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# MicroRNAs Expression Profiles in Romanian Patients with Urinary Bladder Cancer-Preliminary Results

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## Abstract

**Aim:** Bladder cancer is known to be the ninth most common cancer worldwide. Current diagnostic and prognostic tools are insufficient to predict clinical outcome and response to personalized therapy. Carcinogenesis mechanisms involve genetic and epigenetic pathways. The aim of this research was to develop optimal experimental condition to assess the expression of selected miR-145-3p, miR-145-5p, miR-152 and miR-182 in patients with urinary bladder tumors, in order to correlate this with the tumor clinic routine characteristics. This approach would add more information on the etiology of bladder cancer tumors in an attempt to elaborate molecular algorithm.

**Methods:** This work approaches epigenetic modifications in order to investigate the dynamics of certain tissue extracted microRNA species expression correlated with particular tumor characteristics. We have selected: miR-145-3p, miR-145-5p, miR-152 and miR-182 to be evaluated in terms of their expression in tumor tissues samples. A number of 71 Romanian patients undergoing investigations for urinary bladder cancer were introduced in our study. Their clinical characteristics were correlated with the microRNAs expressions in cancer tissues and normal urinary bladder tissues. Upregulation and down regulation of selected microRNAs was revealed using quantitative TaqMan based real-time reverse transcription PCR (RT-qPCR) (Applied Biosystems USA). Data analysis was performed by using $2^{-\Delta\Delta CT}$ method and Student's t-test.

**Results:** We have found different expression profiles showing down regulation for the miR-145-3p, miR-145-5p, miR-152 and upregulation of miR-182. The clinical significance of this profile has emphasized that the investigated bladder tumors have a distinct genetic fingerprint with an impact on pathology and prognosis of the patients. Different mechanisms are involved for significantly reduced expression level of miRNAs in bladder cancers: genetic alterations and Single Nucleotide Polymorphism (SNP), epigenetic silencing and defects in the miRNA biogenesis pathway.

**Conclusions:** Our preliminary results show that selected miRNAs are differently expressed in cancer tissue samples from selected patients in comparison with normal bladder tissues. This epigenetic profile could be used for early diagnosis, response to treatment and prognosis of urinary bladder tumors.

**Keywords:** Epigenetic; MicroRNA; Bladder cancer; Expression profiles; Biomarker; Diagnosis; Prognosis

## Introduction

Bladder cancer is a socially significant and costly healthcare problem. It is also a major cause of morbidity and mortality worldwide, its incidence being rising continuously. More than 356,600 new cases are diagnosed annually worldwide [1]. Risk factors that are incriminated in the development of bladder cancer are: smoking, exposure to aromatic amines and polycyclic aromatic hydrocarbons, exposure to ionizing radiation, abusive consumption of phenacetin containing analgesics, drinking of arsenic contaminated water, nutrition, cyclophosphamide treatment, *Schistosoma haematobium* chronic infections, pollution and genetic predisposition. Age is also a significant risk factor for this type of cancer (70 years mean age). Bladder cancer is more common in males than in women (male: women ratio, 3:1) [2,3].

Current diagnostic and prognostic tools are not appropriate and accurate to predict clinical outcome. Understanding and identifying the different molecular signatures of the disease may offer alternative approaches to improve disease prognosis and support personalized treatment decisions for patients. Recently, the urinary bladder cancer approach involves epigenetic factors such as microRNA species acting as critical regulator of gene expression. MicroRNAs are a small non-coding RNAs around 22 nucleotides, which suppress gene expressions during cytoplasm post-transcriptional gene control via an endoribonuclease Dicer. They are responsible protein coding mRNA degradation by binding to 3'-untranslated regions (3'UTR) [4]. Many studies have demonstrated the importance of microRNAs in both normal and pathologic tissues [5]. They are crucially important in regulating the translation rate of about 60% of the protein coding

genes, thus controlling various metabolic and cellular pathways [6]. There were several reports indicating significantly reduced expression levels of miRNAs. These molecular details could define relevant cancer subtypes, patient survival scores, and treatment responses [7].

