s disease. Extrammary Paget’s disease of the vulva was first described by Dubrenlilh in 1901 [1]. In 2007, just over 250 cases of vulvar Paget’s disease were reported in the literature. It is a rare (1-2%) neoplastic vulvar lesion [2-4] usually described as a very slowly developing invasive adenocarcinoma or adenocarcinoma in situ. It usually affects postmenopausal women and appears as a macular, solitary, reddish circular lesion. The clinician should always remember that invasive vulvar adenocarcinoma coexists in 15-25% of the cases. Furthermore, it should be pointed out that it also coexists with other malignancies, especially breast cancer [5-8]. It is usually found in one of the labia but it can also be found in the clitoris, cervix, perineum or anus [9]. The histological characteristics include pathognomic cells of Paget which are large cells with pale clear cytoplasm and large round hyperchromatic nuclei in clusters or solid nests within the epidermis [10] which show specific immunohistochemical characteristics. Four forms of Paget’s disease are described in the literature: i) intraepidermal vulvar Paget’s disease, ii) minimally invasive vulvar Paget’s disease, iii) invasive vulvar Paget’s disease, iv) vulvar Paget’s disease with an underlying apocrine gland carcinoma [4,10]. The treatment of such a lesion varies and could include wide local excision, partial vulvectomy and radical resection for invasive adenocarcinoma ± inquino femoral lymphadenectomy [11-13]. Margin-controlled surgical excision of all the involved epidermis is the most effective treatment [14, 15]. Recurrence of the disease is often seen (12-58%) which emphasizes the need for careful postoperative follow-up [15, 16]. The high recurrence rates after local excision could be explained due to horizontal or vertical migration of the disease within the epidermis [17, 18].

Method

This is a retrospective study including cases of Paget’s disease of the vulva in the period January 1996 up to December 2005. The epidemiology, clinical manifestations, management, and outcome of each case are presented. Although, our hospital is a tertiary center, the rarity of such a disease explains the small number of our cases. All the patients were examined on an external basis having a suspicious lesion in the labia with itching, burning or pain. We searched the hospital data base including patient records, surgery reports and histology specimens in order to note the patient’s age, clinical manifestations, cytology, chosen surgical treatment, recurrences, coexistence or not of vulvar carcinoma or malignancies in other sites such as the breast or urothelium and finally follow-up. Recurrence of the disease was defined as a new lesion in a period > 6 months after the surgery.

Results

The median age of the patients was 64 years ranging from 53 up to 75 years; one, three and seven patients were nulli-, prima-, secundi-gravida, respectively. The most usual clinical symptom was pruritus of the vulva (9/11 patients) followed by pain (8/11), while five patients found the suspicious lesion themselves. Dysuria and vaginal discharge were noted in two and two patients, respectively. No patient received hormone replacement treatment. Three of 11 patients received treatment for diabetes mellitus type 2 and four of 11 thyroid hormone for hypothyroidism. One patient had a previous history of breast cancer operated on three and a half years before. Papanicolaou smears revealed no serious pathology except for two of 11 patients with cervicitis (the same patients who presented with vaginal discharge). The topography of the lesion was four in the right labium, five in the left labium and two were bilateral. In only one patient was there an underlying invasive vulvar carcinoma, but the pruritus was heavier. It should be mentioned that all the patients had delayed diagnoses due to the use of topical steroids. One, seven and three patients underwent Ultracision, simple vulvectomty and radical vulvectomy with inquino femoral lymphadenectomy, respective-
ly. In all the patients a frozen section was obtained which revealed free surgical margins in ten of 11 patients but the final histology showed nine of 11 women had free surgical margins. The two patients without free margins underwent further surgical excision. In all our cases, the typical characteristics of Paget’s disease were evident (Figures 1, 2). Neoplastic cells showed a negative reaction to melanoma and squamous cell markers and were positive for glandular differentiation. In the case of the patient with a history of breast cancer, an additional marker (GCDF-) was performed, specific for breast epithelia. The negative reaction of the vulvar lesion proved that this case was a primary vulvar lesion rather than metastatic spread from the breast. Three of the 11 patients had a recurrence. The first one 14 months after the first operation (Ultracision), the second (with coexistence of vulvar carcinoma) 23 months after radical vulvectomy and the third 36 months after a simple vulvectomy. A correlation with the absence of free surgical margins was found in the two of these three patients. All of them underwent further surgical excision. The follow-up of the patients ranged from 27 up to 64 months and included a visit in our office every three months for two years, and then annually.

Discussion

Paget’s disease is a disease of postmenopausal women [1]. Although, we did find a case of a 35-year-old patient with the lesion during our Pubmed search [19], in our study all the women were postmenopausal with a median age of 64 years and a minimum of 12 years duration of menopause. It should be mentioned that our patients had delayed diagnosis of disease due to the use of topical corticosteroids for the treatment of pruritus as a misdiagnosis of an eczematous lesion. The median diagnosis period was six months. Late diagnosis was also because many patients preferred not to visit the gynecologist due to personal taboos or even due to fear of a malignancy.

When such a lesion is found by a gynecologist the differential diagnosis should include contact dermatitis, fungal infections, lichen sclerosis and VIN [20]. A biopsy of the suspicious lesion could provide the diagnosis but it should always be remembered that an invasive cancer may coexist nearby. One study showed that diagnoses could be made in 15.8% of patients through suspicious cells in the Papanicolaou smears [21], however we did not find such a correlation.

Our diagnoses were based on clinical manifestations (pruritus, burning, pain, lesion) and biopsy of the lesions. The differential pathological diagnosis includes malignant melanoma and early epidermal cancer. Immunohistochemistry investigation of melanoma markers (Melan A, S100) and cytokeratins of high and low molecular weight specific for adeno- and squamous cell carcinoma is valuable in the final diagnosis.

Treatment of the disease is usually surgical. The nearby clitoris might make the operation technically more difficult but the prognosis is the same. Some authors believe that the recurrence rates could range from 0-31% up to 25-75% in patients with free or positive surgical margins [4, 15, 22-24]. None of our patients with free surgical margins had a recurrence whereas two of 11 (18.2%) with positive surgical margins did. It should be mentioned that this was different from the findings of other studies [22, 25].

Vulvar Paget’s disease may coexist with other malignancies such as breast or urothelium cancers [16, 26]. Only one of our patients had a previous history of breast carcinoma. Although, we did not perform breast scanning or cystoscopy, no woman during our follow-up period revealed such a malignancy.

The limitation of our study is the small number of patients due to the rarity of the disease, however we believe that our study represents an example of the clinical manifestation, management, and prognosis of Paget’s disease.
References


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**Does vaginal intraepithelial neoplasia have the same evolution as cervical intraepithelial neoplasia?**


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**Summary**

**Background:** Vaginal intraepithelial neoplasia is a little known disease which could be related to risk factors different from simple HPV infections. **Objective:** To ascertain whether vaginal lesions have a natural history similar to cervical lesions. **Materials & Methods:** A retrospective study to identify patients with vaginal lesions and synchronous cervical lesions through biopsy. The rate of mild cervical lesions (koilocytosis, warts, CIN I with and without koilocytosis) was compared with the rate of severe cervical lesions (CIN II and III, cervical carcinoma) in patients with mild vaginal lesions (warts and koilocytosis, and low-grade VAIN) and in patients with severe vaginal lesions (high-grade VAIN). Using koilocytosis as a marker, the rate of “active” cervical lesions was compared with the rate of “non active” cervical lesions in patients with “active” versus “non active” vaginal lesions. Finally, the rates of mild and severe cervical lesions were compared among each group of VAIN (low-grade, high-grade, with or without koilocytosis). **Results:** In patients with mild cervical lesions, mild cervical lesions were significantly more frequent than severe cervical lesions. In patients with “active” vaginal lesions the rate of “active” cervical lesions was significantly higher than “non active” cervical lesions. The differences in rates of mild cervical lesions and severe cervical lesions among patients with high-grade VAIN and low-grade VAIN (with and without koilocytosis) were not significant. **Conclusion:** These data suggest that CIN and VAIN may have some common features in certain cases, i.e., if an HPV infection is proved.

**Key words:** VAIN; Koilocytosis; CIN.

**Introduction**

Vaginal intraepithelial neoplasia (VAIN) is a rare and little known disease which could be related to risk factors different from simple HPV infections [1-3]. Such behavior may reflect the intrinsic resistance of vaginal epithelium to HPV infection, leading to the rarity of VAIN developing. In two recent studies [4, 5], the presence of HPV DNA has been reported to be integrated in the squamous cells of vaginal and vulvar lesions in some patients who had previously been treated for cervical dysplasia or squamous cervical carcinoma. Such studies suggest that multicentric cancerogenesis in the lower genital tract related to HPV infection may occur in a very similar way. Under this hypothesis, we can research the features of synchronous HPV-related cervical lesions in patients with vaginal lesions to prove that the more severe the lesion in the cervix is, the more severe the lesion in the vagina is.

**Materials and Methods**

A retrospective study was carried out from 1999 to 2004. Out of 2,854 patients who had undergone colposcopy with vaginoscopy as follow-up because of a previous cervical dysplasia or an abnormal pap test, 240 directed vaginal biopsies were performed which turned out to be positive for viral infection with or without dysplasia. Colposcopic exams were performed after treatment for cervical-vaginal infection or estroprogestin treatment for postmenopausal dystrophy when necessary. Vaginal biopsies were performed in abnormal colposcopic areas (i.e., lugol-negative areas as well). At the same time as the vaginal biopsies, cervical biopsies were taken if abnormal areas were seen. The worse colposcopic areas of both vaginal and cervical abnormal patterns were the preferred biopsy sites. In some cases multiple biopsies were taken. Vaginal specimens were distinguished according to the histologic criteria [6]: warts, koilocytosis, low-grade VAIN (VAIN I) with koilocytosis, low-grade VAIN without koilocytosis, high-grade VAIN (VAIN II and III) with koilocytosis, high-grade VAIN without koilocytosis. Cervical specimens were distinguished as cervical koilocytosis, low-grade CIN (CIN I, with and without koilocytosis), high-grade CIN (CIN II and III with and without koilocytosis) and cervical carcinoma. As reported for the cervix [7] koilocytosis was taken as a marker of “active” replication of HPV, and this feature was used to label cervical and vaginal lesions with an “active” production of viral particles.

Among all the vaginal lesions, patients with a previous total hysterectomy (13 cases), in which synchronous cervical lesions could not be assessed, were excluded. The remaining cases were assessed in the following way. First, the rate of mild cervical lesions (koilocytosis, warts, CIN I with and without koilocytosis) was compared with the rate of severe cervical lesions (CIN II and III, cervical carcinoma) in patients with warts and koilocytosis, patients with low-grade VAIN, and patients with high-grade VAIN. Second, the rate of “active” cervical lesions was compared with the rate of “non active” cervical lesions in patients with “active” and “non active” vaginal lesions. Third, the rate of mild cervical lesions and severe cervical lesions was compared in each group of VAIN.
Statistical analysis was performed using the chi-square test and the Fisher’s exact test, when indicated. To check the differences in age among the groups the Tukey-Kramer test was applied. A level of $\alpha \leq 0.05$ was determined as significant.

Results

After exclusion of the above-mentioned 13 cases, the remaining 227 vaginal lesions were divided in the following way: 93 koilocytosis (mean age 36.9, range 19-71), 16 warts (mean age 31.4, range 19-42), 56 low-grade VAIN with koilocytosis (mean age 34.8, range 25-62), 17 low-grade VAIN without koilocytosis (mean age 35.2, range 28-72), 19 high-grade VAIN with koilocytosis (mean age 32.6, range 21-46), 26 high-grade VAIN without koilocytosis (mean age 39.2 range 19-77). The mean ages of the subgroups of patients were not significantly different.

In patients with vaginal koilocytosis and warts, the rate of mild cervical lesions was 53.2% (58/109) and the rate of severe cervical lesions was 21.1% (23/109). This difference was significant ($p = 0.001$). In patients with low-grade VAIN (with and without koilocytosis), the rate of mild cervical lesions was 61.1% (44/73) and the rate of severe cervical lesions was 33.3% (14/73). This difference was also significant ($p = 0.001$). In patients with high-grade VAIN (with and without koilocytosis), the rate of mild cervical lesions was 42.2% (19/45) and the rate of severe cervical lesions was 44.4% (20/45), with no significant difference. Overall, an odds ratio (OR) of 2.592 (95% C.I. 1.29-5.22, $p = 0.011$) may be calculated for a severe cervical lesion in patients with vaginal dysplasia without koilocytosis.

In patients with “active” vaginal lesions (koilocytosis, warts, low-grade and high-grade VAIN with koilocytosis) the rate of “active” cervical lesions (warts, koilocytosis, low-grade and high-grade CIN with koilocytosis) was significantly higher than “non active” cervical lesions (107/184 vs 35/184, $p < 0.001$). This significance was not reached in patients without signs of “active” vaginal lesions (“active” cervical lesions: 14/43; “non active” cervical lesions 23/43; $p = 0.297$). Overall, an OR of 4.896 (95% C.I. 2.42-9.89; $p < 0.001$) may be calculated for a “non active” cervical lesion in a patient with a “non active” vaginal lesion and an OR of 2.88 (95% C.I. 1.43-5.81; $p = 0.004$) for an “active” cervical lesion in a patient with an “active” vaginal lesion. The differences of rates of mild severe cervical lesions among patients with high-grade VAIN and low-grade VAIN (with and without koilocytosis) were not significant. Just the rate of severe cervical lesions was significantly higher in patients with high-grade VAIN without koilocytosis as compared to patients with low-grade VAIN with koilocytosis (57.7% vs 21.4%, $p = 0.047$).

Discussion

The aim of this study was to ascertain if cervical and vaginal lesions which are HPV-related may have the same features, leading to the conclusion that VAIN may have a similar behavior as CIN. So far, a study on the natural history of VAIN (without any treatment) has been reported only by Aho et al. [8]. Their study on only a few cases reported a 9% invasion in six to nine years without a clear relationship with the grade of VAIN. Our results seem to suggest some common features of vaginal and cervical lesions in relation to the presence of koilocytosis and the severity of the dysplasia. However, the grade of VAIN may not be strongly linked with the severity of the cervical lesions, suggesting a different evolution in some cases. Such results are similar to those reported by Aho et al. [8], and may perhaps be related to the fact that VAIN is not a homogeneous entity, due to HPV infection, as is CIN. Therefore, the absence of koilocytosis may be a marker of worse prognosis. A diagnosis of koilocytosis is hardly reproducible [9], so that this method may be questionable as a reliable marker of “active” HPV infection. However, the presence of koilocytosis is an indication of infection with the production of a high number of viral copies [10], typically “active” infections, and it marks cervical lesions with a good prognosis [9]. This could explain why koilocytosis seems to be an unusual indication for both high-grade CIN and VAIN, where the viral DNA could be integrated and the infection could be “non active” (or “latent”) [4-5, 11-13]. As shown in the cervix [11, 12], a latent infection is more dangerous than an active one and might lead to a worse prognosis of VAIN. Moreover, as the natural history of VAIN is not well defined we are not able to give any prognostic value to each vaginal dysplasia, especially where it is not clearly related to an HPV infection, which women can recover from. Thus the presence of koilocytosis could be encouraging even from this point of view.

An aspect of this work, which could be criticized, is that the sample size we have examined does not allow a strong statistical power. Consequently, the reported data are interesting if they are considered together with those by Vinokurova et al. [4] and by Hampl et al. [5], who provide a cytogenetic model of carcinogenesis HPV related to the female genital tract, but are inconclusive in weighing each risk factor for VAIN.

Conclusion

It seems that synchronous vaginal and cervical lesions have some common features that let us consider a common natural history from a synchronous HPV infection in some cases. Additionally, patients with VAIN and without koilocytosis have an OR of 2.6 to be affected by a severe cervical lesion (including a cervical cancer) at the same time without a relationship with the grade of VAIN. These data confirm those reported by Hampl et al. [5] and let us consider koilocytosis associated with vaginal dysplasia as a favorable prognostic factor as it is for CIN.
Does vaginal intraepithelial neoplasia have the same evolution as cervical intraepithelial neoplasia?

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Past obstetric history and risk of malignant breast neoplasms

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Summary

Many studies indicate hormonal disorders as a crucial reason for breast pathology. They are also probably responsible for the development of benign breast neoplasms and play a role in the origin and development of breast carcinoma. Although the mammary gland is under the influence of many steroid and peptide hormones such as thyroid hormones, prolactin, growth hormone, glucocorticosteroids, it is estrogen that plays an important role in the development of breast cancer. The purpose of the study was to analyze the obstetrical past of patients and the potential influence on the risk of developing malignant breast neoplasms. The participants in the study were healthy women with no changes in mammary glands (control group) and women with diagnosed malignant or benign breast neoplasms (study group). The total number of participants was 555 females aged 35-70 years. The study was carried out in the Greater Poland and Lubuskie province between 2005 and 2006. Hormonal disorders in childhood and puberty symptoms of early menarche play a crucial role in increasing the risk of malignant breast neoplasms. In women who experienced one or more miscarriages the risk of malignant breast neoplasms is significantly increased. On the basis of our study we calculated the odds ratio (OR) of malignant breast neoplasms among women who during lactation experienced problems needing medical intervention (OR = 2.25; 95% CI, 1.20-4.19) in comparison to women who experienced no problems).

Key words: Breast cancer; Risk factors.

