miRNA-100 Inhibits Human Bladder Urothelial Carcinogenesis by Directly Targeting mTOR

Chuanliang Xu1, Qinsong Zeng1,2, Weidong Xu1, Li Jiao1, Yanqiong Chen1, Zhensheng Zhang1, Chengyao Wu1, Taile Jin1, Anyin Pan1, Rongchao Wei1, Bo Yang1, and Yinghao Sun1

Abstract
miRNAs are involved in cancer development and progression, acting as tumor suppressors or onco-genes. In this study, miRNA profiling was conducted on 10 paired bladder cancer tissues using 20 GeneChip miRNA Array, and 10 differentially expressed miRNAs were identified in bladder cancer and adjacent noncancerous tissues of any disease stage/grade. After being validated on expanded cohort of 67 paired bladder cancer tissues and 10 human bladder cancer cell lines by quantitative real-time PCR (qRT-PCR), it was found that miR-100 was downregulated most significantly in cancer tissues. Ectopic restoration of miR-100 expression in bladder cancer cells suppressed cell proliferation and motility, induced cell-cycle arrest in vitro, and inhibited tumorigenesis in vivo both in subcutaneous and in intravesical passage. Bioinformatic analysis showed that the mTOR gene was a direct target of miR-100. siRNA-mediated mTOR knockdown phenocopied the effect of miR-100 in bladder cancer cell lines. In addition, the cancerous metastatic nude mouse model established on the basis of primary bladder cancer cell lines suggested that miR-100/mTOR regulated cell motility and was associated with tumor metastasis. Both mTOR and p70S6K (downstream messenger) presented higher expression levels in distant metastatic foci such as in liver and kidney metastases than in primary tumor. Taken together, miR-100 may act as a tumor suppressor in bladder cancer, and reintroduction of this mature miRNA into tumor tissue may prove to be a therapeutic strategy by reducing the expression of target genes. Mol Cancer Ther; 12(2); 207–19. ©2012 AACR.

Introduction
It is estimated that bladder cancer accounts for 69,250 new cases of cancer and 14,990 cancer-related deaths in the United States in 2011 (1). It is the fourth most common cancer in men and the eighth leading cause of death from cancer. The ratio of men to women who develop bladder cancer is approximately 3:1 (1). The disease presents in 2 different forms: non–muscle-invasive bladder cancer (NMIBC; stage Ta and T1) and muscle-invasive bladder cancer (MIBC; stage T2–4). NMIBC represents a heterogeneous group of tumors, from completely benign non–invasive papillary tumors that rarely progress to invasive bladder cancer. The ratio of men to women who develop bladder cancer is approximately 3:1 (1). The disease presents in 2 different forms: non–muscle-invasive bladder cancer (NMIBC; stage Ta and T1) and muscle-invasive bladder cancer (MIBC; stage T2–4). NMIBC represents a heterogeneous group of tumors, from completely benign non–invasive papillary tumors that rarely progress to invasive bladder cancer.

miRNAs are involved in cancer development and progression, acting as tumor suppressors or oncogenes. In this study, miRNA profiling was conducted on 10 paired bladder cancer tissues using 20 GeneChip miRNA Array, and 10 differentially expressed miRNAs were identified in bladder cancer and adjacent noncancerous tissues of any disease stage/grade. After being validated on expanded cohort of 67 paired bladder cancer tissues and 10 human bladder cancer cell lines by quantitative real-time PCR (qRT-PCR), it was found that miR-100 was downregulated most significantly in cancer tissues. Ectopic restoration of miR-100 expression in bladder cancer cells suppressed cell proliferation and motility, induced cell-cycle arrest in vitro, and inhibited tumorigenesis in vivo both in subcutaneous and in intravesical passage. Bioinformatic analysis showed that the mTOR gene was a direct target of miR-100. siRNA-mediated mTOR knockdown phenocopied the effect of miR-100 in bladder cancer cell lines. In addition, the cancerous metastatic nude mouse model established on the basis of primary bladder cancer cell lines suggested that miR-100/mTOR regulated cell motility and was associated with tumor metastasis. Both mTOR and p70S6K (downstream messenger) presented higher expression levels in distant metastatic foci such as in liver and kidney metastases than in primary tumor. Taken together, miR-100 may act as a tumor suppressor in bladder cancer, and reintroduction of this mature miRNA into tumor tissue may prove to be a therapeutic strategy by reducing the expression of target genes. Mol Cancer Ther; 12(2); 207–19. ©2012 AACR.
in cancer cells seems controversial as it served as an oncogene in acute myelogenous leukemia but as a tumor suppressor in cervical cancer (12, 13). There has been no evidence to confirm the function of miR-100 in bladder cancer.

