

miRNAs can predict prostate cancer biochemical relapse and are involved in tumor progression

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Abstract. Prostate cancer is the leading cancer diagnosed and the second most common cause of cancer related death in the western world. For prognostic monitoring after prostatectomy, recurrent increase of prostate-specific antigen (PSA), the so-called PSA or biochemical relapse remains the leading biomarker. There is currently no biomarker that can accurately predict the risk of relapse at the time of surgery. We analyzed formalin-fixed and paraffin-embedded tissue samples from 52 primary prostate cancers and normal adjacent tissues obtained after radical prostatectomy. Patients were grouped into two categories: i) patients with early biochemical relapse (<1 year after radical prostatectomy) and ii) patients with late or no biochemical relapse (>2 years after surgery). Multiplex real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed to identify a miRNA signature that can predict relapse. Results were validated by quantitative RT-PCR analysis. We identified 63 miRNAs that were differentially expressed among the same categories of patients, of whom 35 miRNAs were up-regulated and 28 were down-regulated. Literature search shows that many of these miRNAs have an established prognostic significance in other cancers and can be actively involved in tumor progression. Target prediction analysis showed that predicted targets of these miRNAs could

be involved in biological processes and pathways that enhance tumor progression. We experimentally validated the role of one of the dysregulated miRNAs (miR-10b) in relapse using proliferation and wound-healing assay. miRNAs can be reliable predictive markers for biochemical relapse of prostate cancer at the time of radical prostatectomy. This should have significant impact on patient managing plans.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed and the second most cause of cancer-related death in the Western world. The most reliable biomarker for PCa is prostate-specific antigen (PSA), which is helpful for screening, diagnosis and follow-up after surgery.

Approximately 15-30% of patients experience a biochemical relapse after curative treatment of radical prostatectomy (RP) or radiation therapy (1,2). It is still a challenging task in PCa to identify patients with aggressive tumors to provide them with closer follow-up and more intensive treatment. For prognostic monitoring after prostatectomy or adjuvant therapy, a recurrent PSA increase, the so-called PSA or biochemical relapse, remains the only available marker. Yet, it has been shown that biochemical relapse is not inevitably leading to disease progression (3). Moreover, there is currently no biomarker that can accurately determine relapse risk pre-operatively.

Various clinical models are established to estimate the risk of biochemical recurrence or clinical failure as has been reviewed recently (4), for example risk stratification models or risk scores. The accuracy of these models is limited as patients with heterogeneous disease characteristics will be clustered in the same risk group, thereby decreasing the predictive accuracy (4). As nomograms allow the use of weighted variables, they have a higher accuracy than classical models. However, most clinical prediction models still lack accuracy. Therefore, much effort is invested into identification of new more accurate prognostic markers for PCa.

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Table I. Patients and tumor characteristics.

	Relapse <1 year (N=24)		Relapse >1-4 years (N=16)		No relapse within 3 years (N=12)	
	Median (range)	N (%)	Median (range)	N (%)	Median (range)	N (%)
Age (years)	61 (51-71)		63.5 (49-73)		65.5 (55-72)	
Pre-operative PSA (ng/ml)	8.9 (0.8-25.0)		7.0 (2.1-41.9)		6.5 (3.7-25.8)	
Pathological tumor stage						
pT2a				1 (6)		
pT2b		3 (13)		4 (25)		1 (8)
pT2c		5 (21)		2 (13)		4 (33)
pT3a		12 (50)		6 (38)		5 (42)
pT3b		4 (17)		3 (19)		2 (17)
Gleason score						
6		3 (13)		1 (6)		3 (25)
7		13 (54)		8 (50)		5 (42)
8				2 (13)		3 (25)
9		7 (29)		5 (31)		1 (8)
10		1 (4)				
Surgical margins						
R0		15 (63)		12 (52)		7 (58)
R1		9 (38)		11 (48)		5 (42)
PSA-free survival (months)	4.57 (1-12)		25.0 (14-50)		51.9 (37-65)	

Molecular markers will help us to move into the promising field of personalized medicine (PM) (5). The concept of PM is to utilize person's genetic information to best plot a course for an individual in terms of disease risk assessment, prevention, treatment or palliation. The era of PM in cancer management promises more efficient treatment based on individual 'tumor characterization' rather than 'tumor anatomical/morphological classification'. Subdividing patients into smaller groups will provide more accuracy of prognosis and treatment efficiency. Furthermore, treatment will be given to only a fraction of patients who will respond, while the others, who gain no clinical benefit, will not need to suffer from severe toxic side-effects. A number of platforms have been developed for PM in cancer, as outlined in our recent review (6). miRNA profiling is an emerging tool for PM in cancer.

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs (7) that are predicted to regulate up to 30% of all messengerRNAs (mRNAs) (8). miRNAs function mainly through post-transcriptional regulation of gene expression and participate in various physiological as well as pathological processes (9).

