Co-Regulated Expression of TGF-β Variants and miR-21 In Bladder Cancer

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Purpose: To investigate a potential alteration in the expression levels of transforming growth factor β (TGF-β) and miR-21 in bladder cancer tissues.

Material and Methods: Using real-time polymerase chain reaction (PCR) method, we examined a potential correlated expression of miR-21 and TGF-β variants in 30 bladder tumors and their marginal/non-tumor biopsy specimens obtained from the same patients.

Results: Our data revealed a significant down-regulation of TGF-β variants ($P = .03$) along with a non-significant alteration in the expression of miR-21 in tumor vs. non-tumor samples. However, in contrast to low-grade tumors, the expression of miR-21 was upregulated in high-grade ones, and the expression level can efficiently discriminate low-grade tumors from high-grade ones ($P = .03$).

Conclusion: In accordance to the observed similarity between TGF-β variants and miR-21 gene expression alterations in bladder tumors, treating 5637 bladder cancer cell line with TGF-β recombinant protein caused a significant upregulation of miR-21. The later finding further confirmed a correlated expression of TGF-β and miR-21 in bladder tumors.

Keywords: biological markers; analysis; urinary bladder neoplasms; miR-21; TGF-β; gene expression profiling

INTRODUCTION

Recently, several high-throughput studies have focused on delineating genomic changes and gene expression alterations in bladder cancer, in hope to find novel markers correlated to different grades and stages of the tumors.[1] The recent studies have also documented a link between the expression of microRNAs (miRNAs or miRs) and cancer pathogenesis. MiRNAs are a class of non-coding RNA molecules with 19–24 nucleotides in length. They contribute to cancer initiation and progression, and are differentially expressed in normal and tumor tissues.[2] MiRNAs exert their effects by silencing the expression of their target mRNAs via a perfect and/or an imperfect complementary base-pairing, causing either mRNA degradation or translational inhibition.[3] Almost 50% of human miRNA genes are located in fragile chromosomal regions, which are frequently amplified, deleted, or translocated in various cancers.[4] Based on accumulating data, some miRNAs can function as either tumor suppressors or oncogenes.[5,6]

Deregulations of miRNA expression have been already reported for several human cancers. For example, elevated expression of miR-21 has been implicated in the acquisition of invasive and metastatic properties of human cancer cells. MiR-21 functions as an oncomir, by targeting multiple tumor suppressor genes such as: PTEN, PDCD4, TPM1, and MASPIN.[7,8] Likewise, overexpression of miR-21 is linked to higher stages and lymph node metastasis in human breast cancer.[9]

MiRNA's biogenesis begins with the transcription of its gene, followed with the maturation of the primary transcript (pri-miRNA) into a hair-pin structure (pre-miRNA), and then processing of a ~20-24 nucleotides mature form. Based on a recent report, miR-21 was rapidly induced by the growth factors bone morphogenetic protein-4 (BMP-4) and transforming growth factor-β (TGF-β) in vascular smooth muscle cells.[10] These factors induced a rapid elevation of mature miR-21, by enhancing the processing of primary transcripts of miR-21 (pri-miR-21) into its precursor form (pre-miR-21).[10] The later finding suggests that the autocrine TGF-β signaling can contribute to an altered expression of miR-21 in cancer cells. TGF-β, a family of multifunctional homodimeric polypeptides, has three different isoforms in mammalian cells: TGF-β1, TGF-β2, and TGF-β3. The 25-kDa mature forms of all isoforms are structurally and functionally similar.[11] This family of cytokines can regulate cell growth, differentiation, inflammatory responses, apoptosis and extracellular matrix (ECM) production.[12] Because of its potent anti-proliferative effects on many cell types, such as endothelial and epithelial cells, TGF-β1 is regarded as a tumor suppressor gene.[11] However, an unexpected overexpression of TGF-β has been described in several tumor tissues, which demonstrated to be associated with the tumor progression and metastasis.[13-16]

In the present study, we employed a quantitative real-time polymerase chain reaction (PCR) method to investigate whether the expression level of TGF-β variants is altered in bladder tumor tissues. We also measured the expression level of mature miR-21, a direct target of TGF-β, in tumor and non-tumor specimens of bladder. We then investigated the possibility of any correlation between TGF-β and miR-21 expression levels in bladder cancer tissues.

