EMT Markers in Locally-Advanced Prostate Cancer: Predicting Recurrence?

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Background: Prostate cancer (PCa) is the second most frequent cause of cancer-related death in men worldwide. It is a heterogeneous disease at molecular and clinical levels which makes its prognosis and treatment outcome hard to predict. The epithelial-to-mesenchymal transition (EMT) marks a key step in the invasion and malignant progression of PCa. We sought to assess the co-expression of epithelial cytokeratin 8 (CK8) and mesenchymal vimentin (Vim) in locally-advanced PCa as indicators of EMT and consequently predictors of the progression status of the disease.

Methods: Co-expression of CK8 and Vim was evaluated by immunofluorescence (IF) on paraffin-embedded tissue sections of 122 patients with PCa who underwent radical prostatectomies between 1998 and 2016 at the American University of Beirut Medical Center (AUBMC). EMT score was calculated accordingly and then correlated with the patients’ clinicopathological parameters and PSA failure.

Results: The co-expression of CK8/Vim (EMT score), was associated with increasing Gleason group. A highly significant linear association was detected wherein higher Gleason group was associated with higher mean EMT score. Additionally, the median estimated biochemical recurrence-free survival for patients with <25% EMT score was almost double that of patients with more than 25%. The validity of this score for prediction of prognosis was further demonstrated using cox regression model. Our data also confirmed that the EMT score can predict PSA failure irrespective of Gleason group, pathological stage, or surgical margins.

Conclusion: This study suggests that assessment of molecular markers of EMT, particularly CK8 and Vim, in radical prostatectomy specimens, in addition to conventional clinicopathological prognostic parameters, can aid in the development of a novel system for predicting the prognosis of locally-advanced PCa.

Keywords: prostate cancer, cytokeratin 8, vimentin, epithelial-to-mesenchymal transition, Gleason group, clinicopathological parameters
INTRODUCTION

Prostate Cancer (PCa) is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males worldwide (1). Screening for PCa is not routinely practiced in the Middle East, which pertains to the rising incidence rates and the high proportion of patients being diagnosed with high-risk locally-advanced and metastatic disease in this region of the world (2, 3).

Radical prostatectomy is an effective therapeutic procedure for men with organ-confined PCa. This modality, however, fails in 30–40% of patients as serum prostate-specific antigen (PSA) levels continue to rise and patients eventually develop biochemical recurrence postoperatively (4). It is of utmost importance to identify the parameters that can accurately predict the prognosis and clinical outcome following radical prostatectomy. To date, several investigators have described the usefulness of various clinicopathological factors—including PSA, Gleason scores, pathological stage, surgical margin status (SMS), perineural invasion (PNI), seminal vesicle invasion (SVI), lymphovascular invasion (LVI), and tumor volume—and their correlation with treatment failure (5–8). However, these studies have carried several limitations, such as the recent stage migration and grade inflation because of the greater aggressiveness of PCa (9), besides the differences in PCa features among diverse ethnic groups (10).

Expression of epithelial-to-mesenchymal transition (EMT) markers represents a crucial step in the malignant progression of several cancers, such as prostate, breast, ovarian, and colon cancers (11–15). This pathological process ensues the breakdown of cell-to-cell or cell-to-extracellular matrix (ECM) adhesions at the polarized epithelium lining prompting conversion into mesenchymal phenotype and enhanced cell mobility, invasion, and metastasis (14, 16). The role of EMT in PCa metastasis has been studied (16) revealing significant interplay between EMT-related genes and tissue invasion on one hand, and alterations in TGF-β (17), IL-6 (18–20), AR variants (21, 22), FGF (23), and Wnt/β-catenin signaling pathways (24–26) on the other hand.

In a previous study by our group, we have reported increased co-expression of epithelial cytokeratin 8 (CK8) and mesenchymal vimentin (Vim) markers in androgen-independent PLum-AI murine PCa cell lines, which represent advanced stages of PCa, referring to a positive EMT status in those cells, when compared to androgen-dependent PLum-AD cells which represent primary PCa (27). CK8/Vim co-expression was also reported in other murine PCa cell lines, including PLum-P and PLum-C Pten−/−TP53−/− murine prostate epithelial progenitor cells (28).