The suite of clinical applications of miRNA in cancer is broad, encompassing diagnostic, prognostic and predictive clinical applications. miRNAs can also serve as predictive indicators of disease relapse and metastasis. miRNAs have been shown to be dysregulated in the majority of solid tumors, including kidney (10,11) and bladder (12). Several reports have documented dysregulation of miRNAs between normal and cancerous tissues in prostate cancer (13-19). Moreover, miRNA expression is related to tumor stage and progression (16,20),

perineural invasion (21) and androgen-dependence (15,20,22). Recent reports indicate that miR-221 and miR-96 can predict biochemical recurrence (17,23).

In this study we aimed using a low density real-time quantitative polymerase chain reaction (RT-qPCR) assay to identify miRNAs that are differentially regulated in prostate cancer patients having an early biochemical relapse, late biochemical relapse or no biochemical relapse. In order to use miRNAs as potential prognostic markers for predicting a recurrence of prostate cancer after radical prostatectomy, we evaluated in this respect as first proof-of-principle two of the most striking miRNAs, miR-10b and miR-222, by quantitative RT-qPCR. Since *in silico* and experimental evidence indicates that miRNAs can influence cell proliferation and migration and that their predicted targets function in tumor progression, we assessed their potential biological significance by testing the effect of miR-10b on these cellular characteristics.

Materials and methods

Tissue specimen and cell culture. Tissues from 52 primary prostate cancers were obtained after RP between 2001 and 2008 at Charité, University Hospital, Berlin, Germany and St. Michael's Hospital, Toronto, Canada. Tissues were routinely formalin-fixed and paraffin-embedded (FFPE) directly after surgery. To obtain a pure tumor population, tumor areas were identified by haematoxylin/eosin staining and punch-biopsied with a 1-mm tissue microarray punch needle. Tumor content was re-confirmed by a pathologist. Patients were initially divided into three study groups for profiling: i) patients with an early

biochemical relapse (<1 year after RP, N=10), ii) patients with a biochemical relapse between 1-4 years after RP, N=10) and patients with no biochemical relapse during the follow-up of at least 3 years (N=10) (Table I). For validation, samples were divided into two groups: i) patients with an early biochemical relapse (<1 year after RP, N=24) and ii) patients with no or late biochemical relapse (at least 2 years after RP, N=22). The latter cohort included 22 of the 28 patients from the 2 patient groups characterized in the middle and right column of Table I, while all patients with a biochemical relapse between 1 and 2 years were excluded from further analysis. Biochemical relapse was defined as the first post-operative PSA of >0.1 ng/ml, as confirmed by at least 1 subsequent increasing value (persistent PSA increase) after achieving undetectable PSA post-operatively, defined as a detection limit of <0.04 ng/ml. Patients with no relapse were censored at the time-point of the last request.

LNCaP and DU-145 prostate cancer cell lines were grown in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO₂ atmosphere.

Total RNA extraction. Total RNA extraction from malignant FFPE tissue was performed using the Recover All total nucleic acid extraction kit (Ambion, Austin, TX, USA), following the manufacturer's protocol. FFPE tissue was minced with a scalpel and deparaffinized. Prior to protease digestion the tissue was homogenized using a TissueLyser (Qiagen, Hilden, Germany). After protease digestion, samples were demodified by adding 26.5 µl 5 M NH₄Cl and incubating the sample at 94°C for 20 min (24). RNA extraction of benign FFPE tissue was performed with the miRNAeasy FFPE kit (Qiagen) according to the manufacturer's instructions.

The yield and quality of extracted RNA was assessed by spectrometrical analyses (ratio of 260-280 nm) with a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA integrity number was calculated on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

Multiplex quantitative RT-PCR. Multiplex quantitative RT-PCR was performed using the TaqMan Array Human MicroRNA A+B Cards v3.0 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A pool of 10 RNA samples was prepared for each relapse group using 400 ng RNA per sample. Pooled RNA (517 ng) were reverse transcribed using Megaplex RT primers A or B from the Megaplex Primer Pool, Human Pools Set v3.0 (Applied Biosystems) and the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. RT-qPCR was performed on the ABI 7900 machine (Applied Biosystems) in 40 cycles. Expression was normalized to RNU6B, which is provided as endogenous control on the arrays. Cq values above 32 were regarded as not expressed according to the manufacturer's recommendation.

Quantitative RT-PCR. Quantitative miRNA RT-PCR was performed with the TaqMan MicroRNA Assay kit according to the manufacturer's recommendations (Applied Biosystems). RT-PCR was performed for two dysregulated miRNAs (hsa-miR-10b, hsa-miR-222) in a total of 46 cases as described

previously (16). Briefly, 6.67 ng total RNA was reverse-transcribed into cDNA using gene-specific miRNA RT-qPCR primers followed by amplification for 40 cycles using ABI Step One System (Applied Biosystems) and miRNA specific probes (Applied Biosystems). Each experiment was done in triplicate. Expression values were normalized to RNU44. Standard curves and efficiencies were measured for each miRNA (miR-10b, E=1.99; miR-130b, E=1.99; miR-222, E=1.98; RNU44, E=2.00). Normalization was done as previously described (16).

Transfection of prostate cancer cell lines. LNCaP and DU145 were transfected with pre-miRNAs and anti-miRNA inhibitors (Applied Biosystems) for miR-10b in a final concentration of 10 nM using siPort NeoFX transfection agent (Applied Biosystems). In all experiments a culture transfected with a negative control (NC) pre-miR (pre-NC#1; Applied Biosystems) and a no transfection control were carried along.

