

The role of the endothelin axis in promoting epithelial to mesenchymal transition and lymph node metastasis in prostate adenocarcinoma

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Abstract

Introduction: Aberrant activation of endothelin (ET) axis has been identified as a key player in tumor growth and metastasis in several tumor types. However, little is known about the possible interaction of the ET with epithelial to mesenchymal transition (EMT), a process that transforms tumor cells in a motile, resistant to apoptosis phenotype prone to invasion and metastasis. The aim of this study was to investigate the activation of the ET axis in prostate adenocarcinoma and examine possible associations with EMT markers, lymph node (LN) metastasis, and other clinicopathological parameters.

Materials and Methods: We immunohistochemically evaluated the expression of ET-1 and its receptors A and B (ET-A, ET-B) in 64 N0 and 23 N1 prostate adenocarcinoma cases. EMT markers E-cadherin, N-cadherin, and β -catenin and the transcriptional factor SNAIL were evaluated. We examined possible correlations of ET pathway members with EMT markers, LN status, Gleason grade, and T stage.

Results: Our results revealed increased expression of ET-1 and ET-A (but not ET-B) in prostate carcinoma; both ET-1 and ET-A were associated with lymph metastasis and T stage but not with Gleason grade. We observed E-cadherin and β -catenin decrease/relocalization and increased N-cadherin expression. SNAIL also showed increased expression in tumor tissue and was associated with LN metastasis (Mann–Whitney test, $P = 0.0032$). Expression of ET-1 and ET-A correlated well with SNAIL expression (Spearman r , $P = 0.0002$ and $P = 0.0176$, respectively).

Conclusions: These findings indicate that activation of the ET pathway may induce EMT through SNAIL activation and correlates with increased metastatic potential.

Keywords: Endothelin, epithelial to mesenchymal transition, metastasis, prostate carcinoma, SNAIL

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INTRODUCTION

The endothelin (ET) axis is well known for its multiple physiological roles in vasomotor tone, cell proliferation, and tissue differentiation and development. Recently, the activation of the ET axis has been implicated in the

development and progression of cancer, enhancing the rationale of the use of selective ET-1 antagonists as potential antitumor agents.^[1] In many tumor types, such as colon, ovarian, kidney, and lung cancer, ET and its receptors ET-A and ET-B have been implicated in tumor

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growth and progression through various mechanisms.^[1] In prostate cancer, the available studies are limited; however, overexpression of ET and its receptors has been found present in all phases of prostate cancer.^[1] Although some preliminary data of ET-A antagonists as monotherapy are not encouraging,^[2] there is still keen interest in the possible antitumor effects of the ET axis inhibition.

An interesting fact of the ET actions is the multiple crosstalk with other important tumor initiation and progression pathways. Such an important pathway is the epithelial to mesenchymal transition (EMT), a molecular mechanism that is physiologically activated during embryogenesis, response to injury, and wound healing.^[3] EMT provides epithelial cells with mesenchymal properties such as increased invasiveness and migration, a process that is especially active at the invasive front of the tumor, increasing the metastatic potential of carcinoma cells.^[3,4] The key event of EMT is the loss of the epithelial molecule E-cadherin and gain of mesenchymal markers, such as N-cadherin and vimentin. Several important transcription factors such as SNAIL and SLUG are important drivers of EMT in cancer cells by repressing E-cadherin expression.^[4] However, the interaction of the ET axis with EMT has not been adequately studied. There are only a few studies available that underline the role of ET axis in promoting EMT in ovarian cancer cells.^[5,6]

The aim of this study was to investigate the activation of ET axis in prostate adenocarcinoma (PCa) and to examine possible associations with EMT markers, lymph node (LN) metastasis, and clinicopathological parameters. Moreover, we tested the hypothesis that increased ET-1/ET-A receptor expression is associated with increased expression of the transcription factor SNAIL.

