Comprehensive Study of Gene and microRNA Expression Related to Epithelial-Mesenchymal Transition in Prostate Cancer

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Abstract

Prostate cancer is the most common cancer in men, and most patients have localized disease at the time of diagnosis. However, 4% already present with metastatic disease. Epithelial-mesenchymal transition is a fundamental process in carcinogenesis that has been shown to be involved in prostate cancer progression. The main event in epithelial-mesenchymal transition is the repression of E-cadherin by transcription factors, but the process is also regulated by microRNAs. The aim of this study was to analyze gene and microRNA expression involved in epithelial-mesenchymal transition in localized prostate cancer and metastatic prostate cancer cell lines and correlate with clinicopathological findings. We studied 51 fresh frozen tissue samples from patients with localized prostate cancer (PCa) treated by radical prostatectomy and three metastatic prostate cancer cell lines (LNCaP, DU145, PC3). The expression of 10 genes and 18 miRNAs were assessed by real-time PCR. The patients were divided into groups according to Gleason score, pathological stage, preoperative PSA, biochemical recurrence, and risk group for correlation with clinicopathological findings. The majority of localized PCa cases showed an epithelial phenotype, with overexpression of E-cadherin and underexpression of the mesenchymal markers. MiRNA-200 family members and miRNAs 203, 205, 183, 373, and 21 were overexpressed, while miRNAs 9, 495, 29b, and 1 were underexpressed. Low-expression levels of miRNAs 200b, 30a, and 1 were significantly associated with pathological stage. Lower expression of miR-200b was also associated with a Gleason score ≥8 and shorter biochemical recurrence-free survival. Furthermore, low-expression levels of miR-30a and high-expression levels of Vimentin and Twist1 were observed in the high-risk group. Compared with the primary tumor, the metastatic cell lines showed significantly higher expression levels of miR-183 and Twist1. In summary, miRNAs 200b, 30a, 1, and 183 and the genes Twist1 and Vimentin might play important roles in the progression of prostate cancer and may eventually become important prognostic markers.

Introduction

Prostate cancer (PCa) is one of the most common tumors in men, and it accounts for 29% of all newly diagnosed cancers [1]. After the adoption of PSA screening, most patients present with localized PCa, but 4% already have metastatic disease at the time of diagnosis [1]. At present, clinicopathological features such as staging, Gleason score (GS), and PSA levels are good prognostic markers [2] and are used to make treatment decisions; however, they are not sufficiently accurate to discriminate between tumors that will remain indolent and those that will later progress to become metastatic. Indeed, the unique biological features and heterogeneous genetic backgrounds of PCa [3] can limit the efficacy of conventional clinicopathological parameters as predictive markers. For these reasons, molecular biomarkers have been increasingly investigated to help understand and predict cancer behavior.

The epithelial-to-mesenchymal transition (EMT) is a reverse biological process that plays a role in invasion and metastasis during carcinogenesis. Epithelial cell-cell adhesion is decreased, and the cells acquire a spindle-shaped, highly motile fibroblast phenotype and a greater capacity for migration and invasion [4]. The main feature of EMT is transcriptional silencing of E-cadherin [5,6], which is controlled by the transcriptional regulators ZEB1, ZEB2, SNAI1 (Snail), SNAI2 (Slug), and TWIST1 [5,7,8]. Additionally, there is also upregulation of mesenchymal markers, such as Vimentin and N-cadherin, a process that is known as cadherin switching [9].

The roles of genes related to EMT in PCa are not completely understood, and previous studies describe the loss of E-cadherin [10] followed by increased expression of N-cadherin, Cadherin-11 and Vimentin [9] in immunohistochemistry analysis. The expression levels of ZEB1, a crucial regulator of EMT in PCa, are
related to the GS [11], and Behnsawy et al proposed the use of EMT gene expression profiles as markers of biochemical recurrence after radical prostatectomy [12].

MicroRNAs (miRNAs), a new class of non-coding, regulatory RNAs, have been shown to participate in many processes related to the development and progression of cancer, including EMT [15]. One of the main miRNAs involved in EMT is the miR-200 family, which is a potent inducer of epithelial differentiation. This group comprises miR-200a, miR-200b, miR-429, miR-200c, and miR-141, which are generated from two transcripts. The first three are derived from chromosome 1, while the latter two are derived from chromosome 12. The members of this group are highly related in sequence, indicating that they likely target a similar complement of messenger RNAs [14].

