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Supplementary Materials and Methods

Cell culture and Androgen treatment. The prostate cancer cell lines LNCaP, 22Rv1, PC-3 and DU145 were maintained in RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% nonessential amino acids and 0.11 mg/ml sodium pyruvate, and cultured at 37°C in 5% CO_2. The immortalized noncancerous prostatic stromal cell line WPMY-1 was cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. All reagents used in cell culture were from Life Technologies (NY, USA).

Transient transfection. Transfection was carried out with Lipofectamine 2000 transfection reagent in accordance with the manufacturer's procedure (Invitrogen). The day before transfection, cells were seeded in six-well plates at a density of 4×10^5 cells/well. A 100 pmol miR-133bm or miR-NC in 250 μl Opti-MEM medium (Gibco, Grand Island, New York) was mixed with 5 μl Lipofectamine 2000 dissolved in 250 μl of the same medium and stand at room temperature for 20 min. The resulting 500 μl transfection solutions were then added to each well, which already contained 1.5 ml of RPMI 1640 without penicillin/streptomycin or FBS. Four hours later, the cultures were replaced with 2 ml fresh RPMI 1640 complete medium. The same procedure was performed for the transfection of anti-miR-133b or anti-miR-NC with double dose.

Dual luciferase assay. LNCaP cells (7.5×10^4 per well) were seeded into 24-well plates and cultured for 1 day. The cells were then cotransfected 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 by Lipofectamine 2000. Each sample was also cotransfected with 8 ng control vector pRL-TK (Promega, Madison, WI) containing Renilla luciferase as an internal control for transfection efficiency. Forty-eight hours post-transfection, cells were rinsed once with PBS and lysed with passive lysis buffer as indicated in Dual Luciferase Reporter Assay System (Promega). Luciferase activity was measured in a GloMax® 96 Microplate Luminometer (Promega). Data were shown as ratio of luc/Renilla to normalize for transfection efficiency. Each assay was replicated 3 times.

Apoptosis assay. The day before transfection, prostate cancer cells were seeded in six-well plates (3.5×10^5 cells/well for LNCaP; 3×10^5 cells/well for PC-3). miR-133bm or miR-NC or anti-miR-133b or anti-miR-NC was transfected as described above. After 48 h of transfection, cells were washed twice with cold PBS and then resuspend in 1× binding buffer at a concentration of 1×10^6 cells/ml. Aliquots of 100 μl from cell culture from each group were transferred to an Eppendorf tube, and added with 5 μl FITC Annexin V and 5 μl PI (FITC Annexin V Apoptosis Detection Kit; BD Biosciences). Cells were gently mixed and incubated for 15 min at room temperature in the dark. Finally, we added 400 μl of 1× binding buffer to each tube, and analyzed the results by FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA).

LNA-ISH and Immunohistochemistry of TMA. Locked nucleic acid in situ hybridization (LNA-ISH) was performed on a set of prostate cancer TMA slides using LNA™ probes for miR-133b (Exiqon, Vedbaek, Denmark) (sequence:

/5DigN/TAGCTGGTTGAAGGGGACCAAA/3Dig_N/). Specificity was verified using probe that was mismatched at three nucleotide positions (Exiqon, sequence: /5DigN/ TA**A**CTGGTTGA**C**GGGGA**G**CAAA/3Dig_N/). TMA slides were incubated at 37°C for 30 min, deparaffinized in xylene, and rehydrated with graded alcohol washes. Slides were then washed at 37°C in PBS 3 times for 5 min each wash, then incubated in proteinase K solution at 37°C for 15 min. Slides were prehybridized in hybridization buffer (without probe) at 37°C for 4 h, then subsequently incubated with miR-133b targeted Digoxigenin 3'and 5' double-labeled, mercury-locked nucleic acid probes (Exiqon, Vedbaek, Denmark) for 18-40 h at 37°C. A parallel set of prostate cancer TMAs were hybridized with a scrambled miRNA probe as a negative control. Following hybridization, slides were washed at 37°C with 2 × SSC (5 min, 3 times), 0.5 × SSC (5 min, 3 times), and 0.2 × SSC (5 min, 3 times). For visualization of bound probes, slides were blocked with sheep serum at 37°C for 30 min, and then were incubated with a mouse anti-digoxigenin antibody at 37°C for 60 min. After washing with PBS, and incubation in NBT/BCIP reagent (Zhong Shan Co., China) for 15-30 minutes, color development was observed and recorded.

