MicroRNA-325 Is a Potential Biomarker and Tumor Regulator in Human Bladder Cancer

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Abstract
Purpose: We evaluated whether human microRNA-325 may be a potential biomarker and tumor regulator in bladder cancer.
Methods: Human microRNA-325 expression was probed by quantitative real-time polymerase chain reaction in both in vitro bladder cancer cell lines and in vivo bladder carcinoma tissues retrieved from patients with cancer. The prognostic potential of human microRNA-325 in predicting postoperative overall survival of patients with bladder cancer was estimated. Endogenous human microRNA-325 was overexpressed by lentiviral transduction in bladder cancer cell lines, T24 and 5637 cells. The tumor regulatory effects of human microRNA-325 upregulation on T24 and 5637 cells were evaluated both in vitro and in vivo. Results: Human microRNA-325 was aberrantly downregulated in both bladder cell lines and human bladder carcinomas. Lowly expressed human microRNA-325 in bladder carcinoma was closely associated with poor postoperative overall survival of patients with cancer. In T24 and 5637 cells, virally transduced cells had markedly upregulated human microRNA-325 expressions. Biochemical assays demonstrated that human microRNA-325 upregulation in bladder cancer had tumor-suppressive functions by decreasing cancer proliferation, cisplatin chemoresistance, and cancer migration in vitro and hindering transplantation growth in vivo and cell cycle transition. Conclusion: Human microRNA-325 is lowly expressed and may serve as a potential prognostic biomarker in human bladder cancer. After further validation, human microRNA-325 may be a novel therapeutic target for suppressing carcinoma in patients with bladder cancer.

Keywords
bladder cancer, miR-325, prognosis, overall survival, proliferation, cisplatin

Abbreviations
ATCC, American Type Culture Collection; FBS, fetal bovine serum; hsa-miR-325, human microRNA-325; hsa-miR325-M, human microRNA-325mimics; hsa-miR-NC, human miRNA-mimics oligonucleotides; miRNA, microRNA; qPCR, quantitative real-time polymerase chain reaction.

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Introduction
Bladder cancer is one of the most commonly diagnosed geriatric cancers in the world. In developed countries such as the United States, nearly 80,000 new cases were diagnosed, and approximately 17,000 patients died of urinary bladder cancer every year.1 Especially in male patients, the incidence and mortality rates of bladder cancer were almost 4 times higher than in female patients.1 In developing countries such as China, the estimated annual number of new bladder cancer cases was similar to the number in the United States, about 80,500.2 However, the estimated bladder cancer-associated deaths among Chinese patients were almost as double as those in United States, nearly 33,000 every year.2 In addition, while systemic chemotherapy (typically platinum-based) and immunotherapy with immune checkpoint blockade (anti-Programmed cell death-1 [PD-1]/Programmed death-ligand 1

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[PDL1] are commonly used for the management of advanced/metastatic bladder cancer, clinical outcomes remain poor and improvements are much needed.3-5 Thus, it is critical to understand the molecular networks underlying human urinary bladder cancer and to identify new prognostic biomarkers and develop novel therapeutic strategies to improve the clinical outcomes of patients with bladder cancer.

MicroRNAs (miRNAs) are families of short (18-22 nucleotide long) noncoding RNAs that bind to the 3'-untranslated region of targeted mRNAs, thus inhibit gene transcription and induce protein degradation in not only animal or plants but also humans.6-8 In human cancers, emerging evidence has demonstrated that miRNAs may be aberrantly expressed (either downregulated or upregulated) in various types of carcinomas and have critical roles in regulating human cancer oncogenesis, maturation, metastasis, or apoptosis.9-11 Specifically, previous studies had shown that groups of miRNAs could be either upregulated or downregulated in either patient's sera or carcinoma tissues, thus may serve as efficient biomarkers for diagnostics and prognostication in bladder cancer.12,13 Thus, identifying aberrantly expressed miRNAs and revealing their functional roles are critical in understanding the epigenetic regulation of miRNA in human bladder cancer.

