

Up-regulation of microRNA in bladder tumor tissue is not common

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Abstract MicroRNAs (miRNAs) have recently been shown to down-regulate gene expression by targeting mRNA translation and to play a critical role in tumorigenesis; how they regulate bladder tumor development, particularly in patients, is, however, poorly understood. The difference in miRNA expression in a bladder tumor compared with healthy tissue from the same patients was examined using microRNA arrays in seven patients. Here, we showed that up-regulation of miRNA was not commonly found in

this limited number of patients, and four miRNAs (miR-26a, miR-29c, miR-30c, miR-30e-5p) were down-regulated as a common marker in patients with a 1–3 grade of disease. Our data suggest that instead of up-regulation of carcinogenic miRNAs, loss of regulation of these miRNA may be critical for bladder tumor development in patients.

Keywords Bladder cancer · MicroRNA · Gene expression

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Introduction

Bladder tumor is a common cancer and has been listed as one of the ten most common malignant tumors in China [1]. It has been documented that invasive transitional cell carcinoma (TCC) of the urinary bladder leads to greater mortality than any other kind of urinary malignancy, aggressively progressing to metastatic disease with a poor prognosis (~50% survival at five years) [2]. The pathological classification of bladder TCC can be graded by following the 1973 WHO grading system, or can be staged according to the 1997 TNM system [3]. To date, many genes have been identified as being involved in bladder cancer development, for example tumor suppressor protein retinoblastoma protein [4] and fibroblast growth factor receptor 3 [5]. However, the molecular regulation of these gene expressions in bladder tumor development from low to high stage or

well (minimal anaplasia) to poor differentiation (severe anaplasia) is poorly understood.

Recently, researchers have identified a group of short (~22 bases) and noncoding RNA molecules, named microRNAs (miRNAs), that can down-regulate protein expression of a target gene either by interference with its translational efficiency or by induction of mRNA cleavage [6–8]. The effect of miRNA regulatory activity has been described in cell apoptosis, proliferation, and differentiation [9–11]. It has been suggested that miRNA can act as oncogenes and/or tumor-suppressors [12, 13], thereby playing an important role in oncogenesis [14]. Furthermore, miRNA expression is also tissue-specific in many human tissues [15, 16], suggesting the possibility of creating the signature for different solid tumors [17]. However, few studies have been published in the attempt to understand the regulatory effects of specific miRNA on the tumorigenesis of bladder cancer. It has been found that a total of ten miRNAs (miR-223, miR-26b, miR-221, miR-103-1, miR-185, miR-23b, miR-203, miR-17-5p, miR-23a, and miR-205) are up-regulated in 25 urothelial tumor samples compared with two normal bladder mucosa [18].

In this study, changes in the miRNA expression profile were analyzed in seven patients with bladder tumors. Each miRNA expression profile was compared with that in healthy bladder mucosa from the same patient to avoid patient-specific differences. We showed there is no commonly up-regulated miRNA in bladder cancer, and that four types of miRNAs are decreased in this group of patients, implying that loss of miRNA regulatory functions may be more important in their contribution to the progression of bladder cancer.

Materials and methods

Patients and tissue samples

Seven patients whose bladder TCC was pathologically graded by following the 1973 WHO grading system or staged according to the 1997 TNM system [3] (Table 1) at Hangzhou First People's Hospital and the First Affiliated Hospital, Zhejiang University School of Medicine, China, during 2006–2007, were recruited into this study. They were four males and three females with a median age of 73 years old. All the patients received one of the following treatments: transurethral resection of the bladder tumor (TURBT) for patients with T1 stage, and partial or radical cystectomy for patients with T2 stage. Both tumor and normal mucous membrane (on the side opposite to the pathological changes) samples of the urinary bladder were biopsied at the same time during surgery. A consent form was obtained from each patient and approval was obtained from our local institutional ethics committee. Tissue samples were frozen immediately in liquid nitrogen after resection and stored at -80°C . The normal mucous tissues were used as controls only after confirmation by pathological evaluation.