Proliferation assay. Proliferation of pre-miR-10b transfected LNCaP cells was assessed by metabolic conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, Oakville, Ontario, Canada). Cells were grown to 80% confluence and seeded into 96-well plates at a final concentration of 6x10³ cells per well and transfected with 10 nM precursor miRNA, anti-miRNA inhibitor or pre-miR-NC#1. Cells were allowed to rest for 24 h and were then serum starved for another 24 h. Cultures were incubated in 5 mg/ml MTT for 4 h and lysed in 10% SDS in 0.01 M HCl. Lysates were incubated at 37°C overnight and absorbance of formazan was measured at 550 nm. Each reaction was performed in triplicate and results are shown as average of three independent assays.

Wound-healing assay. To assess the migrative phenotype of DU-145 cells upon pre-miR-10b transfection we performed a wound-healing assay. Cells were grown to 80% confluence and seeded into 6-well plates and transfected with 10 nM precursor-miRNA, pre-miR-NC#1 or anti-miRNA inhibitor. After reaching confluence, the cells were scratched with a 100-µl pipette tip. Re-migration of cells to the wound was assessed by life cell imaging for 24 h under serum starvation. Percentage of open area was measured with the software TScratch (25) and the velocity of migration or the open area relative to time-point 0 was assessed.

Bioinformatics analysis. Target prediction analysis was performed using miRecords (<http://mirecords.bioclead.org/>). We applied strict inclusion criteria and considered a prediction only if it was concordantly identified by two of the three most common algorithms (miRanda, picTar, TargetScan) as well as two other algorithms.

Predicted targets were forwarded to gene ontology (GO) analyses using the Database for Annotation, Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov/home.jsp>) to test for overrepresentation of functional identity. The over-representation of genes from the same pathway was analysed using Reactome Skypainter 2 (<http://www.reactome.org/cgi-bin/skypainter2>). Chromosomal localization of the miRNA and overlapping transcripts were identified using miRBase (<http://www.mirbase.org/index.shtml>).

Table II. Differential expression of the top dysregulated miRNAs in prostate cancer relapse patients given as fold changes in comparison to the no-relapse cohort.^a

miRNA	No relapse within 3 years	Relapse >1-4 years	Relapse <1 year
hsa-miR-23a	NE ^b	(89.44)	(646.34)
hsa-miR-449a	NE	(22.25)	(247.59)
hsa-miR-449b	NE	(68.46)	(207.79)
hsa-miR-200a	1.00	1.12	2.53
hsa-miR-1233	1.00	2.23	2.36
hsa-miR-10b	1.00	2.53	1.90
hsa-miR-1825	1.00	1.11	1.84
hsa-miR-186	1.00	0.64	0.67
hsa-miR-1275	1.00	1.08	0.65
hsa-miR-532-5p	1.00	0.99	0.64
hsa-miR-193b	1.00	1.10	0.64
hsa-miR-886-3p	1.00	1.13	0.60
hsa-miR-664	1.00	1.29	0.58
hsa-miR-196b	1.00	0.45	0.58
hsa-miR-1274B	1.00	1.19	0.57
hsa-miR-720	1.00	1.19	0.51
hsa-miR-146b-5p	1.00	0.83	0.49
hsa-miR-222	1.00	0.85	0.44
hsa-miR-31	1.00	0.91	0.43
hsa-miR-127-5p	1.00	0.27	NE

^aA full list of differentially expressed miRNAs are available upon request. ^bNE, not expressed.

Statistical analysis. Statistical tests were performed with GraphPad Prism 5.04 (GraphPad Software Inc., San Diego, CA, USA), PASW Statistics 18.0.0 (IBM Corp., Somers, NY, USA) or Medcalc 11.5.1.0 (MedCalc Software, Mariakerke,

Belgium). Kruskal-Wallis test and Spearman rank correlation coefficients were used. All tests were performed two-tailed and p-values <0.05 were considered statistically significant. Receiver operating characteristic (ROC) curves were calculated to determine the potential of miRNAs to discriminate between recurrent and non-recurrent samples. Kaplan-Meier analysis and multivariate Cox regression were performed to identify variables associated with biochemical recurrence.

Results

Patient and tumor characteristic. miRNA expression analysis was performed in 52 prostate cancer specimen. Patient and tumor characteristics are summarized in Table I.

Identification of differentially expressed miRNAs in prostate cancer relapse. To address the hypothesis that miRNAs can be regulated in prostate cancer relapse, we performed a Megaplex RT-qPCR for three pooled RNA samples, that correspondent to a group of early relapse, late relapse and no relapse patients as defined in the material section. In total, 198 of 762 miRNAs showed a detectable signal (>32 Cq) in at least one pool. A miRNA was defined to be differentially expressed if the fold change in comparison to the no relapse pool was >±1.5. According to this definition, 63 miRNAs were differentially expressed (Table II), of these 35 miRNAs were up-regulated and 28 miRNAs down-regulated. We performed a literature search for the 63 miRNAs and thus identified 13 miRNAs (miR-10b, miR-30d, miR-31, miR-133b, miR-135a, miR-143, miR-151-3p, miR-200a, miR-221, miR-222, miR-375, miR-429, miR-516-3p), which have been described as putative prognostic or predictive markers in cancer (Table III).