MicroRNAs coding genes are usually localized at fragile sites that are often characterized by repetitive sequences. Genomic regions that are frequently altered in tumor cells highlight the important role that microRNAs play in cancer through controlling expression of their target coding mRNAs [8]. Epigenetic and genetic alteration in components of miRNA biogenesis pathway causes microRNA depletion which is involved in oncogenic transformation of cells [9]. However the whole molecular biogenesis mechanisms of microRNAs expression are still not entirely understood. It is suggested that they may be activating during pathogenesis in order to facilitate tumor growth, invasion, angiogenesis, and immune evasion [7]. Cancer-associated microRNAs (tumor suppressor microRNAs and oncogenic microRNAs) are differently involved in the oncogenesis and progression of various carcinomas, including bladder cancer [10]. In patients with bladder tumors, it has been shown that the microRNA expression profile is significantly altered not only in tumors but also in blood and urine [11]. Identifying aberrant expression of microRNAs in human cancers is a first step towards elucidating microRNAs-mediated oncogenic molecular pathways [12].

The aim of this research was to reveal the expression of microRNA species selected from literature as relevant epigenetic markers of urinary bladder tumors collected from 71 Romanian patients. We have selected the following microRNA species: miR-145-3p, miR-145-5p, miR-152 and miR-182 for their clinical relevance in the urinary bladder tumorigenesis mechanisms. Previous studies shown the biological functions of these microRNAs by their association with multiple mRNAs targets involved in carcinogenic process (SOCS7, IGF-1R, PAK1, Smad4, PIK3CA) [13-17]. Deregulation of selected microRNAs was reported also in other tumors such sarcoma, colon, breast, prostate, endometrial, ovarian and lung cancers [18-27]. Actually, our results target the clinical impact of selected microRNAs as diagnostic and prognostic biomarkers in order to assess tumor initiation, progression and response to treatment.

## Patients and Methods

### Patient's selection

The study was conducted in 71 patients with bladder cancer (83 % males and 17% women) admitted at Center of Urology, Dialysis and Kidney Transplantation, Fundeni Clinical Institute, Bucharest during a one year period of time (May 2017 to May 2018). Patient average was 63 years (ranging from 36 to 81 years). Twelve of them had lung metastases, one patient had liver metastases, two patients had bone metastases and two of them revealed lymphatic metastases. Evaluation of selected microRNAs expression was performed in normal and tumor tissues. Specimens of bladder tumor tissues from 71 patients with high-grade, invasive, staged pT2a/pT4b urothelial carcinomas were obtained by cystectomy. After the surgery collected tissue samples were immediately stored at -80°C. As control samples, we have used normal peripheral bladder tissues from the same patients. Normal and tumor tissues were first evaluated by routine clinical histopathological method for their inclusion in microRNA expression analysis. Evaluation of each tumor sample by this method was confirmed the diagnosis. Informed written consent was obtained from all patients, and research protocols were approved by the Fundeni Clinical Institute Ethical Committee.

### Methods

**RNA extraction and amplification:** Total RNA was isolated from frozen tissue samples using a mirVana miRNA Isolation Kit with phenol (Invitrogen) according to manufacturer's instructions and the concentration was assessed by a Qubit Fluorometer. Each miRNA cDNA for selected miRNA species was generated using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems USA). MicroRNA reactions were performed as follows: incubation at 16°C for 30 min, elongation at 42°C for 30 min and denaturation at 85°C for 5 min. The quantification of miR-145-3p, miR-145-5p, miR-152 and miR-182 was performed by qRT-PCR using a TaqMan Reagent Kit and 7300 Real Time PCR Applied Biosystems as follow: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min. All reactions were done in duplicate. The expression level values were normalized to the endogen control expression of the small nuclear RNU48. The same reaction with the same protocol was used to assess microRNAs expressions in 22 normal control tissues. Normal control samples were obtained from patients by the surgical procedure using their normal peripheral bladder tissues.

