

Original Research

Deregulation of seven CpG island-harboring miRNAs in bladder cancer: miR-155 and miR-23b as the most promising oncomiRs

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Abstract: Analyses of differential miRNA expressions in tumor and normal tissues can identify specific miRNAs involved in cancer pathogenesis, which can then be used as diagnostic, therapeutic and prognostic biomarkers. In this respect, we aimed to investigate expression levels of seven CpG island-harboring miRNAs in 50 paired UBC tissues by qRT-PCR. miR-21 and miR-155 were found to be significantly upregulated, and miR-23b, miR-126, miR-129-5p, miR-143a and miR-218-5p were downregulated. ROC analysis indicated miR-155 as the most promising candidate for discrimination of tumors from healthy tissue, and miR-23b for the discrimination of early stage from late stage tumors.

Key words: microRNA, deregulation, urinary bladder cancer, miR-155, miR-23b.

Introduction

Bladder cancer is the ninth most common cancer and second most common genitourinary malignancy worldwide, with approximately 47.000 estimated new cases and 180.000 deaths in 2015 (1, 2). It ranks 5th in the cancer incidence among men. Male to female ratio of bladder cancer cases is typically considered as 3:1. Unfortunately, the number of new bladder tumors in males is expected to be five folds in 2015 as compared to females. Besides sex, the most relevant risk factors for bladder cancer are smoking and exposure to certain chemicals like aromatic amines and polycyclic aromatic hydrocarbons (PAHs). (3, 4).

Although many studies have revealed the basic pathways and related key molecules of urinary bladder cancer (UBC) pathogenesis, many more still remain to be identified to clarify its etiology, early carcinogenic events and exact molecular mechanisms of progression. Identification of the molecular alterations will lead the way to explore biomarkers of early detection and prognosis, as well as to determine efficient therapeutic targets. In this respect, micro RNAs (miRNAs) have been the focus of recent interest as promising diagnostic and prognostic biomarkers of cancers. miRNAs are 19-25 nucleotides long non-coding RNA molecules, which regulate gene expression by post-transcriptional silencing, and are involved in modulating various biological processes, including cell proliferation and apoptosis (5, 6). Considering that there is at least one conserved miRNA binding site in more than 60% of human protein-coding genes, it is obvious that most of them are under strict control of miRNA expression (7, 8). In parallel, miRNA expressions are tightly regulated by genetic and epigenetic events. Single nucleotide polymorphisms, mutations, copy number variations, and also methylation patterns of miRNA coding genes or miRNA binding sites are often associated with various cancers (9-14). Aberrant hypermethylation of miRNA genes have been observed in different cancer types including gastric

cancers, cervical cancers and acute myeloid leukemia (15-17). Such abnormal methylation profiles may play a role in the determination of tumor origin or metastatic susceptibility, in addition to causing altered gene expression patterns during carcinogenesis (18-20). As evident by the aforementioned studies, identification of altered miRNA expression profiles in cancers facilitates the detection of prognostic and diagnostic biomarkers, in addition to the clarification of underlying carcinogenic events. Previous findings encouraged us to investigate the expression levels of CpG island-harboring miRNAs in UBC tumors in order to identify differentially expressed miRNAs, which will be useful for early detection and classification of tumors. For this purpose, seven miRNAs were selected by literature search based on their likelihood of affecting bladder carcinogenesis. All the selected miRNAs have experimentally validated cancer associated targets, in addition to be epigenetically regulated and differentially expressed in various cancers (20-33). The list of selected miRNAs and their experimentally validated target genes are shown in Table 1.

