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Identification of EpCAM as a Molecular Target of Prostate Cancer Stroma

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To delineate the molecular changes that occur in the tumor microenvironment, we previously performed global transcript analysis of human prostate cancer specimens using tissue microdissection and expression microarrays. Epithelial and stromal compartments were individually studied in both tumor and normal fields. Tumor-associated stroma showed a distinctly different expression pattern compared with normal stroma, having 44 differentially expressed transcripts, the majority of which were up-regulated. In the present study, one of the up-regulated transcripts, epithelial cell adhesion activating molecule, was further evaluated at the protein level in 20 prostate cancer cases using immunohistochemistry and a histomathematical analysis strategy. The epithelial cell adhesion activating molecule showed a 76-fold expression increase in the tumor-associated stroma, as compared with matched normal stroma. Moreover, Gleason 4 or 5 tumor stroma was increased 170-fold relative to matched normal stroma, whereas the Gleason 3 tumor area showed only a 36-fold increase, indicating a positive correlation with Gleason tumor grade. Since the stromal compartment may be particularly accessible to vascular-delivered agents, epithelial cell adhesion activating molecule could become a

valuable molecular target for imaging or treatment of prostate cancer. (Am J Pathol 2009, 175:2277–2287; DOI: 10.2353/ajpath.2009.090013)

The stromal compartment in tissues is often considered a passive mechanical support for epithelial cells; however, recent evidence indicates that the stroma plays a critical role in many important biological processes.^{1–11} For example, both *in vivo* and *ex vivo* studies have shown that dynamic epithelial-stroma interactions influence branching morphogenesis during glandular development, and affect angiogenesis during tissue specific differentiation.^{12,13} Similarly, macrophage association with the developing mammary gland is critical during embryogenesis as evidenced by the fact that colony stimulating factor-1 or colony stimulating factor-1 receptor null mice (devoid of macrophage) have defective mammary glands.¹⁴

In neoplasia, several lines of evidence suggest that stromal abnormalities contribute to tumorigenesis. Genome-based studies indicate stromal cells are altered in some inherited cancer susceptibility syndromes,¹⁵ genomic rearrangements at several loci are observed in tumor-associated stromal cells,^{16,17} and genetic alterations in the stroma may precede genotypic changes in epithelial tumors.^{16–18} Moreover, heritable genetic defects that affect the stroma have also been identified in

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juvenile polyposis and in syndromes associated with endometrial polyps.^{19–21}

Gene expression changes in stromal cells, or expression alterations that affect stromal-epithelial interactions can also influence the development of invasive epithelial tumors, either positively or negatively.^{22,23} As an example, bone morphogenetic protein antagonist germline 1 is widely expressed by cancer-associated stromal cells and provides a favorable microenvironment for cell survival and expansion.²⁴ Alternatively, attenuation of β 1-integrin (laminin receptor) in highly aggressive human breast cancer cells leads to reorganization of the cytoskeleton, redistribution of β -catenin and E-cadherin, formation of adherens junctions, and alteration in signaling pathways that result in a reversion of the aggressive phenotype.²⁵

In addition to the influence of genomic status and gene expression levels, several experiments have shown that the physical presence of stromal cells, such as tumor-associated fibroblasts can directly influence the malignant progression of cancer. Human prostatic epithelial cells show dramatic changes both in histology and growth rate when grown with human fibroblast cells derived from prostatic carcinoma, and co-injection of fibroblasts with tumor epithelial cells into mice enhances tumor formation.^{26,27}

Taken together, these genomic, gene expression, and cell-based observations suggest that alterations in the stroma can significantly affect cell proliferation and tumor development.

To assess the molecular profile of the tumor-associated stroma in prostate tissues, microdissected epithelial and stromal cells from normal and tumor regions of human prostatectomy specimens were previously analyzed at the transcriptome level. Forty-four genes were differentially expressed in the tumor-associated stroma, including epithelial cell adhesion activating molecule (EpCAM), an epithelial glycoprotein.²⁸ In the present study we analyzed the expression of EpCAM at the protein level in prostate cancer patients using immunohistochemical staining of prostatectomy sections, coupled with a histomathematical analysis that allowed us to quantitatively measure protein amounts in the tumor microenvironment.