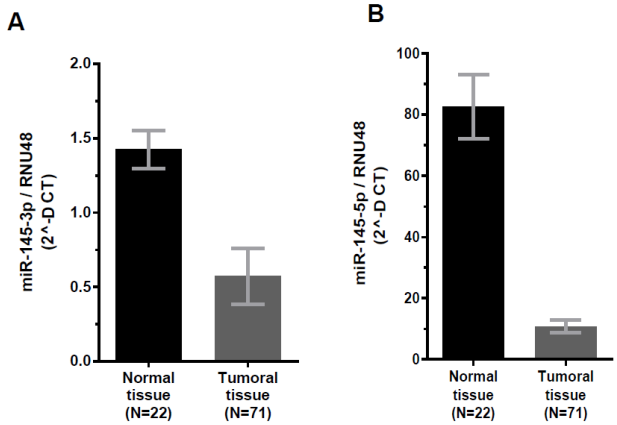


**Figure 1:** Expression of miR-145-3p and miR-145-5p.

### Statistical analysis

The levels of microRNA species in studied patient samples were expressed relative to Control samples using the double delta CT method. Initially, we calculated delta CT values ($_{\Delta}CT_{control}$) in the Control group for each targeted microRNA species as the difference between its CT value and the CT of the endogenous control (RNU48). Further we calculated delta CT values ($_{\Delta}CT$) of the targeted group for each targeted miR species as the difference between its CT value and the CT of the endogenous control (RNU48). The double delta CT was obtained as the difference between $_{\Delta}CT_{targeted}$ samples and $_{\Delta}CT_{control}$ as follow: $_{\Delta\Delta}CT=_{\Delta}CT_{targeted}$ samples-$_{\Delta}CT_{control}$, were $_{\Delta}CT_{targeted}$ samples $CT_{miR}$-$CT_{RNU48}$ in targeted samples and $_{\Delta}CT_{control}=CT_{miR}$-$CTR_{NU48}$ in Control group, for each targeted microRNA species. Finally, the fold-changes relative to control were calculated for the studied patients according to the formula: Fold change=$2^{-\Delta\Delta CT}$. Statistical analysis was performed using GraphPad Prism version 6.01.

The results were expressed as mean ± SEM. Confidence interval of differences were evaluated by performing the two-tailed Student's t-test. The p-value<0.05 was considered statistically significant.

## Results

Characterization of molecular signature of urinary bladder tumors could be improved by association of tumor characteristics with expression pattern changes of microRNAs. In this study we evaluated the expression levels of miR-145-3p, miR-145-5p, miR-152 and miR-182. We obtained changes of selected microRNAs in tumor tissues (N=71) as compared with the normal control tissues (N=22). The results showed that miR-145-3p and miR-145-5p, which have functions in regulation of cell cycle and invasiveness, are significantly down regulated in bladder cancer tissues. Relative expressions of miR-145-3p and miR-145-5p are represented in Figure 1. Interestingly, miR-145-5p it was significantly under expressed in comparison with the expression in control normal tissues. Evaluation of miR-152 expression level indicated its downregulation in tumor urinary bladder tissues (Figure 2). Increase invasiveness of bladder tumor cells could be sustained by overexpression of miR-182. Expression level analyses of miR-182 highlighted their considerably overexpression in the selected patients group (Figure 3).

