Identification of miR-133b and RB1CC1 as independent predictors for biochemical recurrence and potential therapeutic targets for prostate cancer

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Translational Relevance

The identification of novel molecular prognostic biomarkers and therapeutic targets for prostate cancer is fundamental toward improvement of patient outcome. MicroRNAs represent a new class of biomarkers and drug targets. Here, we identify and validate the dual role of microRNA-133b (miR-133b) as a mediator of aggressive phenotype in LNCaP (androgen-dependent) cells and as a tumor suppressor in PC-3 (androgen-independent) cells. We have also identified miR-133b target, RB1CC1 gene, which functions as a tumor suppressor in LNCaP cells. We performed in situ hybridization and immunohistochemistry on tissue microarrays from 178 prostate cancer patients, showing that miR-133b was associated with poor prognosis, whereas RB1CC1 was associated with favorable prognosis. The expression of miR-133b was negatively correlated with RB1CC1 protein levels. Taken together, these results suggested that miR-133b might play crucial roles in cellular processes of prostate cancer progression, and that miR-133b and RB1CC1 are independent biomarkers of prognosis in prostate cancer.
Abstract

Objective: We aimed to investigate the contribution of microRNA-133b (miR-133b) in prostate cancer cell proliferation, cell cycle and apoptosis. We also examined expression of miR-133b in prostate cancer tissues, and evaluate the prognostic significance of miR-133b, as well as its target gene RB1CC1 in patients with prostate cancer after radical prostatectomy.

Experimental Design: miR-133b mimics (miR-133bm) and anti-miR-133b were transfected into LNCaP and PC-3 cells. CCK-8 was employed to look at cell proliferation, and flow cytometric analysis carried out to study cell cycle, and apoptosis determined by caspase-3 activity. MiR-133b expression was assessed by real time RT-PCR and in situ hybridization in prostatic cell lines and 178 prostate tissue samples, respectively. The protein level of RB1CC1 was examined by western blot and immunohistochemistry in prostatic cell lines and prostate tissue samples, respectively.

Results: Overexpression of miR-133b in LNCaP cells boosted cell proliferation and cell cycle progression, but inhibited apoptosis; in contrast, miR-133bm promoted cell apoptosis, but suppressed cell proliferation and cell cycle progression in PC-3 cells. In LNCaP cells, silencing of RB1CC1, a target of miR-133b, inhibited cell apoptosis, and promoted cell cycle progression. Moreover, miR-133b expression was significantly inversely correlated with RB1CC1 expression in prostate cancer tissues. Multivariate Cox analysis indicated that miR-133b and RB1CC1 might be two independent prognostic factors of biochemical recurrence.
Conclusions: MiR-133b might enhance tumor-promoting properties in less aggressive LNCaP cells, whereas this miR may act as a tumor suppressor in more aggressive PC-3 cells. MiR-133b and RB1CC1 were independent prognostic indicators for prostate cancer.
Introduction

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths in the male population of the United States (1). It has been reported that nearly 217,730 new cases of prostate cancer were diagnosed in the United States in 2010, among which 92% are present as local and low stage cases, as indicated by prostate specific antigen (PSA) testing (2). To date, radical prostatectomy (RP) is the standard treatment for localized prostate cancer (3). However, about 25-50% post-RP patients on long-term follow-up have biochemical recurrence (BCR) (4). This is generally the earliest indicator of the recurrence. Therefore, the early risk detection of BCR with sensitive methods and specific tumor markers may effectively improve the prognosis of prostate cancer patients.

Androgen has been reported to play a pivotal role in androgen pathways and prostate carcinogenesis (5). Androgen receptor (AR) regulates androgen-dependent gene transcription (6). Many AR-regulated genes are crucial regulators of prostate development and maintenance (7). Recently, emerging evidence suggests the potential involvement of altered regulation of microRNAs (miRNAs) in AR signaling and the pathogenesis of prostate cancer (8-11).

MiRNAs are short, endogenous RNAs in cells, promoting translational repression and/or destabilizing target mRNAs, to optimize protein levels in numerous biological processes (12). MicroRNA-133b (miR-133b), located at chromosome 6p12.2, is downregulated in several solid cancers (13-15). MiR-133b has been reported to function as an oncomiR or tumor suppressor in different types of cancers (16-18).
However, few studies have focused on the role of miR-133b in prostate cancer, especially the significance of miR-133b expression in clinical prognostic.

*RB1-inducible coiled-coil 1 (RB1CC1)*, identified as a potential regulator of RB1 gene (19), is found in chromosome 8q11, a region containing several loci of putative tumor suppressors. RB1CC1 has been reported as a critical signaling node which coordinately regulates cell growth, cell proliferation, apoptosis, and cell spreading/migration (20). It is also associated with various types of human cancer, especially primary breast cancer (21). However, the role of RB1CC1 in prostate cancer and the significance of RB1CC1 expression in clinical diagnosis were rarely reported.

In this study, we investigated miR-133b expression in cell lines and clinical samples, and analyzed the potential association between miR-133b and various clinicopathological factors of prostate cancer patients. We identified miR-133b displayed tumor-promoting properties in LNCaP cells, whereas acted as a tumor suppressor in PC-3 cells. MiR-133b and RB1CC1, a target of miR-133b, were independent prognostic indicators for prostate cancer.
Materials and Methods

Patients. 135 specimens of prostate cancer patients, 18 prostatic intraepithelial neoplasia (PIN) patients, and 25 normal prostate tissue samples were included in this study. The normal prostate tissue samples were derived from bladder cancer patients after total cystectomy; PIN and prostate cancer samples were from prostate cancer patients after RP and regional lymph node dissection in Tongji Hospital, subsidiary of Shanghai Tongji University, between January 2001 and November 2012. None of the patients received any pre-operation treatment. Cases were classified according to World Health Organization criteria and staged according to Tumor, Node, Metastasis classification and the Gleason grading system. The Research Ethics Committee of Tongji Hospital approved this protocol, and verbal consents were obtained from all patients. BCR was defined as a post-operative serum PSA concentration $\geq 0.2$ ng/ml (22).

