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MiR-576-3p is a novel marker correlated with poor clinical outcome in bladder cancer

F.-M. MENG[1], F.-M. MENG[2], X.-L. SONG[3]

[1]Department of Urology, Yishui Central Hospital of Linyi, Linyi, Shandong, China
[2]Department of Urology, Zoucheng People's Hospital, Zoucheng, Shandong China
[3]Weifang People's Hospital, Weifang, Shandong, China

Fanmin Meng and Fanmei Meng are co-first author.

Abstract. – OBJECTIVE: MicroRNAs play important roles in the regulation of the initiation and progression of bladder cancer. To our best knowledge, the prognostic value of miR-576-3p in bladder cancer has not been reported. Thus, the purpose of the current study was to determine the expression of miR-576-3p and assess the clinical significance of miR-576-3p in bladder cancer.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (RT-PCR) was performed to evaluate miR-576-3p levels in 232 pairs of bladder cancer specimens and adjacent noncancerous tissues. The association of miR-576-3p expression with clinicopathological factors was statistically analyzed. Overall survival was calculated and survival curves were plotted using the Kaplan-Meier method. The influence of each variable on survival was examined by the Cox multivariate regression analysis.

RESULTS: Our data showed that the expression of miR-576-3p was significantly decreased in bladder cancer tissue compared with matched bladder tissues ($p < 0.01$). Low miR-576-3p expression level was significantly associated with advanced tumor stage ($p = 0.004$), lymph node metastasis ($p = 0.027$), and recurrence ($p = 0.018$). The results of Kaplan–Meier survival analysis showed that patients with low miR-576-3p expression presented shorter mean months of overall survival than patients with high miR-576-3p expression ($p < 0.0001$). Finally, the univariate and multivariate analysis showed that miR-576-3p expression was an independent prognostic marker of overall survival of bladder cancer patients.

CONCLUSIONS: Our results demonstrated that down expression of miR-576-3p in bladder cancer was associated with an unfavorable prognosis, and miR-576-3p might be clinically applicable as an indicator of favorable prognosis.

Key Words:
miR-576-3p, Bladder cancer, Prognosis.

Introduction

Bladder cancer ranks the 9th most common malignancy worldwide and is the second most common cause of death among genitourinary tumors[1,2]. Bladder tumors are classified into two distinct categories: non-muscle and muscle invasive bladder cancer[3,4]. It is indicated that the degree of malignancy in muscle invasive bladder cancer is usually higher and the rate of the metastasis and recurrence is usually higher[5]. Despite improvements in surgery and adjuvant chemoradiotherapy, the median survival of patients with bladder cancer is 15 months, and the 5-year survival rate is only 15%[6]. Therefore, identifying prognostic biomarkers are urgently required to improve clinical outcome of patients suffering this disease.

MicroRNAs (miRNAs) are a class of endogenously expressed, small noncoding RNAs of approximately 22 nucleotides in length that negatively regulate gene expression at the posttranscriptional level[7]. miRNAs have been implicated in a wide range of physiological processes, including cell proliferation, metastasis, cell cycle and apoptosis[8]. Recently, accumulating evidence has indicated that miRNAs can function as tumor suppressors or oncogenes depending on the functions of their target mRNAs[9,10]. More importantly, abnormal expression of miRNAs can be used as biomarkers for diagnosis and prognosis[11,12]. The previous study reported that miR-576-3p was down-regulated in patients with bladder urothelial carcinoma[13]. This finding revealed that miR-576-3p may serve as a tumor suppressor in bladder cancer. However, its prognostic value in bladder cancer hasn't been explored. In this work, we examined the expression difference between bladder cancer tissues and normal bladder tissues, and evaluated the clinical significance of miR-576-3p in bladder cancer patients.

Corresponding Author: Xiao-ling Song, MD; e-mail: sxln821@126.com

Patients and Methods

Ethical Statement

For the use of these clinical materials for research purposes, this study was approved by the Research Ethics Committee of Yishui Central Hospital of Linyi and Weifang Hospital and written informed consent was obtained from all of the patients.

Patients and Tissue Samples

A total of 232 pairs of primary bladder cancer and adjacent normal bladder tissues were collected between 209 and 2011 from Yishui Central Hospital of Linyi. Two pathologists independently reviewed the HE-stained tissue section to determine the tumor stage and histologic grade for each specimen. All specimens from resection surgery were frozen and stored in liquid nitrogen until required. The clinical characteristics of all the patients were summarized in Table I.

RNA Preparation and Quantitative Real-time PCR

Total RNA was isolated using TRIzol® reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was randomly primed from 2 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, MA, USA). Quantitative PCR reactions were conducted on the Bio-Rad CFX96 sequence detection system with Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen, Carlsband, CA, USA). GAPDH was used as an internal control. The qRT-PCR data was analyzed using the method of $2^{-\Delta\Delta CT}$ relative expression quantity. Primer sequences were shown in Table II.