In this study, we focused on miR-100, as our previous miRNA array analysis on 10 pairs of human bladder cancer and adjacent noncancerous tissues showed that miR-100 was stably downregulated and presented highly fold-changed expression both in NMIBC and in MIBC. After validation of the altered expression level of miR-100 on 67 paired bladder cancer tissues by quantitative real-time PCR (qRT-PCR), we tested its effects on cell growth, cell-cycle distribution, colony formation, cell migration, and invasion. In addition, we also investigated the potential role of miR-100 in bladder cancer tumorigenesis in 2 nude mice models. Finally, we explored the underlying mechanism of miR-100 functions in bladder cancer and validated its target gene, mTOR both in vitro and in vivo. Our study may hopefully provide a better understanding about bladder cancer pathogenesis.

Materials and Methods

Human tissues and cell lines

The tissue specimens were from 27 patients with bladder cancer who underwent cystectomy or transurethral resection of bladder tumors between 2008 and 2010 in Shanghai Hospital (Shanghai, China). The background and clinicopathologic characteristics of the patients are summarized in Supplementary Table S1. Tissue samples were immediately snap-frozen in liquid nitrogen. Both tumors and adjacent normal tissues were histologically examined, and all tumors were validated as urothelial carcinoma. Our study was approved by the Bioethics Committee of the Second Medical University (Shanghai, China). Written prior informed consent and approval were given by the patients.

Bladder cancer cell lines ScABER, BIU-87, 5637, and T24 were purchased from American Type Culture Collection (ATCC). Other bladder cancer cell lines RT4, J82, HT-1376, EJ, TCCSUP, and SV40-transformed kidney cell line 293T were kindly provided by Prof. Tianxing Lin (Sun Yat-sen Medical Biotechnology). Oligonucleotide transfection pre-miR-100 precursor and its negative control were purchased from Ambion. Cholesterol-conjugated miR-100 mimics for in vitro RNA delivery and its negative controls were from Ribobio Co. miR-100 inhibitor and mTOR siRNA (target sequence: GAAGCTCGCTGATCCAGATG) were synthesized by BioNEER. Oligonucleotide transfection was conducted with Lipofectamine RNAiMAX reagents (Invitrogen).

miRNA array analysis

Ten paired bladder cancer tissues (5 low-grade NMIBCs and 5 high-grade MIBCs) were sent to CapitalBio Corp. for noncoding RNA microarray analysis. miRNA profiling was conducted using 20 GeneChip miRNA Array (Affymetrix). To respectively determine the significant differentially expressed miRNAs in NMIBC pairs and MIBC pairs, RVM / test analysis of microarrays was conducted using the threshold value of ≥2 or ≤0.5. Intersection data of the significant differentially expressed miRNAs were subsequently obtained between the 2 pairs, log-transformed and median-centered by genes using the Adjust Data function of CLUSTER 3.0 software, then further analyzed with hierarchical clustering with average linkage (16), and finally visualized using Java Tree View (Stanford University, Stanford, CA). The microarray platform and data have been submitted to the Gene Expression Omnibus public database 6 at the National Center for Biotechnology Information, following the Minimum Information About a Microarray Gene Experiment guidelines. The accession numbers are GPL8786 (platform) and GSE9093 (samples; release date June 2012).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). miRNA was detected by stem-loop RT-PCR as described previously (17). cDNA was synthesized with the AccuPower RocketScript RT Premix Kit (BioNEER). The primers for RT-PCR are listed in Supplementary Table S2.

Plasmid construction, lentivirus production, and transduction

miR-100/control lentivirus was purchased from Sunbio Medical Biotechnology. Oligonucleotide transfection pre-miR-100 precursor and its negative control were purchased from Ambion. Cholesterol-conjugated miR-100 mimics for in vivo RNA delivery and its negative controls were from Ribobio Co. miR-100 inhibitor and mTOR siRNA (target sequence: GAAGCTCGCTGATCCAGATG) were synthesized by BioNEER. Oligonucleotide transfection was conducted with Lipofectamine RNAiMAX reagents (Invitrogen).

Cell growth, Annexin V assays, wound-healing, and Matrigel invasion assays

Cell proliferation assay was conducted with the Cell Counting Kit-8 (CCK-8; Dojindo) according to the manufacturer’s instruction. Cell migration and invasion assay were conducted as described elsewhere (18). Apoptosis after doxorubicin treatment was examined by using the Annexin V/Propidium Iodide Detection Kit (KeyGEN) according to the manufacturer’s instructions.