Cell culture and Androgen treatment. The prostate cancer cell lines LNCaP, 22Rv1, PC-3 and DU145, and the immortalized noncancerous prostatic stromal cell line WPMY-1 were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China) where they were authenticated by mycoplasma detection, DNA-Fingerprinting, isozyme detection and cell vitality detection. The four prostate cancer cell lines were maintained in RPMI 1640 medium, and WPMY-1 cells were cultured in DMEM medium. The androgen treatment assay was performed as described previously (23). According to previous reports (24-26), 10 nM DHT was found to be the optimal concentration to stimulate LNCaP cell proliferation.
**MiRNA mimics and inhibitor molecules treatment and RNA interference.**

Synthetic miR-133b mimic (miR-133bm) and its scrambled control, control miRNA (miR-NC), siRNA against RB1CC1 gene, and its negative control (a siRNA lacking homology to the genome) were from GenePharma (Shanghai, China), and used at 50 nM concentration. Anti-miR-133b (si133b, Catalog NO. IH-300709-06-0005) and its scrambled control, control anti-miRNA (siNC, Catalog NO. IN-001005-01-05) were from Thermo Fisher Scientific (Waltham, MA, USA), and at 100 nM concentration. All sequences of synthetic oligonucleotides are listed in Supplementary Table S3.

**Transient transfection.** Transient transfection was performed as described previously (27). The Opti-MEM medium and Lipofectamine 2000 were both from Life Technologies.

**Real-time reverse transcription PCR (qRT-PCR).** qRT-PCR for mRNAs was performed as described in (23). For miRNA quantification, cDNA was synthesized from total RNA with MiScript Reverse Transcription kit (Qiagen, CA, USA). Specific primers for mature miR-133b were from Qiagen (Catalog NO. MS00031430). The primers used for qPCR were listed in Supplementary Table S4.

**Chromatin Immunoprecipitation (ChIP) Assay.** ChIP was performed as described in (23). With minor modification, LNCaP cells were treated with vehicle, DHT (100 nM), anti-androgen Casodex (CDX, 10 μM), or a combination of DHT and anti-androgen for 4 h before harvesting. The primers of androgen response element (ARE) for qPCR were listed in Supplementary Table S5 and S6. KLK3 (PSA) enhancer was used as the positive control (28), whereas XBP-1 promoter and a DNA
region adjacent to miR-133b gene without a putative ARE served as the negative controls (29).

**Expression constructs.** Expression construct was performed as described previously (30). Briefly, the wild-type 3’UTR of *RB1CC1* was amplified by PCR from LNCaP cell, and was inserted downstream of the stop codon of luciferase. Two pairs of primers, Mut-1 and Mut-2, were used to generate a 5 nucleotides substitution mutation of the putative miR-133b binding sites with the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan). The primers were listed in Supplementary Table S7.

**Dual luciferase assay.** LNCaP cells were co-transfected in 24-well plates with 20 pmol miR-133bm or miR-NC, together with 0.8 µg firefly luciferase report construct containing the wild-type or mutant *RB1CC1*-3’-UTR and 8 ng control vector pRL-TK (Promega, Madison, WI). 48 h after transfection, firefly and renilla luciferase activities were measured with GloMax® 96 Microplate Luminometer (Promega).

**Western blot.** Western blot was performed as described previously (23) with antibodies against RB1CC1 (1:1000, Sigma–Aldrich, MO, USA), β-Actin (1:4000, Sigma-Aldrich) and EGFR (1:1000, Proteintech, Chicago, USA).

**Cell proliferation assay.** Cell proliferation was performed as described in (27). Briefly, cells were transfected with miR-133bm or anti-miR-133b, and their negative control, respectively, and examined at 0 h, 24 h, 48 h and 72 h. At each time point, CCK-8 reagent was added into cells and incubated for 2 h. The absorbance was measured at 450 nm using a multi-mode microplate reader (BioTek, Winooski, VT).

**Cell cycle assay.** Cell cycle was performed as described in (31). Briefly, cells were
incubated with propidium iodide (10 μg/ml) for 15 min in the dark. The fractions of viable cells in G1, S, and G2 phases of cell cycle were measured with a FACStar flow cytometer (Becton-Dickinson, San Jose, CA).

**Apoptosis assays.** MiR-133bm, miR-NC, anti-miR-133b or anti-miR-NC was transfected as described above. 48 h after transfection, cells were collected and assayed with FITC Annexin V Apoptosis Detection Kit (Becton-Dickinson) on a FACScalibur flow cytometer following the manufacturer’s instructions.

**Intracellular Caspase and Caspase-3 Activities.** Intracellular Caspase and Caspase-3 assays were performed as described previously (32). Activities of the total caspase and caspase-3 were assayed with CaspGLOW Fluorescein Caspase Staining kit and CaspGLOW Fluorescein Caspase-3 Staining kit (BioVision, Mountain View, CA), respectively, and detected by FACScalibur flow cytometer.