Among the targets of the miR-200 family are ZEB1 and ZEB2 [15–17]. miR-200 members inhibit the expression of ZEB at the post-transcriptional level by binding to highly conserved target sites in their 3’UTRs [18,19]. Interestingly, miR-200 members are transcriptional targets of ZEB1 and ZEB2. The close functional link between the ZEB factors and the miR-200 family in a double-negative feedback loop is known as the ZEB/miR-200 feedback loop [18], in which the activation of one group negatively affects the expression of the other group. Depending on the extracellular signals, this loop can switch from one side to the other side and stabilize either the epithelial or mesenchymal phenotype. Other miRNAs have also been shown to participate in EMT, targeting SNAI1 (miR-29b, miR-30a, miR-34a) [20,21], and SNAI2 (miR-34a, miR-1, miR-200b) [22,23]. However, few studies have assessed miRNAs involved in EMT in PCa.

Our aim is to decipher the role of genes and miRNAs related to EMT in PCa to identify a profile that defines PCa behavior.

Materials and Methods

Patient selection

Fifty-one patients who had clinically localized prostate cancer and underwent radical prostatectomy between 2000 and 2002 were selected. All patients were treated by the same surgeon (MS), and all pathological specimens were analyzed by the same uropathologist (KRML). The patients were followed up for a mean time period of 63.06 months.

The control group consisted of ten samples from patients who underwent surgery for benign prostatic hyperplasia, and had no malignancy in the pathological specimen. The final result was obtained by applying the 2−ΔΔct method. The formula employed is ΔΔct = dCt1 − dCt2, where dCt1 = CT of the target (tumor sample) − CT of the mean of the endogenous control (tumor sample), and dCt2 = CT of the mean of the normal controls (benign prostate tissue) − CT of the mean of the endogenous control (benign prostate tissue). For evaluation of the metastatic cell lines, the “control” (dCt2) was considered to be the pT2 tumors. The final result was obtained by applying the 2−ΔΔct method. Findings greater or lesser than 1 were considered to indicate overexpression or underexpression, respectively. All values were standardized relative to the normal control values, which were represented as a value of 1.

Gene and miRNA selection

The choice of miRNAs and genes evaluated in this study was based on their role in the EMT process in various types of cancer. We performed a literature search via PubMed and Web of Science using the terms “epithelial-mesenchymal transition”, “cancer”, and “miRNA”. Based on the data published in the literature, we selected 18 miRNAs that targeted the most important genes involved in EMT. The data are presented in Table 1.

Statistical Analysis

To compare the clinicopathological features among patients with localized PCa, the patients were divided into groups based on their GS (GS ≤ 6 vs GS ≥ 8), pathological stage (pT2 vs pT3), pre-operative PSA (<10 vs ≥ 10 ng/mL), and absence or presence of biochemical recurrence, defined as PSA ≥0.2 ng/mL. The patients were also classified into low-risk and high-risk disease groups according to the presence of any unfavorable feature. In this scenario, the expression values in the tumor tissue were compared to those in the benign prostate tissue.

For the evaluation of metastatic tumors, three metastatic PCa cell lines were analyzed together and designated as the metastatic group. The expression levels of the genes and miRNAs between the cell group and pT3 tumors were compared in relation to pT2 tumors, which were considered the “control” group. The rationale was that the pathological stage might represent a practical evidence of EMT, and by using this method, we could evaluate which EMT markers are involved in the progression of a localized tumor to metastasis.

The Mann-Whitney U and T tests were used to compare the GS, pathological stage, pre-operative PSA levels, biochemical recurrence, and risk groups. The distribution of gene and miRNA expression levels was skewed, and the data were log-transformed.
hand, TWIST1 showed variable patterns of expression. On the other hand, miR-30a and miR-10b were overexpressed in 51% of the samples, respectively. miRNAs 34a, 155, 30a, and 10b showed a variable pattern of expression: miR-34a and miR-155 were underexpressed in 41 (80%), 41 (80%), 36 (71%), and 42 (82%) samples, respectively. miRNAs 200b, 30a, and 10b were overexpressed in 55% and 57% of the samples, respectively, miRNAs 1, 29b, 9, and 495 were underexpressed in 42 (82%), 44 (86%), 38 (74%), 51 (100%), 38 (74%), and 33 (64%) samples, respectively. miRNAs 1, 29b, 9, and 495 were underexpressed in 41 (80%), 41 (80%), 36 (71%), and 42 (82%) samples, respectively. miRNAs 34a, 155, 30a, and 10b showed a variable pattern of expression: miR-34a and miR-155 were underexpressed in 55% and 57% of the samples, respectively, and miR-30a and miR-10b were overexpressed in 51% of the samples (Table S1 in File S1).