Introduction

Malignant breast neoplasms are the most common neoplasms in women in the majority of developed countries [1, 2].

There is much evidence showing that breast cancer has been concomitant with humans for a long time. It was first mentioned in Egyptian papyrus 5000 years ago [3].

Many studies indicate hormonal disorders as a crucial reason for breast pathology. They are also probably responsible for the development of benign neoplasms and play a role in the origin and development of breast carcinoma [4-7].

Although the mammary gland is under the influence of many steroid and peptide hormones such as thyroid hormones, prolactin, growth hormone, glucocorticosteroids, it is estrogen that plays an important role in the development of breast cancer [6, 7].

The influence of ovarian hormonal effects on the origin of breast cancer is proven by the fact that it occurs 100 times more often in women than in men and also that it appears after adolescence [7].

The purpose of this study was to analyze the obstetrical past of patients and the potential influence on the risk of developing malignant breast tumor.

Material and Methods

The participants of the study were healthy women with no changes in mammary glands and women with diagnosed malignant or benign breast neoplasms. The total number of participants was 555 females aged 35-70 years. The study was carried out in the Greater Poland and Lubuskie province between 2005 and 2006.

The inclusion criteria for the first group (control) (n = 292) was an examination performed by a specialist that revealed no pathological changes, and correct mammography and/or ultrasonic examination.

The second and third groups consisted of patients divided according to pathological examination of material gained by breast biopsy or operation: benign changes (n = 184) and malignant lesions (n = 79), respectively.

Every patient voluntarily filled out an anonymous questionnaire consisting of questions about menstrual and obstetric history, breastfeeding, and puerperium.

In the analyzed groups parameters like age, age of menarche, and age at first pregnancy were characterized by arithmetic means, standard deviation (SD) and minimal and maximal values. Agreement with the normal distribution was checked by the Shapiro-Wilk test.

Parameters presented in a nominal scale (miscarriages, medical treatments in puerperium) were described by numbers and percentage. To check relations between those parameters and inclusion in a group, the chi-square test, Fisher’s exact test or the Fisher-Freeman-Halton test were used.

For the chosen parameters we calculated odds ratio (OR) of malignant breast neoplasms, with 95% confidence interval (CI):

\[
\text{OR} = \frac{a \times d}{c \times b}
\]
Statistical analyses were verified on the significance level of \( p \leq 0.05 \).

Statistical analysis was performed using StatSoft, Inc. (2005), STATISTICA (data analysis software system), version 7.1 and Cytel Studio version 7.0.0 (2005).

Permission for the study was obtained from the Bioethical Commission of K. Marcinkowski University in Poznan.

**Results**

Patient ages in the studied groups are shown in Table 1. Mean age differed only between patients suffering from malignant neoplasms (group CA: 53.4 years) and benign breast tumors (group BZ: 52.4 years) compared with group D (control), where it was the lowest – 47.5 years. Other differences were not statistically significant.

Earliest menarche occurred in the group of women with malignant breast neoplasms; the mean age of menarche was 13.1 years. The latest menarche occurred in group D consisting of women with no changes in breasts (control) – mean age 13.5 years. Statistically significant differences were noted only between groups CA and BZ, \( p = 0.02 \) (Table 2).

Patients experiencing menarche after 14 years of age had an OR = 0.12; 95% CI, 0.02-0.49 in relation to women whose menarche occurred before 11 years of age.

Other analyzed parameters included number of pregnancies and deliveries and the age at first conception. In the CA group the mean number of pregnancies was 2.3, mean number of deliveries 2.0 and age at the first conception 24 years. In the BZ group those numbers were 1.9, 1.8 and 23 years, respectively. Results in group D were different: the mean number of pregnancies was lower compared to the previous groups – 1.7 and the mean number of deliveries was 1.6; mean age at conception was the same as in the BZ group (Tables 3, 4, 5).

Women who delivered more than five children had an OR of 1.64; 95% CI, 0.61-4.1 for malignant breast neoplasms in relation to women who delivered one or two children.

Among women who had experienced pregnancy the number of miscarriages were analyzed. In the CA group miscarriages occurred in 21.52%, in group D 17.93% and in group BZ 10.9% (Figure 1). Statistically significant differences were observed between groups CA and BZ (\( p = 0.02 \)) and between groups D and BZ (\( p = 0.04 \)).

Patients who experienced at least one miscarriage had an OR of 2.22; 95% CI, 1.16-4.26 in comparison to women who had no history of miscarriages.

In women who delivered and breastfed the total duration of lactation was analyzed. Mean duration of breastfeeding in group CA was 8.3 months, group D 6.3 months and in group BZ 6.8 months. Differences among groups were not statistically significant.

Women who breastfed more than six months had an OR of 1.65; 95% CI, 0.78-3.48 of malignant breast neoplasms in comparison to women who had not breastfed.

Participants who breastfed were also asked about problems with their breasts during puerperium. In the CA group 24.32% of women needed medical treatment; in group BZ 10.9% (Tables 3, 4, 5). Differences between the CA and BZ groups were statistically significant (\( p = 0.03 \)).

If breastfeeding mothers experienced breast problems needing medical intervention in puerperium the OR was 2.25; 95% CI, 1.20-4.19 for malignant breast neoplasms in comparison to women who did not have any problems during breastfeeding.

### Table 1. Mean age of women in the studied groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
<th>Range (min-max)</th>
<th>ANOVA p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA - group with malignant neoplasms</td>
<td>53.4 ± 9.0</td>
<td>32-73</td>
<td>CA vs BZ</td>
</tr>
<tr>
<td>D - group with benign neoplasms</td>
<td>52.4 ± 8.06</td>
<td>5-73</td>
<td>D vs BZ</td>
</tr>
<tr>
<td>BZ - group with no changes</td>
<td>47.5 ± 7.96</td>
<td>35-71</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Mean age of menarche in the groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
<th>Range (min-max)</th>
<th>ANOVA p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA - group with malignant neoplasms</td>
<td>13.1 ± 1.5</td>
<td>10-16</td>
<td>CA vs BZ</td>
</tr>
<tr>
<td>D - group with benign neoplasms</td>
<td>13.3 ± 1.4</td>
<td>9-18</td>
<td>D vs BZ</td>
</tr>
<tr>
<td>BZ - group with no changes</td>
<td>13.5 ± 1.2</td>
<td>10-18</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Mean number of pregnancies in the groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD</th>
<th>Range (min-max)</th>
<th>ANOVA p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA - group with malignant neoplasms</td>
<td>2.3 ± 1.4</td>
<td>0-1</td>
<td>Not significant</td>
</tr>
<tr>
<td>D - group with benign neoplasms</td>
<td>1.7 ± 1.1</td>
<td>0-6</td>
<td>Not significant</td>
</tr>
<tr>
<td>BZ - group with no changes</td>
<td>1.9 ± 1.1</td>
<td>0-7</td>
<td>Not significant</td>
</tr>
</tbody>
</table>

### Table 4. Mean number of deliveries in the groups.

<table>
<thead>
<tr>
<th>Number of deliveries</th>
<th>Mean ± SD</th>
<th>Range (min-max)</th>
<th>ANOVA p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA - group with malignant neoplasms</td>
<td>2.0 ± 1.2</td>
<td>0-6</td>
<td>CA vs BZ</td>
</tr>
<tr>
<td>D - group with benign neoplasms</td>
<td>1.6 ± 0.9</td>
<td>0-4</td>
<td>D vs BZ</td>
</tr>
<tr>
<td>BZ - group with no changes</td>
<td>1.8 ± 1.1</td>
<td>0-7</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5. Mean age of first contraception in the groups.

<table>
<thead>
<tr>
<th>Mean age of the first contraception (years)</th>
<th>Mean ± SD</th>
<th>Range (min-max)</th>
<th>ANOVA p &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA - group with malignant neoplasms</td>
<td>24.1 ± 4</td>
<td>18-39</td>
<td>CA vs BZ</td>
</tr>
<tr>
<td>D - group with benign neoplasms</td>
<td>23.2 ± 1.8</td>
<td>18-38</td>
<td>D vs BZ</td>
</tr>
<tr>
<td>BZ - group with no changes</td>
<td>23 ± 3.5</td>
<td>17-38</td>
<td></td>
</tr>
</tbody>
</table>

Only differences between groups CA and BZ are statistically significant, \( p = 0.02 \).
breast neoplasms. The risk is two times higher in comparison to women who delivered before 20 years of age [7, 9, 13-17]. The youngest women who delivered in group BZ was 17 years old. Budner and Przybylski [9] consider that the risk for women delivering after they are 30 years old is 1.3-2.2 in relation to those delivering before they are 20.

Some authors are of the opinion that apart from the age at delivery it is also the number of deliveries that matters [2, 3, 7, 10, 12, 18, 19]. The risk for multiparous females and women who delivered after 35 years of age is at the same level as for nulliparous females [3, 7, 19]. According to the study, OR for women who delivered five or more children was increased (OR = 1.64) in comparison to those who delivered only once or twice. However in a study conducted by Ostrowska et al. [20] the number of deliveries was not statistically significant.

According to Godlewski [11], Becher et al. [21], Tavani et al. [12] the first delivery at an early age and higher number of deliveries are protective factors against breast neoplasms. They also consider long breastfeeding to be protective.

Kamarudin et al. [22] revealed that the risk of morbidity was decreased by 61% for women who breastfed at least 13 months in comparison to those who did not experience breastfeeding, OR = 0.39; 95% CI, 0.17-0.87. They also observed that the OR of breastfeeding women who did not use oral contraceptives was decreased by 56% (OR = 0.44; 95% CI, 0.44-0.87) in comparison to breastfeeding women using oral contraception.

On the other hand according to Lee et al. [23], who studied a group of Korean women, the decreased risk occurs in non breastfeeding women (OR = 0.7; 95% CI, 0.5-1.1) in comparison to women who breastfed 14-24 months. What they also observed was that the risk was decreased (OR = 0.6; 95% CI, 0.3-1.0) for women who breastfed more than 24 months in comparison to those who breastfed for a shorter period.

Tessaro et al. [24] did not observe any protective effect for breastfeeding women (OR = 0.9; 95% CI, 0.8-1.2) in comparison to non breastfeeding women.

In our study we did not observe any protective influence of the length of lactation on the risk of morbidity. The mean length of breastfeeding in the CA group was the longest and lasted 8.3 months and in the D and BZ groups – 6.3 and 6.8, respectively. The OR was significantly increased for women who breastfed longer then six months in relation to non breastfeeding women (OR = 1.65; 95% CI, 0.78-3.48).

Jernstromi et al. [25] revealed that the length of breastfeeding was connected with risk reduction and that for each month OR was 0.98; 95% CI, 0.97-0.99. They also found that breastfeeding is protective and decreases risk among patients – carriers of mutated gene BRCA1.

On the basis of our study we calculated the odds ratio of malignant breast neoplasms among women who during lactation experienced problems needing medical intervention (OR = 2.25; 95% CI, 1.20-4.19) in comparison to women who had no problems.)
Conclusions

1. Hormonal disorders in childhood and puberty with early menarche play a crucial role in increasing the risk of malignant breast neoplasms.

2. In women who experienced one or more miscarriages the risk of malignant breast neoplasms is significantly increased.

3. In multiparous females who delivered more than five children and additionally experienced problems with breastfeeding the risk of malignant breast neoplasms is increased.

4. Factors like number of miscarriages and deliveries, length of breastfeeding and medical interventions in puerperium should be taken into account when qualifying women to a group of increased risk of malignant breast neoplasms.

References


Prognostic significance of high-risk HPV persistence after laser CO₂ conization for high-grade CIN: a prospective clinical study

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Department of Gynecology, Perinatology and Human Reproduction, University of Florence, Florence (Italy)

Summary

Purpose of investigation: To estimate the persistence rate of high-risk HPV DNA (HR-HPV DNA) in a population treated totally by laser CO₂ conization for high-grade cervical intraepithelial neoplasia (HG-CIN), and to examine if this persistence might be considered an independent risk factor for relapsing disease. Methods. All women with a histological diagnosis of HG-CIN and planned for laser CO₂ conization from January 2003 to December 2004 were prospectively submitted to a HR-HPV test prior to surgery and at three and six months of follow-up. Women providing written informed consent with 24 months of follow-up were enrolled in the study group. A positive HPV test, involvement of resection margins, age at first intercourse, smoking habits, parity and age at conization > 50 years old were considered as risk factors for relapsing HG-CIN during follow-up, and were univariately and multivariately analyzed to discover any independent influencing factors. Results: Of HG-CIN 15.4% resulted not to be HPV related nor relapsing. The HPV clearance rate after treatment was 78.8%. Involvement of resection margins and HR-HPV DNA persistence post-treatment resulted as the only two statistically significant risk factors for HG-CIN recurrence (rate 3.8%). HR-HPV DNA persistence in follow-up resulted to be independent from other risk factors at multivariate analysis. Conclusions: Although able to reach a low recurrence rate of HG-CIN, laser CO₂ conization does not remove HPV infection completely from the cervix with a case of persistence in every five treated patients. In our experience this persistence in itself represents an independent risk factor for developing relapsing disease and constitutes the basis to introduce HPV testing even in the follow-up of patients treated for HG-CIN by laser CO₂ conization.

Key words: Laser CO₂ conization; HPV persistence; Recurrent CIN.

Introduction

There is general agreement in treating high-grade cervical intraepithelial neoplasia (HG-CIN) by conservative outpatient ablative or excisional procedures.

The main purpose of conservative treatment of CIN is the prevention of invasive cervical cancer along with preservation of the integrity of the genital system among a population of women who have a strong desire for subsequent pregnancies [1].

The use of conservative treatments in women with a diagnosis of CIN produces a reduction in the risk of invasive cancer by 95% during the first eight years after the procedure [2].

However women conservatively treated for HG-CIN represent a subgroup of the population in which the incidence of invasive cervical cancer still remains about five times greater than in the general population requiring a careful, long-term, post-treatment follow-up.

Due to this incremented risk compared with the screened population, the main intention of post-treatment follow-up protocols is maximizing test sensitivity in the early detection of persistent or recurrent diseases, suggesting the introduction of high-risk human papilloma virus (HR-HPV) DNA detection by Hybrid Capture II (HCII) or polymerase chain reaction (PCR) as a matching surveillance tool together with the current recommended cervical cytological checks [3].

The rationale of this association is based on former prospective studies about the natural history of HPV infection conducted following-up untreated lesions that reported a regression rate of 25%, a persistence rate of 61% and a progression rate of 14%, revealing a direct correlation between HPV infection and HPV-related CIN behavior [4].

Clinical trials comparing different conservative treatments have generally failed to show significant differences in outcome [5] and to our knowledge almost all the studies evaluating the risk of post-treatment CIN related to HR-HPV persistence have been conducted on women treated by the loop electrosurgical excision procedure (LEEP) or cold knife conization, with only one published report in which a subgroup of the population was treated by laser CO₂ conization [6-8].

The aim of this prospective cohort study was to evaluate the persistence rate of HR-HPV infection after laser CO₂ conization for HG-CIN and to examine if virus persistence might be considered an independent risk factor for residual/recurrent disease during follow-up.

Materials and Methods

All women submitted to laser CO₂ conization from January 2003 to December 2004 at our institution for a biopsy-proven HG-CIN were submitted to a HPV test before the surgical procedure and during follow-up examinations.

The personal history of established risk factors for cervical cancer (age, smoking habits, age at first intercourse, parity) and...
of comorbidities (other sexually transmitted infections, diabetes, chronic diseases and subsequent therapies) were collected for each patient before treatment.

All procedures were performed in an outpatient setting by two skilled laser surgeons using a SHARPLAN 733A CO2 laser (ESC Sharplan, Yokneam, Israel) with a maximum power output of 50 Watt, used in continuous mode, connected to a Zeiss OPMI colposcope (Carl Zeiss, Oberkochen, Germany).

A preoperative colposcopic evaluation was first performed to identify the external limit of the lesion and to determine the extent of the excision. A 30-gauge needle was used to inject 5-8 ml of a 2% lidocaine solution with epinephrine at the 3, 6, 9 and 12 o’clock positions of the cervix.

The first step of the laser conization procedure was to direct the laser beam perpendicularly to the cervical surface achieving an initial 0.5-1 cm deep circular section and then to guide it obliquely by manipulating the on-going excised specimen using a small steel hook. To assure complete clearance of the lesion after excision, the crater base and the walls were vaporized with a defocused laser beam. Defocalized laser beam action was sufficient in every case to reach hemostasis without need for sutures.

Histological analysis reports of excised specimens were collected recording the status of margins.

Patient follow-up consisted of six close cytological and colposcopic examinations taking place at 3, 6, 9, 12, 18 and 24 months after conization and once per year up to five years before returning to the screening program.

A punch biopsy was performed in all cases of colposcopic abnormality observed during follow-up and only histologically-proven HG-CIN was considered as a persistent/recurrent lesion.

HPV DNA test was introduced at the 3-month and 6-month examinations in patients providing informed consent for HPV testing and was performed as follows, independent of the cytoscopical examination. After speculum insertion cervical cells were collected with a swab and suspended in 3 ml of saline solution prior to colposcopy. After centrifugation the pellet was incubated with proteinase K and digesting buffer at 56° C for 2-5 h followed by automated DNA purification (BioRobot EZ1, QIAGEN, Germany). PCR amplification of HPV-DNA sequences, using commercial multiplex PCR kits (Nanogen), was carried out first by L1 consensus primers for screening (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66 and 68) and by E6-E7 primers for typing (6,11 for low risk; 16, 18, 31, 33, 35, 45, 52 and 58 for high risk). The amplified products were identified by agarose gel electrophoresis. The quality of the sample DNA was validated by detection of the housekeeping gene beta-globin as an internal control.