Tumor formation in nude mice

All animal experiments were undertaken in accordance with the NIH Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Second Medical University. Tumor-bearing female nude mice with subcutaneous passage of EJ cells and intravesical passage of 5637 cells were used for evaluating the antitumor effect of miR-100 in vitro. Bladder cancer cells were resuspended at a concentration of 1 × 10^6
cells/mL of PBS before use. Tumor size was measured as described previously (19). After 6 weeks, these mice were sacrificed and examined.

For preparation of the subcutaneous model, 100 μL suspending EJ cells infected with lenti-miR-100 or lentivector control were injected s.c. into the same side of the flank of the female nude mouse (4 weeks of age, each group with 6 mice, n = 6).

In addition, another subcutaneous model was prepared using wild-type EJ cells for in vivo RNA delivery (each group with 4 mice, n = 4). For intratumoral injection of cholesterol-conjugated RNA, miR-Ribo agomir-100, 2 nmol RNA in 50 μL saline buffer was locally injected into each tumor mass at a 3-day interval for 4 weeks.

The intravesical orthotopic bladder cancer model prepared using the technique described by Watanabe and colleagues (20). Briefly, after anesthesia of the female athymic nude mouse and transurethral insertion of a 22-gauge catheter into the bladder, 100 μL 0.2% trypsin in 0.02% EDTA was infused and retained in the bladder for 30 minutes. After trypsinization, the bladder was recatheterized, washed with PBS, and scratched carefully by the catheter. Subsequently, 100 μL suspending 5637 infected with lenti-miR-100 or vector was instilled into the bladder. The urethra was ligated with 6–0 nylon suture to assure that the cells were retained in the bladder. After 3 hours, the suture was removed, and the bladder was evacuated by spontaneous voiding.

**Tumor metastases in nude mice**

When the animals succumbed to tumor burden and the orthotopic bladder tumor was palpable after intravesical 921T implantation (6 weeks of age, with 3 mice, n = 3), the mice were examined daily and sacrificed at 4 weeks after implantation or earlier if they developed signs of distress. Only obvious metastases of the lungs, liver, spleen, pancreas, bladder, kidneys, or retroperitoneal organs were obtained and treated for cell culturing. After validation of their origin by immunohistochemical staining, epithelial tumor metastasis was minced into small pieces about 0.5 mm³ with scalpels and cultured until tumor cells were present by the technique described previously (15). When the cells presented stable after in vitro passage, they were harvested for intravesical implantation once again to prepare the cancerous metastatic model (6 weeks of age, with 3 mice, n = 3). New metastatic tumor foci and orthotopic bladder cancer tissues of the mice were harvested and used for evaluating the antimetastatic effects of miR-100 and the metastatic effects of its target gene(s) in vivo.

**Bioinformatics**

The predicted target genes and their conserved sites of the seed region binding with each miRNAs were investigated using the TargetScan program (release 5.0, http://www.targetscan.org/). Fisher exact test was used to analyze the target genes according to the Gene Ontology (GO), which is the key functional classification of NCBI (21). False discovery rate (FDR) algorithm (22) was applied to adjust the P values, using the threshold values of FDR < 0.05. Using the KEGG database, the network of genes was built according to the relationship between miRNAs and target genes in the database (23, 24). Technical expertise for systemic bioinformatic analysis was provided by Novel Bioinformatics Co., Ltd.

**Luciferase assay**

About 100 ng/mL of plasmid and 50 nmol/L miRNA mimics were co-transfected into HEK 293T cells. Forty-eight hours after transfection, a Dual-Luciferase Reporter Assay System (Promega) was used to examine the effects of miR-100 on the activity of mTOR reporter according to the manufacturer’s protocol.

**Western blot analysis and immunohistochemical assay**

Anti-mTOR mcAb and anti-p70S6K were purchased from Cell Signaling Technologies. Immunohistochemical staining was conducted on 5-μm sections of paraffin-embedded tissue to determine the expression of AE1/ AE3 (antibody from Zhongshan Goldenbridge Bio), a general stain for normal and neoplastic cells of epithelial origin.

**Statistical analysis**

The results were presented as mean ± SEM. The data were subjected to the Student t test unless otherwise specified (χ² test). P value of less than 0.05 was considered statistically significant.

**Results**

**miR-100 expression is decreased in human bladder cancer tissues**

Twenty GeneChip miRNA array was done on 10 paired bladder cancer tissues and adjacent normal tissues. Of the 6,703 encoded miRNAs analyzed, 20 exhibited significantly differential expression in low-grade NMIBC tissues and 22 in high-grade MIBC samples (Supplementary Tables S3 and S4). Unsupervised hierarchical clustering of these significantly dysregulated miRNAs was able to distinguish the bladder cancers from corresponding normal tissues (Fig. 1A). In addition, 10 miRNAs were found as an intersection set of the 2 sets of significantly dysregulated miRNAs, suggesting that they played a stable role in any tumor stage or grade (Table 1).