MATERIALS AND METHODS

Prostate carcinoma samples

Pathology reports of patients with PCa who underwent radical prostatectomy with pelvic LN dissection during the past 6 years were reviewed. A total of 87 cases with readily available archival material were selected for this study. All 23 cases who were pathologically assessed to have regional LN metastasis (LN⁺, Stage pT2-3, N1, M0) were included in this study while the remaining randomly selected 64 cases formed the LN⁻ group (Stage pT2-3, N0, M0). After Institutional Review Board's approval, representative archival formalin-fixed, paraffin-embedded tissue blocks were selected for each case. Tumors were graded according to the Gleason system and staged according to the TNM (AJCC 2009) staging system for

radical prostatectomy. The cases were further divided into three groups, according to Gleason score as follows.

Group I: $n = 30$, grade <7 (26)

Group II: $n = 38$, grade = 7 (3 + 4 or 4 + 3)

Group III: $n = 19$, grade ≥ 8 .

Immunohistochemistry

Serial 4 μm sections were mounted on SuperFrost[®] Plus slides (Menzel-Glaser, Germany), deparaffinized by incubation in xylene at 60°C, and rehydrated in a series of graded alcohol solutions, followed by washing in tris-buffered saline (TBS) (pH 7.6). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in distilled water. For antigen retrieval, sections were treated in a microwave oven in citrate buffer (pH 6.0). Nonspecific binding was blocked by treating slides for 20 min with 3% bovine serum albumin (BSA) in TBS. The commercially available antibodies for E-cadherin, N-cadherin, β -catenin, SNAIL, ET-1, ET-A, and ET-B were used [Table 1]. Bound primary antibody was detected with the Envision[™] detection kit (DAKO, Hamburg, Germany) and diaminobenzidine was used as chromogen. Finally, tissue sections were counterstained with Mayer's hematoxylin and dehydrated through graded ethanol and xylene.

Negative control slides were treated with TBS/BSA instead of primary antibody and colon carcinoma specimens and vein samples were used for positive control slides, respectively. The noncancerous prostatic tissue was used as internal control in each case.

Immunohistochemical assessment

All slides were independently assessed by one senior pathologist (HP) and one investigator (SP). In cases of discrepant scoring, agreement was reached upon discussion.

The expression of the studied antibodies by the neoplastic cells was evaluated in a semiquantitative fashion, by developing an immunoreactivity score including both intensity and distribution of staining. Distribution was graded from 0 to 3 based on the percentage of positive cells (0: $<10\%$, 1:

Table 1: Antibody characteristics and incubation methodology

Antibody	Type	Source	Dilution	Incubation
E-cadherin	M	BD Biosciences	1:1500	Overnight at 4°C
N-cadherin	P	Acris	1:200	Overnight at 4°C
β -catenin	M	BD Biosciences	1:1500	Overnight at 4°C
SNAIL	P	Abcam	1:300	Overnight at 4°C
Endothelin-1	M	Acris	1:900	Overnight at 4°C
Endothelin receptor A	P	Acris	1:150	Overnight at 4°C
Endothelin receptor B	P	Acris	1:50	Overnight at 4°C

10%–30%, 2: 30%–70%, and 3: >70% of cells). Intensity of staining was scored as follows: Score 0: negative, 1: weak, 2: moderate, and 3: strong staining. “Negative” staining corresponds to complete absence of staining, “strong” corresponds to staining easily recognized at $\times 4$ magnification, “weak” corresponds to staining that can be recognized only at $\times 20$ magnification, and “moderate” is the staining intensity values between weak and strong. The two scores were multiplied and the immunoreactivity score (values from 0 to 9) was determined as follows: Score 0 as negative, Score 1 (values 1, 2, 3) as weakly positive, Score 2 (values 4, 6) as moderately positive, and Score 3 (value 9) as strongly positive.

Statistical analysis

The commercially available GraphPad Prism™ 5.0 statistical software, GraphPad Software, Inc., La Jolla, CA 92037, USA was used for all calculations. Differences between PCa and noncancerous prostatic tissue were evaluated using Wilcoxon test. Mann–Whitney and Kruskal–Wallis tests were used for nonparametric data comparisons between groups. Spearman’s correlation test was used for evaluating correlations of ET and its receptors with EMT markers and SNAIL. A 5% significance level was used for all tests.