Materials and Methods

Tissue Specimens

Prostatectomy cases were obtained from the National Institutes of Health and the National Naval Medical Center under an institutional review board-approved protocol. A total of 20 cases were studied, including nine flash frozen tissues embedded in optimal cutting compound medium, and 11 prostate cases that were ethanol fixed and paraffin embedded as described previously.²⁹ Two pathologists (R.F.C. and J.R.-C.) evaluated the sections and concurred on Gleason scores. All cases contained localized prostate cancer.

Table 1. Summary of EpCAM Protein Levels in Normal and Tumor Regions of the Prostate

Cell type	Stromal EpCAM
Normal, <i>n</i> = 20	Stain-0 (<10%) = 20
Tumor, <i>n</i> = 23	Stain-1+ (10% to 20%) = 2
	Stain-2+ (20% to 50%) = 6
	Stain-3+ (50% to 90%) = 12
	Stain-4+ (>90%) = 3

Immunohistochemistry

Immunohistochemical staining of ethanol-fixed and paraffin-embedded sections for EpCAM expression was performed using a standard immunohistochemistry (IHC) protocol. Each 5- μ m thick section was heated at 60°C for 1 hour and then deparaffinized, rehydrated, and incubated with 0.3% hydrogen peroxide for 30 minutes at room temperature to block endogenous peroxidase activity. Frozen sections were incubated in 70% ethanol for 10 minutes at room temperature and blocked with hydrogen peroxide for 30 minutes before IHC. After rinsing in 0.1 M/L PBS (pH 7.4), sections were incubated for 1 hour at room temperature with EpCAM primary mouse monoclonal antibody (Abcam, cat. #11294) diluted 1:200 with antibody diluent (Zymed). Sections were incubated for 1 hour with goat anti-mouse secondary antibody conjugated to peroxidase (Dako). The chromogen 3, 3'-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution containing 0.005% hydrogen peroxide in 50 mmol/L ammonium acetate-citrate acid buffer (pH 6.0). The sections were lightly counterstained in Mayer's hematoxylin and mounted. Negative controls were established by replacing the primary antibody with antibody diluent and no detectable staining was evident in these

Figure 1. Representative histological section and low power view of EpCAM stained whole-mount human prostate showing staining of normal epithelium, tumor cells, and tumor associated stroma. The primary anti-EpCAM antibody was titrated to provide clean staining of normal epithelium (regions outside of box). At this antibody concentration, intense EpCAM staining of tumor regions (due to both epithelial tumor cells and tumor stroma) was observed as indicated by the box in the figure.

sections. Normal epithelium in the tissue was used as a positive control for EpCAM staining. Positive reactions (ie, positively stained cells) were identified by the presence of a brown precipitate. Negative reactions (ie, negative cells) were identified by the absence of a brown precipitate and only blue counterstain.

IHC on the same sections was performed for pan-cytokeratin AE1/AE3 (Invitrogen, cat. # 18-0132) at a dilution of 1:50 to differentiate epithelial from stromal cells. Additionally, two ethanol-fixed, paraffin-embedded sections were used for IHC staining of CD31 (Dako, cat. #) at a dilution of 1:50 to label endothelial cells.

Data Collection

Two pathologists (R.F.C. and J.R.-C.) analyzed the IHC-stained tissue sections and photographed representative regions with an Olympus microscope and a charged-couple device camera using $\times 20$ magnification and a resolution of 2080×1542 pixels (CCD color bayer mosaic; Q-color-3, Olympus America Inc., Melville, NY). In each case, four images were taken per distinct Gleason focus, as well as one arbitrarily selected field containing only normal cells.