To further confirm the microarray results, several targets with higher fold change in the data (miR-100, -145, -182, and -210) were selected and examined in an expanded bladder cancer cohort consisting of 67 pairs of tumor tissues and corresponding adjacent normal tissues using qRT-PCR assay. It was found that the dysregulated expression level of miR-100, -145, and -182 was consistent with the microarray data. miR-100 and -145 expressions were stably downregulated, whereas miR-182 was upregulated in any tumor stage or tumor grade, judging from
the mean $\Delta\Delta C_t$ values of the 4 miRNAs (Fig. 1B). However, no significant association was found between their expressions in altered tumor stage or grade ($P > 0.05$), even though the mean $\Delta\Delta C_t$ values of both miR-100 and -145 in invasive tumors or high-grade tumors presented slightly lower than those in non–muscle-invasive or low-grade tumors (Fig. 1B). It was interesting to find that these 2 miRNAs were outstandingly downregulated in bladder cancer.

Table 1. The intersection set (10 miRNAs) between the 2 groups of significantly dysregulated miRNAs

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Tumor</th>
<th>Adjacent normal</th>
<th>Fold change</th>
<th>$P$</th>
<th>Tumor</th>
<th>Adjacent normal</th>
<th>Fold change</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-182</td>
<td>86.8</td>
<td>25.3</td>
<td>3.431</td>
<td>0.005865</td>
<td>98.9</td>
<td>23.3</td>
<td>4.245</td>
<td>4.27E-05</td>
</tr>
<tr>
<td>hsa-miR-20a</td>
<td>174</td>
<td>49.6</td>
<td>3.508</td>
<td>0.02246</td>
<td>176.3</td>
<td>59.9</td>
<td>2.943</td>
<td>8.31E-04</td>
</tr>
<tr>
<td>hsa-miR-106b</td>
<td>125.6</td>
<td>35.3</td>
<td>3.558</td>
<td>0.004262</td>
<td>99.5</td>
<td>42.1</td>
<td>2.363</td>
<td>0.003637</td>
</tr>
<tr>
<td>hsa-miR-210</td>
<td>77.5</td>
<td>23.3</td>
<td>3.326</td>
<td>0.045518</td>
<td>54.7</td>
<td>25.3</td>
<td>2.162</td>
<td>0.017549</td>
</tr>
<tr>
<td>hsa-miR-214</td>
<td>68.1</td>
<td>170.5</td>
<td>0.399</td>
<td>0.001812</td>
<td>107.8</td>
<td>216</td>
<td>0.499</td>
<td>0.020883</td>
</tr>
<tr>
<td>hsa-miR-145</td>
<td>311.4</td>
<td>726.2</td>
<td>0.429</td>
<td>0.032092</td>
<td>443.6</td>
<td>901.2</td>
<td>0.492</td>
<td>0.025808</td>
</tr>
<tr>
<td>hsa-miR-143</td>
<td>429.5</td>
<td>1074.6</td>
<td>0.4</td>
<td>0.008212</td>
<td>531.2</td>
<td>1331.5</td>
<td>0.399</td>
<td>0.008439</td>
</tr>
<tr>
<td>hsa-miR-125b</td>
<td>182.7</td>
<td>571.5</td>
<td>0.32</td>
<td>0.014452</td>
<td>228.1</td>
<td>619.1</td>
<td>0.368</td>
<td>0.004022</td>
</tr>
<tr>
<td>hsa-miR-99a</td>
<td>28.4</td>
<td>139.5</td>
<td>0.204</td>
<td>0.011457</td>
<td>54.1</td>
<td>156.9</td>
<td>0.345</td>
<td>0.002054</td>
</tr>
<tr>
<td>hsa-miR-100</td>
<td>82.2</td>
<td>233.8</td>
<td>0.352</td>
<td>0.03338</td>
<td>76.4</td>
<td>242.5</td>
<td>0.315</td>
<td>1.56E-04</td>
</tr>
</tbody>
</table>
Inhibitory Role of MiR-100 in Bladder Cancer

Although several studies (25, 26) clearly identified miR-145 as a tumor suppressor in bladder cancer, the role of miR-100 in bladder cancer pathogenesis was not further studied. It was found in our study that downregulation of miR-100 was almost ubiquitous (53 of 67 tumors, 79.10%) and obvious in bladder cancer (Fig. 1C) either from non-muscle-invasive stage to invasive stage or from low- to high-grade (Fig. 1C).

