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Original Article

Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis

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Abstract: Prostate cancer (PCa) has a predilection to metastasize to bone. Before metastasis can occur there is transition of the sessile epithelial cancer cells to become motile and invasive mesenchymal phenotypes by an important albeit transient process called Epithelial-to-Mesenchymal Transition (EMT). The cascade of molecular events triggered by this process is clinically relevant as they are associated with cancer stem-like cells (CSC), decreased senescence and eventual drug resistance phenotype. We interrogated some EMT markers that have been implicated in primary and bone metastasis of PCa using archived patient samples. Using an immunohistochemical approach, E-cadherin, Vimentin, ZEB1, Notch-1, PDGF-D and NF-κB were analyzed. Cases were microscopically scored using intensity (0, +1, +2, +3) and percentage of positive cells. Data was statistically analyzed using Fisher's Exact Test. Aberrant expression of EMT markers E-cadherin, Vimentin, PDGF-D, NF-κB, Notch-1 and ZEB1 was observed in PCa (primary tumor specimen) and bone metastasis tissues. The aberrant expression pattern varied according to the location within the tumor with higher expression was observed more at the invasive tumor front (ITF) vs. the center of the tumor. Notch-1 was significantly over-expressed in bone metastasis compared to primary PCa ($p=0.057$). The expression levels, intensity and % of positive cells of the remaining markers were not statistically significant in PCa vs. bone metastasis. In conclusion, protein expression analysis revealed the existence of EMT phenotype in the PCa and bone metastasis. Variation in the aberrant expression patterns at the invasive tumor front indicates the role of EMT markers in tumor invasion. Our results suggest that Notch-1 could play a role in PCa bone metastasis. Studies in larger patient cohorts are warranted before these EMT molecular markers can be translated to the clinical use.

Keywords: Prostate carcinoma, epithelial-to-mesenchymal transition (EMT), E-cadherin, Vimentin, PDGF-D, NF-κB; Notch-1, ZEB1, immunohistochemistry

Introduction

Recent statistics reveal that prostate cancer (PCa) continues to remain the most commonly diagnosed lethal malignancy in men in the United States with 1 out of 6 men developing PCa and 1 out of 35 dying from it [1]. This diverse and heterogeneous tumor has a natural history which varies from indolent to highly aggressive tumor behavior. Despite similar histologic presentation at diagnosis, it is difficult to predict the clinical behaviors, including time to disease progression and metastasis [2,3]. Risk stratification of disease poses a significant challenge to the treating physician.

Several risk predictors are currently under investigation in PCa including markers of Epithelial-to-Mesenchymal Ttransition (EMT). This transient but biologically significant phenomenon occurs during cancer progression and increases the innate invasive and metastatic capabilities of cancer cells [4,5]. EMT is associated with histologic, molecular and transcriptional changes [6]. An interplay of several extracellular signals, growth factors, their effectors and transcription factors induce EMT and have the propensity to serve as EMT markers [7] consistent with tumor aggressiveness.

Recently EMT has been mechanistically linked with stem cell signatures in PCa cells [8]. Acquisition of properties of stem cells can lead to increased resistance to apoptosis, diminished senescence and escape from immune surveillance. Eventually these would lead to resistance

Table 1. Immunohistochemistry Methods

Antibody	Company	Catalog #	Dilution	Duration (minutes)	Antigen retrieval method	Staining pattern
E-CADHERIN	Zymed	18-0223	1:50	16	CC1-M	Membranous
VIMENTIN	Ventana	760-2512	prefilled	32	CC1-S	Cytoplasmic
ZEB1	Santa Cruz	sc-25388	1:100	60	EDTA	Nuclear
NOTCH-1	Abcam	ab8925	1:100	120	Citrate	Nuclear
PDGF-D	Zymed	40-2100	1:200	60	EDTA	Cytoplasmic
NF-κB	Cell Signaling	3037	1:10	60	EDTA	Nuclear

to therapy [9]. Since EMT could be a reversible phenomenon, this makes it clinically relevant in preventing metastasis and cancer progression by novel approaches that could cause reversal of EMT to Mesenchymal-to-Epithelial Transition (MET). Additionally with the report of drugs like sialomycin targeting cancer stem cells [10], it is important to investigate EMT markers as possible therapeutic targets.