Total RNA isolation and microRNA array assay

The total RNA was isolated from tissue samples using Trizol reagent (Invitrogen China, Shanghai, China) following the manufacturer's protocol and standard operating procedure (KangChen Bio-Tech, Shanghai, China). The assay with miRNA array was also performed at KangChen Bio-Tech. Briefly,

Table 1 Summary of all seven patients with TCC of bladder

Patient	Gender	Age	Tumor stage	Pathologic grade	Surgery
1	F	50	T1	1	TURBT
2	M	76	T1	1	TURBT
3	M	83	T1	1–2	TURBT
4	M	48	T2	1	Partial cystectomy
5	F	73	T2	1–2	Radical cystectomy
6	F	78	T2	2	Partial cystectomy
7	M	70	T2	3	Radical cystectomy

Bladder TCC was graded as papilloma (no nuclear anaplasia), 1 (minimal nuclear anaplasia), 2 (moderate anaplasia), or 3 (most severe anaplasia) by following the 1973 WHO grading system, or was classified according to the 1997 TNM system [3]. T1, invasive but not into the muscular layer; T2, invasive into the muscle

miRNA in RNA samples was labeled with fluorescent Hy3 using the miRCURYTM array-labeling kit (Exiqon, Denmark). The labeled miRNA was detected by hybridization to a miRNA array containing 464 different miRNA sequences (miRCURYTM array microarray kit, Exiqon) on Bioarray LifterSlip coverslip slides (Genetimes Technology, Shanghai, China). After being washed with wash buffer A, B, and C and dried by centrifugation, the slides were scanned using a microarray scanner Genepix 4000B with a 635 nm laser (Molecular Devices, CA, USA). The fluorescent density data in the images were analyzed using Genepix Pro 6.0 software (Molecular Devices). Data were presented as the *n*-fold change (increase, I; decrease, D) of each miRNA probe fluorescent density in the tumor tissue sample (T) after normalization with that in normal tissue sample (N) from the same patient as follows: $I = T/N$; $D = -N/T$.

Stem-loop real-time reverse transcription (RT)-PCR

The miRNAs (let-7a, miR-129, and miR30c) were quantitated by real time RT-PCR to confirm the reliability of the miRNA array assay. Briefly, RNA was converted into cDNAs by SuperScriptIII reverse transcription kits (Invitrogen China). Real-time PCR was performed using an Applied Biosystems (Shanghai, China) 7000 sequence detection system following a standard TaqMan PCR procedure with appropriate oligonucleotides primers and probes (Table 2) [19]. The TaqMan CT values were converted into absolute copy numbers using a standard curve from synthetic

let-7a miRNA. Each sample was run in triplicate. The mean threshold cycle value of the triplicates and the *n*-fold difference in the expression of each miRNA in both normal and tumor tissues were determined after normalization to the expression level of beta-actin. Comparison analysis of these miRNA levels using real time RT-PCR with miRNA array was performed.

Statistical analysis

Student's *t*-test was used as appropriate for comparisons between groups. A *P*-value of ≤ 0.05 was considered to be significant.

Results

Instead of examining the global miRNA expression profile in bladder tumor tissues from one group of patients as compared with others as controls, the changes in miRNA expression in the tumor from each individual patient were profiled by comparison with that in their own healthy bladder tissue. Overall, expression of a range from 40 to 111 miRNAs had over 1.5-fold change in a total of 464 miRNAs in bladder tumors compared with those in normal tissue in each patient; from 18 to 50 miRNAs were up-regulated whereas from 9 to 89 miRNAs were down-regulated (Fig. 1).

