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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

following mature human miRNAs: miR127-3p, miR-410, miR-152, miR-200b, miR-10a. The 15 μL RT reaction contained 10 ng of total RNA, 1.5 μL of 10× RT-buffer, 0.15 μL of 100 mM dNTPs with dTTPs, 0.2 μL RNAse-Inhibitor (20 units/μL), 1 μL of MultiScribe Reverse Transcriptase (50 units/μL), 2 μL of microRNA specific stem-loop primers. cDNA was transcribed using MJ Research PTC-200 (Bio-Rad Laboratories, Hercules, CA): 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C then held at 4 °C. 5 μL of the RT product was then used with 1 μL specific primers and TaqMan 2X Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) in 20 μL reaction within a 96-well optical plate at 95 °C for 10 min, followed by 40 cycles of 95 °C 15 s and 60 °C for 1 min. qRT-PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). All experiments were done in triplicate for each cell line and treatment. Two normalization steps were used: loading the same quantity of template RNA in each well and normalizing the data against endogenous genes (RNU48 + RNU44). The ABI TaqMan SDS v 2.3 software was utilized to obtain raw Ct values. Relative quantification of miRNA expression was calculated using RQ manager v 1.2. Relative quantification of miRNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems User Bulletin N° 2 (P/N 10303859)).

2.6. Data presentation

Data were presented according to the recommendations for cell-culture statistics by Cumming et al. [22].

3. Results and discussion

3.1. Screening for miRNAs downregulated in bladder cancer cell lines

To screen for miRNAs downregulated in bladder cancer, miRNA levels in all three bladder cancer cell lines (RT4, 5637 and J82) were compared to those in primary urothelial cells, using a TaqMan-low-density-array (TLDA). In total, 197 miRNAs were differentially expressed in all three cell lines compared to HUEPC. Among these, 58 miRNAs were downregulated in J82, 25 miRNAs in 5637, and 38 miRNAs in RT4. Overall, 61.4% of miRNAs were downregulated in cancer cell lines thus confirming previous reports that miRNA downregulation is more common in bladder cancer rather than upregulation [11]. Due to their strong downregulation (<0.5-fold) in cancer cells, miR-10a, miR-127-3p, miR-152, miR-200b and miR-410 were selected for further analyses (Table 1).

3.2. Association of DNA methylation and miRNA expression in untreated cells

To test for an association between miRNA expression and DNA methylation, we measured both parameters in the cancer cell lines and HUEPC for the selected set of miRNAs. Methylation results obtained from the PCR Array system were validated using quantitative methylation analysis with the MassArray system. Further investigation of the DNA stretches within the target regions of the PCR-Array (provided by Qiagen) confirmed the methylation-ranking of cell lines obtained by the PCR-array (Figs. S1–S3).

Table 1
miRNA expression in the three bladder cancer cell lines compared to HUEPC. Fold regulation was calculated according to TLDA-results.

	RT4	5637	J82
miR-10a	0.52	4.34	0.17
miR-127-3p	0.03	0.05	0.03
miR-152	0.04	0.36	0.73
miR-200b	6.25	5.44	0.01
miR-410	0.12	0.19	0.09

Most of the investigated DNA sequences were unmethylated in cultivated primary urothelial cells (Fig. 1). In contrast, our analysis revealed elevated DNA methylation for miR-127-3p and miR-410 in HUEPC although the expression of these miRNAs was within the range of the other miRNAs (Fig. S4) suggesting that a small percentage of unmethylated DNA was sufficient to account for the considerable transcriptional activity observed, at least in primary urothelial cells.

A strong differential methylation compared to HUEPC and an association with miRNA expression, suggesting a causal relationship between both parameters were observed for three miRNAs. There was more than 60% less unmethylated DNA regulative sequences (=gain in methylation) of miR-200b and miR 10a in J82 as well as miR-152 in 5637 (Fig. 1). These striking differences in DNA methylation were accompanied by either strong (for miR-200b and miR-10a in J82) or weak (for miR-152 in 5637) decreases in miRNA expression levels compared to cultured primary cells. Besides these examples of positive associations between higher levels of DNA methylation and correspondingly lower levels of miRNA expression, an additional number of similar but less pronounced associations were generally observed for most of the cell lines and miRNAs investigated. For example, for miR-10a, DNA methylation was found to be about 30% more abundant in RT4 (30% less unmethylated DNA) than in HUEPC, while the corresponding miRNA expression was less than half of that found in normal urothelial cells (Fig. 1C). Furthermore, both the abundance of unmethylated DNA and miRNA expression for miR-127-3p and miR-410 were slightly higher in HUEPC compared to the three cancer cell-lines (Fig. S4).

Only a few cases contradicted a causal association between methylation and expression parameters in untreated cells. For instance, a similar miR-152-expression was observed in HUEPC and J82 cells, although the latter exhibited considerably less unmethylated DNA. Furthermore, the level of miR-10a unmethylated DNA was about 70% lower in 5637 cells than in HUEPC, whereas the corresponding miRNA expression was even twice as high when compared with primary cells.

3.3. Effect of Aza on miRNA expression and DNA methylation

To analyze whether a loss of DNA methylation (=gain in unmethylated DNA) is associated with an increase in miRNA expression, we assessed and compared both parameters in cell lines that were exposed to a demethylating agent (Aza) or to a solvent control (DMSO). As expected, little to no further DNA demethylation could be achieved for miRNAs in cell lines where the DNA was already shown to be exclusively unmethylated in the untreated cells, i.e., for miR-200b in RT4 and 5637. However, levels of DNA methylation also remained unchanged for some DNA sequences of miRNAs and cell lines where considerable levels of DNA methylation could be detected in untreated cells (miR-410 and miR-152, both in J82). A similar unresponsiveness of DNA methylation to the effects of Aza was previously reported for other targets [23].

