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CLINICAL IMPLICATIONS OF ANOMALOUS CD44 GENE EXPRESSION IN NEOPLASIA

Steve Goodison and David Tarin

UCSD Cancer Center 9500 Gilman Drive La Jolla California 92093 USA

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1. ABSTRACT

An intensive search continues for reliable markers that would be clinically useful in the diagnosis of small tumors and in the evaluation of their predicted clinical outcome. One potential marker, extensively studied in human samples is the cell surface adhesion molecule CD44. The single CD44 gene codes for a large family of cell surface proteins by alternative splicing and severe abnormalities have been observed in the patterns of its expression in many types of human tumors using both protein and RNA-based analyses. These abnormalities are manifested by markedly increased levels of unusual transcripts and proteins in tumor cells compared to the corresponding normal tissues. Aberrant processing of immature CD44 transcripts has also been observed in tumor cells and this can lead to the inappropriate retention of introns and to the use of cryptic splice sites in the mRNA. Inappropriate expression patterns of the alternatively spliced exons have also been linked both to tumor progression and to metastatic potential. The clinical relevance of all these observations is demonstrated by the frequent detection of these abnormalities in samples from malignant tumors of many different organs and by their presence in pre-invasive and high risk pre-cancerous lesions. This article reviews the current information regarding the expression of the CD44 gene in tumor cells and its potential use as a marker in clinical evaluation. The overall conclusion is that with the use of the latest assay techniques and perhaps in combination with other molecular markers, analysis of CD44 expression can provide new and powerful assays for the detection of neoplastic disease.

2. INTRODUCTION

As many as one in four people in industrialized countries die from the consequences of malignant tumors. It is widely recognized that the majority of cancers can be cured by surgical resection and appropriate adjuvant therapy if diagnosed at an early stage, before significant tumor cell dissemination occurs. There is, therefore, a strong incentive to find methods of early diagnosis and for assessment of prognosis and treatment of neoplastic lesions.

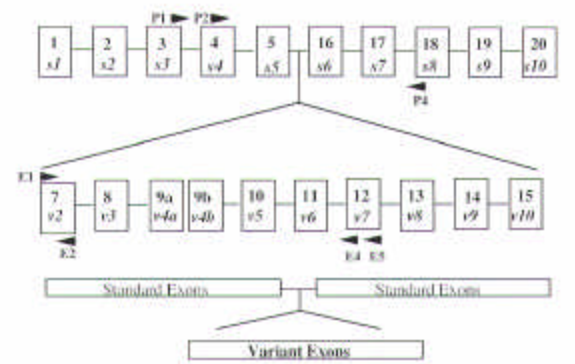

Figure 1. Map of the human CD44 gene. The nomenclature of standard (s) and variant (v) exons is shown. Arrowheads denote primer annealing positions as used for RT-PCR hybridization analysis.

As research advances our understanding of the genetic and biological changes involved in the progression of cancer a variety of molecules become of interest as potential markers. The identification of molecules which have expression patterns specifically related to neoplasia can not only aid the development of diagnostic tests but may also provide targets for potential therapeutic intervention.

Recent studies have provided evidence that the expression of the CD44 gene is specifically altered in many types of tumors. As the disorder is present in the early stages of neoplasia and can be identified in small samples using amplification techniques, it seems a promising candidate as a marker for early diagnosis and for monitoring patients during treatment.

The group of proteins encoded by the CD44 locus first attracted interest as a result of efforts by several research laboratories to identify and characterize cell surface molecules on white blood cells which might be important in cell mediated immunity. These investigations included studies to isolate molecules involved in organizing the circulatory traffic of lymphocytes as they patrol through the organs of the body on surveillance missions (1). The work resulted in the raising of a number of monoclonal antibodies (mAbs) which were eventually recognized to bind to different epitopes on a family of structurally related proteins which were assigned the designation CD44 (Cluster of Differentiation 44) by the International Workshop on Human Leucocyte Differentiation.

Subsequent studies (2, 3) with these antibodies rapidly made it clear that the CD44 family of proteins are not confined to the surfaces of white cells and that some of them appear to be ubiquitously distributed on all cell types. It also emerged simultaneously that they are not just involved in immunological activities, but in a surprising diversity of biological processes (4, 5). Some of the functions in which they have been implicated include lymphocyte recirculation, T lymphocyte activation, adhesion to other cells and to the intercellular matrix, embryonic development, hyaluronan metabolism, signal transduction across the cell membrane and growth factor secretion.

Various regions of the gene were subsequently cloned and sequenced by several different groups (6-9). The human CD44 gene has been mapped to the chromosomal locus 11p13 (10) and confirmed to encode a family of large transmembrane glycoproteins. The gene is composed of two groups of exons (figure 1), one group comprizing exons 1-5 and 16-20, are expressed together on all cell types as the standard form (CD44s). The 10 variable exons (exons 6-15) can be alternatively spliced and included within the standard exons at an insertion site between exons 5 and 16 (6, 7, 11, 12). Transcripts or proteins containing the variable exons or their peptide products are designated CD44v. The variant isoforms differ in which peptide units are included in the extracellular region of the protein, theoretically alternative splicing would allow more than a thousand CD44 variants to be generated. Further diversity of CD44 results from post-translational modifications, including N- and O-linked glycosylation, to create a large polymorphic family of trans-membrane proteins (13-15).

Details of the mechanisms regulating CD44 synthetic activity in different cells and tissues are currently unknown and there are many more details yet to be understood on the relationship between the structure and functions of the many isoforms which can be produced by this gene.

The further complexity of the processes in which this gene may be involved in cell and tissue function was revealed when it became apparent that its expression is characteristically and severely changed in many common types of neoplasia. It has also been shown that the expression of some CD44 isoforms is associated with the acquisition of metastatic ability in some cell lines. The following account will summarize current knowledge of the unusual patterns of CD44 expression in cancer and consider their clinical implications.

3. CD44 EXPRESSION IN NORMAL TISSUES

The CD44 family of proteins is ubiquitously expressed, but the expression of some variant isoforms appears to be tissue-specific. Studies using monoclonal antibodies have shown that the majority of epithelial, hematopoietic and other non-epithelial cells predominantly express the CD44s isoform which lacks any variant exon products. Many epithelial cells also express the larger “epithelial “ isoform known as CD44E which has products of exons v8-10 (figure 1) included and this isoform is often expressed in excess of CD44s (16). A few cell types, including hepatocytes and pancreatic acinar cells and cells of some specific structures within organs such as the ducts and tubules of the kidney and pancreas do not express CD44 at all (3, 17).

The expression of CD44 isoforms containing other variant exons is far more restricted in normal tissues. The expression of v6 has been observed in the ducts of breast (17) and pancreas (18, 19) and v4 may be expressed in normal urothelium (20). The expression of variant isoforms is detectable in the proliferative stem cells within

epithelial tissues (3) and they are also expressed at high levels and in many combinations in hemopoietic cells (21). Isoforms containing many of the variant exons, but predominantly v10, v6 or v3 are expressed in peripheral blood mononuclear cells (22) and in reactive lymph node cells (21, 23). The human CD44 gene contains a stop codon within exon 6 (v1) and so does not code for a protein product as it does in rats and mice.

The major ligand for CD44s is hyaluronan , a component of the intercellular matrix. Although all isoforms include the binding site at exons 2 and 5, the inclusion of variant peptides into the protein is known to alter the affinity for this substrate and to affect the binding of alternative substrates such as fibronectin (24) , collagen (25), serglycin (26) and osteopontin (27). Given the information that in certain circumstances CD44 proteins can also exercise signal transduction functions, it is possible that this group of proteins is active in epithelial mesenchymal interactions which are known to be essential for the establishment and maintenance of normal organotypic histological structure.

4. CD44 EXPRESSION IN NEOPLASIA

The deduction from early studies was that CD44 is involved in cell attachment phenomena such as the binding of circulating lymphocytes to vascular endothelium and of sedentary epithelial and stromal cells to each other or to the intercellular matrix. This induced curiosity about the status of these cell surface molecules in tumors. Initial studies, using Northern blotting (15, 28) suggested that tumor tissues contained additional, unusual CD44 transcripts relative to those present in corresponding normal tissues. Separate work on tumor cell lines (29, 30) indicated that cells with elevated levels of CD44 proteins were capable of making more aggressive tumors in animal experiments. However, more rapid and more sensitive analysis of CD44 gene expression was achieved when the technique of reverse transcription-PCR (RT-PCR) became widely used. Many studies using PCR and/or immunohistochemical analyses have revealed that there is overproduction of a striking array of unusual CD44 species in a wide variety of tumor tissues relative to their normal counterparts. The changes in the patterns of expression of a multi-functional gene in a single tissue of an organ are likely to disrupt essential epithelial-mesenchymal interactions and thus to contribute to the progressive structural and functional disorganization characteristic of cancer. The following discussion focuses on CD44 expression in the commonest neoplasms, on which most information is available.

