Distinct microRNA Expression Profile in Prostate Cancer Patients with Early Clinical Failure and the Impact of let-7 as Prognostic Marker in High-Risk Prostate Cancer

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Abstract

Background: The identification of additional prognostic markers to improve risk stratification and to avoid overtreatment is one of the most urgent clinical needs in prostate cancer (PCa). MicroRNAs, being important regulators of gene expression, are promising biomarkers in various cancer entities, though the impact as prognostic predictors in PCa is poorly understood. The aim of this study was to identify specific miRNAs as potential prognostic markers in high-risk PCa and to validate their clinical impact.

Methodology and Principal Findings: We performed miRNA-microarray analysis in a high-risk PCa study group selected by their clinical outcome (clinical progression free survival (CPFS) vs. clinical failure (CF)). We identified seven candidate miRNAs (let-7a/b/c, miR-515-3p/5p, -181b, -146b, and -361) that showed differential expression between both groups. Further qRT-PCR analysis revealed down-regulation of members of the let-7 family in the majority of a large, well-characterized high-risk PCa cohort (n = 98). Expression of let-7a/b and -c was correlated to clinical outcome parameters of this group. While let-7a showed no association or correlation with clinical relevant data, let-7b and let-7c were associated with CF in PCa patients and functioned partially as independent prognostic marker. Validation of the data using an independent high-risk study cohort revealed that let-7b, but not let-7c, has impact as an independent prognostic marker for BCR and CF. Furthermore, we identified HMGA1, a non-histone protein, as a new target of let-7b and found correlation of let-7b down-regulation with HMGA1 over-expression in primary PCa samples.

Conclusion: Our findings define a distinct miRNA expression profile in PCa cases with early CF and identified let-7b as prognostic biomarker in high-risk PCa. This study highlights the importance of let-7b as tumor suppressor miRNA in high-risk PCa and presents a basis to improve individual therapy for high-risk PCa patients.

Introduction

Prostate cancer (PCa) is the most common malignancy among men in Europe, with an estimated incidence of 345,900 in 2006 [1]. The natural course of the disease is heterogeneous, varying from indolent to highly aggressive cancer that metastasizes at early stage causing pain and untimely death. Current risk stratifications like low-/intermediate-/and high-risk PCa alone are insufficient to predict clinical outcome. Even men with high-risk PCa (PSA ≥20 ng/ml and/or biopsy Gleason Score ≥8 and/or clinical stage ≥ T3) represent a heterogeneous group of patients. Even though characterized as a group that has poorest clinical outcome among all risk groups, only up to 30% develop metastases and die due to their disease [2–4]. Therefore, new prognostic biomarkers are urgently needed to better sub-stratify risk groups, identify the lethal disease, eventually avoid overtreatment, and improve individual therapy. The identification of prognostic markers, especially for the lethal disease, is hardly possible in an unselected PCa collective. Although high-risk PCa cohorts now show much better than expected outcomes, they still represent an ideal group to identify factors specifically correlated with the lethal disease.

There is growing evidence that microRNAs (miRNA) are suitable candidates for the development of such biomarkers. MiRNAs are small non-coding RNA strands that regulate expression of genes at the post-transcriptional and the translational level. Individual miRs have been characterized either as tumor suppressors or oncogenes (oncomiRs) [5].
Several reports describe PCa-specific miRNA expression signatures, however the kind of regulated miRNAs is diverse. Agreement exists among these studies in that the majority of miRNAs are down-regulated in the PCa cohort [6–10]. Although a correlation to tumor stage and grade was described for several miRs their relevance as prognostic markers to predict hard clinical endpoints, like clinical failure or cancer-related death, remains limited [8,11–14]. However, there are promising approaches to detect coherence between altered expression of specific miRNAs and progression of the disease. Larne and coworkers recently identified a miRNA-based multimarker model as prognostic tool for progression in PCa [15]. Our working group previously described miR-221 to be a prognostic marker for disease recurrence in high-risk PCa [16].

Some of the most frequently mentioned miRs that show down-regulation in PCa are members of the let-7 family [6,9,17,18]. This family consists of several members, whose diversity is distinct by isoforms (let-7a-g,-i, miR-98). The expression of some let-7 members was shown to be down-regulated in various other cancer entities as well, such as breast, ovarian, and lung cancer [19–21]. Known relevant targets of let-7 are oncogenes like Ras, cmyc, EZH2 and HMGA2 [17,22–24], indicating a tumor-suppressive function of let-7 by regulating oncogenes specifically involved in growth and self-renewing capacity of PCa cells. More recently it was shown that PCa stem cells are characterized by down-regulation of let-7 family members and that let-7 is critically involved in tumorigenicity by controlling androgen receptor signaling, proliferation and differentiation [25–27]. However, a role of let-7 family members as prognostic markers in PCa has not been described up to now.

The aim of this study was to identify miRNAs differentially expressed in high-risk PCa with diverse clinical outcome. We detected a pattern consisting of 7 miRNAs associated with early clinical recurrence and identified let-7 family members to be progressively down-regulated in aggressive tumors. In a large high-risk PCa cohort we confirmed these results and demonstrated that down-regulation of let-7b is correlated with biochemical recurrence and clinical failure. Furthermore, we confirmed let-7b as independent prognostic marker in high-risk cancer in an independent validation cohort. In addition we showed that expression of HMGA1, a non-histone protein, is regulated by let-7b by binding to the 5’UTR of HMGA1. Our results demonstrate that high-risk PCa is characterized by a specific miRNA profile and that individual let-7 family members are promising prognostic markers in this patient group.

