c-Met and CREB1 are involved in miR-433-mediated inhibition of the epithelial–mesenchymal transition in bladder cancer by regulating Akt/GSK-3β/Snail signaling

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Emerging evidence has suggested that microRNAs (miRNAs) have an important role in tumor development and progression by regulating diverse cellular pathways. Here we describe the function and regulation network of miR-433 in bladder cancer (BCa). miR-433 is frequently downregulated in BCa tissues compared with adjacent non-cancerous tissues. Epigenetic mechanisms may be involved in the regulation of miR-433 expression. Enforced expression of miR-433 significantly inhibits proliferation, colony formation, migration, and invasion in BCa cells. In addition, miR-433 inhibits the epithelial–mesenchymal transition (EMT) in BCa cells by regulating c-Met/Akt/GSK-3β/Snail signaling pathway. Both c-Met and CREB1 are downstream target genes of miR-433. CREB1 can also indirectly regulate c-Met/Akt/GSK-3β/Snail signaling via MITF. Furthermore, CREB1 expression is an independent prognostic factor for overall survival in patients with BCa. Finally, there appears to exist a reciprocal regulation between c-Met and miR-433/miR-409-3p. Taken together, this study reveals that miR-433-c-MET/CREB1/Akt/GSK-3β/Snail signaling is critical to EMT in BCa. Targeting the pathway described here may open up new prospects to restrict metastatic progression of BCa.

Cell Death and Disease (2016) 7, e2088; doi:10.1038/cddis.2015.274; published online 4 February 2016

Bladder cancer (BCa) is the most common malignancy of the urinary tract. It is the 7th most common cancer in males and the 17th most common in females.¹ It is an aggressive epithelial tumor characterized by a high rate of early systemic dissemination.² Approximately one-third of BCa patients develop locally invasive or metastatic disease.³ Although numerous therapeutic strategies have been improved on and used in recent years, radical cystectomy remains the standard of care for invasive BCa,⁴ with a 5-year survival reported to be as low as 62% in the current literature.⁵ These data support continuing efforts to develop novel and more effective therapeutic strategies.

MicroRNAs (miRNAs) are a class of short (19–22 nucleotides) noncoding RNA sequences that suppress gene expression through sequence-specific binding with the 3′-untranslated region (3′-UTR) of a target mRNA.⁶ Emerging evidence reveals that disordered miRNA expression contributes to the initiation and progression of human BCa.⁷ We have previously identified a series of miRNAs, including miR-26a,⁸ miR-101,⁹ miR-124-3p,¹⁰ miR-320c,¹¹ miR-409-3p,¹² miR-490-5p,¹³ and miR-576-3p,¹⁴ that is involved in the proliferation, migration, and invasion of BCa cells. Nevertheless, the biological function of miRNAs in BCa and their downstream mechanisms remain elusive.

To further clarify the role of miRNAs in the pathogenesis of BCa, we have reviewed the published miRNA expression profiles in human BCa.¹⁵,¹⁶ Based on these studies, miR-433 has captured our attention. miR-433 belongs to the DLK1-DIO3 miRNA cluster at 14q32.2 and is associated with the tumorigenicity and progression of gastric cancer,¹⁷ liver cancer,¹⁸ and myeloproliferative neoplasms.¹⁹ However, the exact role of miR-433 in BCa has not been documented.

In this study, we observed that miR-433 could inhibit the epithelial–mesenchymal transition (EMT) in BCa cells by regulating Akt/GSK-3β/Snail signaling. Both c-Met and CREB1, which were involved in the EMT process, were identified as direct target genes of miR-433. Furthermore, we described a reciprocal regulation between c-Met and miR-433/miR-409-3p.

Results

miR-433 is downregulated in BCa. To evaluate the expression of miR-433 in BCa, quantitative real-time PCR (qRT-PCR) was performed in 13 pairs of clinical BCa tissues and adjacent non-cancerous tissues (the clinical characteristics of the patients are shown in Supplementary Table S1), as well as in three types of urinary BCa cell lines T24, UM-UC-3, and 5637. The expression level of miR-433 was generally lower in tumor tissue than in non-tumor tissue (Figure 1a, 11 out of 13 displayed a downregulation pattern).
The examination of miR-433 in three different BCa lines also showed significant downregulation compared with SV-HUC-1 cell line (Figure 1b). These results are in agreement with a previously published data set, implying that miR-433 might have an important role in BCa progression.