4.1. Breast

Abnormalities in CD44 expression in breast cancer were initially observed using the technique of RT-PCR (31). This sensitive technique showed over-expression of an extensive variety of alternatively spliced CD44v transcripts in fresh tissue samples from most primary breast cancers (figure 2) and from their lymph node metastases. Tumors from patients with no clinical evidence of metastases showed similar but less severe disturbances in CD44 activity when studied by this method. Immunohistochemical evaluation has demonstrated increased expression of CD44s and variant isoforms v5 (32), v6 (33, 34) and v9 (33) in carcinoma cells relative to normal breast epithelium, which does not express CD44v epitopes other than in myoepithelial cells surrounding the luminal epithelium (32, 35) (figure 3). Another study reported that there was a significant correlation between expression of v3, v4 and v6 and increased tumor grade in breast cancers (36). Benign breast tumors (fibroadenomas) have not been studied extensively but the data available, obtained with RT-PCR (11), indicate that CD44 expression is raised and abnormal in these neoplasms.

Aberrant CD44 expression has also been demonstrated in breast cancer cell lines. The patterns of expression of the variant isoforms differ between the several lines so far examined and can also be altered by culture conditions, including treatment with hyaluronidase (37). Some authors report that cell lines with higher estrogen receptor levels show more expression of the epithelial variants of CD44, than those with low estrogen receptor status (38) However, others (39) have indicated that in a different group of cell lines overexpression of CD44 was associated with low levels of estrogen receptor production, elevated vimentin expression and higher invasive and chemotactic activity in assays *in vitro*. In a study using micro-dissected tumor samples, the levels of CD44s showed no correlation with ER status. However, the pattern of expression of larger forms of CD44 incorporating variant exons v7 and v10 was significantly different between ER+ve and ER-ve tumors, suggesting a link between the profile of CD44v expression and cellular differentiation as indicated by the ER phenotype (40). The findings on cell lines may in themselves have no direct clinical implications, but they do indicate that tumor cells can display diverse phenotypes and that multivariate analysis of several tumor markers may therefore be needed to allow more accurate assessment of the malignant potential of a given lesion.

4.2. Lung

There have been relatively few studies to date on CD44 expression in fresh tissue samples of the lung, considering the prevalence of these tumors in industrialized countries. In a study using polyclonal antibodies to undefined epitopes of human CD44, Penno *et al* (41) described high expression of CD44 proteins in the majority (10 of 14) of non-small cell carcinomas (NSCLC), but little or no expression in any of 9 small cell tumors (SCLC). However, in more recent studies, staining for CD44 v4/5 was markedly increased in SCLCs (72%) as compared with adenocarcinomas (2.2%) (42) and expression of the CD44 isoforms v5, v7, v8, and most notably that of v6, were found to strongly correlate with tumors of squamous cell and bronchio-alveolar carcinoma origin (43). Intense immunoreactivity for CD44v6 has also shown to be present in 19 of 20 (95%) metastatic lung carcinomas (42) and its expression is maintained throughout tumorigenesis in squamous cell carcinoma and bronchio-alveolar cell carcinoma, (44). In non-neoplastic lung tissue, moderate v6 staining can be seen in lymphocytes and macrophages in

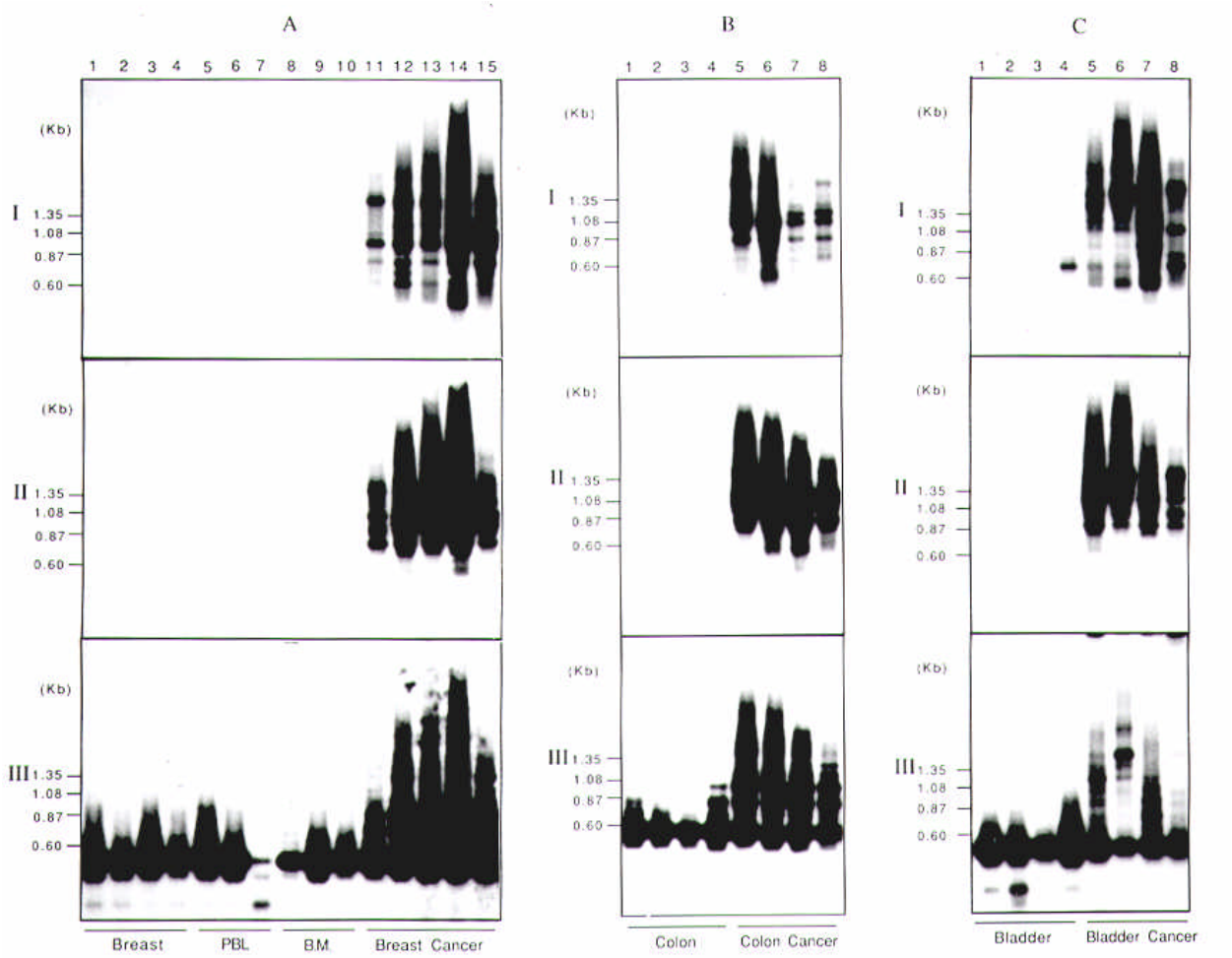

Figure 2. Autoradiograph of filters hybridized with CD44 exon specific probes. RT-PCR products, amplified using primers P1 and P4 (see Figure 1) were electrophoresed and blotted to nylon membranes. Hybridization was performed with DNA probes specific for variant exon 7 (row I), variant exon 12 (row II) and standard exon 4 (row III). Panel a: Normal breast tissue (lanes 1-4), peripheral blood leukocytes from healthy volunteers (lanes 5-7), sternal bone marrow from patients with heart disease (lanes 8-10), and breast cancer tissue (lanes 11-15). Panel b: Non-neoplastic colon tissue (lanes 1-4), normal tissue in lanes1 and 3, inflamed colonic mucosa from Crohn's disease in lane 2 and ulcerative colitis in lane 4. Tissue from primary colon cancer (lanes 5-8). Panel c: Normal bladder tissue (lanes 1-4) and primary bladder cancer tissue (lanes 5-8).

the parenchyma, and is strongly detected in type II pneumocytes and the bronchiolar basal cells (41, 42, 44). Therefore, as in other tissues, care must be taken in interpretation of analyses that use material from homogenized samples.

More studies have been conducted on lung tumor cell lines (6, 41, 45). Using immuno-fluorescence and RT-PCR, Penno *et al* (41) found that NSCLC cell lines expressed detectable amounts of CD44s but that 5 out of 6 SCLC lines expressed little or none. This is in general agreement with the observations of other investigators (6, 45) but occasional LCLC or squamous cell cancer lines which express several CD44 splice variants have been described. There have not as yet been enough studies conducted on lung tumor tissues to reach a consensus regarding CD44 expression patterns. The contradictory data obtained by the IHC studies also highlights another problem of human tissue analysis. For direct comparison, tumors need to be categorized and dealt with experimentally in the same way. Differences in tumor type, staging and grading of specimens and with IHC, the use of different antibodies and the way in which antigen retrieval protocols are performed often vary between laboratories.

4.3. Gastrointestinal

Several studies have provided evidence that CD44 gene expression is abnormal in gastrointestinal tumors. Most of these have been conducted on carcinomas of the colon and stomach, which are the commonest sites affected by neoplasia in the GI tract.