Validation of dysregulated miRNAs. The array results are a proof-of-principle that miRNA might be differentially expressed in prostate cancers that relapse in comparison to non-relapsing tumors. We validated expression of two miRNAs, miR-10b and miR-222 in 46 prostate cancer specimen (Fig. 1A and B). miRNAs for validation were chosen according to the following criteria: i) at least 1.5-fold expression change, ii) expression

Table III. miRNAs with a previously described prognostic significance in cancer.

miRNA	Prognostic significance	Refs.
miR-516-3p	Correlated with pathological grade, associated with ER(+)/LNN breast tumor aggressiveness	(36)
miR-200a	Associated with survival in cervical tumors	(37)
miR-10b	Predicts survival in gastric cancer	(38)
miR-429	Associated with prognosis in ovarian cancer	(39)
miR-30d	Associated with overall survival in NSCLC	(40)
miR-375	Associated with prognosis in esophageal cancer with Barrett's	(41)
miR-143	Correlated with tumor stage in prostate cancer and tumor size in colorectal cancer	(42,43)
miR-151-3p	Associated with CLL harboring 17p deletion and CLL with IGHV unmutated genes	(44)
miR-222	Associated with poor outcome in pancreatic tumors	(45)
miR-31	Correlated with Gleason score in prostate cancer and invasion in colorectal cancer	(16,46)
miR-135a	Associated with relapse and disease-free survival in cHL	(47)
miR-221	Associated with biochemical recurrence in prostate cancer	(17)
miR-133b	Associated with prognosis in bladder cancer	(48)

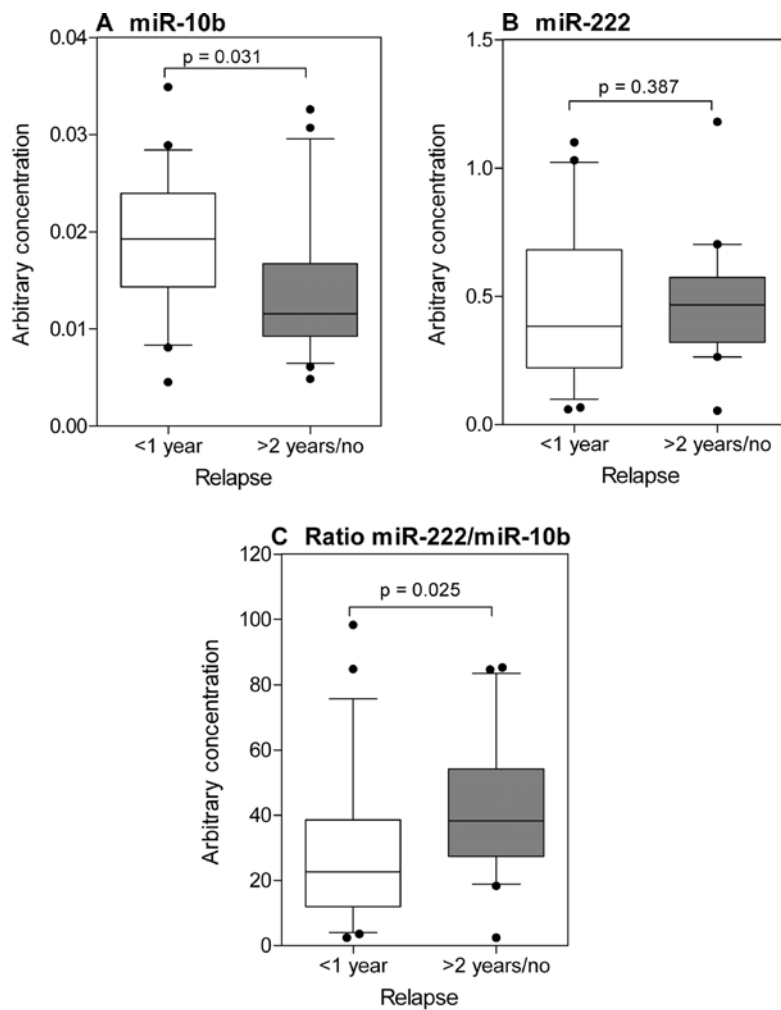


Figure 1. Expression of (A) miR-10b, (B) miR-222 expression and (C) expression ratio of miR-222/miR-10b. Boxes represent the lower and upper quartiles with medians, whiskers the 10 and 90 percentiles. miRNA expression was measured by TaqMan RT-qPCR. Expression was normalized to the geometric mean of miR-130b and RNU44, interplate controls and RT-qPCR efficiency. Significances, calculated by the Kruskal-Wallis test, * $p < 0.05$.

values below 30 Cq values and iii) description of regulation in progression/metastases in another malignancy.

miR-10b showed a 1.67-fold higher median expression in the patients with a biochemical relapse in <1 year in comparison to patients with no biochemical relapse for at least 2 years (Fig. 1A; Mann-Whitney test, $p=0.031$). miR-222 showed a slightly lower median expression of 1.21-fold, this trend was not statistically significant (Fig. 1B; Mann-Whitney, $p=0.387$). The expression ratio of miR-222 to miR-10b was calculated to enhance the power of the variates to predict biochemical relapse. The miR-222/miR-10b ratio was 1.69-fold lower in the patients with a relapse in <1 year (Fig. 1C; Mann-Whitney test, $p=0.025$).