Materials and Methods

Patient Cohorts

We worked with three diverse PCa collectives for our analyses:

Cohort A consists of 98 formalin fixed and paraffin embedded (FFPE) tissue specimen of a well-characterized group of high-risk PCa patients [16]. See Table 1 for clinic-pathological data. Tissue samples and clinical data were utilized for microarray and qRT-PCR analyses on microRNAs as well as subsequent correlation and association studies.

Cohort B consists of 92 FFPE samples from RP. Tissue specimen and clinic-pathological data were obtained from the Department of Urology at the University Hospital Leuven, Belgium (Table 1). This group served as validation collective to confirm the role of let-7b as prognostic marker.

Preoperative staging in cohort A and B included DRE, an abdominopelvic-computed tomography (CT) scan and a bone scan. Neoadjuvant hormonal treatment, radiation or chemotherapy was an exclusion criterion. Lymph node metastasis and prostate specimens (whole mount sections, 4 mm intervals) were staged and graded according to the 2002 TNM classification and the Gleason grading system as previously described [16]. Follow-up was performed every 3 months for the first 2 years after surgery, every 6 months in the following 3 years, and annually thereafter. Biochemical relapse (BCR) was defined as PSA ≥0.2 ng/ml on 2 consecutive follow-up visits. Clinical failure was declared when either local or distant metastases were histologically proven or confirmed by CT or bone scan. Overall survival (OS) was defined as time from RP to death attributed to PCa or complications of the disease.

Benign prostatic hyperplasia (BPH) samples were derived from prostate adenomectomy specimens. Samples were paraffin-embedded as well; regions with >80% adenoid tissue were used. All patients had normal PSA levels before surgery and carcinoma was excluded by histopathology.

Cohort C consists of 21 cancer samples obtained from the Department of Urology at the University Hospital Wuerzburg, Germany. Pairs of fresh frozen PCa tissue and adjacent benign tissue, were used for mRNA and miRNA isolation. This group contains patients with unselected histopathological PCa specimen (high-, intermediate-, and low risk cancer). Since miRNA isolation of fresh frozen tissue specimen is more effective compared to isolation on FFPE samples we worked with this cohort for correlation studies on expression of HMGA1 and let-7b. For this analysis clinic-pathological data were irrelevant and therefore not shown. Histological evaluation was performed by a Senior pathologist (P.S.). Cancerous samples containing at least 80%
malignant cells were used for further analyses. Areas with at least 80% ducts and no cancerous cells were selected for adjacent benign tissue.

The studies were approved by the local ethics committee of the medical faculty of the University of Wuerzburg, Germany (no. 59/04) and the Catholic University Leuven, Belgium (UZ Leuven Study number S54424; Belgian Study number B322201214832); all patients provided written informed consent.

Microarray Analysis

To screen for candidate miRs, being correlated with poor outcome 6 BPH tissues and 13 high-risk PCa specimens were hybridized to microarrays (cohort A). High-risk PCa specimens were subdivided into two groups (group 1: CPFS (n = 7); group 2: CF (n = 6) (see Table 2 for details). A set of 668 miRs (Probe Set 1564V2 miRVana Applied Biosystems) was spotted on Nexter-ion® HiSense E microarray slides in quadruplicates. We used the Pure-Link FFPE Total RNA Isolation Kit and the RiboMinus Concentration Module (Invitrogen) for RNA purification. Slide processing was performed according to the Applied Biosystems miRVana™ manuals. The microarray data is available under GEO accession number GSE18671.

RNA Extraction and Reverse Transcription

Total RNA extraction from paraffin-embedded or frozen tissue samples and PCa cell lines were performed using the Recover all Total Nucleic Acid Isolation Kit and the Total RNA Extraction Kit respectively (Ambion and microEasy Mini Kit, Qiagen). Specific cDNA was synthesized from total RNA with stem-loop reverse transcription primers according to the TaqMan® miR assay protocol (PE Applied Biosystems). Unspecific cDNA synthesis was performed with ImProm-II™ Reverse Transcription System (Promega) according to the ABsolute QPCR SYBR Green protocol (Thermo Scientific).

qRT-PCR

Confirmation of the microarray results on independent samples and on an increased sample size was done using qRT-PCR. M(i)RNA expression in tissue samples and cell lines was quantified with either TaqMan® (miR) or SYBR Green (mRNA) assay kits and the BioRad OPTICON 2, following the manufacturer’s instructions (BioRad). Primers for let-7a-d, miR-16, -29a, -221, -146b, and -181b were obtained from Applied Biosystems; small nuclear RNA RNU6B was applied for normalization. β-Actin served as housekeeper for HMGA1 expression (Applied Biosystems). Relative m(i)RNA-expression was calculated with the

### Table 2. Clinic-pathological features of 13 high-risk PCa patients (cohort A) separated by clinical outcome (group 1: high-risk PCa+CPFS; group 2: high-risk PCa+CF).