Table 1. Clinicopathological characteristics of patients with bladder cancer

Variables	Value.
Number of patients	30
Age groups, years	
30-60	10
61-90	20
Mean age at diagnosis, year	63.8
Gender	
Male	28
Female	2
Number of specimens	
Tumor	30
Non-Tumor	30
Tumor stage at diagnosis	
PT_a-Pt_1	26
PT_2-PT_3	3
CIS	1
Tumor grade at diagnosis	
High grade	10
Low grade	20

MATERIAL AND METHODS

Table 2. The sequences of the designed primers and the PCR product sizes for each primer pairs

	Gene Bank access number	Forward primer sequence	Reverse primer sequence	Amplicon size
TGF-β1	NM_000660	5´-TGGCGATACCTCAGCAAC-3´	5´-ACCCGTTGATGTCCACTTG-3´	181-bp
TGF-β2	NM_001135599 (variant 1) NM_003238 (variant 2) 169 bp (variant 2)	5´-AGGAGCGACGAAGAGTACTAC-3´	5´-ACTCTGCTTTCACCAAATTG-3´	169 bp (variant 1)
TGF-β3	NM_003239	5´-TGTCCATGTCACACCTTTCAG-3´	5´-TGTGGTGATCCTTCTGCTTC-3´	145 bp
GAPDH	NM_002046	5´-GTGAACCATGAGAAGTATGACAAC-3´	5´-CATGAGTCCTTCCACGATACC-3´	123 bp

Patients and clinical samples

Thirty pairs of human tumor and non-tumor (apparently normal tissue biopsy from the same patients) specimens of bladder were obtained from Labbafi-Nejad hospital in Tehran, Iran. The samples had been immediately snap-frozen in liquid nitrogen and had been stored in -70ºC, until being used for RNA extraction. For each patient, the clinicopathological information including: gender, age, grade, and stage of tumors were gathered (Table 1). The experimental procedure was approved by the ethical committee of Tarbiat Modares University.

Cell culture

The 5637 bladder cancer cell line was obtained from the national cell bank of Iran (Pasteur institute of Iran, Tehran) and was cultured in RPMI-1640 (Gibco, USA) medium, supplemented with penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively) and 10% fetal bovine serum (FBS), at 37°C in a humidified atmosphere of 5% CO_2. After reaching to a confluency of 70-80%, cells were treated with 400 µM/ml of TGF-β recombinant protein (Peprotech, USA) for different time-points. The cells were then lysed for RNA extraction and performance of real-time PCR.

RNA extraction and real-time PCR

Total RNA was isolated from the homogenized cell culture or tissue specimens; using Trizol solution (Invitrogen, USA), according to the manufacturer's instructions. The 260/280 absorbance ratio was determined by the Nano Drop ND-100 spectrometer (Nano Drop Technologies, Wilmington, DE). RNase-free DNase (TAKARA, Japan) treatment of the total RNA was performed to eliminate any potential contamination with genomic DNA. cDNA synthesis was carried out by using two commercial kits: "Universal cDNA synthesis kit" (Exiqon, Denmark) for miR-21 cDNA synthesis, and "PrimeScriptTM 1st strand cDNA Synthesis Kit" (TAKARA, Japan) for TGF-β cDNA synthesis.

For miR-21 amplification, the real-time PCR reactions were performed using 8µL of diluted cDNA(20X) products, miR-21 LNA™ primers (Exiqon, Denmark), and SYBR Premix Ex Taq II (Perfect Real Time) (TAKARA, Japan), according to the manufacturer's protocol. The PCR reactions were conducted at 95˚C for 30 seconds, followed by 40 cycles of 95˚C for 10 seconds, and 60˚C for 1 minute, in an ABI 7500 real-time quantitative PCR system (Applied Biosystems, USA). U6 snRNA gene was used as a housekeeping internal control. All real-time PCR reactions were done in duplicates. To minimize the data variation in separate runs, paired tumor and non-tumor samples from the same patient were always examined on the same runs.