The tumor tissues collected in this study were classified into either the T1 or T2 group. In the T1 group, the differentiated grade was 1, 1, and 1–2 (Table 1). As shown in Fig. 2a, a total of nine miRNAs (miRNA-129, miRNA-141, miRNA-494,

Table 2 Oligonucleotides for stem-loop reverse-transcription (RT) real-time PCR

miRNA ID	Primers/probes	Sequence (5' → 3')
hsa-let-7a	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATA
	Forward	GCCGCTGAGGTAGTAGGTTGTA
	Probe	(6-FAM)-TGGATACGACAATACTATAC(MGB)
hsa-miR-30c	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTGA
	Forward	GCCGCTGTAAACATCCTACACT
	Probe	(6-FAM)-TGGATACGACGCTGAGA(MGB)
hsa-miR-129	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTTG
	Forward	GCCGCCAAAAAGCGGACAGG
	Probe	(6-FAM)-TGGATACGACGCTTGC(MGB)
Universal reverse primer		GTGCAGGGTCCGAGGT

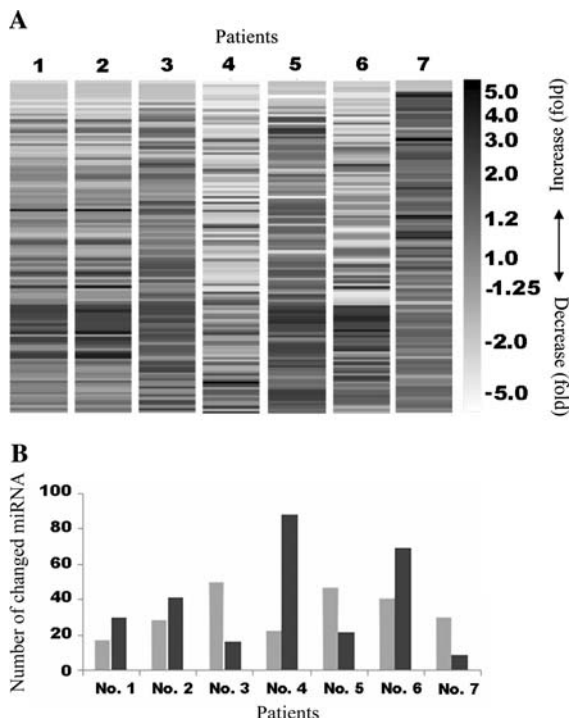


Fig. 1 The changes in the miRNA expression profile in bladder tumors as compared with normal tissue in each patient. **a** The level of change for each miRNA expression, as indicated by different darkness from white (−5 fold decrease) to black (fivefold increase). The data were visualized using Genespring computer software (version 7.5, Agilent Technologies Canada Inc., Mississauga, ON). **b** The number of changed miRNAs that were up-regulated (grey bar) or down-regulated (black bar) as determined by a 1.5-fold cutoff line in each patient

miRNA-498, miRNA-500, miRNA-513 and three unknown miRNAs) were found to be up-regulated in every patient, whereas 13 miRNAs (miR-let-7a, 7b, 7c, 7d, miR-143, miR-199a*, miR-21, miR-24, miR-26a, miR-29c, miR-30a-5p, miR-30c, and miR-30e-5p) were down-regulated (Fig. 2b). In the up-regulated group, most of miRNAs had a mean twofold increase except for miRNA-141, which was increased more than fivefold. In the down-regulated group, the mean decrease was 2 to 3-fold for most miRNAs, and a sixfold increase was seen for miR-143. In the T2 group, the differentiated grade ranged from 1, 1–2, 2, and 3 (Table 1), which was not significantly different from the T1 group. But as all the changes in miRNA expression were screened in the tumor tissue relative to healthy tissue in this group of patients, no any up-regulated miRNA was commonly seen in every patient. However, four miRNAs (miR-26a, miR-29c,

miR-30c, and miR-30e-5p) were commonly down-regulated (Fig. 2c) and were also found to be down-regulated in the T1 group (Fig. 2b), suggesting that no specific miRNA expression change was detected in the T2 group. Overall, the common change in miRNA expression in the bladder tumor over that in normal tissue in these seven patients was a decrease in the expression of these four miRNA, as indicated by the 1.5-fold cutoff line.

To evaluate the reliability of quantitation by the miRNA array assay in this study, the levels of three miRNAs (miRNA-let-7a, miRNA-30c, and miRNA-129) in all seven patients were examined by real-time RT-PCR. As shown in Table 3, although the change value of each miRNA expression was not exactly matched as measured by real-time RT-PCR versus miRNA array in each individual patient, there was no significant difference in the measurement of these three miRNA expression changes by these two methods, as pooled from all seven patients.

