Identification of miR-133b and RB1CC1 as Independent Predictors for Biochemical Recurrence and Potential Therapeutic Targets for Prostate Cancer

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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 evaluated 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 used to look at cell proliferation, flow cytometric analysis was carried out to study cell cycle, and apoptosis was determined by caspase-3 activity. miR-133b expression was assessed by real-time reverse transcription 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% to 50% post-RP patients on long-term follow-up have biochemical recurrence (BCR; ref. 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 patients with prostate cancer.

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 (miRNA) 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 biologic processes (12). MicroRNA-133b (miR-133b), located at chromosome...
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 tissue specimens, 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.

**Materials and Methods**

**Patients**

A total of 135 specimens of patients with prostate cancer, 18 patients with prostatic intraepithelial neoplasia (PIN), and 25 normal prostate tissue samples were included in this study. The normal prostate tissue samples were derived from patients with bladder cancer after total cystectomy; PIN and prostate cancer samples were derived from patients with prostate cancer after RP and regional lymph node dissection in Tongji Hospital, subsidiary of Tongji University (Shanghai, China), 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 postoperative serum PSA concentration ≥ 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 Dulbecco’s Modified Eagle’s medium. The androgen treatment assay was performed as described previously (23). According to previous reports (24–26), 10 nmol/L dihydrotestosterone (DHT) was found to be the optimal concentration to stimulate LNCaP cell proliferation.

**miRNA mimics and inhibitor molecule 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 (an siRNA lacking homology to the genome) were from GenePharma (Shanghai, China), and used at 50 nmol/L concentration. Anti-miR-133b (si133b; catalog number IH-300709-06-0005) and its scrambled control, control anti-miRNA (siNC; catalog number IN-001005-01-05) were from Thermo Fisher Scientific, and at 100 nmol/L 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 quantitative reverse transcription PCR**

Real-time quantitative reverse transcription PCR (qRT-PCR) for miRNAs was performed as described in (23). For miRNA quantification, cDNA was synthesized from total RNA with the miScript Reverse Transcription Kit (Qiagen). Specific primers for mature miR-133b were from Qiagen (catalog number M500031430). The primers used for qPCR were listed in Supplementary Table S4.
**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChiP) was performed as described in (23). With minor modification, LNCaP cells were treated with vehicle, DHT (100 nmol/L), anti-androgen Casodex (10 μmol/L), or a combination of DHT and antiandrogen for 4 hours before harvesting. The primers of androgen response element (ARE) for qPCR were listed in Supplementary Tables 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 (untranslated region) 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). The primers are listed in Supplementary Table S7.

**Dual luciferase assay**

LNCaP cells were cotransfected 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). Forty eight hours 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 (1:1,000; Sigma-Aldrich), β-actin (1:4,000; Sigma-Aldrich), and EGFR (1:1,000; Proteintech).

**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, 24, 48, and 72 hours. At each time point, CCK-8 reagent was added into cells and incubated for 2 hours. The absorbance was measured at 450 nm using a multimode microplate reader (BioTek).

**Cell-cycle assay**

Cell cycle was performed as described in (31). Briefly, cells were incubated with propidium iodide (10 μg/mL) for 15 minutes 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).

**Apoptosis assays**

miR-133bm, miR-NC, anti–miR-133b, or anti–miR-NC were transfected as described above. Forty eight hours after transfection, cells were collected and assayed with the 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 the CaspGLOW Fluorescein Caspase Staining Kit and CaspGLOW Fluorescein Caspase-3 Staining kit (BioVision), respectively, and detected by FACScalibur flow cytometer.

**Tissue microarray construction**

Tissue microarray (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.), as described by Kononen (34).

**Locked nucleic acid in situ hybridization 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; sequence: /5DigN/TAGCTGGTTGAAGGGGAAC- CAAA/3Dig_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) 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 until November 2012. The differential expressions of RB1CC1 between groups were analyzed using the Wilcoxon rank-sum test. 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, χ2 analysis, Mann–Whitney U test, and Fisher exact test were used 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.).