Observations on samples of tumor tissue from patients with stomach cancer (46, 47) have demonstrated

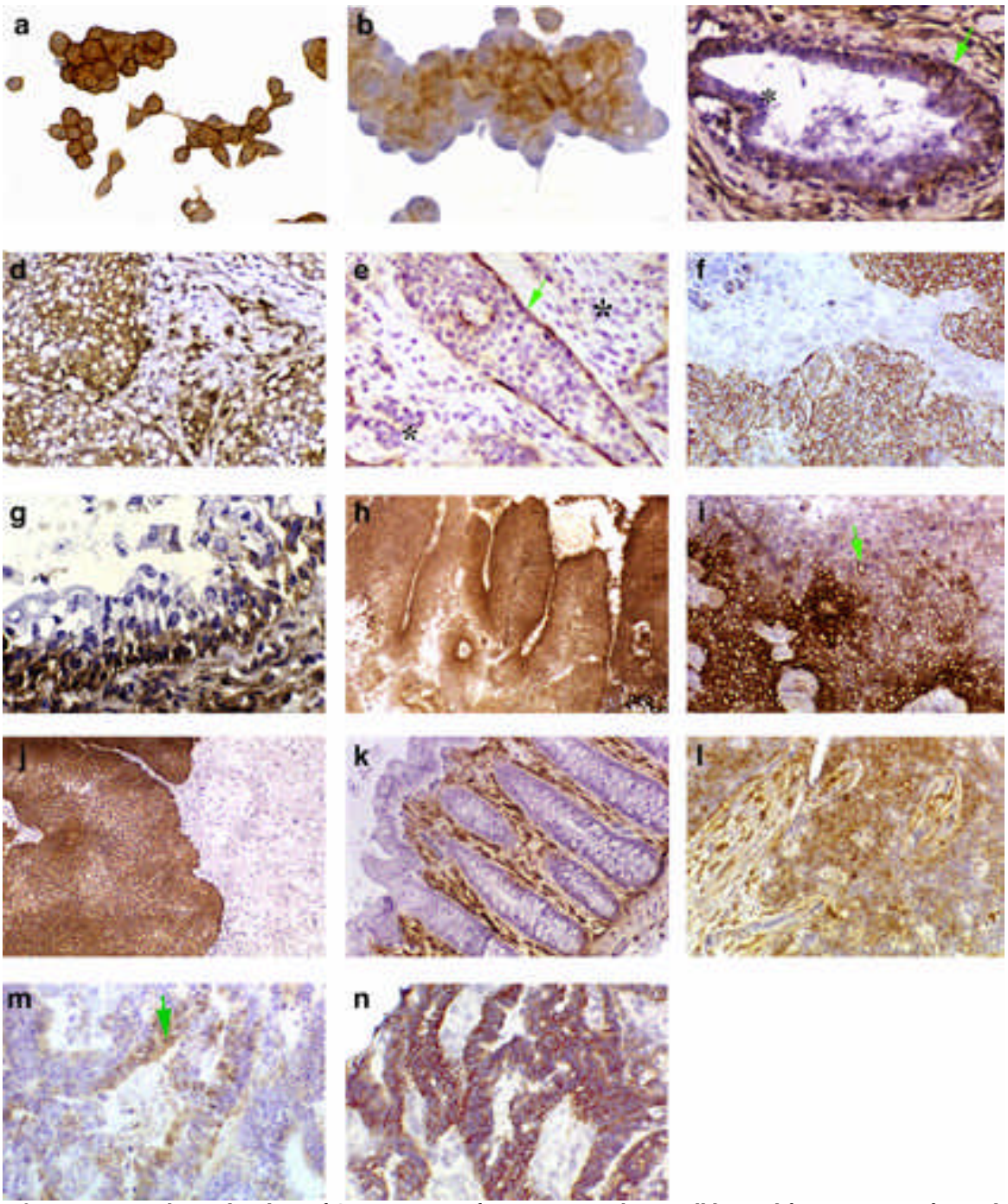

Figure 3. Immunocytochemical analysis of CD44 protein isoforms in ZR75-1 breast cell line and frozen sections of normal and malignant breast, bladder and colon tissue. In ZR75-1 cells, strong membrane and cytoplasmic staining was observed with (a) mAb Hermes 3 (exon 5 epitope), and (b) mAb 23.6.1 (exon 7 epitope). Plates (c-n) show representative immunohistochemical staining in normal and malignant tissues. (c) shows a normal breast duct stained with Hermes 3 (exon 5 epitope); myoepithelial cells showing positive immunoreactivity are arrowed, whilst negative ductal epithelial cells are marked with an asterisk. Plates (d)-(f) show malignant breast tumors stained with Hermes 3, mAb 23.6.1 (exon 7 epitope) and mAb 2F10 (exon 11 epitope) respectively. The arrow in plate (e) shows positively stained myoepithelium and the asterisks mark the position of negatively stained tumor cells. Plate (g) shows normal bladder stained with Hermes 3, (h)-(j) malignant bladder stained with Hermes 3, mAb 23.6.1 and mAb 2F10 respectively. (k) shows normal colon stained with Hermes 3, (l)-(n) malignant colon with Hermes 3, mAb 23.6.1 and mAb 2F10 respectively. The arrow in (m) marks malignant cells exhibiting immunoreactivity with Mab 23.6.1.

elevated and abnormal expression of several CD44s and CD44v isoforms. Tumors of the gastric mucosa display increased expression of isoforms containing v8-10 and the expression of these has been correlated with invasion and the presence of metastases in these lesions (48, 49). Some correlation with the expression of CD44v5 and v6 with histological subtype has been suggested (50) and these isoforms are also present in intestinal metaplasia of the stomach, a condition which is considered to be premalignant. Different patterns of unusual CD44 transcripts have been observed in intestinal- and diffuse-types of adeno-carcinomas (50) supporting the interpretation based upon previous evidence (51) that these two types of tumors, which have different behavior patterns and prognoses, may also have different genetic pathways of induction.

Although normal cells of the intestinal epithelium predominantly express CD44s, as in the gastric mucosa, the proliferative stem cells at the base of the crypts have been shown to express variant isoforms (52, 53). Immunohistochemical observations confirm that small amounts of CD44s proteins can be detected in the epithelium at the bases of the intestinal crypts (figure 3) and in the lymphocytes, fibroblasts and macrophages in the lamina propria (32, 35, 54). In colonic tumor tissues, however, there is a marked increase in both CD44s and CD44v, detectable by RT-PCR (31, 55, 56) (figure 2) and IHC (47, 52). Both methods show that many variant isoforms are overproduced in colonic tumor tissues in an inconsistent and disorganized manner. These patterns of expression are not seen in control samples from patients with inflammatory or other non-neoplastic conditions (31).

Colonic adenomas and carcinomas express a large range of CD44v isoforms (32, 57) but v5 and v6 isoforms are predominant (47, 56) and there appears to be a correlation with the quantity and range of CD44v expression with increase of the size of lesion (58). The alterations in CD44 expression also seem to precede the altered expression of other colorectal tumor markers such as K-ras mutation, DCC deletion and p53 expression (59). CD44v5 has been reported to be an early tumor marker, expressed before v6, expression of which has been shown to rise with progression of the tumor (60). A recent study also showed that proximal tumors express more v5/6 than distal tumors (61).

Contradictory data is available (62) in that CD44 staining diminished through progression from adenoma to carcinoma to metastasis. Another study showed that v10 was required for metastasis in colorectal cancer (63). There are very few reports so far on CD44 activity in malignant tumors in other parts of the gastro-intestinal tract. This reflects the lower incidence of tumors in such sites.

4.4. Uterine cervix

Expression of the CD44 standard isoform protein has been shown immunohistochemically in the normal cervical subepithelial stromal cells and throughout all the cervical epithelial layers (64). The expression of epitopes encoded by the variant exons 9 (v4), 11 (v6) and 14 (v9) of this gene was also detected in both the basal and supra-basal epithelial cells. However, these epitopes are absent in the epithelial mid-zone and in the stratum superficiale (64-66). In a recent study, we have confirmed this pattern of standard and variant (v6) protein expression and obtained further data showing that variant protein isoforms containing v2 and v5 epitopes are also present in the basal and mid zone of normal cervical tissue (Gorham and Tarin-unpublished data). These CD44 epitopes are absent in the superficial layers.

Some studies have reported a decrease in expression of variant isoforms in invasive cervical carcinomas. In a study of forty-five cervical intraepithelial neoplasias (CIN) which analyzed CD44v5, v6, and v7-8 expression by IHC, a negative correlation of CD44v5 expression with the grade of CIN was found and the authors suggest that a loss of v5 occurs progressively during dysplastic transformation (67). A study using RT/PCR demonstrated the presence of CD44s and v6 in all samples of normal cervix as well as those with invasive carcinomas (66) but they reported that tumor specimens appeared to contain exon 11 (v6) in higher molecular transcripts than did specimens of normal cervix. In the same study immunohistochemistry confirmed the presence of CD44s and v6 and revealed v9 expression in normal cervical epithelium. In malignant samples, heterogeneity in staining was apparent and poorly differentiated and carcinomas from patients with poor prognosis did not stain at all.