RESULTS

Endothelin axis activation is mediated through endothelin-1 and endothelin-A but not endothelin-B receptor

Immunohistochemical reactivity for ET-1 was present in 86 of 87 cases of prostate carcinoma (99%). The immunostaining

was diffuse and cytoplasmic [Figure 1a and b], varying from medium to intense (mean \pm standard deviation [SD]: 2.06 ± 0.61) in carcinoma, compared to weak in noncancerous prostatic tissue (mean \pm SD: 1.04 ± 1.15 , Wilcoxon test, $P < 0.0001$). A higher ET-1 expression was associated with LN⁺ (Mann–Whitney test, $P = 0.0005$) and pT stage (T3, Mann–Whitney test, $P = 0.025$) but not Gleason grade when three grade groups were utilized [Figure 2]. ET-A receptor immunoreactivity displayed a similar to ET-1 expression pattern. In 98.6% of PCa specimens, cytoplasmic staining for ET-A was evident [Figure 1c and d], varying from medium to intense, in comparison to weak staining of noncancerous prostatic tissue (mean \pm SD: 2.19 ± 0.59 vs. mean \pm SD: 1.04 ± 0.15 , respectively, Wilcoxon test, $P < 0.0001$). Similarly, higher expression of ET-A was associated with LN⁺ (pN1, Mann–Whitney test, $P = 0.0003$) and pT stage (T3, Mann–Whitney test, $P = 0.027$) but not Gleason grade [Figure 2].

However, in an attempt to further evaluate possible association of ET-1 and ET-A expression with tumor grade, we developed two grade groups (low < 7 and high ≥ 7). According to this separation, statistically, significant higher ET-1 and ET-A immunoreactivity was present in high-grade cases [Mann–Whitney test, $P = 0.0026$ and $P = 0.0108$, respectively, Figure 3].

Despite repeated immunohistochemical attempts in several dilutions and conditions, no immunoreactivity for ET-B was present in any PCa case while a limited and uneven expression in noncancerous tissue was observed.

Epithelial to mesenchymal transition is present in prostate carcinoma cases

The EMT phenomenon was verified in our PCa series: Reduced membranous expression along with increased cytoplasmic and nuclear staining of E-cadherin was observed in tumor cells compared to the adjacent

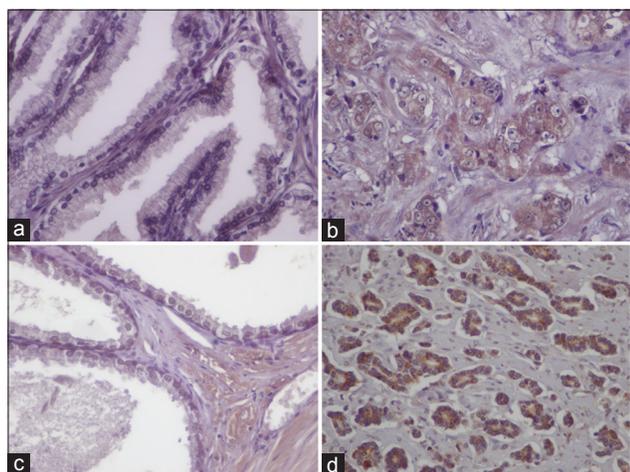


Figure 1: Increased immunohistochemical expression of ET-1 and ET-A in human prostate acinar adenocarcinoma: (a) adjacent nonneoplastic prostatic acini with negative immunoreactivity for ET-1, (b) cytoplasmic immunoreactivity of ET-1 in a representative case of prostate adenocarcinoma, (c) negative immunoreactivity for ET-A in adjacent nonneoplastic prostatic acini, (d) representative case of prostate adenocarcinoma with increased cytoplasmic immunohistochemical expression of ET-A ($\times 400$). ET-1: Endothelin-1

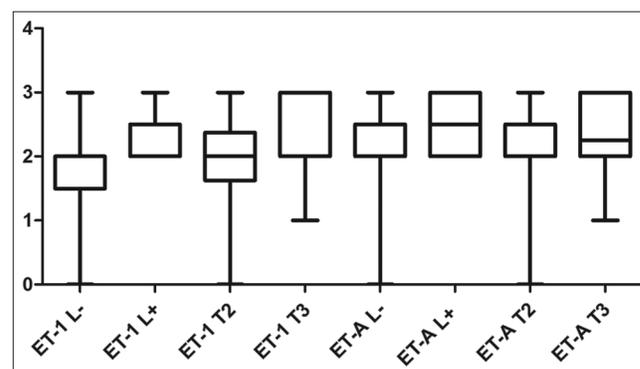


Figure 2: Box and Whiskers graph of ET-1 and ET-A expression versus lymph node status (L) and tumor stage (T). Whiskers: Minimum to maximum. ET: Endothelin