Only women with both 3-month and 6-month positive HR-HPV tests were considered as having persistent post-treatment infection.

A retrospective evaluation of the prospectively collected data was performed enrolling in the study group only women providing written informed consent to HPV testing with 24 months of follow-up without having missed more than one of the scheduled examinations. Diabetes, HIV and chronic steroidal therapy were considered as exclusion criteria commonly related to potential immunodepression.

Data about risk factors for relapsing disease were firstly analyzed in an univariate fashion applying Pearson’s chi-square test for categorical variables, with p < 0.05 considered as significant. The same variables were then included in a multiple logistic regression analysis to assess if they were likely influencing HR-HPV persistence at follow-up or if, otherwise, this characteristic might have an independent role in predicting relapsing CIN. Effect estimates are expressed as odds ratio (OR) with a profile likelihood-based on 95% confidence limits.

### Results

Ninety-two women underwent laser CO2, conization for HG-CIN during the study period. Among these, 78 patients matched the inclusion criteria and constituted the study group.

The mean age of the studied population was 38.3 years (22-62); the mean age at first intercourse was 17.8 years (range 12-25); at the time of laser conization 35/78 (44.9%) were nulliparous and 50/78 (64.1%) were smokers.

Histological analysis of cone-specimens resulted as HG-CIN in all cases with a margin-involvement rate of 12.8% (10/78).

Twelve women of 78 (15.4%) had a negative HPV test before the procedure. In this subgroup resection margins were negative in all cases, no patient presented a positive HPV test at follow-up and no relapsing CIN was detected after conization.

Among the remaining 66 women (84.6%), who presented a positive HPV test before treatment, 52 (78.8%) became negative at HPV testing in the follow-up, while 14 (21.2%) continued to be HPV-positive at the three- and six-month evaluations.

Among HPV-negative women in the follow-up, 48 of the 52 (92.3%) were still negative at the test performed within three months of the treatment, while four (7.7%), resulting positive at three months, were cleared of HPV infection at the six-month evaluation.

No negative test at three months became positive afterwards.

Three women of 66 (3.8%) developed a relapsing HG-CIN during follow-up and were submitted to a second laser conization; one case (1.9%) was observed six months after conization in the subgroup of 52 negative women at follow-up HPV testing and two (at 9- and 12-month evaluations) in the subgroup of 14 persistent positive patients (14.3%).

Involvement of margins and HPV infection persisting at follow-up (Table 1), resulted to be the only two statistically significant risk factors for relapsing HG-CIN on univariate analysis (p < 0.01 and p < 0.05, respectively).

Multivariate analysis revealed that none of the evaluated risk factors enhanced the likelihood of the HPV test to

### Table 1.

<table>
<thead>
<tr>
<th>Recurrence (n: 3)</th>
<th>No recurrence (n: 63)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive margins</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>HPV DNA at follow-up</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Smoking</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>Parity</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>Age &gt; 50 at conization</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>&gt; 17 yrs old at first intercourse</td>
<td>1</td>
<td>20</td>
</tr>
</tbody>
</table>
remain positive in the follow-up so that HPV DNA persistence after laser conization for HG-CIN appeared to be an independent risk factor for relapsing disease (Table 2).

Discussion

Persistence of HR-HPV infection after conservative treatment of CIN has already been widely pointed out to be strongly correlated with relapse of the disease. This is probably due to conservative treatment which, by excision of infected epithelium, is able to reduce HPV DNA presence – as demonstrated by the declining HPV antibody levels – but not to completely remove it [9].

The persistence rate of HPV infection after conservative treatment of CIN varies in the literature from 6% [10] to 50% [11]. This large variability probably derives from differences in patient selection criteria and treatment methods. For example Nagai et al. [8] evaluated HPV DNA persistence in a group of patients consistent in the indications for treatment (histologically proven CIN III in all cases), but not in the method of treatment since they were differentially submitted to cold knife or laser CO₂ conization. Conversely Kucera et al. [10] evaluated the rate of HPV persistent infection in a group of patients treated totally by electrosurgical excision of the transformation zone (LLETZ) for CIN, without distinguishing between cases of low- and high-grade disease. In the same way Bollen et al. [12] evaluated a group of patients treated by LEEP for cervical dysplasia without classifying them into grades of disease severity.

Moreover some of the studies reporting high rates of HPV DNA persistence include patients treated by destructive techniques with higher rates of relapsing CIN compared with patients treated by excisional methods. Distefano et al. [11] found that only 75% of their patients treated by electrosurgical or diathermic ablation were negative for CIN at follow-up and furthermore that 50% of them remained HPV DNA positive.

To our knowledge with the exception of Nagai et al. [8], who included in their series some patients treated by laser CO₂ conization, all the previous studies evaluating the role of persisting HR-HPV infection after conservative treatment for CIN were conducted in patients treated by LEEP/LLETZ, cold knife conization or destructive techniques.

For the first time our study has provided an evaluation of persisting HR-HPV infection in an homogeneous group of patients treated totally by laser CO₂ conization for HG-CIN.

We observed that in approximately 21% of HPV infected patients, HPV DNA was persistently detected in the cervix after therapeutic conization and that it can be considered an independent risk factor for residual/recurrent disease.

The recurrence rate observed in our experience by laser CO₂ conization (3.8%) is one of the lowest reported in literature where rates range from 0.3% to 23% of women with uninvolved excisional margins up to 84.8% of women with positive margins [13, 14]. This is probably due to the fact that our study population can be considered consistent from a methodological view point, in that conization was technically standardized and performed by only two skilled laser surgeons. The low rate of recurrence may be correlated even to the advantage brought about by the conization using the laser technique, which guarantees a sufficient depth of resection and vaporization of the crater walls, combining the advantages of excisional and destructive procedures, as previously observed [15].

The close selection of patients and the standardization of the operative technique overcome methodological bias in analyzing risk factors for persistence of HPV DNA, thus reducing to a minimum the impact of differences in treatment modalities and selection criteria observed in previous experiences.

Moreover an HPV test before conization was introduced into the study to exclude a population subgroup from the analysis whose cervical lesions were not HPV-related and which presumably had different biology in response to the treatment and persistence/recurrence rate. This hypothesis was derived from Alonso et al.’s results where a linear relationship between pretreatment HR-HPV load and risk of residual/recurrent disease was described [6].

In the present study 15.4% of patients with HG-CIN resulted to be HPV negative, in agreement with some previous studies such as the one by Kucera et al. [10] who observed that HPV tests resulted negative before treatment in approximately 16% of their case series.

In our experience among pretreatment negative HPV patients, no CIN recurrence or HPV DNA persistence developed, confirming Alonso et al.’s hypothesis that absence of HPV DNA prior to treatment might be considered a positive prognostic factor.
In our study HPV testing was performed at the third and sixth month evaluations to obtain data comparable with previous studies reporting conflicting outcomes on time of clearance of HPV DNA after treatment for CIN [7, 9, 16]. In our series 92.3% of the clearance rate was reached within three months of the treatment and only 7.7% of patients achieved absence of HPV DNA afterwards. Moreover none of these ultimate patients was complicated by relapsing lesions which occurred only in the case of persisting HPV DNA up to six months from the conization. This suggests, in agreement with Costa et al. [7], that HPV DNA tests might be introduced at least singularly at six-month evaluations to improve follow-up sensitivity in distinguishing high-risk patients for relapsing CIN.

Together with some established risk factors for cervical cancer we decided to include age at the time of treatment as part of the analyzed risk factors for relapsing disease on the basis of a previous study by Verguts et al. [17] which described women older than 50 at conization having a higher recurrence rate than younger ones with a statistically significant difference.

The present study did not confirm Vergut et al.’s result and none of the chosen risk factors for cervical cancer (age at first intercourse, smoking and parity) resulted to statistically influence residual/recurrent HG-CIN after laser CO2 conization.

In our experience HPV persistence was not the only significant risk factor for CIN at follow-up because involvement of the resection margins, in agreement with others [18-22], also appeared to be a predictor of disease recurrence. However, multivariate analysis cleared up that HPV DNA persistence at follow-up was not influenced by any of the other analyzed risk factors, including involvement of margins. This confirms that women with persistent HPV infection after laser CO2 conization for HG-CIN have a higher risk of residual/recurrent disease – aside from the fact that this risk could be enhanced by other variables such as involvement of the resection margins – and deserve closer follow-up than women with negative HPV tests after treatment.

Conversely to Gok et al. [23] and Verguts et al. [17] who found a 100% negative predictive value (NPV) (absence of recurrent disease among the population with negative HPV tests at follow-up), our study revealed that approximately 2% of patients with negative HPV tests after treatment will have relapsing HG-CIN at a subsequent follow-up. This result is in agreement with the meta-analysis of Zielinsky et al. [19] who described a NPV of HPV testing alone in the follow-up of treated patients of 98%.

Even if our study is biased by a small case series, 2% of false negatives is, in our opinion, too large a risk to run in promoting HPV testing as only a single initial follow-up evaluation and to skip subsequent controls for patients resulting negative. We could agree with Parasekevaidis et al. [24] who favor the use of HPV tests only in conjunction with cytology to primarily select a precise population subgroup at major risk for relapsing disease, and eventually skip visits only for those resulting negative to both HPV tests and cytology.

In conclusion, laser CO2 conization, although able to reach a low recurrence rate of HG-CIN – taking advantage of being a mixed excisional and destructive technique – does not remove HPV infection completely from the cervix with a case persistence of every five treated patients. In our experience this persistence in itself represents an independent risk factor for developing recurrent disease and constitutes the basis to introduce HPV tests at six-month evaluations even in the follow-up of patients treated for HG-CIN by laser CO2 conization.

References


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A retrospective analysis of borderline ovarian tumors in a Greek University Hospital

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Summary
Purpose: The aim of this retrospective study was to analyze the pathologic and clinical characteristics of borderline ovarian tumors. Methods/Results: During the period from January 1993 up to December 2002 we found 93 cases of borderline ovarian tumors. The mean age of patients was 44.3 years (range 28.9-59.7 years); 77.4%, 10.8% and 11.8% of patients had Stage I, II and III, respectively. The histological outcomes revealed 52.7% of serous and 41.9% of mucinous origin; 44.1% underwent radical surgery, whereas 55.9% had unilateral salpingo-oophorectomy or cystectomy. The mean follow-up was 84 ± 22 months. The overall five-year survival was 97.4% and 98% for mucinous and serous tumors, respectively. The survival rate was 100%, 90% and 81.8% in Stages I, II and III, respectively. Conclusion: From our results it can be concluded that borderline ovarian tumors have a favorable prognosis even after conservative management.

Key words: Borderline ovarian tumors; Management; Prognosis; Low malignant potential.

Introduction
Borderline ovarian tumors (BOTs) are a puzzling subset of epithelial ovarian tumors. These tumors were first described in 1929 by Taylor [1] as semi-malignant tumors but they were classified by FIGO and WHO in the early 1970s [1]. The WHO definition included tumors lacking stromal invasion, with budding, multilayered epithelium, mitotic activity and nuclear atypia [2]. More than two of the above criteria classified the tumor as borderline or of low malignant potential according to the 2003 WHO classification [3]. BOTs account for 15% of epithelial ovarian carcinomas with the majority diagnosed in early stages and being non aggressive. They usually occur in younger ages (1/3 in women under 40 years) [4]. Factors linked with such tumors are oral contraceptive use, menarche, age at first pregnancy and at first delivery, menstrual history, smoking, and family history of ovarian cancer. BOTs may arise from benign serous cystadenomas but the theories of progression to invasive carcinoma are still controversial. Although these lesions might arise from tumors of different origin, many believe that they could develop into invasive carcinoma after progression to an intermediate lesion called micropapillary serous carcinoma [5]. On the other hand, many believe that borderline tumors are characterized by the high frequency of KRAS and BRAF mutations, whereas p53 is often mutated in high-grade tumors [6-8]. Borderline tumors can be managed by more conservative techniques with a favorable prognosis.

Materials and Methods
This was a retrospective analysis of women diagnosed with BOTs and treated between January 1993 and December 2002 at the 2nd Department of Obstetrics and Gynecology, Medical School, University of Athens, Aretaieion Hospital, Athens, Greece. The necessary data were collected by reviewing patient records or hospital electronic bases and by contacting the physicians and patients regarding the age of diagnosis, stage and histological type of the tumor, treatment, relapse rate and 5-year overall survival. Ethical review approval was achieved by the ethical committee of our hospital. Disease staging and tumor characteristics were carried out according to the FIGO criteria.

Results
Ninety-three patients were included in the study. The mean age was 44.3 years (range 28.9-59.7 years). Preoperatively CA125 and CA19-9 measurements were performed in all patients and were positive in 61 (65.6%) and 22 (23.7%) women, respectively; 72 (77.4%), ten (10.8%) and 11 (11.8%) patients had Stage I, II and III disease, respectively. The histological outcomes revealed 49 (52.7%) serous, 39 (41.9%) mucinous, one (1.1%) endometrioid, one (1.1%) Brenner and three (3.2%) of clear cell origin. Eighteen (12.9%) patients were partially staged. It
should be mentioned that 52 (55.9%) women were under 40 years old and had fertility sparing surgery. Twelve (23.1%) women conceived spontaneously and four (7.7%) after IVF use. The mean follow-up was 84 ± 22 months. The relapse rates were one out of 41 (2.5%) in cases of radical surgery, four out of 45 (8.9%) in cases of salpingo-oophorectomy and finally three out of seven (42.9%) in cases of cystectomy. The relapse rate was one out of 19 (5.3%) and seven out of 19 (36.8%), respectively, in cases of non-invasive and invasive implants. Progression into invasive carcinoma was found in three out of 93 cases (3.2%). The type of surgical approach (laparotomy or laparoscopy) did not influence the recurrence rates and/or the survival. The overall five-year survival was 38/39 (97.4%) and 48/49 (98%) for mucinous and serous tumors, respectively. The survival rate was 72/72 (100%), 9/10 (90%) and 9/11 (81.8%) for Stage I, II and III disease, respectively.

Discussion

Our retrospective study revealed similar results to other previous studies on the subject [1, 9, 10]. However, some differences were also found in comparison to such studies.

According to the literature, a preoperative high level of CA125 is usually found in serous tumors (56%), whereas elevation of CA19-9 is usual in mucinous tumors (57%) [9, 10]. According to our findings of CA125 and CA19-9, 65.6% and 23.7%, respectively, had positive results. It should also be noted that these values were increased in larger tumors over 8 cm according to the histopathology findings.

The prognostic factors of borderline ovarian tumors are DNA ploidy, stage, histological type and patient age, and also the micropapillary pattern [5, 11, 12]. From our study, it was also shown that invasive implants are a significant risk factor for disease recurrence (36.8% vs 5.3%).

For many years the treatment of borderline tumors was the same as for ovarian carcinomas including peritoneal washing, hysterectomy with bilateral salpingo-oophorectomy, omentectomy, and multiple peritoneal biopsies [13, 14]. Today, the management of such tumors is more conservative. The current management includes peritoneal washing, preferably unilateral salpingo-oophorectomy or cystectomy when the tumor is bilateral; otherwise salpingo-oophorectomy when the history includes, omentectomy, peritoneal biopsies and resection, and appendectomy when frozen section reveals mucinous tumors [1]. Based on the fact that node involvement in Stage I tumors could be found in 0-36% of cases [15] leading to restaging as Stage III, and moreover that the survival rate could reach 98% [16], we did not perform lymph node sampling.

In our cases radical surgery was preferred in older women (> 40 years) whereas conservative techniques were used in younger patients with Stage I tumors according to FIGO or in selected cases with noninvasive implants. However, it should be noted that cystectomy had a higher risk of intraoperative cyst rupture and recurrence. When fertility-sparing treatment [1, 17] was implemented the need for prolonged and careful follow-up (with clinical examination, CA125 measurements, ultrasound and CT scanning) was essential to prevent recurrences. Careful selection of such candidates is always necessary in these cases. The need for close follow-up is significant but counseling the patient remains difficult as the tumor pathogenesis is still not well understood. Although, a diameter less than 10 cm makes laparoscopic management feasible with fewer complications and shorter hospital stay [18], such treatment was not the rule in our hospital. This was because of the fear of elevated risk of inadequate initial staging, tumor cell contamination, cyst rupture and wound metastasis in comparison to exploratory laparotomy.

The necessity of completing the operation (with removal of the remaining ovary) post pregnancy is still debated. In our patients we preferred to complete the radical treatment when the woman had completed her family planning, except in cases of earlier recurrences. Finally, a study by Zanetta et al. [19] showed progression into invasive carcinoma in 2% (7/339 patients), whereas we found an increased percentage (3/93). It should be mentioned that chemotherapy was only used in these three cases of invasive carcinoma and not in borderline tumors.

Taking into account that our study was retrospective, from a single institution, based on patient databases and included only a small number of patients may lead to special limitations of our research. However, we believe that our study could be representative in the understanding of borderline ovarian tumors.

Conclusion

As presented in previous studies, the prognosis of BOTs is excellent with low risk of recurrence even after the use of more conservative management, but there is a need of longer follow-up periods. Fertility-sparing treatment could be performed but careful follow-up is essential. We propose the organization of randomized controlled studies by different oncologic centers in the world which could consequently answer many controversial aspects of ovarian borderline tumors.