It was interesting to note that there was one patient (No. 7), whose tumor was graded 3 with a poorly differentiated phenotype (Table 1). Although it shared the down-regulation of four miRNAs as described above, the miRNA expression change profile in this tumor was remarkably different from those in the other six patients; there were fewer down-regulated miRNAs and the presence of its specific up-regulated miRNAs differed (Fig. 1). In particular, as shown in Table 4, a group of miRNAs was increased in this grade 3 tumor tissue; miRNA-21 was extremely elevated, close to tenfold, but either down-regulation or no significant change in the expression of this miRNA was noted in others. The miRNA-let-7 group (hsa-let-7a, hsa-let-7c, and hsa-let-7d) was significantly increased (2.07, 1.71, and 4.20-fold) whereas others displayed a common decrease in these miRNAs.

Discussion

Recent studies have reported that a group of miRNA molecules regulate gene expression as an emerging regulatory factor, and this has been suggested to play an important role during tumorigenesis [11–14]. However, little is understood about how these miRNAs regulate tumor development and whether there is a specific group of miRNAs for a specific tumor. In this study, we examine the changes of miRNA expression

Fig. 2 Common changes in miRNA expression in bladder tumors compared with patients' normal tissues. **a** Up-regulated miRNAs in tumors with T1 clinical stage. **b** Down-regulated miRNAs in tumors with T1 clinical stage. **c** Down-regulated miRNAs in tumors with T2 clinical stage. The cutoff line for miRNA expression change was 1.5-fold. Data are presented as mean ± SD (*n* = 3–4 patients)