Each slide was blinded scored by two independent pathologists. For detection of miR-133b each TMA spot was scanned and scored based on the intensity of hybridization [e.g., 0 (negative), 1 (weak), or 2 (strong)] and the percentage of positive epithelial cells detected [e.g., 0 (< 1%), 1 (focal, 1-50%) or 2 (diffuse, > 50%), respectively]. An 'ISH-score' for each lesion was then calculated by multiplying intensity by the area (1), and classified as 'negative' (score 0) versus

'positive' (score ≥ 1), thereby representing low versus high levels of miR-133b expression, respectively. This relatively simple, reproducible scoring method provided highly concordant results between independent evaluators.

Expression of RB1CC1 was detected in immunohistochemistry assays performed on a set of prostate cancer TMA slides. Polyclonal antibody for RB1CC1 (Proteintech, Chicago, USA) was used. Briefly, slides were initially deparaffinized in xylenes, then rehydrated in graded alcohols. Endogenous peroxidases present were then quenched with 0.3% hydrogen peroxide in methanol at room temperature, and slides were placed in 0.01 M sodium citrate buffer (pH 6.0) at 120°C for antigen retrieval. Primary antibodies were subsequently applied and included RB1CC1 (1:100). The Dako Envision System provided secondary antibodies, and bound antibodies were visualized with diaminobenzidine (DAB). Slides were then counterstained with hematoxylin, dehydrated, and mounted. Identical slide sections stained in the absence of primary antibodies served as negative controls. Specificity of the antibody against *RB1CC1* was demonstrated by western blotting (1:500).

Scoring of immunohistochemically stained tissue sections was based the fraction of positively stained cells present (e.g., score 0, 0%; score 1, 1-25%; score 2, 26-50%; score 3, 51-75%; score 4, 76-100%). An additional intensity score was recorded according to the following criteria: score 0, no staining; score 1, weak positive signal; score 2, moderate positive signal; score 3, strong positive signal (2). The sum of these two sets of scores provided a final staining score (range: 0-7), and a score ≥ 3 was considered positive.

Reference

1. Habbe N, Koorstra JB, Mendell JT, Offerhaus GJ, Ryu JK, Feldmann G, et al. MicroRNA miR-155 is a biomarker of early pancreatic neoplasia. Cancer Biol Ther. 2009;8:340-6.
2. Soumaoro LT, Uetake H, Higuchi T, Takagi Y, Enomoto M, Sugihara K. Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. Clin Cancer Res. 2004;10:8465-71.

Supplementary Figure Legends

Supplementary Figure S1. The expression of *PSA* and *KLK2* in DHT treated LNCaP cells and the transfection efficiency of cell proliferation in prostate cancer cells. (A-B) LNCaP cells were starved for 3 days, and then treated with 10 nM DHT or vehicle control in a time-course manner. The expression levels of *PSA* (A) and *KLK2* (B) were examined by qRT-PCR. (C-E) The transfection efficiency of cell proliferation was analyzed by qRT-PCR in LNCaP (C), 22Rv1 (D) and PC-3 cells (E), respectively. Each experiment was performed in triplicate. Values are mean ± SD.