References

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Effect of intraoperative irrigation with alteplase on adhesion formation associated with intraperitoneal chemotherapy (experimental study)

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Summary

Objective: To examine the efficacy of intraoperative irrigation with alteplase, a tissue plasminogen activator, for the prevention of adhesion formation associated with intraperitoneal chemotherapy. Material and Methods: Rats, in which serosal injury was induced in the right uterine horn and ipsilateral parietal peritoneum, were randomly divided into four groups. Group 1 (n = 10) had intraoperative intraperitoneal irrigation with alteplase following the standard operation. Group II (n = 10) had irrigation with normal saline, while the rats in Group III (n = 10) and Group IV received no peritoneal irrigation. All rats, except for those in Group IV, received intraperitoneal (IP) paclitaxel plus carboplatin chemotherapy on the seventh postoperative day, and all rats were sacrificed seven days after chemotherapy. Total adhesion scores in the induced standard defects were calculated by evaluating percentage of adhesion formation, as well as the severity and degree of the adhesions. The scores were compared among the groups. Results: Comparison of the severity, percentage, degree and total score of adhesions among the groups demonstrated that subjects in Group I, where intraoperative alteplase irrigation was used, had fewer adhesion components (severity, percentage, degree) and a lower total adhesion score when compared to the other groups (p < 0.05). Adhesion components and the total adhesion score in Group IV, which did not receive chemotherapy, were found to be significantly lower when compared to Groups II and III (p < 0.05). Conclusion: Intraoperative Alteplase irrigation may reduce adhesion formation associated with intraperitoneal chemotherapy. Thus, intraperitoneal chemotherapeutic agents may be ensured to reach all peritoneal surfaces easily.

Key words: Intraperitoneal chemotherapy; Adhesion formation; Paclitaxel; Carboplatin; Alteplase.

Introduction

Recently, intraperitoneal chemotherapy has been reported to significantly increase survival rates when compared to intravenous chemotherapy in both ovarian and other cancers [1]. More specifically, it has been reported that intraperitoneal use of cisplatin plus paclitaxel combination in ovarian cancer significantly increases survival rates when compared to standard intravenous chemotherapy [2]. However, this route of chemotherapy administration is not perfect yet, and has some specific disadvantages, side-effects and complications. Intrapertoneal chemotherapy requires experience, causes port-associated complications and intraabdominal adhesions. The intraabdominal adhesion rate associated with intraperitoneal chemotherapy has been reported to be as high as 21.8% [3].

Experimental studies investigating the physiopathology of adhesion formation have shown that there is a direct relation between a decrease in fibrinolytic activity and an increase in adhesion formation [4]. In previous studies, tissue plasminogen activators (TPA), as fibrinolytic agents, have been used to prevent postoperative adhesion formation, and produced successful results [5, 6]. However, TPAs have not been used to prevent adhesion formation after intraperitoneal chemotherapy procedures.

The present study investigated the efficacy of alteplase, a TPA, in adhesion formation associated with intraperitoneal paclitaxel-carboplatin administration.

Material and Methods

Care and feeding of animals

The present study was carried out in the “Experimental Animals Study Unit of Firat University”. The local ethics committee approved the study protocol (no: 24-2005). The study included 39 Wistar-Albino rats weighing between 200 and 300 grams. Before the operation, all rats were kept in an environment with 12-hour light and 12-hour dark periods and 50-60% moisture. Feed and water for all rats were given ad libitum. “Ethical guidelines for experimental studies on animals” as identified in the 1993 Helsinki declaration were followed throughout the study period.

Formation of groups and the experiment

The rats were randomly divided into four groups. Of these, the first three included ten rats each (Groups I, II, III), and the last one was comprised of nine rats (Group IV). The standard operation was performed in all groups. Following the standard operation, intraabdominal irrigation was conducted immediately after surgery using 1 mg Alteplase (Actilyse®, Boehringer Ingelheim) in 5 ml normal saline (NS) in Group I, and the same volume of NS without alteplase in Group II. No intraoperative irrigation was performed in Groups III and IV. The first three groups received intraperitoneal chemotherapy (paclitaxel: 3.5
Effect of intraoperative irrigation with alteplase on adhesion formation associated with intraperitoneal chemotherapy

Standard Surgical Operation

All rats were anesthetized with xylazine (10 mg/kg) and ketamine (40 mg/kg) after six hours of fasting. Under sterile conditions and using sterile techniques, the intraperitoneal area was accessed through a 5 cm midline incision, and the standard operation was performed. In the standard operation, a 2-cm long by 1-cm wide area on the peritoneum of the right paracolic gutter was scraped using a bistoury to induce peritoneal injury, and a standard serosal incision of about 1 cm was made on the right uterine horn. A buffer was applied to the induced defects for two to three minutes to control bleeding. Layers of abdomen were sutured with 4/0 vicryl, and the skin with 4/0 silk.

Statistical analysis

The total score of each subject was calculated by adding all components (percentage, degree, severity). The scores obtained were compared among the groups. Kruskal Wallis and Mann-Whitney U-tests were employed as the statistical method, where appropriate. Level of significance was set at $p < 0.05$.

Results

Throughout the study period, there was no complication associated with surgery or intraperitoneal chemotherapy. No deaths nor decrease in the activity of rats were recorded during the study. Consistency between the inde-
to the tumor tissue at a high concentration [3, 8]. This has been shown in clinical studies through the significant differences between survival rates in intravenous and intraperitoneal chemotherapy procedures. To ensure easy circulation of the intraperitoneal fluid, and thus the chemotherapeutic agents in the intraperitoneal area is the cornerstone for the rationale of this procedure. Thus, apart from the known complications of adhesion formation (like need for a reoperation, pain, ileus, etc.), lack of adhesions associated with intraperitoneal chemotherapy theoretically means that that the efficiency of the drug will increase [9]. Our study is an important preliminary study to eliminate chemotherapy-associated adhesion formation, which is one of the main obstacles for intraperitoneal chemotherapy administration.

Although previous clinical studies reported significant levels of adhesion formation in cases who received intraperitoneal chemotherapy [3], the intraperitoneal adhesions in these cases might have been associated with surgery and probably directly with cancer, besides chemotherapy. There are many studies in the literature which cover all these three conditions [3, 4, 8]. The rats used in our study did not have cancer, and therefore adhesion formation associated with cancer has not been discussed. It is obvious that further studies are needed at this point. However, the design of our groups clearly demonstrated the effect of intraperitoneal chemotherapy added to surgery on adhesion formation. When Groups III and IV were compared, it was observed that although standard surgery was performed in both groups, carboplatin plus paclitaxel-combination chemotherapy significantly elevated adhesion scores in Group III. This finding is consistent with the literature. Likewise, it was demonstrated in previous studies that use of intraperitoneal paclitaxel reduced adhesion formation, whereas cisplatin and carboplatin increased it [10-12]. When our findings are interpreted in light of these previous studies, it can be stated that the increase in adhesion formation might be associated with carboplatin.

TPAs have been used in many studies to prevent postoperative adhesions and produced successful results. Studies that comprehensively investigated the pathophysiological events related with postoperative intraabdominal adhesion formation reported that this process was initiated by fibrin clots, or more specifically, “fibrin gel matrix”, originating from fibrinogen [13]. Peritoneal trauma and ischemia are the starting points for tissue factor expression and adhesion formation, and the peritoneum responds fairly rapidly to such events as trauma and ischemia [14]. Fibrin is broken down by fibrinolytic enzymes like plasmin in the healthy intraabdominal environment, in the presence of inflammation. However, these fibrinolytic enzymes become inactive, which leads to local reproduction of fibroblasts and formation of permanent adhesions [15]. The direct relation between the decrease in fibrinolytic activity and the increase in adhesions has been also demonstrated experimentally [16]. In light of this information, it is possible to say that intraoperative irrigation with alteplase prevents adhesion formation by increasing fibrinolytic activity. Even though

**Table 2. Distribution of total adhesion scores by groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage of adhesion formation</th>
<th>Severity of adhesion formation</th>
<th>Degree of adhesion formation</th>
<th>Total screening of adhesion formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.1 ± 0.5 ±</td>
<td>1.1 ± 0.5 ±</td>
<td>1 ± 0.4 ±</td>
<td>3.3 ± 1.2 ±</td>
</tr>
<tr>
<td>II</td>
<td>1.9 ± 0.3 ±</td>
<td>2.2 ± 0.4 ±</td>
<td>2.2 ± 0.4 ±</td>
<td>6.3 ± 0.3 ±</td>
</tr>
<tr>
<td>III</td>
<td>2.1 ± 0.3 ±</td>
<td>2.0 ± 0.6 ±</td>
<td>2.1 ± 0.1 ±</td>
<td>6.2 ± 1.2 ±</td>
</tr>
<tr>
<td>IV</td>
<td>1.8 ± 0.8 ±</td>
<td>2.6 ± 0.5 ±</td>
<td>2.7 ± 0.4 ±</td>
<td>8.1 ± 0.9 ±</td>
</tr>
</tbody>
</table>

Different upper symbols in the same column shows statistical significance.

**Discussion**

Two important findings were obtained at the end of the study. The first is that intraperitoneal paclitaxel plus carboplatin combination significantly increased adhesion formation in experimental conditions, and the second is that intraoperative intraabdominal irrigation with alteplase, a TPA, significantly reduced adhesion formation associated with surgery plus intraperitoneal chemotherapy.

The purpose of intraperitoneal chemotherapy in ovarian cancer is to ensure direct penetration of the drug to the tumor tissue at a high concentration [3, 8]. This has...
this mechanism proposed for alteplase concerns postoperative adhesion formation, there is also peritoneal irritation associated with intraperitoneal chemotherapy, and the mechanism by which alteplase reduces adhesion formation may hold true for the latter case, as well [17]. In our study, alteplase was utilized in light of the literature information cited above, and significantly reduced adhesion formation.

In previous studies, the degree of peritoneal reactive angiogenesis reached a maximum on postoperative days [8-12]. In this period, expansion of the adhesions is restructured and reduced, whereby angiogenesis reaches a maximum [16, 17]. Therefore, it can be speculated that, theoretically, it may be more appropriate to use agents employed to prevent adhesion formation during the increase in peritoneal neo-angiogenesis. As fibroblasts differentiate in mesothelial cells on the eighth day, use of agents before this day may be thought to affect fibroblast differentiation, and could be effective against adhesion formation [18]. That is why TPA was used during the operation in the present study, that is on day 0. There are two reasons why chemotherapy was administered on the seventh day in our study. The first is that the critical steps in adhesion formation reach maximum levels in this period; and the second is that chemotherapy administration was delayed to avoid any complications like incision opening [11, 16].

In conclusion, the fact that subjects irrigated with alteplase had lower adhesion scores in comparison to other groups demonstrates that use of intraoperative alteplase in the primary surgery is quite effective. Although our study is experimental, the results obtained clearly demonstrate that intraperitoneal irrigation with alteplase during primary surgery can reduce adhesion formation. This effect is fairly important in terms of the chemotherapeutic agent coming into contact with more peritoneal surfaces. The doses reported in our study did not cause any bleeding complications. However, intraoperative intraperitoneal alteplase doses that would not cause such complications in humans need to be determined.

References

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Case Reports

Two rare cases of methotrexate-induced pneumonitis and pleurisy in patients with gestational trophoblastic neoplasms

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Summary

Background: Pneumonitis is a serious and unpredictable side-effect of treatment with methotrexate (MTX) that may result in a life-threatening outcome. Pulmonary toxicity occurs in 0.5% to 14% of patients receiving low-dose MTX [3]. Methotrexate (MTX) is a folate antagonist used for several chronic inflammatory and neoplastic conditions, as well as for a gestational trophoblastic neoplasm (GTN). Pneumonitis is a serious and unpredictable side-effect of treatment with methotrexate (MTX) which may result in a life-threatening outcome [1, 2]. Pulmonary toxicity occurs in 0.5% to 14% of patients receiving low-dose MTX [3]. Rheumatoid arthritis (RA) is the most frequent underlying disease [4, 5]. To our knowledge, methotrexate pneumonitis and pleurisy in patients with a GTN have rarely been reported before, only one case report of respiratory failure due to pneumocystis carinii (PCP) following methotrexate therapy for GTN was presented in 2005 [2]. For the first time two cases of GTN patients of Chinese ethnicity who developed pneumonitis and pleurisy following single-regimen therapy with methotrexate are presented.

Case Reports

Case 1

A 47-year-old woman presenting with amenorrhea (for 55 days) and an elevated $\beta$-hCG level of 40.642 mIU/ml, who had undergone suction evacuation of the uterus on July 1, 2005, was diagnosed with hydatidiform mole. The $\beta$-hCG level decreased in the first three weeks, but unfortunately rose again and then remained at 830-1000 mIU/ml in the following four weeks. Pulmonary nodular shadows (0.5 cm in diameter) were found by pulmonary computed tomography (CT). Hepatic ultrasonography (US), cranial CT and chest X-ray were normal. The diagnosis of a gestational trophoblastic neoplasm with FIGO Stage III and a WHO score of 2 was confirmed. The methotrexate-citrovorum factor rescue (MTX-CF) protocol (methotrexate 1.0 mg/kg IM every other day for four doses with leucovorin 0.1 mg/kg 24 hours after each dose of methotrexate) was initiated on August 21, 2005. After two courses of MTX chemotherapy (total dosage 500 mg), the patient had chest pain, fever (38.8°C), cough, and pharyngalgia. The peripheral leucocyte count was 7.1×10^9/l, neutrophilic granulocytes were 79% and eosinophilia was 0.3%; chest radiography was rejected by the patient. Clindamycin (1.8 g/day IV) was prescribed. Two days later her symptoms disappeared and hCG levels decreased to less than 5 mIU/ml. The patient underwent the third MTX course on September 20, 2005. On the sixth day of the third course the patient merely presented fever (38.4°C), without chest pain or cough. The practitioner prescribed levofloxacin (400 mg/day IV). Her body temperature was normal on the seventh day, but in the early dawn of the eighth day the patient felt sharp chest pains, especially beneath the arch of the ribs, and shortness of breath. It was even hard for her to lie down. Clinical examination revealed that her blood pressure was 125/80 mmHg, heart rate 90 beats per minute and respiratory rate at 30 per minute with a temperature of 37.5°C. Dyspnea was relieved after a few moments, but the chest pain continued. The peripheral blood cell count, urine routine, hepatic function and renal function were normal. Pulmonary CT revealed bilateral pleurisy and diminished pulmonary lesions compared to the previous scan. Cefuroxime (1.5 g IV/day) was prescribed and oxygen therapy was given. The symptoms were alleviated five days later. The following two courses of chemotherapy were switched to actinomycin-D (Act-D) for a 5-day course (10 ug/kg/day × 5 days) because of the above-mentioned symptoms caused by MTX. No chest pain, dyspnea, fever or cough occurred after switching to the Act-D course.

Case 2

A 42-year-old woman presenting with amenorrhea (for 58 days) and elevated $\beta$-hCG levels (> 10000 mIU/ml), had undergone suction evacuation of the uterus on February 2, 2007. She was pathologically diagnosed as having hydatidiform mole. The
β-hCG level decreased in the first three weeks, but rose again in the fourth week and kept rising to 936 mIU/ml in the fifth week. A 2.5 cm lesion was found by uterine US, while chest X-ray, pulmonary CT, hepatic US and cranial CT were normal. The diagnosis of GTN was made and FIGO stage/WHO score 1:1 were confirmed. A single regimen therapy of MTX (0.4 mg/kg daily for 5 days IM) was initiated on March 11, 2007. HCG declined to 3 mIU/ml after two courses of MTX chemotherapy. On the last two days of the fourth course of MTX therapy (total dosage 440 mg), the patient presented with fever (37.8°C), and levofloxacin (400 mg/d IV) was prescribed. On the sixth day when the fourth course finished, the temperature declined to normal, but she complained of chest and back pain. Auscultation revealed moist rale and pleural rale. The white blood cell count was 5.0 × 10^9/l, and C-reactive protein was 20.0 mg/l. Pulmonary CT revealed bilateral pleurisy with right pleural effusion (Figure 1 A/B). The practitioner prescribed prednisone (15 mg daily). The symptoms were alleviated a few days later and the patient was discharged (the prednisone was decreased gradually over seven days).

Discussion

Pneumonitis is a serious and unpredictable side-effect of treatment with MTX that may result in life-threatening complications [1]. To our knowledge, MTX pneumonitis and pleurisy in patients with GTN has rarely been reported before. There was only one case report of respiratory failure due to PCP following MTX therapy for GTN by French doctors in 2005 [2]. MTX pneumonitis has not been reported in GTN patients of Chinese ethnicity.

We report two cases of MTX-induced pneumonitis and pleurisy in Chinese patients with GTN, aged 42 and 47, respectively, who had no history of interstitial lung disease before chemotherapy. They were both categorized as the low-risk group, and underwent single regimen therapy of methotrexate: one MTX-CP protocol, and the other the 5-day MTX protocol. Total dosage was 440 mg and 500 mg, respectively, when pulmonary symptoms such as fever, chest pain, acute nonproductive cough, dyspnea and hypoxemia appeared. Their symptoms did not respond to antibiotics immediately, but were alleviated several days later. The first patient’s pulmonary function clearly improved after the treatment of corticosteroids. Based on the symptoms and clinical examinations, we considered the diagnosis of interstitial pneumonitis and pleurisy induced by MTX.

