Relative Expression of Type IV Collagenase, E-cadherin, and Vascular Endothelial Growth Factor/Vascular Permeability Factor in Prostatectomy Specimens Distinguishes Organ-confined from Pathologically Advanced Prostate Cancers

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ABSTRACT

The tumor grade (Gleason score) in the biopsy and pretherapy prostate-specific antigen level do not accurately predict disease outcome of individual patients’ prostate cancer. We used a rapid colorimetric in situ hybridization technique to evaluate the expression level of E-cadherin (which affects cell cohesion); matrix metalloproteinases (MMPs) types 2 and 9 (which affect invasion); and vascular endothelial growth factor/vascular permeability factor (which affects angiogenesis) in archival prostatectomy specimens from 40 patients. Intratumoral heterogeneity for gene expression (edge versus center versus perineural area) was more pronounced in advanced cancers than in those that were organ confined. Regardless of Gleason score, the highest expression level for E-cadherin was found in the center or perineural area of the tumors, whereas the highest expression levels for MMP-2 and MMP-9 were associated with the invasive edge. The relationship between advancing pathological stage and expression of all four metastasis-related genes was highly significant. Decreased expression of E-cadherin and increased expression of MMP-2, MMP-9, and vascular endothelial growth factor/vascular permeability factor were associated with the Gleason score of the tumors. Irrespective of serum prostate-specific antigen level or Gleason score, the ratio between expression of MMPs and E-cadherin at the invasive edge of tumors exhibited the strongest association with nonorgan-confined prostate cancer. These data suggest that the relative expression of metastasis-related genes in radical prostatectomy specimens can distinguish between organ-confined and advanced prostate cancers and provides the rationale for a prospective study correlating gene expression in pretherapy core biopsies with outcome.

INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of cancer death in men in the United States (1). Despite earlier diagnosis and presumably smaller tumor volumes, 35–50% of patients with clinically organ-confined prostate cancer will be shown to have extraprostatic disease subsequent to radical prostatectomy (2–4). The strongest predictive factors for advanced disease are the Gleason score, serum PSA, and clinical stage (5). Of the three, Gleason score and pretherapy PSA levels are the most important.

The Gleason grading system in biopsy or prostatectomy specimens is a measure of biological aggressiveness and correlates well with final pathological stage and the prognosis of prostate cancer patients (5–9). Serum PSA is strongly associated with tumor volume and several other factors and also correlates with stage (5–12). Both serum PSA and Gleason score provide significant prognostic information as individual variables when their values are at the very high (PSA level >20 ng/ml; Gleason score ≥8) or low ends (PSA level <4 ng/ml; Gleason score 2–4) of the spectrum (13, 14). However, most patients present with intermediate PSA levels and Gleason scores (5, 9). Recently, several groups have combined clinical stage, serum PSA level, and Gleason score to generate “nomograms” that predict for pathological stage or prognosis (5, 9, 15). Although these efforts allow a “ballpark” estimate of prognosis to be made with readily available clinical data, they do not predict with accuracy disease outcome of individual patient’s prostate cancer.

Recent advances in the understanding of the molecular regulation of cancer metastasis and the design of molecular
diagnostic tools have provided new procedures with which to predict the malignant potential of individual human cancers (16). The outcome of metastasis is determined by multiple interactions between metastatic tumor cells and host factors (16, 17). To produce clinically relevant metastases, tumor cells must complete all steps in the metastatic cascade (18, 19). Thus, the failure to produce a metastasis can be attributable to different single or multiple deficiencies (19). We have developed a rapid colorimetric ISH technique to evaluate gene expression in formalin-fixed, paraffin-embedded surgical specimens of human tumors (20–25). We used this technique to study the expression level of several genes that regulate particular steps of metastasis in human prostate cancer cells implanted into the prostate of nude mice (26). Highly metastatic cells expressed higher mRNA levels of type IV collagenase (which affects invasion; Refs. 27–29); basic fibroblast growth factor and interleukin 8 (which affect angiogenesis; Refs. 30–32); and the multidrug resistance gene (33, 34) compared with cells of lower metastatic potential (27). No difference in the epidermal growth factor receptor expression (which affects growth; Ref. 35) was found between the cells, but the expression of E-cadherin (which affects cell cohesion; Refs. 36 and 37) was decreased in the metastatic cells (27). VEGF/VPF, which affects tumor angiogenesis (38–41), has also been found to be overexpressed in prostate cancer in comparison with normal epithelium or benign prostatic hyperplasia (42, 43). We found that VEGF/VPF levels correlated with microvessel density and metastatic potential of human prostate cancer cells growing in the prostate of nude mice (44). Similarly, treatment with anti-VEGF monoclonal antibody was shown to inhibit the growth of DU 145 human prostate cancer cells in nude mice (45).