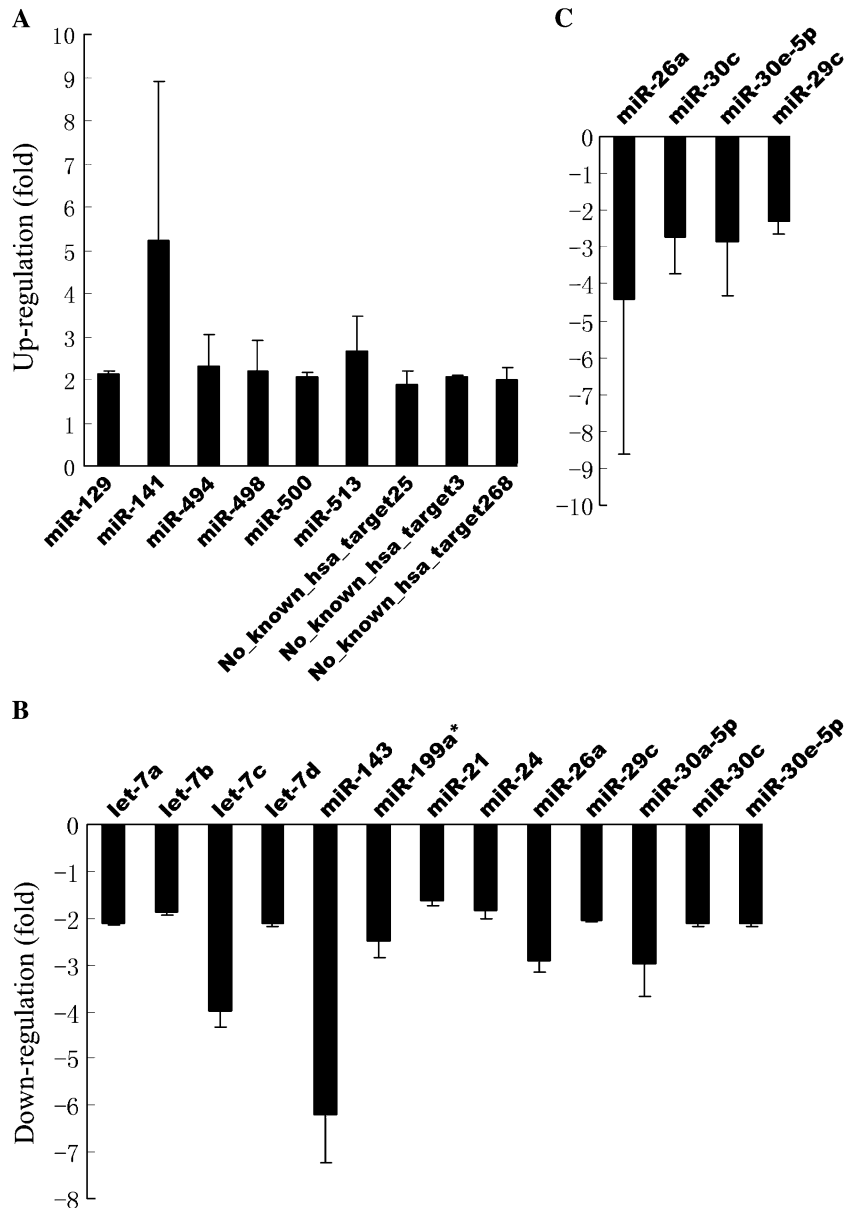


Table 3 Changes in miRNA expression as determined by miRNA array versus quantitative RT-PCR

miRNA	Assays	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	<i>P</i> value
let-7a	Array	-2.16	-2.10	-2.04	-4.25	-2.07	-6.97	2.07	0.74
	PCR	-4.00	-2.46	-8.00	1.41	-3.48	-1.32	-3.48	
miR-30c	Array	-2.17	-2.10	-2.00	-4.160	-2.530	-2.00	-2.17	0.77
	PCR	-2.00	-3.25	-6.50	-11.31	1.00	-1.23	-2.83	
miR-129	Array	-1.01	2.18	2.06	-1.11	2.16	2.23	1.09	0.84
	PCR	-1.15	2.64	1.41	-1.41	1.23	2.46	3.03	

The change in miRNA expression was calculated as follows: *n*-fold increase = T/N; *n*-fold decrease = -N/T (T, level in tumor tissue; N, level in normal tissue)

Table 4 The specific change in miRNA expression in poorly differentiated bladder tumor (grade 3)

miRNA	Patient No. 1 (1 ^a)	Patient No. 2 (2 ^a)	Patient No. 3 (1–2 ^a)	Patient No. 4 (1 ^a)	Patient No. 5 (1–2 ^a)	Patient No. 6 (2 ^a)	Patient No. 7 (3 ^a)
let-7a	–2.16	–2.10	–2.04	–4.25	–2.07	–6.97	2.07
let-7c	–3.73	–4.38	–3.38	–5.80	–2.24	–2.74	1.71
let-7d	–2.05	–2.2	–2.09	–2.62	–27.67	–5.07	4.20
miR-21	–1.61	–1.51	–2.04	1.56	–1.04	–2.78	9.67
miR-26a	–3.01	–2.63	–3.57	–10.70	–2.12	–2.83	–2.02
miR-29c	–2.08	–2.02	–2.01	–2.10	–2.171	–2.81	–2.02
miR-30c	–2.17	–2.10	–2.00	–4.16	–2.53	–2.00	–2.17
miR-30e-5p	–2.19	–2.09	–2.04	–5.07	–2.02	–2.32	–2.10

^a The pathological grade of the tumor

The change in miRNA expression was calculated as follows: n -fold increase = T/N ; n -fold decrease = $-N/T$ (T, level in tumor tissue; N, level in normal tissue)

in bladder tumors compared with that in normal tissue in each patient. Our results show that out of 464 different miRNAs, down-regulation of four miRNAs (miR-29c, miR-26a, miR-30c, and miR-30e-5p) is a common change in all the bladder tissue samples, irrespective of tumor stage or grade. The decrease in the let-7 family (let-7a, b and c) is only found in well to moderately differentiated tumors, whereas in a poorly differentiated tumor miR-21 and these let-7 mRNAs are up-regulated, which needs to be further confirmed in more patients.