E-cadherin was overexpressed in 50 cases (90%). The genes N-cadherin, TGFβ1, and ZEB1 were underexpressed in 36 (71%) patients, while SNAI2 and Vimentin were underexpressed in 42 (82%) and 41 (80%) patients, respectively. ZEB2, SNAI1, and PDGF showed variable patterns of expression. On the other hand, TWIST1 was the only EMT-induced gene that showed overexpression in the majority of cases (73%) (Table S1 in File S1).

miRNAs and genes associated with clinicopathological features

Tables 3 and 4 illustrate the data regarding miRNA and gene expression in relation to clinicopathological features, respectively. Low levels of miR-200b, miR-30a, and miR-1 were associated with pT3 disease. Of the 18 miRNAs studied, three were significantly underexpressed in pT3 disease (miR-200b - 7.73 vs 23.86, P = 0.02; miR-30a - 1.73 vs 3.79, P = 0.048; and miR-1 - 0.72 vs 1.97, P = 0.04). However, regarding the genes, we could not find any association between their expression and pathological stage.

We assessed the association of GS with the miRNAs excluding GS 7 because of their uncertain behavior. Fifteen patients (29%) had a GS ≤6 and 23 (45%) had a GS ≥8. We found that miR-200b expression was significantly lower in patients with a GS ≥8 when compared to patients with a GS ≤6 (6.94 vs 18.67, P = 0.035). No association was found between GS and the other miRNAs and genes.

When patients were grouped according to low-risk and high-risk disease, the high-risk disease had significantly lower levels of miR-30a (1.70 vs 6.37, P = 0.039). Also high levels of Vimentin and TWIST1 were significantly associated with high-risk disease (0.27 vs 0.90, P = 0.017; 1.81 vs 8.89, P = 0.018).

Due to the significant association between miRNAs 200b, 30a, and 1 with pathological stage and their potential as prognostic markers, a survival analysis was performed. Kaplan-Meier analysis revealed that patients with lower levels of miR-200b had significantly shorter biochemical recurrence-free survival (P = 0.049) (Figure 1).

Moreover, miR-183 and TWIST1 expression levels were significantly higher in metastatic PCa cell lines compared to the levels in patients with pT3 disease and high-grade tumors (Table S2 in File S1). In cell lines, the miR-183 and Twist1 levels were 2.64 and 3.54, respectively, while in pT3 tumors, their levels were...
The importance of the EMT in carcinogenesis has been extensively studied in the last few years, and it is now considered one of the main mechanisms responsible for tumor progression and metastatic dissemination. Our study aimed to evaluate the significance of the expression patterns of multiple miRNAs and genes involved in EMT in clinical specimens of localized prostate cancer and in metastatic cell lines. Our findings are summarized in Figure 2, which shows the main miRNAs and genes involved in EMT in the progression of PCa and their possible mechanism of action.

We have shown that miR-200b, miR-30a, and miR-1 were significantly underexpressed in non-organ-confined tumors and could constitute interesting prognostic factors. A recent study supports our findings by showing that miR-200b and miR-1 induce mesenchymal-epithelial transition (MET) in mouse and human PCa cells and are important regulators in prostatic tumorigenesis and tumor progression [23].