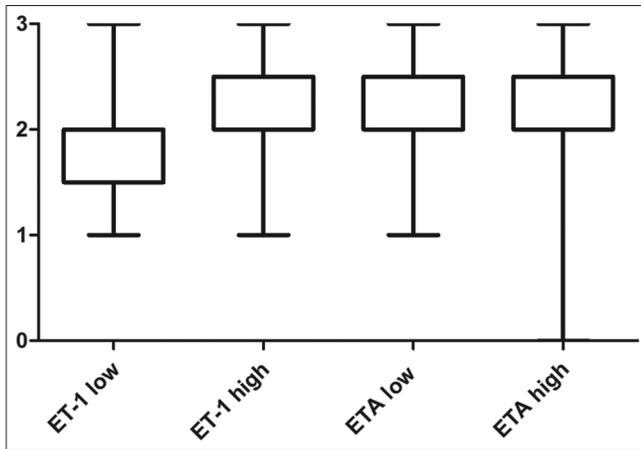


Figure 3: Box and Whiskers graph of ET-1 and ET-A expression versus tumor grade (two groups: Low-high). Whiskers: Minimum to maximum. ET: Endothelin

nonneoplastic epithelium, where E-cadherin showed strong membranous immunostaining [mean \pm SD: 0.92 ± 0.56 vs. 3 ± 0 , Wilcoxon test $P < 0.0001$, Figure 4a and b]. When the cytoplasmic and nuclear expression of E-cadherin was compared according to the Gleason grade, a positive association was present (Kruskal–Wallis test, $P = 0.0002$ and $P = 0.0002$, respectively). No statistically significant results occurred when E-cadherin immunoreactivity was evaluated toward pT stage and LN status.

Immunopositivity for N-cadherin was detected in 85 of 87 tumors (97.7% of cases). The cytoplasmic expression of N-cadherin was significant increase in tumor cells of PCa in comparison to nonneoplastic cells [mean \pm SD: 2.56 ± 0.63 vs. 2.06 ± 0.6 , Wilcoxon test, $P < 0.0001$, Figure 4c and d]. Both cytoplasmic and membranous expression of N-cadherin was associated with pT stage (Mann–Whitney test, $P = 0.0368$ and $P = 0.0377$) and Gleason score (Kruskal–Wallis test, $P = 0.0003$ and $P < 0.0001$, respectively).

The immunostaining of β -catenin in the adjacent “normal,” nonneoplastic epithelium showed strong membranous localization while no membranous staining at all was present in any tumor specimen. In contrast, medium and strong cytoplasmic and nuclear staining were present in 95.1% and 84.1% of PCa cases, respectively [Figure 5a and b]. Cytoplasmic expression of β -catenin was significantly correlated with the presence of LN metastasis (Mann–Whitney test $P = 0.0230$), but not pT or Gleason grade.

The transcription factor SNAIL showed medium and intense nuclear immunostaining in 95% of carcinoma cases while a weak expression was limited to the 74% of