Several studies have evaluated the expression of E-cadherin (46–51), type IV collagenase (29, 52, 53), and microvessel density (surrogate marker of angiogenesis; Refs. 55–62) in human cancers as single prognostic factors. Most of these correlative studies reached the inevitable conclusion that the expression of a given gene is necessary but insufficient to account for the multistep process of metastasis (19). Because each of the discrete steps in the pathogenesis of metastasis is regulated by one or several independent genes, the identification of cells with metastatic potential in heterogeneous primary human prostate cancer requires multiparametric-multivariate analysis of gene expression (19–25).

The present study analyzed expression of metastasis-related genes in 40 archival prostatectomy specimens (59 tumors). We show that increased expression of collagenase type IV (MMP-2 and MMP-9), VEGF/VPF, and decreased expression of E-cadherin are associated with increasing Gleason score. The ratio of MMP-2 and MMP-9 to E-cadherin, however, exhibited the strongest association with advanced prostate cancer.

**MATERIALS AND METHODS**

**Surgical Specimens and Patient Characteristics.** Forty formalin-fixed, paraffin-embedded, archival radical prostatectomy specimens from patients treated at the University of Texas M. D. Anderson Cancer Center were examined. Fifty-nine tumors from the 40 cases were included (15 cases with multiple tumors). The cases were selected at random, and no patients received any therapy prior to prostatectomy. Methods for handling specimens, including gross examination, sample processing, and assignment of Gleason score and pathological stage have been published previously (11). The specimens were classified by the TNM system (63), where pT1 cancer is organ-confined, pT2a cancer exhibits extraprostatic extension, and pT2b cancers invade the seminal vesicles. N+ cases exhibit regional metastasis to the lymph nodes irrespective of primary T stage. In our study, the primary tumor in all node-positive cases was pT2a or pT2b. Tumors were graded according to the Gleason system (6). In cases with multiple tumors, the Gleason score and TNM stage of each tumor was noted. When multiple tumors were present in a case with lymph node metastasis, we arbitrarily denoted the tumor of the highest histological grade in the prostate as the tumor from which the dissemination occurred. Clinical stage in the 40 patients was assigned by retrospective chart review with 38 of 40 patients having clinically confined prostate cancer (T ≤2) by digital rectal exam and transrectal ultrasound and 2 of 40 patients with suspected extraprostatic extension (T3). Preoperative serum PSA levels in the 40 patients were determined in the laboratory of The University of Texas M. D. Anderson Cancer Center using the Tosoh AIA assay.

**Histopathology.** Thin sections (4 μm) from the prostatectomy specimens were stained with H&E and evaluated histopathologically for further correlation with the ISH findings. We examined the expression of metastasis-related genes in serial sections of individual tumors and normal epithelium by mRNA ISH. Previous reports analyzing the expression of metastasis-related genes in surgical specimens of human gastric carcinomas (24), human colon carcinomas (21–23), and human pancreatic carcinomas (25) concluded that the expression level of collagenase type IV and E-cadherin varied between the edge and center of the lesions. For this reason, we examined the expression level of E-cadherin, MMP-9, MMP-2, and VEGF/VPF at the invasive edge (toward the prostate’s periphery) and the center of the cancers. In addition, in 21 tumors, we studied the expression of metastasis-related genes in tumor foci invading nerves (perineural invasion), because this has been associated previously with locally advanced prostate cancer (64). Within a specimen, tumors of different Gleason scores and pathological stage were studied. Within individual tumor foci, areas of the tumor with different Gleason grades were selected for analysis. When the Gleason score was uniform, we studied multiple random areas.

**Oligonucleotide Probes.** Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts of four metastasis-related genes, based on published reports of the cDNA sequences (65–68). The specificity of the oligonucleotide sequences was initially determined by a GenEMBL database search using the FastA algorithm (69), which showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The sequences and working dilution of the probes are as follows: MMP-9, 5’-CCG GTC CAT CTC GCT GGC CCG GA-3’ (1:200); MMP-2, 5’-GGC CAC ATC TGG GTT GGC GC-3’ (1:200); E-cadherin, mixture of 5’-TGG AGC GGG CTG GAG TCT GAA CTG-3’ (1:200) and 5’-GAC GCC GCC GGC CCC TTC ACA GTC-3’ (1:200); and VEGF/VPF, 5’-TGG TGA TGT TGG ACT CCT CAG TGG GC-3’ (1:200). A dT(D)20
oligonucleotide was used to verify the integrity of mRNA in each sample (70). All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3’ end via direct coupling using standard phosphormidine chemistry (Research Genetics, Huntsville, AL; Refs. 70 and 71). The lyophilized probes were reconstituted to a 1× stock solution in 50 mM Tris-HCl (pH 7.6) and 1 mM EDTA. The stock solution was diluted with Probe Diluent (Research Genetics) immediately before use.

ISH. ISH was performed as described previously (72, 73) with a minor modification. The Microprobe manual staining system (Fisher Scientific, Pittsburgh, PA) was used to stain tissue sections mounted on Silane-coated ProbeOn slides (Fisher Scientific). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics).