Of many of the potential prognostic miRNAs in human cancers, mature human microRNA-325, or hsa-miR-325, has been demonstrated to be aberrantly expressed in various carcinoma types, such as liver cancer, thyroid cancer, lung cancer, or head and neck cancer.14-18 However, little is known about the expression, prognostic implication, or mechanistic role of hsa-miR-325 in urinary bladder cancer. More interestingly, hsa-miR-325 was upregulated in some carcinoma types, such as head and neck cancer and squamous cell carcinoma of tongue,15,18 but downregulated in other carcinoma types, such as lung cancer, thyroid cancer, or liver cancer.14,17,18 Thus, it seems like there was no predominant or universal expression pattern of hsa-miR-325 in human cancers.

In this study, we firstly used quantitative real-time polymerase chain reaction (qPCR) to probe hsa-miR-325 expressions in both in vitro bladder cancer cell lines and in vivo bladder carcinoma tissues. We then used statistical analysis to evaluate the prognostic potential of hsa-miR-325 in predicting postoperative overall survival of patients with bladder cancer. Also, we used lentiviral transduction to endogenously overexpress hsa-miR-325 bladder cancer cell lines, T24 and 5637 cells. The corresponding tumor regulatory effects of hsa-miR-325 upregulation on T24 and 5637 cells were further examined in both in vitro and in vivo assays.

Materials and Methods

Ethics Statement

The clinical and laboratory protocols of the current study were thoroughly reviewed and approved by the Clinical Research Boards and Ethic Committees at The Affiliated Hospital of Qingdao University in Qingdao (#170540091) and Liaocheng People’s Hospital in Liaocheng (#173207737), China. All experiments were performed in accordance with the Declaration of Helsinki. All participating patients, prior to the enrollment of this study, signed consent forms.

Bladder Carcinoma Cells and Clinical Tissues

In our study, bladder cancer carcinoma cell lines, T24, RT4, 5637, HT-1376, J82, UM-UC-3, and TCCSUP, were purchased from American Type Culture Collection (ATCC, Manassas, Virginia). In addition, 2 noncarcinoma urinary bladder cell lines, HT-1197 and HS228.T, were also purchased from ATCC. All cells were cultured in 6-well plates in a culture medium consisting of minimum essential medium (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin (Invitrogen), and 0.1 mg/mL streptomycin (Invitrogen). The 6-well plates were maintained in a humidified tissue culture chamber with 95% O2/5% CO2 at 37°C.

Clinical samples, including carcinoma or noncarcinoma (normal) bladder epithelial tissues, were obtained from 164 patients with bladder cancer who received transurethral resection of bladder tumor at The Affiliated Hospital of Qingdao University or Liaocheng People’s Hospital during the time between June 2008 and March 2012. Among them, 42 patients also received partial or radical cystectomy. Staging of patients’ tumors was performed according to the 7th edition of the International Union Against Cancer and the American Joint Committee on Cancer.20 The medium postoperative follow-up time was 61.2 ± 5.4 months. All clinical tissues, upon retrieval, were immediately snap-frozen in liquid nitrogen and stored at −70°C prior to RNA extraction.

RNA Extraction and qPCR

Total RNA was extracted from bladder cancer cell lines or clinical tissues using a mirVana miRNA Isolation Kit (Invitrogen) according to the manufacturer’s recommendation. For each sample, a total of 200 ng purified RNA was used to convert to complementary DNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) according to the manufacturer’s recommendation. Detection of mature hsa-miR-325, or qPCR, was performed using a TaqMan MicroRNA Assay (Applied Biosystems) on an ABI automated 7800HT sequence detection system (Applied Biosystems) according to the manufacturer’s recommendation. U6 small nuclear RNAs (snRNAs) was used as internal control and relative hsa-miR-325 expression level was characterized using the 2−DDCt method.