**Tissue microarray (TMA) construction.** TMA construction was performed as described previously (33). Two replicate tumor samples (1 mm in diameter) were taken from the donor tissue blocks in a highly representative fashion and arrayed into a recipient paraffin block (35 mm × 622 mm × 65 mm) with a tissue microarrayer (Beecher Instrument Inc, WI, USA), as described by Kononen (34).

**LNA-ISH and Immunohistochemistry of TMA.** Locked nucleic acid in situ hybridization (LNA-ISH) was performed as described previously (33) on a set of prostate cancer TMA slides using LNA™ probes for miR-133b (Exiqon, Vedbaek, Denmark; sequence: 5′DigN/TAGCTGGTTGAAGGGGACCAAA/3′Dig_N/). Expression of RB1CC1 was detected in immunohistochemistry assays performed as
described previously (35), with polyclonal antibody for RB1CC1 (1:100, Proteintech).

**Image acquisition and management.** Digital images were captured using Nikon DS-Ri1 ECLIPSE Series (Nikon, Tokyo, Japan) with a 10 × objective. TMA images were managed using NIS Element D4.00 software (Nikon).

**Statistical analysis.** The BCR-free survival time was defined as the time from the date of surgery to that of BCR, and the follow-up data were updated till November 2012. The differential expressions of RB1CC1 between groups were analyzed using $\chi^2$ analysis. The probability of survival was estimated according to the Kaplan-Meier method. With the log-rank test, we analyzed the differences between the survivals of different patients groups. To analyze the correlation between miR-133b and RB1CC1 expression and clinicopathologic characteristics, the T-test, $\chi^2$ analysis, Mann-Whitney U test and Fisher’s exact test were employed according to the test condition. A Cox proportional hazards model was performed to establish independent factor(s) for patients’ survival. Statistical significance was defined as $p<0.05$, and all tests were two-tailed. The statistical analysis was performed based on the SPSS software, version 15.0 (SPSS Inc, Chicago, IL).

For experiments in cell lines, means and standard deviations of individual groups (n ≥3) were calculated. $P$ values were assessed by performing two-tailed Student’s t-test.
Results

Androgen induces the expression of miR-133b. We have previously analyzed miRNA and mRNA expression in LNCaP cells treated with 10 nM DHT at different time points (23). cDNAs reverse transcribed from total RNAs at each time point were hybridized to Illumina Sentrix Human WG-6_V2 expression BeadChip arrays (for mRNA) or MicroRNA Expression Profiling Panels (for miRNA). The raw data have been uploaded to the Gene Expression Omnibus public repository (http://www.ncbi.nlm.nih.gov/geo/; Gene Expression Omnibus series no. GSE21245). Data pre-processing, filtering criteria and statistical analysis were as described in (23). Of the 137 differentially expressed miRNAs, miR-133b was found to be androgen-responsive in early stage after DHT stimulation (20 min, 8 h), and had higher ARE enrichment according to genomatix database prediction (36). Further, miRDB database was applied to predict the sequence-based miRNA targets (37), which were also androgen-responsive genes, and GenMAPP for the pathway enrichment of miR-133b. As a result, miR-133b was valued with significant pathway enrichment in key cellular processes, such as regulation of cell size, cell growth, and cell cycle ($p<0.05$, data not shown).

Our microarray data analysis identified miR-133b to be up-regulated before 4 h, with a peak at 1 h after DHT stimulation (Fig. 1A). Further validation of miR-133b expression was performed by qRT-PCR in LNCaP cells under the same experimental conditions. The expression of miR-133b was increased compared to the vehicle control (Fig. 1B). The expressions of two androgen-responsive genes PSA and KLK2
were used as positive controls (Supplementary Fig. S1A-B).

**Differential expression of miR-133b in prostatic cell lines.** Next, we evaluated the basal expression of miR-133b in noncancerous prostatic cells WPMY-1 and four prostate cancer cell lines LNCaP, 22Rv1, PC-3 and DU145. LNCaP is androgen-dependent prostate cancer (ADPC) cell line; 22Rv1 is weakly androgen-dependent; PC-3 is androgen-independent prostate cancer (AIPC) cell line without AR expression; and DU145 is AIPC cell line with AR expression. We observed that the less aggressive LNCaP cells had the least expression of miR-133b, whereas the more aggressive PC-3 cells showed the highest expression, which was 395.5-fold over that of LNCaP cells (Fig. 1C). The finding raised the possibility that differentially expressed miR-133b might contribute to the progression of prostate cancer. In this study, we focused on miR-133b and investigated its involvement in prostate cancer.

**Verification the binding of AR to miR-133b.** As we have confirmed the expression of miR-133b is up-regulated by androgen, we wondered whether AR could bind to the DNA region of the miR-133b locus. We used Genomatix database (36) to predict AREs within the upstream and downstream 10 kb from pre-miR-133b’s transcription start site. Seven AREs, formed 5 clusters (named A–E), were detected in the proximal DNA regions of miR-133b (Fig. 1D). By ChIP-qPCR, we found that AR recruitment onto six out of seven AREs was induced significantly after 100 nM DHT stimulation.
for 4 h compared with the vehicle (Fig. 1E). Treatment with solo-CDX or a combination of DHT and CDX significantly reduced AR recruitment to levels comparable to vehicle. The PSA enhancer and XBP-1 promoter served as positive control and negative control for AR-binding, respectively (Fig. 1F). Taken together, our results suggested that androgen-AR signaling mediated the regulation of miR-133b in LNCaP cells.