Statistical Analysis

All statistical analyses were carried out by using the SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL, USA). The relationships between clinical parameters and the expression of miR-576-3p were calculated using χ^2-tests. Kaplan-Meier analysis and the log-rank test were performed to identify survival differences in bladder cancer patients. A Cox proportional hazard regression analysis was used for univariate and multivariate analyses of prognostic values. Differences were considered statistically significant when p was less than 0.05.

Table I. Primer sequences for RT-PCR.

Primers	Sequences
miR-576-3p (forward)	5'-AAGATGTGGAAAAATTGGAATC-3'
miR-576-3p (Reverse)	5'-ATTCTAATTTCTCCACGTCTTT-3'
GAPDH (forward)	5'-GGGAAACTGTGGCGTGAT-3'
GAPDH (Reverse)	5'-GGGTGTCGCTGTTGAAGT-3'

Table II. Association between miR-576-3p expression and clinicopathological characteristics of bladder cancer.

Variable	No.	miR-576-3p expression level: High (n =113)	Low (n = 119)	*p*-value
Age (y)				0.073
≤50	98	41	57	
>50	134	72	62	
Gender				0.166
Male	133	70	63	
Female	99	43	56	
Tumor size (cm)				0.075
< 3	132	71	61	
≥ 3	100	42	58	
Multiplicity				0.606
Single	119	56	63	
Multiple	113	57	56	
Tumor stage T				0.004
T1-T2	169	92	77	
T3-T4	63	21	42	
Lymph nodes metastasis			0.027	
No	183	96	87	
Yes	50	17	32	
Recurrence				0.018
Yes	57	20	37	
No	175	93	82	

Results

MiR-576-3p is Down Expressed in Bladder Tissues

We first examined the expression of miR-576-3p in bladder cancer tissues and matched non-cancerous bladder tissues. Real-time PCR analysis showed that the expression levels of miR-576-3p were lower in bladder cancer tissues when compared with non-cancerous bladder tissue samples (Figure 1, $p < 0.01$). The results revealed that miR-576-3p may function as a tumor suppressor in bladder cancer.

MiR-576-3p Associations with Clinic and Pathological Characteristics

Next, we further explored the clinicopathological correlation of miR-576-3p downregulation in bladder cancer tissues. As shown in Table II,

Table III. Univariate analyses of different prognostic parameters on bladder cancer survival.

Variables	Risk ratio	95 % CI	*p*-value
Age (y)			
≥50 vs. <50	1.455	0.681-2.341	NS
Gender			
Male vs. Female	1.933	0.623-3.138	NS
Tumor size (cm)			
< 3 vs. ≥ 3	2.144	0.616-2.997	NS
Multiplicity			
Single vs. Multiple	1.882	0.891-2.562	NS
Tumor stage			
T3-T4 vs. T1-T2	3.881	1.147-6.632	**0.004**
Lymph nodes metastasis			
Yes vs. No	3.115	0.782-5.883	**0.009**
Recurrence			
Yes vs. No	8.321	2.113-13.356	**0.003**
miR-576-3p expression			
High vs. Low	0.256	0.172-0.883	**0.001**

miR-576-3p expression levels were observed to correlate with tumor stage, lymph nodes metastasis and recurrence (p = 0.004, 0.027 and 0.018, respectively). However, However, there were no significant correlations of miR-576-3p expression with other clinical features such as gender, age, tumor size, and multiplicity (all $p > 0.05$). These findings strongly indicated that miR-576-3p expression played a critical role in bladder cancer development and may be a valuable marker for this disease.

Correlations Between miR-576-3p and Prognosis

To explore the relationship between miR-576-3p expression and bladder cancer patients' prognosis, we performed Kaplan-Meier curves for overall survival by comparing the patients with high and low miR-576-3p expression. The results showed that that the overall survival time of patients with high miR-576-3p expression was significantly longer than those with low miR-576-3p expression (Figure 2, $p < 0.0001$).

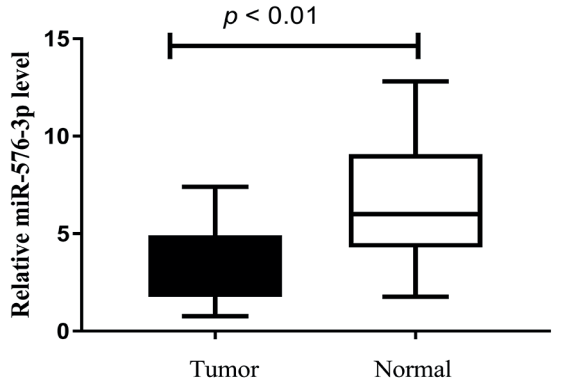

Figure 1. Relative expression of miR-576-3p in bladder cancer specimens and adjacent normal bladder tissues determined using qRT-PCR and normalized against endogenous control (GAPDH).