Human microRNA-325 Upregulation Assay

We used lentiviral transduction to upregulate endogenous hsa-miR-325 in bladder cancer cell lines, T24 and 5637 cells. A lentivirus containing the synthetic oligonucleotides of hsa-miR-325 mimics (hsa-miR325-M) and a lentivirus containing the synthetic oligonucleotides of hsa-miR-325 bladder cancer cell lines, T24 and 5637 cells. The clinical and laboratory protocols of the current study were thoroughly reviewed and approved by the Clinical Research Boards and Ethic Committees at The Affiliated Hospital of Qingdao University in Qingdao (#170540091) and Liaocheng People’s Hospital in Liaocheng (#173207737), China. All experiments were performed in accordance with the Declaration of Helsinki. All participating patients, prior to the enrollment of this study, signed consent forms.

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miR-NC) were purchased from Sunbio (Sunbio Technology, Beijing, China). Two of the examined bladder cancer cell lines, T24 and 5637 cells, were transduced with hsa-miR-NC or hsa-miR325-M, in the presence of polybrene (8 μg/mL, Invitrogen), at multiplicity of infection of 15 for 48 hours. After removing floating dead cells, the attached cells were further selected by blasticidin (2 mg/mL, Sigma-Aldrich, St Louis, Missouri) for 72 hours. Then, healthy colonies were lifted off, mixed, and cultured in a new 6-well plate in fresh culture medium (without lentivirus or selection reagent). After 3 passages, qPCR was performed to evaluate hsa-miR-325 expression in virally transduced T24 and 5637 cells.

**In Vitro Proliferation Assay**

In vitro proliferation of bladder cancer cells was evaluated using a Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, Oregon) according to the manufacturer’s recommendation. Briefly, virally transduced T24 and 5637 cells were lifted off from a 6-well plate and recultured in a 96-well plate (3000 cells/well). For a total length of 120 hours, MTZ (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay medium was mixed into tested wells every 24 hours, followed by brief treatment with SDS-HCl medium. Relative proliferation rate for each tested well was characterized using a Bio-Tek microplate reader (ELX800 Universal; Bio-Tek, Tucson, Arizona) at absorbance of 540 nm.

**Cisplatin Chemoresistance Assay**

Virally transduced T24 and 5637 cells were lifted off from a 6-well plate and recultured in a 12-well plate (30 000 cells/well). Once cells reached ~85% confluence, they were treated with cisplatin (Sigma-Aldrich) at concentrations (μM) of 0.01, 0.1, 0.2, 0.5, 1, 2, and 5. Forty-eight hours later, cisplatin was removed and cells were evaluated using an alamarBlue Cell Viability Assay (Invitrogen) according to the manufacturer’s recommendation. For each tested well, luminescence was measured using a Bio-Tek microplate reader (ELX800 Universal; Bio-Tek) at absorbance of 490 nm. Relative cell viability was then characterized by normalizing luminescence of each tested well against the luminescence in control wells (treated with 0.01 μM cisplatin).

**In Vivo Transplantation Assay**

Virally transduced 5637 cells were subcutaneously injected into left flanks of 6-week-old male athymic nu/nu mice. One group of mice received hsa-miR-NC-transduced 5637 cells and the other group received hsa-miR325-M-transduced 5637 cells (3 million cells/injection, n = 6 for each group). Cancer-cell-bearing mice were then provided with ample food/water and put on a 12 hour/12 hour day/night cycle for 5 weeks. Each week, in vivo tumor sizes of subcutaneous 5637 transplants were measured using a formula, \( v = \frac{(l \times w^2)}{2} \), where \( v \) represents volume (mm\(^3\)), \( l \) represents length (m), and \( w \) represents width (m) of subcutaneous tumor. After 5 weeks, mice were killed. The 5637 transplantations were extracted and examined under a broad-light microscope.