To amplify TGF-β variants, the real-time PCR reactions were performed using 2µL of cDNA products, specific primers for each variant (table 2), SYBR Premix Ex Taq II (Perfect Real Time) (TAKARA, Japan), and the ABI 7500 real-time quantitative PCR instrument. The specific primers for TGF-β variants as well as GAPDH (as an internal control) were designed by Gene Runner software, version 3.5 (Hastings Software, New York, USA), and synthesized by TAG company (Copenhagen A/S, Denmark), as high purified salt-free grade. To make sure about the uniqueness of all PCR products, all designed primers were blasted against human genome.

Statistical analysis:

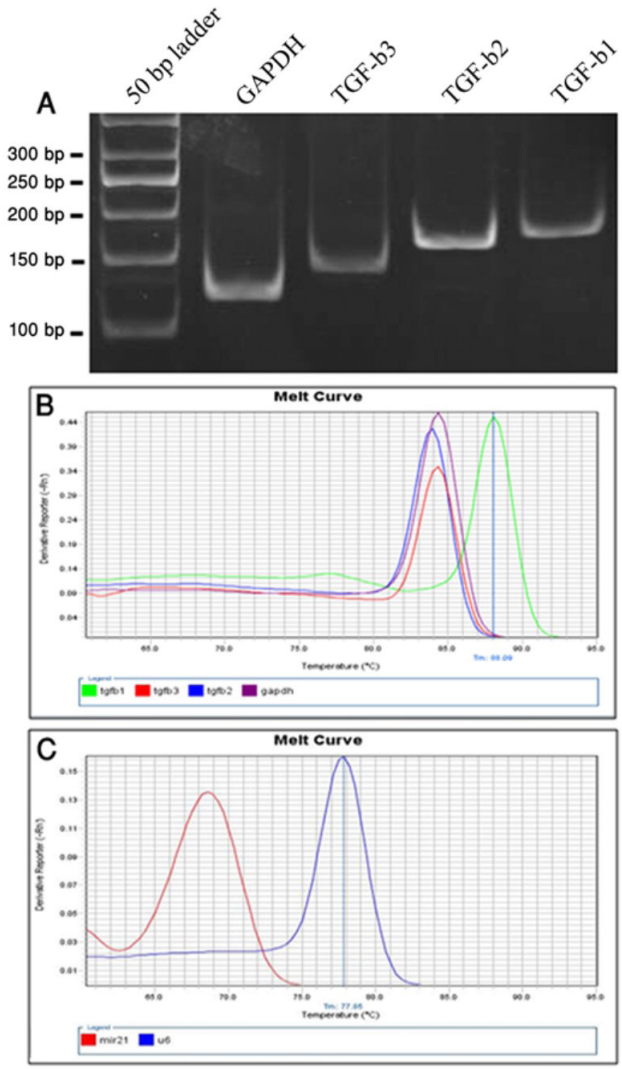

Figure 1. **(A)** A representative acryl-amid gel electrophoresis result demonstrating a single amplified product for each TGF-β variant, as well as the internal house-keeping control gene, GAPDH. The sizes of all PCR products appeared to be identical to the ones expected by primer designing. Representative melt curve graphs of real-time PCR products of TGF-β variants and GAPDH **(B)**, as well as miR-21 and the internal control U6 **(C)** confirmed the authenticity of amplified products.