**MiR-133b influenced cell proliferation and cell cycle status of prostate cancer.** Since miR-133b was directly regulated by AR, we analyzed the role of miR-133b on various cellular processes of prostate cancer. Firstly, we evaluated the effect of miR-133b on cell proliferation of LNCaP, 22Rv1 and PC-3 cells. These cells were transfected with miR-133b mimics (miR-133bm), or anti-miR-133b (si133b). We observed that when cells were treated with miR-133bm, cell proliferation was significantly increased, compared with miR-NC in LNCaP and 22Rv1 cells (Fig. 2A and 2C); whereas anti-miR-133b partially decreased cell proliferation, compared with anti-miR-NC in these two cell lines (Fig. 2B and 2D). In contrast, in PC-3 cells, miR-133bm inhibited cell proliferation; whereas si133b boosted cell proliferation (Fig. 2E-F). The transfection efficiency of these assays was analyzed by qRT-PCR (Supplementary Fig. S1C-E).

As cell cycle distribution is a parameter reflecting the growth of cells, we assessed the function of miR-133b on cell cycle profile of LNCaP and PC-3 cells. Overexpression of miR-133b in LNCaP cells induced an increase in S phase, and a
decrease in G1 phase, compared with miR-NC; whereas knockdown of miR-133b resulted in a reduction in S phase, and an increase in G1 phase (Fig. 2G and Supplementary Fig. S2A). However, in PC-3 cells, miR-133bm caused an increase in G1 phase and a decrease in S phase; si133b decreased the proportion of cells in G1 phase and increased the proportion in S phase (Fig. 2H and Supplementary Fig. S2C). The transfection efficiency of these assays was shown in Supplementary Fig. S2B and S2D. Collectively, miR-133b boosted cell proliferation and growth of ADPC cells LNCaP by promoting cell cycle progression, but suppressed cell proliferation and growth of AIPC cells PC-3.

**MiR-133b affected cell apoptosis of prostate cancer.** We further explored the role of miR-133b on cell apoptosis of prostate cancer. LNCaP and PC-3 cells were treated with miR-133bm, si133b, or their negative control, respectively, and followed by flow cytometry analyses. In LNCaP cells, miR-133bm boosted the fraction of living cells, and decreased that of late apoptotic cells, compared with the miR-NC; whereas si133b increased both the fraction of early apoptotic and late apoptotic cells (Fig. 3A and Supplementary Fig. S3A). On the other hand, in PC-3 cells, miR-133bm reduced the fraction of living cells, and boosted that of late apoptotic cells; but si133b increased the proportion of living cells, and decreased that of late apoptotic cells (Fig. 3B and Supplementary Fig. S3C). The transfection efficiency of these assays was shown in Supplementary Fig. S3B and S3D.

In order to clarify the mechanism by which miR-133b affected cell apoptosis of
prostate cancer, we detected the activity of total caspase and caspase-3 in LNCaP and PC-3 cells. In LNCaP cells, miR-133bm significantly reduced the fractions of activated caspase (Fig. 3C and Supplementary Fig. S4A) and caspase-3 (Fig. 3E and Supplementary Fig. S4E), as compared with miR-NC; however, si133b boosted the proportion of activated caspase. In PC-3 cells, miR-133bm significantly increased the fractions of activated caspase (Fig. 3D and Supplementary Fig. S4C) and caspase-3 (Fig. 3F and Supplementary Fig. S4G); whereas, si133b decreased the proportion of activated caspase. The transfection efficiency was shown in Supplementary Fig. S4B, S4D, S4F, and S4H, respectively. Taken together, we found that miR-133b functioned as an oncomiR, which promoted cell proliferation and suppressed apoptosis of ADPC cells LNCaP; however, this miR acted as a tumor suppressor by inhibiting cell proliferation and promoting apoptosis of AIPC cells PC-3.

**RB1CC1 is a target of miR-133b.** To identify potential target of miR-133b, we conducted a computational screen for genes with complementary sites of miR-133b in their 3’UTR using open-access software (TargetScan (www.targetscan.org), PicTar (http://pictar.bio.nyu.edu), and miRDB (www.mirdb.org)). We have performed expression profile in LNCaP cells and found several androgen-responsive miRNAs and mRNAs. To precisely evaluate the actual regulation of each predicted miRNA-mRNA pair, the Modulation Score (MS) was proposed (23). For each predicted miRNA-mRNA pair whose members were both androgen-responsive, MS was calculated and the p value for each MS was evaluated. Subsequently, four
mRNAs were identified as candidate targets of miR-133b: \textit{RB1CC1, PTPRK, SESN1, and CPNE3}. qRT-PCR analysis in LNCaP cells indicated that the expression of \textit{RB1CC1} was the most significantly affected by overexpression or knockdown of miR-133b among these putative targets (Supplementary Fig. S5A). Further, we focused on \textit{RB1CC1}, which was reported to be a tumor suppressor and activate the expression of \textit{RB1} in breast cancer (38), but its role in prostate cancer is unclear.

In order to confirm \textit{RB1CC1} as the direct target of miR-133b, we performed luciferase assays in LNCaP cells. There is a miR-133b eight-nucleotide seed match region at position 252-259 of the \textit{RB1CC1} 3'-UTR (Fig. 4A). Co-transfection of LNCaP cells with the wild-type 3'-UTR-reporter and miR-133bm showed significantly decreased luciferase activity as compared with miR-NC (Fig. 4B). Interaction between the binding-site in \textit{RB1CC1} 3'-UTR and miR-133b was sequence-specific, since mutation of the seed sequence restored luciferase activity to values comparable to the empty vector (Fig. 4B). These results denoted that the 3' UTR of \textit{RB1CC1} transcript was a target of miR-133b.