**Cell Cycle Assay**

Virally transduced T24 and 5637 cells were lifted off and processed with centrifugation. Pellets were quickly fixed in 70% ethanol and resuspended in PBS solution containing 1 mg/mL RNase and 50 μg/mL propidium iodide for 30 minutes at room temperature. Percentages of cancer cells at G0/G1, S, and G2/M cell cycle phases were measured using a fluorescence-activated cell sorter (FACS; BD, San Jose, California) according to the manufacturer’s recommendation.

**Statistical Analysis**

In our study, all procedures to generate mean values were independently repeated for at least 3 times. Data were then shown as mean ± standard error of mean. A Windows-based SPSS software (version 13.0, SPSS, Chicago, Illinois) was used to perform statistical analysis. The postoperative overall survival of patients with bladder cancer was recorded using the Kaplan-Meier method. Statistical difference between patients with high and low hsa-miR-325 expressions was analyzed by log-rank test. Other data were analyzed using a 2-tail, unpaired Student t-test. Significant difference was defined if \( P < .05 \).

**Results**

**Human microRNA-325 Is Lowly Expressed in Human Bladder Cancer**

In our study, we used qPCR to evaluate whether there was an aberrant expressing pattern of hsa-miR-325 in human bladder cancer. Firstly, we examined several in vitro human bladder cancer cell lines, including T24, RT4, 5637, HT-1376, J82, UM-UC-3, and TCCSUP. Their endogenous hsa-miR-325 expressions were compared against a noncarcinoma urinary bladder cell line, HT-1197. The result of qPCR showed that hsa-miR-325 was universally downregulated, or lowly expressed, in all tested human bladder cancer cell
Endogenous hsa-miR-325 in Bladder Tumor May Predict Overall Survival of Patients With Cancer

We then evaluated the aberrant expression pattern, or downregulation, of hsa-miR-325 in bladder tumors may be associated with clinical outcome of patients with cancer. According to the qPCR result of endogenous hsa-miR-325 expression levels in carcinoma tissues of patients with bladder cancer, they were separated into 2 groups. One group of patients had bladder carcinomas whose endogenous hsa-miR-325 expression levels were higher than mean value. The other group of patients had bladder carcinomas whose endogenous hsa-miR-325 expression levels were lower than mean value. Then, those patients’ postoperative overall survival was followed up using the Kaplan-Meier method and compared between 2 groups using the log-rank test. It showed that, the group of patients with bladder cancer, whose carcinomas had low endogenous hsa-miR-325 expression levels, had significantly worse overall survival than the other group of patients, whose carcinomas had high endogenous hsa-miR-325 expression levels (Figure 2, \( P = .0114 \), log-rank test).

**Figure 1.** Human microRNA-325 (hsa-miR-325) is downregulated in bladder cancer cells and human carcinomas. A, Quantitative real-time polymerase chain reaction (qPCR) was used to evaluate endogenous hsa-miR-325 expression levels in *in vitro* bladder cancer lines, T24, RT4, 5637, HT-1376, J82, UM-UC-3, and TCCSUP, which were compared to endogenous hsa-miR-325 expression level in a noncarcinoma urinary bladder cell line, HT-1197 (*\( P < .05 \)). In addition, hsa-miR-325 expression was evaluated in another noncarcinoma urinary bladder cell line, HS228.T, also compared to HT-1197 (\( \Delta P > .05 \)). B, In *in vivo* clinical tissues extracted from patients with bladder cancer, qPCR was also used to compare endogenous hsa-miR-325 expressions between noncarcinoma (normal) and carcinoma bladder epithelial tissues (*\( P < .05 \)).
Overexpressing hsa-miR-325 Decreased Proliferation, Cisplatin Chemoresistance, and Migration in Bladder Cancer

We then suspected that hsa-miR-325 might have a mechanistic role in human bladder cancer. To evaluate this hypothesis, we transduced human bladder cancer cell lines, T24 and 5637 cells, with a hsa-miR-325 overexpressing lentivirus, hsa-miR325-M. In controlled transduction, cells were transduced with a nonspecific human miRNA-mimics lentivirus, hsa-miR-NC. After viral transduction was stable, qPCR demonstrated that endogenous hsa-miR-325 expressions were significantly upregulated in T24 or 5637 cells transduced with hsa-miR325-M, rather than in cells transduced with hsa-miR-NC (Figure 3A, *P < .05).