For experiments in cell lines, mean and SDs of individual groups (n ≥ 3) were calculated. P values were assessed by performing the two-tailed Student t test.
Results

Androgen induces the expression of miR-133b

We have previously analyzed miRNA and mRNA expression in LNCaP cells treated with 10 nmol/L 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 miRNA) or MicroRNA Expression Profiling Panels (for mRNA). The raw data have been uploaded to the Gene Expression Omnibus public repository (http://www.ncbi.nlm.nih.gov/geo/; Gene Expression Omnibus series number GSE21245). Data preprocessing, 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 minutes, 8 hours), 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 upregulated before 4 hours, with a peak at 1 hour 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 with the vehicle control (Fig. 1B). The expressions of two androgen-responsive genes PSA and KLK2 were used as positive controls (Supplementary Fig. S1A and S1B).

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 of the binding of AR to miR-133b

As we have confirmed the expression of miR-133b is upregulated 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 of seven AREs was induced significantly after 100 nmol/L DHT stimulation for 4 hours compared with the vehicle (Fig. 1E). Treatment with solo-Casodex or a combination of DHT and Casodex significantly reduced AR recruitment to levels comparable with 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

Because miR-133b was directly regulated by AR, we analyzed the role of miR-133b on various cellular processes of prostate cancer. First, we evaluated the effect of miR-133b on cell proliferation of LNCaP, 22Rv1, and PC-3 cells. These cells were transfected with 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 C), whereas anti–miR-133b partially decreased cell proliferation compared with anti–miR-NC in these two cell lines (Fig. 2B and D). In contrast, in PC-3 cells, miR-133bm inhibited cell proliferation, whereas si133b boosted cell proliferation (Fig. 2E and F). The transfection efficiency of these assays was analyzed by qRT-PCR (Supplementary Fig. S1C–S1E).

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, whereas 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 is 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

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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 is shown in Supplementary Fig. S3B and S3D.

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

Figure 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 nmol/L DHT stimulation in LNCaP cells. The Y-axis referred to normalized signal intensity of the miRNA microarray. B, LNCaP cells were treated with 10 nmol/L DHT or ethanol as vehicle at different time points as indicated. Total RNA was prepared as described in Materials and Methods and analyzed for miR-133b expression by 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 is indicated by arrows. E and F, occupancy of AR on candidate AREs of miR-133b, PSA enhancer, and XBP-1 promoter with antiandrogen treatment. LNCaP cells were treated with vehicle, 10 μmol/L antiandrogen Casodex (CDX), 100 nmol/L DHT, or a combination of 100 nmol/L DHT and 10 μmol/L Casodex (indicated as DHT+Casodex) for 4 hours 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 (9982–9895-bp 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 is defined as P < 0.05 (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
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 is shown in Supplementary Fig. S4B, S4D, S4F, and S4H, respectively. Taken together, we found that
Figure 3. miR-133b involved in prostate cancer cell apoptosis. Cells were transfected with miR-133bm or miR-NC, si133b or siNC for 48 hours, 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 increased the proportions of both early and 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. E, miR-133b 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.
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.mdc-berlin.de), 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 both members were 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: RB1CC1, PTBPK, SESN1, and CPNE3. qRT-PCR analysis in LNCaP cells indicated that the expression of RB1CC1 was the most significantly affected by overexpression or knockdown of miR-133b among these putative targets (Supplementary Fig. S5A). Further, we focused on RB1CC1, which was reported to be a tumor suppressor and activate the expression of RB1 in breast cancer (38), but its role in prostate cancer is unclear.

To confirm RB1CC1 as the direct target of miR-133b, we performed luciferase assays in LNCaP cells. There is an miR-133b eight-nucleotide seed match region at position 252–259 of the RB1CC1 3′-UTR (Fig. 4A). Cotransfection 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 RB1CC1 3′-UTR and miR-133b was sequence-specific, because mutation of the seed sequence restored luciferase activity to values comparable with the empty vector (Fig. 4B). These results denoted that the 3′ UTR of RB1CC1 transcript was a target of miR-133b.

**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 RB1CC1 relative to miR-NC, whereas si133b increased the mRNA and protein level of RB1CC1 compared with siNC (Fig. 4C and D and Supplementary Fig. S5C).

To further address the physiologic 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).