miR-200b was overexpressed in PCa specimens, and this finding is in agreement with previous studies on PCa [25,26]. The members of the miR-200 family are the most important miRNAs involved in EMT [27], and studies in PCa cells have shown that miR-200b inhibits EMT, growth, and metastasis [23,28]. We hypothesize that miR-200b has the greatest potential to become a prognostic marker because lower expression of miR-200b was significantly associated with a high GS, pT3 disease, and shorter biochemical recurrence-free survival. The role of miR-200b has been described in other tumors, and its downregulation is related to advanced disease stage [29] and shorter overall survival [30–32]. Similar to our findings, Barron et al found that miR-200a levels were reduced in patients who relapsed by...
| Pathological Stage | Gleason Score | Pre-operative PSA | Biochemical Recurrence | Risk Group |
|-------------------|---------------|-------------------|------------------------|------------|
|                   | Mean          | Mean              | Mean                   | Mean       |
|                   | (SD)          | (SD)              | (SD)                   | (SD)       |
| miRNA             | pT2           | pT3               | ≤6                     | ≥8         | <10       | ≥10       | No        | Yes       | P         |
|                   | Mean          | Mean              | Mean                   | Mean       |
|                   | (SD)          | (SD)              | (SD)                   | (SD)       |
| 200a              | 4.03          | 11.22             | 0.388                  | 2.41       | 11.77     | 0.401     | 4.61      | 18.67     | 0.938     |
|                   | (6.51)        | (38.20)           | (2.47)                 | (42.35)    | (8.17)    | (56.29)   | (35.50)   | (4.38)    | (3.82)    |
| 200b              | 23.86         | 7.74              | 0.02                   | 18.67      | 6.94      | 0.035     | 16.85     | 8.98      | 0.412     |
|                   | (42.40)       | (8.47)            | (18.05)                | (6.36)     | (33.99)   | (8.03)    | (35.16)   | (6.19)    | (19.54)   |
| 200c              | 4.10          | 3.35              | 0.636                  | 3.52       | 3.17      | 0.821     | 3.59      | 4.18      | 0.743     |
|                   | (6.31)        | (4.91)            | (3.88)                 | (5.05)     | (5.41)    | (6.10)    | (6.29)    | (3.49)    | (3.34)    |
| 429               | 9.45          | 6.46              | 0.505                  | 5.99       | 8.10      | 0.625     | 8.68      | 5.29      | 0.436     |
|                   | (17.82)       | (14.02)           | (6.59)                 | (15.61)    | (18.16)   | (4.34)    | (19.03)   | (4.11)    | (7.34)    |
| 141               | 21.98         | 14.85             | 0.440                  | 18.64      | 13.88     | 0.416     | 20.23     | 11.82     | 0.321     |
|                   | (44.95)       | (17.85)           | (17.32)                | (17.57)    | (36.79)   | (12.05)   | (37.07)   | (20.38)   | (13.96)   |
| 205               | 31.09         | 8.39              | 0.159                  | 29.41      | 10.75     | 0.555     | 19.85     | 13.78     | 0.561     |
|                   | (50.98)       | (10.19)           | (51.68)                | (12.13)    | (40.96)   | (15.85)   | (30.50)   | (45.39)   | (61.38)   |
| 203               | 11.47         | 2.79              | 0.193                  | 2.40       | 3.23      | 0.405     | 7.72      | 3.42      | 0.587     |
|                   | (38.21)       | (3.13)            | (2.48)                 | (3.25)     | (29.63)   | (2.68)    | (30.83)   | (3.66)    | (1.62)    |
| 183               | 10.53         | 7.70              | 0.479                  | 7.79       | 7.72      | 0.989     | 9.03      | 8.64      | 0.958     |
|                   | (15.60)       | (12.72)           | (11.37)                | (14.20)    | (13.86)   | (15.15)   | (16.22)   | (5.74)    | (13.21)   |
| 21                | 108.96        | 67.34             | 0.432                  | 60.48      | 105.61    | 0.417     | 85.29     | 85.30     | 0.950     |
|                   | (213.46)      | (161.82)          | (106.85)               | (193.84)   | (169.55)  | (237.02)  | (214.95)  | (104.17)  | (126.44)  |
| 373               | 0.29          | 0.28              | 0.231                  | 0.34       | 0.28      | 0.108     | 0.28      | 0.30      | 0.297     |
|                   | (0.37)        | (0.31)            | (0.39)                 | (0.28)     | (0.34)    | (0.31)    | (8.29)    | (4.32)    | (0.37)    |
| 1                 | 1.97          | 0.72              | 0.040                  | 2.47       | 0.68      | 0.101     | 1.42      | 0.82      | 0.504     |
|                   | (3.32)        | (1.39)            | (3.91)                 | (1.48)     | (2.70)    | (1.79)    | (2.79)    | (1.59)    | (4.43)    |
| 29b               | 0.74          | 0.42              | 0.123                  | 0.76       | 0.31      | 0.224     | 0.51      | 0.68      | 0.546     |
|                   | (0.77)        | (0.70)            | (0.96)                 | (0.32)     | (0.66)    | (0.98)    | (0.72)    | (0.81)    | (0.64)    |
| 9                 | 1.44          | 1.13              | 0.622                  | 0.83       | 1.75      | 0.930     | 0.92      | 2.17      | 0.415     |
|                   | (2.86)        | (1.69)            | (0.84)                 | (3.03)     | (1.39)    | (1.59)    | (1.27)    | (3.41)    | (0.89)    |
| 495               | 1.03          | 0.81              | 0.623                  | 0.66       | 0.92      | 0.536     | 0.81      | 1.13      | 0.479     |
|                   | (0.35)        | (0.33)            | (0.41)                 | (0.33)     | (0.37)    | (0.31)    | (1.73)    | (1.18)    | (0.39)    |
| 34a               | 3.81          | 4.92              | 0.690                  | 2.79       | 5.44      | 0.433     | 4.53      | 4.20      | 0.976     |
|                   | (7.93)        | (10.92)           | (7.69)                 | (11.31)    | (10.31)   | (8.35)    | (8.11)    | (12.47)   | (0.58)    |
| 155               | 2.83          | 1.89              | 0.405                  | 3.90       | 1.65      | 0.260     | 1.97      | 3.16      | 0.281     |
|                   | (4.22)        | (3.73)            | (5.84)                 | (2.95)     | (3.41)    | (5.28)    | (3.69)    | (4.49)    | (4.56)    |
studying miR-200a expression in formalin-fixed paraffin-embedded tissue from patients with pT3 disease [33], supporting the potential of miR-200 family as a marker of biochemical recurrence.