Previous studies have suggested that methylation may participate in the regulation of miR-433 expression. After treatment with 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, the expression of miR-433 was significantly increased in the T24, UM-UC-3, and 5637 cell lines (Figure 1c). Two CpG islands were identified by the CpG Island Searcher program (http://www.urogene.org/methprimer/) from the 1000-bp upstream region of miR-433 (Figure 1d). The results of bisulfite-sequencing PCR (BSP) revealed that the T24, UM-UC-3, and 5637 cell lines showed a high methylation level (Figure 1e). These results supported the notion that DNA methylation might contribute to the deregulation of miR-433.

**Overexpression of miR-433 inhibits proliferation and colony formation in BCa cells.** T24 and UM-UC-3 cells were transfected with miR-433 mimics. The ectopic expression of miR-433 was confirmed by qRT-PCR (Supplementary Figure S1). Although there was no significant dosage effect, miR-433 suppressed the growth of cultured BCa cells at different concentrations and time points (Figure 2a). In parallel, miR-433 also had a marked impact on the colony-forming ability of BCa cells. The colony-formation capacity of miR-433 transfected cells was much lower than in cells transfected with NC (Figure 2b). These results suggested that miR-433 could regulate proliferation in BCa cells.

**Overexpression of miR-433 inhibits cell motility and induces the EMT.** Next, we examined whether miR-433 had any effect on migration and invasion capacity. A wound-healing assay indicated that the overexpression of miR-433 in UM-UC-3 cells was associated with a retardation of wound closure compared with the control group (Figure 4a). A migration chamber assay was undertaken to verify the role of miR-433 in cell motility. As shown in Figure 2c, miR-433 significantly suppressed the migration and invasion of T24 and UM-UC-3 cells. As EMT is a key event in cancer invasion and metastasis, the effect of miR-433 on EMT was analyzed by detecting the
protein levels of EMT markers. As shown in Figure 2d and Supplementary Figures S2 and S3, overexpression of miR-433 increased the expression of E-cadherin (an epithelial marker) but decreased fibronectin, Vimentin (mesenchymal markers), and Slug and Snail (EMT-related transcription factors). We further explored the underlying mechanism for miR-433-suppressed EMT. It has been well documented that the activation of AKT increases the nuclear expression and transcriptional activity of Snail by inhibitory phosphorylation of GSK-3β, thereby triggering cell migration and EMT.20 We detected AKT/GSK-3β signaling-related proteins by western blot analysis and found that the expression of phosphorylated Akt and phosphorylated GSK-3β (inactive form) were decreased in miR-433-transfected cells (Figure 2d). These results indicated that the overexpression of miR-433 could suppress the EMT phenotype of T24 and UM-UC-3 cells by regulating Akt/GSK-3β/Snail signaling.

**CREB1 and c-Met are direct targets of miR-433.** We used two methods to screen for potential direct targets of miR-433: bioinformatics prediction (http://www.targetscan.org/) and gene expression microarray analysis. Based on the results of bioinformatics prediction and microarray analysis (Supplementary Table S2), we identified several candidate target genes and subsequently tested the expression of these genes after treatment with miR-433 mimics by qRT-PCR. A decrease in the expression of c-Met, CREB1, GCLC, TGFβR1, and ARMC1 was observed in miR-433-transfected BCa cells (Figure 3a).

We then performed luciferase reporter assays to examine the direct interaction between miR-433 and the 3′-UTRs of the five genes (c-Met, CREB1, GCLC, TGFβR1, and ARMC1). We cloned the 3′-UTRs into pmirGLO Dual-Luciferase miRNA Target Expression Vectors. As shown in Figure 3b, overexpression of miR-433 significantly decreased the relative luciferase activity of c-Met and CREB1 but had no effect on the other genes. Western blot analysis revealed a reduced protein expression of c-Met and CREB1 in miR-433-transfected cells (Figure 3d). To verify that miR-433 directly binds to the 3′-UTRs of c-Met and CREB1, we mutated the miR-433-targeting sites (Figure 3d). As expected, the luciferase activity of the mutated vectors was unaffected by the transfection of miR-433 (Figure 3e). Collectively, these data supported the notion that CREB1 and c-Met were direct downstream targets of miR-433.