Association of miRNAs to clinicopathological parameters and biochemical relapse. miR-10b did not correlate with the histological grade (Gleason score), the tumor stage and the surgical margin, while miR-222 was inversely correlated with the histological grade (Table IV). Also, the miR-222/miR-10b ratio was significantly correlated with histological grade ($r_s=0.41$, $p \leq 0.01$) and tumor stage ($r_s=-0.31$, $p=0.04$).

To investigate the capacity of the dysregulated miRNAs to discriminate patients with a biochemical relapse in <1 year from the remaining patients, we performed ROC analysis.

Table IV. Correlation of miRNA expression with biochemical relapse and clinicopathological parameters.

	miR-10b		miR-222	
	r_s	p-value	r_s	p-value
Gleason score	0.24	0.11	-0.31	0.04
Pathological stage	0.17	0.28	-0.22	0.16
Surgical margins	-0.02	0.92	0.17	0.27
Pre-operative PSA	0.14	0.37	-0.08	0.91

miR-10b and the miR-222/miR-10b showed a good discrimination with areas under the curve of 0.72 and 0.70, respectively, while miR-222 was not able to discriminate between the groups (AUC=0.56).

We then performed Kaplan-Meier analysis and multivariate Cox regression to determine, which miRNAs were significantly associated with biochemical relapse. miR-10b, miR-222, miR-222/miR-10b ratio and the pre-operative PSA were dichotomized according to their medians. The Gleason