Virally transduced T24 and 5637 cells were lifted off from 6-well plate culture and plated in a new 96-well plate. Their growth rates were evaluated by an in vitro proliferation assay for 5 consecutive days. It showed that cancer proliferation rates were significantly decreased in bladder cancer cells transduced with hsa-miR325-M, rather than in cells transduced with hsa-miR-NC (Figure 3B, *P < .05).

In addition, virally transduced T24 and 5637 cells were reevaluated on their chemoresistance to cisplatin. T24 and 5637 cells were treated with cisplatin at concentrations ranging from 0.01 to 5 μM. Forty-eight hours later, cisplatin was removed. An in vitro viability assay showed that cisplatin chemoresistance was significantly decreased in bladder cancer cells transduced with hsa-miR325-M, rather than in cells transduced with hsa-miR-NC (Figure 3C, *P < .05).

Moreover, migrating capability was evaluated in virally transduced T24 and 5637 cells. Using a 24-well Transwell migration assay, the imaging results demonstrated that considerably less bladder cancer cells migrated while they were transduced with hsa-miR325-M, rather than those transduced with hsa-miR-NC (Figure 3D). Measurement on relative migration showed that cancer migrating capabilities were significantly inhibited in bladder cancer cells transduced with hsa-miR325-M, rather than in cells transduced with hsa-miR-NC (Figure 3E, *P < .05).

Overexpressing hsa-miR-325 Inhibited In Vivo Growth of Bladder Cancer Transplantation and Cell Cycle Transition

We also evaluated the effect of hsa-miR-325 on in vivo growth of bladder cancer cells. Virally transduced 5637 cells were subcutaneously injected into left flanks of male athymic nu/nu mice. Tumor transplantations were evaluated weekly, by calculating their in vivo volumes for 5 consecutive weeks. It showed that subcutaneous tumor sizes were significantly decreased in mice bearing hsa-miR325-M-transduced 5637 cells, rather than in mice bearing hsa-miR-NC-transduced 5637 cells (Figure 4A, **P < .05, 1-way analysis of variance). Five weeks later, tumor-bearing mice were killed and 5637 transplantations were extracted. It demonstrated that overexpressing hsa-miR-325 did markedly inhibit the in vivo growth of bladder cancer cells (Figure 4B).

Moreover, cell cycle transition was evaluated in virally transduced T24 and 5637 cells. It showed that G0/G1 phases were significantly extended in bladder cancer cells transduced with hsa-miR325-M, rather than in cells transduced with hsa-miR-NC (Figure 4C, *P < .05).