| Clinic-pathol. features | Group 1 | Group 2 |
|-------------------------|---------|---------|
| age, years (range)      | 64 (52–74) | 66 (57–71) |
| follow-up, months (st.dev) | 106 (±34.1) | 72.6 (±25.8) |
| pre-operative PSA (ng/ml) | 52.27 (21–79) | 63.96 (24–125) |
| Gleason Score           |         |         |
| 6                       | 3       | 0       |
| 7                       | 4       | 0       |
| 8                       | 0       | 0       |
| 9                       | 0       | 1       |
| 10                      | 0       | 5       |
| pathol. tumor stage     |         |         |
| pT2b                    | 1       | 0       |
| pT3a                    | 1       | 0       |
| pT3b                    | 3       | 1       |
| pT4                     | 2       | 5       |
| surgical margin status  |         |         |
| pos.                    | 6       | 6       |
| neg.                    | 1       | 0       |
| biochemical relapse     |         |         |
| yes                     | 3       | 6       |
| no                      | 4       | 0       |
| clinical failure        |         |         |
| yes                     | 0       | 6       |
| no                      | 7       | 0       |
| cancer-related death    |         |         |
| yes                     | 0       | 3       |
| no                      | 7       | 3       |

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The expression was determined by 

Comparative ΔCt-method (ΔCt sample = Ct sample - Ct RNU6B). The 2^ΔΔCt method was used to assess fold changes in m(i)R-expression between samples and controls. Mean Ct was determined from triplicate PCR experiments. Standard deviation was ≤0.4, p values < 0.05 were considered significant.

Cell Culture and Transient Transfection
LNCaP and PC-3 cells were obtained from ATCC, Germany using passages 5 to 35. Cells were grown at 37°C in a humidified incubator at 5% CO2. Cells were seeded and simultaneously transfected in 6-well plates at a density of 3.5 x 10^5 per well using RPMI 1640 Medium Gibco without Phenolred, containing 10% fetal calf serum (Invitrogen), Glutamax (Invitrogen), NEAA (Gibco), HEPES Buffer solution (PAA), and Sodium pyruvate solution (PAA). Transient transfection was performed using Lipofectamine™ reagent (Invitrogen) and Opti-MEM® (Invitrogen-Gibco) following the supplier’s instructions. Precursor-respectively antiog-let-7b was applied to induce or suppress let-7b expression, pre-and anti-negative let-7b (Applied Biosystems) were used for control transfection. Cells were harvested 48 h after transfection.

Western Blot
Cells were lysed in Cytobuster or Phosphosafe (Novagen) after preparation and determination of equal amounts proteins were separated on SDS-PAGE and later transferred to Nitrocellulose membrane (Bio-Rad). For protein expression of HMGA1 we used 1 mg/ml goat polyclonal antibodies (HMGA1a/b, Abcam); β-Axin (Abcam) served as housekeeper. For visualization we used horseradish peroxidase-coupled secondary antibodies (Abcam and Dako) and the ECL Plus kit (GE Healthcare). For quantification of band intensity we used Image J (http://imagej.nih.gov/ij/).

 Luciferase Assay
The 3’UTR of the human HMGA1 gene was PCR-amplified using the following primers that contained additional Hind III or Spe I sites: HMGA1/for: 5’-GATC AAGCTTCA-TATTTGGTGATGGAG-3′; HMGA1/rev: 5’-CAAT GC-TAGT GACATTTTGGCCGTTGAG-3′. The resulting HMGA1 PCR fragment containing the two most downstream located let-7b complementary sites to HMGA1 was cloned downstream of the Renilla luciferase stop codon into a luciferase reporter plasmid (pMIR-REPORT-Luciferase, Applied Biosystems) using the Hind III and Spe I sites. To perform luciferase assays LNCaP cells were seeded in 6-well plates at a density of 3.5 x 10^5 per well and incubated for 24 hours. The reporter vector was co-transfected into LNCaP cells with a control non-targeting RNA oligonucleotide or let-7 precursor miRNA. Transient transfection was performed using Lipofectamine™ reagent (Invitrogen). 48 hours post transfection the luciferase activity was analyzed by Dual-Lucerases® Reporter Assay System (Promega). Renilla activity was used for normalization and as a control for Lipofectamine™ reagent (Invitrogen) and Opti-MEM® fetal calf serum (Invitrogen), Glutamax (Invitrogen), NEAA RPMI 1640 Medium Gibco without Phenolred, containing 10% fetal calf serum (Invitrogen), Glutamax (Invitrogen), NEAA (Gibco), HEPES Buffer solution (PAA), and Sodium pyruvate solution (PAA). Transient transfection was performed using Lipofectamine™ reagent (Invitrogen) and Opti-MEM® (Invitrogen-Gibco) following the supplier’s instructions. Precursor-respectively antiog-let-7b was applied to induce or suppress let-7b expression, pre-and anti-negative let-7b (Applied Biosystems) were used for control transfection. Cells were harvested 48 h after transfection.

Statistical and Bioinformatics Analysis
Microarray-Analysis: Spot intensities from scanned slides were quantified using ScanAlyze Software (http://rana.lbl.gov/EisenSoftware.htm). Data were analyzed with different R packages from the Bioconductor project (www.bioconductor.org). Resulting signal intensities were normalized by variance stabilization [28]. Differentially expressed genes were selected from the microarray data by limma (Linear models for microarray Analysis) package [29].
total, 148 miRs were diversely expressed within all three tissue types. The expression of seven miRNAs (namely let-7a/c, miR-315-3p/5p, -181b, -146b, and -361) were found to be significantly different between all three groups analyzed (BPH, group 1 and 2) indicating a specific miRNA profile for high-risk PCa with progressive disease. 47 miRNAs showed different expression in group 1 vs. group 2; among the top ten most differentially expressed miRNAs we found several members of the let-7 family (Fig. S1).