Previous studies indicate that downregulation of miR-200 may contribute to the progression of PCa [15] [34]. Xu et al observed an 80% reduction in miR-200b levels in chemically castrated LNCaP cells via RNA sequencing [35]. Emerging evidence supports the involvement of EMT processes in the deregulation of the androgen signaling axis, but data are still controversial. Zhu and Kyprianou observed that androgens induce independently EMT patterning within prostate cancer cells, resulting in substantial changes in cellular invasion and motility [36]. The activated androgen receptor (AR) has recently been shown to promote EMT activation via suppression of E-cadherin expression within breast cancer cells [37]. On the other hand, Sun et al found that androgen deprivation causes EMT in vivo and acquisition of mesenchymal features [38].

This is the second study relating miR-1 and prognosis in PCa. Hudson et al previously found that lower expression levels of miR-1 were associated with earlier biochemical recurrence in PCa [39]. Now we showed that miR-1 was downregulated in the primary tumor compared to benign prostate tissue, and was significantly reduced in non-organ confined disease. It is thought that miR-1 regulating Slug [23], through histone methylation and acetylation [39] and also having as target genes related to proliferation, migration and invasion [40] plays an important role in EMT in PCa.

Data regarding the role of miR-30a in PCa are scarce and contradictory. In our study, miR-30a showed a variable pattern of expression. miR-30a was described as being downregulated in the study conducted by Porkka et al [41], while Carlsson et al reported the upregulation of this miRNA [42]. Recently, Kao et al showed that the ETS-related gene (ERG), which is the most frequently overexpressed oncogene in PCa, is a direct target of miR-30 and that overexpression of miR-30 in PCa cells suppresses EMT phenotypes and inhibits cell migration and invasion [43]. miR-30 family also inhibits cell migration, invasiveness, and metastasis in vitro in other tumors, such as lung, breast, and hepatocellular cancer [21,44–46], by targeting SNAI1 [21,44] and Vimentin [45,46]. In this study, the relationship observed between decreased expression of miR-30, advanced pathological stage, and high-risk disease confirms miR-30 as a tumor suppressor miRNA in PCa.

Cheng et al observed that low levels of miR-30a were predictors of advanced stage and lymph node metastasis in invasive breast cancer [45]. Wang et al showed that low expression levels of miR-30a were significantly associated with a higher incidence of portal vein tumor thrombus in hepatocellular carcinoma [46].

Regarding the genes, we observed overexpression of E-cadherin in virtually all cases, and the majority of the mesenchymal markers, including N-cadherin, TGFBI, ZEB1, Vimentin, and SNAI2, were downregulated. This gene expression profile strongly suggests that localized PCa maintains the epithelial phenotype despite tumor differentiation and increasing stage. However, TWIST1 was overexpressed in 73% of the cases. TWIST1 is a helix-loop-helix transcription factor that activates EMT through indirect inhibition of E-cadherin [47]. TWIST1 has been shown to be overexpressed in PCa on immunohistochemistry assays and to positively correlate with the GS [48,49]. It is interesting that a gene with such importance in EMT and with prognostic value in PCa is overexpressed in localized tumors. The early overexpression of TWIST1 may be attributed to its regulation by the NKX3-1 gene [50], a tumor suppressor that was found to be underexpressed in the early stages of PCa [51,52]. However, the early upregulation...
| Pathological Stage | Gleason Score | Pre-operative PSA | Biochemical Recurrence | Risk Group |
|--------------------|--------------|-------------------|------------------------|------------|
|                    | Mean         | Mean              | Mean                   | Mean       |
|                    | (SD)         | (SD)              | (SD)                   | (SD)       |
| Gene               | pT2          | pT3               | ≤6                     | ≥8         | <10       | ≥10       | No | Yes | P     | Low | High | P     |
| E-cadherin         | 4.61         | 6.10              | 0.343                  | 5.07       | 5.75      | 0.736     | 5.74| 4.72| 0.644 | 5.70| 4.98| 0.667 | 3.06| 6.04| 0.126 |
| TGFB1              | 0.68         | 1.04              | 0.123                  | 0.77       | 1.06      | 0.497     | 0.89| 0.87| 0.994 | 0.83| 1.01| 0.335 | 0.69| 0.94| 0.263 |
| ZEB1               | 1.15         | 1.10              | 0.903                  | 1.03       | 1.13      | 0.803     | 1.21| 0.90| 0.520 | 1.19| 0.98| 0.580 | 1.13| 1.12| 0.980 |
| ZEB2               | 1.23         | 1.45              | 0.459                  | 1.48       | 1.38      | 0.764     | 1.53| 0.89| 0.50  | 1.41| 1.24| 0.577 | 1.32| 1.36| 0.904 |
| TWIST1             | 6.51         | 8.25              | 0.665                  | 3.62       | 4.78      | 0.543     | 4.76| 1.47| 0.445 | 9.48| 3.53| 0.153 | 1.81| 8.89| 0.018 |
| SNAI1              | 2.80         | 2.44              | 0.728                  | 1.47       | 2.66      | 0.637     | 2.73| 2.24| 0.761 | 3.11| 1.58| 0.072 | 1.21| 2.94| 0.602 |
| N-cadherin         | 0.86         | 1.61              | 0.288                  | 1.74       | 1.19      | 0.561     | 1.39| 1.01| 0.641 | 1.44| 0.97| 0.525 | 0.81| 1.40| 0.496 |
| Vimentin           | 0.67         | 2.80              | 0.533                  | 0.55       | 0.63      | 0.760     | 0.76| 0.83| 0.692 | 0.88| 0.58| 0.315 | 0.27| 0.90| 0.017 |
| SNAI2              | 0.76         | 0.93              | 0.684                  | 0.75       | 0.48      | 0.422     | 1.01| 0.46| 0.382 | 1.08| 0.39| 0.369 | 0.29| 0.99| 0.176 |
| PDGFD              | 1.91         | 1.30              | 0.985                  | 1.06       | 1.39      | 0.423     | 1.68| 1.25| 0.596 | 1.85| 0.98| 0.562 | 1.21| 1.65| 0.582 |