EMT is regarded as an important step in cancer metastasis [11]. Bone metastases occur in more than 90% of patients with advanced PCa and a high burden of metastatic disease is associated with poor survival [12]. The primary PCa tumor epithelial cells undergo EMT with activation of the embryonic programs of epithelial plasticity and switch from a sessile, epithelial phenotype to a motile, mesenchymal phenotype [9]. Growth factors and molecular alterations that play a prominent role in EMT induction in the primary tumor have been identified as important stimulators of skeletal metastasis formation [13]. The PCa metastatic tumor cells interact with the bone microenvironment which plays a pivotal role in metastasis. Novel agents targeting these EMT molecules can be designed to efficiently disrupt these interactions among cancer cells and bone microenvironment [9]. In this study, we interrogated the most significant EMT factors and signaling pathways that are emerging to be associated with PCa bone metastasis. This study represent a proof-of-concept study in a limited number of samples, and as such suggest that Notch-1 expression could become the signature for the acquisition of EMT in PCa and its bone metastasis, which requires further in-depth investigation.

Materials and methods

Case selection

We obtained archived, formalin fixed, paraffin-embedded tissue from surgically resected PCa specimens (primary and bone metastasis) containing tumor and adjacent normal tissues as part of a collaborative effort with the University of Michigan Prostate SPORE program (P50 CA69568). Institutional review board approval and a waiver of consent for a retrospective review of archived material were obtained. The tissue specimens were histologically examined. We selected 20 PCa tissue samples (10 primary and 10 PCa bone metastasis). Four microns tissue sections were cut from the representative tumor blocks and placed on positively charged slides for immunohistochemical expression of EMT markers in PCa progression.

Immunohistochemical analysis and evaluation

Tissue sections were stained by immunohistochemistry (IHC) using specific antibodies for E-cadherin, Vimentin, NF-κB, Notch-1, ZEB1 and PDGF-D. These markers were chosen based on existing evidence from our laboratory documenting the role of these markers in the acquisition of EMT in prostate cancer cells [14,15,16, 8,17,18,19,7,20,21]. Standard staining protocols according to the laboratory manual were established using the avidin-biotin complex (ABC) staining procedure. Initial trials used the manufacturer's suggested specimen preparation and staining conditions. Each protocol was then optimized for antigen retrieval, antibody dilution, and incubation conditions as outlined

Table 2. Marker expression as intensity and percent of positive cells

Marker	levels	Intensity			% of positive cells		
		Prostate Cancer			Prostate Cancer		
		Primary	Bone Metastasis	P value‡	Primary	Bone Metastasis	P value‡
E-CADHERIN	0	1	3	0.433	0	3	0.112
	1	0	1		1	0	
	2	8	6		4	1	
	3	1	0		5	6	
VIMENTIN	0	6	6	1	6	6	1
	1	2	1		3	3	
	2	2	3		0	1	
	3	0	0		1	0	
NF-κB	0	2	4	0.659	2	4	0.386
	1	2	3		4	5	
	2	5	2		4	1	
	3	1	1		0	0	
PDGF-D	0	2	4	0.7	2	4	0.864
	1	2	1		2	1	
	2	6	5		4	3	
	3	0	0		2	2	
NOTCH-1	0	1	1	0.721	1	1	0.033
	1	1	0		0	0	
	2	8	7		5	0	
	3	0	2		4	9	
ZEB1	0	2	4	0.314	2	4	0.536
	1	5	6		3	4	
	2	3	0		5	2	
	3	0	0		0	0	

‡ Fisher's Exact test

in **Table 1.** A known positive tissue for the antigen of interest was used to titer the antibody and subsequently was stained with each investigative case for the current study. Immunohistochemical staining was performed as follows: tissue sections were deparaffinized, hydrated to phosphate-buffered saline (PBS) buffer (pH 7.4) and pretreated with hydrogen peroxide (3%) for 10 minutes to remove endogenous peroxidase. This was followed by antigen retrieval in a steamer for 20 minutes with EDTA. The slides were then incubated with the primary antibody at ambient temperature and washed with PBS followed by incubation with biotin-labeled secondary antibody for 30 minutes at room temperature. Finally slides developed with 0.05% 3',3-diaminobenzidine (DAB) tetrahydrochloride, which has been freshly prepared in 0.05mol/L Tris buffer at pH 7.6 containing 0.024% H_2O_2 and then counterstained with Mayer hematoxylin, dehydrated and mounted.