ISH Control Experiments. Controls for endogenous alkaline phosphatase activity included treatment of the samples in the absence of the biotinylated probe and use of chromogen in the absence of any oligonucleotide probes. In addition, to analyze the specificity of the hybridization signal, the following controls were performed: RNase pretreatment of tissue sections, a biotin-labeled sense probe, and competition assays with unlabeled antisense probe. A markedly decreased or absent signal was obtained under all of these conditions.

**Image Analysis.** To quantify intensity of the reaction, stained sections were examined in a Zeiss photomicroscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a three-clip charged coupled device color camera (model DXC-960 MD; Sony Corp., Tokyo, Japan). The images were analyzed using the Optimas image analysis software (version 5.2; Bothell, WA). The slides were prescreened to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue mode of the color camera. All subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined based on their equivalence to the mean log inverse gray scale value multiplied by the area of the field. The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the ISH reaction.

For each tumor focus analyzed, we measured the expression of metastasis-associated genes at the invasive edge (edge facing the outer surface of the prostate), at the center of the tumor and, in selected cases (21 separate tumor foci), in areas of perineural tumor invasion (located at the edge of the tumor facing the outer surface of the prostate). The areas to be measured were selected and identified with ink on the corresponding H&E stained section as follows: (a) normal glands of peripheral or transition zone distant from the tumor; (b) at least five...
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<table>
<thead>
<tr>
<th></th>
<th>Edge</th>
<th>Center</th>
<th>Normal</th>
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<td><img src="image17.png" alt="Image" /></td>
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150 μm
separate fields (approximately one field every 2 mm) irrespective of the Gleason score from the invasive edge (toward the periphery of the prostate) of the tumor; (c) Two to four separate fields, including at least one representative field of each of the different Gleason grades present from the center of the tumor; and (d) areas of perineural tumor invasion. Within each 1-mm² field, we analyzed at least 10 cells (range, 10–20) with adequate cytoplasm, permitting measurement of staining intensity. Areas of nuclear staining and necrotic cells were avoided. Whenever possible, measurements for each probe were performed in the same tumor cell cluster/cells. This was particularly important for the values of MMP-9 and MMP-2 and E-cadherin; therefore, the corresponding ratio reflected values for the same cells. For each tumor, multiple data points were recorded. The values assigned to a given tumor for E-cadherin and MMP-2 and MMP-9 expression were those of the “representative field” providing the highest MMP:E-cadherin ratio. The VEGF/VPF value assigned reflected the highest value among the measured fields.

Controls for Image Analysis. To minimize experimental variations in staining intensities, normalization of mRNA expression levels was performed. The levels of E-cadherin, MMP-2, MMP-9, and VEGF/VPF for each field were normalized by subtracting background staining and then dividing by the expression level of the poly d(T)₂₀ probe (mRNA integrity) for the same area. To allow for a comparison of samples run on different days, the staining intensity of each probe was further normalized for the mRNA expression level in histologically normal prostate glands on the same slide.

Statistical Analysis. The mean of the assigned expression levels (± SD, range) for E-cadherin, MMP-2, MMP-9, VEGF/VPF, and the MMP:E-cadherin ratio for the 59 tumors was stratified according to pathological stage, Gleason score (6, 7), and location of the measured area. To assess the statistical significance of differences in mean expression levels, ANOVA (with the Tukey honestly significant difference multiple comparison post-hoc test) was performed (74). P ≤ 0.05 was considered a significant difference. The MMP:E-cadherin ratio was used to express the invasive profile of a tumor and was calculated using the following formula: (MMP-2 + MMP-9)/2 ÷ E-cadherin expression level. To determine the significance of the mRNA expression levels for the above genes that were representative of the whole radical prostatectomy specimen, a separate analysis was performed using only the mRNA expression levels of the tumor focus of the highest Gleason score or pathological stage in the specimen (dominant tumor). For these 40 cases, mRNA expression as well as the preoperative serum PSA levels were related to the final pathological stage. In addition, logistic regression analysis was used on the same data set to define the most important variables predicting organ-confined versus nonorgan-confined (i.e., more advanced) prostate cancer (SPSS, Inc., Chicago, IL; Ref. 74).

RESULTS

Intratumoral Heterogeneity of the Expression of Metastasis-related Genes. The integrity of mRNA in each sample was first verified using a poly d(T)₂₀ probe (22–25). All samples had an intense reaction, indicating that the mRNA was preserved. Normalization of mRNA expression intensities for poly d(T)₂₀ probe intensity and also for normal prostate glands on the same slide allowed for a comparison of expression intensities of multiple samples analogous to loading controls (i.e., glyceraldehyde-3-phosphate dehydrogenase) used for Northern blot analysis.