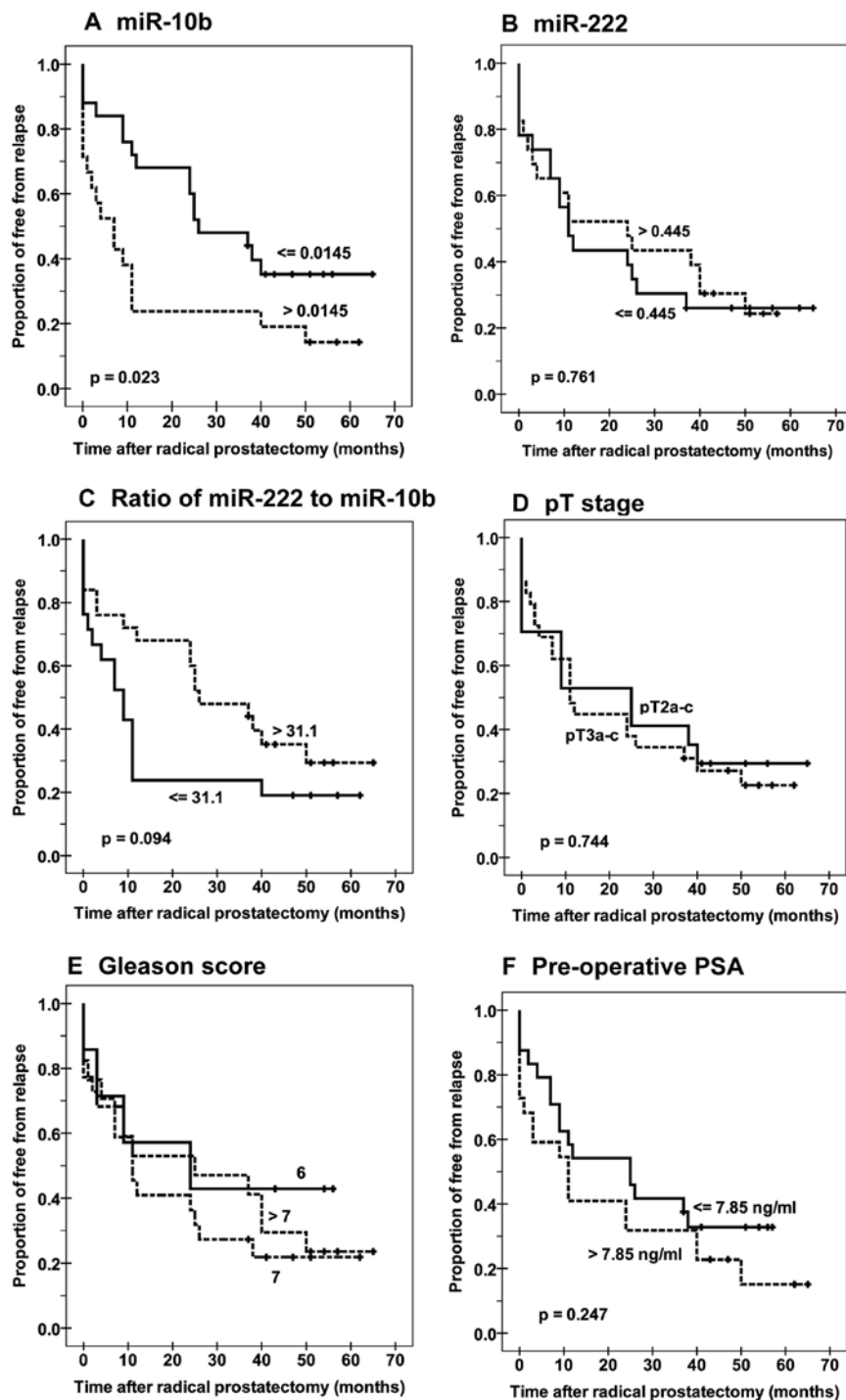


Figure 2. Kaplan-Meier analysis of recurrence-free survival according to various variables. Kaplan-Meier plots of (A) miR-10b, (B) miR-222, (C) ratio of miR-222 to miR-10b, (D) tumor stage, (E) Gleason score and (F) pre-operative PSA. The time of recurrence was defined as the first post-operative PSA value $>0.1 \mu\text{g/l}$ PSA confirmed by at least one subsequent rising value after the patients had reached an undetectable PSA level (detection limit $<0.04 \mu\text{g/l}$) after surgery.

score was trichotomized into a group of 6, 7, and above 7. Pathological stage was dichotomized so that all samples with pT2 were grouped as well as all samples with pT3.

Kaplan-Meier analysis revealed a significant association of miR-10b expression with biochemical relapse (Fig. 2A; log-rank test, $p=0.023$), while miR-222 was not significantly associated with biochemical relapse (Fig. 2B). Surprisingly, none of the clinicopathological variables were significantly associated with biochemical relapse (Fig. 2D-F). In a multivariate Cox

regression model, only miR-10b was an independent variable and remained the only variable in the model using forward and backward elimination methods (Table V).

Chromosomal location of regulated miRNAs. The 63 miRNAs are located on 67 chromosomal locations. Let-7f, miR-135a, miR-516b* and miR-1233 were found in two different locations each. Twenty-eight miRNAs were intronic, while 35 miRNAs were intergenic. Two miRNAs were located in introns as well

Table V. Cox regression of dysregulated miRNAs and clinicopathological variables.

Method	Variable	β	HR	95% CI	p-value
Inclusion	miR-10b	0.76	2.15	1.02-4.51	0.044
	Gleason score	-0.22	0.80	0.37-1.72	0.575
	Pathological stage	-0.11	0.90	0.40-1.99	0.787
	Surgical margins	0.07	1.07	0.50-2.28	0.864
	Pre-operative PSA	0.25	1.28	0.64-2.58	0.485
Forward LR	miR-10b	0.74	2.10	1.06-4.17	0.033
Backward LR	miR-10b	0.74	2.10	1.06-4.17	0.033

HR, hazard ratio; CI, confidence interval; LR, likelihood ratio elimination approach of the Cox regression.

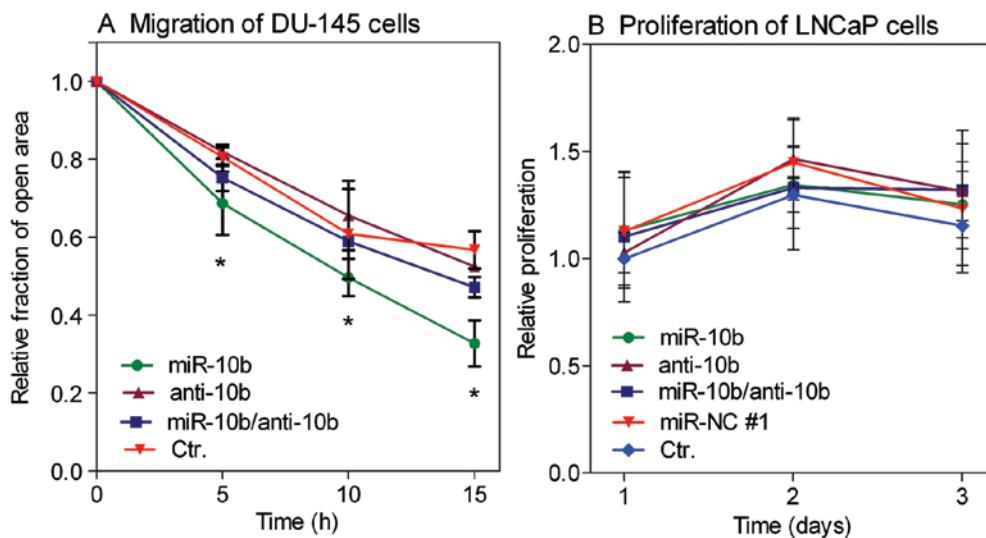


Figure 3. Effect of miR-10b on cell proliferation and migration. Cells were transfected with 10 nM pre-miR-10b, anti-miR-10b, pre-miR-NC#1 or the combination of pre-miR-10b and anti-miR-10b. (A) DU-145 cells were transfected and grown to 100% density. Monolayers of the transfected and untreated (Ctr.) cells were scratched with a pipette tip and re-migration to the wound were analyzed for 15 h by life cell imaging. Pictures were taken every 10 min. Percentage of open area was determined every 5 h using TScratch software. Data are presented as mean values from three independent assays (\pm SD). Significance: Two-way ANOVA, * $p < 0.05$. (B) The effect on cell proliferation of LNCaP cells was measured by MTT assay over three days. Mean values (\pm SD) of three independent experiments. No statistical significance: Two-way ANOVA, $p = 0.732$.

as exons of splice-variants, while two miRNAs were only located in an exon.