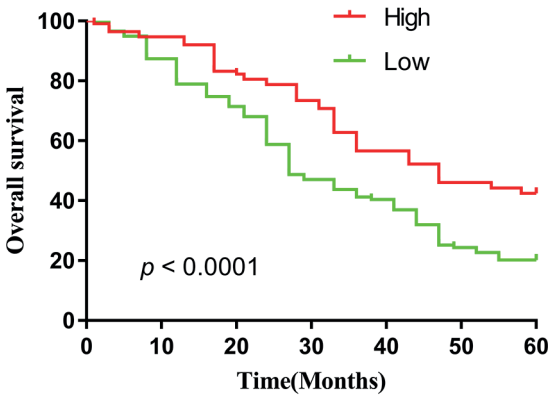

Figure 2. Survival analysis comparison of bladder cancer patients with relative low (*below mean*) and high (*above mean*) expression of miR-576-3p.

To figure out whether miR-576-3p expression was an independent prognostic factor in bladder cancer patients, we performed univariate and multivariate analyses by Cox's proportional hazard model. The univariate analysis indicated that tumor stage, lymph nodes metastasis, recurrence, and miR-576-3p expression were significantly associated with overall survival of bladder cancer patients ($p < 0.004$, < 0.009, < 0.003 and 0.001, separately; Table III). Furthermore, the multivariate Cox regression analyses confirmed the miR-576-3p expression was an independent prognostic indicator for overall survival (RR = 0.256; 95% CI, 0.172-0.883; p = 0.001) in

Table IV. Multivariate analyses of different prognostic parameters on bladder cancer survival.

Variables	Risk ratio	95 % CI	*p*-value
Tumor stage			
T3-T4 vs. T1-T2	4.146	1.334-7.724	**0.002**
Lymph nodes metastasis			
Yes vs. No	3.872	1.422-6.442	**0.001**
Recurrence			
Yes vs. No	9.872	3.766-16.762	**<0.001**
miR-576-3p expression			
High vs. Low	0.304	0.123-0.994	**0.001**

patients with bladder cancer (Table IV). The findings revealed that miR-576-3p expression could be developed as a powerful independent prognostic factor.

Discussion

As one of the most prevalent carcinomas worldwide, About 40% of the patients with bladder cancer experience multiple recurrences, which has a significant impact on the quality of life[14]. Because there is no reliable diagnostic biomarker for detection of early bladder cancer at present, most bladder cancers are found at advanced stage[15]. Given that recurrences were related to the stage of bladder cancer, novel biomarkers, with high sensitivity and specificity, are urgently needed as diagnostic and prognostic tools in bladder cancer.

Increased evidence has strongly suggested that abnormal miRNA expression is a common and important feature in cancer development[16]. More and more studies showed that miRNAs regulate the expression of many invasion and metastasis-related genes in various types of malignancies including bladder cancer[17]. For instance, Zhao et al[18] showed that miR-29c could inhibit the proliferation, migration, and invasion of bladder cancer cells via regulating CDK6. Xu et al[19] found that overexpression of miR-124-3p significantly repressed the capability of migration and invasion of bladder cancer cells. They further identified ROCK1 as a new target of miR-124-3p. Another study by Peng et al[20] revealed that miR-155 served as a tumor promoter in bladder cancer by repressing the tumor suppressor DMTF1. Those results strongly suggested that miRNAs played a key effect in the progression of bladder cancer. The potential role of miRNAs as a prognostic biomarker in bladder cancer has been investigated. For example, Ma et al[21] showed that miR-148a expression was decreased in bladder cancer specimens and reduced miR-148a expression was associated with poorer survival time. Andrew et al[22] found that down-regulation of miR-34a was associated with a reduced risk of bladder cancer recurrence. A new miRNA was identified as a tumor suppressor by Liang et al[23]. They performed cells experiment and found that miR-576-3p inhibits proliferation in bladder cancer cells by targeting Cyclin D1. Thus, we wondered whether miR-576-3p could influence the prognosis of patients suffering bladder cancer.

In the present work, we aimed to investigate the clinical significance of miR-576-3p in bladder cancer. We first detected the expression of miR-576-3p in 15 bladder cancer tissues and corresponding noncancerous tissues and showed that miR-576-3p was aberrantly down expressed in bladder cancer tissues. Then, we observed that decreased miR-576-3p expression was associated with the advanced clinical stage, recurrence and distant metastasis, indicating that miR-576-3p down expression may promote an aggressive phenotype of bladder cancer. Moreover, we estimated the prognostic role of miR-576-3p in bladder cancer. Our results showed the overall survival time of patients with lower miR-576-3p expression levels was shorter than that of patients with higher miR-576-3p expression levels. These findings were further supported by the univariate and multivariate analyses of Cox proportional hazards regression model, suggesting that miR-576-3p was an independent prognostic factor for bladder cancer.

Conclusions

To our best knowledge, this is the first report about the clinical value of miR-576-3p in bladder cancer. We studied the role of miR-576-3p in bladder cancer, and found that a lower level of miR-576-3p in bladder cancer tissues correlated with a reduced patient survival rate. Our data revealed that miR-576-3p might be useful as a prognostic biomarker for bladder cancer.

Conflict of interest

The authors declare no conflicts of interest.

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