Intratumoral heterogeneity for gene expression was observed for E-cadherin, MMP-2, and MMP-9. Pathologically advanced (pT₃a₋b, N₀₋ N+ ) cancers exhibited a greater degree of intratumoral heterogeneity than organ-confined cancers (pT₂; Table 1; Fig. 1). Specifically, E-cadherin expression was highest at the center and lowest at the edge of the tumors [significant difference, edge versus perineural (organ-confined tumors) P = 0.009; edge versus central, or perineural area (advanced tumors) P = 0.032]. In contrast, the expression of type IV collagenase (MMP-2 and MMP-9) was significantly elevated at the edge as compared with the central or perineural areas for both organ-confined and advanced cancers. Because down-regulation of E-cadherin and up-regulation of MMP-2 and MMP-9 were found at the tumor edge, the MMP:E-cadherin ratio was also highest at the edge of 52 of the 59 tumors (88%). In seven cases (12%), however, the ratio was highest in the center of the tumor. Overall, intratumoral heterogeneity of gene expression with respect to the MMP:E-cadherin ratio was highly significant for both organ-confined (P = 0.001) and advanced (P < 0.001) cancers. This was not the case, however, for VEGF/VPF because the expression varied throughout the tumors and did not differ significantly among the edge, center, or perineural areas (Table 1).

Perineural invasion is characteristic of aggressive cancers, such as pancreatic carcinoma (25), and is also thought to be a poor prognostic feature in prostate cancer (64). Although most of the areas of perineural invasion were found on the edge of tumors, the expression of E-cadherin and type IV collagenase genes in these tumor cells was essentially identical to that of tumor cells in the center of lesions (Table 1).

Intratumoral Heterogeneity for Expression of Metastasis-related Genes. Next, we related the expression level of metastasis-related genes to the tumor pathological stage (Table 2). The expression levels of VEGF/VPF, E-cadherin, MMP-2, and MMP-9 significantly differed (P = 0.015 -< 0.001) between organ-confined (pT₂), and advanced cancers (pT₃a₋b, pT₄, and pT₃a₋bN+). Advanced tumors expressed lower E-cadherin but higher VEGF/VPF, MMP-2, and MMP-9 than organ-confined tumors. The calculated MMP:E-cadherin ratio at the tumor edge also showed clear differences between organ-confined and ad-
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The expression of metastasis-related genes was next compared with the Gleason score of the tumors (Table 3). The expression levels of VEGF/VPF, E-cadherin, MMP-2, and MMP-9 differed significantly between tumors with a Gleason score of 5–6 (well differentiated) and those with a Gleason score of 8–10 (poorly differentiated). The high grade, poorly differentiated tumors expressed a lower level of E-cadherin mRNA, a higher level of MMP-2 mRNA ($P < 0.001$), and a higher level of MMP-9 mRNA ($P < 0.001$), and a higher level of VEGF/VPF RNA ($P = 0.015$) than Gleason score 5–6 tumors. The MMP:E-cadherin ratio was also significantly higher in high-grade tumors (Gleason score 8–10) than in low-grade tumors (Gleason score 5 and 6; $P < 0.001$).

Table 2 Relationship between the expression of metastasis-related genes and pathological stage

<table>
<thead>
<tr>
<th>Pathological stage</th>
<th>No. of tumors</th>
<th>VEGF/VPF</th>
<th>MMP-9</th>
<th>MMP-2</th>
<th>E-cadherin</th>
<th>MMP:E-cadherin ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT2</td>
<td>34</td>
<td>113 ± 40</td>
<td>154 ± 56</td>
<td>151 ± 66</td>
<td>73 ± 14</td>
<td>2.2 ± 0.9</td>
</tr>
<tr>
<td>pT3a</td>
<td>13</td>
<td>181 ± 69</td>
<td>327 ± 131</td>
<td>318 ± 125</td>
<td>40 ± 11</td>
<td>8.5 ± 1.9</td>
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<tr>
<td>pT3b</td>
<td>5</td>
<td>208 ± 74</td>
<td>216 ± 57</td>
<td>283 ± 77</td>
<td>31 ± 7</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>pT3a–b N+</td>
<td>7</td>
<td>195 ± 63</td>
<td>330 ± 85</td>
<td>438 ± 110</td>
<td>35 ± 7</td>
<td>11 ± 1</td>
</tr>
</tbody>
</table>

Table 3 Relationship between the expression of metastasis-related genes and Gleason score

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>No. of tumors</th>
<th>VEGF/VPF</th>
<th>MMP-9</th>
<th>MMP-2</th>
<th>E-cadherin</th>
<th>MMP:E-cadherin ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5, 6</td>
<td>18</td>
<td>107 ± 36</td>
<td>146 ± 52</td>
<td>150 ± 75</td>
<td>72 ± 169</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>157 ± 70</td>
<td>218 ± 118</td>
<td>230 ± 143</td>
<td>61 ± 20</td>
<td>4.7 ± 3.6</td>
</tr>
<tr>
<td>8–10</td>
<td>16</td>
<td>168 ± 65</td>
<td>302 ± 102</td>
<td>333 ± 109</td>
<td>36 ± 8.5</td>
<td>9.2 ± 2</td>
</tr>
</tbody>
</table>