Materials and Methods

Subjects

50 UBC patients, comprising 45 men and 5 women have been enrolled in this study. The study protocol was approved by Istanbul University Istanbul Medical Faculty Clinical Research Ethics Committee, and all subjects have provided a written informed consent prior to their inclusion in the study. Paired tumor and healthy bladder tissue samples have been obtained as surgical

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Table 1. Selected miRNAs and their targets.

microRNAs	Target Genes
miR-155	<i>TP53-1NP1</i>
miR-23b	<i>ZEB1</i>
miR-218-5p	<i>ROBO1, RICTOR</i>
miR-126	<i>EGFL7, CRK</i>
miR-129-5p	<i>CDK4, SOX4</i>
miR-143	<i>FNDC38, BCL2</i>
miR-21	<i>PDCD4, PTEN</i>

specimens and were snap frozen immediately in liquid nitrogen before being stored at -80°C until RNA isolation. Tumor staging and grading were performed by two pathologists according to Union for International Cancer Control (UICC) and World Health Organization/International Society of Urological Pathology (WHO/ISUP) consensus clarifications of 2004. All patients had pathologically confirmed transitional cell carcinoma (TCC) of the bladder. Healthy tissue specimens were collected by the resection of sample tissue at least 2 cm away from tumor margins. All healthy tissue specimens were histologically confirmed to be cancer free.

RNA extraction

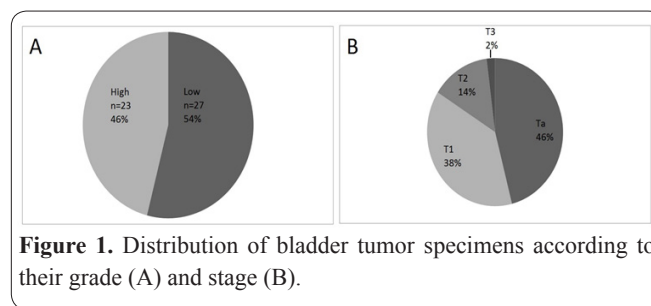
Total RNAs were extracted from bladder tissues with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quantitation and purity analyses were performed by optical density measurement using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). RNA integrities of the samples with sufficient concentration and purity were validated on 1.5% agarose gels.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Reverse transcription of the isolated RNAs were performed by polyA tail addition with Ambion PolyA Tailing Kit (Ambion), followed by cDNA conversion using ABI miRNA EasyScript cDNA synthesis kit (ABM Inc.). miRNAs were quantified with the Eco RT-PCR thermal cycler (Illumina) using EvaGreen qPCR miRNA mastermix (ABM Inc.) and miRNA-specific primer pairs (Qiagen). U6 RNA was used as the housekeeping gene. All reactions were performed in triplicates and checked for specificity with melting curve analysis. Livak's delta delta Ct ($\Delta\Delta\text{Ct}$) method was utilized for the evaluation of the qPCR results (34). miRNA expression levels were compared both between paired tumor and healthy bladder tissues, and tissues of early (Ta and T1) and late stages (T2 and T3).

Statistical analysis

Expression levels were calculated as relative expression and reported as fold changes. Significant up/down regulations were determined with t-test and $p < 0.05$ was considered significant. The discriminative power of miRNA expression levels were analyzed by receiver operating characteristic (ROC) curve analysis and calculation of the area under ROC curve (AUC). Normalized expression levels, and their logarithms were used as the test variables for the upregulated and downregulated miRNAs, respectively. All statistical analyses were performed using SPSS 21.

**Figure 1.** Distribution of bladder tumor specimens according to their grade (A) and stage (B).

Results

50 paired healthy and urothelial bladder tumor samples from 45 men and 5 women UBC patients were included in the study. The mean age of the patients were 67 ± 11.47 years (range: 31-85 years). The distribution of tumor samples according to their stage and grade is provided in Figure 1. The majority of them was of low grade (54%), and early stage (84%).

miR-21 and miR-155 were shown to be significantly upregulated in bladder tumors by 1.2 and 11.19 folds, respectively, compared to healthy bladder tissue (control). On the other hand, miR-23b, miR-126, miR-129-5p, miR-143a and miR-218-5p were significantly downregulated, with miR-23b showing the highest decrease in expression in bladder tumors by 12.5 folds (Table 2, Figure 2). Comparison of miRNA expression levels in early and late stage tumors yielded similar results (Table 2, Figure 3). miR-21 and miR-155 were more upregulated in late stage tumors than in early stage