miR-100 suppresses bladder cancer cell proliferation in vitro and in vivo

To determine the impact of miR-100 on bladder cancer cell proliferation, we established EJ and 5637 transfectants stably expressing miR-100 using lentivirus infection. In addition, J82 and HT-1376 were transfected with miR-100 inhibitor and nonspecific control miRNA (NC). The expression levels of miR-100 were determined by qRT-PCR (Supplementary Fig. S1). The CCK-8 assays and colony formation assays revealed that overexpression of miR-100 significantly decreased bladder cancer cell proliferation (P < 0.05; Fig. 2A and B), whereas RNA interference–mediated silencing of miR-100 increased the cell growth ratio (P < 0.05; Fig. 2A).

To further confirm the above findings, 3 in vivo models were used. Tumors in the subcutaneous nude mouse model became palpable around 7 days in the control group, whereas those in the lenti-miR100–treated group were palpable about 2 weeks after inoculation. All the mice developed tumors at the end of the experiment (Fig. 2C). The growth rate of transplanted tumors was significantly different between the 2 groups in as early as 2 weeks after implantation (P < 0.01). At 4 weeks after implantation, mice injected with lenti-vector carried larger burdens. The mean tumor volume of the lenti-miR100–treated group reduced markedly by more than 70% as compared with the control (P < 0.001; Fig. 2C). No significant difference in body weight was found between the lenti-miR100–treated and the control mice (P = 0.056; Fig. 2C).

Three mice in the lenti-miR100–treated group of the intravesical orthotopic bladder cancer model, and all the mice in the control group developed tumors at the end of the experiment. Tumors became palpable around 3 weeks. At 5 weeks after implantation, the mean tumor volume of the lenti-miR100–treated group reduced markedly by more than 60% as compared with the control group (P < 0.001; Fig. 2C). No significant difference in body weight was found between the 2 groups (P = 0.212; Fig. 2C).

Tumors in the subcutaneous model using wild-type EJ cells for in vivo RNA delivery became palpable between 6 and 8 days, and all the mice developed tumors. At 3 weeks after intratumoral injection of cholesterol-conjugated agomiR-100, the mean tumor volume of the agomiR-100–treated group reduced significantly (P = 0.028; Fig. 2D) as compared with the control group. Taken together, our data indicate that miR-100 exerted an inhibitory effect on human bladder cancer growth.

mTOR is a direct target of miR-100

All the 10 miRNAs within the intersection set were rendered for systemic bioinformatic analysis. Totally, 2,208 target genes were found, and target genes of miR-100 included HS3ST2, SMARCA5, ZZZF1, EIF2C2, FRAP1 (mTOR), and BMPR2. The high-enrichment GOs targeted by the interested miRNAs are shown in Supplementary Fig. S2. On the basis of the TargetScan and GO analysis, the potential target gene mTOR (FRAP1), whose 3′-untranslated region (UTR) contains putative miR-100 complementary sites, was rendered for further study (Fig. 3A).

mTOR is a central element in the evolutionary conserved signaling pathway that regulates proliferation, cell growth, and survival, orchestrating signals originating from growth factors, nutrients, or particular stress stimuli. The AKT and ERK/MAPK signaling pathways are 2 important modulators of mTOR activity (27). On the basis of the network of genes that we built on the basis of the KEGG database, the network of genes within the mTOR pathway (mTOR-miR-Network) was finally built (Fig. 3B). miR-100, -125b, -182, -143, -106b, -20a, -214, -143, and -145 target their corresponding gene(s) within this pathway directly or indirectly, in which miR-100 plays a central role among them (Fig. 3B).

When confirming whether mTOR is a bona fide target of miR-100, the relative luciferase activity of the reporter containing wild-type mTOR 3′-UTR was significantly suppressed when miR-100 was cotransfected. In contrast, the luciferase activity of the reporter containing the mutant miR-100–binding site was unaffected, indicating that miR-100 may suppress gene expression through miR-100–binding sequences at the 3′-UTR of mTOR (Fig. 3A).

To find out the relationship between miR-100 expression and mTOR mRNA amplification level, 10 bladder cancer cell lines were examined by qRT-PCR. Pearson correlation test indicated that there was an inverse correlation between miR-100 and the mTOR mRNA level (r = −0.579, P < 0.05; Fig. 3C).