Discussion

Mounting evidence has demonstrated that miRNAs are efficient biomarkers and effective tumor regulators in human urinary bladder cancer, thus holding promising implications in both clinical and therapeutic practices. As for hsa-miR-325, although it is implicated in other types of human carcinoma, neither its expression nor its function was characterized in human urinary bladder cancer. Thus, in this study, we firstly used qPCR to evaluate mRNA expression pattern of hsa-miR-325 in human urinary bladder cancer. By examining both the in vitro bladder cancer cells and the in vivo human bladder carcinoma samples, we discovered that there was an aberrant expression pattern or downregulation of hsa-miR-325 in bladder cancer. Previous publication had demonstrated that hsa-miR-325 was also aberrantly expressed in other human cancers. Interestingly, it was downregulated in carcinomas in lung, thyroid, or liver but upregulated in carcinomas in head and neck or tongue. The result of this study thus further confirms that the expression patterns of hsa-miR-325 in human cancers vary among different organs, and complex molecular
Figure 3. Human microRNA-325 (hsa-miR-325) upregulation has a tumor-suppressing effect on bladder cancer proliferation and chemoresistance. A, T24 and 5637 cells were transduced with a lentivirus containing the synthetic oligonucleotides of hsa-miR-325 mimics (hsa-miR325-M) or a lentivirus containing the nonspecific human miRNA-mimics oligonucleotides (hsa-miR-NC). After viral transduction was stabilized, quantitative real-time polymerase chain reaction (qPCR) was used to evaluate the endogenous hsa-miR-325 expressions in bladder cancer cells (*P < .05). B, Virally transduced T24 and 5637 cells were evaluated by an in vitro proliferation assay for 5 days. Relative proliferation rates of T24 and 5637 cells were measured at an absorbance of 540 nm (**P < .05, 1-way analysis of variance [ANOVA]). C, Virally transduced T24 and 5637 cells were treated with cisplatin at concentrations (μM) of 0.01, 0.1, 0.2, 0.5, 1, 2, and 5. Forty-eight hours later, relative cell survival was evaluated by an in vitro viability assay (***P < 0.05, 1-way ANOVA). D, Virally transduced T24 and 5637 cells were evaluated by an in vitro migration assay for 24 hours. Representative images of migrated cells are shown. E, Relative migrations of virally transduced T24 and 5637 cells were measured (***P < .05, 1-way ANOVA).
networks may be differentially associated with hsa-miR-325 in human cancers. However, caution may be taken to conclude the expression pattern of hsa-miR-325 in bladder cancer, as the in vivo normal tissues collected in this study may include heterogeneous cell populations.

Then, in this study, we evaluated whether hsa-miR-325 may have any clinical implication in human urinary bladder cancer. After patients with bladder cancer received surgeries, they were followed up to record their overall survival. We discovered that, for patients who had bladder carcinomas with low hsa-miR-325 expressions, their overall survival was significantly worse than patients who had bladder carcinomas with high hsa-miR-325 expressions. This result immediately suggests that hsa-miR-325 may be potentially used as a prognostic biomarker to predict bladder cancer. However, caution is needed before we can jump to this conclusion. First, to truly serve as a non-invasive biomarker, it is better to learn whether hsa-miR-325 may also be aberrantly expressed in patient’s circulating system, or sera. It would be much easier to draw blood samples, rather than perform surgery to extract bladder epithelial samples from patients with cancer. Second, much advanced statistical analyses are needed to confirm the correlation between bladder carcinoma’s hsa-miR-325 expression and clinical outcomes of patients with bladder cancer. It would be much better to learn whether aberrant expression pattern of hsa-miR-325 may be statistically associated with patients’ clinicopathological features or may be independently used a prognostic factor to predict patients’ clinical outcome.

Also in this study, we took further steps to ectopically overexpress hsa-miR-325 in bladder cancer cells and then evaluate the functional role of hsa-miR-325 upregulation on cancer development. Through lentiviral transduction, we successfully generated T24 and 5637 cells with stable hsa-miR-325 upregulation. Then, through several in vitro and in vivo assays, we showed that hsa-miR-325 upregulation had a significant tumor-suppressing effect in bladder cancer cells, by decreasing in vitro proliferation, cisplatin chemoresistance, migration, and cell cycle transition, as well as inhibiting in vivo growth of transplanted 5637 carcinoma. Upregulation of hsa-miR-325 was also shown to have anticancer effects in other human carcinomas, such as non-small cell lung cancer or liver cancer. However, our results provided first-ever and convincing
evidence that hsa-miR-325 may be an important cancer modulator in human urinary bladder cancer.

Conclusion
To conclude, this study demonstrated a novel finding that hsa-miR-325 was aberrantly expressed or downregulated in human urinary bladder cancer. Low expression of hsa-miR-325 was significantly correlated with clinical outcome of patients with bladder cancer, suggesting a potential biomarker role of hsa-miR-325 in predicting bladder cancer. In addition, this study showed that hsa-miR-325 was a functional tumor suppressor in bladder cancer. After further validation, hsa-miR-325 can be an interesting molecular candidate for targeted therapy for treating patients with bladder cancer.

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