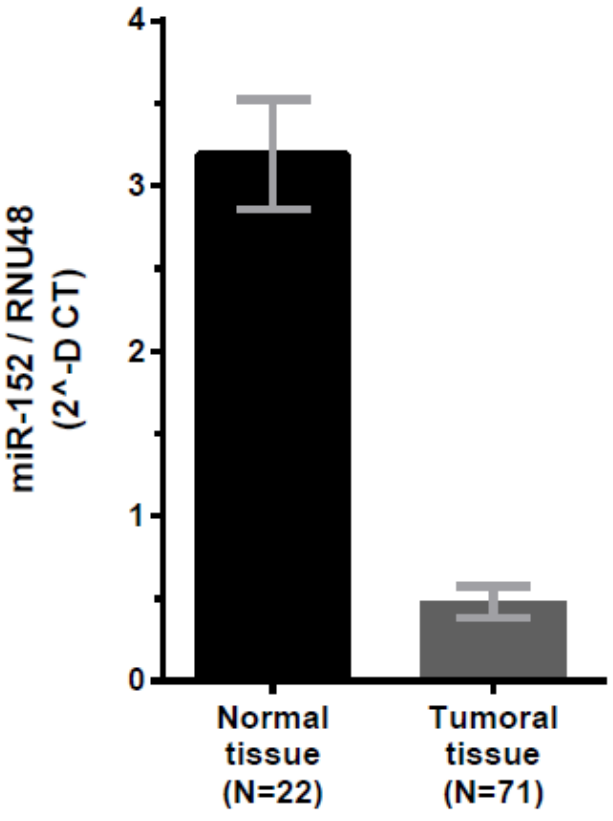


**Figure 2:** Expression of miR-152.

Statistical significance and fold changes of selected microRNA expressions, between different tumor tissues and normal tissues, are represented in Figure 1. The differential expression analysis was statistically performed with SEM and negative inverse transformation for down-regulation genes.

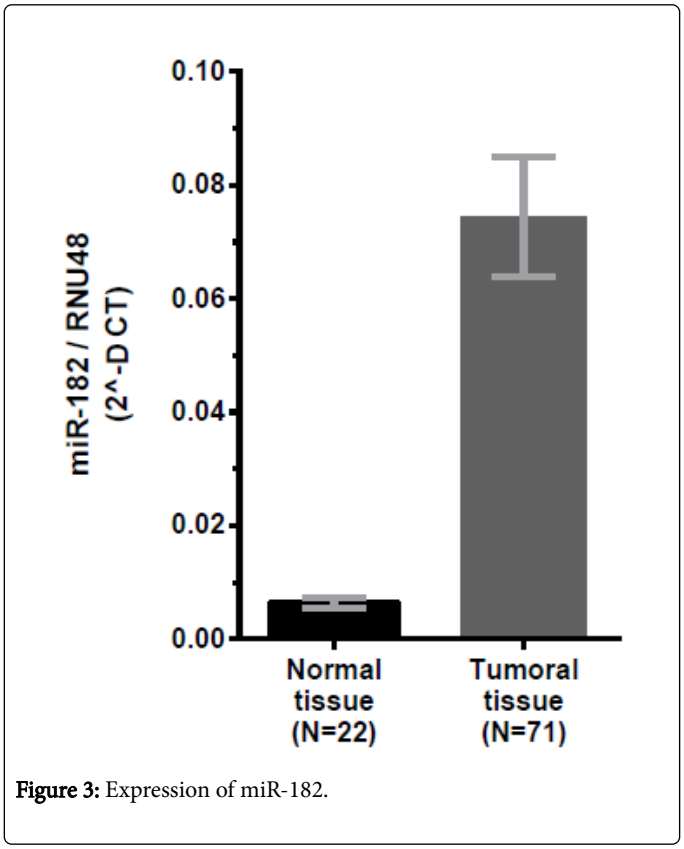


**Figure 3:** Expression of miR-182.

## Discussions

The search for improved and accurate molecular biomarkers is one of the most important priorities in order to fight against cancer. Early detection strategies are necessary as urinary bladder tumors are usually clinically silent in the first stage of the disease. In this study we have investigated the potential of microRNAs to discriminate between non-malignant and malignant bladder tissues. The expression of miRNA-145-3p, miRNA-145-5p, miRNA-152 and miRNA-182 were significantly modified in the selected group of patients. The identification of dynamically regulated microRNAs networks underlines their great potential to serve as biomarkers and offers promising opportunities to improve bladder cancer diagnosis and prognosis. The significance of each microRNA expression revealed in our preliminary result is underlined in other research studies. Other authors showed that urine levels of miR-145, miR-126 and miR-182 help in identifying bladder cancer within a range of 72%-87% sensitivity and 82%-89% specificity [28].