MTX is a folate antagonist used in several chronic inflammatory and neoplastic conditions. Pulmonary toxicity occurs in 0.5% to 14% of patients receiving low-dose MTX [3]. Manifestations of pulmonary toxicity are protean and include parenchymal inflammation, pneumonia, airway hyper-reactivity, air trapping and possibly neoplasm. Rheumatoid arthritis (RA) was the most frequent underlying disease [4, 5]. There were also case reports of MTX pneumonitis in psoriatic patients [6] and acute lymphoblastic leukemia [7], causing acute respiratory failure and fatal results. Most patients present subacute symptoms over several weeks, which include dyspnea, dry cough, fever, and bibasilar crackles. The chest radiograph is normal in a small number of cases, but more commonly reveals bilateral interstitial or mixed, interstitial and alveolar infiltrates with a predilection for the basis. CT scans demonstrate ground-glass opacities, interstitial infiltrates, septal lines or widespread consoli-
dation. Pulmonary function studies reveal a restrictive ventilatory defect and/or impaired gas exchange [4-7]. Bronchoalveolar lavage (BAL) may be helpful in ruling out an infectious etiology and in supporting the diagnosis of MTX-induced pneumonitis. Cellular interstitial infiltrates, granulomas, fibrosis, atypical epithelial cells, and diffuse alveolar damage (DAD) are the main histologic features. Lung biopsy reveals cellular interstitial infiltrates, granulomas or a diffuse alveolar damage pattern accompanied by perivascular inflammation [8]. These clinical and pathological findings are not specific to MTX pneumonitis but can also be seen in other drug-induced lung toxicities. The pathogenesis of MTX pneumonitis is still uncertain. It is believed to be related to hypersensitivity and direct toxicity of MTX.

Once MTX-induced pneumonitis (MIP) is suspected, MTX should be withdrawn. Corticosteroids may accelerate resolution and are recommended in severe or fulminating cases. Cyclophosphamide may successfully cure some cases of interstitial pneumonitis resistant to steroids [9]. The prognosis of MIP is usually favorable, but occasionally the outcome may be fatal.

Gynecologists, as well as the managers of GTN patients receiving MTX, should be aware of this potentially life-threatening complication. The prompt evaluation of new pulmonary symptoms in patients receiving MTX is important in the early recognition of this drug-induced complication. It is also important that all patients receiving MTX be educated concerning this potential adverse reaction and instructed to contact their physicians when significant new pulmonary symptoms develop while undergoing therapy. If MTX pneumonitis is suspected, MTX should be discontinued, supportive measures instituted, and careful examination for different causes of respiratory distress conducted.

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Ovarian endometriosis associated with carcinoma and sarcoma: case report

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Summary

Endometriosis is a common clinical disorder that shares certain characteristics, metastasis and recurrence, with malignant neoplasms. Most malignant ovarian tumors arising from endometriosis are clear cell carcinoma or endometrioid adenocarcinoma. Few reports exist of sarcoma associated with endometriosis, and even fewer exist of multiple types of malignancies occurring simultaneously. Here, we report the case of a 32-year-old woman who presented with infertility and a pelvic mass. She underwent exploratory laparotomy and bilateral salpingo-oophorectomy. She was then referred to our institution for treatment recommendation. The pathologic findings revealed bilateral endometrioid adenofibroma of low malignant potential, which was associated with endometrioid intraepithelial carcinoma in the left ovary and high-grade sarcoma in the right ovary. Both tumors seemed to have arisen from endometriosis. She was treated with 75 mg/m² of doxorubicin and 10 g/m² of ifosfamide every three weeks for eight courses. She was later found to have bilateral brain metastases, which were resected and treated by whole-brain irradiation. She was again treated with doxorubicin and ifosfamide. The optimal treatment for endometriosis-associated ovarian cancer depends on the type of malignancy; simultaneously occurring multiple tumor types should be treated individually.

Key words: Ovary, endometriosis; Carcinoma; Sarcoma.

Introduction

Endometriosis is a common clinical disorder, found in 10%-15% of women [1]. Although endometriosis is benign, malignant ovarian tumors arising from endometriosis have been found in approximately 1.0%-1.5% [2].

In 1925, Sampson [3] reported the first case series of cancer arising in endometriosis and proposed three criteria for its diagnosis: 1) clear evidence of endometriosis found close to the tumor, 2) histopathologic appearance consistent with that of endometriosis (resembling endometrial stroma surrounding characteristic glands), and 3) no other primary tumor site found. In 1953, Scott [4] added a fourth criterion: microscopic continuation, cellular progression from one type to another, between benign endometrioid tissue and malignant tumor tissue. Most reported cases meet Sampson’s criteria, but only few reports meet Scott’s criteria [5, 6].

Several case reports and reviews exist of endometriosis-associated ovarian cancer (Tables 1 and 2) [7-19]. However, bilateral endometriosis associated with ovarian cancer of multiple types is rare. Here, we report a case of bilateral endometrioid adenofibroma of low malignant potential, which was associated with endometrioid intraepithelial carcinoma in the left ovary and high-grade sarcoma in the right ovary. The tumors seemed to have arisen from endometriosis.

Case report

A 32-year-old, gravida 1, para 0 white woman presented in July 2006 with a pelvic mass and a history of infertility at the University of Alabama Hospital. She had a history of pelvic endometriosis for years without other medical diseases. She has been on a careful screening program due to a significant familial history of breast cancer. In July 2006, she underwent a hysterosalpingogram; the procedure was complicated by a pelvic abscess. Exploratory laparotomy of the pelvis revealed 300 cc of ascitic fluid and a 17-cm solid right ovarian mass. On palpation, the surface of the left ovary was normal. Both ovarian masses were thought to be malignant; therefore, she underwent bilateral salpingo-oophorectomy, partial omentectomy, pelvic and periaortic lymph node sampling. The uterus was left in place for future fertility. The patient was then referred to The University of Texas M. D. Anderson Cancer Center for treatment recommendation.

A review of the pathologic findings revealed high-grade sarcoma in association with endometrioid adenofibroma of the right ovary; the mass had arisen from an ovarian endometriosis (Figure 1). High-grade sarcoma was also found in one of the three examined right pelvic lymph nodes and one right periaortic lymph node. In the left ovary, we found endometrioid adenofibroma of low malignant potential and endometrioid intraepithelial carcinoma that had arisen from an ovarian endometriosis (Figure 2). These findings met both Sampson’s and Scott’s criteria. A postoperative computed tomography scan revealed lymphadenopathy of the thoracic inlet and left supraclavicular fossa, a subcentimeter pulmonary nodule in the lingula, right intraperitoneal adenopathy, and a small focus of residual tumor in the right common iliac region.
The patient was treated with systemic chemotherapy, which consisted of 75 mg/m² of doxorubicin and 10 g/m² of ifosfamide every three weeks for eight courses. She was later found to have bilateral brain metastases, which were resected and treated by whole-brain irradiation in April 2007. She was again treated with doxorubicin and ifosfamide. Three months after surgery, she was still on treatment.

Discussion

Endometriosis has been found in 10-15% of ovarian cancer cases [7]. Malignant transformation of endometrioid lesions occurs in 1.0%-1.5% of cases [2], although this rate may be higher because the tumor could destroy the tissue of origin, eliminating any histopathologic evidence of endometriosis.

The clinical characteristics of endometriosis-associated ovarian cancers are distinct from those of typical ovarian cancer: the patients tend to be younger (45-50 years old) [16, 20-24] and nulliparous [21], like our patient. In addition, at the time of surgery, many endometriosis-associated ovarian tumors are Stage I or II and can be completely resected, with no postoperative residual disease [16, 21, 23, 24]. Women with endometriosis-associated ovarian cancer may also have longer disease-free survival durations, and possibly longer overall survival durations than non-endometriosis-associated ovarian cancer [21, 24].

The predominant histologic cell types in endometriosis-associated ovarian cancer are clear cell (8%-70%) and endometrioid carcinoma (9%-43%) (Table 1) [7-17], and most endometrioid tumors are grade 1 or 2 [16, 21, 23, 24]. The patient in our case had endometrioid adenofibroma of low malignant potential in association with endometrioid intraepithelial carcinoma in the left ovary and high-grade sarcoma in the right ovary; both of these appeared to have arisen from endometriosis. Primary ovarian sarcoma is rare, and to our knowledge, no data exists on the relationship between adenofibroma and ovarian sarcoma. Endometrioid stromal sarcoma, on the other hand, is associated with endometriosis (Table 2) [12, 18, 19], and endometriosis-associated malignant mixed mullerian tumor and adenosarcoma have been reported [12].
Table 1. — Summary of incidence of endometriosis in epithelial ovarian cancer patients.

<table>
<thead>
<tr>
<th>Study</th>
<th>Histologic tumor types/total (%)</th>
<th>Endometrioid</th>
<th>Clear cell</th>
<th>Mucinous</th>
<th>Serous</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auer et al. 1977 [7]</td>
<td>20/212 (9%)</td>
<td>14/59 (24%)</td>
<td>1/203 (1%)</td>
<td>0/357 (0%)</td>
<td>35/831 (4%)</td>
<td></td>
</tr>
<tr>
<td>Kornman et al. 1972 [8]</td>
<td>4/37 (11%)</td>
<td>1/12 (8%)</td>
<td>0/33 (0%)</td>
<td>2/118 (2%)</td>
<td>7/200 (4%)</td>
<td></td>
</tr>
<tr>
<td>Russel 1979 [9]</td>
<td>20/72 (28%)</td>
<td>16/33 (49%)</td>
<td>3/69 (4%)</td>
<td>7/233 (3%)</td>
<td>46/407 (11%)</td>
<td></td>
</tr>
<tr>
<td>Vercellini et al. 1993 [10]</td>
<td>30/114 (26%)</td>
<td>8/38 (21%)</td>
<td>6/94 (6%)</td>
<td>8/220 (4%)</td>
<td>52/166 (11%)</td>
<td></td>
</tr>
<tr>
<td>Toki et al. 1996 [11]</td>
<td>16/54 (30%)</td>
<td>22/44 (50%)</td>
<td>3/33 (9%)</td>
<td>9/88 (10%)</td>
<td>50/219 (23%)</td>
<td></td>
</tr>
<tr>
<td>Fukunaga et al. 1997 [12]</td>
<td>13/31 (42%)</td>
<td>27/50 (54%)</td>
<td>2/35 (6%)</td>
<td>6/103 (10%)</td>
<td>46/172 (27%)</td>
<td></td>
</tr>
<tr>
<td>Jimbo et al. 1997 [13]</td>
<td>3/13 (23%)</td>
<td>13/32 (41%)</td>
<td>1/35 (3%)</td>
<td>8/92 (9%)</td>
<td>25/172 (15%)</td>
<td></td>
</tr>
<tr>
<td>Erzen et al. 1998 [14]</td>
<td>2/13 (15%)</td>
<td>1/50 (2%)</td>
<td>0/7 (0%)</td>
<td>0/31 (0%)</td>
<td>3/56 (6%)</td>
<td></td>
</tr>
<tr>
<td>Ogawa et al. 1999 [15]</td>
<td>3/7 (43%)</td>
<td>30/43 (70%)</td>
<td>0/3 (0%)</td>
<td>4/46 (7%)</td>
<td>37/127 (29%)</td>
<td></td>
</tr>
<tr>
<td>Vercellini et al. 2000 [16]</td>
<td>1/16 (6%)</td>
<td>5/5 (14%)</td>
<td>1/30 (3%)</td>
<td>2/61 (3%)</td>
<td>21/192 (11%)</td>
<td></td>
</tr>
<tr>
<td>Takahashi et al. 2001 [17]</td>
<td>4/10 (40%)</td>
<td>2/11 (18%)</td>
<td>0/3 (0%)</td>
<td>1/15 (7%)</td>
<td>7/39 (14%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>128/629</td>
<td>139/359</td>
<td>17/569</td>
<td>47/1338</td>
<td>331/2895</td>
<td></td>
</tr>
</tbody>
</table>

Heaps et al. [25] reviewed 195 previously reported cases of malignant tumors arising in the foci of endometriosis and added their own ten cases in 1990. Of the 205 cases, 183 were carcinomas and 24 were sarcomas; two of these cases involved both types [26, 27]. Our case is similar to the latter two, in that she had two histopathologically different tumors, sarcoma and endometrioid adenofibroma. These findings are suggestive of gradual progression from benign to malignant tumors.

The most important treatment for endometriosis-associated ovarian cancer is surgical resection of the tumor. Postoperative treatment of this disease has varied considerably, however, as yet no concise treatment guidelines exist. Postoperative treatment is especially challenging in patients with multiple histologic tumor types. However, adjuvant chemotherapy has been found to result in longer survival duration and better prognosis than in patients who do not undergo adjuvant chemotherapy [21, 24]. A retrospective study evaluated the use of adjuvant treatments for endometriosis-associated ovarian cancer [21, 24], including chemotherapy alone, pelvic irradiation alone, chemotherapy and pelvic irradiation combined, and hormonal therapy. The chemotherapy regimens included platinum-based drugs alone and platinum-based drugs with paclitaxel and melphalan. No relationship was found between overall survival and postoperative treatment modality (chemotherapy vs radiation) on univariate analysis. However, significant predictors of overall survival were identified: the stage, grade, and histologic type of the tumor and the type of postoperative chemotherapy. Women treated with platinum alone had poorer overall survival rates than did those treated with platinum-based combined chemotherapy. In the multivariate analysis, however, only stage remained an independent significant predictor of the rate of overall survival [21, 24].

Sarcoma is more difficult to treat. Young et al. [18] reported the largest series of ovarian endometrioid stromal sarcoma associated with endometriosis. Low-grade sarcoma was associated with longer survival, with no adjuvant treatment, in four patients. Two patients treated with chemotherapy and progesterone were alive after one year and five years. Pelvic irradiation was effective in four patients, leading to survival durations of one, two, four and ten years. Only two of the 19 patients with low-grade tumor, compared to three of four patients with high-grade tumor, died of their disease. Two patients with high-grade disease, who had been treated postoperatively with chemotherapy, died of their disease after two and three years. Another patient with high-grade disease was treated with chemotherapy and radiation; she died five months later. Only one patient with high-grade disease – who was treated with radiation and methotrexate – was still alive after five years [18].

Marchevsky and Kaneko [27] reported a case of bilateral endometriosis associated with carcinosarcoma of the right ovary and endometrioid carcinoma of the left ovary. The patient was successfully postoperatively treated with doxorubicin and ifosfamide. Cooper [26] reported a case of mixed mesodermal tumor and clear cell carcinoma arising in ovarian endometriosis but did not provide treatment information. The patient in our report received eight courses of doxorubicin and ifosfamide but subsequently experienced brain metastasis. She underwent surgical resection, whole-brain irradiation, and salvage chemotherapy. Three months after surgery, she was still on treatment.

The optimal treatment for endometriosis-associated ovarian cancer depends on the type of malignancy; simultaneously occurring multiple tumor types should be treated individually.

References


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A rare case of low-grade endometrial stromal sarcoma with myxoid differentiation and atypical bizarre cells

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Summary

Endometrial stromal sarcoma (ESS) is a rare mesenchymal tumor with characteristic histological appearances, consisting of diffuse infiltrate of small uniform endometrial stromal cells with a multinodular arrangement and distinct vascular pattern. Less common variants of ESS include “mixed endometrial stromal and smooth muscle tumors”, “endometrial stromal tumors resembling ovarian sex cord tumors” and “endometrial stromal neoplasms with endometrial glands”, and “aggressive endometriosis”. Rarely do endometrial stromal tumors have a prominent fibrous or myxoid appearance which causes confusion and possible misdiagnosis as myxoid leiomyosarcoma.

In this report we present a very unusual subtype of ESS in a 32-year-old woman. The tumor revealed atypical pleomorphic bizarre cells which were stained positive only with vimentin and CD10 in an abundant myxoid matrix. A low-proliferative rate was established with MIB-1 staining. To our knowledge such appearance has not been previously reported.

Key words: Uterus, Endometrial Stromal Sarcoma, Myxoid change, Bizarre cells.

Introduction

Endometrial stromal tumors account for 2-4% of uterine malignancies [1]. Histologically they are classified as endometrial stromal nodules, endometrial stromal sarcoma (ESS) and undifferentiated stromal sarcoma. ESS is the most frequent form, characterized by tumor cells which closely resemble endometrial stromal cells of the proliferative phase [2].

Less common variants of ESS include those which contain smooth muscle differentiation – “mixed endometrial stromal and smooth muscle tumors”, and those associated with epithelial structures [3, 4]. The most common epithelial patterns resemble those seen in ovarian sex cord stromal tumors – “endometrial stromal tumors resembling ovarian sex cord tumors” [5]. Endometrial stromal tumors rarely have a prominent fibrous or myxoid appearance which causes confusion and possible misdiagnosis as myxoid leiomyosarcoma and those showing osteoclast-type giant cells [6-9]. In the diagnosis of ESS, the number of mitotic figures per 10 HPF should not exceed 10. This criterion is important in making the differential diagnosis with undifferentiated stromal sarcoma.

We present a case of ESS with abundant myxoid stroma and atypical pleomorphic bizarre cells.