**Repression of c-Met inhibits cell motility and induces EMT.** We previously reported that knockdown of c-Met by
siMet inhibited migration and invasion in BCa cells, but the underlying mechanism was not explored. Considering the importance of c-Met/AKT/GSK-3β/Snail signaling in EMT and metastasis, we used western blottings to examine the expression of several proteins involved in this signaling pathway. Decreased expression of phosphorylated Akt, phosphorylated GSK-3β, fibronectin, and Snail, as well as increased expression of E-cadherin, were observed in siMet-transfected cells (Figure 4c). Accordingly, the migration and invasion of BCa cells were significantly suppressed (Figures 4a and b). These results phenocopied the effect of miR-433 on cell motility and EMT.

We further investigated the expression patterns and subcellular localizations of c-Met protein in BCa tissues and adjacent non-tumor tissues by immunohistochemical (IHC) analysis. c-Met showed a membranous and cytoplasmic location (Figure 4d). Statistical analysis indicated that the expression level of c-Met protein in BCa tissues was significantly higher than in adjacent non-tumor tissues (P < 0.001, Figure 4e). To evaluate the prognostic role of c-Met, a survival analysis was executed. Kaplan–Meier survival curves indicated that the protein expression of c-Met was not associated with the overall survival rate of BCa patients (P = 0.788, Figure 4f).

Repression of CREB1 inhibits cell proliferation and induces EMT. The exact role of CREB1 in BCa is unclear. We first analyzed the effect of CREB1 on cell proliferation. The CCK-8 assay showed that knockdown of CREB1 by siCREB1 inhibited the growth of T24 and UM-UC-3 cells at
different concentrations and time points (Figure 5a). In parallel, the colony-forming ability of BCa cells was decreased. The colony-formation capacity of siCREB1-transfected cells was much worse than in cells transfected with NC (Figure 5b). Next, we explored the role of CREB1 in the migration and invasion of BCa cells. The wound-healing assay indicated that the downregulation of CREB1 in UM-UC-3 cells led to a retardation of wound closure compared with the control group (Figure 4a). A migration chamber assay was also performed and revealed that migration and
invasion were suppressed in siCREB1-transfected cells (Figure 5c). These data suggested that CREB1 could regulate BCa cell proliferation and motility.

We were curious as to whether CREB1 also participated in EMT, which has not been well established. It has been reported that CREB1 can directly bind to the MITF promoter region and stimulate MITF transcription. On the other hand, c-MET transcription can be directly regulated by MITF. Thus, we speculated that CREB1 might indirectly regulate the expression of c-Met. This hypothesis was supported by western blot analysis, which showed that knockdown of CREB1 by siCREB1 reduced c-Met protein expression (Figure 5d). As expected, downregulation of CREB1 also inhibited the AKT/GSK-3β/Snail pathway. Decreased expression of phosphorylated Akt, phosphorylated GSK-3β, fibronectin, and Snail, as well as increased expression of E-cadherin, were detected in siCREB1-transfected cells (Figures 5e and f). Collectively, these data implied that the
repression of CREB1 could suppress the EMT phenotype of T24 and UM-UC-3 cells at least partially by regulating c-Met/Akt/GSK-3β/Snail signaling.

We further examined the expression patterns and subcellular localizations of CREB1 proteins in BCa tissues and adjacent non-tumor tissues by IHC analysis. CREB1 localized in the nucleus and cytoplasm (Figure 5g). Statistical analysis indicated that the expression level of CREB1 protein in BCa tissues was significantly higher than in adjacent non-tumor tissues (P < 0.001, Figure 5h). Kaplan–Meier survival curves indicated that the protein expression of CREB1 was significantly associated with the overall survival rate of BCa patients (P = 0.029, Figure 5i). Finally, in a multivariate Cox model including sex, age, tumor grade, T stage, lymph node status, c-Met expression, and CREB1 expression, we found that the overexpression of CREB1 expression was a good independent prognostic factor for overall survival in patients with BCa (P = 0.039, Supplementary Table S3).