Supplementary Figure S2. Quantitative analyses of cell cycle in LNCaP and PC-3 cells and the transfection efficiency of cell cycle assays in these two cell lines. The fractions of viable cells in the G1, S, and G2 phases of the cell cycle were quantified by flow cytometry analysis of propidium iodide-stained cells in LNCaP cells (A) and PC-3 cells (C). The transfection efficiency of cell cycle was validated by qRT-PCR in LNCaP cells (B) and PC-3 cells (D), respectively. Each experiment was performed in triplicate. Values are mean ± SD.

Supplementary Figure S3. Quantitative analyses of cell apoptosis in LNCaP and PC-3 cells and the transfection efficiency of cell apoptosis assays in these two cell lines. The fractions of living cells, early apoptotic cells, and late apoptotic cells were quantified by flow cytometry analysis of Annexin V/PI-stained cells in LNCaP cells (A) and PC-3 cells (C). The transfection efficiency of cell apoptosis was validated by

qRT-PCR in LNCaP cells (B) and PC-3 cells (D), respectively. Each experiment was performed in triplicate. Values are mean ± SD.

Supplementary Figure S4. Quantitative analyses of activated caspase and caspase-3 in LNCaP and PC-3 cells and the transfection efficiency in these two cell lines. The activity of total caspase and caspase-3 was detected by flow cytometry, and calculated as the percentage of M2 phase population compared to the total cells. (A) Caspase in LNCaP. (C) Caspase in PC-3. (E) Caspase-3 in LNCaP. (G) Caspase-3 in PC-3. The transfection efficiency of caspase and caspase-3 activity assay was validated by qRT-PCR in LNCaP (B for caspase, F for caspase-3) and PC-3 cells (D for caspase, H for caspase-3), respectively. Each experiment was performed in triplicate. Values are mean ± SD.

Supplementary Figure S5. Identifying putative targets of miR-133b. (A) qRT-PCR analysis of putative targets of miR-133b: *RB1CC1, PTPRK, SESN1* and *CPNE3*. qRT-PCR showed the relative expression of these four genes in LNCaP cells transfected with miR-133bm, miR-NC, si133b or siNC. (B) qRT-PCR and western blotting showed the endogenous expression of *RB1CC1* in LNCaP cells after 10 nM DHT stimulation for 24 h. The results revealed that DHT induced the endogenous expression of miR-133b, and decreased the endogenous expression of *RB1CC1* compared with that of the vehicle group (ethanol). (C) qRT-PCR and western blotting showed the endogenous expression of *RB1CC1* in 22Rv1 cells transfected with

miR-133b, miR-NC, si133b or siNC. The results revealed that overexpression of miR-133b repressed the endogenous expression of *RB1CC1* compared with that of the control, and anti-miR-133b partially restored the expression of *RB1CC1*. (D-E) qRT-PCR and western blotting showed the endogenous expression of *EGFR* in LNCaP cells (D) and PC-3 cells (E) transfected with miR-133bm, miR-NC, si133b or siNC. The results revealed that overexpression of miR-133b repressed the endogenous expression of *EGFR* compared with miR-NC, and anti-miR-133b partially restored the expression of *EGFR* in both cell lines. Each experiment was performed in triplicate. Values are mean ± SD.

Supplementary Figure S6. *RB1CC1* affected cell cycle and apoptosis of LNCaP cells. (A) Flow cytometry analysis of cell cycle of LNCaP cells with *RB1CC1* knockdown. The fractions of viable cells in the G1, S, and G2 phases of the cell cycle were quantified by flow cytometry analysis of propidium iodide-stained cells. The results revealed that knockdown of *RB1CC1* caused a decrease in G1 phase, and an increase in S phase. (B) Statistics of the results in (A). (C) LNCaP cells were transfected with *RB1CC1* siRNA, miR-133bm, si133b or NC separately for 48 h. The fractions of living cells, early apoptotic cells, and late apoptotic cells were quantified by flow cytometry analysis of Annexin V/PI-stained cells. The results revealed that knockdown of *RB1CC1* caused an increase in living cells, and a decrease in late apoptotic cells, which was concordant with the results of miR-133b overexpression. Instead, si133b contributed to a decrease of living cells, and an increase of late

apoptotic cells. (D) Statistics of the results in (C). (E) The transfection efficiency of *RB1CC1* siRNA was verified by qRT-PCR analysis in LNCaP cells. Each experiment was performed in triplicate. Values are mean ± SD.