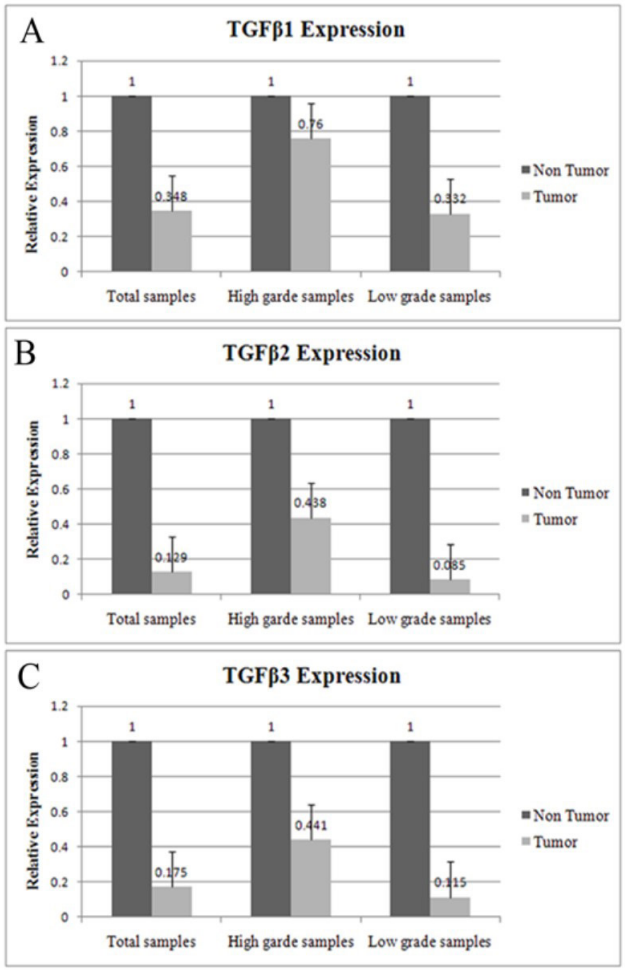

Figure 2. The real-time RT-PCR quantification of the expression levels of TGF-β1 **(A)**, TGF-β2 **(B)**, and TGF-β3 **(C)** variants is compared between tumor vs. non–tumor (total samples), and in high-grade vs. low-grade tumors of bladder. The expression of each variant is normalized to that of GAPDH (the internal control). Histograms show the mean values of TGF-β's relative expression, with standard deviation as error bar. Note that the expression of all TGF-β variants is significantly down-regulated in tumor samples, compared to their non-tumor counterparts.

All real-time PCR data were analyzed with Representational State Transfer (REST) 2008 (Corbett research Pty Ltd, Australia) software, and Statistical Program for Social Sciences (SPSS) software version 13.0 (SPSS Inc., Chicago, USA). REST software was used to analyze and normalize the real-time PCR data. Kolmogorov–Smirnof normality test (KS-test) was used to examine the normal distribution of the samples. Statistical differences between tumor and non-tumor samples were determined by paired t-test (if KS-test was passed) or Mann-Whitney non-parametric test (if KS-test wasn't passed). The probability of a statistically significant difference between the clinicopathological parameters and the gene of interest expression fold change was tested by Mann-Whitney non-parametric test. Receiver operating characteristics (ROC) curve was plotted to determine the suitability of the miR-21 expression level as a potential tumor marker for bladder cancer. All tests were performed as two-tailed, and a P-value of $<.05$ was considered as statistically significant.

RESULTS

Overall, tissue specimens from 30 patients were collected (30 tumor and 30 non-tumors) and examined. To categorize tumor samples based on their stages and grades, the clinico-pathological characteristic of all patients were obtained and further confirmed by an expert urologist (Table1).

Optimization of real-time PCR experiments

After RNA extraction with TRIZOL reagent, to assure that equal amounts of RNA were used for each PCR, we used GAPDH and U6 as internal controls (reference genes), and normalized the relative TGF-β and miR-21 expression of each sample with its own reference expression. The specificity of all primers was determined by examining the melting curves and the sizes of PCR products (Figure1). Statistical analysis of primers efficiencies was done by LinReg PCR software. Direct sequencing of the PCR products was further confirmed the accuracy and authenticity of each PCR product.