\textit{Ectopic expression of miR-133b affected endogenous RB1CC1.} After we transiently transfected LNCaP, 22Rv1 and PC-3 cells with miR-133m, si133b, or their negative controls, respectively, miR-133bm was shown to decrease the mRNA and protein level of \textit{RB1CC1}, relative to miR-NC; whereas si133b increased the mRNA and protein level of \textit{RB1CC1}, compared with siNC (Fig. 4C-D and Supplementary Fig. S5C).
To further address the physiological relationship between endogenous miR-133b and RB1CC1 in prostate cancer cells, RB1CC1 protein level and miR-133b expression were evaluated by western blot (Fig. 4E) and qRT-PCR (Fig. 4F) in four prostatic cell lines, respectively. A significantly inverse correlation between RB1CC1 protein and miR-133b expression was found, with a Pearson correlation coefficient of -0.831 ($p<0.05$, Fig. 4G).

Since androgen induced expression of miR-133b, and miR-133b downregulated RB1CC1, we went further to evaluate changes of RB1CC1 mRNA and protein levels after 10 nM DHT stimulation for 24 h in LNCaP cells. As a result, androgen significantly decreased mRNA and protein level of RB1CC1, relative to vehicle control (Supplementary Fig. S5B). Collectively, these results suggested that expression of RB1CC1 and miR-133b were closely linked in prostate cancer cells, supporting RB1CC1 as an endogenous target of miR-133b.

**Knockdown of RB1CC1 expression boosted cell cycle progression and inhibited cell apoptosis.** To evaluate how RB1CC1 functioned in cell cycle and cell apoptosis processes of prostate cancer, we used RB1CC1 siRNA to knock down the expression of RB1CC1. As shown in LNCaP cells, knockdown of RB1CC1 significantly decreased the proportion of cells in G1 phase and increased that of cells in S phase (Supplementary Fig. S6A-B), similar to that observed in the overexpression of miR-133b (Fig. 2G).

Cell apoptosis analysis revealed that knockdown of RB1CC1 boosted the fraction of
living cells, and reduced that of late apoptotic cells, compared with negative control, similar to that observed in the overexpression of miR-133b (Supplementary Fig. S6C-D). The transfection efficiency of RB1CC1 siRNA was shown in Supplementary Fig. S6E.

Taken together, we found that RB1CC1, a target of miR-133b, functioned as a tumor suppressor by inducing G1-S phase arrest and promoting cell apoptosis of LNCaP cells.

**Expression of miR-133b and RB1CC1 in prostate tissue samples.** To assess the clinical relevance of these observations in cell lines, we evaluated the expression of miR-133b and RB1CC1 on prostate cancer tissue microarray. LNA-ISH was performed to detect miR-133b expression for 178 prostate tissue specimens mounted on a set of prostate cancer TMA slides. ISH score of miR-133b was estimated by scanning each TMA spot. Fig. 5A-B showed representative miR-133b staining in TMAs. Among the 135 prostate cancer samples, 92 cases (68.1%) showed positive expression of miR-133b, compared with 14 out of 18 PIN (77.8%) samples and 7 out of 25 normal prostate tissues (28.0%), respectively, which was statistically significant ($\chi^2 = 16.432, p < 0.0001$).

Immunohistochemistry assay was conducted to analyze RB1CC1 protein level in 178 prostate tissue specimens. Fig. 5C-D showed the representative staining of RB1CC1 protein in TMAs. Among the 135 prostate cancer samples, 54 cases (40.0%) cases showed positive expression of RB1CC1 protein, compared with 6 (33.3%) out
of 18 PIN samples and 20 (80.0%) out of 25 normal prostate tissues, respectively, which was statistically significant ($\chi^2=14.730; p<0.001$).

**Correlation between expression of miR-133b and RB1CC1 protein in prostate cancer samples and their relationship with clinical variables.** In order to explore the correlation between miR-133b and RB1CC1 expression, we performed both the Pearson's and Spearman's rank correlation coefficient analyses. A significantly inverse correlation between RB1CC1 protein and miR-133b expression was found, with a correlation coefficient of -0.180 ($p=0.036$, Supplementary Table S8 and S9). We also examined the relationship between the expression of miR-133b, RB1CC1 and various clinicopathologic features in prostate cancer patients after RP. As shown in Supplementary Table S1, positive miR-133b expression was significantly associated with pathological stage ($p=0.012$), and BCR ($p=0.038$); whereas positive RB1CC1 protein expression was significantly associated with pre-operation PSA ($p=0.021$), Gleason Score ($p=0.012$), and BCR ($p=0.024$).

**MiR-133b and RB1CC1 were prognostic factors for BCR after RP.** To further examine whether there was significantly different outcome between patients with negative and positive miR-133b expression, the risk of BCR was evaluated using the Kaplan-Meier method, and a statistically significant separation of survival curves was observed (Fig. 5E). Furthermore, the 5-year BCR-free survival probability rate and mean survival time were estimated (Supplementary Table S2). We also estimated the
probability of survival in patients with negative and positive RB1CC1 expression, and the median BCR-free time and 5-year BCR free survival rates. A statistically significant separation of survival curves was observed (Fig. 5F).