Slide evaluation

Immunostained slides were blindly evaluated under a transmission light microscope. Areas of highest staining density were identified for evaluating the expression in tumors.

Microscopic scoring of expression

Expression was scored for each antibody separately and semiquantitatively by assessing the stain localization, intensity and the percentage of stained cells in the tumors. Stain localization was classified as nuclear, cytoplasmic and membranous. Staining intensity was scored as 0 (*negative*), 1 + (*weak*), 2 + (*medium*) or 3 +

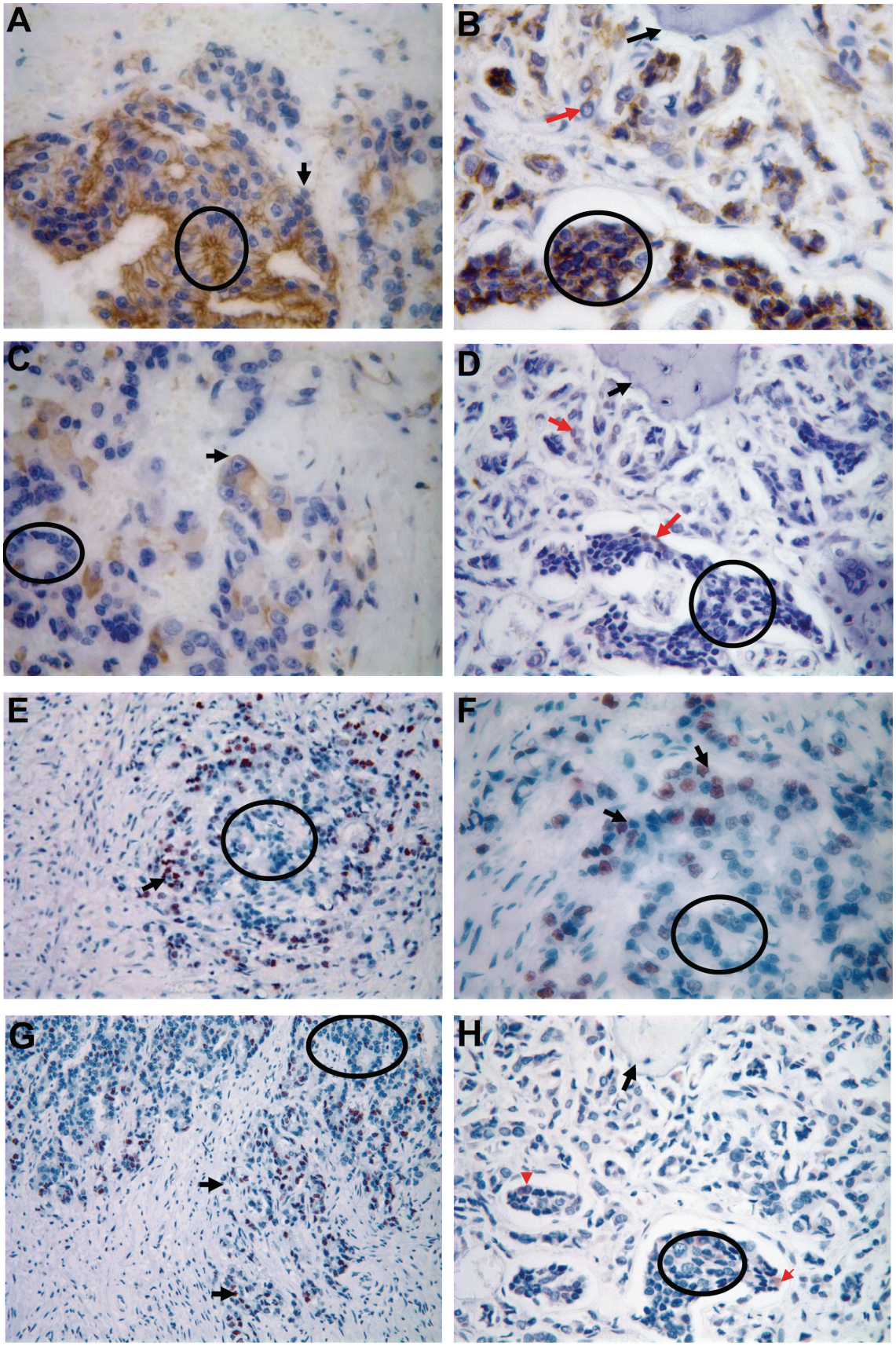
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Figure 1. Photomicrophotographs of EMT markers: (A) E-cadherin in PCa showing membranous expression in the center of the tumor cell clusters (circle), and loss of expression at the invasive tumor front (arrow) x 400. (B) E-cadherin in bone metastatic PCa (bone is shown by the black arrow) showing reduced E-cadherin expression in invading tumor cells (red arrow) with reappearance of E-cadherin expression in the metastasized tumor cell clusters (circle) by a reversal of the EMT to MET phenomenon x 400. (C) Vimentin expression seen at the invasive tumor front of the primary Pca (arrow) with minimal expression in the tumor cell clusters (circle) x 400 (D). In bone metastatic PCa (bone is shown by the black arrow), the invasive tumor fronts (red arrows) show Vimentin expression with none in the tumor cell clusters (circle) once they have reached their destination x 200; (E) NF-κB expression at the invasive tumor front of the primary Pca (arrow); however at the center of the tumor cell cluster (circle) there is no NF-κB expression x 200. (F) Higher magnification of PCa showing absence of NF-κB expression at the center of the tumor cell cluster (circle) and NF-κB expressing invasive tumor cells at the ITF x 400. (G) Another case of PCa showing NF-κB expression at the invasive tumor front (arrow) with absence of NF-κB expression at the center of the tumor cell cluster (circle) x 200. (H) In bone metastatic PCa (bone is shown by the black arrow) there is variable NF-κB expression at the center of the tumor cell cluster (circle) compared to the invasive tumor front (red arrows); x 200.