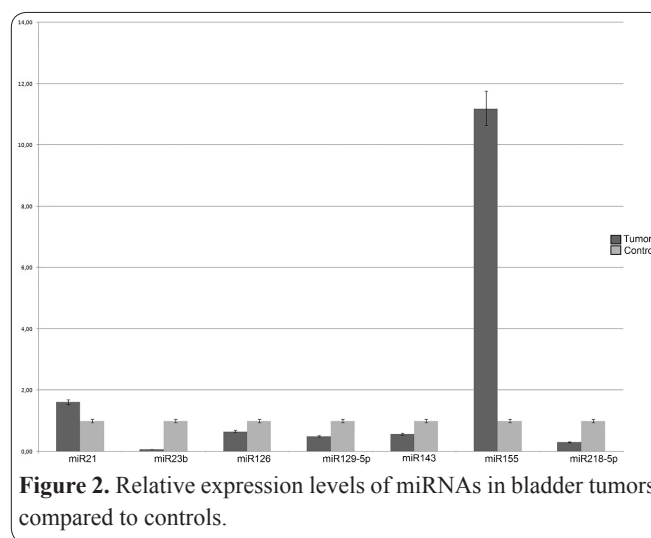
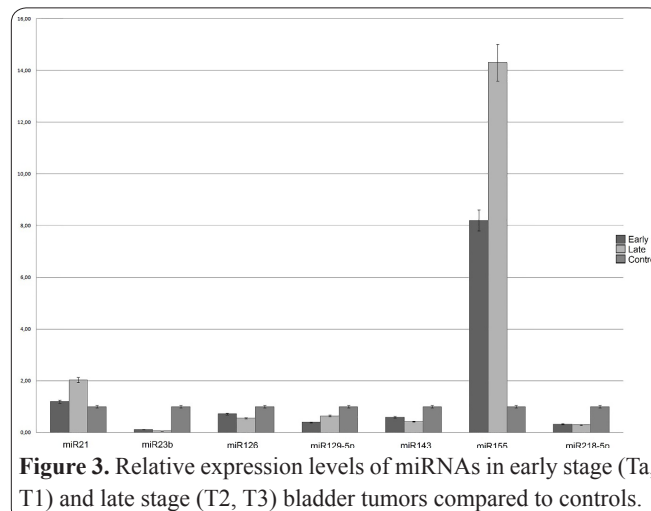
**Figure 2.** Relative expression levels of miRNAs in bladder tumors compared to controls.**Figure 3.** Relative expression levels of miRNAs in early stage (Ta, T1) and late stage (T2, T3) bladder tumors compared to controls.

Table 2. Fold changes in miRNA expression.

	Tumor vs Control		Early Tumor vs Control		Late Tumor vs Control	
	Fold Change	<i>p</i> value	Fold Change	<i>p</i> value	Fold Change	<i>p</i> value
miR-21	1.62	0.013	1.2	0.027	2.04	0.0053
miR-155	11.19	0.025	8.20	0.0076	14.30	0.004
miR-126	-1.51	0.021	-1.38	0.015	-1.78	0.0097
miR-143	-1.75	0.0024	-1.60	0.00062	-2.32	0.0078
miR-129-5p	-2.00	0.017	-2.50	0.0085	-1.54	0.043
miR-218-5p	-3.22	0.038	-3.12	0.029	-3.33	0.011
miR-23b	-12.5	0.042	-19.23	0.034	-16.6	0.045

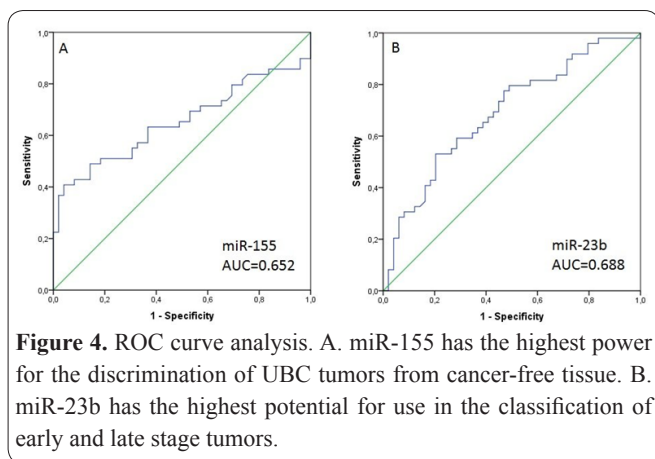


Figure 4. ROC curve analysis. A. miR-155 has the highest power for the discrimination of UBC tumors from cancer-free tissue. B. miR-23b has the highest potential for use in the classification of early and late stage tumors.