Because 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 nmol/L DHT stimulation for 24 hours 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 was 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 and S6B), 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 and S6D). The transfection efficiency of RB1CC1 siRNA is 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 TMA. 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. Figure 5A and B showed representative miR-133b staining in TMA slides. Among the 135 prostate cancer samples, 92 cases (68.1%) showed positive expression of miR-133b, compared with 14 of 18 PIN (77.8%) and 7 of 25 normal prostate tissues (28.0%), respectively, which was statistically significant (χ² = 16.432; P < 0.0001).

Immunohistochemistry assay was conducted to analyze RB1CC1 protein level in 178 prostate tissue specimens. Figure 5C and D showed the representative staining of RB1CC1 protein in TMA slides. Among the 135 prostate cancer samples, 54 cases (40.0%) showed positive expression of RB1CC1, compared with 6 (33.3%) of 18 PIN samples and 20 (80.0%) of 25 normal prostate tissues, respectively, which was statistically significant (χ² = 14.730; P < 0.001).
Figure 4. miR-133b directly targeted RB1CC1. A, the binding site of miR-133b to the 3'-UTR of RB1CC1. Vertical arrows represent 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 cotransfected with miR-133b miRNA mimics and pRL-TK, or cotransfected 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). (Continued on the following page.)
Correlation between expression of miR-133b and RB1CC1 protein in prostate cancer samples and their relationship to clinical variables

To explore the correlation between miR-133b and RB1CC1 expression, we performed both the Pearson and Spearman rank correlation coefficient analyses. A significantly inverse correlation between RB1CC1 protein and miR-133b expression was found, with a correlation coefficient of $R = -0.180$ ($P = 0.036$; Supplementary Tables S8 and S9). We also examined the relationship between the expression of miR-133b, RB1CC1, and various clinicopathologic features in patients with prostate cancer after RP. As shown in Supplementary Table S1, positive miR-133b expression was significantly associated with pathologic stage ($P = 0.012$) and BCR ($P = 0.038$), whereas positive RB1CC1 protein expression was significantly associated with preoperation 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 mean 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 preoperation PSA, Gleason score, surgical margin, lymph node metastasis, capsular invasion, pathologic 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 patients with prostate cancer 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], preoperation 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 and RB1CC1 protein level could independently predict the risk of BCR for patients with prostate cancer after RP.

Discussion

The miRNA expression profiles of many solid malignancies have been reported (33). Compared with traditional mRNA and protein markers, miRNA expression patterns are more reliable and sensitive to changes in cell biology. miRNA 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; ref. 13), esophageal SCC (14), and colorectal cancer cells (15).

Our previous study had shown that 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 that 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 that miR-133b might display tumor-suppressing properties in ADPC 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 that miR-133b downregulated the expression of

Table 1. Multivariate Cox regression analysis of BCR–free survival for all patients with prostate cancer after RP

<table>
<thead>
<tr>
<th>Variables</th>
<th>P</th>
<th>HR for recurrence</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>Preoperation 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>
</tbody>
</table>

NOTE: Significant contributing factors in multivariate Cox regression analysis were miR-133b expression, RB1CC1 expression, tumor margins, preoperation PSA, and Gleason score.
EGFR in LNCaP and PC-3 cells (Supplementary Fig.S5D and S5E).

In 2012, Patron and colleagues performed whole-genome microarray expression analysis after transfection of miR-133b in HeLa cells and matched the list of downregulated genes with miRecords (41). However, RB1CC1 was not on the list, because 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). On the basis of 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 was inversely correlated. Because none of the patients involved in our study received any preoperation 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 property 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 patients with prostate cancer 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 and colleagues reported that distant or local recurrence of about one third of patients following RP (46). Therefore, to achieve long-term disease-free survival for patients with prostate cancer, 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 biologic potential of individual tumor (48). On the basis of this study, patients with prostate cancer 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 antiapoptotic and pro-growth molecule with oncomiR 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 is 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: X. Li, X. Wan, Y. Li
Development of methodology: X. Li, H. Chen, H. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Li, X. Wan, H. Chen, Y. Liu, W. Du, H. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Li, X. Wan, S. Yang, Y. Liu, W. Mo, D. Meng, W. Du, J. Wang, T. Li
Writing, review, and/or revision of the manuscript: X. Li, Y. Huang, Y. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Li, X. Wan, W. Mo, W. Du, Y. Li
Study supervision: Y. Li

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