Table 3. EMT Marker expression levels in PCa & bone metastasis

EMT Marker	Expression	PCa		
		Primary	bone metastasis	p value*
E-CADHERIN	LOW	5	4	1
	HIGH	5	6	
VIMENTIN	LOW	9	10	1
	HIGH	1	0	
NF-κB	LOW	9	9	1
	HIGH	1	1	
PDGF-D	LOW	9	8	1
	HIGH	1	2	
NOTCH-1	LOW	6	1	0.057
	HIGH	4	9	
ZEB1	LOW	10	10	NA
	HIGH	0	0	

(*strong*). The percentage of stained cells was categorized into: 1=0-10% stained cells; 2=11-50% stained cells; 3=>50% stained cells. Final score was obtained by multiplying the 2 scores. Cases with a score of 0-4 were classified as low expressers and those with a final score of 5-9 were classified as high expressers.

Statistical analysis

The differences in the expression levels of the EMT markers among the PCa primary and bone metastasis cases were statistically analyzed using the Fischer's Exact Test.

Results and discussion

Microscopic evaluation of the immunohistochemically stained slides from primary PCa and PCa bone metastasis revealed membranous expression of E-cadherin; cytoplasmic expression of Vimentin and PDGF-D, and nuclear of NF-κB, Notch-1 and ZEB1 (**Figure 1 and 2**). The expression pattern of the markers varied according to the location within the tumor with high expression of the EMT phenotype at the invasive tumor front (ITF) compared to the center of the tumor cell clusters. The difference in the expression levels of EMT markers between PCa and PCa bone metastasis are detailed in **Table 2 and 3.** Upon statistical analysis of the EMT markers' expression levels among the PCa and bone metastasis, using the Fisher's Exact test, if the intensity and % of positive cells were considered separately, only the % of positive Notch-1 cells were statistically significantly