In this study, we evaluated the co-expression of two potential molecular markers of EMT, namely CK8 and Vim, in radical prostatectomy specimens of locally-advanced PCa patients using immunofluorescent (IF) staining. Accordingly, we developed a novel scoring system to quantify EMT expression (EMT score) and explored the correlation between this score and the different clinicopathological outcomes. Our results confirmed that the EMT score can predict PSA failure, and thus biochemical recurrence, irrespective of Gleason group and other conventional PCa diagnostic and prognostic parameters.
in a citrate buffer in a steamer at 100°C for 40 min. This was followed by protein blocking using the blocking buffer (3% BSA, 0.1% Triton x-100, and 10% Normal Goat Serum in PBS) for an hour at room temperature. Slides were stained using the different primary antibodies: anti-CK8 overnight, and anti-Vim for 2 h; then tissues were incubated with the corresponding secondary antibodies. Finally, slides were mounted with the anti-fade Fluoro-gel II with DAPI.

### Microscope Specifications

Indirect immunofluorescence microscopic analyses were performed using Carl Zeiss Axio Observer.Z1 and LSM710 laser scanning confocal microscopes. All images were acquired and analyzed using the Carl Zeiss ZEN 2012 image software.

### IF Evaluation and EMT Scoring

EMT scoring was performed manually using a 40× objective and a Carl Zeiss Axio Observer.Z1 microscope. It was done by screening the whole tissue section in a systematic manner and counting the total number of glands, then counting the number of glands with at least one cell co-expressing CK8 and Vim. Then, the percentage was calculated by dividing the number of glands with at least one double positive cell by total number of glands, multiplied by 100. This percentage is referred to as EMT score. CK8/Vim staining was graded as double positive only when cytoplasmic staining was detectable.

### Statistical Analysis

The EMT score was categorized into <25% and more than or equal to 25%. This cutoff of 25% was assigned based on the EMT score distribution where 95.1% (116) of the total population clustered in the “less than or equal to 50% EMT score.” Chi-square test and two-tailed unpaired Student’s t-test of independent variables were used to compare the mean EMT score (as a continuous variable) between the different Gleason groups. A Mantel–Haenszel test of trend was run to determine whether a linear association existed between the EMT score categories and the different Gleason groups. In a secondary analysis, a linear regression model was built to examine the effect of the Gleason group on the EMT score while adjusting for the pathological stage and the surgical margins. EMT score in addition to the Gleason group, pathological stage, and surgical margins (the three clinicopathological variables which showed statistically significant difference between the two EMT score categories) were entered as covariates in the cox regression model. $P \leq 0.05$ were considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences statistical package 21.0 software (SPSS, Inc.).

### RESULTS

#### Clinicopathological Characteristics of PCa Patients and Their Correlation With the EMT Score

A total of 122 radical prostatectomy specimens were analyzed. Table 1 summarizes the clinicopathological characteristics of the 122 patients. Association of several clinicopathological variables and EMT score is shown in Table 2. The specimens were analyzed by IF and examined for CK8/Vim co-expression (EMT score) (Figure 2).

In studying the sample distribution statistics between the two categories of the EMT score, a significant statistical difference was detected between the two categories in terms of Gleason group ($p = 0.014$), pathological stage ($p = 0.014$), and surgical margins ($p = 0.006$). No significant differences in the patient’s age, pre-operative PSA, PSA failure (defined by an increase in blood PSA...
level at or above 0.2 ng/mL following surgery), and tumor volume were observed (Table 2).

**High Mean EMT Score Is Significantly Associated With Higher Gleason Group**

To investigate the difference in the mean EMT score between the assigned Gleason groups, an independent t-test was run. There were 60 patients in group A, 30 patients in group B, and 28 patients in group C. There was no statistical difference in the mean EMT score between group A and B. Nonetheless, the mean EMT score was higher in group B ($M = 15.3\%$, $SD = 21.3\%$) than group A ($M = 10.7 \%, SD = 11.6\%$), with a mean difference ($M = -4.62$, $95\% CI [-13.04;3.81], p = 0.274$). When comparing the mean EMT score of the 60 patients in the Gleason group A ($M = 10.7 \%, SD = 11.6\%$) to the 28 patients in group C ($M = 26.8\%, SD = 29.1\%$), a significant difference with quite high mean difference was recorded ($M = -16.09$, $95\% CI [-27.71; -4.47], p = 0.008$). The mean EMT score comparison between groups B and C revealed no significant difference, although a higher mean was recorded in the higher Gleason group ($M = -11.47$, $95\% CI [-24.99; -2.06], p = 0.091$) (Table 3).