Image Analysis

The immunostaining of all of the images was evaluated by three authors (S.M., R.F.C., and J.R.-C.) and EpCAM expression in the stroma was analyzed initially

using a semiquantitative method, followed later by a more quantitative histomathematical analysis to measure the fold-change in different tumor grades compared with normal.

For semiquantitative analysis, stromal staining was visually classified into four categories: 1+ (10% to 20% tumor stroma stained), 2+ (20% to 50%), 3+ (50% to 90%), 4+ (more than 90%). For quantitative analysis, all images were initially scanned and analyzed using the ImagePro Analysis System (ImagePro 4.5; Cybernetics, Chevy Chase, MD) as described previously.³⁰ Images were examined using the ACDSee program (ACD Systems of America; Miami, FL) and the IHC-positive staining was evaluated according to the most intensely stained and the least intensely stained image. The data were collected based on the brown staining in the intensely stained image (ie, positively stained cells) and for the blue staining in the least intensely stained case (ie, negative cells). The subsequent epithelial measurements were based on positive versus negative cell count; whereas, the stromal measurements were based on topographical regions that were positive or negative, thereby including both cell- and matrix-associated EpCAM staining.

The ImagePro watershed separation was used for the image analysis. Each measurement was exported to an MS Excel (Microsoft Excel 2000; Seattle, WA) spreadsheet and the mean staining was calculated from the four images analyzed for each set.

Tumor foci that showed different Gleason grades within each tissue were identified and photographed.

Figure 2. Serial sections of two tissue specimens from a patient with prostate cancer. The first sample contains normal epithelium and stroma (A–C), and the second sample contains normal epithelium, stroma, and tumor cells (D–F). **A, D:** H&E-staining. **B, E:** Cytokeratin staining. **C, F:** EpCAM staining. Strong EpCAM staining is seen in both normal and tumor epithelium, but is selectively observed in the stroma associated with tumor (F, bottom half).

One distinct tumor was identified in 17 cases and two tumors were identified in three cases. Therefore, a total of 23 tumors were analyzed (Table 1).

Data Analysis

Four multiple subregion staining values were averaged across each normal region and each tumor region. To obtain a single average measurement for both normal and tumor staining, we also averaged staining values across multiple tumors on the same subject. A Wilcoxon signed rank test was used to determine whether the staining was different between tumor and matched normal tissue. We examined whether the difference between tumor and matched normal tissue varied by Gleason score using a Wilcoxon rank sum test. In this latter analysis, we dichotomized Gleason score and compared changes between tumor and matched normal tissue for tumors with Gleason 3 versus Gleason 4/5. All tests were two-sided and a P value <0.05 was considered statistically significant.

Results

EpCAM protein levels in twenty human prostate specimens containing 23 independent tumors were studied.

In each case, IHC measurements of EpCAM were taken from whole-mount sections containing normal stroma, normal epithelium, tumor stroma, and tumor epithelium, and analyzed using both a semiquantitative and a quantitative histomathematical approach (Figure 1). To ensure the results were not significantly affected by tissue processing, both frozen samples and ethanol-fixed, paraffin-embedded specimens were included in the study and similar results were obtained for both. IHC for cytokeratin was also performed as a control to identify possible tumor cells in the stroma and to distinguish their staining from true stromal staining (Figure 2A–F, Figure 3A–F).

Consistent and reproducible EpCAM staining was observed in all 20 cases. Epithelium showed a membranous and diffuse pattern both in normal and tumor cells with no difference in staining intensity (data not shown). No stromal staining was observed in histological fields containing only normal cells (Figure 2C). However, in the tumor regions strong staining was present in the stroma (Figures 2F, 3C, 3F, 4, B–F; Table 1; Table 2).

An initial semiquantitative analysis of EpCAM levels in the tumor-associated stroma showed: Four+ expression in 3/23 tumors (13%). Twelve out of the 23 samples were scored as 3+ (52%). A staining score of 2+ was observed in 6/23 (26%) tumors, and weak staining

Figure 3. Serial sections of a tissue sample from a second patient with prostate cancer. The sample contains normal epithelium, stroma, and tumor cells. Images were taken at $\times 10$ magnification (A–C) and $\times 40$ magnification (D–F). A, D: H&E staining. B, E: Cytokeratin staining. C, F: EpCAM staining. EpCAM staining is seen in both normal and tumor epithelium, but is selectively observed in the stroma associated with tumor (C and arrows in F).