Of interest, the Gleason score 7 tumors were heterogeneous and included 15 (60%) organ-confined tumors and 10 (40%) tumors that were associated with extension into extraprostatic tissue (7 cases), seminal vesicles (2 cases), or lymph node metastasis (1 case). This difference in tumor aggressiveness prompted us to categorize the analysis of the Gleason score 7 tumors to pathologically organ-confined or advanced disease (Table 4; Fig. 2). The difference in expression levels of VEGF/VPF, E-cadherin, MMP-2, and MMP-9, and the MMP:E-
cadherin ratio between Gleason 7 tumors that were organ confined or advanced was highly significant (Table 4, P < 0.001). In fact, organ-confined Gleason score 7 tumors exhibited a pattern of gene expression that was similar to Gleason score 5–6 cancers (Tables 3 and 4), whereas the pattern of gene expression of advanced Gleason score 7 tumors was identical to high-grade tumors (Tables 3 and 4). Thus, an assessment of metastasis-related genes was very informative in cancers with similar histology.

Shown in Table 5 are the mRNA expression levels of VEGF/VPF, MMP-9, MMP-2, and E-cadherin as well as the MMP:E-cadherin ratios for the dominant tumor in the radical prostatectomy specimen. Also shown are the mean serum PSA levels stratified by pathological stage. Significant overlap in serum PSA between the various pathological stages precluded separation with the exception of organ-confined cancer from seminal vesicle involvement. On the other hand, the expression levels of VEGF/VPF, MMP-9, MMP-2, and E-cadherin correctly separated the patients with extraprostatic extension and lymph node metastasis from those with pathologically organ-confined disease. Furthermore, the MMP:E-cadherin ratio separated patients into three different groups: (a) organ-confined cancer (pT2); (b) those with extraprostatic extension (pT3a,b); and (c) those with lymph node metastasis (pT4, N+; Table 5).

The specific gene expression was clinically significant because of the feasibility of determining metastasis-related gene expression using an ISH technique in archival radical prostatectomy specimens; (b) to ascertain the distribution of gene expression in a cancer known for its histological heterogeneity; and (c) to determine whether the expression of metastasis-related genes correlates with aggressive behavior in individual patients as assessed by the tumor pathological stage. The present results show that the ISH technique is feasible and that normalization of gene expression for mRNA integrity [poly d(T)20] as well as for expression in the normal epithelium allows for quantitation as well as for comparisons between samples that were performed on different days.

As with other neoplasms, human prostate carcinomas consist of multiple subpopulations of tumor cells interspersed with host fibroblasts, epithelial cells, endothelial cells, and leukocytes (56, 57, 64). Because metastases originate from a small subpopulation of preexisting tumor cells (16, 75, 76), the identification of these cells requires a sensitive technique that preserves zonal heterogeneity. Northern blot analysis represents the average level of mRNA of all of the cells in a sample and thus cannot identify a small subpopulation of cells in a heterogeneous tumor (22, 23). Moreover, in many human tumors, the expression of E-cadherin, collagenase type IV, and other genes varies between the center and the edge of the tumor (21–25). Our present data agree with these findings and show that the difference in expression levels was more marked in advanced prostate cancers, suggesting a causal relationship between invasion and low expression of E-cadherin (37, 46, 49) and high expression of MMPs (25, 28, 52, 53).

E-cadherin, a cell surface glycoprotein involved in calcium-dependent homotypic cell-to-cell adhesion, is responsible for the organization, maintenance, and morphogenesis of epithelial tissues (36, 77). Reduced levels of E-cadherin are associated with decreases in cellular and tissue differentiation and a resulting higher histological grade in various epithelial neoplasms (37, 46, 49, 77). The transfection of an E-cadherin-encoding cDNA into invasive cancer cells has been shown to inhibit their motility and invasiveness (80, 81), and immunohistochemical studies in patient specimens demonstrate that re-

### Table 4 Comparison between organ-confined and advanced Gleason 7 tumors

<table>
<thead>
<tr>
<th>Pathological stage</th>
<th>No. of tumors</th>
<th>VEGF/VPF</th>
<th>MMP-9</th>
<th>MMP-2</th>
<th>E-cadherin</th>
<th>MMP:E-cadherin ratio</th>
</tr>
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<tbody>
<tr>
<td>Organ confined</td>
<td>15</td>
<td>120 ± 47(^b)</td>
<td>159 ± 58</td>
<td>146 ± 49</td>
<td>75 ± 9</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>Advanced</td>
<td>10</td>
<td>(93–220)(^b)</td>
<td>(97–298)</td>
<td>(77–265)</td>
<td>(61–90)</td>
<td>(1.3–3.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(177–454)</td>
<td>(143–500)</td>
<td>(123–573)</td>
<td>(20–59)</td>
<td>(6.4–12.0)</td>
</tr>
<tr>
<td>(p(^d)</td>
<td></td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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</table>

\(^a\) TNM classification: Organ confined (pT2); Advanced (pT3\(_a\), n = 7; pT3\(_b\), n = 2; pT4,N+, n = 1; Ref. 63).
\(^b\) The numbers are expression intensities compared with normal epithelium of prostate glands (expression at normal glands, 100). The data are shown as mean ± SD (range).
\(^c\) Mean of MMP:E-cadherin expression ratios. The data shown are mean ± SD (range).
\(^d\) Examined by two-sided \(t\) test.