Western blotting and qRT-PCR were carried out to examine the effect of overexpression or knockdown of miR-100 on the mRNA and protein levels of mTOR in 2 bladder cancer cell lines. mTOR mRNA and protein expressions were significantly decreased in EJ, 5637, and J82 cells after transfection with pre-miR-100 (Supplementary Fig. S3). Meanwhile, inhibition of endogenous miR-100 by miR-100 inhibitor resulted in upregulated expression of mTOR in 5637 and J82 cells (Supplementary Fig. S3). In addition, EJ and 5637 cells were transduced with lenti-miR-100 at 3 different multiplicity of infection (MOI) of 10, 40, and 100 to examine mTOR expression level. As shown in Fig. 3D, ectopic expression of miR-100 decreased mTOR and p70S6K (p70 S6 kinase-1, its direct downstream gene, important in cellular growth) protein levels in a dose-dependent manner. At MOI of 100, the mTOR protein level decreased by approximately 60% to 70% as compared with that of MOI of 10. These results indicate
that miR-100 may act as a tumor suppressor through downregulating the expression of mTOR in bladder cancer cells.

Cell cycle and apoptosis

EJ cells were transfected with pre-miR-100, si-mTOR, or control RNA (50 nmol/L), collected after 48 hours, and...
stained with propidium iodide (PI) for analysis of the cell cycle by fluorescence-activated cell sorting (FACS). It was found that cells in miR-100–treated group and si-mTOR–treated group were arrested in the G0–G1 phase. This was accompanied by a corresponding decrease in the fraction of cells in the S-phase (Fig. 4A). However, no evidence of apoptosis was seen in any of the 3 groups as tested by Annexin V assays 48 hours after cell treatment (Fig. 4B). Similarly, 5637 cells were infected by lenti-miR-100 or lenti-vector, and cell-cycle assay was done at 72 hours. It was also found that cells infected with lenti-miR-100 were arrested in the G0–G1 phase, compared with the lenti-vector–infected cells (Fig. 4C). In addition, these cells were retreated with doxorubicin (0.15 μg/mL) 72 hours after 5637 infection by lentivirus at MOI of 40. The results of Annexin V assays conducted 24 and 36 hours after doxorubicin treatment showed that overexpression of miR-100 induced by pretreatment with lenti-miR-100 made 5637 cells more sensitive to the exposure of doxorubicin setting and enhanced the cell apoptotic effect of doxorubicin (Fig. 4D).

Overexpression of miR-100 reduces tumor cell migration and invasion

The result of the scratch/wound-healing assay showed that lenti-miR-100 treatment did reduce migration of EJ and 5637 cells (Fig. 5A and B). Furthermore, miR-100 upregulation, like knockdown of mTOR gene, reduced the invasive ability of the aggressive EJ or J82 cells by approximately 30% compared with control cells (P < 0.01), whereas inhibition of miR-100 could gently enhance the invasive ability (P < 0.05; Fig. 5C and D).

The cancerous metastatic model further suggests the function of miR-100 and mTOR

All the 3 mice receiving intravesical 921T implantation developed bladder tumors, including one that developed bilateral kidney metastases (without ureter metastasis) 20 days after the orthotopic tumor became palpable (Fig. 6A). The metastatic tumor tissues of the kidneys in the mouse showed similar AE1/AE3 expression when compared with the primary bladder cancer tissue of the patient, suggesting that they were of epithelial origin (Fig. 6B). Cells from the metastases of the
right kidney were obtained and cultured, which were named 921T-2.

All the 3 mice receiving intravesical 921T-2 implantation developed tumors. Two of them also developed metastatic tumors, including one with simultaneous liver and kidney metastases, and the other only with retroperitoneal nodal metastases (Fig. 6A). Cells derived from these metastatic foci were cultured. It was found that these metastatic cells presented characteristic morphologic features compared with 921T cells (Fig. 6A). They were usually large with abundant granular cytoplasm and/or intracytoplasmic vacuoles, suggesting that they had some cytoplasmic features of glandular differentiation. Most nuclei were large with more than 2 prominent nucleoli. Another distinctive feature was the presence of spindled and/or racquet-shaped malignant cells.

921T-2 cells and metastatic bladder cancer cells from the liver, kidney, and retroperitoneal lymph nodes were collected and assayed for miR-100 and mTOR expression by qRT-PCR. As shown in Fig. 6C, miR-100 was more than 5-fold downregulated in all the 3 metastatic bladder cancer cells (P < 0.01), whereas mTOR expression was upregulated correspondingly (P < 0.01), compared with that in 921T-2 cells. Especially, mTOR was 8.3-fold upregulated in liver metastatic cells. In addition, Western blotting assay on these cells suggested that protein levels of mTOR and p70S6K were higher both in liver and in kidney metastatic cells (Fig. 6D).