A mean plot of the EMT score vs. the three Gleason groups is shown in Supplementary Figure 1. A Mantel–Haenszel test of trend was run to determine whether a linear association existed between EMT score categorized into two groups (<25% and more than or equal to 25%) and the assigned Gleason groups. The Mantel–Haenszel test of trend showed a statistically significant linear association between them ($\chi^2_{(1)} = 7.547$, $p < 0.007$, $r = 0.254$), where higher Gleason group was associated with a higher EMT score (Supplementary Table 1). A scatterplot simplifying the linear association between EMT score and the Gleason groups is presented in Supplementary Figure 2.

**Gleason Groups Can Predict EMT Score Irrespective of the Pathological Stage and Surgical Margins**

A multiple regression model was built to study if Gleason group can predict EMT score while adjusting for the pathological stage and surgical margins, the variables which showed statistically significant difference between the two EMT score categories (Table 2). The multiple regression model significantly predicted EMT score, $F_{(3,112)} = 7.037$, $p < 0.001$. $R^2$ for the overall model was 15.9 % with an adjusted $R^2$ of 13.6%. Only Gleason group added statistical significance to the prediction, $p = 0.001$. Regression coefficients and their $P$-values can be found in Supplementary Table 2.

**EMT Score Can Predict PSA Failure Irrespective of Gleason Group, Pathological Stage, or Surgical Margins**

To study the correlation between EMT score and PSA failure, a Cox regression model was built. Time to PSA failure was considered time to event, and EMT score, Gleason group, pathological stage, and surgical margins were added as covariates to the model using forward method. EMT score was found to be an independent predictor of PSA failure. Biochemical recurrence was higher in patients with EMT score $\geq 25\%$ (OR: 2.23, $95\% CI [1.018; 4.895], p = 0.045$). The overall model has a $\chi^2$ of 4.221, with a $P$-value of 0.04. Biochemical recurrence-free survival curve estimating PSA failure based on the patients’ EMT score is shown in Figure 3.

**DISCUSSION**

Despite the advances in the treatment of metastatic PCa, most patients eventually die from their disease. This is due to the rapid and poorly understood progression of PCa from a primary stage to an advanced and metastatic castration-resistant PCa (mCRPC) stage which involves several mechanisms, including epithelial-to-mesenchymal transition (EMT). The latter is recognized in endorsing the invasiveness of PCa cells due to increased mobility and migration of mesenchymal cells (16). In addition to the role of EMT in PCa progression, it has been identified as playing a substantial role in PCa therapeutic resistance to anti-androgens and radiotherapy (30). Therefore, it has...
been postulated that targeting EMT may improve the overall survival of patients with PCa (16). The main cause of PCa mortality is the progression to metastatic castration-resistant PCa (mCRPC); therefore, identifying the onset of metastatic dissemination through assessment of molecular markers of EMT can aid in the development of a novel system for predicting the prognosis of PCa. Nonetheless, the translation of EMT into clinical applicability presents substantial challenges (31). This can be attributed to tumor heterogeneity and diverse metastatic behavior, which is underrepresented in
TABLE 3 | Comparison of the mean EMT scores between Gleason groups.

| Gleason group     | N   | Mean (±SD)   | Mean difference (95% CI)       | P-value |
|-------------------|-----|--------------|--------------------------------|---------|
| Mean EMT score A: | 60  | 10.7 (±11.6) | −4.62 [−13.04; 3.8]            | 0.274   |
| B: Gleason score 7(4 + 3) | 30 | 15.3 (±21.3) | −11.47 [−24.99; 2.06]          | 0.091   |
| C: Gleason scores 8 and 9 | 28 | 26.8 (±29.1) | −16.09 [−27.71; −4.47]         | 0.008   |
| A: Gleason scores 6 and 7(3 + 4) | 60 | 10.7 (±11.6) | −16.09 [−27.71; −4.47]         | 0.008   |
| C: Gleason scores 8 and 9 | 28 | 26.8 (±29.1) | −16.09 [−27.71; −4.47]         | 0.008   |

Bold values represent statistically significant data.