However, some studies suggest that, in contrast to normal cervical epithelial differentiation, cervical carcinogenesis may be characterized by the up-regulation of CD44 splice variant expression. In addition, a novel abnormal CD44 isoform has been described in cervical cancer patients. The splice variant features a transcript containing exons 12 (v7) and 13 (v8) and this pattern of variant exon splicing was not detectable in normal cervical epithelium (64). Such divergent observations could be due to differences in the stages of the tumors under study or their degrees of histological differentiation. Reduced expression of CD44 variant protein isoforms, particularly v6 (exon 11) has been observed in other invasive squamous cell carcinomas, such as those of the head and neck (68).

4.5. Lymphoid tissue

Substantial evidence now indicates that CD44 is involved in numerous lymphoid cell activities, These include lymphocyte recirculatory traffic, cytokine release and T-cell activation (4). It is also known to be involved in directing immature thymocytes from the marrow to the thymus (69, 70) and the maturation of B-lymphocytes in the bone marrow (71).

In lymphoproliferative disease, there is a high expression of the standard form in most cases of non-Hodgkins lymphoma (NHL) (70, 72). The immunohistochemical evidence suggests that in the more aggressive, high grade, malignant lymphomas there is strong up-regulation of expression, both in the tumor tissue (70, 72, 73) and in the serum (74), of several CD44 splice variant isoforms, particularly those which include products from variant exons 8 (v3), 11 (v6) and 14 (v9) (72, 73). While the exact patho-physiological significance of the up-regulated CD44 isoform expression remains undefined, it is possible that splice variant analysis may help to stratify the high grade lymphomas with respect to individual prognosis (21).

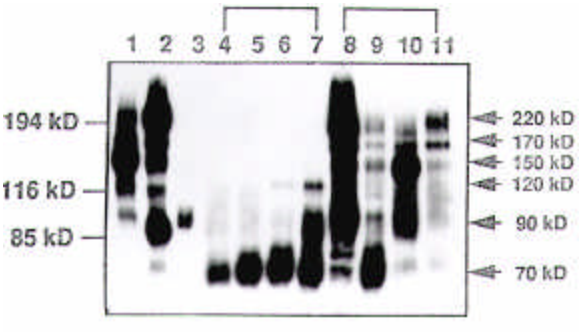

Figure 4. Western blot analysis of cell lysates with Hermes 3 (CD44s): Arrows show dominant CD44 proteins. Lanes 1, 2 and 3 show results with cell lysates from HT29 colon cancer cell line (0.5mg protein/lane), RT112 bladder cancer cell line (0.5mg/lane) and normal human leukocytes (1mg/lane), respectively. Major protein bands of ~150kD, ~220kD (plus ~85kD) and ~90kD are seen in HT29, RT112 and leukocytes, respectively. The 90kD band corresponds to CD44s, the 150kD band to the epithelial isoform and the 220kD band to the largest variant isoform. The urine specimens (5mg/lane) from normal people (lanes 4-7) contain one to three band(s) of MW ~70kD to ~120kD, whereas several bands of ~70kD to ~220kD are seen in specimens from cancer patients (lanes 8-11).

4.6. Urological

The urinary tract is lined by a continuous mucosa covered by a layer of transitional cells and is a common site for the formation of malignant tumors. The most common site affected is the bladder, where concentrated urine is stored for hours at a time. The normal urological mucosa expresses CD44s on the cells in the basal layer of the epithelium as well as on the fibroblasts and other cells in the underlying connective tissue (32, 75) (figure 3). Immunohistochemical studies show that small quantities of CD44v5 and CD44v6 are also detectable in this tissue but their distribution, like that of the standard form is confined to the basal layer. As the differentiating cells migrate towards the surface of the epithelium they begin to shed CD44 with the result that desquamated epithelial cells show no reactivity with CD44 mAbs (75). In contrast, RT-PCR studies show a marked increase in CD44s and CD44v mRNA transcripts (figure 2) and a relative increase in the corresponding CD44v proteins in the cells obtained from the patients with cancer, as shown by comparative Western blot analysis (32, 75) (figure 4). In a study by Muller *et al* (76) using mAbs and ELISA, all investigated transitional cell carcinomas expressed CD44v2 protein and no differential expression between invasive and noninvasive carcinoma was observed. Because of difficulties in direct comparisons between protein-based investigations, assay of CD44 expression at the RNA level may be as yet, the more useful marker for urothelial cancer (32, 76).

4.7. Other tumors

CD44 expression has been analyzed in many other human tumors including renal cell carcinomas (77), leukemias (78), pancreatic carcinomas (18) hepatocellular carcinoma and melanomas (79, 80) with the general theme of CD44 variant isoform over-expression in tumor samples in comparison to their normal counterparts being observed. However, there are a number of human tumor tissues which appear to down-regulate CD44 expression. Reports indicate that ovarian carcinoma cells collected from ascites do not express CD44 (81), variant isoforms are not expressed in small-cell lung cancers (82) and squamous cell carcinomas down-regulate CD44v6 expression (68). There also appears to be an inverse correlation between CD44s expression and differentiation in prostate cancers (83). CD44 is not expressed in advanced neuroblastomas (84) and the down-regulation of CD44s has been shown to occur as tumor cell differentiation progresses (85). Variant CD44 isoforms do not appear to be present at any stage in neuroblastoma (86) and CD44 expression has been identified as a favorable prognostic factor for this form of cancer (87-89). There are reports of CD44v expression on a few neoplastic cells of gliomas and glioblastomas (90, 91) but overall the pattern of expression observed on non-epithelial neoplasms is confined to CD44s isoforms.

4.8. Aberrant Processing of CD44 transcripts

Much of the data regarding CD44 gene expression has been gathered using RT-PCR techniques and with this form of analysis distinctive large molecular weight amplicons are consistently observed in tumor cell-containing samples. Indeed, some of these appear to be larger than would be predicted even if all possible variant exons were included in the transcript, indicating the presence of abnormal gene transcripts. This highly characteristic abnormality could result from incompletely or mis-processed mRNA species, possibly containing retained intronic sequences. This hypothesis was investigated by characterization of CD44 introns and looking for retention in CD44 mRNA transcripts in tumor samples. Matsumura *et al*, described for the first time that detection of tumor cells in a sample could be achieved by monitoring intron retention in CD44 mRNA (9). Retention of the normally removed variant intron 9 was first observed in exfoliated urothelia of bladder cancer patients and was subsequently found also to occur in breast carcinoma tissues (38) and in colon carcinoma (92) (figure 5).

We have recently assessed whether such retention is specific to certain introns, or is a more general phenomenon affecting CD44 gene expression in tumor cells. Intron 18 was cloned and sequenced from genomic DNA and the novel sequences analyzed and used to create intron 18-specific probes. Retention of the newly characterized intron 18 was found in 15 of 20 (75%) of colonic tumor tissues, whilst it was retained in only 3 of the 20 (15%) matched normal tissues. In the previous study (92), the abnormal retention of intron 9 in colonic tissue transcripts was found in 16 (80%) out of 20 patients with carcinoma and in 4 of the corresponding normal tissues. The incidence of intron 9 and/or intron 18 in these samples did not appear to follow a pattern. Transcripts containing introns 9, or 18 alone, or both introns together, or in combination with intron 17 were all found in tumor cell mRNA (Goodison and Tarin-unpublished data). These results show that aberrant splicing is not intron-specific and intron retention therefore now joins inappropriate and

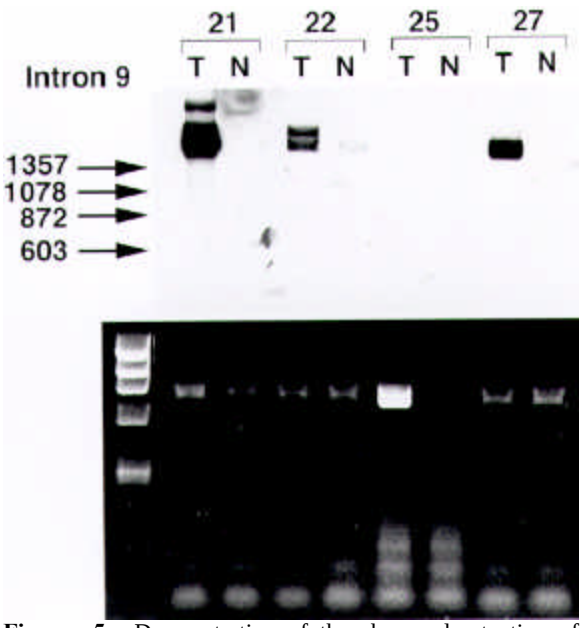

Figure 5. Demonstration of the abnormal retention of CD44 intron 9 in mRNA transcripts from human colon carcinoma tissues (T) and matched adjacent normal mucosa (N), (cases 21-27). RT-PCR was performed with primers annealing to exons 7 and 12, hybridization was performed with a DNA probe specific for intron 9. Ethidium bromide staining of the gel before blotting is presented in the lower panel.

disorderly over-expression of variant exons as a characteristic feature of CD44 gene expression in neoplasia. The consequences of abnormal intron retention for CD44 function could be profound. In the event of introns upstream of exon 17 being retained, the encoded isoform would contain only part of the extracellular portion of the protein and could be soluble. The protein could then be retained in the cytosol and/or secreted because of the absence of the short anchoring transmembrane region encoded by exon 18.