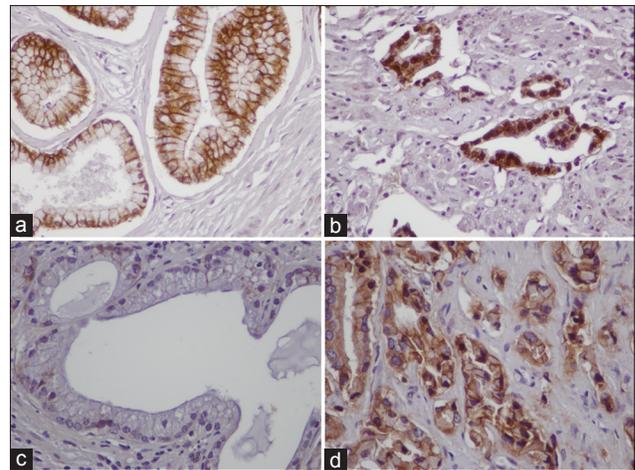


Figure 4: Immunohistochemical expression of E-cadherin and N-cadherin in human prostate acinar adenocarcinoma (a) adjacent nonneoplastic prostatic acini with membranous immunostaining for E-cadherin, (b) cytoplasmic and nuclear immunoreactivity of E-cadherin in a representative case of prostate adenocarcinoma, (c) negative expression of N-cadherin in adjacent nonneoplastic prostatic acini, (d) representative case of prostate adenocarcinoma with increased membranous and cytoplasmic immunohistochemical expression of N-cadherin ($\times 400$)

nonneoplastic epithelium [mean \pm SD: 2.60 ± 0.54 vs. 0.83 ± 0.71 , Wilcoxon test, $P < 0.0001$, Figures 5c, d and 6]. The increased nuclear levels of SNAIL in the tumoral compartment were associated with the presence of LN metastasis (Mann–Whitney test, $P = 0.0032$), pT stage (Mann–Whitney test, $P = 0.0375$), and the Gleason score (Kruskal–Wallis test, $P = 0.0402$).

Endothelin expression correlates with epithelial to mesenchymal transition markers in prostate carcinoma cases

In an attempt to reveal possible associations between the ET axis and EMT, the ET-1 and ET-A receptor expression was evaluated according to the expression of EMT markers. Both ET-1 and ET-A showed positive correlation with cytoplasmic E-cadherin expression (Spearman rank-order correlation, $r = 0.288$, $P = 0.006$ and $r = 0.236$, $P = 0.027$, respectively). Similarly, a positive correlation of ET-1 with “cytoplasmic” and negative correlation with “membranous” expression of β -catenin was present (Spearman correlation, $r = 0.177$, $P = 0.044$, and $r = -0.216$, $P = 0.0446$, respectively). The expression of ET-1 and ET-A correlated also well with SNAIL expression (Spearman r , $r = 0.394$, $P = 0.0002$ and $r = 0.254$, $P = 0.0176$, respectively).

DISCUSSION

The understanding of the pleiotropic actions of ET signaling in cell proliferation and survival, tumor neovascularization, and invasion has enhanced the interest

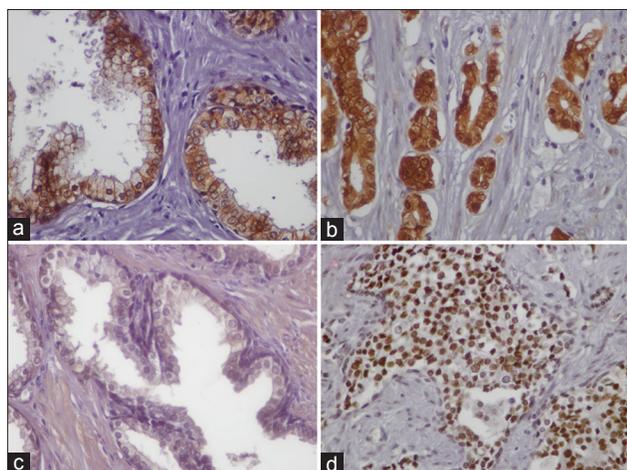


Figure 5: Immunohistochemical expression of β -catenin and SNAIL in human prostate acinar adenocarcinoma: (a) adjacent nonneoplastic prostatic acini with membranous immunostaining for β -catenin, (b) increased cytoplasmic and nuclear immunoreactivity of β -catenin in a representative case of prostate adenocarcinoma, (c) negative expression of SNAIL in adjacent nonneoplastic prostatic acini, (d) representative case of prostate adenocarcinoma with nuclear immunopositivity for SNAIL ($\times 400$)

in the central role of the ET axis in tumorigenesis and progression. Studies in several carcinoma types, including ovarian, colon, breast, bladder, and lung cancers, have revealed an activation of the ET axis that is also associated with pathological outcomes, such as decreased patient survival and metastasis.^[7] In PCa, the overexpression of ET-1 and ET-A receptor has been demonstrated in even early phases of prostate cancer, including high-grade prostate intraepithelial neoplasia.^[8] Similarly, the action of ET-1 through ET-A receptor has been implicated in the aggressiveness of prostate carcinoma.^[9] Our results are in accordance with these findings. A statistically, significantly increased expression of ET-1 and ET-A receptor was present in all PCa cases compared to nonneoplastic epithelium. An earlier study was unable to reveal any difference in the intensity of staining for ET-1 between BPH and PCa; however, this may be due to the small number of cases used.^[10]