### DISCUSSION

The purpose of the present study was 3-fold: (a) to define the feasibility of determining metastasis-related gene expression using an ISH technique in archival radical prostatectomy specimens; (b) to ascertain the distribution of gene expression in a cancer known for its histological heterogeneity; and (c) to determine whether the expression of metastasis-related genes correlates with aggressive behavior in individual patients as assessed by the tumor pathological stage. The present results show that the ISH technique is feasible and that normalization of gene expression for mRNA integrity [poly d(T)\(_{20}\)] as well as for expression in the normal epithelium allows for quantitation as well as for comparisons between samples that were performed on different days.

As with other neoplasms, human prostate carcinomas consist of multiple subpopulations of tumor cells interspersed with host fibroblasts, epithelial cells, endothelial cells, and leukocytes (56, 57, 64). Because metastases originate from a small subpopulation of preexisting tumor cells (16, 75, 76), the identification of these cells requires a sensitive technique that preserves zonal heterogeneity. Northern blot analysis represents the average level of mRNA of all of the cells in a sample and thus cannot identify a small subpopulation of cells in a heterogeneous tumor (22, 23). Moreover, in many human tumors, the expression of E-cadherin, collagenase type IV, and other genes varies between the center and the edge of the tumor (21–25). Our present data agree with these findings and show that the difference in expression levels was more marked in advanced prostate cancers, suggesting a causal relationship between invasion and low expression of E-cadherin (37, 46, 49) and high expression of MMPs (25, 28, 52, 53).

E-cadherin, a cell surface glycoprotein involved in calcium-dependent homotypic cell-to-cell adhesion, is responsible for the organization, maintenance, and morphogenesis of epithelial tissues (36, 77). Reduced levels of E-cadherin are associated with decreases in cellular and tissue differentiation and a resulting higher histological grade in various epithelial neoplasms (37, 46, 49, 77). The transfection of an E-cadherin-encoding cDNA into invasive cancer cells has been shown to inhibit their motility and invasiveness (80, 81), and immunohistochemical studies in patient specimens demonstrate that re-
**Fig. 2 A, ISH analysis of E-cadherin, type IV collagenase (at the edge of the tumor), and VEGF/VPF (at the center of the tumor) mRNA of an organ-confined (pT2) tumor.** H&E staining shows that the tumor has a Gleason score 7. Hybridization with the poly d(T)20 probe confirmed mRNA integrity. A positive reaction in this assay stains red. The numbers for E-cadherin, MMP-9, MMP-2, and VEGF/VPF indicate expression intensities as compared with the epithelium of normal glands, which were assigned a value of 100. The expression intensity values for E-cadherin, MMP-9, MMP-2, and VEGF/VPF were 90, 122, 111, and 93, respectively. The MMP:E-cadherin ratio \[
\left( \frac{122 + 111}{290} \right) = 1.3.
\]

**B, ISH analysis of E-cadherin, type IV collagenase (at the edge of the tumor), and VEGF/VPF (at the center of the tumor) mRNA of a tumor exhibiting extraprostatic extension (pT3a).** H&E staining shows that the tumor has a Gleason score 7. Hybridization with the poly d(T)20 probe confirmed mRNA integrity. The numbers for E-cadherin, MMP-9, MMP-2, and VEGF/VPF indicate expression intensities as compared with the epithelium of normal glands, which were assigned a value of 100. The expression intensity values for E-cadherin, MMP-9, MMP-2, and VEGF/VPF were 33, 359, 368, and 192, respectively. The MMP:E-cadherin ratio \[
\left( \frac{359 + 368}{233} \right) = 11.0.
\]
Fig. 2 Continued.

Gleason score 7 / pT3a

Tumor

Normal

H & E

Poly d(T)20

E-cadherin

MMP-9

MMP-2

VEGF/VF

300 μm

33

359

368

192

100

100

100

100
duced expression of E-cadherin predicts for advanced disease and poor prognosis (37, 46–51).