Studies by Ermak *et al* have shown that thyroid and breast tumor cells also produce CD44 transcripts which have been aberrantly processed using "cryptic" splice sites. They described a novel CD44 transcript containing a junction between subsegments of exons 4 and 13 whilst maintaining the correct reading frame throughout the cDNA (93) and found that the presence of this isoform was greatly increased in goiters, adenomas and cancers of the thyroid compared to normal tissue (94). They went on to show that breast tumor cells also produce abnormally spliced isoforms with a junction between exons 3 and 13, although this transcript did not contain a complete open reading frame. The use of cryptic splice sites is thought to occur when the correct sites are unavailable through incorrect folding or complexing resulting in "second-choice" splicing events. The fact that abnormal splice site choices and intron retention occurs in dysplastic cells suggests that alternative splicing mechanisms are severely compromised in tumor cells.

Studies correlating CD44 mRNA and protein profiles simultaneously in the same populations of tumor cells (32) have provided further intriguing insights into the degree of misregulation of this gene in neoplasia. Investigations have revealed that the production of transcripts and the translation of these gene products appear to become uncoupled. Analysis of a number of tumor cell lines revealed that many prominent transcripts identified by PCR did not have protein counterparts as analyzed by Western blots using mAbs which recognize epitopes encoded by the corresponding exons (32). It is possible that the discrepancy is due to the sensitivity of PCR amplification but some of the mRNA transcripts that were translated were no more prominent than those that were not. The retention of introns or cryptic splice site usage in some of the transcripts would result in abrogation or truncation of the translation process or possibly inappropriate degradation or secretion of products containing incorrect compartmentation-targeting domains. This possibility gains some support from reports that soluble truncated forms of CD44 can be detected in culture media (32) and in elevated amounts in clinical samples of body fluids such as blood (95) and urine (Woodman and Tarin-unpublished data) from cancer patients.

4.9. Summary of CD44 in neoplasia

The majority of publications have described elevated CD44 transcription and translation in the most common types of cancers. RT-PCR consistently reveals an extensive array of amplicons of unusual sizes including many that are much larger than would be predicted from the known structure of the gene and the primers chosen for amplification (figures 1 and 2). Southern blot hybridization analysis with probes for specific exons indicate that the abnormality affects the expression of all regions of the CD44 gene, but it is clear that the expression of the variant exons is particularly elevated. Detailed exon-junction analysis by PCR has revealed that the order of assembly of the exons in the transcripts is also altered in tumors relative to comparable normal tissue and that many different variations can be seen in the same specimen (57, 93, 94).

Immunohistochemical studies have revealed that there is not only an overall increase in the amount and diversity of CD44 protein production in tumors, but also a marked heterogeneity in the pattern of expression within the neoplasm. In carcinoma cell populations the normal orderly, localized expression of CD44 isoforms is lost and these changes are expected to disrupt essential epithelial-mesenchymal interactions and thus contribute to the progressive structural and functional disorganization characteristic of cancer.

The data which is available on early pre-malignant lesions such as dysplastic areas in the bladder (8) and adenomas of the colon (96, 97), indicate that the disorderly expression of this gene begins when the neoplasm is first forming and increases as the tumor becomes larger, invasive and metastatic. However, it has been observed that deeply invasive cells in late stage, advanced, aggressive cancers of the bladder (98) and the colon (Tarin-unpublished observations) uniformly show

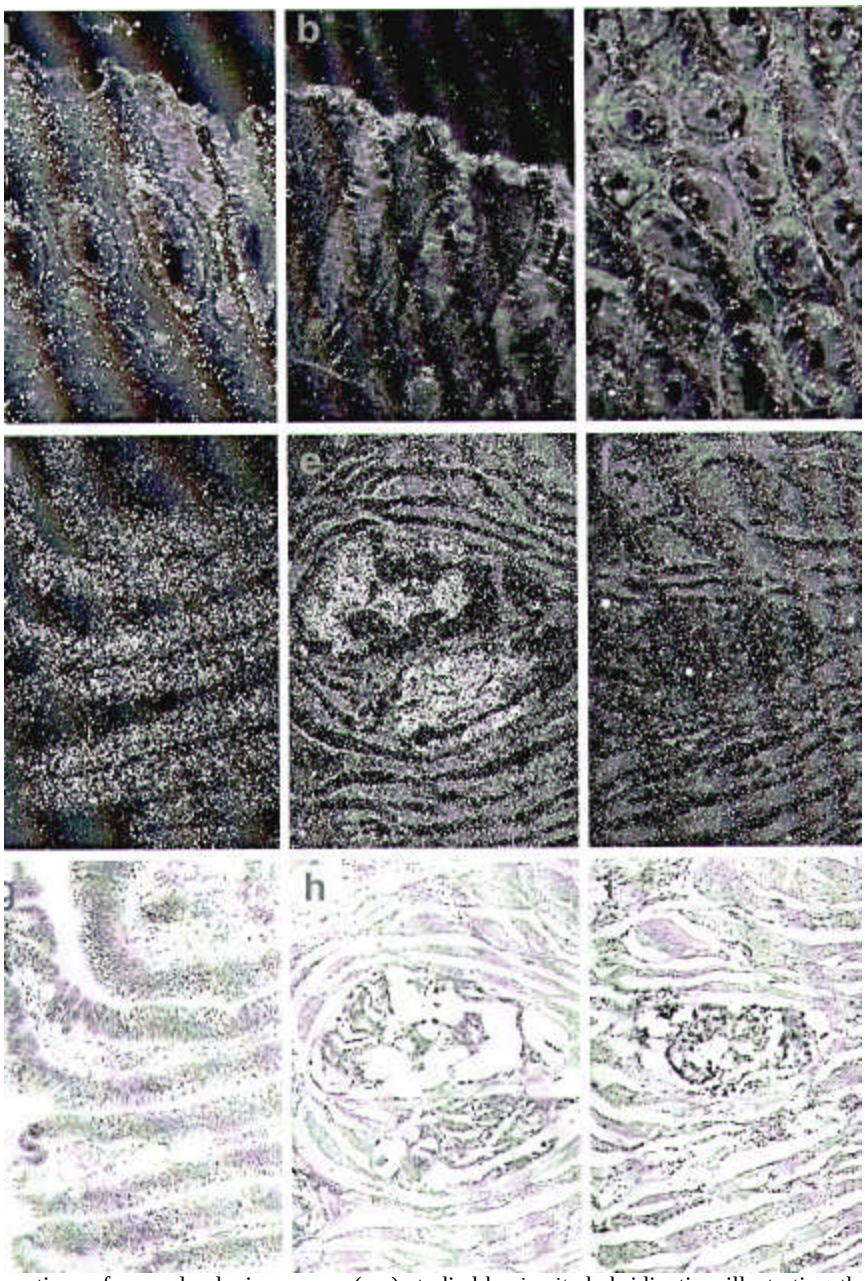

Figure 6. Frozen sections of normal colonic mucosa (a-c) studied by *in situ* hybridization illustrating the presence of CD44 standard mRNA transcripts in basal crypts, infiltrating lymphocyte and macrophages. Plates (a) and (b) hybridized with CD44(s) antisense and sense riboprobes respectively. In comparison plate (c) CD44(v) antisense riboprobe gave minimal signal. (Darkfield images, magnification x 430). Plate (d) shows a darkfield image of colonic adenoma hybridized with CD44(v) antisense riboprobe showing increased signal compared to normal colonic mucosa. Plate (g) shows the corresponding brightfield image. Plates (e) and (f) show darkfield images of colonic carcinoma hybridized with CD44(v) antisense and sense riboprobes respectively, the elevated signal located over carcinoma cells relative to normal colonic epithelium (plate c) and to surrounding non-malignant cells being clearly demonstrated. Plates (h) and (i) are corresponding brightfield images (magnification x 215).

little or no expression of any isoform as they reach the boundaries of the organ or penetrate surrounding tissues. This suggests that although the expression of CD44 is disorderly in tumor cells, the regulation of CD44 transcription is not beyond the influence of the extracellular environment. This has recently been confirmed in studies using tumor cell lines cultured under various growth conditions (99).

When analysis of expression is performed on RNA extracted from a tissue sample the possibility exists for the contamination or dilution of the tumor cell

transcripts by RNA from non-neoplastic cell types in the homogenized sample. However, *in situ* hybridization studies performed on specimens of tumors and corresponding normal tissues from the colon, breast, bladder and cervix (97, 98, 100), have confirmed that the over-expression of CD44 transcripts occurs only in the tumor cells, not in reactive inflammatory cells which may be in the vicinity nor in non-neoplastic resident cells of the supporting stroma (figure 6).