Figure 4. Cell cycle and apoptosis monitored by flow cytometry. A and B, cell cycle and apoptosis distribution tested 48 hours after transfection of EJ cells with pre-miR-100, si-mTOR, or control RNA. C, cell-cycle distribution tested 48 hours after transfection of 5637 cells infected with lentivirus at MOI of 40, cells were retreated with doxorubicin and Annexin V assays were conducted in 24 and 36 hours. FITC, fluorescein isothiocyanate.
Discussion

Recent advances have suggested that dysregulation of miRNAs is a common event in human cancers, including bladder cancer. miR-182 (28, 29), -20a (30), -106b (31), -210 (32), -214 (33), -143 (8, 34), -145 (25), and -125b (6) have been reported in bladder cancer (6, 8, 25, 29) and other cancers (28, 30–34), suggesting that these miRNAs may play common roles in tumorigenesis. A preliminary study (11) suggested that aberrant expression of miR-100 was almost ubiquitous in low-grade bladder cancer. However, no clear information about the function or molecular mechanism of miR-100 in human bladder cancer has been reported.

On the basis of our previous preliminary functional tests, this study focused on miR-100, finding that miR-100 was frequently downregulated in bladder cancer tissues and that miR-100 could pleiotropically inhibit cell growth and colony formation, suppress cell migration and invasion, induce G1 arrest in bladder cancer cells, and suppress tumorigenesis in nude mice models of bladder cancer xenograft with 2 different approaches (Fig. 2), suggesting that miR-100 may act as a potential tumor suppressor in bladder cancer. This is similar to the findings in lung cancer (35), cervical cancer (13), and ovarian cancer (36), in which miR-100 was downregulated, and ectopic expression of miR-100 suppressed cell proliferation and cycle. Interestingly, as predicted from Fig. 1, there was a potential trend that the expression level of miR-100 in low-grade/stage adjacent normal tissues seemed higher than that in high-grade/stage samples (P = 0.112 and 0.489, respectively). The mean ΔΔCt value of miR-100 in high-grade/stage tissue pairs presented slightly lower than that in low-grade/stage pairs (P > 0.05). Further studies with more clinical samples are warranted to validate the speculation that the microenvironment of miRNA level may be altered.

Figure 5. Overexpression of miR-100 significantly reduces cell migration and invasion. A and B, the scratch migration assay. Serial photographs were obtained at indicated times posttransfection in EJ and 5637 cells. C and D, invasion rates were determined by counting the number of cells invading through Matrigel. Magnification, ×100. *, P < 0.05; **, P < 0.01.
and miR-100 may represent a more downward expression trend in adjacent noncancerous bladder tissues of more aggressive bladder cancer cases, although it remains normal in histopathologic examinations.

Our in vivo data from the tumor metastasis model established with primary bladder cancer cells suggest that altered expression of miR-100 played a role in bladder cancer cellular metastasis. Downregulated expression of miR-100 was implicated with nodal/distant metastasis. However, recent studies (37, 38) showed that miR-100 was amplified and a high level of miR-100 was related to biochemical recurrence of localized prostate cancer in patients. These controversial results suggest that the role of miR-100 is possibly tumor-specific and highly dependent on its targets in different cancer cells. Indeed, the tissue- and time-dependent expression of miRNAs may

Figure 6. The cancerous metastatic model further suggests the function of miR-100 and mTOR. A, top, one mouse developed orthotopic tumors and bilateral kidney metastases 20 days after intravesical 921T implantation. After intravesical 921T cell implantation, one mouse developed liver and kidney metastases simultaneously and the other with retroperitoneal nodal metastases. Bottom, characteristic morphologic features of metastatic cells obtained from the live and retroperitoneal nodes compared with 921T cells. B, AE1/AE3 expression detected by immunohistochemistry in the metastatic tumor tissues of the kidneys and the primary bladder cancer tissue (921T-originated) of the patient. C, miR-100 expression downregulated in all the 3 metastatic bladder cancer cells (P < 0.01), whereas mTOR upregulated correspondingly (P < 0.01) compared with that in 921T-2 cells. D, Western blotting assay suggested that protein levels of mTOR and p70S6K were higher both in liver and in kidney metastatic cells. Magnification, ×200.
influence protein translation during distinct cellular processes, and the aberrant expression of their target genes may affect different biologic pathways with diverse functions (39).