The production of type IV collagenase (gelatinase, MMP) in metastatic tumor cells also correlates with the invasive capacity of human cancer cells (26–28, 52, 53, 65, 80). The MMPs degrade the basement membrane and extracellular matrix and hence facilitate invasion of the stroma. In prostate cancer, increased levels of type IV collagenase have been associated with increasing Gleason score (28). Moreover, the balance of the expression of type IV collagenase and one family of inhibitors (tissue inhibitor of metalloproteinase-1 and tissue inhibitor to metalloproteinase-2) correlates with the invasive and metastatic capacity of human prostate cancer (53). The present data confirm these findings because the concurrent relationship of expression of type IV collagenase to E-cadherin in radical prostatectomy specimens was a measure of the invasive phenotype.

Tumor foci surrounding nerves (perineural invasion) exhibited a pattern of metastasis-related gene expression similar to that of the center of tumors, where we noted lower levels of type IV collagenase and higher levels of E-cadherin. This was somewhat surprising because perineural invasion in radical prostatectomy and prostate biopsy specimens has been reported to be associated with extraprostatic extension of the tumor (37, 81, 82). However, in several recent studies where a multivariate statistical analysis was performed, perineural invasion did not predict extraprostatic extension when serum PSA level, Gleason score, or ultrasound contact length were accounted for (81–83). Similarly, perineural invasion does not predict survival in pros-

<table>
<thead>
<tr>
<th>Pathological stagea</th>
<th>No. of cases</th>
<th>Serum PSA level</th>
<th>VEGF/VPF</th>
<th>MMP-9</th>
<th>MMP-2</th>
<th>E-cadherin</th>
<th>MMP:E-cadherin ratioa</th>
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</thead>
<tbody>
<tr>
<td>pT2</td>
<td>18</td>
<td>8 ± 6</td>
<td>111 ± 43</td>
<td>174 ± 67</td>
<td>152 ± 75</td>
<td>72 ± 13</td>
<td>2.4 ± 1</td>
</tr>
<tr>
<td>(2.5–27)</td>
<td></td>
<td>(72–220)</td>
<td>(97–324)</td>
<td>(34–322)</td>
<td>(47–93)</td>
<td>(1.2–5.5)</td>
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</tr>
<tr>
<td>pT3a</td>
<td>12</td>
<td>10 ± 6.5</td>
<td>181 ± 72</td>
<td>318 ± 133</td>
<td>323 ± 129</td>
<td>41 ± 11</td>
<td>8.2 ± 1.8</td>
</tr>
<tr>
<td>(2–21)</td>
<td></td>
<td>(67–308)</td>
<td>(143–584)</td>
<td>(123–550)</td>
<td>(20–59)</td>
<td>(6.4–12.4)</td>
<td>(6.4–12.4)</td>
</tr>
<tr>
<td>pT3b</td>
<td>5</td>
<td>18 ± 5</td>
<td>208 ± 74</td>
<td>216 ± 57</td>
<td>284 ± 77</td>
<td>31 ± 7</td>
<td>8.2 ± 1.1</td>
</tr>
<tr>
<td>pT3a,b,N1</td>
<td>5</td>
<td>5 ± 1</td>
<td>217 ± 58</td>
<td>351 ± 83</td>
<td>465 ± 104</td>
<td>37 ± 8</td>
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</tr>
</tbody>
</table>

Table 6 Prediction of organ-confined prostate cancer subsequent to radical prostatectomy by logistic regression analysis

<table>
<thead>
<tr>
<th>Log-likelihood</th>
<th>P</th>
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<tbody>
<tr>
<td>Serum PSAa</td>
<td>0.4</td>
</tr>
<tr>
<td>Gleason scoreb</td>
<td>15.1</td>
</tr>
<tr>
<td>VEGF/VPFa</td>
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<td>MMP-2a</td>
<td>16.5</td>
</tr>
<tr>
<td>E-cadherina</td>
<td>24.0</td>
</tr>
<tr>
<td>MMP:E-cadherin ratioa</td>
<td>29.0</td>
</tr>
</tbody>
</table>

a Assessed as continuous variables.
b Assessed as categorical variable: Gleason score <7, 7, or >7.
c MMP:E-cadherin ratio is the sole variable necessary to predict organ-confined prostate cancer using multivariate regression analysis.

expression of type IV collagenase and one family of inhibitors (tissue inhibitor of metalloproteinase-1 and tissue inhibitor to metalloproteinase-2) correlates with the invasive and metastatic capacity of human prostate cancer (53). The present data confirm these findings because the concurrent relationship of expression of type IV collagenase to E-cadherin in radical prostatectomy specimens was a measure of the invasive phenotype.

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Table 5 Relationship between expression of metastasis-related genes, PSA, and pathological stage in the prostatectomy specimen

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</tr>
</tbody>
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a TNM classification (63).
b The numbers are expression intensities compared with normal epithelium of prostate glands (expression at normal glands, 100). The data are shown as mean ± SD (range).
c Mean of calculated MMP:E-cadherin expression ratios. The data shown are mean ± SD (range).
d Examined by Tukey HSD multiple range test.
*NS, no significant difference.