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of TWIST1 does not appear to be sufficient to initiate the EMT process. According to Casas et al, TWIST1 induces SNAI2 to promote EMT [53], but depletion of SNAI2 completely blocks the ability of TWIST1 to suppress E-cadherin and induce EMT.

We have shown that high levels of TWIST1, as well as Vimentin, are significantly associated with patients in the high-risk group and TWIST1 were also significantly higher in the metastatic cell lines. In a recent study, Behnsawy et al [12] showed that high expression levels of TWIST1 and Vimentin evaluated by immunohistochemistry is an independent factor related to shorter biochemical recurrence-free survival, suggesting that these genes might be potential markers of biochemical recurrence after radical prostatectomy.

TWIST1 appears to play a role in various steps of EMT, and its role in the progression of PCa [49]. In the study by Kwok et al, TWIST1 expression was higher in tissues derived from metastatic lesions from bones and lymph nodes [48]. The role of TWIST1 in this later step of EMT might be explained by the activation of its target, miR-10b. miR-10b not only represses E-cadherin [54] but also inhibits the translation of the HOXD10 protein, permitting the expression of the pro-metastatic gene product, RHOC [53].

We have also observed that expression levels of miR-183 were significantly higher in the metastatic group. Ueno et al observed that higher expression levels of miR-183 were significantly associated with higher PSA, higher stage and shorter overall survival after radical prostatectomy, but its behavior in PCa is absolutely controversial some showing that miR-183 promotes migration and invasion [56–58], while others indicate that it inhibits migration, invasion, and metastasis [59–61]. Some targets of miR-183 have been proposed, DKK3, SMAD4 [56], EGR1 and PTEN [57], which turns miR-183 a context dependent miRNA. Based on our results and according to previous studies in the literature, we believe that miR-183 acts as an oncomiR in PCa, and the mechanism might involve PTEN which is related to PCa progression and development of metastasis [62]. Ding et al also showed that concomitant PTEN and SMAD4 inactivation in the prostatic epithelium is able to produce a fully-penetrant invasive and metastatic PCa phenotype in mice [63].

In conclusion, it is important to understand that EMT influences tumor progression in different steps through several markers. Here, we described a comprehensive study of miRNAs and genes related to EMT in PCa and found that the expression levels of miR-200b, miR-30a, miR-1, TWIST1 and Vimentin could be used in decision-making processes related to primary or adjuvant treatments in the future.
Expression levels of miRNAs and genes from each case in relation to metastatic, an increase in the expression levels of miR-183 and TWIST1 is observed. The dotted lines indicate the genes where these miRNAs or genes might act, based on previously published data. BR = Biochemical Recurrence.