Case Report

A 32-year-old woman was admitted to the hospital with abnormal uterine bleeding of six months duration. She had been treated with progestin for the previous two months. Pelvic computed tomography (CT) revealed a well circumscribed mass, 8.5 cm in diameter, arising from the uterine corpus and resembling a fibroid (Figure 1). Dilatation and curettage was performed. Histopathological examination of the curettage material revealed the presence of atypical bizarre cells with pleomorphic nuclei and abundant cytoplasm, some of which had long cytoplasmic processes. These were set in a fibromyxoid matrix among mucosal fragments of proliferative endometrium (Figure 2). On histological ground alone, the possibility of a pseudosarcoma-tous lesion was entertained and excision of the mass was suggested to rule out malignancy. The excised fibroid-like circumscribed mass was 8.5 cm in diameter with fish-meat like softening of the cut surfaces. The same histological features were observed in the excisional biopsy. The tumor exhibited less than three to four mitotic figures per 10 HPF. There was focal invasion into the myometrium (Figure 3). Immunohistochemically tumor cells reacted for CD10 and vimentin, but there was no reaction for smooth muscle actin, cytokeratin 7 and low molecular weight cytokeratin. With these findings, our diagnosis was ESS. Since the surgical borders were focally positive with neoplastic cells, the patient underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy and appendectomy. No residual tumor was seen in the hysterectomy material. The patient had no evident recurrence or metastatic disease 11 months after the operation.

Discussion

Endometrial stromal sarcoma accounts for 2-4% of all uterine corpus malignancies [1]. The average age for ESS is 40-49 and it is generally diagnosed during the premenopausal period, but has also been reported in young women and girls [10]. In our case the patient was 32 years old. In most cases, the presenting symptom is
abnormal uterine bleeding, uterine enlargement or pelvic pain. The usual preoperative diagnosis is uterine leiomyoma [10]. Diffuse CD10 immunoreactivity is common in endometrial stromal tumors, as it was in our case. CD10 positivity and smooth muscle actin negativity are important to distinguish ESS from myxoid leiomyosarcoma.

Like our case, ESS with abundant myxoid stroma has been reported but the association with large bizarre epithelial-like cells has not, to our knowledge, been described [6, 7].

We have presented an extremely rare example of low-grade ESS including both fibromyxoid stroma and atypical bizarre tumor cells. Such myxoid ESS with bizarre cells may create diagnostic difficulties, especially in curettage material. It may be mistaken as myxoid leiomyoma, myxoid leiomyosarcoma, inflammatory myofibroblastic tumor and myxoid malignant fibrous histiocytoma. Clinicopathological and immunohistochemical features may be helpful in the differential diagnosis.

References


Invasive squamous carcinoma of the vulva in women aged less than 40 years: report of two cases and a third case diagnosed during pregnancy


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Summary

Purpose of investigation: Invasive squamous cell cancer of the vulva (ISCC) is a rare disease in young patients and in pregnant women. The purpose of this paper was to investigate this type of cancer in women less than 40 years old and to present three cases, one which was diagnosed in the third trimester of pregnancy. Methods: Three cases of invasive squamous cell cancer in women under age 40 among the retrospectively analyzed 52 vulvar cancer cases diagnosed between 1995-2002 were investigated. Results: Women aged 25, 39 and 31, respectively, had Stage 1, 2 and 3 ISCC of the vulva. The first two cases had been spared by surgery and radiotherapy. The third patient was diagnosed during the last trimester of pregnancy. Although she was treated by radical surgery and post-operative radiotherapy, she had a recurrence in the inguinal region at 36 months, and died of disease 12 months later. Conclusion: Vulvar ISCC in young women may occur in association with or without predisposing factors. Although HPV-related type is predominant in the literature, keratinizing type of carcinoma may also be seen in this group of patients. Biopsy from suspected lesions is of paramount importance.

Key words: Vulva; Vulvar cancer; Pregnancy; Young.

Introduction

Vulvar cancer is a rare disease, seen most often in older women, accounting for 3-5% of all gynecologic malignancies [1]. The most frequent histologic type is invasive squamous cell carcinoma (ISCC) comprising around 90% of the cases. Other types are basal cell carcinoma, melanoma, adenocarcinoma, Paget’s disease and sarcomas. They all are rare in patients younger than 40 years old [2].

Invasive carcinoma of the vulva seems to derive from two separate entities [3]. The more common is a keratinizing carcinoma associated with lichen sclerosus, squamous hyperplasia, and p53 mutation in older women. The other is human papilloma virus (HPV)-linked warty or basaloid carcinoma in younger women. The latter patients appear to have a significantly better prognosis than the first group [4].

Vulvar cancer in women less than 40 years of age is not a thoroughly studied topic in the literature. An increase in the incidence of vulvar cancer, especially squamous type, has been recently reported [5, 6] and immunosuppression has been suggested to have a contributory role in this age group [7]. In order to assist in future research and treatment recommendations in this group, we have discussed clinical and pathologic findings, treatment and outcomes of three cases of ISCC in women aged less than 40 years in a tertiary care university hospital setting.

Materials and Methods

We conducted a retrospective review of the medical records of 52 women treated at our university for vulvar cancer from 1995-2002. In situ carcinoma cases were not included in these numbers. We identified six cases occurring in women less than 40 years of age accounting for 11.5% of all vulvar cancer cases. Three of these cases were ISCC and the rest were sarcomatous in origin. The relevant clinical data and follow-up information of ISCC cases were gathered. Since one of the cases was a pregnant woman diagnosed to have cancer in the third trimester, association of pregnancy and vulvar ISCC is also discussed.

Results

The clinical data and outcome of cases are outlined in Table 1.

The first case was a 25-year-old woman who presented with a lesion 1 x 1.5 cm in size on the right labium major. After biopsy revealed that it was a squamous carcinoma, the patient had a radical vulvectomy with bilateral inguinofemoral lymphadenectomy. The tumor was a well-differentiated squamous cell carcinoma and eight lymph nodes were negative in terms of metastasis. Evaluated as having been treated for Stage I disease, she received adjuvant postoperative radiotherapy. After attending her regular follow-up visits, she was still free of her disease after five years.

The second case was a 39-year-old woman with chronic itching of the vulva. Her clinical examination revealed a 2.5 x 2 cm ulcerative lesion on the superior...
portion of the left labium major, around 1 cm away from the clitoris. Radical vulvectomy with bilateral inguinofemoral lymphadenectomy was performed. Pathologic evaluation reported a grade 1 keratinizing squamous cell carcinoma with clear surgical margins of at least 2.5 cm in all directions, and 14 lymph nodes were all negative for disease. She received 28 fractions of pelvic radiotherapy postoperatively. Ninety months later she presented with a 3 x 1.5 cm recurrent ulcerative lesion at the former location of the clitoris. She underwent wide local excision and the defect was covered by bilateral subinguinal perineal flaps. The recurrence was a grade 2 lesion with focal pseudosarcomatous differentiation. Surgical margins were negative. After 24 months of follow-up, she had no evidence of recurrent cancer.

The third case was a 31-year-old pregnant woman, gravida 3, para 2, who presented at our clinic in the 31st week of gestation with a slowly growing lesion on the vulva. Her antenatal evaluation was normal, the fetus was appropriate for gestational age and no anomalies were detected during ultrasound. However, a 2 x 3 cm tumoral lesion on the right labium major extending to the clitoris was encountered. Biopsy revealed ISCC. The pregnancy was terminated and after one week definitive surgery comprising radical vulvectomy and bilateral inguinofemoral lymphadenectomy was carried out. The pathology reported that the tumor was a well differentiated squamous cell carcinoma but Stage 3 disease was present since there were unilaterally positive inguinal lymph nodes. The surgical margins of the specimen were clear of disease. The patient had adjuvant pelvic radiotherapy treatment and was closely followed-up. At the 36th month of follow-up she had a recurrence in the inguinal region and despite salvage therapy, succumbed to her disease at 48 months.

Discussion

ISCC of the vulva is not commonly seen in young patients and most of the literature consists of small case series, with large series being rare [1, 8-10]. It has been reported that 3.3-15% of vulvar tumors occur in women less than 40 years old [1, 11]. Although our numbers are limited and derived from a single institution, this ratio is roughly in accordance with our result of 11.5%. Of note, two of the cases were associated with HPV while the other was a keratinizing carcinoma.

The coexistence of pregnancy and ISCC vulvar cancer is very rare and around 30 cases have been published to date [12, 13]. Due to the paucity of cases, a consensus on the treatment plan of these patients has not been established. In some patients definitive surgery was postponed until the postpartum period to avoid increased vascularity of the region, like in our case in which definitive surgery was performed one week after termination of the pregnancy. However the treatment was instituted promptly in our case and the decision to terminate the pregnancy in the 31st week of gestation was based on the patient’s young age, presence of a midline structure and a lesion greater than 2 cm, availability of fetal intensive care, and demonstration of fetal maturation. Of the reported cases, nine were surgically treated during pregnancy and did not develop recurrence. Delaying the treatment until the postpartum period would have had grave complications and result in recurrence and death [14]. Our case turned out to have Stage III carcinoma, which undoubtedly negatively affected survival; she developed recurrence at the 36th month after the operation and eventually succumbed to her disease. Since there was no delay in the definitive treatment of our patient, whether the effect of delivery and/or early puerperium itself may worsen the prognosis of the disease is subject to discussion and should be further investigated.

Although it has been suggested by Bakou et al. [15] that vulvar cancer in pregnancy and by Carter et al., [9] that vulvar cancer in the young may be associated with immune system failures, there was no evidence of defective immune function in any of our patients. This finding suggests that mechanisms other than known causes of immunodeficiency could be operating to result in ISCC. One of the largest population-based series to date by Al-Ghamdi et al. also reported that immunocompromised hosts accounted for only a small percentage of young patients with vulvar ISCC [10]. It is also interesting to note that Ogunleye et al. have published an ISCC diagnosed and treated during pregnancy which recurred, just 11 weeks after surgery, at the 34th week of gestation. They concluded that although their case did not have any immune deficit, vulvar ISCC may recur in the setting of pregnancy and should be carefully followed-up [16].

Conclusion

Vulvar ISCC in women less than 40 years of age may occur in association with or without predisposing factors. Although HPV-related type is predominant in the literature, keratinizing type of carcinoma may also be seen in this group of patients. Although very rare, vulvar lesions during pregnancy or in the young may indeed be a carcinoma; therefore consideration for biopsy is of paramount importance. If diagnosis of vulvar cancer is confirmed, treatment should be started without delay.
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Meningeal carcinomatosis as a late complication of brain metastases of epithelial ovarian carcinoma

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Summary

The brain represents a rare site of metastasis in patients with epithelial ovarian carcinoma (EOC). In recent decades there has been an apparent increase in the number of EOC patients diagnosed with brain metastases, probably as a result of improved prognosis of patients with advanced tumors, but cases of meningeal carcinomatosis complicating EOC remain rare. A patient with Stage III EOC had brain metastases diagnosed 31 months after primary surgery. The isolated brain metastases were controlled with radiosurgery, surgery and chemotherapy. Forty-five months after the diagnosis of brain metastases, meningeal carcinomatosis was diagnosed which led, despite intrathecal therapy, to a fatal outcome. At autopsy, the disease was limited to the central nervous system. Meningeal carcinomatosis may represent a late fatal complication of brain metastases of EOC.

Key words: Brain metastasis; Epithelial ovarian carcinoma; Meningeal carcinomatosis.

Introduction

The survival of patients with epithelial ovarian carcinoma (EOC) has improved substantially in recent decades. The peritoneal cavity represents the most common site of metastatic spread in EOC, and in advanced EOC the disease is frequently limited to the peritoneum. However, the marked prolongation of survival resulting from multimodality treatment is changing the natural history of EOC, and more patients live long enough to develop distant metastases. Among sites of distant relapse, central nervous system (CNS) metastases, once considered very rare, are now being diagnosed with increasing frequency. In hospital population series, estimates of the frequency of CNS metastases in EOC range between 0.5 and 12 %. The frequency of CNS metastases in EOC has increased since the 1970s, and this increase is thought to reflect a change in the clinical course of the disease resulting from the introduction of more effective local and systemic therapy [1]. In most cases of EOC, CNS metastasis of brain parenchyma is involved, but metastatic spread to the meninges is rather exceptional.

We present a case of an EOC patient with metachronous brain metastases that were controlled with radiosurgery, surgery and chemotherapy for 45 months, but were later complicated by fatal meningeal carcinomatosis.

Case Report

A 48-year-old woman presented with Stage III serous EOC treated by bilateral salpingo-oophorectomy, hysterectomy and debulking surgery in February 1998. After the surgery, four courses of the systemic combination of paclitaxel (135 mg/m² 24-hr infusion) and cisplatin (75 mg/m²) were administered. Subsequently, an intraperitoneal catheter with subcutaneous port system was implanted, and the patient received another three courses of paclitaxel and cisplatin intraperitoneally. Serum CA125 levels normalized and the patient subsequently received three courses of intraperitoneal cisplatin with systemic cyclophosphamide and four courses of intraperitoneal carboplatin and etoposide. Six courses of intraperitoneal interferon-γ and interleukin-2 were then administered as consolidation therapy. Treatment was completed in 1999, and complete clinical remission was achieved.

In September 2000 the patient presented with generalized tonic seizures and light paresis of the left upper extremity and left facial nerve. A right frontal mass (18 x 14 x 13 mm) and a small metastasis in the gyrus cinguli (3 mm) were detected by magnetic resonance imaging (MRI), and the patient was treated with radiosurgery in October 2000. Both brain metastases in the right frontal region and right gyrus cinguli were irradiated using the gamma knife in a single session with minimal dose (D min) to the periphery of 21 Gy on 50% isodose curve. The patient was subsequently well under prophylactic therapy with sodium valproate and was able to return to her work as a nurse. In September 2002 the patient had repeated generalized tonic seizures. Progression of metastasis in the frontal lobe was detected by MRI. Biopsy confirmed EOC metastasis, and a complete resection of the brain metastasis was performed in December 2002. The patient was subsequently treated with a combination regimen including 5-fluorouracil, gemcitabine and cisplatin [1]. Between January and May 2003 the patient received four cycles of this combination regimen. She was asymptomatic until March 2004 when she complained about headaches and reported seizures. Control MRI revealed two lesions located in the right frontal lobe. These lesions were retreated by radiosurgery in April 2004 with single D min 19 Gy on 50% isodose curve. Subsequently, chemotherapy with the combination of 5-fluorouracil, gemcitabine and cisplatin was reinstituted. Three cycles of this regimen were administered, but the condition of the patient deteriorated. The patient complained about instability, impaired movement of the lower extremities, and falls. Cog-
nitive impairment was also evident. Control MRI in June 2004, 45 months after the initial diagnosis of brain metastases, revealed regression of the metastasis in the right frontal lobe.

Because of the conflict between radiologic improvement and neurological deterioration, the diagnosis of meningeal carcinomatosis was suspected, and spinal fluid was obtained for cytological analysis. Cytological examination confirmed the presence of tumor cells (Figure 1). As – apart from the brain metastases that were controlled by radiosurgery – the metastatic involvement of the meninges was isolated, an Ommaya reservoir was implanted for intrathecal therapy. By that time, left hemiparesis manifested and cognitive functions further deteriorated. Before the start of therapy cerebrospinal fluid CA125 concentrations were increased compared to serum (Figure 2). Methotrexate (20 mg) was administered intrathecally on June 17, 2004, and five doses of the combination of methotrexate (15 mg), cytarabine (50 mg) and hydrocortisone (15-30 mg) were administered between July 1 and July 13. The intrathecal application of combination of methotrexate and cytarabine resulted in a decrease of serum and cerebrospinal fluid CA125 concentrations, but clinically the condition of the patient further deteriorated. She was unable to sustain active movement, confined to bed and somnolent. Moreover, the administration of intrathecal therapy was later complicated by leukopenia, thrombocytopenia and gastrointestinal toxicity (diarrhea). Leucovorin was administered, and antibiotic therapy was initiated. The final course was complicated with pneumonia which did not respond to antibiotic therapy. The patient died on July 21, 2004. Autopsy revealed isolated metastatic involvement of the CNS with carcinomatosis involving most of the meningeal surface of the brain and spinal cord, recurrent metastasis in the right frontal lobe, bilateral pneumonia and deep vein thrombosis with pulmonary embolism that did not seem to be hemodynamically significant.

Discussion

Meningeal metastases in the present patient developed late in the course of CNS metastases, as an apparent sequela to brain metastases that were controlled for more than three years with radiosurgery, surgery and systemic chemotherapy. The survival of our patient was remarkably long compared to survival of most other patients with EOC brain metastases reported in the literature. It is evident that the increased frequency of CNS metastases in EOC is linked with the advent of effective therapy, resulting in significant prolongation of survival that allows for the manifestation of distant metastases. Similarly, it is possible that metastatic involvement of the meninges was associated with long survival after diagnosis of brain metastasis in our patient. We have recently observed meningeal metastases in another EOC patient who survived more than three years after diagnosis of brain metastases [1]. Another factor that could be linked to metastatic spread to the meninges could be brain surgery which was performed during the course of the disease in both of these patients [2].

CNS metastases are still a rare complication in patients with EOC, and cases of meningeal carcinomatosis are even more exceptional. EOC also represents an unusual primary among patients with CNS metastases. The brain is by far the most common site of CNS metastases in EOC, and more than 200 cases of EOC brain metastases have been reported [1]. We have recently performed a pooled analysis of the survival of patients with EOC brain metastases reported in the literature. The most favorable outcome was observed in patients treated by surgery combined with radiotherapy and/or chemotherapy, and median survival of patients treated by combined modality therapy was more than one year [1]. Although the prognosis of brain metastases is, in general, rather unfavorable with median survival between three and four months, patients with solitary brain metastasis or a chemosensitive primary, e.g. breast carcinoma or EOC, have a more favorable prognosis [3]. In the largest series of patients with EOC brain metastases, the median survival was six months [4].