We screened the set of differentially regulated miRNAs for expression clusters. A cluster was defined as a set of miRNAs that were located < 10 kb from each other. We identified 6 clusters whose members are dysregulated in prostate cancer relapse. Of them, 4 miRNA clusters show concordant expression.

In case of miR-126*, miR-151-3p, miR-196b and miR-449a and miR-449b a coexpression with their host genes (EGFL7, PTK2, HOXA9, CDC20b) has been described previously (26-30). Interestingly, the EGFL7 transcript, which hosts the miR-126 precursor, can be transcribed in three different variants each of them under control of its own promoter. Promoter studies identified one promoter, which is methylated in prostate cancer and treatment with methyltransferase and histone deacetylase inhibitors were able to up-regulate EGFL7 as well as miR-126 (29).

We further investigated if miRNAs are located in chromosomal aberration sites in prostate cancer relapse. A frequent chromosomal aberration in prostate cancer is 8p loss and 8q gain (31). The 8q24.3 region, which is amplified in prostate cancer, harbors miR-151-3p and miR-661.

Target prediction analysis and functional clustering of miRNA targets. To elucidate a potential function of the dysregulated miRNAs in prostate cancer relapse, we performed *in silico* target research using the software miRecords. Targets were filtered as described in Materials and methods. In total we identified targets for 36 miRNAs, ranging from 4-347 targets per miRNA (data available upon request). The predicted targets were used to perform gene ontology (GO) analysis using DAVID as well as pathway analysis using Reactome Skypainter.

Pathway analysis for miR-10b revealed, that mainly proteins involved in cell-cell junctions (ACTG1 and CADM1) and regulation of transcription (ESRRG, NR4A3, HNRNPK,

GTF2H1, TBX5, NFAT5, HOXA1, KLF13) are over-represented in the set of samples. DAVID allows clustering of GO terms, which are concordantly identified in the sample. Again, regulation of transcription seems to be the most identified biological process in the set of target genes for miR-10b.

Reactome Skypainter analysis for miR-222 indicated that the regulation of protein stability (EIF5A2, SEMA6D, EIF3J) and the TRKA signaling pathway are over-represented in the set of target genes (CDKN1B and RALA). These results are in accordance with literature as CDKN1B is an experimentally proven miR-222 target (32). The most redundantly observed GO terms in DAVID analysis were related to regulation of transcription and apoptosis.

miRNAs dysregulated in relapse play a functional role in tumor progression. miR-10b showed a significant up-regulation of expression in patients with biochemical relapse. Therefore, we addressed the question how miR-10b influences tumor progression *in vitro*.

For wound-healing assays, we transfected DU-145 with 10 nM pre-miR-10b, anti-miR-10b or combination of both and monitored remigration to the wound for 15 h (Fig. 3A). DU-145 cells transfected with pre-miR-10b showed a 70% higher remigration to the wound in comparison to the transfection control (Fig. 3A; Two-way ANOVA, $p < 0.0001$). The combined transfection of miR-10b and anti-miR-10 leads to a partial recovery from miR-10b function with only a 44% faster migration in comparison to the control, while transfection with anti-miR-10b has no significant effect on the remigration.

Proliferation was monitored by MTT assay (Fig. 3B). LNCaP cells were transfected with pre-miR-10b, anti-miR-10b, combination of both or pre-miR-NC#1. After serum starvation, cell proliferation was monitored for 3 days. However, no significant effect on proliferation was observed (Fig. 3B; Two-way ANOVA, $p = 0.732$).

Discussion

This study is a proof-of-principle investigation for the utilization of miRNAs as predictors of biochemical relapse. Using pooled RNA from patients with i) early biochemical relapse, ii) late biochemical relapse and iii) no biochemical relapse we were able to show that a set of miRNAs is differentially expressed between the study groups. These results were validated for two miRNAs in a set of 46 prostate cancer specimens for miR-10b and miR-222.

FFPE tissue is routinely archived from all prostatectomized patients and is therefore the ideal sample for detection of prognostic markers. Expression profiling of mRNA fails to be reliable in FFPE tissue due to their high level of degradation in these tissue samples (33). miRNAs are in contrast to mRNAs considerably more stable in degraded RNA samples (34) and are reliably detectable in FFPE tissue, probably due to their small size and their cooperation into large ribonucleoprotein complexes (35). They might therefore be ideal markers for detection in FFPE tissue.