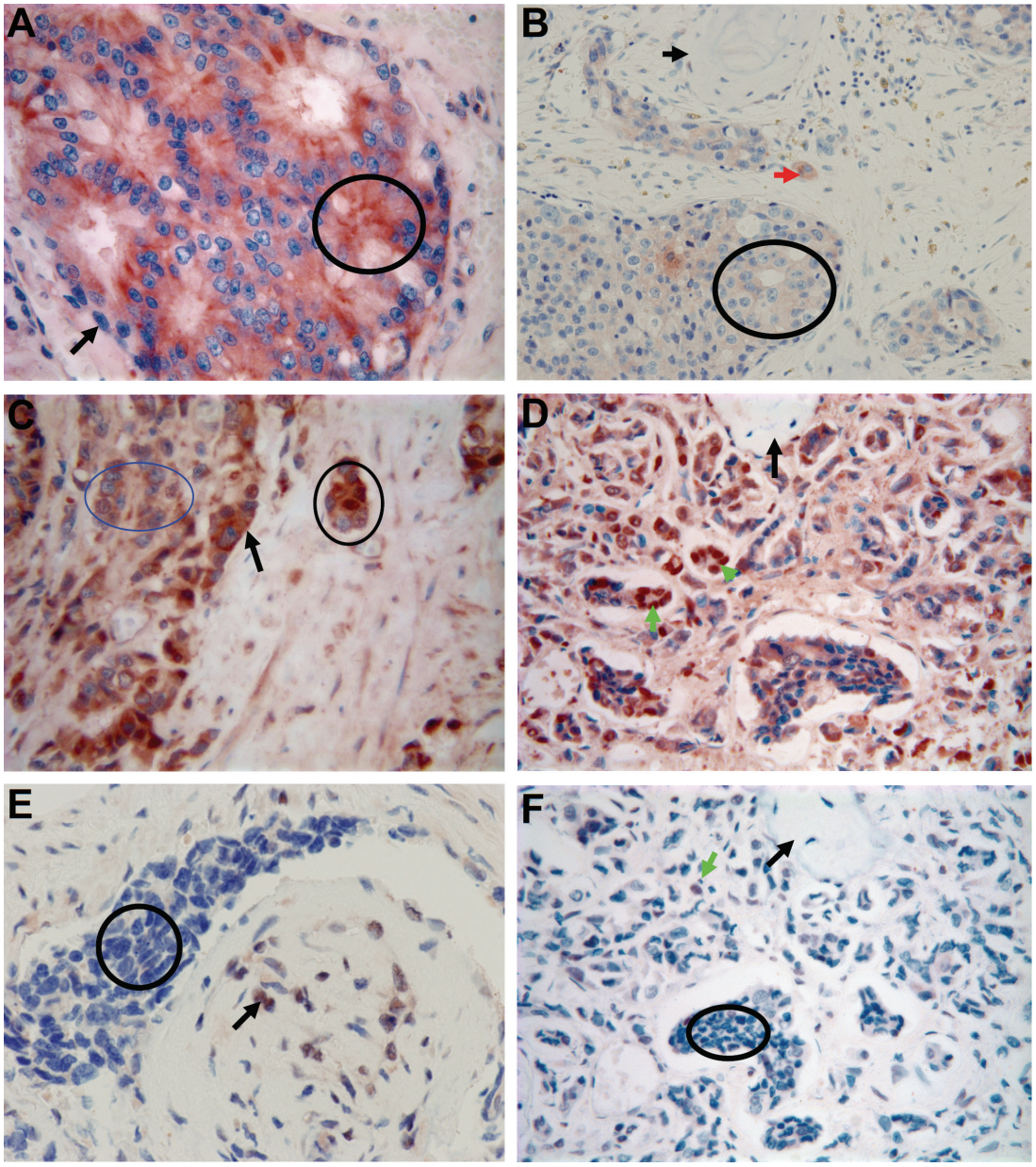

Figure 2. Photomicrographs of EMT markers: (A) PCa showing PDGF-D over-expression at the center of the tumor cell clusters (circle) and low expression at the invasive tumor front (arrows) x 400. (B) Bone metastatic PCa (arrow) showing variable PDGF-D expression at the center of the metastatic tumor clusters (circle) and in the invading cells (red arrow) x 200; (C) Notch-1 in PCa showing differential expression at the ITF (black arrow & circle) compared to the center of the tumor cell clusters (blue circle) x 400 ; (D) In bone metastatic PCa (bone is showing by the black arrow), there is Notch-1 over-expression in the metastatic tumor cells (green arrow) x 200. (E) PCa shown ZEB1 nuclear expression in invading tumor cells (arrow) while there is no expression in the center of the tumor cell clusters (circle) x 400. (F) Bone metastatic PCa (bone is shown by the black arrow) showing ZEB1 nuclear expression in the invading tumor cells (green arrow) as compared to the center of the tumor cell clusters (circle) x 200.