CREB1 can indirectly regulate the expression of c-Met via MITF. We compared the expression of CREB1, c-Met, and MITF in BCa tissues. As shown in Figures 6a and c, CREB1 was significantly positively correlated with c-Met (r = 0.340, P = 0.021) and MITF (r = 0.364, P = 0.013). A significantly positive correlation between c-Met and MITF (r = 0.291, P = 0.049) was also observed (Figure 6b). Furthermore, either overexpression of miR-433 or repression of CREB1 in UM-UC-3 cells inhibited the mRNA level of MITF (Figures 6d and e). On the other hand, inhibition of MITF suppressed the mRNA level of c-Met (Figure 6f). At the protein level, repression of CREB1 and MITF also inhibited the MITF and c-Met, respectively (Figure 6g). Finally, we performed a rescue experiment, which showed that MITF overexpression was able to, at least partially, rescue the level of c-Met in the presence of siCREB1 (Figure 6h).

miR-409-3p is involved in miR-433-mediated inhibition of cell motility. A previous study has suggested that c-Met could regulate the 14q32.2 miRNA cluster (e.g., the DLK1-DIO3 miRNA cluster),25 which includes miR-433 and miR-409-3p. On the other hand, our previous study12 and this study indicated that miR-409-3p and miR-433 could directly regulate c-Met. Thus, we speculated that there might be reciprocal regulation between miR-433/miR-409-3p and c-Met. Consistent with this assumption, downregulation of c-Met by siMet increased the expression of miR-433 and miR-409-3p (Figures 7b and d), whereas upregulation of c-Met by an overexpression vector (pc-Met) reduced the expression of miR-409-3p (Figure 7c). Overexpression of miR-433 could also significantly increase the expression of miR-409-3p (Figure 7a), at least partially, by inhibiting the expression of c-Met. A genetic regulatory network is depicted in Figure 7e and summarizes the key findings of our study.

Discussion

Recent evidence suggests that the deregulation of miRNAs has an important role in tumorigenesis and progression in BCa and other malignancies. Numerous studies have characterized the miRNA signatures of BCa, but the exact role of miRNA dysregulation in the pathogenesis of BCa remains elusive. In this study, the expression of miR-433 was significantly lower in BCa tissues than in adjacent non-cancerous tissues. Furthermore, gain-of-function analyses showed that miR-433 suppressed the proliferation, migration, and invasion of BCa cells. These findings have highlighted the tumor suppressor role of miR-433 in BCa.

Human miR-433, located at chromosomal region 14q32.2, belongs to the DLK1-DIO3 genomic imprinted miRNA cluster and functions as a tumor suppressor in several different types of human cancers.17–19 Epigenetic programming has an important role in the regulation of miR-433. Guo et al.17 and Yang et al.18 showed that miR-433’s surrounding CpG islands were hypermethylated, and that treatment with the DNA methylation inhibitor 5-aza-CdR could induce miR-433 expression. Similarly, the results of our study suggested that epigenetic mechanisms might be involved in the regulation of miR-433 expression in BCa. Furthermore, Sato et al.26 showed that the expression of miR-127, the paired gene that is clustered with miR-433, is regulated epigenetically in BCa cells. Collectively, the deregulation of miR-433 in human cancers may be partly attributed to the epigenetic modification of chromatin.