Table 2. EpCAM IHC Staining in the Tumor Microenvironment (Epithelial and Stromal) and in the Tumor-Associated Stroma

Sample	Case	Total staining (epithelial and stromal)			Tumor stromal staining		
		Staining value	Average	SD	Staining value	Average	SD
1	N-1	0.350475	0.5177	0.114494	0.0000468	0.000318	0.0003735
	N-2	0.538864			0.000135		
	N-3	0.600926			0.0002234		
	N-4	0.5808504			0.000868		
1-(Gl: 3)	T-1	0.40238	0.4230	0.050942	0.042744	0.022391	0.013735
	T-2	0.4985			0.12452		
	T-3	0.3865			0.018099		
	T-4	0.4049			0.016240		
1-(Gl: 4)	T-1	0.5499679	0.4995	0.06286	0.045978	0.0377435	0.0076948
	T-2	0.45584			0.035979		
	T-3	0.435514			0.027935		
	T-4	0.557006			0.04097		
2	N-1	0.3303763	0.3168	.082681	0.00044	0.0001903	0.0001707
	N-2	0.3835467			0.0001427		
	N-3	0.3562809			0.000124		
	N-4	0.1971748			0.0000545		
2-(Gl: 3)	T-1	0.219683	0.2314	0.053122	0.0094746	0.01441	0.005023
	T-2	0.164621			0.008177		
	T-3	0.290488			0.03022		
	T-4	0.250834			0.009783		
3	N-1	0.389129	0.2954	0.07854	0.0000644	0.0001289	0.0004421
	N-2	0.243924			0.000988		
	N-3	0.330038			0.00031		
	N-4	0.218758			0.000041		
3-(Gl: 4)	T-1	0.307758	0.21496	0.06413	0.0197405	0.0121536	0.0056938
	T-2	0.163382			0.008668		
	T-3	0.204816			0.0132022		
	T-4	0.183927			0.00701		
4	N-1	0.128635	0.0823	0.038884	0.0000755	0.0000684	0.0000169
	N-2	0.0976			0.0000859		
	N-3	0.039908			0.0000657		
	N-4	0.063381			0.0000462		
4-(Gl: 4)	T-1	0.301010	0.23548	0.069158	0.007896	0.0073379	0.0018856
	T-2	0.289457			0.00599		
	T-3	0.176823			0.00976		
	T-4	0.17465			0.005702		
5	N-1	0.373312	0.3507	0.167772	0.0002538	0.0002087	0.000162
	N-2	0.11707			0.0001275		
	N-3	0.51568			0.0000404		
	N-4	0.396851			0.0004129		
5-(Gl: 4)	T-1	0.48244	0.4839	.04292	0.047419	0.02675	0.0150277
	T-2	0.526183			0.01176		
	T-3	0.50161			0.02608		
	T-4	0.425393			0.021736		
6	N-1	0.24116	0.1444	0.09852	0.0005291	0.000222	0.0002175
	N-2	0.21320			0.0002196		
	N-3	0.08725			0.000094		
	N-4	0.03604			0.000045		
6-(Gl: 3)	T-1	0.34258	0.3766	0.02902	0.02143	0.02222	0.0062448
	T-2	0.371976			0.015980		
	T-3	0.413275			0.030870		
	T-4	0.378558			0.020603		
7	N-1	0.21949	0.1415	0.05987	0.0003952	0.0001853	0.0001478
	N-2	0.073653			0.0001718		
	N-3	0.13350			0.0001210		
	N-4	0.13953			0.0000542		
7-(Gl: 4)	T-1	0.06764	0.1510	0.100582	0.0013318	0.0057	0.0071163
	T-2	0.14047			0.0032546		
	T-3	0.29521			0.0163074		
	T-4	0.10103			0.001912		
8	N-1	0.14068	0.1371	0.036695	0.0007268	0.0002882	0.0002953
	N-2	0.11707			0.0002011		
	N-3	0.1872			0.0001087		
	N-4	0.1037			0.0001163		
8-(Gl: 3)	T-1	0.42453	0.4421	0.01816	0.0070605	0.020878	0.0193542
	T-2	0.46603			0.0231231		
	T-3	0.44572			0.0332446		
	T-4	0.43234			0.0200845		