tumors when compared to controls. The most obvious downregulation was yet again observed for miR-23b with a fold change of 19.23 in early stage and of 16.6 in late stage tumors. Comparison of miRNA expressions among low and high tumor grades yielded no significant results ($p > 0.05$). ROC analyses (Figure 4) pointed out miR-155 as the most promising candidate for the discrimination of bladder tumors from healthy bladder tissue (AUC= 0.652, 95% CI= 0.540–0.765, $p=0.09$) and miR-23b for the discrimination of early stage from late stage tumors (AUC= 0.688, 95% CI= 0.583–0.793, $p=0.001$).

Discussion

miRNAs, acting like tumor suppressors or oncogenes, are clearly deregulated in human cancers, and affect carcinogenic pathways. Several miRNAs with altered expression have been identified to have a role in UBC pathogenesis. In the present study, we investigated the expression profiles of seven miRNAs in 50 TCC of bladder and paired healthy tissues via qRT-PCR. The miRNAs were chosen to harbor CpG islands, and according to their previously reported altered regulation and functions in various cancers. All the selected miRNAs have shown significant deregulation in this study, confirming previous findings. miR-23b and miR155 have the highest fold changes in tumors when compared to cancer-free tissue.

According to our results miR-23b is the most deregulated miRNA with 12.5 folds downregulation in UBC tumors. miR-23b is a cell-specific miRNA with cell type dependent functions. It has been reported to be overexpressed and to target proline oxidase, a tumor suppressor, in renal cancers (20). In contrast to renal cancers, miR-23b was shown to be downregulated in colon cancers, which results in overexpression of its target, MAP3K, leading to metastasis (21). A recent study reported miR-23b as a tumor suppressor in blad-

der cancer by regulating ZEB1, a key gene in epithelial to mesenchymal transition (EMT) (22). Our results indicate a similar role for miR-23b, corroborating these findings. The magnitude of miR-23b downregulation was decreased in our study as tumors progressed from early to advanced stage, suggesting an inhibitory role on bladder cancer progression for miR-23b.

miR-155, shown to be significantly upregulated in bladder tumors by 11.2 folds in this study, is a highly conserved miRNA with various roles in a wide range of different cellular mechanisms. It has multiple targets, among which TP53-INP1 attracts the most attention due to its pro-apoptotic features. Since miR-155 silences a pro-apoptotic gene, overexpression of this miRNA is thought to induce apoptotic resistance leading to advanced cancer. This assumption has been confirmed in two recent studies, where they revealed the association between increased miR-155 expression and cancer progression (23,24). Our findings corroborate these results and indicate a similar role for miR-155 in bladder cancer. According to the results of this study, miR-155 is the second most deregulated miRNA in bladder tumors compared to controls, among others tested. The upregulation was more distinct in late stage tumors, with 14.3 folds increase compared to cancer free tissue. Another recent study suggested miR-155 as a urine biomarker for bladder cancer (25), however, although this study reported miR-155 also as one of the most deregulated miRNAs, it was reported to be repressed in urine samples of bladder cancer patients (25). This disparity can be partly attributed to the difference in the source of starting sample materials. However, it is not sufficient by itself to explain this controversy, since tumor cells are considered to be the main source of miRNAs released in the urine of bladder cancer patients.

miR-218-5p is coded on a genomic loss region, 4p15.31. It was shown to be hypermethylated in oral squamous cancers (26). In nasopharyngeal tumors miR-218-5p has shown decreased expression, and its exogenous expression inhibited tumor growth by targeting ROBO1 and survivin (BIRC5) genes (27). Similar results have been obtained by Tatarano *et al.* (2011) in bladder cancer cell lines, supporting a tumor suppressor role for miR-218-5p (28). We also observed decreased expression of miR-218-5p in bladder tumors in this study. However, the level of downregulation was almost the same, about 3 folds, for all comparison groups (Table 2). The consistency of the level of downregulation across various tumor stages indicates a more general role for this miRNA, and suggests that the loss of miR-218-5p expression as a result of genomic loss or hypermethylation may be an early event in bladder carcinogenesis.