Using RT-PCR analysis expression differences between BPH, group 1 and 2 for let-7a, let-7c, miR-146b, and -181b could be confirmed (Fig. 2B). Since expression of let-7b and -d was shown to discriminate at least two of the three tissue types we included both let-7 family members in further analyses. As predicted by the array data, the let-7 family members showed a progressive down-regulation from benign to malignant (group 1) and further to the aggressive disease (group 2), whereas expression of miR-146b and -181b was lowest in group 2 (Fig. 1B). Let-7d expression was hardly detectable by RT-PCR and was, therefore, excluded in further investigations.

Expression of let-7a/b/c is Significantly Down-regulated in High-risk PCa

By microarray and RT-PCR analyses we identified some let-7 family members (let-7a-c) as potential candidate miRs for diagnostic and prognostic markers in high-risk PCa.

We now wanted to confirm these results by qRT-PCR experiments on our entire high-risk cohort A (n = 98). Statistical analysis of the qRT-PCR data confirmed a significant down-regulation of let-7a-c in the high-risk PCa cases compared to benign hyperplasia (p≤0.001) (Fig. 3A). In about 81/84/96% of the tissue samples analyzed, the expression of let-7a/b and -c, respectively, was below the median expression of the BPH samples (Fig. 3B), indicating the tumor-specific value of the let-7 family members in patients with high-risk prostate cancer.

Down-regulation of let-7b is Associated with Aggressive Cancer Characteristics

Based on the assumption that some let-7 family members are progressively down-regulated in the more aggressive PCa cases (group 2), we postulated that down-regulation of let-7 might also be associated with clinic-pathological features or prognosis of PCa patients. Therefore, we looked for correlation between let-7a/b/c expression and tumor characteristics of the PCa collective A like Gleason Score (7 ≤ GS ≤ 8), pathological tumor stage (3a ≤ pT ≤ 3b) and clinical endpoints like BCR, CF, and CRD (see Table 2). We found progressive down-regulation of let-7b in patients with poor cancer characteristics like high Gleason Score (≥8), biochemical relapse and clinical failure (p<0.05) (Fig. 3C). However, expression of let-7c was correlated with CF only. No correlation was seen between let-7b or -c expression and pathological tumor stage (3a ≤ pT ≤ 3b) or CRD (Fig. 3C). Since let-7a lacked any significant results in terms of cancer characteristics and clinical endpoints we neglected this miR in our further analyses (Fig. S2).

Let-7b and let-7c as Prognostic Marker for Progression in a High-risk PCa Collective (cohort A)

To determine whether let-7b or -7c could serve as a prognostic indicator for biochemical relapse or clinical failure we dichotomized a high-risk study group (cohort A) in low and high let-7b/c expression based on z-scores. Kaplan-Meier estimates showed a significant difference between groups of high and low let-7b expression in BCR (log rank p = 0.01), while let-7c showed borderline significance (log rank p = 0.08) (Fig. 4A). The 10 yr biochemical progression-free survival rate was 65% and 37% for high and low let-7b expression, respectively. For let-7b and let-7c Kaplan-Meier estimates also predicted a significant difference between groups of high and low expression in CF (log rank p<0.001) (Fig. 4A). 14 of 38 patients (37%) with low let-7b expression, but only 5 of 60 patients (8.3%) in the high let-7b expression group were found to have experienced CF.

Cox regression analysis showed that let-7b but not let-7c expression was univariately significant for the prediction of BCR (HR 0.36 (0.16–0.82)). When let-7b expression and Gleason Score were considered together in a multivariate model both variables independently predicted BCR. Eventually, we evaluated whether let-7b or let-7c are independent markers for clinical failure in cohort A. The expression of both miRs are univariately significant for the prediction of clinical failure (Table 3). However, in a multivariate Cox regression model only high Gleason Score but not let-7b or -c were significantly correlated to poor prognosis.

In summary the Kaplan Meier estimates and Cox regression analysis indicate that low let-7b and let-7c expression might be correlated to BCR and CF in cohort A.

Validation of let-7b and let-7c as Prognostic Markers in an Independent, External Testing Collective (cohort B)