The regulation of miRNAs in tumorigenesis has become a new focus in cancer research, but miRNAs as dysfunction factors for tumor suppressor genes or inducing factors for oncogenes are poorly understood. Many studies have demonstrated anti-onco mRNAs and negative regulators of oncogenes in various hematopoietic and solid tumors. Both miR-143 and miR-145 are extremely reduced in colon cancer cells and significantly inhibit cell growth in DLD-1 and SW480 cells [20]. We also noted a sixfold decrease of miR-143 in the T1 group, but not all tumors (Fig. 2b). Also, similar to our observation of a decrease in miR-26a, miR-29c, and miR-30c in bladder tumors, down-regulation of miR-29c is found in lung cancer [21] and nasopharyngeal carcinoma [22], and of miR-26a in thyroid papillary carcinomas [23]. In Myc-induced B cell lymphoma, the expression of miR-26a, miR-29c, and miR-30c is repressed, and enforced expression of these miRNAs diminishes the tumorigenic potential of lymphoma cells [24]. The anti-tumorigenic activity of miR-26a may also be

supported by an experimental study of myogenesis using C2C12 cells, where overexpression of miR-26a positively regulates myogenesis via a process of transdifferentiation from proliferating myoblasts to differentiated myotubes [25]. Taken together, the loss of miRNAs found in this study, including miR-143 (T1 group), miR-26a, miR-29c, and miR-30c, may be required for development of bladder cancer, as demonstrated for other types of cancer.

Expression of the let-7 family was also decreased in all the bladder tumor tissues, except for poorly differentiated tumor (Table 4), suggesting that the loss of the regulation of this group of miRNAs may significantly contribute to bladder tumor development. Indeed, reduced expression of miR-let-7s has been found in lung cancer [26] and colon cancer [27]. The anti-oncogenic activity of let-7 miRNA is confirmed by the fact that this miRNA is required for the repression of oncogenic high mobility group A2 (Hmga2) [28] and RAS [29]. At present, it is difficult to understand why up-regulated let-7 miRNA is present in poorly differentiated tumors, although this needs to be confirmed in more patients. However, one study showed that the reduction of let-7 is only associated with an early occurrence of carcinogenesis, but this is not the prognosis in lung cancer [30]. Taken together, the loss of let-7 anti-oncogenic activity may play a part in the early development of bladder cancer.

A tenfold increase in miR-21 expression was associated with poor differentiation in bladder tumors (Table 4). Many studies have demonstrated this

miRNA is an oncogenic RNA, as indicated by an increase of miR-21 expression in many types of solid tumors, for example breast cancer [31], and lung, prostate, head and neck, ovarian, colon, esophagus, and stomach cancer [32]. Further mechanism studies have indicated that miR-21 down-regulates the phosphatase and tensin homolog (PTEN) gene, leading to constitutive activation of the downstream phosphoinositide 3-kinase (PI3K) pathway and Akt, resulting in the transformation and increase of tumor cell survival [33]. More importantly, miR-21 also plays a role in cell invasion and tumor metastasis. It has been shown that suppression of miRNA-21 in metastatic breast cancer MDA-MB-231 cells significantly reduces invasion and metastasis. MiR-21 probably affects cell invasion and metastasis by regulating multiple target genes, for example tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), and maspin [34]. Our data indicate that the up-regulated miR-21 in bladder tumors may have a similar effect, regulating bladder tumor differentiation from a large to a poor degree.

In conclusion, our data are the first to reveal the complicated changes of miRNA expression in bladder tumors; up-regulation of some miRNAs was seen but not in every tumor sample even from a limited number of patients. The decrease in expression of four miRNAs is common for all the bladder tumors regardless of cancer stage or tumor differentiation. A similar finding was seen in breast cancer, as recently demonstrated by loss of miR-126 and/or miR-335 expression associated with primary breast tumors from patients who relapsed and with poor distal metastasis-free survival [35]. Taken together, these data indicate that instead of up-regulation of onco-miRNA expression leading to alternation of anti-oncogene expression, repression of anti-onco miRNA expression may be the most common mechanism of bladder tumor development. Our preliminary study suggests the potential of de-repression of the anti-onco-miRNA family (e.g., miR-26a, miR-29c, miR-30c, and miR-30e-5p) as a novel therapy for patients with bladder cancer.

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