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# Analyses in human urothelial cells identify methylation of miR-152, miR-200b and miR-10a genes as candidate bladder cancer biomarkers

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ABSTRACT

Urinary miRNAs are discussed as potential biomarkers for bladder cancer. The majority of miRNAs, however, are downregulated, making it difficult to utilize reduced miRNA signals as reliable diagnostic tools. Because the downregulation of miRNAs is frequently associated with hypermethylation of the respective regulative sequences, we studied whether DNA hypermethylation might serve as an improved diagnostic tool compared to measuring downregulated miRNAs. miRNA expression arrays and individual qPCR were used to identify and confirm miRNAs that were downregulated in malignant urothelial cells (RT4, 5637 and J82) when compared to primary, non-malignant urothelial cells (HUEPC). DNA methylation was determined by customized PCR-arrays subsequent to methylation-sensitive DNA-restriction and by mass spectrometry. miRNA expression and DNA methylation were determined in untreated cells and in cultures treated with the demethylating agent 5-Aza-2′-deoxycytidine. miR-200b, miR-152 and miR-10a displayed differential expression and methylation among untreated cancer cell lines. In addition, reduced miRNA expression of miR-200b, miR-152, and miR-10a was associated with increased DNA methylation in malignant cells versus HUEPC. Finally, the demethylation approach revealed a causal relationship between both parameters for miR-152 in 5637 and also suggests a causal connection of both parameters for miR-200b in J82 and miR-10a in 5637. In conclusion, our studies in multiple bladder cancer cell lines and primary non-malignant urothelial cells suggest that hypermethylation of miR-152, miR-10a and miR-200b regulative DNA sequences might serve as epigenetic bladder cancer biomarkers.



## 1. Introduction

Bladder cancer ranks among the ten most common cancers in the world [1], and due to its high rates of recurrence and cost intensive follow-up care, it belongs to the most expensive. Cystoscopy and urine cytology are the gold standards for bladder tumor detection. However, cystoscopy is accompanied with considerable pain, high costs and the risk of misdiagnosing a tumor *in situ* for urocystitis. Cytology, though non-invasive, has limited sensitivity, especially for low-stage-tumors [2]. Therefore, numerous approaches have been undertaken to find non-invasive blood or urine biomarkers at different molecular levels [3,4]. Due to the increasing knowledge on the epigenetic alterations in bladder cancer [5], DNA methylation and miRNA expression are gaining more and more attention as potential biomarker candidates.

miRNAs are small, non-coding RNAs that negatively regulate gene-expression by degrading or translationally inhibiting mRNA molecules depending on the degree of complementarity [6]. For bladder cancer, several miRNA-molecules have been reported to interfere with important signal transduction-pathways [7]. Recently, miRNA molecules in the urinary sediment were discussed as promising biomarkers [8–10]. However, the predominant number of miRNAs discussed as cancer biomarkers are downregulated in bladder cancer [11], thus rendering them controversial as reliable diagnostic tools.

Besides miRNA expression, DNA methylation was found to be altered in bladder tumors [12]. Some of these alterations were also detected in urine, suggesting them as potential non-invasive candidate biomarkers for bladder cancer [13–16]. The most promising genes among the candidates belong to the class of tumor suppressor genes. Methylation induced silencing of the latter is one of the most important epigenetic mechanisms promoting tumor growth

*Abbreviations:* Aza, 5-Aza-2′-deoxycytidine; HUEPC, human urothelial epithelial cells.

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and de-differentiation [17]. Moreover, miRNAs with tumor suppressor activity have been reported to be silenced by DNA hypermethylation thus contributing to the global reduction of miRNA levels observed in cancer. The interplay between these two epigenetic factors has gained attention in recent publications [18–20].

As the diagnostic value of "positive" cancer-specific biomarkers (i.e., those which are upregulated in cancer cases compared to healthy controls) is always superior to that of "negative" biomarkers (which are downregulated or absent in cancer), the described link between miRNA expression and miRNA gene methylation gives rise to the idea of "translating" the downregulation of individual miRNAs into an increased methylation of miRNA genes. In this way, researchers could benefit from valuable miRNA expression information–even if it primarily disqualifies a miRNA molecule as a potential biomarker because of down-regulation–by transforming it into a more promising, "positive", methylation-biomarker.