In contrast, fewer than 20 cases of meningeal carcinomatosis in EOC patients have been reported so far [5, 6]. The published reports focused on patients with isolated meningeal carcinomatosis, and little has been published
so far about meningeal carcinomatosis as a late complication of parenchymal brain metastases. In our patient, signs of meningeal carcinomatosis were not obvious on MRI, and the diagnosis was established by cytological examination of cerebrospinal fluid. The cerebrospinal fluid CA125 concentration was increased compared to serum. There are only anecdotal data on the use of cerebrospinal fluid CA125 measurement in EOC meningeal carcinomatosis [5], but the present observation suggests, in agreement with previous reports, that increased cerebrospinal fluid CA125 (compared to serum) concentrations may be helpful in establishing the diagnosis. Both cerebrospinal fluid and serum CA125 levels decreased after intrathecal chemotherapy. However, in contrast to a recent report on patients with breast carcinoma and meningeal carcinomatosis [7], the decrease in tumor marker concentrations was not accompanied by clinical improvement. Our patient had clinical signs of carcinomatous encephalopathy that progressed in spite of an apparent response detected by serial CA125 measurements. Terminal pneumonia was also considered a consequence of progressive carcinomatous encephalopathy. The present experience indicates that tumor cell destruction in patients with extensive meningeal carcinomatosis may not lead to functional improvement. Inflammatory phenomena accompanying tumor cell destruction could also explain the neurological deterioration. It is therefore likely that some cases of carcinomatosis involving most of the meningeal surface may be refractory to any currently available therapy. In fact, similarly to the present case, most patients with EOC meningeal metastases have died within one month of presentation [8-10], although individual cases of patients with longer survival after therapy have also been reported [5, 6], including patients treated with intrathecal chemotherapy. Among 13 cases reviewed by Khalil et al. [5], seven patients died within one month of diagnosis of meningeal metastases.

In conclusion, meningeal carcinomatosis may represent a late fatal complication of brain metastases of EOC. It is possible that with the improvement in management of EOC patients, including the patients with brain metastases, the diagnosis of meningeal carcinomatosis will be more frequent.

Acknowledgement

Supported by a research project of the Ministry of Health of the Czech Republic MZO 00179906 and a grant from the Internal Grant Agency of the Ministry of Health of the Czech Republic NR 8363-3.

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Cervical cancer coexisting with small lymphocytic lymphoma detected during positron emission tomography/computed tomography simulation: a case report

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Summary

Background: Positron emission tomography (PET)/computed tomography (CT) simulation in cervical cancer may help radiation oncologists to better define the target volumes. It may also detect extrapelvic lesions and incidental second malignancies, leading to significant changes in treatment management. Case: A 63-year-old woman who was deemed inoperable due to carcinoma of the cervical stump extending to the parametria and paraaortic lymph nodes detected on MR images presented for extended field radiotherapy. PET/CT simulation revealed an FDG avid mass in the cervical stump, and an enlarged axillary lymphadenopathy showing moderate FDG uptake. The excisional biopsy was consistent with small lymphocytic lymphoma (SLL). Conclusion: In our case, PET/CT simulation not only led to changes in treatment management, but also revealed a very rare coexistence of SLL and invasive squamous cell carcinoma of the cervix.

Key words: PET/CT/simulation; SLL; Uterine cervix.

Introduction

Although many cases of multiple malignant neoplasms involving the female genital tract have been reported, synchronous gynecologic and hematologic malignancies are extremely rare [1, 2]. Synchronous malignancies may also be the reason for misdiagnosis and subsequent treatment. In recent years, functional imaging with positron emission tomography (PET) has proved to be superior to computed tomography (CT) or magnetic resonance (MR) imaging in the diagnosis and staging of many types of cancer. PET scanning can result in a change of staging and thus patient management in 20-30% of cases, mainly due to detection of metastatic disease or nodal involvement. Treatment changes occur most frequently in patients with head and neck cancer, lung cancer and gynecologic malignancies [3].

We present the case of a patient with cervical cancer in whom PET/CT simulation revealed a second malignancy and totally altered the treatment management.

Case Report

A 63-year-old woman presented to her gynecologist with postmenopausal vaginal bleeding. She had a history of subtotal hysterectomy due to leiomyomas 20 years before. Physical examination revealed an ulcerative mass in the cervical stump with parametrial involvement. Biopsy was consistent with invasive squamous cell carcinoma. To evaluate for metastatic disease an MR scan of the whole abdomen was performed, which demonstrated multiple lymphadenopathies measuring up to 1.5 cm in diameter in the paraaortic, bilateral internal, external, and obturator areas (Figure 1). In the cervical stump a mass lesion measuring 4.5 cm with involvement of the parametrial space posteriorly and laterally was observed. With these findings the patient was deemed inoperable due to Stage IIB disease with paraaortic metastasis and was referred to our department for definitive radiotherapy.

Our initial plan was to treat the patient with intensity modulated radiotherapy (IMRT) using a simultaneous integrated boost technique to escalate the dose at involved lymph node areas while keeping the dose to the surrounding critical structures at acceptable levels. To better define and delineate the primary target volume in the pelvic and paraaortic region we decided to perform PET/CT simulation.

The patient underwent a PET/CT scan in the supine position. The patient’s arms were placed over her chest and her pelvis was immobilized using a dual leg positioner (MEDTEC, Orange City, IO, USA). For planning purposes the scan was first acquired from the diaphragm to 3 cm below the ischial
tuborosities, and then a whole body scan was obtained for metastatic workup. The PET/CT scan revealed no uptake in the pelvic and para-aortic enlarged lymph nodes while showing a hypermetabolic mass at the cervical stump. Additionally, multiple enlarged bilateral axillary lymph nodes were detected on CT scans from which only a left-sided lymph node showed moderate F18-fluoro-2-deoxy-D-glucose (FDG) uptake (Figure 2). Therefore, an excisional biopsy of the left sided enlarged axillary lymph node was performed, which was consistent with diffuse small lymphocytic non-Hodgkin’s lymphoma (SLL). Immunohistochemical staining showed positivity for CD 20, CD 5, CD 23, and negativity for CD 3 with a KI 67 index of 20%. Immunophenotyping using blood revealed no abnormal cell clusters, nor any increase in monoclonal antibodies. Her LDH, β2-microglobulin, and complete blood count levels were within normal limits, and she was considered to have Stage IIIA indolent lymphoma. The patient was discussed at the monthly meeting of the Istanbul Lymphoma Group and it was decided that she did not need any further treatment for SLL till symptomatic progression.

We treated her for Stage IIB cervical cancer with pelvic IMRT to 45 Gy in 1.8 Gy fractions with concurrent weekly cisplatin. She tolerated the treatment well and had a good response at the end of treatment. She then received high-dose rate intracavitary treatments. The total dose to point A, including both external beam and brachytherapy, was 75 Gy.

Discussion

In the case presented, PET/CT aided treatment planning revealed a very rare, incidental coexistence of cervical cancer and SLL, and led to changes in the management of the patient.

MR imaging plays an important role in the staging of cervical cancer. Assessment of metastatic lymphadenopathy in patients with endometrial and cervical carcinoma on MRI is based on the size of the lymph nodes. Nodes with a size exceeding 1 cm short axis diameter are considered pathologic [4]. Signal intensity characteristics have not been useful in differentiating metastatic from hyperplastic lymphadenopathy in the pelvis. Our patient had multiple enlarged lymphadenopathies in the pelvis and paraaortic region with the greatest diameter of 1.5 cm. Therefore, they were considered metastatic and the patient was referred to the Radiation Oncology Department for extended field radiotherapy.

Functional imaging with FDG has been increasingly used for staging purposes in many different tumor sites. FDG is an analog of glucose and is rapidly absorbed by cancer cells that possess an increased glucose need compared with non malignant cells because of greater blood flow, glucose phosphorylation, and cell membrane transporters [5]. Applied in the clinic, PET can be useful for tumor staging, prediction of tumor response, selection and delineation of target volumes in radiotherapy planning, assessment of tumor response to treatment, and for the detection of early recurrences [6]. In cervical cancer the superior specificity of PET support the inclusion of FDG positive nodes into target volumes during the treatment planning process. In addition, its ability to detect extrapelvic lesions may help to determine the appropriate treatment fields and change treatment management [3].

Grigsby et al. compared PET/CT and CT in the staging of cervical cancer and demonstrated that PET studies
detected abnormalities in 99% of patients versus 76% imaging these cancers using CT. PET revealed abnormal pelvic/paraaortic nodes in 67%/21% of patients versus 20%/7% using CT scans, respectively. Total-body PET scans were also able to detect occult metastatic disease in the supraclavicular region in 8% of patients [7]. Although based on a limited number of patients, other investigators also reported that PET was much more specific than CT or MRI in detecting paraaortic lymph node metastases [8, 9].

FDG uptake in lymphoma patients is correlated to histologic grade and proliferative activity. There is limited and conflicting data in the literature about the sensitivity and specificity for FDG-PET of 87% and 100%, respectively, in 36 indolent lymphoma patients [10]. In contrast, Jerusalem et al. studied 36 patients with low-grade NHL [11]. PET detected 40% more abnormal lymph node areas than conventional staging in follicular lymphoma, but was inappropriate for the staging of small lymphocytic lymphoma for which it detected less than 58% of abnormal lymph node areas.

In the present case the pathologic paraaortic and pelvic lymph nodes which were revealed by MR imaging were not found FDG avid during the PET/CT planning process. However, the whole body PET/CT scan showed additional bilateral axillary lymphadenopathies from which a left-sided scan showed moderate FDG uptake. The excisional biopsy was consistent with indolent lymphoma. Because of the high node specificity of FDG-PET in cervical cancer, and low specificity in detecting abdominal and pelvic lymph nodes in small lymphocytic lymphoma we decided that the pelvic and paraaortic lymphadenopathies originated from lymphoma, rather than cervical cancer. The patient had two primaries, a locally advanced cervical stump cancer and indolent lymphoma involving supra- and infradiaphragmatic sites.

Patients with SLL have been reported to have an increased risk of developing a secondary malignant tumor due to immunosuppression which may be related to B-cell dysfunction or to chemotherapeutic agents. The most frequent sites of secondary malignancy are the lung, gastrointestinal tract and prostate. Uterine tumors account for about 1% of secondary tumors associated with SLL. Our literature search resulted in two case reports of the synchronous appearance of uterine tumors and SLL, one in the international and the other one in the national database. Mikami et al. [1] reported the case of a woman who was followed for stage 0 SLL who developed invasive squamous cell carcinoma of the uterine cervix. The microscopic evaluation of the hysterectomy specimen established the diagnosis of invasive squamous carcinoma of the cervix and monotonous populations of small lymphoid cells with proliferative centers, which were consistent with SLL, in the cervix as well as parametrium. The prognosis was dismal, and the patient died of disseminated disease 23 months after the initial diagnosis.

In conclusion, the use of PET/CT aided treatment planning is a hot topic in radiation oncology. Although it is recommended to select the target volumes for tumor types like non-small cell lung cancer and esophageal cancer where FDG-PET shows superior specificity to CT, for other sites the data are still immature. For cervical cancer, especially with involved pelvic and paraaortic lymph nodes, PET/CT simulation may help in directing more focused external beam irradiation to these nodes. PET/CT scanning during the treatment planning process may also detect extrapelvic abnormalities and incidental secondary primaries leading to significant changes in treatment management, as was the case in our patient.

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In vitro fertilization in a patient with ovarian cancer (Stage IC) following conservative surgery and chemotherapy: a case report

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Summary

A 30-year-old female underwent left salpingo-oophorectomy followed by chemotherapy for Stage IC adenocarcinoma of the ovary. Three years later she had ovarian hyperstimulation and in vitro fertilization (OH-IVF) resulting in a singleton pregnancy. During cesarean section peritoneal washings and biopsies were negative for recurrence. Seven years after the initial diagnosis, the patient is still free of any disease. In conclusion, OH-IVF may be considered in young patients with early ovarian cancer treated with conservative surgery and chemotherapy.

Key words: Ovarian cancer; Chemotherapy; IVF.

Introduction

The rate of ovarian tumor diagnoses in reproductive age woman has increased parallel to the improvements in diagnostic methods and regular gynecological visits. Because of this, conservative surgeries for the preservation of reproductive function have gained more interest. Data on the role of in vitro fertilization (IVF) and ovarian stimulation in patients with established diagnoses of ovarian cancer and who want to get pregnant are still limited. Most of the reported cases are described in patients with low malignant potential (LMP) ovarian tumors and have shown encouraging fertility outcome [1, 2]. However, the situation seems to be more complicated for IVF treatment in patients with early-stage invasive cancer needing chemotherapy. The role and safety of IVF and ovarian stimulation in these patients are still controversial [3, 4]. This lack of consensus is attributed to the complex interactive nature of infertility, the potential effects of the medications, and other inherent risk factors in this patient population. This has left little guidance for women who have an established diagnosis of ovarian cancer and who want to conceive through fertility drugs. The problem becomes more complex in patients with invasive metastatic implants who require adjuvant chemotherapy.

We report a case of a young woman with a borderline epithelial ovarian tumor and invasive implants (Stage IC adenocarcinoma of the ovary) who underwent conservative surgery and chemotherapy followed by one successful IVF pregnancy, and another spontaneous pregnancy thereafter.

Case Report

The patient was a 30-year-old woman when she had surgery in another hospital for a persistent left complex ovarian cyst. She underwent open ovarian cystectomy (with intraoperative rupture of the cyst) in May 2000 and the pathology was consistent with a borderline mucinous ovarian tumor with negative peritoneal washings. No tumor markers were taken at that time. She was seen in our hospital in July 2000 for a recurrence of the cyst with an elevated CA125 of 742 with normal CA19-9 and CEA. CT scan of the abdomen was unremarkable except for a 5 x 3 cm left ovarian complex cyst.

In August 2000, she underwent an exploratory ovarian staging laparotomy, peritoneal washing, left salpingo-oophorectomy, and appendectomy. There was now evidence of a residual adenocarcinoma (grade 1) limited to the left ovary. All the other specimens were negative. Following surgery and because of prior manipulation of the left ovary which now contained an invasive cancer (thus staged as IC), she received four adjuvant chemotherapy cycles of paclitaxel and carboplatin. She had amenorrhea during and three months following the chemotherapy. She then spontaneously had resumption of her menses. She has been followed since then with examinations, vaginal ultrasonography (US), and CA125 monitoring as well as a yearly computed tomography (CT) scan of the abdomen and pelvis with no recurrence of the tumor.

The patient got married in December 2003 and wanted to conceive. Her baseline day 3 FSH was 12.6 IU/ml and estradiol was 13 pg/ml. Hysterosalpingography showed a right patent tube and her husband had a normal semen analysis. She was unable to conceive spontaneously for seven months so a repeat day 3 FSH was done resulting as 18.6 IU/ml with estradiol of 32 pg/ml. After counseling she was referred to our IVF Unit. Controlled ovarian hyperstimulation was performed by a long standard protocol with GnRH agonist. In brief, GnRH agonist was started in the mid-luteal phase at a daily dose of 0.05 mg until the day of hCG injection. Recombinant FSH (200 U/day) was started on the third day of her cycle. Oocyte aspiration was performed approximately 36 hours after hCG administration. A total of six metaphase II oocytes were aspirated. Four embryos were transferred two days after oocyte pickup resulting in a singleton pregnancy. The patient had a smooth antenatal course.
August 2005, at term, she delivered by cesarean section for a breech presentation a live healthy female newborn weighing 3,200 g. She also had washing of the peritoneum in addition to biopsy of the right periovarian adhesion, remnant of left the infundibulopelvic ligament, anterior and posterior cul-de-sac peritoneum, peri-colic gutters, and remnants of the omentum. All were unremarkable.

One year later she had a spontaneous singleton pregnancy and again had a term repeat cesarean section and delivered a live female newborn weighing 3,100 g. Peritoneal washings and biopsies were again unremarkable.

Discussion

The role of IVF in patients with ovarian cancer has been described in few reports, mostly in patients with LMP tumors [1, 2, 5, 6]. In addition, spontaneous pregnancies following chemotherapy for ovarian cancer have been reported in several patients [3, 7]. However, the use of IVF in patients with established ovarian cancer treated with conservative surgery and adjuvant chemotherapy has been reported recently in only one patient, making our patient the second case to be reported in the literature [4]. Our patient was diagnosed initially as a case of LMP ovarian tumor and underwent conservative surgery. Unlike their invasive counterparts, LMP ovarian tumors tend to occur more frequently during a woman’s reproductive years. Because the diagnosis for patients with borderline ovarian tumors is excellent, particularly in women with the most common stage - Stage I - there has been a trend over the past several years toward fertility sparing surgery in women of reproductive age who have not completed childbearing. The reproductive performance of women who underwent conservative surgery for borderline ovarian tumors was found to be adequate, and spontaneous pregnancies with good outcomes have been reported. Moreover, no relationship between pregnancy and recurrence has been demonstrated [1, 2].