Supplementary Tables

Table S1. Correlation between miR-133b expression and RB1CC1 protein level and clinicopathologic features in prostate cancer patients after radical prostatectomy (RP).

Features	miR-133b expression		*p*-Value	RB1CC1 expression		*p*-Value
	Negative	Positive		Negative	Positive	
[a]Age at Surgery (n = 135)			0.368			0.366
Median	68.00	68.00		68.0	67.5	
(Range)	(54.00-77.20)	(54.00-75.35)		(54.0-73.9)	(55.0-77.3)	
Mean	67.35	66.30		66.2	67.2	
[b]Pathological Stage (pT) (n = 135)			0.012			0.071
pT2-pT3a	41	72		64	49	
pT3b	2	20		17	5	
[c]Lymph Node Status (n = 135)			0.725			1.000
Positive	4	6		6	4	
Negative	39	86		75	50	
[c]Tumor Margins (n = 135)			0.803			0.394
Positive	6	16		15	7	
Negative	37	76		66	47	
[c]Capsular Invasion (n = 135)			0.718			0.324
No Invasion	41	85		77	49	
Invasion	2	7		4	5	
[d]Pre-operation PSA Level (ng/ml) (n = 135)			0.088			0.021
Median	15.00	21.44		23.00	16.10	
(Range)	(4.17, 63.60)	(4.38, 93.50)		(4.11, 98.70)	(5.18, 81.20)	
Mean	24.67	31.20		32.42	24.17	
[b]Gleason Score (n = 135)			0.694			0.012
Gleason ≤ 6	14	27		18	23	

Gleason ≥ 7	29	65		63	31	
[b]Biochemical Recurrence (n = 135)			0.038			0.024
Yes (≥ 0.2ng/ml)	17	54		49	22	
No (< 0.2ng/ml)	26	38		32	32	

[a]: Mean (t-test)

[b]: chi-square test

[c]: Fisher's Exact Test

[d]: Mann-Whitney U test.

Table S2. 5-year biochemical recurrence (BCR)-free survival rates and mean BCR-free time for negative and positive miR-133b expression (a) and RB1CC1 expression (b) groups of prostate cancer patients after RP.

A

miR-133b expression	No. of patients	5-year BCR-free survival rates	BCR-free	
			Mean (months)	95% CI
Negative	43	49.0%	36.78	29.20-44.35
Positive	92	18.9%	27.00	22.08-31.91
p=0.032				

B

RB1CC1 expression	No. of patients	5-year BCR-free survival rates	BCR-free	
			Mean (months)	95% CI
Negative	81	22.5%	25.63	20.41-30.85
Positive	54	37.9%	37.13	30.35-43.90
p=0.011				

Table S3. Sequences of synthetic oligonucleotides.

Oligonucleotides	**Sense (5'to 3')**	**Anti-sense (5'to 3')**
miR-133bm	UUUGGUCCCCUUCAACCAGCUA	GCUGGUUGAAGGGGACCAAAUU
miR-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
RB1CC1 siRNA	GAGAGAAGUUGUUGAGAAAUU	UUUCUCAACAACUUCUCUCUU

Table S4. Primers used for real-time PCR amplification.

