

A Significant Upregulation of miR-886-5p in High Grade and Invasive Bladder Tumors

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Purpose: To investigate the expression alteration of miR-886-5p in bladder tumors and evaluating its expression level as a potential biomarker in this type of cancer.

Materials and Methods: Formalin-fixed paraffin-embedded (FFPE) samples of bladder tumors belonging to 70 patients whom had been referred to the Shahid Labbafi-Nejad medical center were obtained from the archival collection of pathology department. After RNA extraction and cDNA synthesis, expression levels of miR-886-5p were quantified by a real-time reverse transcription polymerase chain reaction (RT-PCR) approach.

Results: Our data revealed a significant upregulation (~3 times) of miR-886-5p in high grade bladder tumors, compared to the low grade ones ($P < .05$). Moreover, its expression level could significantly discriminate noninvasive (Ta, T1) from invasive (T2-T4) tumor stages.

Conclusion: Our data suggests a potential role for miR-886-5p in progression of bladder cancer.

Keywords: carcinoma; transitional cell; gene expression regulation; microRNAs; genetics; urinary bladder neoplasms.

INTRODUCTION

Bladder cancer is the most common form of malignancy in the urinary tract, however, its molecular pathogenesis is incompletely understood.⁽¹⁾ It develops in a multistep process with a variety of distinct biological and functional features.⁽²⁾ Conventional clinical and pathological parameters are widely used to classify bladder tumors with different grades and stages, and also to predict the clinical outcome of the disease. Nevertheless, the predictive ability of these parameters is limited.⁽³⁾ Therefore, there has been a great effort in the field to discover novel molecular pathways involved in bladder cancer, to improve its diagnosis, prognosis and treatment. microRNAs (miRNA) are small (~19-25 nucleotides) single-stranded RNA molecules, with an important role in post-transcriptional regulation of their targets via repressing gene translation or degrading target mRNAs. miRNAs are involved in development as well as in progression of a number of human cancers, including bladder cancer.⁽⁴⁻¹⁰⁾ Based on a vast number of profiling experiments, the miRNA signatures are tumor type- and tissue-specific. Moreover, the signature is often related to the grade and stage of the tumors. Thus, miRNA expression analysis could be used to classify tumors according to their grades of

malignancies. It has been recently proposed that the combined expression of stem cell associated factors and specific oncogenes could induce a non-differentiated state in cancer cells which can then progress into high-grade ones.⁽¹¹⁻¹³⁾ Strikingly, histologically poorly differentiated tumors display a preferentially elevated expression of genes normally enriched in embryonic stem (ES) cells.⁽¹¹⁾ These molecular markers, alone or in combination with conventional approaches, have the capacity to improve diagnosis, identifying patients who will respond to chemotherapy, and finding molecular targets for novel therapeutic interventions.⁽¹⁴⁻¹⁹⁾ Regarding the potential role of miR-886-5p in stem cell self-renewal, pluripotency and differentiation, we encouraged to investigate its potential expression in bladder cancer tissues, and also its potential expression alteration in tumors with different grades of malignancies.

MATERIALS AND METHODS

Sample Collection and Preparation

Formalin-fixed paraffin-embedded (FFPE) specimens of bladder cancer and associated patients' data were collected from Labbafi-Nejad hospital (Tehran, Iran). A total of 70 specimens were obtained from patients who had been undergone operations between

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Table 1. The sequence of primers used in this study.

Name	Primer	Primer Sequence (5' to 3')	PCR Product Length (nucleotides)
miR-886-5p	Stem-loop (for cDNA synthesis)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCCG CACTGGATACGACCCGCTT	
	Forward	CGGGTCGGAGTTAGCTCA	58
	Reverse	GTGCAGGGTCCGAGGT	
	RNU6	Forward	GAACGATACAGAGAAGATTAGC
	Reverse	GAATTGCGTGTCACTCCTTG	

Abbreviation: PCR, polymerase chain reaction.

2005 and 2011. The histopathological features of the samples were re-examined and confirmed by an expert pathologist (M.P.), according to the grading and TNM system for stage classification of the World Health Organization. Written informed consent had been obtained from all subjects prior to sampling. The samples with inadequate tissue size, incomplete clinicopathological information, unclear tumor cells from pathological view and multiple samples from recurrent patients were excluded from the study. The Medical Ethics Committee of Tarbiat Modares University approved the experiment design. FFPE blocks were cut into thin sections and prepared for RNA extraction.