Meanwhile, the Cox proportional hazards model was used to assess independent predictors of BCR-free survival, the factors including pre-operation PSA, Gleason score, surgical margin, lymph node metastasis, capsular invasion, pathological stage, and the expression of miR-133b and RB1CC1 protein. As a result, miR-133b was identified as an independent prognostic factor of outcome for prostate cancer patients who underwent RP (HR (95% CI): 1.775 (1.013 – 3.108), p=0.045), as well RB1CC1 (HR (95% CI): 0.534 (0.316 – 0.902), p=0.019), pre-operation PSA (HR (95% CI): 1.009 (1.002 – 1.016), p=0.012), Tumor Margins (HR (95% CI): 0.470 (0.224 – 0.984), p=0.045) and Gleason score (HR (95% CI): 2.242 (1.215-4.137), p=0.010) (Table 1). Thus, our findings suggested that miR-133b expression and RB1CC1 protein level could independently predict the risk of BCR for prostate cancer patients after RP.
Discussion

The miRNA expression profiles of many solid malignancies have been reported (33). Compared to traditional mRNA and protein markers, miRNA expression patterns are more reliable and sensitive to changes in cell biology. mRNA and its translated protein level are not often proportional, an important reason for which is the regulatory influences of epigenetic mechanisms, including those mediated by miRNAs (39).

Specifically, miRNAs can act as oncomiRs or tumor suppressors in a multitude of cancers (40). As referred to miR-133b, in cervical carcinoma cells, it enhanced cell proliferation and colony formation, suggesting that it could act as an oncomiR (16). On the other side, miR-133b was reported to be a tumor suppressor in tongue squamous cell carcinoma (SCC) (13), esophageal SCC (14), and colorectal cancer cells (15).

Our previous study had shown miR-133b significantly boosted AR-induced cell viability of LNCaP cells, and miR-133b was required for mediating AR signaling to prostate cancer cell viability and survival (23). In this study, we found that miR-133b stimulated proliferation of LNCaP and 22Rv1 cells, whereas inhibited apoptosis of LNCaP cells. Therefore, miR-133b played an oncogenic role in ADPC cells LNCaP and 22Rv1. On the other hand, two previous articles demonstrated tumor suppressive functions of miR-133b in AIPC cells PC-3 and DU145 (41, 42). Both of these studies showed miR-133b contributed greatly to the suppression of proliferation in PC-3 cells. Consistent with their reports, we observed that miR-133b inhibited cell proliferation
and cell cycle progression, whereas promoted apoptosis of PC-3 cells. It has been shown that the same miRNA could have opposing roles in different cellular settings (43). Collectively, these data indicated miR-133b might display tumor-suppressing properties in AIPC cells PC-3, but act as an oncomiR in ADPC cells LNCaP and 22Rv1.

At present, we asked which factor affects or regulates the cellular abundance of miR-133b in prostate cancer cells. We have found that androgen stimulation induced an enhanced abundance of miR-133b in LNCaP cells. Furthermore, we observed that the AR is recruited to the ARE region of miR-133b in the presence of DHT. These findings suggest that androgen-AR signaling may mediate the regulation of miR-133b in AR-positive prostate cancer cells. AR may associate with additional regions or may require other factor(s) to associate efficiently with the miR promoter. Further characterization of androgen-dependent and androgen-independent regulations of miRNA expression is currently in progress in our group.

A previous study demonstrated that EGFR was a target of miR-133b in prostate cancer (42). In line with their results, we found miR-133b downregulated the expression of EGFR in LNCaP and PC-3 cells (Fig.S5D-E).

In 2012, Patron et al. performed whole genome microarray expression analysis after transfection of miR-133bm in HeLa cells, and matched the list of downregulated genes with miRecords (41). However, RB1CC1 was not on the list. Since HeLa cells are cervical cancer cells, and our data were obtained in prostate cancer cells. As we know, the same miRNA can target different mRNAs in different cellular context (43).
Further experiments will be necessary to clarify whether RB1CC1 is the target of miR-133b in HeLa cells.

Notably, recent studies suggested that RB1CC1 could represent a potential diagnosis and drug target for a broad range of cancers. Nuclear expression of RB1CC1 was shown to predict a better clinical outcome in salivary gland cancers (44). In breast cancer, RB1CC1 was reported to predict long-term survival together with RB1 and p53 (45). Based on our results, we speculated that RB1CC1 might function as a tumor suppressor, which was opposite to the oncomiR role of miR-133b in ADPC cells LNCaP, yet further study is needed to fully illustrate the function of RB1CC1 in AIPC cells.

To our knowledge, this is the first study showing the significance of miR-133b and RB1CC1 in relation to clinical data on patients with prostate cancer. We found that the expression of miR-133b and RB1CC1 protein were inversely correlated. Because none of the patients involved in our study received any pre-operation treatment, the clinical samples can be regarded as androgen dependent. Hence, our findings in patients’ specimens confirmed the results we obtained in ADPC cells LNCaP, which indicated an oncomiR role of miR-133b and a tumor suppressor function of RB1CC1. Finally, multivariate Cox hazard regression analysis revealed that miR-133b and RB1CC1 were identified as independent prognostic factors of outcome for prostate cancer patients after RP.