Interestingly, both ET-1 and ET-A were expressed in similar patterns in PCa specimens and were both associated with LN⁺ and increased T stage. As far as the LN status is concerned, this finding has not been previously described in detail. Although older studies have found an association of ET axis activation with increased “stage” as a general term, LN metastasis has not been studied separately in PCa.^[11] In other tumor types (breast, thyroid, and others), increased lymphatic invasion and metastasis have been associated with activation of the ET axis.^[12,13] Several mechanisms may be implicated

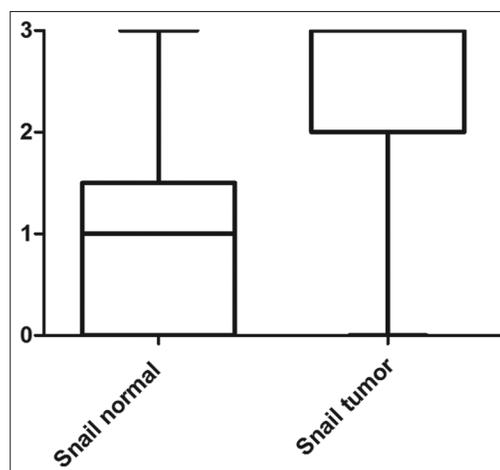


Figure 6: Box and Whiskers graph of SNAIL expression in nonneoplastic versus adenocarcinoma tissue. Whiskers: Minimum to maximum

in this process, including activation of members of the vascular endothelial growth factor (VEGF) family that promote lymphangiogenesis and hypoxia-induced aberrant expression of ET-1.^[13-16]

In this study, higher immunoexpression for ET-1 and ET-A was associated with higher T stage. In accordance with our findings, increased immunoexpression of ET-1 has also been associated with increased T stage.^[11,17] Moreover, increased immunoreactivity of ET-1 has been strongly associated with extracapsular extension of the tumor, a finding that might be used as a prognostic factor in needle biopsy specimens.^[18] Possible explanations include the effect of the ET axis on EMT, which leads to deconstruction of cell junctions and polarity and the acquisition of an invasive phenotype,^[19] as it will be further discussed.

A striking initial finding in our study was the lack of statistically significant differences in ET-1 and ET-A expression between the three grade groups. Most studies agree that, generally, poorly differentiated PCa is associated with higher ET-1 and ET-A immunoexpression.^[10,11,18,20,21] However, the results of these studies do not use a uniform way of stratifying cases according to Gleason score: the method used varied from exact Gleason scores to low-high scores. Hence, we classified our cases in two major groups (low <7 and high ≥ 7 Gleason score) as often used in other studies.^[18] In this case, differences for both ET-1 and ET-A proved statistically significant. This may indicate the problematic nature of grouping different Gleason scores for easier statistical calculations.

Despite repeated immunohistochemical attempts, we were unable to show any significant ET-B expression in

our series. This finding might represent a methodological artifact or a loss of tissue antigenicity; however, control slides were successfully used in all cases. In other immunohistochemical studies, the expression of ET-B was not completely diminished but was certainly reduced when compared to ET-A.^[11,20] Interestingly, Nelson *et al.* have described a decreased expression of ET-B in PCa (or even absence in prostatic cancer cell lines), suggesting that hypermethylation of the ET-B receptor gene, EDNRB, may be responsible for the downregulation of receptor expression.^[22,23] This finding has also been verified in other tumors such as lung, colon, and bladder cancer, suggesting a more “universal” role of ETB silencing in carcinogenesis.^[23] It has been proposed that ETB downregulation in fact further activates the ET-1/ET-A axis by reducing the clearance of ET-1.^[1,23]