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Supporting Information

File S1 Combined file of supporting tables. Table S1: Expression levels of miRNAs and genes from each case in relation to BPH samples. Table S2: Expression levels of miRNAs and genes in pT3 tumors and in cell lines (in relation to pT2 tumors) (DOCX)

References

1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63: 11–30.
2. Quinn DJ, Henshall SM, Haynes AM, Brenner PC, Kooner R, et al. (2001) Prognostic significance of pathologic features in localized prostate cancer treated with radical prostatectomy: implications for staging systems and predictive models. J Clin Oncol 19: 3692–3705.
3. Mackinnon AC, Yan BG, Joseph LJ, Al-Ahmadie HA (2009) Molecular biology underlying the clinical heterogeneity of prostate cancer: an update. Arch Pathol Lab Med 133: 1033–1040.
4. Thiery JP, Acloque H, Huang RY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139: 871–890.
5. Huber MA, Kraut N, Beng H (2005) Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 17: 548–550.
6. Hallbleib JM, Nelson WJ (2006) Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. Genes Dev 20: 3199–3214.
7. Moreno-Ruano O, Cubillo E, Sarrio D, Peinado H, Rodriguez-Pinilla SM, et al. (2006) Genetic profiling of epithelial cells expressing E-cadherin repressors reveals a distinct role for Snail, Slug, and E47 factors in epithelial-mesenchymal transition. Cancer Res 66: 9543–9556.
8. Balle E, Sanchez E, Franci C, Dominguez D, Monfar M, et al. (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2: 84–89.
9. Tomita K, van Bokhoven A, van Leenders GJ, van Leenders GJ, Ruijter ET, Jansen CF, et al. (2008) The ZEB1 switch in human prostate cancer progression. Cancer Res 68: 6162–6170.
10. Putzke AP, Ventura AP, Bailey AM, Akute C, Opoluk-Anah J, et al. (2011) Metastatic progression of prostate cancer and e-cadherin regulation by zeb1 and SRG family kinases. Am J Pathol 179: 400–410.
11. Graham TR, Zhao HE, Odor-Marah VA, Onoukoya AO, Kimbro KS, et al. (2008) Insulin-like growth factor-1 receptor-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. Cancer Res 68: 2479–2488.
12. Behesnavy HM, Miyake H, Harada K, Fojisawa M (2013) Expression patterns of epithelial-mesenchymal transition markers in localized prostate cancer: significance in clinicopathological outcomes following radical prostatectomy. BJU Int 111: 30–37.
13. Barthel DP (2004) MicroRNA: genomics, biogenesis, mechanism, and function. Cell 116: 201–207.
14. Rendorake A, Knoof EC, Garg KS, Parkin RK, Kroh EM, et al. (2010) Regulation of miR-200 family microRNAs and ZEB transcription factors in ovarian cancer: evidence supporting a mesothelial-to-epithelial transition. Gynecol Oncol 116: 117–125.
15. Gregory PA, Bert AG, Paterson EL, Barry SC, Trykin A, et al. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 10: 593–601.
16. Korpal M, Lee ES, Hu G, Kang Y (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 283: 14910–14914.
17. Park SM, Gaur AB, Lengyel E, Peter ME (2008) The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 22: 894–907.
18. Burke U, Schubert J, Wellner U, Schmalhofer O, Vincan E, et al. (2006) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 9: 582–589.
19. Bracken CP, Gregory PA, Kolesnikoff N, Bert AG, Wang J, et al. (2008) A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res 68: 7846–7854.
20. Ru P, Steele R, Newhall P, Phillips NJ, Toh K, et al. (2012) miRNA-29b suppresses prostate cancer metastasis by regulating epithelial-mesenchymal transition signaling. Mol Cancer Ther 11: 1166–1173.
21. Zhang J, Zhang H, Liu J, Tu X, Zang Y, et al. (2012) miR-30 inhibits TGF-beta-induced epithelial-to-mesenchymal transition in hepatocytse by targeting Snail1. Biochem Biophys Res Commun 417: 1100–1105.
22. Siemens H, Jaakstad R, Hunter S, Kaller M, Munsen A, et al. (2011) miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. Cell Cycle 10: 4256–4271.
23. Liu YN, Yin JJ, Ahou-Khiweit H, Hynes PG, Casey OM, et al. (2013) MR-1 and miR-200 inhibit EMT via Slug-dependent and tumorigenesis via Slug-independent mechanisms. Oncogene 32: 296–306.
24. Epstein JL, Alishbrook WC, Jr., Amin MB, Egevad LL, Committee IG (2005) The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol 29: 1228–1242.
25. Kong D, Li Y, Wang Z, Banerjee S, Ahmad A, et al. (2009) miR-200-regulates PDGF-D-mediated epithelial-mesenchymal transition, adhesion, and invasion of prostate cancer cells. Stem Cells Dev 17: 1712–1721.
26. Ambh S, Prueitt RL, Yi M, Hudson RS, Howe TM, et al. (2008) Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. Cancer Res 68: 6162–6170.