(table continues)

Table 2. *Continued*

Sample	Case	Total staining (epithelial and stromal)			Tumor stromal staining		
		Staining value	Average	SD	Staining value	Average	SD
8-(Gl: 4)	T-1	0.93621	0.92485	0.12972	0.035453	0.031075	0.0041912
	T-2	0.67608			0.029356		
	T-3	0.649186			0.033150		
	T-4	0.77104			0.002594		
9	N-1	0.598548	0.4563	0.11980	0.0034380	0.0040835	0.0008508
	N-2	0.40325			0.005336		
	N-3	0.32223			0.003798		
	N-4	0.501448			0.0037612		
9-(Gl: 3)	T-1	0.53086	0.5892	0.04650	0.045568	0.0576275	0.01018
	T-2	0.57413			0.052780		
	T-3	0.63454			0.0661355		
	T-4	0.617475			0.065969		
10	N-1	0.04415	0.0538	0.049176	0.0000496	0.0002809	0.0004707
	N-2	0.00780			0.0000232		
	N-3	0.03993			0.0000642		
	N-4	0.12346			0.0009865		
10-(Gl: 4)	T-1	0.2727656	0.2072	0.080686	0.017280	0.010369	0.0055567
	T-2	0.270118			0.009389		
	T-3	0.104056			0.003756		
	T-4	0.182009			0.0108056		
10-(Gl: 5)	T-1	0.44411	0.5156	0.13903	0.0655667	0.0486066	0.0249433
	T-2	0.72843			0.083281		
	T-3	0.645329			0.025990		
	T-4	0.4633			0.072222		
11	N-1	0.059744	0.0896	0.037292	0.0000755	0.0000618	0.0000291
	N-2	0.1441345			0.0000859		
	N-3	0.07739			0.0000657		
	N-4	0.077128			0.0000199		
11-(Gl: 3)	T-1	0.1411165	0.1459	0.065539	0.0029926	0.0045357	0.0029695
	T-2	0.214203			0.0059906		
	T-3	0.1700213			0.0078955		
	T-4	0.05863			0.0012639		
12	N-1	0.19147	0.1828	0.032241	0.0000864	0.0001241	0.0000377
	N-2	0.2222			0.0000991		
	N-3	0.14567			0.0001419		
	N-4	0.1722			0.0001689		
12-(Gl: 4)	T-1	0.5029	0.4633	0.038481	0.0474195	0.03249	0.0135662
	T-2	0.4681			0.0360844		
	T-3	0.4106			0.0317362		
	T-4	0.4719			0.01472		
13	N-1	0.2314	0.2026	0.038213	0.0001119	0.0000995	0.0000212
	N-2	0.14629			0.0000803		
	N-3	0.21537			0.0000827		
	N-4	0.21734			0.000123		
13-(Gl: 3)	T-1	0.3513	0.3653	0.056592	0.0164733	0.017122	0.0072624
	T-2	0.28007			0.0109118		
	T-3	0.3285			0.0274695		
	T-4	0.4163			0.0136334		
14	N-1	0.069322	0.0855	0.036803	0.0002864	0.00012	0.0001169
	N-2	0.10436			0.0000821		
	N-3	0.125644			0.000099		
	N-4	0.04267			0.000012		
14-(Gl: 5)	T-1	0.3052	0.4767	0.30358	0.029926	0.04581	0.028999
	T-2	0.39154			0.059906		
	T-3	0.33890			0.078955		
	T-4	0.9482			0.014612		
15	N-1	0.2904	0.2571	0.062375	0.0002538	0.000104	0.0001151
	N-2	0.2925			0.0000296		
	N-3	0.2817			0.0000275		
	N-4	0.1638			0.0000149		
15-(Gl: 3)	T-1	0.7560	0.7238	0.032056	0.0312058	0.035453	0.0064386
	T-2	0.7059			0.037982		
	T-3	0.6885			0.0433648		
	T-4	0.7457			0.0292594		
16	N-1	0.2763	0.2182	0.092544	0.0001119	0.000081	0.0000443
	N-2	0.3175			0.0000803		
	N-3	0.1336			0.0000082		
	N-4	0.1454			0.0000488		