To validate the role of let-7b and -c as prognostic markers we worked with an independent collective of primary high-risk PCa cases (cohort B). As already observed for cohort A we detected a significant down-regulation of let-7b and let-7c in PCa cases when compared to BPHs (Table S2). We created z-score levels based on the analyzed AC expression data of both let-7s in the testing cohort. Subsequently we dichotomized the testing cohort using the cut-off levels identified for collective A. Each sample was predicted to be either on high or low risk for BCR or CF based on these thresholds. We could not confirm the prognostic role of let-7c as marker for progression in the testing cohort as indicated by Kaplan Meier estimates and Cox regression analysis (Fig. 4b and Table 4). Nevertheless, Kaplan Meier estimates indicated that patients with low let-7b expression showed significantly shorter cumulative time to BCR or CF than those with high let-7b expression (Fig. 4b). To identify whether let-7b is an independent prognostic covariate for progression of PCa we performed Cox proportional hazard analysis. The multivariate Cox regression model revealed let-7b, but not let-7c, to be an independent prognostic marker for both: BCR (HR = 0.3 (0.15–0.61)) and CF (HR = 0.23 (0.08–0.70) (Table 4).
Figure 2. MiRNA expression in high-risk PCa (cohort A) with early clinical failure. A. Venn diagram showing relationships between human miRs that were expressed significantly different (≥1.5 fold) in PCa group 1 vs. PCa group 2 vs. BPH (adj p < 0.05). Circles include numbers of up- or
down-regulated human miRs of each pair-wise comparison. Common miRs between different comparisons are shown in intersections. Grey boxes indicate expression status of the let-7 family members. The table below lists all human miRs in which expression was significantly different either: between (gr.1 vs. gr.2), (gr.2 vs. BPH) and (gr.1 vs. BPH) (green column); between (gr. 1 vs. gr. 2) and (gr. 2 vs. BPH) (red column); between (gr. 1 vs. gr. 2) and (gr. 1 vs. BPH) (blue column). All miRNAs are ranked based on the maximum expression fold change. The two columns aside the list of miRs indicate an up- or down-regulation in expression (see arrows) and the dimension of expression fold change. B. Validation of the microarray data by qRT-PCR. Relative expression of miR-146b, -181b, and let-7a/b/c and -c was analyzed in 6 BPH (white), 7 high-risk PCa samples with CPFS (group 1) (light grey), and 6 high-risk PCa samples with CF (group 2) (dark grey). MiRNA expression is shown as means of expression in BPH and high-risk tissue, respectively with error bars for standard deviation. *indicates p<0.01, p values were calculated using the Welch 2 sample t-test.

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Figure 3. Let-7a/b/c expression in a large high-risk PCa collective (cohort A) and its association to clinical data. A. Box- and whisker-plot of relative expression of let-7a/b/c in 6 BPH (white bar) and 98 high-risk PCa (grey bar) tissue specimens. Expression was assessed by qRT-PCR. Figure 3B summarizes the median expression of let-7a/b/c in 6 BPH and 98 PCa samples and shows corresponding standard deviation and the p value. The median expression of the BPH samples was used as threshold. The percentage of PCa samples with down-regulated let-7 expression are shown in the 6th column of the table. B. Box- and whisker-plot show expression of let-7b and -7c in 98 high-risk PCa tissue and 6 BPH specimens; assessed by qRT-PCR. Subgroups are based on: • pathologic tumor features like Gleason Score and pathologic tumor stage (GS ≤7, pT ≤Ta – light grey), (GS ≥8, pT ≥T3b – dark grey) and • clinic-pathological characteristics like BCR, CF, CRD (no BCR/no CF/no CRD – light grey), (BCR/CF/CRD – dark grey). A. + C. Let-7a/b/c expression is shown as means with error bars for standard deviation. *indicates p<0.01 (A) or p<0.05 (C). p values were calculated using the Welch 2 sample t-test.

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Let-7 in High-Risk Prostate Cancer

HMG1 is a Direct Target of let-7b in PCa Cells

Expression and correlation studies of the high-risk cohort had revealed let-7b to be a potential prognostic marker for progression in high-risk PCa. Therefore, we searched for potential new target genes using database target search programs (pictar, miRanda). A binding site for let-7b was found in the 3’UTR of HMG1A suggesting that this oncogene might be a target of let-7b (Fig. 5A). To determine the relative level of let-7b and HMG1A in prostate cancer cell lines we performed qRT-PCR on LNCaP and PC-3 cells. We confirmed results from a previous study showing that let-7b is expressed at very low levels in PC-3 when compared to hormone sensitive LNCaP cells [18]. Our results showed that HMG1A expression is reduced in LNCaP when compared to PC-3 cells indicating a reciprocal expression of HMG1A and let-7b in these PCa cell lines (Fig. 5B).

In addition, we analyzed the HMG1A expression in PC-3 cells 48 hours after transient transfection with pre- and anti-let-7b by qRT-PCR and Western blot analysis. HMG1A-mRNA expression was significantly lower after let-7b over-expression (p = 0.004) (Fig. 5C). On protein level an inverse correlation was detected by Western blot analysis in PC-3 cells. Reintroduction of let-7b lead to diminished HMG1A protein expression compared to cells transfected with pre-negative control (down-regulation to 64.4% in PC-3). On the other hand, suppression of let-7b resulted in an up-regulation of HMG1A expression in PC-3 cells (Fig. 5D). On protein level an inverse correlation was detected by Western Blot analysis in PC-3 cells. Reintroduction of let-7b lead to diminished HMG1A protein expression compared to cells transfected with pre-negative control (down-regulation to 64.4% in PC-3). On the other hand, suppression of let-7b resulted in an up-regulation of HMG1A expression in PC-3 cells (Fig. 5C). On protein level an inverse correlation was detected by Western Blot analysis in PC-3 cells. Reintroduction of let-7b lead to diminished HMG1A protein expression compared to cells transfected with pre-negative control (down-regulation to 64.4% in PC-3). On the other hand, suppression of let-7b resulted in an up-regulation of HMG1A expression in PC-3 cells (Fig. 5D).

To compare whether HMG1A expression was correlated to let-7b in primary human PCa we isolated m(i)RNA of PCa and adjacent benign tissue samples (cohort C) and performed qRT-PCR analyses on let-7b and HMG1A. We utilized 21 pairs of PCa samples in which expression of let-7b was significantly suppressed (≥0.5 cycles) in cancerous compared to benign tissue (Fig. S3). The balance of m(i)RNA expression in PCa and BPH was used for assessment of the correlation coefficient (ΔCt, PCaΔCt/HMG1AΔCt, benign tissue ΔCt). Pearson’s correlation coefficient confirmed a negative correlation between HMG1A and let-7b expression (r = −0.71) suggesting that expression of HMG1A in primary PCa cases is at least partially regulated by let-7b (Fig. 5E).