Recent studies reported miR-129-5p to be epigenetically silenced and downregulated in endometrial, bladder and gastric cancers (13, 29, 30). The foremost target of miR-129-5p is SOX4, a transcription factor regulating EMT and invasion (31). While the overexpression of SOX4 in bladder cancer is reported by two independent studies, the direct relation of miR-129-5p hypermethylation with increased SOX4 expression leading to cancer progression remains to be studied (32, 33). In accordance with the findings of Dyrskjøt *et al.* (2009) (29) the tumor samples included in our study have also shown decreased miR-129-5p expression, indicating a tumor suppressor role for miR-129-5p in urothelial bladder cancer. The association between the methylation levels of miR-129-5p, SOX4 overexpression and bladder cancer invasion should be further investigated. The discovery of a direct relation between miR-129-5p hypermethylation and bladder cancer progression may provide new therapeutic targets.

miR-126 is a tumor suppressor miRNA reported to target ADAM9 (disintegrin and metalloproteinase domain-containing protein 9) gene. ADAM9 was identified to be crucial for EMT, and its silencing by miR-126 has been shown to be a key event in pancreatic cancer invasion. (35). These findings were later corroborated in a more recent study, where they suggested miR-126/ADAM9 interaction as a biomarker for UBC aggressiveness (36). The same study also reported miR-126 as the most down regulated miRNA in UBC samples. While our results are in accordance with these findings, we observed miR-126 as the least downregulated one among chosen miRNAs.

In contrast to miR-126, miR-21 targets primarily tumor suppressors, including PTEN, PDCD4 and RECK (37, 38). As evident by its main targets, it has been shown to promote invasion in glioma and breast tumors (39, 40). miR-21 is overexpressed in UBC and its expression increases in higher grade tumors (41). According to our results miR-21 is upregulated in UBC tumors with a higher expression level in late stage tumors.

miR-143 is another miRNA with strictly tissue specific roles varying from cardiac morphogenesis to metastasis (42, 43). It has been shown to be repressed by RAS oncogenes, suggesting a protective role for miR-143 in cancers (44). In addition to that, miR-143 is revealed to inhibit growth and invasion in UBC and NSCLC (45, 46). According to a recent study miR-143 downregulation is associated with tumor progression and clinical outcome (47). Consistent with these findings, we also observed downregulation of miR-143 in tumors. Downregulation was more prominent when cancer-free tissues were compared to late stage tumors.

One of the major necessities in cancer research in terms of early diagnosis, prediction of disease course and prevision of potential treatment outcomes is the identification of stable and distinct biomarkers obtained by non-invasive methods. Recent studies point out tumor-associated circulating miRNAs as both diagnostic and prognostic biomarkers of many cancers, owing to their easy extraction from various biological fluids, like serum and urine, and their high stability. In order to define distinct miRNAs or miRNA signatures as biomarkers of cancers, their deregulation in tumors compared to control tissues should first be demonstrated. In

this respect, the results of this study are promising since all the studied miRNAs were found to be deregulated significantly in bladder tumors. Especially miR-155 can be regarded as the most promising candidate for the discrimination of bladder cancer status, and miR-23b for the discrimination of early stage from late stage tumors. However, these results are of 50 paired bladder tissues, and should be verified in future studies with larger number of UBC tumor samples. In parallel, serum-isolated miRNA expressions of UBC patients and controls should be investigated in order to explore the potential of circulating miRNAs, especially of miR-155 and miR-23b, as biomarkers of early detection and prognosis of UBC, respectively.

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