Despite the combination of increasingly refined surgical techniques and reduced incidence of surgical complications, the variable disease course leads to eventual
recurrence of about one third of patients following RP (46). Pound et al. reported that distant or local recurrence of prostate cancer has not occurred in males without BCR (47). Therefore, in order to achieve long-term disease-free survival for prostate cancer patients, BCR risk should be assessed. Recent investigations have tried to apply the improved understanding of tumor cell biology to determine additional tumor characteristics as potential prognostic factors. Such prognostically important factors might help determine the optimal treatment strategy based on the biological potential of individual tumor (48). On the basis of this study, prostate cancer patients with low expression of miR-133b or high protein level of RB1CC1 should undergo regular monitoring of serum PSA and clinical symptoms. In contrast, patients with high expression of miR-133b or low protein level of RB1CC1 could benefit from obtaining additional clinical data including ultrasound-guided biopsy, computed tomography, magnetic resonance imaging, and bone scans.

In conclusion, our results ascertained miR-133b as a direct target of AR in LNCaP cells. MiR-133b acted as a potent anti-apoptotic and pro-growth molecule with oncomiRs properties in ADPC cells LNCaP, but functioned as a potential tumor suppressor in AIPC cells PC-3. MiR-133b targeted and downregulated RB1CC1 in prostate cancer cells. The expression of miR-133b and RB1CC1 protein are inversely correlated, and they can be used to predict the risk of BCR in patients with prostate cancer after RP. Although evaluations of miR-133b expression and RB1CC1 protein in larger populations are still needed, our results indicated that miR-133b and RB1CC1 might be promising candidates as molecular prognostic biomarkers and
therapeutic targets for prostate cancer.

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Reference

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Table 1. Multivariate Cox regression analysis of biochemical recurrence (BCR)-free survival for all prostate cancer patients after radical prostatectomy (RP).

<table>
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<th>Variables</th>
<th>p-Value</th>
<th>Hazard ratio for recurrence</th>
<th>95% CI</th>
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<tr>
<td>miR-133b expression</td>
<td>0.045</td>
<td>1.775</td>
<td>1.013, 3.108</td>
</tr>
<tr>
<td>RB1CC1 expression</td>
<td>0.019</td>
<td>0.534</td>
<td>0.316, 0.902</td>
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<td>Pre-operation PSA</td>
<td>0.012</td>
<td>1.009</td>
<td>1.002, 1.016</td>
</tr>
<tr>
<td>Tumor Margins</td>
<td>0.045</td>
<td>0.470</td>
<td>0.224, 0.984</td>
</tr>
<tr>
<td>Gleason score</td>
<td>0.010</td>
<td>2.242</td>
<td>1.215, 4.137</td>
</tr>
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</table>

Note: Significant contributing factors in multivariate Cox regression analysis were: miR-133b expression, RB1CC1 expression, Tumor Margins, Pre-operation PSA and Gleason score.
Figure Legend

Fig. 1. MiR-133b expression to response androgen and the basal expression in different prostatic cell lines. (A) Time course profile of miR-133b expression in response to 10 nM DHT stimulation in LNCaP cells. The y-axis referred to normalized signal intensity of the microRNA microarray. (B) LNCaP cells were treated with 10 nM DHT or ethanol as vehicle at different time points as indicated. Total RNA was prepared as described in Materials and Methods section and analyzed for miR-133b expression by quantitative RT-PCR (qRT-PCR). All expression levels were normalized to vehicle control at each time point, each experiment was performed in triplicate, and values are mean ± SD. (C) qRT-PCR analysis of miR-133b expression level in different prostatic cell lines as indicated. The data were expressed as fold change of miR-133b expression in each cell line versus LNCaP cells. (D) Schematic diagram of miR-133b’s ARE sites at the 5’ and 3’ regions. ARE site was indicated by arrows. (E-F) Occupancy of AR on candidate AREs of miR-133b, PSA enhancer and XBP-1 promoter with anti-androgen treatment. LNCaP cells were treated with vehicle, 10 μM anti-androgen CDX, 100 nM DHT, or a combination of 100 nM DHT and 10 μM CDX (indicated as DHT+CDX) for 4 h before harvesting for ChIP assays. Chromatin samples were immunoprecipitated with anti-AR antibody, and bound DNA was quantified as described in Materials and Methods. (E) AR occupancy on the candidate AREs of miR-133b. A DNA region adjacent to miR-133b gene (9982~ 9895bp upstream from pre-miR-133b transcription start site) that does not contain a putative ARE served as negative control (indicated as NC). (F) AR
occupancy on the PSA enhancer (positive control) and XBP-1 promoter (negative control). Values, expressed as percentages of input DNA, are the mean ± SD of at least three independent experiments. Significance was defined as $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

**Fig. 2.** MiR-133b played critical roles in cell proliferation and cell cycle in prostate cancer cells. (A-F) Cell proliferation analysis. Cells were seeded into a 96-well plate at 5000 cells/well and examined at 0, 24, 48 and 72 h after transfection. Each experiment was performed in sextuplicate ($n = 6$). MiR-133b overexpression boosted cell proliferation in LNCaP (A), 22Rv1 (C), and inhibited proliferation in PC-3 cells (E); Knockdown of miR-133b suppressed cell proliferation in LNCaP (B), 22Rv1 (D), and promoted proliferation in PC-3 cells (F). (G-H) Cell cycle analysis. Cells were transfected with miR-133bm, miR-NC, si133b, or siNC for 48 h, and stained with Propidium Iodide (PI), and then subjected to cell cycle analysis. (G) MiR-133b overexpression caused a decrease in G1 phase and an increase in S phase in LNCaP cells; knockdown of miR-133b caused an increase in G1 phase and a decrease in S phase. (H) MiR-133b overexpression caused an increase in G1 phase and a decrease in S phase in PC-3 cells; anti-miR-133b caused a decrease in G1 phase and an increase in S phase.