Currently used homogenous cell lines and preclinical models (32). Yet, several studies have addressed the changes in the expression levels of genes and/or proteins associated with EMT in human tumor samples to establish an association with clinical significance.

In carcinoma, invasion and metastasis are associated with transition of cancer cells from an epithelial keratins-expressing phenotype to a mesenchymal vimentin (Vim)-expressing phenotype (33, 34). The importance of assessing the EMT status through investigating Vim overexpression was highlighted in
Relationship between an E-cadherin to N-cadherin switch and shown to be associated with a worse prognosis and a more was significantly higher compared to other subtypes, and was

In our patients, representing a cohort of high-risk locally-
advanced PCAs from the Middle East region, looking at co-expression patterns of CK8 and Vim revealed that the mean EMT score increases significantly as disease becomes more poorly differentiated reflected by higher Gleason group (Table 3). Our results show that there is a highly significant difference in the mean EMT score between Gleason groups A and C (10.7 ± 11.6% in Gleason group A vs. 26.8 ± 29.1% in Gleason group C, p = 0.008). Furthermore, there is a highly significant linear association based on Mantel–Haenszel test (p = 0.007) whereby higher Gleason groups were associated with higher EMT scores (Supplementary Figure 2). The added value of this EMT scoring system is the fact that it can predict PSA failure irrespective of Gleason group, pathological stage, and surgical margins (41). As PSA recurrence is a powerful predictor of distant metastasis, cancer-specific survival, and overall survival, these results suggest that the EMT score can be used to estimate the biochemical recurrence-free survival of a patient irrespective of other clinicopathological parameters.

A possible explanation of the link between EMT status and disease progression is the fact that cells with hybrid epithelial/mesenchymal phenotypes possess a large repertoire of survival strategies under many stress conditions (42). EMT has been linked to circulating tumor cells (CTCs) generation and subsequently metastasis. In colorectal cancer, for instance, the presence of biophenotypic and mesenchymal CTCs, rather than epithelial CTCs, is indicative of a more advanced disease stage and metastasis (43).

**CONCLUSIONS**

In conclusion, this study underscores the importance of EMT markers (increased Vim and decreased CK8 expression) for predicting the prognosis of PCAs. Whereas, previous studies have indicated reduced expression of epithelial markers and increasing expression of mesenchymal markers, an EMT phenotype and the co-expression of such markers specifically CK8 and Vim and their association with outcome data have not been described. Since these markers could have a significant effect on the management of PCa patients, including projections of targeted therapy, we suggest the extrapolation of this study to larger cohorts of patients from different ethnicities to further validate our findings. Besides, since androgen receptor (AR) expression and EMT have been recently reported to be mutually exclusive (44), future studies are indeed warranted to evaluate expression levels of AR and PSA in the PCa tissue samples and their correlation with EMT score.

**STUDY LIMITATIONS**

We recognize that our study has some limitations. First, as a clinical study the sample size is relatively small, therefore the results obtained require further investigation on a larger cohort. Second, samples were collected retrospectively over the period of 18 years with around 75% of the samples having a positive margin and around 70% with a pathological stage.
greater than pT3. The latter identified the study sample as a high-risk cohort thus restricting the results obtained to such sample characteristics. Third, the retrospective collection of data led to missing information regarding the SVI, PNI, and LNM status of the patients; this might explain the lack of significant correlation between the EMT score and the metastatic status.

AUTHOR CONTRIBUTIONS

KC, HB, OH, ES, CD, MH, MS, ZM, SN, AT, MB, RK, WW, RN, AS, ST, and ME-S contributed to the project design and execution of experiments. KC, HB, OH, and ES contributed to the analysis of results and writing of manuscript. AE-H, DM, and WA-K contributed to overlooking and following up with experiments, result analysis, and manuscript proofreading. AE-H, DM, and WA-K contributed to project design, result analysis, manuscript writing, and proofreading. All authors critically revised and edited the manuscript and approved the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00131/full#supplementary-material

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