higher in the PCa bone metastasis (p=0.033). However when both intensity and % of positive cells were analyzed together to give a final score of high vs. low expression, then Notch-1 expression was found to be approaching statistical significance in bone metastatic PCa com-

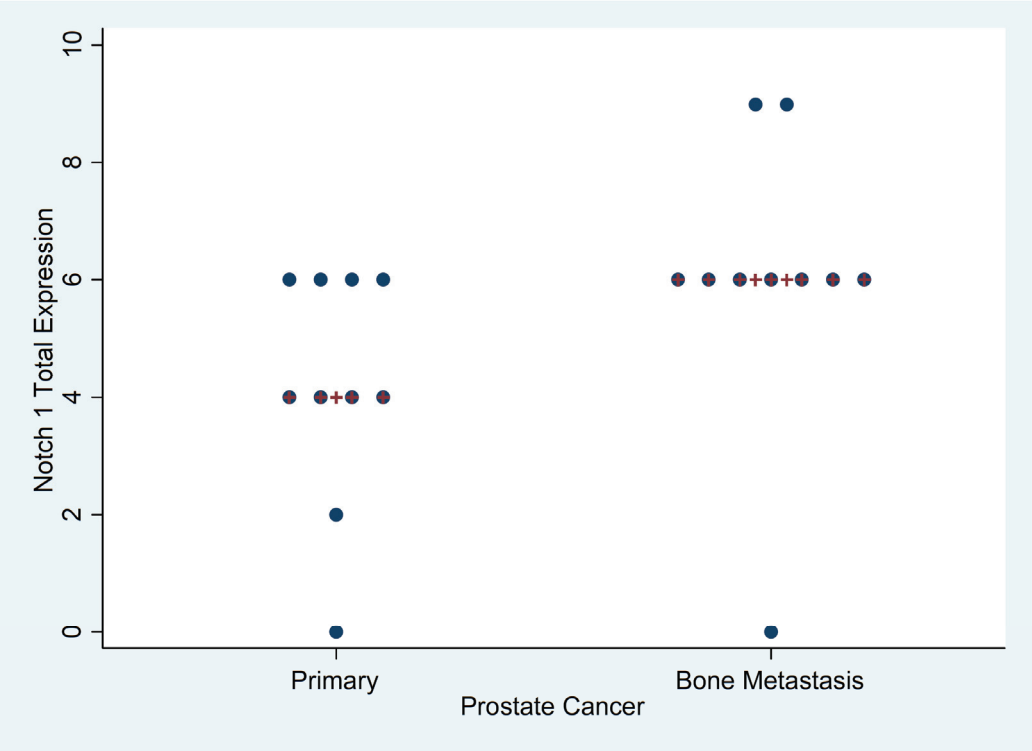

Figure 3. Dot-plot graph showing Notch-1 expression in the primary and bone metastatic PCa.

pared to primary PCa, suggesting its role in PCa bone metastasis (p=0.057) (**Figure 3**). For the remaining markers there was no significant difference in the expression, intensity, % of positive cells and scores in PCa vs. PCa bone metastasis.

Cancer is the cause of death in up to 25% of the population in the United States, and the vast majority of these are of epithelial cell origin [1]. During carcinogenesis, the immotile, polarized epithelial cells acquire highly migratory, apolar fibroblast-like features through a transient phenomenon referred to as Epithelial-to-Mesenchymal Transition (EMT). This indispensable process empowers the epithelial cancer cells with invasive and metastatic capabilities through acquisition of molecular alterations characteristic of mesenchymal phenotypes. It is known that EMT is associated with altered expression of growth and transcription factors and modified expression of cytoskeletal and adhesion molecules[22]. These can serve as potential targets for EMT for both cancer prevention and/or treatment. Inhibition of EMT, targeted killing of EMT phenotypic cells or the reversal of EMT phenotype, which will sensitize the cancer cells to conventional therapeutics are becoming a great promise for the treatment management of cancer patients.