Discussion

Over the last years specific miRNA signatures for PCa have been described in several studies suggesting that miRNAs or miRNA profiles can be used as diagnostic or prognostic markers in this disease [5–9,11,12,15,16]. Nevertheless, there is not much knowledge about the applicability of these as prognostic markers in PCa. To screen for miRNAs that show specific expression for high-risk disease we analyzed 13 high-risk PCa samples by a microarray experiment. Initially, we compared the expression of all 13 high-risk samples to the expression of 6 BPH samples and found a miRNA expression profile that allowed a clear distinction between the malignant and the benign disease. This scatter, with the majority of miRNAs down-regulated in the high-risk PCa samples, was comparable to results seen in further microarray analyses using contemporary PCa study groups. Among the most highly de-regulated microRNAs we primarily found miRNAs that were previously described as PCa specific in other studies e.g. miR-15a, -26a, -125b, -143, -205, and miR-221, indicating that the high-risk PCa cases show comparable miRNA expression alterations as observed in contemporary PCa collective [6–12].

Subsequently, we analyzed the miRNA expression alterations among PCa samples with and without CF to screen for prognostic

Table 3. Uni- and multivariate Cox regression analysis of high-risk cohort A.

| variable + BCR | univariate | multivariate |
|----------------|------------|--------------|
|                | n | HR | 95% CI | p value | HR | 95% CI | p value |
| let-7b dichot. | high 47, low 51 | 0.36 | 0.161 - 0.823 | 0.02 | 0.44 | 0.193 - 1.022 | 0.05 |
| let-7c dichot. | high 7, low 91 | 2.81 | 0.840 - 9.388 | 0.09 | |
| Gleason Score  | 98 | 1.62 | 1.149 - 2.296 | <0.01 | 1.50 | 1.059 - 2.125 | 0.02 |
| comb. pT       | 98 | 1.58 | 0.717 - 3.496 | 0.26 | |
| pre.PSA        | 98 | 1.01 | 0.999 - 1.021 | 0.07 | |

| variable + CF | univariate | multivariate |
|---------------|------------|--------------|
|               | n | HR | 95% CI | p value | HR | 95% CI | p value |
| let-7b dichot. | high 47, low 51 | 0.21 | 0.076 - 0.588 | <0.01 | 0.46 | 0.148 - 1.411 | 0.17 |
| let-7c dichot. | high 7, low 91 | 0.23 | 0.095 - 0.578 | <0.01 | 0.53 | 0.189 - 1.475 | 0.22 |
| Gleason Score  | 98 | 2.93 | 1.741 - 4.916 | <0.01 | 2.15 | 1.276 - 3.615 | <0.01 |
| comb. pT       | 98 | 2.05 | 0.737 - 5.683 | 0.17 | |
| pre.PSA        | 98 | 1.02 | 1.003 - 1.028 | 0.01 | 1.01 | 0.997 - 1.024 | 0.13 |

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relevant miRNAs. Interestingly, hierarchical clustering of microarray data using a selected set of miRs differentially expressed in BPH and PCa samples generated a tree with additional separation of these both PCa groups suggesting that high-risk PCa cases with early CF could be characterized by a distinct miRNA expression profile. As expected from these results, we found seven miRNAs that were differentially expressed between both PCa groups and the non-malignant BPH samples. Three of these, namely miR-361, -515-3 and -515-5, have not been described as oncomiRs until now and their relevance in development and progression of PCa remains to be tested in the future. Four other miRNAs (miR-146b, -181b, let-7a and let-7c) are known oncogenic or tumor suppressor miRNAs [30–33]. Our results demonstrate that miR-146b and -181b are down-regulated in all PCa cases, but showed up-regulation when we compared expression in PCa patients with CF to patients with CPFS. These results are supported by studies implicating that miR-146b up-regulation has prognostic effects in cancer patients: Expression of miR-146b is significantly lower in low-risk myelodysplastic syndrome (MDS), whereas patients with high-risk MDS show an elevated expression of this miRNA [30]. It was also demonstrated that squamous cell lung cancer patients with high miR-146b expression have significantly worse overall survival compared to patients with low expression [31]. Moreover, down-regulation of miR-146b is described to correlate with development of hormone refractory PCa [32]. Altogether, these results implicate that deregulation of miR-181b or miR-146b might be critically involved in development and progression of PCa.

However, our microarray-based miRNA expression study is limited to a low number of analyzed cases, since it was primarily designed as a screening approach. To test the clinical relevance of the identified candidate miRNAs as prognostic markers larger cohorts have to be tested. In addition our expression profiling is limited by the use of BPH samples as control tissue. Based on the highly invasive growth of various high-risk PCa cases (data not shown) we were not able to isolate adjacent normal prostate tissue with sufficient amounts of benign prostatic ducts for analyses. We are aware that gene expression in BPH and cancer samples might differ in regard to the different origin of the tissues (transition zone vs. peripheral zone). Therefore, the results obtained by the microarray study need to be interpreted cautiously and await further experimental clarification by validation in independent study cohorts and by functional studies in the future. However, our current study focused on expression differences of let-7 family members between samples of cancer patients with diverse outcome rather than between cancerous and non-cancerous tissue.