Altered expression of TGF-β variants and miR-21 in bladder tumors

Real-time PCR was performed as duplicates for all samples, under similar conditions. The obtained data revealed a significant down-regulation of TGF-β variants in tumor samples, in comparison to non-tumor ones (Figure 2). The relative (normalized to that of internal control, GAPDH) down-regulation for each variant of TGF-β in tumor vs. non-tumor samples was as follow: TGF-β1 (0.35 times, $P = .029$), TGF-β2 (0.13 times, $P = .001$), and TGF-β3 (0.17 times, $P = .000$). The down-regulation of TGF-β variants was more significant in low grade samples: TGF-β1 (0.33 times, $P = .041$), TGF-β2 (0.08 times, $P = .006$), and TGF-β3 (0.11 times, $P = .000$). However, the down-regulation of all variants was much less in high grade specimens, where there was no statistically significant difference between the expression of all variants in high-grade tumors and their non-tumor counterparts (Figure 2).

We observed a similar down-regulation of miR-21 in total bladder tumors (0.33 times) as well as in low-grade tumors (0.17 times), compared to their non-tumor counterparts (Figure 3A). interestingly, miR-21 expression was much higher in high-grade tumors, compared to their non-tumor counterparts (2.2 times); nevertheless, the difference was not statistically significant. But, a comparison between high-grade and low-grade tumor samples revealed that miR-21 is significant-

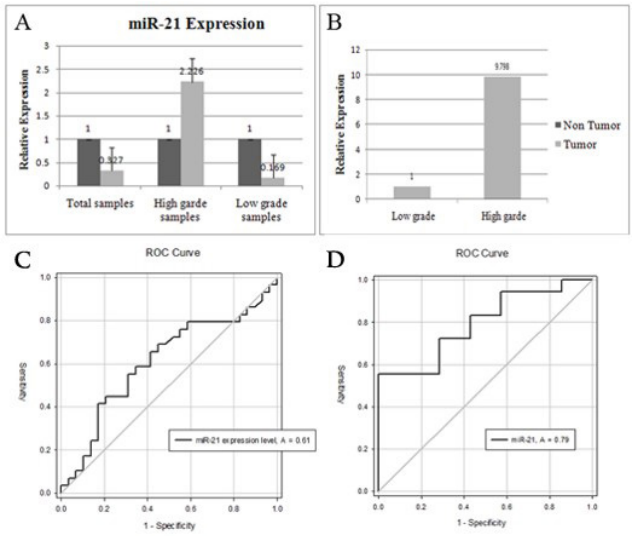

Figure 3. **(A)** The relative expression of miR-21 in tumor vs. non-tumor tissue samples and high-grade vs. low-grade tumors, normalized to the expression level of U6, as the internal control. Note that the altered expression between tumor and non-tumor samples is not statistically significant. However, in contrast to low-grade tumors, miR-21 is upregulated in high-grade ones, in a way that it could significantly discriminate between high-grade and low-grade tumor samples **(B)**. C and D) ROC curve analysis was performed to determine the sensitivity and specificity of miR-21 expression level to discriminate between tumor and non-tumor **(C)**, as well as low-grade and high-grade states of the samples (D). Note that the area under curve (AUC) is much bigger in **(D)**, suggesting that miR-21 could discriminate high-grade tumors from low-grade ones much better than tumor from non-tumor states of the samples.

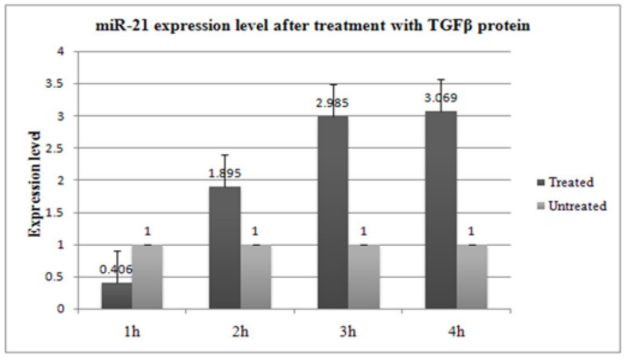

Figure 4. miR-21 expression level is significantly elevated after treatment of 5637 bladder cancer cell line with TGF-β protein, at different time points.