RNA Extraction

Sections of 15 µm thickness were prepared from each FFPE specimen. Paraffin was removed by xylene (Merck KGaA, Darmstadt, Germany) treatment and tissues were washed out for three times with absolute ethanol (Merck KGaA, Darmstadt, Germany) to remove xylene. After drying, tissues were treated with proteinase K (Fermentas, Vilnius, Lithuania) at 56°C for 3 hours. The homogenized tissues were then employed for total RNA extraction, which was performed by acid guanidinium phenol chloroform procedure using Trizol solution (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA purity and quantity were assessed by means of spectrophotometry (Gene Quest), where A260/A280 and 230/260 ratios were used to monitor any potential contamination with genomic DNA and proteins.

DNase Treatment and cDNA Synthesis

To remove any possible genomic DNA contamination, total RNAs were treated with DNase I (Fermentas, London, UK) at 37°C for 30 minutes. Reverse transcription (RT) reaction was performed on 2 µg of purified total RNA by reverse transcriptase enzyme (Takara Holdings, Kyoto, Japan), as described previously. (20) RT reactions also contained 0.15 µM stem-loop RT primer (Table 1), 3 µM random hexamer (Macrogen Inc. Seoul, South Korea), and 1 × RT buffer (Takara Holdings, Kyoto, Japan). The 10 µL reactions were then incubated at 16°C for 30 minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes and then held at 4°C till being used. All RT reactions, including no-template and no-RT controls, were run in duplicate.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Quantitative real-time RT-PCR was performed using the ABI7500 System (Applied Biosystems, CA, USA).

The relative expression of miR-886-5p was assessed in comparison to U6 snRNA, as a reference internal control, using specific primers. All primers were designed as shown in Table 1. Real-time PCRs were performed in a final reaction volume of 20 µL including, 10 ng cDNA, 10 µL of SYBR Green I master mix (Takara Holdings, Kyoto, Japan), and 200 nM of forward and reverse primers, according to the manufacturer's instructions. The PCR reactions were carried out as follows: an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. Authenticity of the PCR products was examined by examining the sizes of the PCR products by polyacrylamide gel electrophoresis, as well as by inspecting the uniqueness of the products melt curves. To compensate for the inter-PCR variations, the expression of the target gene was normalized of that of endogenous control U6 snRNA. For this analysis, the comparative Ct (threshold cycle number) method (Δ Ct) was used.

Cloning and Sequencing of the Amplicons

Table 2. Demographic and clinical characteristics of the patients with bladder cancer.

Variables	Score
Age (minimum-maximum), years	38-87
Gender, No.	
Male	65
Female	5
Stage, No.	
Ta/T1	40
T2-T4	25
Undetermined	5
Grade, No.	
Low grade	28
High grade	35
Undetermined	7
Surgical procedure, No.	
TUR-BT	48
Radical cystectomy	22

Abbreviation: TUR-BT, transurethral resection of bladder tumor.

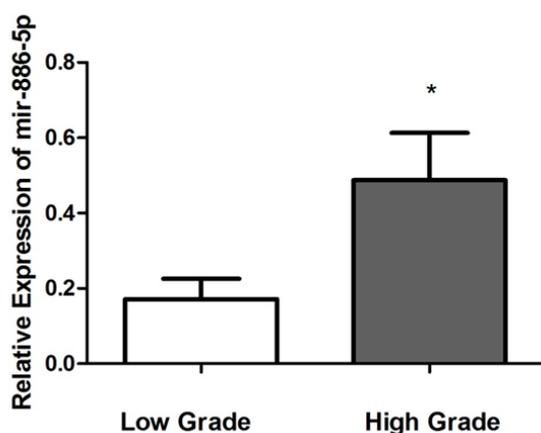


Figure 1. Comparing the expression level of miR-886-5p in bladder tumors with different grades of malignancies.

The amplified product of the real-time PCR with the expected size was cloned via the InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA). The vector was then amplified through transformation into DH5 α , and the isolated clones were then sent for direct sequencing (Macrogen Inc. Seoul, South Korea).

Statistical Analysis

The obtained data were statistically analyzed by Graphpad software (La Jolla, California, USA, www.graphpad.com). The data were presented as mean \pm standard deviation (SD) and the student unpaired *t*-test was used to determine the significance of the observed differences between different groups. A *P* value less than .05 was considered statistically significant. In addition, (ROC) Receiving Operating Characteristic curve analysis, with calculation of both the area under the curve and the corresponding 95% confidence intervals (CI), was used to assess the specificity and sensitivity with which the expression level of miR-886-5p could discriminate between low and high grade/stage tumors.