MicroRNA-145 is located in chromosome 5q32-33, a well-known fragile locus in the human genome [29]. In humans, it is abundant in germ cells and in tissues derived from mesoderm [19]. In different cancer types, including bladder cancer, miR-145 has an indirect tumor suppressor function by targeting and suppressing oncogenes. It was described the association of miR-145 with apoptotic characteristics during oncogenesis. Its low expression would provide an advantage for cell survival during tumorigenesis [30]. Other studies also indicate that p53, an important tumor suppressor gene, is indirectly related with miR-145. P53 is involved in the triggering of the enzymatic

mechanisms controlling of microRNAs, mainly the RNase III Drosha, promoting expression of some microRNAs, including miR-145. Thus, under expression of miR-145 observed in our experiment may be a good indicator of loss of p53 function, and this is particular in tumorigenesis [31]. Also, Kou et al. described the role of miR-145 in bladder carcinogenesis. It was showed that miR-145 expression is negatively correlated with *PAK1* gene expression, promoting the EMT transition [15]. Other reports indicated that UHRF1 gene, which plays a relevant role in controlling gene expression by regulating epigenetic mechanisms, was targeted by miR-145-3p and miR-145-5p [32,33]. It was suggested that in urinary bladder cancer, UHRF1 overexpression determines hyper-methylation, thus suppression of tumor suppressor genes, contributing to increased cellular proliferation, apoptosis inhibition and metastasis [32,34]. Also, Minami et al. [35] reported that axis is one of the signaling pathways which maintain Warburg effect in bladder carcinogenesis. miR-145 targets c-Myc, which regulates PTBP1, leading to damage Warburg effect. Moreover, expression levels of KLF4 was correlated with PTBP1 expression and hence inversely correlated with miR-145 expression [35].

MicroRNA-182 belongs to a polycistronic microRNA cluster located in chromosome 7q32.2 [36]. In tumor tissues, miR-182 plays an oncogenic role. Other research is reporting numerous mRNA species targeted by miR-182 [37]. Its overexpression in malignant tissues is correlated with increased proliferation and invasiveness of cancer cells, inhibition of apoptosis and metastasis at a distance [37]. In urothelial malignant tissues, miR-182 exerts its oncogenic function by inhibiting the expression of RECK and Smad4 genes, as well as by regulating Wnt-beta-catenin associated signaling pathways, whose perturbation plays an important role in progression and metastasis [36]. Some research reports found controversial results, sometimes no statistically significant association between miR-182 expression and tumor clinical and pathological parameters. However, a robust and independent correlation between miR-182 expression and the prostate cancer prognosis was proved [38]. Our results are in accordance with the same study that comprehensively demonstrates the only miR-182 overexpression in prostate cancer tissues [38]. Regarding clinical proved bladder cancer tissues, it is reported that miR-182 is upregulated [39]. Segura et al. described how miR-182 directly antagonized FOXO3 and inhibited microphthalmia-associated transcription factor thus inducing its frequent amplification in melanoma metastasis [40]. Investigations of Neely et al. group revealed that the ratio of miR-182/miR-152 was significantly altered in urine samples of urinary bladder cancer patients compared with healthy group [41]. Chen et al. reported another ratio of miR-182/miR-100 with significant novel promising biomarker for diagnosis and survival prediction in bladder cancer [42]. Recently, numerous other tumors were investigated based on miR-182 expression which was found upregulated: medullary thyroid carcinoma, soft tissue sarcoma [18,43].