ly upregulated (9.8 times, $P = .031$) in high grade specimens (Figure 3B). To determine the suitability of miR-21 expression level in discriminating tumor and non-tumor samples of bladder, their ROC curve plots were created and analyzed. As shown in Figure 3C, the area under the curve (AUC) for miR-21 expression level in tumor vs. non-tumor samples was 61% (Larger AUC value means better overall performance of the medical test to correctly discriminate two groups of samples). The finding suggests that miR-21 expression level is not a good tumor marker in discriminating tumors from non-tumor samples ($P = .1548$). In contrast, the AUC was much bigger (0.79) and statistically significant ($P = .0251$), when miR-21 expression level were used to discriminate high-grade from low-grade tumors (Fig 3D). However, despite this significant result, we couldn't define a valid and applicable cut-off range to determine the specificity, sensitivity, and predictive value for expression level of miR-21 as a grading marker.

Co-regulation of TGF-β and miR-21 expression

The similar expression pattern of TGF-β and miR-21 suggested a potential co-expression regulation of the genes. To examine this possibility, we treatedthe 5637 bladder cancer cell line with recombinant TGF-β protein. The addition of TGF-β significantly elevated the expression of miR-21 in treated cells. As it is evident in figure 4, following a slight decline at the beginning of the experiment, miR-21 was rapidly upregulated at 2, 3, and 4 hours after treatment of the cells.

DISCUSSION

In this study, we examined a potential alteration in the expression of TGF-β and miR-21 in bladder tumors. Our data revealed that all variants of TGF-β were down-regulated in bladder tumors. The finding is in accordance with the common knowledge about TGF-β function as a potent inhibitor of normal epithelial cell proliferation.[17] TGF-β is probably one of the regulatory factors that are genetically or epigenetically altered during bladder tumorigenesis.[11] An altered expression of TGF-β, its receptors (TGF-βR1 and TGF-βR2), and its signaling components (the smad family) are already reported for several tumors.[13,14,18-26] Our finding is in conflict with a previous report,[16] which claimed a significant elevation of serum TGF-β1 levels in patients with invasive bladder cancer. The inconsistency between the two reports could be due to examining different samples, as the same group failed to show similar finding in the urine of the same patients.[16] Moreover, the tumor cells are not the sole source of TGF-β1 release within the serum. Consistence with our finding, and in a follow-up research, the same group reported a down-regulation of TGF-β variants in bladder tumor tissues.[27] In this study, we discriminate the expression of each variant of TGF-β in our samples. The data revealed that the variants of the gene had a similar pattern of expression in tumor vs. non-tumor samples. The later finding implies that all variants of TGF-β are under the same regulatory elements inside the cells, despite being located on different chromosomes. The most significant down-regulation of TGF-β was observed in low-grade tumors. A logical interpretation of this observation is that the alteration of the gene is a vital step in tumor initiation, but not that critical in tumor progression. We also investigated a potential alteration in the expression of miR-21, which its biogenesis is directly regulated by TGF-β. Despite previous reports on upregulation of miR-21 in most cancer types and cancer-derived cell lines,[28,29] the expression level of miR-21 failed to show any statistically significant difference between tumor and non-tumor samples of bladder. However, while miR-21 showed a slight down-regulation in low-grade tumors, its expression was noticeably elevated in high-grade ones. Indeed, the expression level of miR-21 could strongly discriminate high-grade from low-grade tumors. In contrast to the conclusion we made for TGF-β, miR-21's upregulation seems to be a vital step for tumor progression, but not for tumor initiation. Our data demonstrated a significant correlation between expression levels of miR-21 and TGF-β variants. In low-grade tumor samples which TGF-β expression was significantly down-regulated, miR-21 expression was also down-regulated. And in high grade samples, where the TGF-β expression level was increased, the expression level of miR-21 was accordingly elevated.

CONCLUSION

Our data suggest a significant down-regulation of all TGF-β variants in bladder cancer samples, mostly in low-grade tumors. We also observed a non-significant down-regulation

of miR-21 in low-grade bladder tumors. In contrast, miR-21 showed upregulation in high-grade bladder tumors, in a way that the expression level of miR-21 could discriminate low-grade tumors from high-grade ones. Similar pattern of gene expression for TGF-β variants and miR-21 suggests a co-regulated expression for these genes.

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CONFLICT OF INTEREST

None declared.

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