Following this approach, we performed an initial miRNA expression array to identify miRNAs strongly downregulated in at least one human bladder cancer cell line. For this purpose, we compared for the first time the results obtained in the cancer cell lines to those obtained in primary, non-malignant urothelial cells rather than an immortalized cell line such as UROtsa. Among the identified down-regulated miRNAs, we selected five miRNAs with regulatory sequences that were previously described as hypermethylated under relevant biological conditions or in relevant biological samples. For these miRNAs, we investigated the relationship between expression and DNA methylation to evaluate whether the latter might be a candidate biomarker for future clinical studies.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

#### 2.1.1. Cell lines

As model systems for bladder cancer tissue, we used RT4 (CLS Cell Lines Service GmbH, Eppelheim, Germany, Cat.-No. 300326, obtained in October 2010), 5637 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, Cat.-No. ACC 35, obtained in April 2011) and J82 (ATCC Services, Wesel, Germany, Cat.-No. ATCC-HTB-1, obtained in September 2010) as established human bladder cancer cell lines originating from tumors of three different histopathological grades of differentiation (G1, G2 and G3, respectively). For the present study, DNA and RNA from cells cultured between October 2010 and November 2011 were used. We routinely performed STR analysis upon at regular 6–12 month intervals (dependent on their use in the laboratory). To model miRNA expression and miRNA-gene-methylation in non-malignant urothelium, primary human urothelial epithelial cells, HUEPC (Provitro, Berlin, Germany, Cat.-No. 1210721, Lot-No. 536X090206) were used. All cell lines and primary cells were successfully cultured according to the recommendations of the manufacturer.

#### 2.1.2. Demethylation-approach

RT4, 5637 and J82 were seeded in 175 $cm^2$ dishes at densities of 15, 5 and 2.5 million cells per dish in medium containing 5 μM 5-Aza-2′-deoxycytidine (Aza) or 0.05% DMSO as a solvent control. The medium with these additives was replenished every 24 h. After 72 h, cells were harvested by detachment, counted and aliquoted in different buffers for subsequent DNA or RNA isolation. For each cell line, the experiment was repeated three times.

### 2.2. Isolation of nucleic acids from cell pellets

For each cell line, isolation of DNA was performed from 4 million cells reconstituted in 200 μL PBS using the QIAamp DNA Mini Kit (Qiagen, Cat.-No. 51304) according to the recommendations of the manufacturer. Total RNA (including miRNAs) was isolated from $10^6$ untreated cells per probe using the miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction, and concentration of total RNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA quality was determined using a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). For Aza-treated cell lines, RNA was isolated from 4 million cells and reconstituted in 350 μL RA1-buffer using the NucleoSpin RNA II Kit (Machery Nagel, Cat.-No. 740955.50) according to the recommendations of the manufacturer.

### 2.3. Screening for miRNAs suppressed in cancer cells

The TaqMan Low Density Array (TLDA) Human microRNA Panel v 2.0 from Applied Biosystems was used for qPCR according to the manufacturer's instruction. This TLDA included 377 miRNA assays plus three selected endogenous controls. cDNA was transcribed using MJ Research PTC-200 (Bio-Rad Laboratories): 2 min at 16 °C, followed by 40 cycles at 42 °C for 1 min and 50 °C for 1 s, 5 min at 85 °C then held at 4 °C. qPCR was performed using an Applied Biosystems 7900HT Sequence Detection System at 94.7 °C for 10 min, followed by 40 cycles at 97 °C for 30 s, and 59.7 °C for 1 min. A downregulation of less than 0.5-fold expression in the TLDA-Array compared to HUEPC was used as a selection criterion for further studies.

### 2.4. Analysis of DNA methylation in cultured cells

#### 2.4.1. PCR-Array (Qiagen)

To quantitate the degree of DNA methylation, we used a customized version of the EpiTect Methyl qPCR Array-System from Qiagen according to the recommendations of the manufacturer. The test is based on a Real-Time-PCR with a set of gene-specific primers subsequent to the treatment of DNA with methylation-sensitive and/or -dependent enzymes in comparison to a respective mock-digestion. By comparing the resulting Ct-values, the system allows the quantification of the degree of DNA being unmethylated (0% methylated), methylated (>60% methylated) and hemi-methylated in the amplified DNA-stretch. In our custom-array, we investigated the methylation of regulative DNA-sequences for miR-127-3p, miR-410, miR-200b, miR-152 and miR-10a.

#### 2.4.2. MassArray EpiTyper Assay (Sequenom)

To validate the methylation results obtained by the Qiagen PCR-Array system, DNA of untreated cultured cells was analyzed by the MassArray EpiTyper system (Sequenom GmBH, Hamburg, Germany), a MALDI-TOF mass spectrometry-based quantitative method for measuring CpG methylation down to a single dinucleotide resolution [21]. In short, 1 μg of DNA from untreated cells was bisulfite-converted and subjected to PCR with primers (Table S1) designed by Sequenom's EpiDesigner software (http://www.epidesigner.com/index.html). Following shrimp alkaline phosphatase treatment, fragments were ligated to a T7 promoter segment, and subsequently transcribed into RNA. After cleavage of the latter with RNase A, the cleavage products were analyzed by MassArray.

### 2.5. Analysis of miRNA expression in cultured cells

Individual TaqMan microRNA Assays (Applied Biosystems, Darmstadt, Germany) were used to analyze the expression of the