**Fig. 3.** MiR-133b involved in prostate cancer cell apoptosis. Cells were transfected with miR-133bm or miR-NC; si133b or siNC for 48 h, and then subjected to cell
apoptosis (stained with PI and FITC-Annexin V), caspase (stained with FITC-VAD-FMK), and caspase-3 (stained with FITC-DEVD-FMK) analyses. (A) In LNCaP cells, miR-133b overexpression caused a decrease in late apoptotic cells and an increase in living cells; knockdown of miR-133b caused an increase in early apoptotic cells, and a decrease in late apoptotic cells. (B) In PC-3 cells, miR-133b overexpression caused a decrease in living cells and an increase in late apoptotic cells; anti-miR-133b caused an increase in living cells, and a decrease in late apoptotic cells. (C) In LNCaP cells, miR-133b overexpression suppressed the activities of intracellular caspase; knockdown of miR-133b promoted the activity of intracellular caspase. (D) In PC-3 cells, miR-133b overexpression promoted the activities of intracellular caspase; anti-miR-133b suppressed the activity of intracellular caspase. (E) MiR-133b overexpression suppressed the activities of caspase-3 in LNCaP cells. (F) MiR-133b overexpression boosted the activities of caspase-3 in PC-3 cells. Each experiment was performed in triplicate.

**Fig. 4.** MiR-133b directly targeted *RB1CC1*. (A) The binding site of miR-133b to the 3’-UTR of *RB1CC1*. Vertical arrows represented the mutated bases in the *RB1CC1*-3’UTR mutant reporter constructs. (B) LNCaP cells were seeded into a 24-well plate, *RB1CC1* reporter construct (wild-type or mutant) or the empty reporter vector was co-transfected with miR-133bm and pRL-TK, or co-transfected with miR-NC and pRL-TK. miR-133b overexpression decreased the luciferase activity of *RB1CC1*-3’UTR wild-type (*RB1CC1*), but not that of *RB1CC1*-3’UTR-mutant (MUT).
or the empty reporter vector (empty). (C-D) qRT-PCR and western blotting showed the endogenous expression of \textit{RB1CC1} in LNCaP cells (C) and PC-3 cells (D) followed by transfected with miR-133bm, miR-NC, si133b or siNC. The results revealed that overexpression of miR-133b repressed the endogenous expression of \textit{RB1CC1} compared with that of miR-NC control, and anti-miR-133b could partially restore the expression of \textit{RB1CC1}. (E) Western blot analysis of RB1CC1 protein levels in different prostatic cell lines as indicated. Image quantification was performed using the Quantity One software. Data were normalized using \(\beta\)-actin protein level. Fold change was presented as relative RB1CC1 protein level of each cell line versus LNCaP cells. (F) qRT-PCR analysis of miR-133b basal expression in different prostatic cell lines as indicated. Data were normalized using \(\text{RNU6B}\) expression. Fold change was presented as relative miR-133b expression (log10) of each cell line versus LNCaP cells. (G) Correlation between RB1CC1 protein levels and miR-133b expression showed a high degree of reverse correlation \((R=-0.831, p=0.023)\). Each experiment was performed in triplicate. Values are mean \(\pm\) SD.

**Fig. 5.** MiR-133b and \textit{RB1CC1} as potential prognostic markers for prostate cancer. (A-D) MiR-133b and RB1CC1 expression in prostate cancer samples. (A) The representative positive expression of miR-133b was mainly detected in prostatic epithelial cells. (B) The representative negative staining of miR-133b in prostatic epithelial cells. (C) The representative positive staining of RB1CC1 on tissue arrays. (D) RB1CC1 staining was negative in prostate cancer samples. The magnification is
10 X. (E-F) Kaplan-Meier analysis showed biochemical recurrence (BCR)-free survival of prostate cancer patients. (E) miR-133b. (F) RB1CC1.
Figure 1
Figure 2
Site (TargetScan and miRDB database)

hsa-miR-133b/RB1CC1 Alignment

3’ AUGCACCAACUCCCUUUGGUUU 5’ hsa-miR-133b
5’ CAACCUUGAAUUGGCGAATTTA 3’ Position 252-259 of RB1CC1 3’ UTR
5’ CAACCUUGAAUUGGCGAATTTA 3’ RB1CC1 3’ UTR Mutant

B

Relative luciferase expression over negative control

RB1CC1 MUT empty

p=0.017

C

Fold Change Over Negative Control

qRT-PCR in LNCaP Cells

p=0.043

p=0.021

miR-133b miR-NC si133b siNC

D

Fold Change Over Negative Control

qRT-PCR in PC-3 Cells

p=0.004

p=0.021

miR-133b miR-NC si133b siNC

E

Western Blotting

LNCaP PC-3 WPMY-1 DU145

β-actin

RB1CC1

F

Relative miR-133b level (log10)

qRT-PCR

LNCaP PC-3 WPMY-1 DU145

G

Relative RB1CC1 Protein Level

Relative miR-133b level (log10)

LNCaP DU145 WPMY-1 PC-3

R=-0.831, p=0.023
Figure 5

A. miR-133b positive

B. miR-133b negative

C. RB1CC1 positive

D. RB1CC1 negative

E. Survival Functions

F. Survival Functions

Biochemical recurrence-free probability

Time after radical prostatectomy (months)

Log Rank p=0.032

Log Rank p=0.011
# Clinical Cancer Research

## Identification of miR-133b and RB1CC1 as independent predictors for biochemical recurrence and potential therapeutic targets for prostate cancer

Xia Li, Xue Chao Wan, Hong Bing Chen, et al.

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