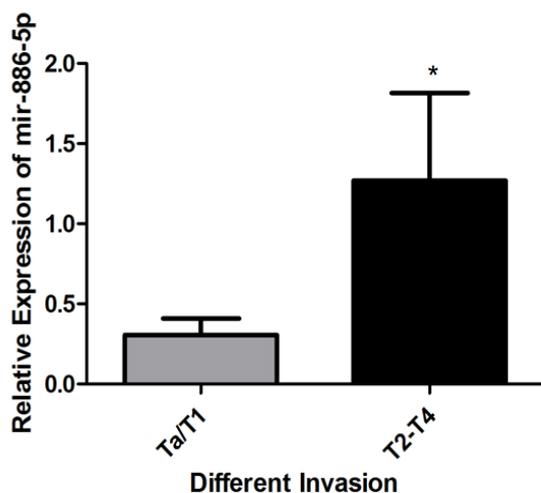


Figure 2. Comparing the expression of miR-886-5p in bladder tumors with different stages.

RESULTS

miR-886-5p is Upregulated in High Grade Bladder Tumors

To evaluate the expression alteration of miR-886-5p in different bladder tumors, we collected FFPE samples from 70 patients. The age of the patients was 38-87 years old (mean, 61 years), of whom there were 65 male and 5 female. All tumor types were transitional cell carcinoma, from which 35 were high grade, and 28 were low grade. Total RNA extraction and real-time PCR performed on all samples and the authenticity of the PCR products were confirmed by direct sequencing of the PCR products. All data were normalized to the expression of U6, as a house-keeping internal control. There were no non-specific products or primer-dimer peaks in melt-curve analysis by ABI-7500 PCR instrument. Analysis of gene expression among different grades of malignancies of bladder tumors revealed a significant upregulation (fold change: 2.84, *P* = .0187) of miR-886-5p in high grade tumors, compared to that of low-grade ones (**Figure 1**).

miR-886-5p Expression in Cancer Tissues with Different Stages

As shown in **Figure 2**, there was a significant difference in the expression level of miR-886-5p in tumors with different stages. Considering the invasiveness, bladder cancer can be categorized either as noninvasive (Ta/T1) or invasive tumors (T2-T4). Comparing the expression level in tumors with different stages revealed that miR-886-5p is significantly upregulated (with a fold change of 4.147, *P* = .036) in invasive tumors.

Analyzing the Validity of miR-886-5p as a Tumor Marker for Bladder Cancer

We used the ROC curve analysis to estimate the sensitivity and specificity by which the miR-886-5p expression level could discriminate between bladder tumors with different grades and stages. As depicted in **Figure 3**, ROC curve analysis yielded an AUC (the areas under the curve) of 0.742 (95% CI: 0.6198-0.8642) and 0.67 (95% CI: 0.535-0.799) for miR-886-5p to discriminate tumors with different stages and grades, respectively. An AUC > 0.70 indicates a good ability of a marker to discriminate two groups of samples.

DISCUSSION

In recent years, tremendous advances have been made in the discovery of new markers associated with alterations at the molecular level of bladder cancer. The studies have shown considerable clinical relevance in different areas such as tumor classification and prognosis. There are some studies which have been focused on expression analysis of some microRNAs involved in bladder cancer progression and tumor behavior. Saito and colleagues reported that miR-127 is down regulated in bladder carcinoma.⁽¹⁷⁾ Furthermore, Gottardo and colleagues⁽¹⁸⁾ identified 10 up regulated miRNAs in cancer samples such as miR-185, miR-203, miR-205, miR-221 and etc. In this study, we found a significant upregulation of miR-886-5p in high grade urinary bladder cancer tissues, compared to the low grade ones. In addition, the expression of miR-886-5p showed a significant elevation in invasive tumors, compared to the non-invasive samples. Employing the ROC curve analysis, we further discovered that this microRNA could po-