(table continues)

Table 2. *Continued*

Sample	Case	Total staining (epithelial and stromal)			Tumor stromal staining		
		Staining value	Average	SD	Staining value	Average	SD
16-(Gl: 4)	T-1	0.8474	0.5978	0.17448	0.0312058	0.040972	0.01246
	T-2	0.5597			0.0533648		
	T-3	0.4410			0.0379818		
	T-4	0.5431			0.0413354		
17	N-1	0.0957	0.1060	0.009916	0.0000755	0.000088	0.0000286
	N-2	0.1136			0.0000859		
	N-3	0.1134			0.0001275		
	N-4	0.0970			0.0000628		
17-(Gl: 4)	T-1	0.4319	0.4821	0.115142	0.036072	0.02581	0.0116898
	T-2	0.3751			0.0203954		
	T-3	0.6427			0.034866		
	T-4	0.4787			0.0119062		
18	N-1	0.2319	0.2175	0.056839	0.0002538	0.0001893	0.000129
	N-2	0.1784			0.0001275		
	N-3	0.1680			0.0000404		
	N-4	0.2917			0.00033		
18-(Gl: 5)	T-1	0.3780	0.4751	0.075066	0.0343808	0.03796	0.0321204
	T-2	0.5563			0.0379818		
	T-3	0.4636			0.078955		
	T-4	0.5025			0.00052		
19	N-1	0.1286	0.0921	0.031324	0.0000755	0.000096	0.0000413
	N-2	0.0572			0.0000859		
	N-3	0.1053			0.0000657		
	N-4	0.0773			0.0001567		
19-(Gl: 4)	T-1	0.4208	0.3328	0.073813	0.049324	0.02832	0.0141159
	T-2	0.2512			0.0214745		
	T-3	0.2986			0.0234028		
	T-4	0.3606			0.0190785		
20	N-1	0.0943	0.1144	0.035523	0.0002864	0.00012	0.0001137
	N-2	0.1457			0.0000991		
	N-3	0.1430			0.0000419		
	N-4	0.0746			0.0000525		
20-(Gl: 4)	T-1	0.2333	0.3833	0.162232	0.0234028	0.0264	0.0297138
	T-2	0.2833			0.0067544		
	T-3	0.4211			0.0693240		
	T-4	0.5955			0.0061186		

was present in 2/23 (9%) (Table 1). With respect to topography, the distribution of stromal staining was maximal near the tumor epithelium and gradually decreased as the distance from tumor cells increased.

The staining was next measured using a quantitative image scoring system (Tables 2 and 3). Overall, there was a statistically significant ($P < 0.001$) two-fold increase in EpCAM staining in the tumor microenvironment compared with normal regions when both epithelial and stromal components were included in the analysis (Table 3). However, when only stromal staining was analyzed,

there was a 76-fold increase in EpCAM staining in the tumor stroma (0.0266) compared with normal (0.000352) ($P < 0.001$).