MicroRNA-152 has been frequently found involved in multiple cancers and diseases, through different mechanisms. It is confirmed [44] that miRNA-152 targets the mRNA of DNA methyltransferase 1 (DNMT1) directly, which explains its role in multiple cancers [45] and it also seems to modulate chemotherapy susceptibility in endometrial and ovarian cancer [46]. Restoration of miR-152 expression in endometrial cancer cell lines was sufficient to inhibit tumor cell growth *in vitro* and *in vivo* [44]. Also, an increased cisplatin sensitivity of SKOV3/DDP and A2780/DDP ovarian cancer cells by inhibiting proliferation and promoting apoptosis is explained [43] by these mechanisms. It was found that TGFα is overexpressed in prostate cancer cells [45] which correlated with miR-152 under-expression. The results were validated by knockdown experiments with Western Blot and dual-luciferase reporter assays. You et al. proved another role of miR-152 involved in gastric cancer during carcinogenesis. It was showed that miR-152-3p and miR-152-5p has synergistic effects in down-regulating PIK3CA, a key driver in gastric cancer cells [46]. MicroRNA-152-5p is hypothesized to act as a tumor suppressor in SGC-7901 gastric cancer cells by down-regulating PIK3CA. In NSCLC it is involved in suppression and proliferation of cancer cells by downregulating FGF2 [47,48]. MicroRNA-152 was found also in the colon cancer cells [49]. Urinary miR-152 was also suggested as a biomarker for bladder cancer showing diagnostic and prognostic value in urothelial cell carcinoma [50]. The mechanism in this case seems to involve aberrant hyper-methylation of CpG dinucleotide repetitive flanking regions of some micro-RNA, including miR-152 [51].

Selection of microRNAs with functions in modulation of various cellular processes, such as cell cycle, proliferation, apoptosis and invasion, can have an important contribution to the diagnosis and evaluation of urinary bladder tumors. Expression patterns of selected microRNAs in our study are able to distinguish between malignant and non-malignant bladder tissues.

This study showed that apparently, downregulation of miR-145-3p, miR-145-5p, miR-152 and overexpression of miR-182 can be associated with tumor invasion and unfavorable prognosis of patients with urothelial carcinomas. In high grade bladder tumors, overexpression of miR-182 is linked to aggressive pathological features of tumors and poor survival rates. This result is in accordance with other studies [14-16].

According to previous researches [52,53] the mechanisms by which selected microRNAs are low expressed include: deletion of genomic sites comprising microRNAs coding genes, abnormal epigenetic pattern including aberrant hyper methylation, abnormal transcriptional control of microRNAs and, also, abnormalities of enzymes that are involved in their biogenesis.

## Conclusions

Our preliminary results have shown that selected miRNAs were differentially expressed in normal bladder tissues and bladder tumors. The expressions of downregulated and upregulated miRNAs are statistically significant. Our miRNAs expression data were highly consistent with those reported in other studies, which indicated that miR-145-3p, miR-145-5p, miR-152 were downregulated while miR-182 was upregulated in high grade bladder cancer tissues studied. Expression pattern of some distinct or combined microRNAs could predict the risk of recurrence of bladder tumors. Also, they could provide an overview of tumor's pathological characteristics which can be useful in therapeutic decisions. The approached epigenetic model is further prone to be optimized on enlarged patients groups with different clinical features. The addition of more miRNA species would provide more valuable information on molecular pathways in bladder carcinogenesis.

Independent validation studies for microRNAs expression alterations in patients with urinary bladder tumors having a wide variety of stages and grades are further needed in order to validate their clinical impact. Future investigation of microRNAs should evaluate their adequacy in clinical decisions and may lead to novel diagnostic and therapeutic approaches for personalized treatment of urinary bladder cancer. Therefore the development of innovative non-invasive approaches using such kind of biomarkers could allow an

accurate early diagnosis, a personalized molecular follow up of the patients with a significant clinical impact on therapy.

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