The results of this study confirm the process of EMT in PCa, including a transition of E-cadherin from the membrane to the cytoplasm and the nucleus of the cancer cell in addition with an increased cytoplasmic and membrane expression of N-cadherin. This plasticity of the two cadherins is best described as a “cadherin switch” that facilitates the acquisition of stemness and metastatic properties of carcinoma cells. This cadherin switch has been verified in other tumor models, including breast, pancreas, colon, and ovary.^[24] It is worth mentioning that, in our study, the nonneoplastic epithelium used for comparison to the PCa showed (albeit limited) traces of EMT process as well. This is not an unexpected finding since the nonneoplastic epithelium demonstrated benign prostatic hyperplasia (BPH) changes in most cases. This has been verified in other studies where BPH has been associated with EMT-like molecular changes.^[25,26]

The “cadherin switch” in our study was associated with poor differentiation and T stage. These findings are in accordance with previous studies where diminished E-cadherin membrane expression and increased N-cadherin cytoplasmic/membrane expression are common findings related to increased tumor stage and grade.^[27-29] The loss of membrane E-cadherin denotes not only a transition to the cytoplasm and nucleus but also a possible downregulation by factors such as the activated androgen receptor.^[30] Interestingly, it has been proposed that the “cadherin switch” in whole may be more important than the expression of E- and N-cadherin separately as it correlates better with biochemical and clinical recurrence in prostate cancer.^[29] Along the same lines, in our study, the loss of membrane β -catenin and increased expression in the cytoplasm have been associated with positive LN status. Similar results have been shown in early gastric cancer,

mammary cancer, and melanoma where β -catenin has been found to be strongly associated with LN metastasis.^[31-33] The interaction of VEGF receptors (VEGFRs) with the EMT pathways may explain this finding as VEGFR-1 activation has been shown to enhance translocation of β -catenin from its usual cell membrane-bound location to the cytoplasm and nucleus.^[34]

Several transcription factors (including SNAIL/SLUG, TWIST, FOX, and ZEB) are master regulators of the EMT. In particular, the transcription factor SNAIL (also known as SNAIL1) plays an important central role in activating EMT programming by early downregulation of E-cadherin.^[19] In our series, moderate and strong immunopositivity for SNAIL was present in the majority (95%) of PCa cases while the nonneoplastic epithelium showed weak expression. SNAIL expression was positively associated with poor differentiation, advanced T stage, and LN metastasis. These findings are in line with a few other studies where increased SNAIL expression was associated with clinicopathologic variables of progressive disease in PCa.^[35,36] LN metastasis in particular was strongly associated with increased SNAIL expression in our study. This is an important finding that has not been adequately described in the existing literature for prostatic cancer. However, data from studies in other tumor types, including cervical and colorectal carcinoma, support our findings by emphasizing the strong relation between high SNAIL expression in the tumor and promotion of LN metastasis.^[37,38]

Activation of the ET axis through ET-1/ET-A was strongly associated with the classic findings of EMT changes in our series, i.e., increased cytoplasmic E-cadherin and loss of membrane β -catenin immunoexpression. In addition, ET-1/ET-A was also strongly associated with higher SNAIL expression. Taken together, these findings may suggest a crosstalk between the ET axis and the process of EMT. To the best of our knowledge, this has never been studied in prostate carcinoma; it has only been demonstrated in ovarian carcinoma cell lines.^[5,6] The exact mechanism remains unclear; however, ET-1 has been shown to increase SNAIL messenger RNA (mRNA) levels and SNAIL protein stability, a phenomenon accompanied by the downregulation of E-cadherin mRNA.^[39] Moreover, ET-1 stabilizes β -catenin, further enhancing the EMT cascade.^[39,40] Taking these findings into account, it appears that the ET1 pathway contributes to the complex procedure of EMT in prostate carcinoma in a similar fashion. It would be interesting to verify our initial immunohistochemical findings in further functional studies utilizing prostate carcinoma cell lines.

CONCLUSIONS

In summary, the results of this study highlight a possible ET-1/ET-A-induced mechanism of EMT in PCa. In addition, we have demonstrated that the transcription factor SNAIL may enhance LN metastasis. Verification of our findings by further studies might pave the way for the rational use of ET receptor inhibitors in certain prostate carcinoma clinical settings.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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