The EpCAM stromal staining was most pronounced in histological fields that contained high-grade Gleason foci as is shown in Figure 4, A–F. Tumors were segregated according to their total Gleason grade sum into high-grade (Gleason grade 4 or 5) and moderate/low-grade (Gleason grade 3 or less) and analyzed for stromal staining as well as total (epithelium + stroma) staining (Table 4). Stromal staining for Gleason 4/5 foci was increased 170-fold relative to matched normal stroma, whereas the lower-grade Gleason tumors showed only a 36-fold increase in staining compared with matched normal stroma. Although these differences were not statistically significant ($P = 0.38$), the direction of the effect suggests that EpCAM protein levels in the tumor stroma increases with Gleason grade. The lack of statistical significance may be due to the small sample size.

Table 3. Summary of EpCAM Levels in Normal and Tumor Areas

All cells (epithelial + stromal)		Stromal cells	
Normal	Tumor	Normal	Tumor
0.203	.412	0.000352	.0266

Figure 4. Representative histological sections showing EpCAM expression in tumor-associated stroma related to histological grade. **A:** Normal area showing no stromal staining. **B:** Tumor region with low Gleason pattern showing patchy and weak staining in the tumor associated stroma. **C:** Tumor region with moderate Gleason pattern with perineurial invasion (center of the picture) and extensive EpCAM staining in the stroma. **D–F:** Tumor region with high Gleason pattern showing more intense tumor associated stromal staining. All images were taken at a magnification of $\times 20$.

We performed endothelial cell IHC labeling with anti-CD31 antibody to analyze the distribution of vessels in both the normal and tumor areas with special attention on the relationship of neovessels and EpCAM staining in tumor stroma. Most of the small vessels in normal areas were distributed around glandular epithelium or lobules, as expected. However, rich neovascularization was seen in tumor areas, with little or no organization and irregularly shaped vessels throughout the tumor stroma (Figure 5, A–F).

Discussion

In the present study, we show that EpCAM protein is up-regulated in tumor-associated stroma of the prostate. All of the primary cases analyzed showed an increase of EpCAM in the tumor areas compared with matched normal regions from the same patients. These results are consistent with our previous mRNA data, where EpCAM

was one of the highest up-regulated transcripts in tumor versus normal stroma.²⁸

The precise role of EpCAM in tumorigenesis is not currently known. EpCAM is an adhesion molecule usually expressed on the basolateral membrane of epithelial cells and has been found to be overexpressed on carcinomas and cancer-initiating cells.³¹ Recently, Maetzel et al³² identified EpCAM as a potent mitogenic signal transducer, promoting cell cycling and enhancing proliferation. Signaling by EpCAM requires regulated intramembrane proteolysis catalyzed by two proteases, TACE and PS-2, thus EpCAM growth-promoting effects may potentially be inhibited by pharmacological inhibition of TACE and/or PS-2. Interestingly, EpCAM seems to have different regulatory pathways in different tumor types. It can be overexpressed as in most colon cancers, or it can be lost as in poorly differentiated colon cancers, or it can be “newly” acquired as in squamous cell carcinoma of the esophagus.³³ Ep-

Table 4. EpCAM Levels Correlated with Gleason Grade

Gleason grade	Number of samples	All cells (epithelium+stroma)		Stroma	
		Tumor fold increase over matched normal	P value	Tumor fold increase over matched normal	P value
3	8	1.6	0.08	36	0.008
4/5	15	2.3	0.001	170	0.001

Figure 5. Serial sections of two tissue samples from a third patient with prostate cancer. The first sample (**A** and **B**) contains normal epithelium and stroma, and the second sample (**C** and **D**) contains tumor cells and tumor-associated stroma. Images were taken at $\times 10$ magnification. H&E-staining of normal (**A**) and tumor regions (**C**). Immunohistochemical staining for CD31 in normal (**B**) and tumor regions (**D**). CD31-positive small vessels in normal areas are seen distributed around glandular epithelium (**B**), whereas the tumor regions showed rich neovascularization with little or no organization and irregularly shaped vessels throughout the tumor stroma (**D**). Panels **E** and **F** show a higher magnification view of the tumor stroma labeled for CD31 (**E**) and EpCAM (**F**) on the same region from consecutive serial sections. **Arrows** on panel **F** indicate the EpCAM positive tumor associated stroma. The close relationship between a CD 31-positive vessel (**E**) and EpCAM-positive stroma (**F**) can be seen.