4.10. CD44 and metastasis

Much interest and investigative effort has centered on the possible involvement of CD44 isoforms in tumor metastasis. Because of its known role in adhesion to components of the connective tissue matrix including hyuloranan, fibronectin and collagen, Stamenkovic *et al* suggested that the increased production of CD44 proteins, which they observed in several carcinoma samples, could facilitate invasion and metastasis (28). Subsequently, Gunthert *et al* demonstrated that an antibody which inhibited metastasis of a rat pancreatic adenocarcinoma cell line, specifically recognized an epitope on exon v6 (exon 11) of this gene (101). Furthermore, transfection of a non-metastatic cell clone from the same rat pancreatic cancer, with a construct containing this exon, induced it to become metastatic. Additionally, two other groups provided evidence indicating that, in experiments with cultured human melanoma (29) and lymphoma cell lines (30), there appeared to be a correlation between raised CD44 expression and metastatic capability. However, in these reports it appeared that it could be CD44s rather than CD44v isoforms which was responsible for, or associated with, the metastatic behaviour. Another group has reported that, at least in animals models, there is evidence that CD44 alone is not responsible for metastatic capability. A murine lymphoma cell line with a CD44 gene double knockout, was shown to retain its local invasiveness and metastatic potential despite losing all hyaluronan-binding ability (102). However, as these studies were performed on different rodent cell lines the issue of whether expression of CD44 plays an important role in natural tumor metastasis remains an open question.

5. CLINICAL IMPLICATIONS

The majority of cancers can be cured by surgical resection, if diagnosed at an early stage and this has led to the introduction of cancer screening programs in many countries. It is also becoming evident that identification of pre-malignant states and of genetic susceptibilities to certain types of tumors can lead to the development of medical strategies which prevent or substantially delay the onset of frank neoplasia.

The histological and cytological analysis of solid tissue biopsy material is considered to be the gold standard for the detection of malignant disease but this requires evaluation by skilled pathologists and can be somewhat subjective as visual assessment is based on morphological cellular changes. The surgical procedures which are used to obtain the material are obviously uncomfortable for the patient and also carry the risk of increasing dissemination of cells from the lesion. There is a need to identify molecular markers which could aid the pathologist in diagnosing early neoplastic changes and to develop molecular-based assays which can be applied to the routine analysis of samples obtained by non-invasive procedures. Such assays could be used for high throughput population screening, evaluation of high risk individuals or for the monitoring of tumor burden or recurrence.

5.1. Early cancer diagnosis

The profound changes in the pattern of CD44 expression in neoplasia identify it as a promising candidate marker for early cancer detection. The clinical relevance of the observations discussed above is demonstrated by the frequent detection of these abnormalities in fresh tissue samples from tumors of many different organs and by their presence in pre-invasive and high-risk pre-cancerous lesions. It remains unknown at present whether these profound disturbances in CD44 gene expression are causally involved in cancer pathogenesis or are a consequence of the disease process. However, although it is clearly of considerable biological interest to resolve this uncertainty, the issue does not affect the promising implications of these findings for clinical cancer diagnosis. Although the pattern of CD44v expression in human tumors has been defined, in the majority of cases, using excised biopsy tissue, we and others have been able to use CD44 expression analysis to detect tumor cells in minimally-invasively obtained samples such as fine needle aspirates of the breast and thyroid (38, 103) and in samples of cervical smears.

Immunohistochemical (64, 65, 67, 104, 105), RT-PCR (64, 66), and *in situ* hybridization studies on the stratified squamous of the cervix uteri (Gorham and Tarin-unpublished data) have confirmed that CD44 expression is down-regulated in normal cervical cells which have ascended into the superficial layers, away from the basal membrane. These events may have a role in regulating the movement of epithelial cells towards the luminal surface. The data discussed above suggests that distinctive changes in CD44 mRNA splicing, resulting in variant isoforms of the CD44 protein, occur during the process of cervical carcinogenesis, although the issue of whether this is a causal relationship remains formally unresolved at present. Despite intense Public Health promotion, cervical cancer remains a significant cause of morbidity/mortality among the gynecological malignancies. Curative therapies are largely dependent upon early diagnosis by qualitative cytological screening of cervical smears. Thus, the development of a biochemical marker capable of distinguishing the early invasive process of cervical carcinoma in a quantitative manner, would be likely to exert a major effect on the early diagnosis and management of these patients. The observed alterations in CD44 mRNA splicing and CD44 epitope expression in cervical carcinoma could conceivably lead to such a marker, one which has been shown to be applicable to cervical smear samples (66).

The airway is another anatomical site from which exfoliated tumor cells could potentially be recovered non-

invasively, as evidenced by the use of sputum cytology to aid diagnosis of bronchial lesions. The incentive for further investigation of CD44 activity in pulmonary tumor tissue and in cells exfoliated into the sputum is therefore high and the results of new work in this area will be of considerable clinical interest.

Of particular clinical interest, it has also been shown that it is possible to achieve non-invasive detection of malignancy by identification of aberrant CD44 expression in exfoliated cells in body fluids and waste products. Samples obtained from naturally voided waste products are perhaps the best source of cellular material for detection of tumor markers. They are the most easily obtained, with the advantage of repeat sampling availability and may be used for diagnostic purposes in symptomatic individuals and potentially for mass screening of the population.

5.1.1. Colon

A non-invasive method for the screening for colorectal cancer is urgently needed because current investigative methods, such as testing for occult blood in the stools, are too non-specific and regular colonoscopy is too labor intensive, uncomfortable and expensive to be suitable for routine evaluation. In a recent study (106) we reported that colonic cancer cells exfoliated into the lumen of the organ can be detected by identification of their abnormal CD44 gene products. Exfoliated cells were obtained by centrifugation of saline wash-outs of 27 surgically resected colon specimens obtained from 15 patients with carcinoma, 7 with ulcerative colitis and 5 with Crohn's disease. After extracting cellular mRNA, (RT-PCR) and Southern blot hybridization were performed to examine the levels and patterns of transcription of exons 11 (v6), 12 (v7) and intron 9 of the CD44 gene. Abnormal expression of exons 11 (v6) and 12 (v7) was detected in exfoliated cells from 11 (73%) of 15 carcinoma cases, but not in any patients with inflammatory bowel disease. The retention of intron 9 in CD44 mRNA transcripts was also detected in 27% of carcinoma cases but not in samples from non-malignant specimens. Examination of the transcription of these CD44 components in snap-frozen solid tissue specimens from the same patients confirmed that the pattern of expression found was the same as that seen in the exfoliated material. These findings suggest that detection of abnormal expression of variant exons and/or introns of the CD44 gene in exfoliated tumor cells in the colonic lumen may be helpful markers for the early, non-invasive, diagnosis of colorectal cancer.

The clinical implication of this result is that the modification of this technique to apply it to evacuated stool specimens could result in a new non-invasive test for colorectal cancer. In preliminary experiments, we have successfully detected abnormal CD44 expression, using RT-PCR on mRNA from exfoliated cells in stools from a small group of patients (6/11) with colorectal cancer, but not in ones from normal subjects (unpublished observations). However, for the sensitivity and specificity of this approach to be accurately evaluated, it will be necessary to develop reliable techniques for retrieving viable exfoliated cells from evacuated stools. Clinical bowel preparation regimens which render the stools liquid and evacuate the lumen in preparation for surgery or radiological and endoscopic examination may be helpful for this purpose.

5.1.2. Urine

Bladder cancer is a common form of malignancy in which early diagnosis of primary tumors is difficult because of inaccessibility. Furthermore, as bladder lesions have a strong tendency to recur, the monitoring of patients following treatment is also problematical. Currently, check cystoscopy, contrast urography and ultrasound are the main methods used for investigating symptomatic disease. However, these are uncomfortable and labor-intensive procedures and are not optimal for routine monitoring of asymptomatic individuals. Urine cytology is simple and highly specific but has insufficient sensitivity for routine use (107, 108). Thus, the development of reliable, sensitive, molecular methods for the detection of urothelial malignancy are urgently required.