It was reported that mutation or overexpression of FGFR3 gene occurs in approximately 80% patients with NMIBC. It can activate the Ras kinase signaling pathway, leading to increased proliferation and motility. In contrast, MBCs mainly contain mutations in TP53 gene. Thus, a well-established 2-pathway model of bladder cancer has been established (40). In recent years, mTOR, a key downstream effector of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, has been recognized to play a crucial role in controlling cancer cell growth (41). AKT and mTOR function as “master switch” proteins in cancer cells to modulate metabolism, cell cycle, and apoptosis (42). Inhibition of mTOR results in a wide variety of effects on normal and malignant cells, including induction of apoptosis and inhibition of cell-cycle progression, cell growth, angiogenesis, endothelial cell proliferation, and protein translation. As a hot research point, the mTOR signaling pathway has also been increasingly implicated in bladder cancer tumorigenesis (43, 44).

On the basis of the systemic bioinformatic analysis, the mTOR-miR network that we built showed that miR-100 played a central role in this pathway (Fig. 3B). Then, we showed that miR-100 inhibited mTOR expression in a dose-dependent manner and confirmed that mTOR was a direct target of miR-100 in bladder cancer cells (Fig. 3). Knockdown of mTOR induced cell growth inhibition and G1 phase arrest without stimulating apoptosis, which was similar to the phenotypes induced by miR-100 restoration (Fig. 4). These results suggest that the inhibitory effect of miR-100 on bladder cancer cell growth was mediated by repressing mTOR expression and was attributed to the regulation of cell-cycle progression, particularly at the G1 to S-transition. This may occur by blocking the downstream messenger, p70S6K, and preventing the translation of some key mRNAs required for cell-cycle progression (45). Our results expand the horizon of previous preclinical studies on the role of the mTOR signaling pathway in bladder cancer (43, 44). Particularly, rapamycin and its derivative everolimus (RAD001) have been noted to inhibit bladder cancer cell proliferation and induce G0-G1-cell-cycle arrest without apoptosis in bladder cancer cell lines. The efficacy of mTOR inhibition on bladder cancer proliferation and tumor growth has also been shown previously in mouse xenografts (44).

Increasing evidence has shed new insights into the role of mTOR in the regulation of cell motility (46). It is known that mTOR functions as 2 complexes, mTORC1 and mTORC2. Earlier studies have shown the participation of mTORC2 signaling in cytoskeletal events and cell movement (47). Recent studies (48, 49) have further shown that rapamycin-sensitive mTORC1 also plays a crucial role in cell regulation by phosphorylating 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and p70S6K. Both mTORC1-mediated p70S6K and 4E-BP1 pathways are involved in the regulation of cell motility. P70S6K regulates cell motility, probably by regulating phosphorylation of the focal adhesion proteins (48). However, little is known about how 4E-BP1 pathway regulates cell motility. We found that knockdown of mTOR reduced the cell-invasive ability similar to the phenotype as shown by miR-100 overexpression (Fig. 5). In addition, data from the cancerous metastatic model further suggest that miR-100 and mTOR regulated cell motility and were associated with tumor metastasis. Both mTOR and p70S6K presented higher expression levels in distant metastatic foci such as in liver and kidney metastases.

Taken together, this study identified miR-100 as a growth-suppressive miRNA in human bladder cancer, at least partly, through repression of mTOR. Our data provide further evidence that miRNAs play a pivotal role in bladder cancer development and progression. As miR-100 is downregulated in bladder cancer, reintroduction of this mature miRNA into tumor tissue may prove to be a therapeutic strategy by reducing the expression of target genes. Although miRNA-based therapeutics is still in their infancy, our findings on miR-100 are encouraging and suggest that this miRNA could be a potential target for the treatment of bladder cancer in future.

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

Authors' Contributions
Conception and design: C. Xu, Q. Zeng, Y. Sun
Development of methodology: C. Xu, Q. Zeng, Y. Cheng, C. Wu, Y. Sun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q. Zeng, W. Xu, T. Jin, A. Pan, R. Wei
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Zeng, L. Jiao, Z. Zhang, A. Pan, R. Wei
Writing, review, and/or revision of the manuscript: Q. Zeng, L. Jiao, C. Wu, B. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Cheng, Y. Sun
Study supervision: C. Xu, Y. Sun

Grant Support
This work was supported by the National Natural Science Foundation of China (grant no. 81172425 to C. Xu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 15, 2012; revised November 9, 2012; accepted December 3, 2012; published OnlineFirst December 27, 2012.

References


Molecular Cancer Therapeutics

miRNA-100 Inhibits Human Bladder Urothelial Carcinogenesis by Directly Targeting mTOR

Chuanliang Xu, Qinsong Zeng, Weidong Xu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0273

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/01/02/1535-7163.MCT-12-0273.DC1

Cited articles
This article cites 48 articles, 10 of which you can access for free at:
http://mct.aacrjournals.org/content/12/2/207.full.html#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.