Fig. 3 MMP:E-cadherin ratios of organ-confined (pT2) versus advanced (pT3a, pT3b, pT3a,b-N+) prostate cancers. The MMP:E-cadherin ratio was calculated by the following equation: (MMP-2 + MMP-9)/2 ÷ E-cadherin.
tate cancer (81). Whether the affinity of prostate cancer cells for growth around nerves is mediated via paracrine growth factors produced by nerve or nerve-associated cells is unknown. Candidate growth factors include NGF, NGF-like protein, and neural cell adhesion molecules (84–86). NGF has been shown to inhibit apoptosis in non-neuronal cells, and in one study perineural prostate cells in the perineural space exhibited a lower apoptotic rate than prostate cancer cells in nonperineural areas (87). Alternatively, perineural migration may represent the path of least resistance for prostate cancer spread, implying that such cells may not have enhanced invasive capacity (88). Indeed, recent data showed that in vitro treatment of human prostate cancer cells (PC-3 and DU-145) with NGF led to re-expression of the KAI1 metastasis suppressor gene, decreased telomerase activity, reduced cell growth, and reduced invasive capacity and that treatment of nude mice with NGF inhibited s.c. tumors (89).

Intratumoral heterogeneity for gene expression was not observed for all metastasis-related genes. Tumors of higher pathological stage exhibited higher VEGF/VPF expression levels than tumors of low pathological stage. Regardless of stage, VEGF/VPF levels were similar within different areas of a given tumor, agreeing with a published study using immunohistochemical staining of human prostate cancer tissue with a polyclonal anti-VEGF antisera (39). VEGF/VPF-induced neovascularization plays a prominent role in tumor progression. Elevated levels of VEGF/VPF have been noted in glioblastomas, breast, ovarian, gastrointestinal, and prostate carcinomas (reviewed in Refs. 41 and 42). A causal relationship between cancer progression, neovascularity, and expression of VEGF/VPF has been shown in several animal models in which VEGF/VPF was overexpressed from full-length cDNA (90), VEGF/VPF mRNA (antisense mRNA transection) was down-regulated (91), or neutralizing VEGF/VPF antibodies were used (45). Studies are in progress to ascertain the relationship of VEGF/VPF expression levels within individual tumors to the microvessel density of the same area.

The finding that the expression levels of E-cadherin, MMP-2, MMP-9, and VEGF/VPF correlated with tumor stage supports the roles of angiogenesis, cell cohesion, and invasion in the metastatic cascade. Furthermore, the relationship among all four genes correlated with the tumor Gleason score, another clinically used histological prognostic marker (5–9, 13–15). In our own data set as well as others, Gleason ≤6 prostate cancers were often organ confined (7, 8), whereas Gleason ≥8 cancers were associated with extraprostatic disease and hence poor prognosis. Histopathological examination of prostate cancers with a Gleason score of 7 revealed both organ-confined and advanced cancers. The present study using ISH for metastasis-related genes clearly distinguished between the Gleason score 7 cancers that were or were not organ-confined (Table 4). This was even true when Gleason score 7 cases were categorized as to the dominant pattern being Gleason 3+4 = 7 or Gleason 4+3 = 7 (data not shown).

The level of PSA in the serum is often used in the prognosis and clinical management of prostate cancer (5, 9–10, 13). In our study, however, the level of serum PSA did not distinguish between the patients with organ-confined cancer and many patients with advanced disease. In contrast, the expression ratio between the tumor-invasive profile, i.e., the MMP:E-cadherin ratio, was particularly informative in that it separated organ-confined from advanced prostate cancers with virtually no overlap (at a cutoff of <6; Fig. 3) and could do so independently of the tumor Gleason score, serum PSA, and VEGF/VPF expression levels. Moreover, an extremely high MMP:E-cadherin ratio (>10) was often associated with lymph node metastasis.

In summary, we used an ISH technique to examine the concurrent expression of metastasis-related genes in formalin-fixed, paraffin-embedded radical prostatectomy specimens. Decreasing expression of E-cadherin and increasing expression of VEGF/VPF, MMP-2, and MMP-9 characterized pathologically advanced prostate cancers as well as those of high histological grade (Gleason score ≥8). The MMP:E-cadherin ratio, however, exhibited the greatest ability to distinguish organ-confined cancer. To determine the ultimate prognostic value of such measurements (considering the intratumoral heterogeneity of metastasis-related gene expression in the present study), the correlation between gene expression in pretreatment biopsies of prostate cancer and subsequent radical prostatectomy specimens in a large series of patients with long-term follow-up is under way.

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REFERENCES


Relative Expression of Type IV Collagenase, E-cadherin, and Vascular Endothelial Growth Factor/Vascular Permeability Factor in Prostatectomy Specimens Distinguishes Organ-confined from Pathologically Advanced Prostate Cancers

Hiroki Kuniyasu, Patricia Troncoso, Dennis Johnston, et al.


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