Recent studies have demonstrated that analysis of urine for signs of abnormal CD44 gene activity can signal the presence of small numbers of exfoliated cancer cells from tumors in the bladder. CD44 mRNA extracted from urine cell pellets and amplified with the polymerase chain reaction showed numerous abnormal CD44v transcripts in 40 of 44 patients with bladder cancer. Over-expression of CD44v transcripts was not found in exfoliated urothelial cells obtained from 38 of 46 people with no urinary symptoms or evidence of neoplasia (8). Furthermore, it was subsequently shown that immature, intron-containing CD44 mRNA transcripts accumulate in exfoliated bladder tumor cells and detection of this abnormal expression can be used to identify the presence of neoplastic lesions (9). Using Western blot analysis of the proteins extracted from urine cells pelleted by centrifugation, we have also confirmed that the abnormal CD44 gene activity detected by RT-PCR in cancer specimens translates into abnormal CD44v protein profiles (75)(figure 4). In desquamated cells from unaffected individuals the dominant proteins run at 90kD and 120-150kD which correspond to the standard and epithelial isoforms of this protein family. However, in 75% of patients with bladder cancer the profile includes dominant isoforms of ~220kD. Using this approach we achieved identification of bladder cancer in single specimens from a group of 90 people (44 bladder cancer patients, 46 tumor-free patients) with a sensitivity of 75% and a specificity of 100% (75). It is interesting to note that samples from 66% of patients with very early stage tumors (pTaG1) and severe dysplasia showed the abnormal CD44 protein pattern seen in more advanced bladder malignancy (75). Muller *et al* recently reported that in 21 bladder cancer patients CD44v2 or CD44s protein levels in cells obtained from voided urine sediments, as assayed by ELISA, was not correlated with diagnosis (109). However, a study using RT-PCR analysis (110) reported that CD44v transcripts exfoliated urothelia collected from bladder cancer patients was indicative of neoplasia. The most recent study, which applied a competitive RT-PCR method to pleural effusion specimens and voided urine samples

(111), revealed that CD44v expression was diagnostic of neoplasia if the ratio v8-10/v10 was >1.0. In this study, the CD44v8-10 expression was found to be predominant in 46 of 61 patients with malignant disease.

5.1.3. Soluble CD44

Another type of clinical sample obtained relatively non-invasively is that of whole blood or serum. CD44 expression can be analyzed on the surface of blood-borne tumor cells such as lymphomas (discussed above) but recent studies have suggested that the level of circulating soluble CD44 present in the serum can also be indicative of the presence of cancer.

Concentrations of serum CD44 between 10 and 200ng/ml (74, 95) circulate in normal individuals. Detailed antibody-mediated analysis of soluble CD44 isoforms has shown that they lack the cytoplasmic tail and the circulating CD44 does not appear to be associated with other proteins or with its ligand hyaluronan (112). The shedding of CD44 from the cell surface is most likely mediated by protease activity as experiments using protease inhibitors have been able to decrease CD44 release from neutrophils (113). Although the natural proteases responsible for shedding are unknown, metalloproteases and serine proteases have been implicated as inhibitors as these inhibit CD44 cleavage (114). There has been much interest in circulating CD44 in cancer patients recently as results suggest that there is a correlation between soluble CD44 (sCD44) levels and tumor burden. The mechanism by which tumors produce more sCD44 are unknown but it could result from the mis-processing of transcripts, including intron retention or exon skipping resulting in frame shifts. There has also been a report describing a previously unrecognized exon in the murine CD44 gene (115) that contains a stop codon within it, but it is not clear whether the same exon is in the human genome. Splicing errors or inclusion of stop codons would lead to the translation of truncated proteins that may lack the transmembrane region and therefore would be secreted.

Patients suffering from lymphomas have significantly elevated levels of serum CD44 at the time of diagnosis compared with healthy individuals and furthermore, the level returns to normal ranges if the patient responds to treatment (116, 117). Measurements of serum CD44 from patients with advanced gastric or colon cancers have been reported to be up to 10-fold higher than normal controls (95). In contrast to patients suffering from lymphoma, the dominant soluble protein isoforms contain variable exon products, reflecting differences in gene expression patterns in tumors of epithelial origin.

Kittl *et al* evaluated the possible utility of soluble CD44 splice variant v5 (sCD44v5) as a circulating, tumor-associated marker in breast cancer patients (118). Although the levels between tumor stages I-IV, benign disease, and a female control group were not significantly different, in patients with active metastatic disease, elevated levels of sCD44v5 were detected in 50% of cases. In some cases, sCD44v5 correlated with the extent of metastatic disease and fell during clinical response to therapy. In another study, node-positive breast cancer patients showed significantly elevated levels of sCD44v5 and -v6 splice variants in the blood in comparison to node-negative patients and healthy controls (119). In a study of 57 breast cancer patients with single organ metastases, immunoblot analysis of sCD44v5 and v6 revealed that serum levels paralleled the amount of the variants seen in the primary tumor (120). The authors also found that the site of metastases influenced the sCD44 levels, with metastases in bone and liver causing the most elevation of sCD44v6. Soluble forms of CD44 variants may promote migration of tumor cells, possibly through interference with tumor cell adhesion.

Conversely, in studies on patients with ovarian cancer serum levels of sCD44s or -v6 have not been found to correlate with tumor burden (121) and opposite conclusions have been drawn with regard to prognosis correlated with sCD44v5 (122, 123). Likewise, serum CD44 standard and CD44v6 levels were not significantly different in 45 non-small cell lung cancer (NSCLC) patients from those in benign lung disease patients. However, serum CD44v6 levels in squamous cell carcinoma were significantly higher than the benign control group (124). Reduced sCD44 levels appear to be indicative of urological malignancies. The sCD44v5 concentrations, as measured by ELISA in patients with prostate cancer, benign prostatic hyperplasia and men with renal cell cancer and bladder cancer were significantly lower than those in the male control group. Only the concentration of sCD44 standard in renal cell carcinoma differed significantly from the others (125). So it appears that the determination of soluble CD44 proteins in serum cannot be recommended as a marker for certain malignancies, such as urological or ovarian and some lung carcinomas.

Circulating serum CD44 is as yet of limited diagnostic use as a stand-alone marker because the levels of soluble CD44 do not indicate the site of an unknown tumor, however, this could improve if tumor and/or tissue-specific isoforms could be identified. There are also wide concentration value ranges in the population such that the normal range would have to be obtained in an asymptomatic individual prior to investigation for neoplasia. To compound the problem of defining normal ranges there have been reports that soluble CD44 levels increase in smokers (126) and in other disease states such as arthritis (127). However, the application of serum CD44 monitoring to the follow-up of patients in treatment regimens or for the assessment of tumor recurrence is promising.

5.1.4. Summary of diagnostic applications

The figures obtained in studies using CD44 abnormalities for detection of neoplasia compare favorably with figures published using other molecular markers. For example, Sidransky *et al* reported the identification of p53 gene mutations in exfoliated cancer cells in urine of bladder cancer patients (128). In parallel studies on solid tissue samples they found mutations in these genes are present in 61% of bladder tumors. Haliassos *et al* sought evidence of H-ras gene mutations in the urine of 21 patients with

bladder cancer and reported mutations at codon 12 in 10 (47%) of them (129). K-ras mutations were found in DNA retrieved from stool samples from 8 of 9 cases with colorectal cancer (130). DNA-based diagnostic methods are useful for epidemiological and family studies on cancer etiology because they detect predisposition and risk. However, RNA and protein-based methods may be more suited to the screening, diagnosis and clinical assessment of patients who currently have neoplastic disease, because they evaluate disturbances in the functional activity of relevant genes.

More recently, another marker of neoplasia, shown to be as consistently indicative of the presence of tumor cells as CD44, is that of the enzyme telomerase. Studies by our group and others (131-133) have shown the presence of telomerase activity to be correlated with neoplasia in the majority of tissue, in up to 85% of cases. Telomerase is present in some non-neoplastic cells of the body and the assay of the molecule is as yet not routine as it involves an active enzyme assay. However, the recent characterization of telomerase cDNA may make analysis of this most promising marker more convenient. The studies performed in the development of telomerase as a marker demonstrate that diagnosis of neoplasia by molecular genetic analysis of minute clinical samples is feasible and that new assays for accurate non-invasive cancer diagnosis are achievable.

It is important to recognize that the majority of CD44 expression data were obtained with techniques that are not optimal for daily use in routine clinical practice. They therefore do not yet constitute a readily available diagnostic test. We and others are attempting to develop assays which can be used routinely and which would provide digital data for ease of interpretation. ELISA based systems for identification of CD44v protein isoforms and/or mRNA species (PCR-ELISA) are under evaluation.

These can be performed by laboratory technical staff and can be semi-automated for high throughput protocols. This would be particularly suitable for evaluation of lesions in organs from which specimens of waste products and secretions can readily be obtained. Although it would be premature to claim that abnormal CD44 gene products constitute the best analytes for cancer detection, the results do show that they are promising candidates and provide the incentive for further work.

5.2. Prognosis

Prognostic evaluation of patients who already have established tumors but no current clinical evidence of metastatic spread, is an extremely important area of cancer management. Chemo-prevention of this aspect of malignant disease is highly desirable, even if it is of subordinate priority to attempts to block the whole process of neoplasia.

It is well established that vascularization is essential for tumors to grow beyond a few millimeters in size and for dissemination of tumors by blood and lymphatic routes. Accordingly, the counting of the number of capillaries in the most vascularized area of a tumor might give a useful pragmatic indication of patient survival or of tumor recurrence in breast cancer and non-small cell lung cancer (133-135). Other variables such as tumor size, estrogen receptor status, expression of molecular markers such as epidermal growth factor receptor (EGFR) and the neu oncogene have been reported (134) to be useful discriminators for judging which patients might have earlier progression of disease, shorter survival and poorer response to hormones. The work of Tahara and colleagues (51, 135) has demonstrated the association of increased expression of certain cellular growth factor-related genes including EGFR and c-met with poor prognosis in patients with stomach cancer.