Thus, we analyzed the expression of let-7 family members in a large high-risk PCa collective to elucidate their potential role as prognostic biomarker. As expected from the microarray data we saw down-regulation of let-7a/b and -c in the majority of the high-risk PCa cases analyzed. However, let-7a showed no clear impact as prognostic marker in association analysis with clinical parameters. Even though, we found some correlation of low let-7c expression with progression of PCa in a high-risk cohort, we could not validate its role as prognostic marker for biochemical or clinical progression using an independent testing cohort. We therefore concluded that let-7c is frequently down-regulated in PCa, but its clinical use as prognostic marker is very limited. With regard to the role of let-7b as potential prognostic marker we showed that patients with poor cancer characteristics like BCR or CF have lower let-7b expression than patients with better clinical outcome. As prognostic factor let-7b is independent from other tested clinical factors and is closely related to PSA relapse and recurrence of the disease. Altogether our findings suggest that reduced expression of let-7-a/b and -c may play a role in pathogenesis of prostate cancer and strongly support that reduced let-7b expression may have a prognostic impact on biochemical and clinical progression of PCa patients.

Our results implicating that let-7b is a useful biomarker to predict clinical outcome are in line with studies in patients with lung cancer. Takamizawa et al. analyzed tissue specimen of 143 lung cancer patients and found that patients, irrespective of disease stage, had significantly worse post-operative survival, if let-7b was down-regulated [19]. In addition, the potential of let-7 as biomarker in various cancer types was recently implicated in a systematic review demonstrating that let-7 is associated most frequently and significantly with clinical outcome parameters in cancer patients [34]. Martens-Uzunova and colleagues recently also found let-7b to be significantly dys-regulated in PCa. They

### Table 4. Uni- and multivariate Cox regression analysis of high-risk cohort B

| variable + BCR | univariate | multivariate |
|----------------|------------|--------------|
|                | n          | HR 95% CI    | p value | HR 95% CI    | p value |
| let-7b dichot. | high 44, low 48 | 0.25 0.125 - 0.493 | < 0.01 | 0.30 0.152 - 0.610 | < 0.01 |
| let-7c dichot. | high 5, low 87 | 0.00 0 - 1.0 | 1.00 |
| Gleason Score  | 92         | 1.54 1.169 - 2.022 | < 0.01 | 1.36 1.012 - 1.820 | 0.04 |
| comb. pT       | 92         | 2.20 1.221 - 3.968 | < 0.01 | 1.24 0.640 - 2.398 | 0.53 |
| pre.PSA        | 92         | 1.02 1.002 - 1.030 | 0.03 | 1.01 0.995 - 1.024 | 0.19 |

| variable + CF  | univariate | multivariate |
|----------------|------------|--------------|
|                | n          | HR 95% CI    | p value | HR 95% CI    | p value |
| let-7b dichot. | high 44, low 48 | 0.24 0.084 - 0.703 | < 0.01 | 0.23 0.078 - 0.700 | < 0.01 |
| let-7c dichot. | high 5, low 87 | 3.67 0.485 - 27.80 | 0.21 |
| Gleason Score  | 92         | 3.98 2.327 - 6.823 | < 0.01 | 4.00 2.076 - 7.712 | < 0.01 |
| comb. pT       | 92         | 5.38 1.946 - 14.84 | < 0.01 | 0.88 0.801 - 7.220 | 0.12 |
| pre.PSA        | 92         | 1.02 1.00 - 1.041 | 0.05 |

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Figure 5. **HMGA1 is a direct target of let-7b in prostate cancer.**

A. 3' UTR-sequence of binding sites for let-7b are indicated by vertical lines towards HMGA1.

B. qRT-PCR for let-7b and HMGA1 in untreated LNCaP and PC-3 cells. While let-7b shows higher expression in LNCaP compared to PC-3 cells, HMGA1 expression shows inverse expression to let-7b.

C. qRT-PCR and Western Blot analysis for HMGA1 using lysates from PC-3 cells transiently transfected with pre-let-7b and negative control, respectively. Western Blot analysis was performed in two independent experiments. Band intensity was measured by Image J in regard to housekeeping gene (β-Actin) band intensity.

D. Luciferase construct containing the 3' UTR of HMGA1 were transfected with either pre-let-7b or pre-miR control into PC-3 cells. Relative expression of firefly luciferase was determined 48 h post transfection and normalized to the transfection control. Error bars represent ± standard deviation and were calculated using three independent experiments.

E. Correlation analysis of HMGA1 and let-7b in vivo. In 21 pairs of PCa and adjacent benign tissue qRT-PCR analysis for let-7b and HMGA1 were performed. Expression levels, normalized by RNU6B or β-Actin, were used to calculate the Pearson's correlation coefficient.
created a miRNA-derived prognostic predictor, consisting of 25 miRNAs, including let-7b, that forecasted post-operative outcome [10]. There is also clear evidence that members of the let-7 family function as tumor suppressor in various cancer types and that over-expression of let-7 suppresses growth of cancer cells in vivo and in vitro including PCa [35]. More recently down-regulation of let-7 was also demonstrated in prostate cancer stem cells indicating an important role of this miRNA in tumorigenicity [25]. Altogether, this further supports our findings of let-7b being an important predictor in clinical outcome in various cancer types including PCa.