In prostate cancer relapse patients, 63 miRNAs were identified to be dysregulated between the groups. For a subset of 13 miRNAs (miR-10b, miR-30d, miR-31, miR-133b, miR-135a, miR-143, miR-151-3p, miR-200a, miR-221, miR-222, miR-375,

miR-429, miR-516-3p) a previous deregulation in tumor progression or metastasis has been described in cancer (16,17,36-48). Of them 6 miRNAs (miR-31, miR-133b, miR-143, miR-221, miR-222 and miR-375) have been previously described to be dysregulated in prostate cancer (13,15,17,18,49,50). Therefore the deregulation of these miRNAs might be highly interesting in terms of biochemical relapse and should be further investigated in future studies.

As the results are based on the measurement of three pools from ten samples each, the risk of type I and II errors is high. The profiling step therefore clearly needs to be regarded as a proof-of-principle. To confirm that miRNAs are indeed regulated in prostate cancer, we confirmed expression of two miRNAs in 46 prostate cancer samples in patients, who experienced a biochemical relapse in <1 year (N=24) and patients, who experienced no or late biochemical relapse (>2 years after surgery; N=22). Expression analysis by RT-qPCR confirmed the up-regulation of miR-10b and revealed a trend to a lower expression of miR-222 in patients with early biochemical relapse. These results support the hypothesis that miRNAs can predict biochemical relapse. Statistical analysis was performed to assess whether those miRNAs can separate patients with early biochemical relapse from the other patients. As is to be expected, miR-222 does not differentiate patients well. Yet, miR-10b as well as miR-222/miR-10b expression ratio were comparably good discriminators with AUCs of approximately 0.70. Kaplan-Meier confirmed the association of miR-10b with biochemical relapse. Interestingly, none of the clinicopathological variables was able to distinguish early relapse patients. Expression of miR-10b is not correlated with the clinicopathological parameters and it is an independent marker of biochemical relapse in a multivariate Cox regression model. Therefore, miR-10b expression might be an ideal prognostic marker that can accurately detect patients with biochemical relapse, especially where the available clinicopathological data fail to correctly classify patients.

This is the first study describing a deregulation of miR-10b in prostate cancer relapse. miR-10b has been described in relation to tumor progression and metastases in several tumor entities. It has been reported to be overexpressed in metastatic breast cancer (51), is further associated with high grade gliomas (52), and is higher expressed in hepatocellular carcinoma in comparison to adenocarcinomas (53). It is also part of a seven miRNA signature predicting survival in gastric cancer (38). A comparison of primary and metastatic tumors of breast, bladder, lung and colon revealed miR-10b to be overexpressed in bladder transitional cell carcinomas (54). Expression ratio of miR-221/222 was reported to be negatively associated with poor survival in ovarian cancer (55) and high miR-222 expression is associated with poor survival in pancreatic cancer (45) and invasive tumors in urothelial carcinomas (56). Thus, previous studies in other cancer entities support the regulation of miR-10b and miR-222 in tumor progression.

In silico target analysis was performed for all dysregulated miRNAs in prostate cancer relapse patients and we identified targets for 36 miRNAs. Analysis was further narrowed down on characterization of miR-10b, which is up-regulated in patients with biochemical relapse. Our *in silico* results indicate that miR-10b mainly targets genes involved in transcriptional regulation. We further transfected DU-145 prostate cancer

cells with miR-10b precursor and identified a role of miR-10b in promoting migration of prostate cancer cells. Function of miR-10b has mainly been studied in breast cancer. *In vitro* it has been shown to promote migration and invasion in breast and esophageal cancer cell lines, as well as malignant peripheral nerve sheath tumor cell lines (51,57,58). *In vivo* studies also highlight the importance of miR-10b in metastasis, since mice treated systemically with antagomiR-10b developed less metastasis than their non-treated counterparts (59). Several targets for miR-10b repression have been identified which further pinpoint the central role of miR-10b in migration and invasion. miR-10b directly targets Hoxd10 (60), a transcription factor, thereby regulating important pro-metastatic genes as RhoA, RhoC and urokinase-type plasminogen activator receptor (61). miR-10b further targets KLF4 another regulator of migration (57) and activates Ras-signaling (58).

In silico analysis of predicted miR-222 targets suggest a function of this miRNA in TRKA signaling. The direct binding of miR-221 and miR-222 to CDKN1B is experimentally proven in prostate cancer, thereby supporting the *in silico* results (32). miR-221 and miR-222 are strongly up-regulated in androgen-independent cells and induce androgen-independent growth and lower the response of the PSA-promotor to 5 α -DHT treatment (62). Expression of this miRNA cluster is not only dependent on androgen status, but can also be regulated by concordant binding of c-Jun and NF- κ B to its promotor (63). The miR-221/miR-222 cluster functions mainly in cell cycle transition (32) and promotes tumor growth in mice (64).

In conclusion, we present here the first proof-of-principle study that miRNAs are regulated in prostate cancer patients experiencing a biochemical relapse. Only few studies have before addressed the question of dysregulated miRNA in biochemical relapse, yet they studied only a small number of RNAs and provided only indecisive results (18,65). We further show that the detection of differentially expressed miRNAs is a suitable approach and that these miRNAs might serve as prognostic markers for patients with an early biochemical relapse to provide them with closer follow-up and earlier treatment.

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