So far, the associations with prognosis for all of these markers are based on statistical correlations that although highly significant, do not specifically indicate individual prognosis. Research therefore needs to continue for the identification of some marker that is invariably associated with good or bad prognosis, so that therapy can be more accurately and appropriately adjusted to the particular patients needs.

As a consequence of the studies showing that CD44v6 can confer metastatic potential in a rat tumor cell line (101), the majority of prognostic studies have concentrated on evaluating clinical outcome with the expression of the v6 isoform. Several groups have reported data indicating that over-expression of CD44 variants containing epitopes encoded by CD44 exon v6 correlates with poor prognosis of patients with various neoplasms. However, as observed in diagnostic evaluations, the expression pattern of CD44 clearly differs between tissues and this causes data variation when analyzed with regard to prognosis. One study has suggested that the presence of CD44v6 is an independent prognostic factor in non-Hodgkins lymphoma (21). Conversely, a study of CD44v5 and v6 found that expression of neither isoform was useful for prediction of survival in patients with gastric cancer, nor was their expression capable of identifying high risk sub-groups (109). In lung tissue, a study which analyzed only CD44s immunohistochemically showed that a poor prognosis was associated with decreased expression (136) and this pattern of expression was also correlated with the presence of metastases in prostate cancer (83).

The majority of CD44 prognostic evaluation studies have been performed on samples from tumors of the colon, breast and uterine cervix.

5.2.1. Colon

CD44v6 expression, as assessed by immunohistochemistry has been shown to be consistently up-regulated in colorectal tumor cells and found to be correlated with tumor stage (52, 92, 137, 138). However, as yet, no consensus has been reached on the relationship between CD44v expression and the prognosis of patients with colorectal cancer. Over-expression of both CD44s and CD44v protein isoforms has been suggested to correlate with poor prognosis, with regard to survival (54, 139) One group reported that the expression of v6 correlates with Dukes staging (137) but another reported

this not to be so in a larger prospective study (140). The same study revealed that there is a down-regulation of v6 in metastatic lesions of colorectal origin, a finding supported by an increased 5-year survival rate observed in patients with CD44v6-negative colorectal tumors (141).

5.2.2. Breast

Investigations into the clinical significance of CD44v expression in breast carcinoma (33, 142) have led to divergent conclusions. In a study by Kaufmann *et al*, of 91 patients, CD44 expression was found to be an indicator of poor overall survival (142). In a larger study of 227 patients, a significant association between CD44v6 expression and relapse free period was found, implying that CD44v6 was associated with less aggressive tumors (33). However, the study revealed no significant correlation of CD44v6 expression with the patients overall survival period. The same report found that v6 expression did correlate with tumor grade and steroid receptor status.

In a study carried out on 52 patients by de la Torre *et al*, the authors found no association between CD44s expression and any of the prognostic factors such as, age, tumor size, tumor grade, DNA ploidy hormone receptor levels or lymph node metastases (143). In an earlier study which used a mAb to CD44 standard protein on paraffin embedded breast cancer tissue, tumors containing >50% positive cells were associated with aggressive breast tumor markers, such as grade and lower estrogen receptor values (144). However, CD44s expression was not an independent prognostic factor in these subgroups in a multivariate analysis. Similar results, confirming a lack of correlation with patient survival have also been reported recently by another group. CD44v6 and CD44s expression was analyzed in 218 frozen samples of primary breast carcinomas (145). CD44 expression in tissue sections was shown to be independent of the patient age, tumor size, histological types and grades, and the lymph node status. CD44v6 was independent of markers of hormone dependence, expression of p53, c-erb B-2 and of markers of multidrug resistance.

5.2.3. Cervix

Patients with invasive cervical carcinoma and lymph node metastasis appear to exhibit a more complex pattern of CD44 splice variant expression and a higher level of expression of large molecular weight isoforms containing variant exons 8 (v3) to 15 (v10) (42).

In a study of 200 cases of stage-IB cervical cancer, multivariate analysis of immunohistochemical data showed that CD44v6 expression was an independent prognostic factor for overall survival of patients with early-stage cervical cancer (146). However, Shimabukuro *et al* reported that CD44v6 was expressed in normal cervical epithelia as well as in invasive carcinoma samples. Furthermore, patients with undifferentiated carcinomas, with a poor prognosis did not stain immunohistochemically for CD44 at all (66).

5.2.4. Summary of prognostic applications

The majority of prognostic studies use fixed-tissue archival material and therefore immunohistochemistry is the technique of choice. The use of RT-PCR allows more sensitive detection of CD44 expression and can determine splicing preferences in variant transcripts but results obtained by groups using this technique (31, 56, 63) have generally supported the conclusions of immunohistochemical studies. Finn *et al* showed that increased CD44v expression in patients with colorectal cancer indicated a poorer prognosis than those with predominantly CD44s transcripts, as judged by survival at five years (56). The presence of CD44v8-10 and –v6 transcripts in colorectal cancer has also been shown to be correlated with the presence of liver metastases (147). A study which analyzed CD44v6 in breast tissue by RT-PCR suggested that disease-free survival is more likely if v6 in present in transcripts with other variant exons (148).

There are considerable difficulties in trying to evaluate and compare the local staining intensities and overall distribution of immunoreactivity in histological preparations. At least some of the apparent discrepancies described above may reflect problems in assessment of essentially qualitative information, as well as differences in fixation, tissue processing, variation between the affinity of antibodies used and observer interpretation. Although it is the most convenient method of assessment, non-quantitative immunohistochemical evaluation of CD44 expression in archival tissue is not an optimal method for evaluation of the prognosis of a given individual. For more accurate knowledge of the prognostic potential of abnormal CD44 expression it will be necessary to use more objective and quantitative methods of measurement, such as quantitative sandwich-ELISA systems, but prospective studies using such new methods will take considerable time. Collectively, although some data suggests an overall trend in the degree and pattern of CD44 expression in some tissues correlating with the aggressiveness of cancer, it is presently not a sufficiently reliable indicator of the outcome of disease in an individual patient for use in treatment decisions. This situation may change when more accurate, reliable and routinely available methods of CD44 expression monitoring are developed.

6. PERSPECTIVE

This overview describes just some of the information that has emerged in the last few years about the expression of the CD44 gene in neoplasia. Theoretically, there are several hundred isoforms that can be produced by this gene and although it is not yet known exactly which of these are biologically functional, many different isoforms containing various exon combinations have so far been detected in human tissue. It is likely that an understanding whether the observed disturbances in CD44 regulation are causally involved in tumor induction and/or progression, or are merely consequences of other events, will take much time and effort.

As understanding of the genetic processes involved in the progression to malignant disease increases, the possibility of multiple marker analyses becomes feasible. This is analogous to the cytological approach to diagnosis. There are a number of criteria that only a few

cells on a pathologists slide will satisfy for diagnosis and in order to be as accurate as possible with alternative assays, molecular markers need to be used in the same way. As the knowledge of coordinate molecular patterns increases, the specificity of tumor marker assays will markedly improve and with the development of expression assay techniques which are capable of automation, sensitivity will be such that rapid and reliable diagnosis will be feasible on samples containing just a few cells. As yet, analysis of CD44 expression alone has not been shown to be reliable enough for routine clinical use but the large amount of data compiled from studies such as those described above make it a prime candidate to be included in multiplex molecular assays in the future.

Further study of the expression of this gene in benign tumors, borderline pre-cancerous lesions and *in situ* carcinomas is needed to define whether there are distinctive changes that characterize the earliest irreversible commitment to neoplasia and/or the onset of aggressive or invasive phenotypes. As well as the potential for diagnostic use, the identification of tumor-related CD44 isoforms could facilitate targeted gene therapy or anti-sense RNA treatment approaches. As an example of this, Dall *et al* have shown the potential of CD44 targeting by experimentally testing an immunotherapeutic approach for cervical cancer based on the expression of a CD44v7/8 epitope (149).

Some of the earlier studies of tumor-related CD44 expression suggested that the detection of particular variant transcripts or protein isoforms may be indicative of neoplastic transformation. However, the summation of results obtained from numerous tumor samples from many different organs leads to the conclusion that there is no specific pattern of expression of any individual exon, nor of any detectable combination of exons, that is characteristic of malignant neoplasia. It is therefore the detection of irregular "patterns" of assembly and of excessive amounts of oversized mRNA species in a given lesion that is itself a more characteristic marker of malignancy. The development of assays that can identify these patterns by molecular weight sizing or by quantitative summation of abnormal transcripts or proteins would lead to the more useful application of CD44 as a clinical marker.

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Send correspondence to: Professor David Tarin, Director, UCSD Cancer Center, 9500 Gilman Drive, La Jolla, California 92093-0658, USA, Tel:(619)-822 1222, Fax:(619)-822 0207, E-mail: dtarin@ucsd.edu