Figure 2. Main miRNAs and genes involved in epithelial-mesenchymal transition in prostate cancer. doi:10.1371/journal.pone.0113700.g002

Author Contributions

Conceived and designed the experiments: BK STR MS KRML. Performed the experiments: BK STR NIV DRM CMM ND IAS AI. Analyzed the data: BK STR. Contributed reagents/materials/analysis tools: BK STR NIV. Wrote the paper: BK STR KRML.
27. Paterson EL, Koleunik N, Gregory PA, Bert AG, Khew-Goodall Y, et al. (2008) The microRNA-200 family regulates epithelial to mesenchymal transition. ScientificWorldJournal 8: 901-904.

28. Williams LV, Veliccosa D, Vinokour E, Volpert OV (2013) miR-200b inhibits prostate cancer EMT, growth and metastasis. PLoS One 8: e83991.

29. Kurashige J, Kamohara H, Watanabe M, Inyoshi Y, Iwasaki M, et al. (2012) MicroRNA-200b regulates cell proliferation, invasion, and migration by directly targeting ZEB2 in gastric carcinoma. Ann Surg Oncol 19 Suppl 3: S656-664.

30. Marchini S, Cavalieri D, Calura E, Garavaglia D, et al. (2011) Association between miR-200c and the survival of patients with stage I epithelial ovarian cancer: a retrospective study of two independent tumour collections. Lancet Oncol 12: 273-285.

31. Nam EJ, Yoon H, Kim SW, Kim H, Kim YI, et al. (2008) MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 14: 2690-2695.

32. Hu X, Macdonald DM, Huettenrauch R, Peng Z, El Naqa IM, et al. (2009) A miR-200 microRNA cluster as prognostic marker in advanced human cancer. Gynecol Oncol 114: 457-464.

33. Barron N, Keenan J, Gammell P, Martinez VG, Freeman A, et al. (2012) Biochemical relapse following radical prostatectomy and miR-200a levels in prostate cancer. Prostate 72: 1193-1199.

34. Vernon AE, LaBonne C (2004) Tumor metastasis: a new twist on epithelial-mesenchymal transition. Curr Biol 14: R719-721.

35. Xu G, Wu J, Zhou L, Chen B, Sun Z, et al. (2010) E-cadherin gene expression and prognosis in human prostate cancer cell line by deep sequencing. PLoS One 5: e15519.

36. Zhu ML, Kyprianou N (2010) Role of androgens and the androgen receptor in prostate cancer. Cancer Res 70: 9570-9580.

37. Liu YN, Liu Y, Lee HJ, Hsu YH, Chen JH (2008) Activated androgen receptor downregulates E-cadherin gene expression and prompts tumor metastasis. Mol Cell Biol 28: 7096-7108.

38. Sun Y, Wang B, Leong KG, Yue P, Li L, et al. (2012) Androgen deprivation causes epithelial-mesenchymal transition in the prostate: implications for androgen-deprivation therapy. Cancer Res 72: 527-536.

39. Hudson RS, Yi M, Esposito D, Watkins SK, Huvitz AA, et al. (2012) MicroRNA-1 is a candidate tumor suppressor and independent prostate cancer predictor. Cancer Res 72: 461-469.

40. Kojima S, Chiyomaru T, Kawakami K, Yoshino H, Enokida H, et al. (2012) Tumour suppressors miR-1 and miR-133a target the oncogenic function of purine nucleoside phosphorylase (PNP) in prostate cancer. Br J Cancer 106: 1143-1149.

41. Porikli KP, Pfeffer MJ, Waltering KK, Vessella RL, Tammela TL, et al. (2007) MicroRNA expression profiling in prostate cancer. Cancer Res 67: 6130-6135.

42. Carlsson J, Davidson S, Helenius G, Karlsson M, Lohovac Z, et al. (2011) A miRNA expression signature that separates between normal and malignant prostatic tissues. Cancer Cell Int 11: 14.

43. Kao CJ, Martinez A, Shi XB, Yang J, Evans CP, et al. (2013) miR-30 as a tumor suppressor connects EGF/Src signal to ERG and EMT. Oncogene.

44. Kumaraswamy R, Mukherjui G, Ceppi P, Mappala S, Koulwani M, et al. (2012) MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snail and is downregulated in non-small cell lung cancer. Int J Cancer 130: 2044-2053.

45. Cheng CW, Wang HW, Chang CW, Chu HW, Chen CY, et al. (2012) MicroRNAs-30a inhibits cell migration and invasion by downregulating vimentin expression and is a potential prognostic marker in breast cancer. Breast Cancer Res Treat 134: 1081-1093.

46. Wang W, Lin H, Zhou L, Zhu Q, Gao S, et al. (2013) MicroRNA-30a-3p inhibits tumor proliferation, invasiveness and metastasis and is downregulated in hepatocellular carcinoma. Eur J Surg Oncol.