Although EMT has been described in normal tissue development during organogenesis, during tissue remodeling and in wound healing, it is inappropriately reactivated during the development and progression of cancer [21]. There is an association between EMT and stem cell transformation in cancers which has been implicated in development of resistance to chemotherapeutic agents [23]. Two thirds of the cancer-related deaths in the US involve bone metastasis [24,25]. Bone is a favored site of PCa metastasis. The epithelial PCa cells undergo EMT changes which confer invasive potential and lead to metastasis. When metastatic PCa cells reach within the bone milieu, they trigger a cascade of molecular events that produce osteolytic and/or osteoblastic phenomena; and thus, obliteration of EMT alterations at the molecular level has the potential to prevent PCa

metastasis.

In the present study we found transformation of the cuboidal epithelial morphology of the PCa cells into the elongated mesenchymal type upon microscopic examination of the tissue sections. Our findings are consistent with previous studies [5,4] emphasizing the loss of 'top-bottom' (apical-basal) polarity that normally limits the movement of epithelial cells. Due to this morphological change, the cancer cells loose their ability to adhere to the adjacent cells, eventually resulting in cells that are much more motile [26]. The morphological changes of EMT are accompanied by alterations in the signaling pathways manifested by changes in protein expression. The altered expression levels were prominent at the invasive tumor front (ITF) than at the center of the cancer cell clusters. Our findings are consistent with previous studies [27,28]. The localization of EMT at the ITF is important as most aggressive cells and many crucial molecular interactions that enhance or inhibit tumor progression, occur at the tumor-host interface [29]. Due to EMT molecular alterations, the PCa cancer cells become discohesive at the ITF, migrate out of the tumor cell clusters, travel through lymphovascular system to metastasize to bone [30]. Once the metastatic PCa cells reach the bone, the EMT adhesion molecules re-express by a counter phenomenon referred to as Mesenchymal-to-Epithelial Transition (MET) wherein there is a reversal of the epigenetic mechanism of gene silencing promoting an epithelial phenotype [30].

In this study we observed altered expression of EMT markers E-cadherin, Vimentin, PDGF-D, NF-κB, Notch-1 and ZEB1 in PCa and bone metastasis tissues. The over-expression of Notch-1 was statistically significant in bone metastasis as compared to primary PCa ($p=0.057$). The expression levels, intensity and % of positive cells of the remaining markers were not statistically significant in PCa vs. PCa bone metastasis perhaps due to limited number of cases, suggesting that this proof-of-concept study warrants further in-depth investigation. Notch is a type 1 transmembrane protein which plays a key role in many fundamental cellular processes such as proliferation, stem cell maintenance and differentiation during embryonic and adult development [31]. Our findings are consistent with previous studies demonstrating Notch-1 over-expression contributing to PCa invasion [32], metastases [18] and osteoblastic bone metastases and osteoblastogenesis of human mesenchymal stem cells [33]. In the bone microenvironment, PCa metastatic cells acquire osteoblastic properties through independent activation of Notch signaling [34]. Notch-1 and its related genes can serve as novel targets for PCa therapy.

In summary, EMT is a crucial step in the progression and metastasis of PCa. The aberrant expression and localization of E-Cadherin, Vimetin, PDGF-D, NF-κB, Notch-1 and ZEB1, appears to be is important in PCa invasion and bone metastasis. These have the potential to serve as molecular markers of signaling pathways involved in EMT. Most EMT changes were seen at the invasive tumor front indicating the localization of this transient process at the TIF. Notch-1 up-regulation appears to play a significant role in PCa bone metastasis. These preliminary results offer great promise in the clinical arena. However, further validation in a larger cohort of PCa patients are needed to define the cross-talk between EMT signaling pathways in PCa and bone metastasis before these potential molecular markers of EMT can have clinical and translational relevance in the era of personalized medicine.

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