CAM expression has been reported to be a possible marker of early malignancy and has become an important target for immunotherapy with monoclonal antibodies because of its specificity in some cancerous lesions.^{34,35}

In prostate, EpCAM does not appear to be regulated by androgens; however, the heterogeneous distribution of EpCAM expression within DU145 and PC3 prostatic cell lines suggests that EpCAM expression may be lost in a subpopulation of cells within androgen independent prostate cancer, indicative of the overall loss of cellular differentiation with progression.³⁶ Studies of EpCAM expression in normal and tumor prostate epithelium have generated conflicting results. One group found up-regulation of EpCAM in hormone-refractory and untreated prostate cancer epithelial cells, but this was not confirmed by another study that exam-

ined hormone-refractory cancers, localized cancers, and metastases.³¹ Moreover, of three analyses of the relationship between EpCAM levels in tumor epithelium and Gleason score, two found no correlation whereas a third study found a positive correlation of EpCAM levels and tumor grade. Finally, a tissue microarray-based study showed up-regulation of EpCAM staining in tumors.³⁷

The present study is the first to report a difference in EPCAM protein levels in tumor versus normal stroma. However, in contrast to our findings, Poczaitek et al³⁶ reported EpCAM immunostaining did not increase significantly in the stroma of formalin-fixed, paraffin-embedded, archival tissue specimens from patients diagnosed with prostatic carcinoma. Possible explanations include differences in tissue processing and the use of different primary anti-EpCAM antibodies. Moreover, the availability of whole mount sections in our study allowed for a thorough three-dimensional analysis of the tumor microenvironment. In some cases EpCAM staining was localized to only a subregion of the tumor field and would not have been easily identified in small specimens due to sampling effects.

The use of stromal markers in the tumor microenvironment has been proposed for both diagnostic and therapeutic purposes.^{38,39} Possible targets for clinical intervention include cancer-associated fibroblasts, infiltrating macrophages, tumor endothelial cells, and extracellular matrix. In patients with multiple myeloma, bone marrow stroma is being targeted using a proteasome inhibitor to attenuate the disease, and anti-angiogenic drugs (Avastin [bevacizumab] and thalidomide) are being used to target endothelial cells.^{40–42} A novel immunological approach to colon cancer therapy targets fibroblast activating protein, that is highly expressed by stroma cells of this tumor type.⁴³ Recently, disruption of the epithelial-stroma interaction has been demonstrated to decrease carcinoma cell proliferation when it is accomplished through chemotherapeutic agents.⁴⁴ Similarly, Kammertoens and colleagues⁴⁵ showed that, in addition to direct tumor killing, T-cell-mediated tumor rejection occurs in part due to disruption of stromal infrastructure.

Since the stromal compartment is relatively accessible to vascular-delivered agents, up-regulation of targets in the tumor-associated stroma makes them attractive candidates to consider as clinical tools.^{46–50} Specifically in the case of EpCAM in the stroma of the prostate tumor micro-environment, IHC analysis of CD31 on whole mounts confirmed a close spatial relationship between stromal EpCAM and neovessels, thus providing the basis for targeting of the tumor microenvironment via systemic delivery of anti-EPCAM directed imaging or therapeutic agents (Figure 5). Moreover, although not statistically significant, EpCAM protein expression showed a trend toward increased stromal levels with Gleason grade as its expression in Gleason 4/5 tumors was higher than in Gleason 3 neoplasms. This observation could have particular importance as molecular markers that increase with tumor

grade would be useful clinically. However, a larger set of cases will need to be studied to validate this relationship.

In summary, emerging data support the concept that the stroma in the tumor microenvironment functions to significantly affect tumorigenesis in epithelial carcinomas. EpCAM mRNA and protein are both up-regulated in prostate tumor stroma and represent potential targets for new diagnostics or therapeutics.

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