Down-regulation of several let-7 isoforms in high-risk disease indicates their potential involvement in pathogenesis and progression of PCa. But how let-7 family members contribute to this is poorly understood. It was previously shown that members of this family simultaneously inhibit multiple oncogenic pathways in cancer cells regulating the expression of relevant oncogenes like cmyc, ras, CDC25A, and HMGA2 among others [22–24]. Moreover, it was shown that the developmentally regulated RNA-binding protein Lin28 selectively blocks the maturation of let-7 members, which in turn repress the expression of Lin28 [35]. Based on the connection of Lin28/let-7 several line of evidence support a role of let-7 in stem cell biology and development. Deregulation of this feedback mechanism is critical in controlling self-renewal, a feature of cancer stem cells, which again is associated with aggressiveness and progression in various cancer types [36,37]. Interestingly, it was shown that the developmentally regulated gene HMGA1 is inversely correlated to let-7 in gastro-pancreatic carcinoma and retinoblastoma [38,39]. HMGA1 is a member of the non-histone nuclear binding proteins that alter chromatin structure by inducing DNA confirmation and thus regulating the transcriptional activity of several genes [40]. HMGA1 over-expression plays a critical role in carcinogenesis and its up-regulation was observed in many cancer entities like retinoblastoma, pancreatic, colorectal, lung and prostate cancer for example [41–46]. Increased levels of HMGA1 were shown to be correlated with the development of high-grade tumors in various cancer types [47] and more recently it was demonstrated that overexpression of HMGA1 is also associated with late stage prostate cancer [48]. Furthermore, over-expression of HMGA1 in PCa cells was associated with enhanced proliferation and metastasis as well as with development of androgen-independent growth [49]. Interestingly, it was recently shown that HMGA1 is regulated by miR-296, but not by let-7c [48]. We report in the current study that let-7b represses HMGA1 expression by inhibiting translation and additionally showed that HMGA1 is a direct target of let-7b. We performed correlation studies ex vivo on primary PCa specimens and confirmed an inverse relation between let-7b and its target HMGA1. Based on these data we suggest that let-7b mediated regulation of HMGA1 might partially explain the role of let-7b in progression of PCa in context with its role in the development of cancer stem cells. Identification of the interaction between let-7b and HMGA1 will provide promising therapeutic opportunities in PCa treatment.

In summary, we defined a distinct miRNA expression profile in high-risk PCa with early CF. Our study further gives evidence that one of the detected miRNAs, namely let-7b, works as a relevant prognostic biomarker predicting clinical recurrence in high-risk PCa. We identified the oncogene HMGA1 as direct target of let-7b in PCa and found an inverse correlation of both in primary PCa cases. It will be of great interest to understand the role of let-7b/ HMGA1 interaction for the development of cancer stem cells in context with the regulation of others potential target genes of the let-7 family members. Altogether, our study represents a basis to develop novel strategies for the prognosis and therapy of PCa patients at high risk for clinical recurrence of the disease.

Supporting Information

Figure S1 MiRNA expression in high-risk PCa (cohort A) with early clinical failure. Venn diagram showing relationships between human miRs that were expressed significantly different (±1.5 fold) in PCa group 1 vs. PCa group 2 vs. BPH (adj p<0.05). Circles include numbers of up- or down-regulated human miRs of each pair-wise comparison. Common miRs between different comparisons are shown in intersections. The table below lists all human miRs in which expression was significantly different either: between (gr.1 vs. gr.2) (blue), (gr.2 vs. BPH) (red), (gr.1 vs. BPH) (green), between (gr. 2 vs. BPH) and (gr. 1 vs. BPH) (yellow). (Complementary data to Fig. 2) All miRNAs are ranked based on the maximum expression fold change. (XLSX)

Figure S2 Association of let-7a expression and clinical data. Box- and whisker-plot show expression of let-7a in 98 high-risk PCa tissue and 6 BPH specimens; assessed by qRT-PCR. Subgroups are based on: • pathologic tumor features like Gleason Score and pathological tumor stage (GS ≤7, pT ≤T3a-light grey), (GS ≥8, pT ≥T3b-dark grey) and • clinic-pathological characteristics like BCR, CF, CRD (no BCR/no CF/no CRD-light grey), (BCR/CF/CRD-dark grey). Let-7a expression is not associated to clinical data in high-risk PCa. Expression is shown as means with error bars for standard deviation. p values were calculated using the Welch 2 sample t-test. (TIF)

Figure S3 Let-7b expression in cancerous and adjacent benign prostatic tissue (cohort C). Scatter Plot shows expression of let-7b in 21 pairs of PCa tissue and adjacent benign tissue. Median expression of let-7b is significantly lower in the cancerous compared to the benign tissue. p values were calculated using the Welch 2 sample t-test. (TIF)

Table S1 MiRNA expression in high-risk PCa (cohort A) with early clinical failure compared to expression in BPH. Microarray analysis of 6 BPH and 13 high-risk PCa tissue specimens (cohort A). The table lists all 82 differentially expressed miRNAs (fold change >1.5 and an adjusted p value <0.05) The majority of miRNAs showed decreased expression in high-risk PCa compared to benign prostatic tissue. (XLSX)

Table S2 MiRNA expression in high-risk PCa (cohort B) and BPH. Table 2 summarizes the median expression of let-7b/c in 6 BPH and 92 high-risk PCa samples and shows corresponding standard deviation and the p value. The median expression of the BPH samples was used as threshold. The percentage of PCa samples with down-regulated let-7b expression are shown in the 6th column of the table. (XLSX)
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