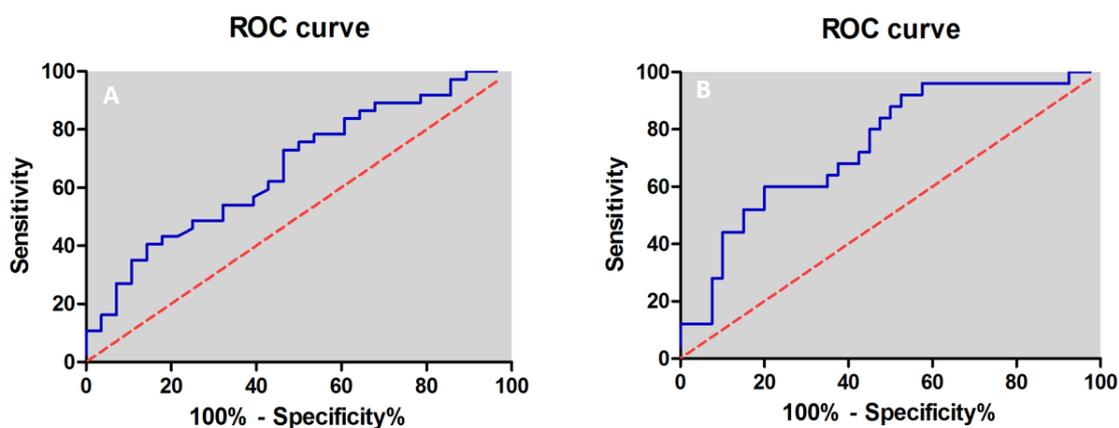


Figure 3. Receiver operating characteristic (ROC) curve analysis to examine the suitability of miR-886-5p expression to discriminate bladder tumors with: **A)** Different grades of malignancies with the areas under the ROC curve of 0.67 (95% CI: 0.535-0.799) and a *P* value of .02, with sensitivity of 73% and specificity of 54%. **B)** Different stages of progression with the areas under the ROC curve of 0.742 (95% CI: 0.6198-0.8642) and a *P* value of .001, with sensitivity of 60% and specificity of 80%.

tentially being used as a good tumor marker to discriminate between high and low grade bladder cancer, as well as for discriminating invasive bladder tumors from noninvasive ones. To the best of our knowledge, these findings are the first report on the expression of miR-886-5p in bladder cancer. Therefore, we could not compare our results with previously reported data. As another limitation to our study, we failed to collect enough normal bladder tissue to compare miR-886-5p expression between normal and tumor tissues. miR-886-5p is known as one of the miRNAs associated with the pluripotency state of stem cells. Wilson and colleagues reported the expression of miR-886-5p in human embryonic stem cells (hESC) and induced pluripotent stem (iPS) cells, where it is down regulated upon the induction of differentiation in both types of pluripotent cells.⁽¹⁵⁾ As indicated by Ben-Porath and colleagues a stem cell signature is present in poorly differentiated and high grade bladder tumors.⁽¹¹⁾ According to their findings, specific transcriptional regulators which are normally active in stem cells are overexpressed in poorly differentiated tumors arising in bladder. Based on our obtained data, we hypothesized that miR-886-5p regulate some pathways involved in progression, invasion and metastasis of bladder tumors, probably by down regulating the expression of some mRNAs functioning in preventing these pathways. An oncogenic role for miR-886-5p has already been provided by Li and colleagues' work in which miR-886-5p inhibits apoptosis of cervical cancer cells by down-regulating the expression of Bax.⁽¹⁶⁾ There are controversies in literatures on the exact nature of miR-886-5p. In some reports it has been designated as a Vault RNA or a non-coding RNA. Stadler and colleagues claimed that the sequence of this microRNA is a part of a longer RNA named VtRNA2.⁽¹⁹⁾ Later, Lee and colleagues introduced the pre-mir-886 as a 102-nucleotide long RNA which is abundantly presents in the cytoplasm of the cells with unique features which differs from those of a genuine pre-microRNAs or vault RNAs.⁽²¹⁾ They found a much lower percentage of mature miR-886-5p compared to its precursor form, pre-miR-886, in lung cancer cell lines. The later finding could be due to either a low rate of cleavage of pre-mir-886 into mature

miR-886-5p, or that, mature miR-886-5p is only a degradation intermediate of pre-mir-886. They also found that pre-mir-886 is suppressed in some cancer cell lines and clinical specimens, where it functions through regulation of protein kinase RNA-activate (PKR).⁽²¹⁾ Part of the aforementioned controversies arises from the innate differences in the strategies and methods used to amplify microRNAs, and most importantly how to discriminate mature form the precursor from. In the current study, we designed a stem-loop RT primer to specifically amplify the mature form of miR-886-5p. The stability of the stem-loop structure of the RT primer precludes its annealing to the pri- or pre-miRNA, due to a steric hindrance.^(22,23) Despite the fact that stem-loop primers are more difficult to design,⁽²³⁾ they are highly specific for amplifying mature miRNAs.^(24,25) The later claim was further reinforced by our direct sequencing of the real-time PCR products which proved the specific amplification of mature miR-886-5p. Regardless of the nature of amplified product in our study, as either a genuine miRNA or a piece of a longer non-coding RNA, its differential expression in tumors with different grades and stages is of great interest. Regardless of our findings on the potential role of miR-886-5p in progression and invasion of bladder tumor, more studies in other cancer types (especially tumor vs. non-tumor states of samples) are necessary in order to determine whether this short non-coding RNA is suitable as a molecular marker for diagnosis and prediction of prognosis of cancers.

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CONFLICT OF INTEREST

None declared.

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