Genes	Forward primer (5'to 3')	Reverse primer (5'to 3')
RNU6B	CGCTTCGGCAGCACATATACTAA	TATGGAACGCTTCACGAATTTGC
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
RB1CC1	ACATCTTGAGAATCAAATAGCAAAAA	TGAAGTTCAGCAACTAAGCTT
EGFR	TTCCTCCCAGTGCCTGAA	GGGTTCAGAGGCTGATTGTG
PSA	GTGCTTGTGGCCTCTCGT	AGCAAGATCACGCTTTTGTTC
KLK2	CCTCACGTTCTGGCATCACTT	CGGCCAGGTGAGTTCCAA

Table S5. Locations and primer pairs for candidate AREs of miR-133b and negative control (a region of the DNA adjacent to miR-133b gene without a putative ARE).

locus	Amplicon (ARE) position relative to TSS	primer-sense	primer-antisense
a	-3689 - -3411 (-3574~-3556)	TTTTAGGGTGAGT GTGTGTGAG	GTTTTCTTTTGTG GTTTTGTTTA
b	-2350 - -2067 (-2228~-2210)	GGAGAGGACAAG CCAAGACA	AAGCAGGGCACC ACAGAAC
c	57 - 310 (145~163)	CCCAGGAAGGAA GAGAAGAGG	GTTGGCACAAAC TCCATCCTC
d	7410 - 7661 (7523 ~7541)	CTCCCTAACCAAG GACACTCAG	ATTCCAGTTTGGG TAAGTCAGGT
e1	13910-14280 (14028 ~14046)	TAGAGGCAAACAC AGTCGGAATC	TATCAACTGCTGT CATCTGGTGG
e2	14325-14587 (14402 ~14420)	TTATTAGTTGGTCC TTATGCG	TGTGGAGAAATG TGATTGGAC
e3	14523-15010 (14606 ~14624)	CTGTCGCATAAGG ACCAAC	TCAGACCTGGGG ACTTCA
NC	-9982- -9895 (None)	GAGATGTAGACGC ATTAC	TTCTTAGTTCCTA TGTCTG

Table S6. Primers used for positive and negative DNA controls of ChIP assay.

Primer name	**Sequence**
KLK3 enhancer_forward	TGGGACAACTTGCAAACCTG
KLK3 enhancer_ reverse	CCAGAGTAGGTCTGTTTTCAATCCA
XBP-1 promoter_forward	TCTGGAAAGCTCTCGGTTTG
XBP-1 promoter_ reverse	AATCCCTGGCCAAAGGTACT

Table S7. Primers used for cloning the wild-type and mutation of 3'-UTR of *RB1CC1* gene into pGL3-promoter Luciferase vector downstream of the Luciferase gene.

Primer name	**Sequence**
*RB1CC1*_forward	CGGAATTCAGAAATGCGGACCAAACTACT
*RB1CC1*_ reverse	GGAATTCCATATGCTTTAGAACCCAGATGACCAAT
Mut-1_forward	CTACCAAACTACTTCATTTTCTCAAAG
Mut-1_ reverse	GCATTTCTAGAGTTGTCTGGGTA
Mut-2 _forward	GAGAAACTACTTCATTTTCTCAAAGGGC
Mut-2 _ reverse	AGGCATTTCTAGAGTTGTCTGGGTA

Table S8. Pearson's rank correlation coefficient analysis.

		total staining score miR-133b	total staining score RB1CC1
total staining score miR-133b	Pearson Correlation	1	-0.180(*)
	Sig. (2-tailed)		0.036
	N	135	135
total staining score RB1CC1	Pearson Correlation	-0.180(*)	1
	Sig. (2-tailed)	0.036	
	N	135	135

* Correlation is significant at the 0.05 level (2-tailed).

Table S9. Spearman's rank correlation coefficient analysis.

			total staining score miR-133b	total staining score RB1CC1
Spearman's rho	total staining score miR-133b	Correlation Coefficient	1.000	-0.180(*)
		Sig. (2-tailed)	.	0.036
		N	135	135
	total staining score RB1CC1	Correlation Coefficient	-0.180(*)	1.000
		Sig. (2-tailed)	0.036	.
		N	135	135

* Correlation is significant at the 0.05 level (2-tailed).