Although several studies have characterized the function and mechanism of miR-433, the molecular basis of the miR-433-mediated antitumor effect is still not well elucidated. In this study, two direct targets of miR-433, namely, c-Met and CREB1, were identified and experimentally verified. c-Met is a well-characterized oncogene and is frequently overexpressed in a variety of human cancers.27 We have previously confirmed that miR-409-3p can inhibit the migration and invasion of BCa cells by targeting c-Met.12 In this study, we found that the miR-433 downregulation is a new mechanism responsible for the abnormal activation of c-Met. We further showed that c-Met has a crucial role in miR-433-induced inhibition of EMT. Reduction of c-Met by miR-433 suppressed the activating phosphorylation of AKT and the inhibitory phosphorylation of GSK-3β, leading to the nuclear accumulation of Snail and increased expression of E-cadherin. Interestingly, we observed that c-Met could reversely regulate the expression of miR-409-3p and miR-433, which was in accordance with the findings of a previous study that reported c-Met could regulate the 14q32.2 miRNA cluster.25 This reciprocal regulation, therefore, maximizes the activation of the c-Met signaling pathway in BCa cells in response to HGF stimulation.

As a transcription factor, CREB1 controls numerous downstream genes and has been implicated in cancer cell migration and invasion in various malignancies.18,28 Nevertheless, the exact role of CREB1 in EMT remains elusive. In this study, we showed that downregulation of CREB1 could suppress the AKT/GSK-3β/Snail pathway at least partially via decreasing the expression of c-Met. The molecular mechanisms underlying CREB1-induced inhibition of c-Met are not well defined in the literature. As CREB1 can directly promote the transcription of MITF, which directly regulates the expression of c-Met,29 we speculate that MITF may be involved in CREB1-mediated activation of c-Met/AKT/GSK-3β/snail signaling. In addition, CREB-VEGF signaling has been implicated in the process of EMT in prostate cancer.30 All these data support the notion that CREB1 has a crucial role in EMT and metastasis.
Furthermore, CREB1 is also a direct target of miR-433; thus, the expression of c-Met is directly and indirectly regulated by miR-433 in BCa cells. Finally, it should be noted that a single miRNA can regulate the mRNA transcripts of hundreds of target genes, and we cannot rule out the possibility that signaling pathways mediated by other targets, apart from c-Met and CREB1, may have a role in miR-433-mediated inhibition of EMT.

In survival analyses, a significantly better overall survival was observed in patients with high CREB1 expression, which seems contradictory to the tumor-promoting role of CREB1. Similarly, Liu et al.\textsuperscript{31} reported that overexpression of p-CREB (Ser133) and CREB correlated with a favorable survival in squamous cell lung carcinoma. They speculated that overexpression of CREB might mainly occur in early-stage tumors.

Based on this hypothesis, we reviewed the existing BCa microarray data sets deposited in the Oncomine database.\textsuperscript{32} Seven data sets that compared superficial BCa with infiltrating BCa were identified from Oncomine. As shown in Supplementary Figure S4, the CREB1 expression in the superficial samples was generally higher than that in the infiltrating samples in six of seven data sets, of which three data sets have statistically significant differences. These results implied that CREB1 might have a more important role in the progression of early-stage BCa. Nevertheless, as the size of our patient cohort is limited, further prognostic study is warranted to confirm the findings of our study. Of note, Chhabra et al.\textsuperscript{33} and Yu et al.\textsuperscript{34} showed that overexpression of CREB1 predicted an unfavorable prognosis in patients with breast cancer and hepatocellular carcinoma, respectively. The prognostic effect of CREB, therefore, seems to be cancer-type specific and context specific.
In conclusion, we report the following new findings: (i) miR-433 is frequently downregulated in BCa at least partially due to altered DNA methylation; (ii) miR-433 functions as a tumor suppressor in BCa cells; (iii) c-Met and CREB1 are identified as downstream target genes of miR-433; (iv) miR-433 inhibits EMT in BCa cells by regulating c-Met/Akt/GSK-3β/Snail signaling; (v) CREB1 can also indirectly regulate c-Met/Akt/GSK-3β/Snail signaling via MITF; (vi) CREB1 expression is an independent prognostic factor for overall survival in patients with BCa; and (vii) a reciprocal regulation between c-Met and miR-433/miR-409-3p may exist. Our study underscores the important role of miR-433 in BCa progression and we expect that our findings on the signaling axis of miR-433-CREB1/c-Met/Akt/GSK-3β/Snail will provide useful information for the development of more effective and promising therapies against BCa.