Following her second surgery, our patient was discovered to have a frankly invasive mucinous ovarian adeno-carcinoma rather than just a borderline tumor as previously diagnosed. Review of the original pathology confirmed the borderline nature, although the diagnosis of borderline versus malignant mucinous tumor is more difficult than the rest of the borderline epithelial tumors. Moreover, because of prior manipulation of the ovary and intraoperative rupture with her first surgery she was staged as Stage IC. During her second surgery, she wanted to preserve her fertility potential so a unilateral salpingo-oophorectomy with full staging was done. This was followed with four cycles of chemotherapy because of prior intraoperative spillage and the short time interval between the original diagnosis of the borderline and the diagnosis of the frankly malignant tumor. She underwent IVF with ovarian stimulation because of an increase in her day 3 FSH and her inability to conceive after a seven month trial. Data in the literature, mainly based on case reports, are not sufficient to argue about the safety of the use of induction of ovulation or IVF in patients with advanced borderline tumors or those which are malignant. Several studies that examined the role of ovulation induction in the development of borderline ovarian tumors reported a possible positive association [8]. However, more recent data have provided reassuring evidence on the absence of a strong association between fertility drugs and subsequent risk of developing invasive epithelial ovarian cancer [9].

Data on the efficacy and safety of IVF procedures in patients with advanced LMP ovarian tumor or early frank ovarian cancer are still limited. Most of the cases reported did not have any significant progression of the disease [1, 2]. However, Attar et al. reported a rapid progression of peritoneal disease in a patient with a Stage IIIC serous tumour with a micropapillary pattern [10]. Thus, it does not seem possible to give guidelines concerning hyperstimulation and IVF in patients with advanced stage disease and/or micropapillary patterns. Although no definite conclusions can be drawn regarding the safety of infertility treatment in this group of patients, mainly because of the retrospective character of the studies and the small number of patients included, the results show that IVF after the diagnosis of a borderline ovarian tumor does not appear to affect survival. The recurrence rate after IVF treatment is reported to be 20%, similar to the rate reported for patients who underwent conservative treatment without any subsequent fertility therapy [6, 9]. Our patient has been disease free for seven years since her initial diagnosis and treatment, during which she had two successful pregnancies one of them a product of IVF.

Spontaneous pregnancy following chemotherapy for ovarian cancer has been reported in several patients [7]. The return of ovarian function and subsequently the fertility potential following chemotherapy varies with the patient’s age, type of chemotherapy used, and her ovarian reserve. It is expected that most young patients who receive chemotherapy for ovarian cancer resume ovarian function and can expect normal fertility rate. Our patient took four cycles of chemotherapy that caused transient amenorrhea. Six months after finishing chemotherapy her menstrual cycles resumed. However, three years later, despite having regular ovulatory cycles, her FSH was increasing reflecting a decrease in her ovarian reserve. This could be due to chemotherapy and/or having only one ovary. Despite this she had adequate response to ovulation induction and successful IVF treatment, and had a spontaneous pregnancy one year after the delivery of her first child. One of the main concerns of patients who received chemotherapy is the effect of toxic chemotherapy in their offspring. The data available so far have not shown any significant increase in the frequency of congenital anomalies in the offspring [3].

In conclusion, IVF with ovarian stimulation may be considered in young patients with early ovarian cancer treated with conservative surgery and chemotherapy, and who fail to conceive spontaneously.
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Primary double invasive cervical carcinoma, squamous cell carcinoma and adenocarcinoma - case report

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Summary

A case of a 56-year-old woman with double primary invasive cervical carcinoma, squamous cell carcinoma and endometrioid adenocarcinoma is presented. The patient was subjected to radical abdominal hysterectomy with pelvic and paraaortic lymphadenectomy. Surgery was followed by radiotherapy. Since the treatment the patient has been doing well and is free of any signs of relapse of the disease.

Key words: Cervical carcinoma; Squamous cell carcinoma; Adenocarcinoma.

Introduction

Cervical carcinoma is the second most common carcinoma of the female reproductive system. Squamous cell carcinoma accounts for 85%-90% of all cervical carcinomas, while adenocarcinoma accounts for about 10%-15% of all cervical carcinomas [1, 2]. The incidence of cervical carcinoma is 7.25/100,000 women and the mortality is 2.04/100,000 women [3]. The mean age for cervical carcinoma is 52.2 years, and the distribution of cases is bimodal, with peaks at 35-39 years and 60-64 years [4]. The major risk factor for development of invasive cervical carcinoma is HPV infection, predominantly types 16 and 18, high parity, increasing number of sexual partners, low socioeconomic status, and positive smoking history [5, 6, 7]. The first symptoms of invasive cervical carcinoma are vaginal bleeding and unusual vaginal discharge. The main types of cervical carcinoma treatment are surgery and radiotherapy [8, 9]. The prognosis in patients with cervical carcinoma is markedly affected by the extent of disease at the time of diagnosis. The major factors that influence prognosis include stage, tumor size, histologic type, degree of stromal invasion, lymph-vascular space invasion, parametrial invasion, and pelvic lymph node status [10, 11]. The prognosis is less favorable in patients with adenocarcinomas in comparison to those with squamous cell carcinomas, regardless of the stage of the disease [12].

Case Report

A 56-year-old nulliparous patient was admitted to our clinic due to vaginal bleeding. Bleeding started ten days before admission to the clinic. She had been menopausal for six years and had normal weight and blood pressure values. Laboratory blood analyses evidenced mild anemia and a high erythrocyte sedimentation rate of 60/110. The lung X-ray and cardiogram were normal. She did not have regular gynecological check-ups and her last examination had been performed six years before. Family history showed no evidence of malignancies. On vaginal examination, mild cervical hemorrhage was noted, while the cervix was barrel-shaped, hypertrophic and of solid consistency. The uterine corpus was of normal size and solid consistency. The right and left adnexal areas were clear. Transvaginal color Doppler ultrasound (US) examination revealed that the uterine cervix was 35 x 40 mm, the uterine corpus 50 x 33 x 30 mm, while endometrial thickness was 1.3 mm. The size of the right and left ovary was 28 x 19 mm and 27 x 22 mm, respectively. In the tumor blood vessels, the flow was registered with a resistance index of 0.40. The peritoneal cavity was free of ascitic fluid. US of the abdominal organs was normal. The pelvic and paraaortic lymph glands were not enlarged. Colposcopy and biopsy of the uterine cervix were performed. The CA 125 tumor marker value was elevated to 80 mIU/ml. Magnetic resonance imaging (MRI) of the small pelvis revealed that the pelvic lymph nodes were not enlarged nor did the parametria have any pathological signs. The final pathological diagnosis of the uterine cervix biopsy was invasive squamous cell carcinoma of the uterine cervix (G2N2). The patient was subjected to radical abdominal hysterectomy with pelvic and paraaortic lymphadenectomy. No suspicious liver, gastric, intestinal, omental and peritoneal changes were evidenced intraoperatively. The final histopathological findings were primary double invasive cervical carcinoma, invasive keratinizing squamous cell carcinoma of the cervix, G2, and invasive adenocarcinoma of the endometrioid type, G1. Depth of invasion was approximately 3.5 cm. The histological finding of the neoplasm is presented in Figure 1. Other findings included endometrial cystic atrophy (Figure 2). The ovary, fallopian tubes, parametria, pelvic and paraaortic lymph nodes were without malignant signs. Staging was based on the FIGO clinical practice guidelines [13]. According to FIGO classification, the tumor was IB1 in our case. After surgery, the patient underwent combined stage radiotherapy, i.e., external teletherapy and intracavitary brachytherapy. Since treatment the patient has been well without any signs of relapse of the disease.
Discussion

Squamous cell carcinoma is the most common histological type of invasive cervical carcinoma. The incidence of invasive disease in the United States and in other developed countries is decreasing because it is being diagnosed earlier [14]. The incidence of cervical adenocarcinoma appears to be increasing relative to that of squamous cell carcinoma [12]. Adenocarcinoma may be detected by cervical sampling but less reliably so than squamous carcinomas. A definitive diagnosis may require cervical conization. Concomitant onset of both types of carcinomas is rare. Invasive adenocarcinoma may be pure or mixed with squamous cell carcinoma. Based on the World Health Organization data, approximately 500,000 women are affected with cervical carcinomas each year, out of whom approximately 250,000 die of the disease [15]. The mean age for cervical carcinoma is 52.2 years [4]. Our patient was 56 years old. The reported risk factors for development of squamous cell carcinoma include obesity, high parity, and long-term application of oral contraceptives, HPV infection, and smoking [5, 6, 7]. Long-term application of oral contraceptives is a risk factor for onset of adenocarcinoma, while smoking has had no influence on increased risk for onset of this type of carcinoma [6]. HPV infection types 16 and 18 increase the risk of development of both squamous cell carcinoma and adenocarcinoma of the uterine cervix [7], while Chlamydia trachomatis serotype 6 infections are most commonly associated with the consequential development of cervical squamous cell carcinoma [16]. Our patient has never been pregnant, has never used oral contraceptives, and was a non-smoker. Cervical carcinoma is most frequently detected in the early phase of development due to accessibility of the cervix for colposcopic examination and performing Pap smears. In our case, the carcinoma was diagnosed in the invasive form since the patient had failed to undergo regular gynecological check-ups. Vaginal bleeding is the most common symptom in the invasive stage of the disease which was also present in our patient. Based on FIGO classification, our patient had Stage IB1 disease. At the time of disease detection, 38% of patients are in Stage I, 32% in Stage II, 26% in Stage III and 4% in Stage IV [17]. In addition to colposcopy and Pap, color Doppler US also plays an important role in the detection of carcinoma. Application of transvaginal color Doppler US led to detection of low values of the resistance index (RI) in intratumoral blood vessels of approximately 0.40 in our case. The RI is significantly lower in patients with cervical carcinoma than in healthy women [18]. CA 125 was elevated to 80 mIU/ml in our case. Other authors reported increased values of the marker in 33% of patients with cervical carcinomas [19]. The main treatment for cervical carcinoma is surgery and radiation therapy. Radiation therapy is most commonly applied postoperatively. Early-stage cervical adenocarcinoma primarily treated by surgical intent has a very low risk of paraaortic metastases. Nerve-sparing radical hysterectomy with pelvic lymph node dissection and pre- and postoperative irradiation remains the treatment of choice for most patients with early-stage and even Stage IIB cervical cancer [20]. Squamous cell carcinoma has a better prognosis than adenocarcinoma, regardless of the stage of disease. Five-year survival rates were 90% versus 60%, 62% versus 47%, and 36% versus 8% for Stage I, II and III, respectively [12]. The absolute 5-year survival rates for the patients in Stage IA2, IB1, IB2, IIA and IIB were 94.4%, 90.7%, 84.1%, 71.1% and 55.4%, respectively [20].

Our patient was subjected to radical hysterectomy with paraaortic and pelvic lymphadenectomy. After surgical treatment, the patient underwent radiotherapy and since treatment the patient has been well.
Primary double invasive cervical carcinoma, squamous cell carcinoma and adenocarcinoma - Case report

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References


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Bilateral struma ovarii:
a case mimicking an ovarian neoplasm

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Summary

We present a case of bilateral struma ovarii which developed postoperatively and was histopathologically diagnosed after the patient was hospitalized for investigation and treatment of tumoral anexal bilateral formations. There was no evidence of clinical malignancy or metastases. Data from the literature, together with histopathologic, diagnostic and therapeutic aspects of the disease were checked again taking into account the scarcity of this lesion, especially bilaterally.

Key words: Struma ovarii; Germ cell tumors; Bilateral.

Introduction

Struma ovarii was first described in 1895 by Von Kalden and is extremely rare with only 150 cases reported in literature [1]. It is an ovarian teratoma made up entirely or predominantly by thyroid tissue (representing > 50% of tumor) with follicles of different sizes with a greenish or brownish yellow gelatinous content. It represents 0.85%-1.3% of all ovarian tumors. Most patients are asymptomatic, which is why the diagnosis is hard to establish preoperatively and is usually randomly discovered when magnetic resonance imaging (MRI) is performed [2, 3]. Struma ovarii represents an unusual tumor with extremely rare malignant changes occurring in less than 1% of cases [5] up to 5% [9], and in 90% of cases when it was situated unilaterally [10]. Most often the diagnosis has been made postoperatively by using macroscopic criteria with histopathologic confirmation [4-6].

Case Report

We present a case of bilateral struma ovarii histopathologically diagnosed from the surgical material. Based on data from the literature we tried to find the optimal therapy for this disease which was associated with uterine fibroleiomyoma and metrorragia. A 41-year-old woman, was hospitalized at the Department of Gynecology of the Clinical Hospital “Filantropia” of Craiova, with a diagnosis of bilateral anexal tumors, uterine fibroma, and metrorragia. Concerning the history, the patient presented with metrorragia and for about two months she had had diffused abdominal pain, abdominal distension, constipation and irregular menses. There was no objective or subjective data to confirm thyroid hyperfunction. A local examination revealed a slightly increased abdomen, especially in the lower part, slightly diffused when superficially palpated, with maximum intensity in the same area. A gynecologic exam found a moderately increased uterus and bilateral adnexal tumors. Abdominal pelvic ecography revealed two ovarian cystic formations, one was pluriseptate, hypoechogenic and/or hyperechogenic with small nodular fibroma and endometrial thickness of 0.8 cm. Tumor markers were normal: CA-125 measurement presented a value of 19 U/ml, while hCG, βhCG, and AFP revealed normal values. Laparotomy was performed and intraoperatively a slightly increased uterus was established with a deformed surface due to the presence of some small fibromatous nodules; the left ovary was increased to 6.5 x 5.5 x 4 cm, with variable consistency to which the left salpinx was closely joined. The right ovary was increased 7.5 x 5.7 x 4.2 cm and also had variable consistency, a globulous appearance, with the right salpinx joined to the right ovary. The two ovaries were sent for frozen section and the result was nonmalignant.

We decided to perform total hysterectomy with bilateral salpingo-oophorectomy. The material was processed and sent to the Laboratory of Pathological Anatomy of the same hospital. After the macroscopic exam, the next histopathologic technique was to parafin embed the microscopic pieces which were stained by H&E and Van Gieson. Macroscopic examination of the surgically removed piece helped us to reveal an almost normal sized uterus 10.55 x 8.4 x 6.3 cm, diminished consistency, a brown-grayish color, with the external surface slightly deformed due to the presence of some small nodular formations. On the section surface formations of variable sizes and white-necreous color were present with a slightly thickened and bleeding endometrium (0.8 cm). The left ovary was adherent to the salpinx and ovary, without any remarkable macroscopic changes. The left ovary 6.5 x 5.5 x 4.5 cm in size with a globulous appearance, yellow-greyish color, and variable consistency from fluctuant to a hard. On a section of the surface of a polymorphous aspect, with many cystic cavities ranging in diameter from 0.8 to 0.3 cm, some with serous or gelatinous contents, as the white of an egg, yellowish-orange, brownish or pistachio color. The right ovoid-shaped ovary had an irregular external surface, variable consistency presenting multiple cystic formations with a serous but also gelatious content of variable color from pistachio to red-brownish on a section presenting a white-necrous nodular formation in the center. Microscopic examination of the uterus revealed a simple hyperplasia aspect without atypia at the level of the endometrium, leiomyofibroma at the level of the uterine body and an aspect of bilateral struma ovarii. Microscopic examination of the seriate section from both ovaries presented ovarian histologic structures containing thyroid glandular tissue with variable fibrotic areas, inflammatory infiltrates, erythrocytes here and there, and cystic spaces lined by cuboidal or flat unstratified epithelium, with an eosinophilic content (Figures 1-6).
Discussion

Struma ovarii is an unusual tumor which rarely becomes malignant. It is dominated by increased thyroid tissue in an ovarian teratoma. Teratomas are considered as tumors made up of tissues originating from all three germ cell layers [7, 8]. Ovarian teratomas are imature and mature. Imature teratomas are most often malignant representing about 1% of all ovarian cancers [9, 10]. Mature teratomas are made up of adult type tissues derived from the three embryo sheets. Most are cystic representing about 25% of ovarian tumors. Mature teratomas are generally benign, and rarely do they...
become malignant but they can suggest a malignant appearance by their macroscopic aspect [11, 12]. Struma ovarii is the most frequent monodermal teratoma, a highly specialized form of a mature ovarian teratoma with maximum incidence in the fifth decade of life. Some cases present hyperthyroidism signs and often Meigs syndrome [13]. Macroscopically, struma ovarii is of a thyroid consistency and thyroid tissue aspect, predominantly solid and gelatinous [14]. Rarely does it appear associated with a dermoid cyst, or as a component of a stromal carcinoid [15]. Microscopically, stroma look like thyroid tissue to which any thyroid change, nodular or diffuse hyperplasia, but rarely carcinomas can be associated. Malignant transformation of the thyroid tissue may be papillary, follicular or mixed and it can include elements of mucinous cystadenocarcinoma [16]. In difficult cases, immunohistochemical stainings for thyroglobulin, triiodothyronine (T3) and tyroxine (T4) can confirm the diagnosis.

Conclusions

Because struma ovarii is extremely uncommon, each case must be individually treated. The diagnosis of struma ovarii should be suspected when a multicystic ovarian tumoral formation with a brownish colored gelatinous content – associated or not to clinical thyroidtoxicosis – is found. Surgical treatment is suggested. If it is associated with a thyroidtoxicosis, treatment of thyroid substitution should be performed but, in our case, after the surgical intervention the patient had a thyroid scan that was normal with normal thyroid function.

References