Materials and Methods

Reagents and transfection. The miR-433 mimic (named miR-433, sense; 5′-AUCAUGAGUGCCUCUCUUGU-3′) and the negative control duplex (named NC, sense; 5′-ACUACUGAGAGACAGA-3′) with no significant homology to any known human sequences were used for gain-of-function studies. The small interfering RNA targeting human c-Met mRNA (named siMet, sense; 5′-GGAGGUGUUGGAAAGAUAdTdT-3′), CREB1 mRNA (named siCREB1, sense; 5′-GAGAGAGUCUCUCUAAUGdTdT-3′), and MITF mRNA (named siMITF, sense; 5′-AGCAAGUCUCCUACCACdTdT-3′) were used for the RNA interference study. The RNA duplexes were chemically synthesized by GenePharma, Shanghai, China. Oligonucleotide transfection was performed using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocol.

Cell lines and cell culture. The human BCa cell lines T24, UM-UC-3, and 5637, as well as one normal bladder cell line SV-HUC-1, were purchased from the Shanghai Institute of Cell Biology, Shanghai, China. These cell lines were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) under a humidified atmosphere of 5% CO₂ at 37 °C.

Human clinical samples. Paired BCa tissues and adjacent non-neoplastic bladder mucosal tissues were obtained from patients undergoing radical cystectomy. The samples were collected between January 2011 and October 2013 at the First Affiliated Hospital of Zhejiang University, after informed consent and Ethics Committee’s approval. The clinical data of the patients has been listed in our previous studies.11 Tissue samples were snap frozen in liquid nitrogen until RNA extraction.

RNA isolation and qRT-PCR. miRNA was extracted from clinical samples and cultured cell lines using RNAiso for Small RNA (Takara, Dalian, China) and reverse transcribed using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara). Total RNA was isolated with RNAiso Plus (Takara) and transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara). qRT-PCR was performed using an ABI 7500 FAST Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and a SYBR Green PCR Kit (Takara, Dalian, China). The relative expression level of miRNA or mRNA was quantified with the 2−ΔΔCT method after normalization with the endogenous reference U6 small nuclear RNA or GAPDH, respectively. The primers are shown in Supplementary Table S4.

Dual-luciferase reporter assay. Oligonucleotide pairs that contained the desired miR-433 target region or mutant target region were designed and ordered from Sangon, Shanghai, China. After annealing, these double-stranded segments were inserted into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA), between the SacI and SalI sites. The insertions were verified by sequencing. HEK293T cells were seeded in 24-well plates and co-transfected with 50 nM miR-433 or NC and 100 ng reporter pmirGLO. The relative luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) 48 h after transfection.

Cell growth/cell viability assay. T24 or UM-UC-3 cells were plated in 96-well plates with ~4 × 10³ cells per well. After overnight incubation, the cells were co-transfected with the RNA duplex (miR-433, siCREB1, or NC) for 2–3 days with concentrations ranging from 10 to 50 nM. At different time points, the medium was removed and WST-8 (Dojindo Laboratories, Kumamoto, Japan) was added to each well. After the 96-well plate was incubated at 37 °C for 1 h, the absorbance of the solution was measured spectrophotometrically at 450 nm with a MRX II absorbance reader (Dynex Technologies, Chantilly, VA, USA).

Figure 7 miR-409-3p is involved in miR-433-mediated inhibition of cell motility. (a) Overexpression of miR-433 significantly increased the expression of miR-409-3p. (b) Knockdown of c-Met increased the expression of miR-409-3p. (c) Upregulation of c-Met by an overexpression vector (pc-Met) reduced the expression of miR-409-3p. (d) Knockdown of c-Met increased the expression of miR-433. (e) Schematic diagram showing the signaling network in which miR-433 is involved. Error bars represent the S.E. obtained from three independent experiments; *P < 0.05.
Colony-formation assay. Cells were trypsinized to a single cell suspension 24 h after transfection with 2′-O-methyl-modified duplexes (50 nM). Next, the cells were seeded in six-well plates (500 cells per well) and maintained under standard culture conditions for 2 weeks. Colony counts were performed after the colonies were fixed with absolute methanol and stained with 0.1% crystal violet.

Wound-healing assays. After transfection, the cells were grown to 100% confluence in six-well plates. A micropipette tip was used to make a cross wound and wound healing was observed after 24 h. Photographs were taken under phase-contrast microscopy (Olympus, Tokyo, Japan) immediately or 24 h after wounding.

Cell migration and invasion assay. The cell migration and invasion assay was performed using transwell chambers (Millipore, Billerica, MA, USA). For the invasion assay, the inserts were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) on the upper surface. After transfection, 8 × 10^4 cells were suspended in 0.2 ml serum-free medium and added to the inserts. Next, 0.6 ml RPMI-1640 medium with 10% FBS was added to the lower compartment as a chemoattractant. After incubation at 37 °C for 24 h, the cells on the upper surface of the membrane were carefully removed using a cotton swab and cells on the lower surface were fixed with 100% methanol and stained with 0.1% crystal violet. Five visual fields of ×200 magnification of each insert were randomly selected and counted under a light microscope (Olympus).

DNA methylation analysis and 5-aza-CdR treatment. BSP was performed as previously described.25 Following bisulfite conversion, the CpG islands of miR-433 were amplified by PCR using the primers 5′-TTTTGGGTTGGGATGTTGTTG-3′ (forward) and 5′-ACACCACTACACTAACACACCTG-3′ (reverse). The PCR products were cloned into the pUC18 Tvector. After bacterial amplification, eight clones were subjected to DNA sequencing (Sangon, Shanghai, China), and anti-β-catenin (Cell Signaling Technology), β-actin (Sigma, St Louis, MO, USA) and anti-β-actin (Sangon, Shanghai, China) were used in the analysis. DNA was extracted and analyzed for the expression of miR-433.

IHC staining. Tissue microarrays (TMAs) containing 46 cases with paired tumor and non-tumor tissues and 13 cases without corresponding non-tumor tissues were analyzed in this study. TMA was obtained from Xinchao Biotech, Shanghai, China. Paraffin sections were dewaxed and rehydrated. Antigen retrieval was performed by heating the slides in sodium citrate buffer (10 mM, pH 6.0). After antigen retrieval, the slides were incubated with 0.1% hydrogen peroxide for 15 min and blocked with 5% normal goat serum. The sections were incubated with the primary antibodies for 1 h at room temperature. After washing with PBS, the sections were incubated with biotinylated secondary antibody for 30 min, followed by incubation with 0.1% diaminobenzidine (DAB) solution for 5 min. The sections were counterstained with Mayer’s hematoxylin solution for 1 min and mounted with a dehydrating solution. The paraffin sections of human bladder cancer specimens were stained with hematoxylin and eosin (H&E) and were used as a positive control.

Statistical analysis. The data were expressed as the mean ± S.D. Differences between groups were estimated using the χ^2-test or Student’s t-test. Overall survival rates were calculated according to the Kaplan–Meier method with log-rank test. A Cox regression analysis (proportional hazards model) was performed for the multivariate analyses of prognostic factors. All analyses were performed using SPSS16.0 software (IBM, Armonk, NY, USA) and a two-tailed value of P < 0.05 was considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements
This study was supported by grants from the National Key Clinical Specialty Construction Project of China, Key Medical Disciplines of Zhejiang Province, Health Sector Scientific Research Special Project (201002010), Combination of Traditional Chinese and Western Medicine Key Disciplines of Zhejiang Province (2012-XK-A23), Zhejiang Province Key Project of Science and Technology (2014C40018-2), Public Welfare Technology Application Research project of Zhejiang Provincial Science Technology Department of China (2014C33198), Zhejiang Provincial Medical Science Foundation of China (2014KYB084), Zhejiang Provincial Natural Science Foundation (LY13H160009), National Natural Science Foundation of China (81472375 and 81372773), and the Scientific Research Foundation of the Ministry of Public Health of China (WKJ2012-2-009).
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Supplemental information accompanies the paper on the Cell Death and Discovery website (http://www.nature.com/cddiscovery)