For most combinations of miRNAs and cell lines investigated, Aza-treatment had a considerable effect on the level of unmethylated DNA-sections. Herein, an increase in unmethylated DNA for miR-200b in J82 (17% increase), miR-152 in 5637 (53% increase) and miR-10a in 5637 (45% increase) was reflected by a concomitant (2.3-fold, 4.5-fold and 3.1-fold) increase in miRNA expression, respectively (Fig. 2). These links between the levels of unmethylated DNA and miRNA expression upon experimental demethylation suggest a methylation-dependent miRNA expression for miR-200b

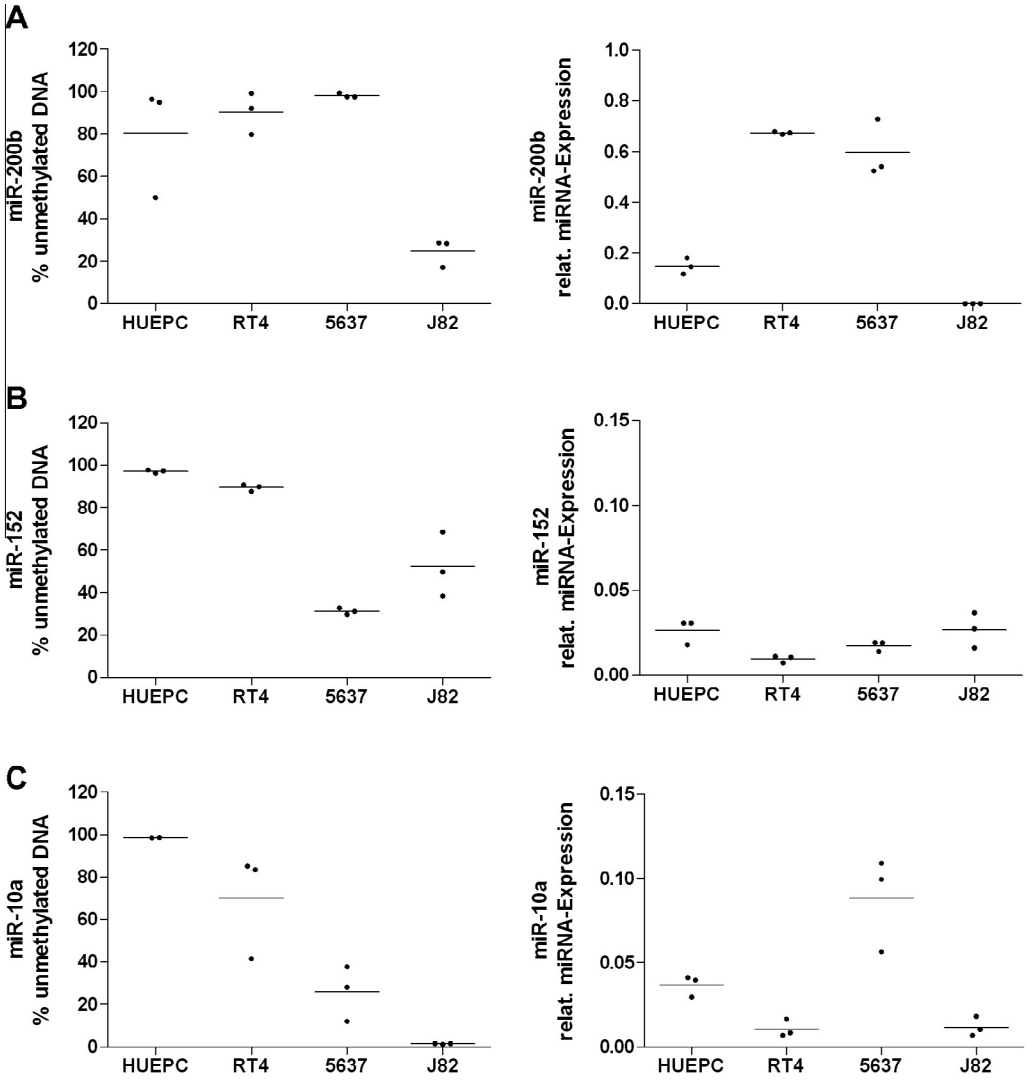

Fig. 1. Absolute percentage of unmethylated DNA (left diagrams) and relative miRNA expression (right diagrams) in untreated cells. Individual values and medians of three independent experiments are shown for miR-200b (A), miR-152 (B), miR-10a (C).

in J82 and for miR-152 in 5637. The latter is in line with the strong methylation of miR-152 in untreated 5637 cells, which was accompanied by a decrease in miRNA expression. A small gain in unmethylated DNA below 15%, as it was observed in all three cancer cell lines for miR-127-3p and in RT4 and 5637 for miR-410, was not accompanied by an increase in miRNA expression in the majority of the cases.

In contrast to the results obtained for untreated cells, a dependency of miRNA expression on DNA methylation could not be established for miR-10 in J82, as no changes could be induced by Aza-treatment for both parameters. However, considerable increases in the levels of unmethylated DNA and miRNA expression were measured in both RT4 and 5637 cells. Thus, for miR-10a, Aza-treatment revealed a strong and conclusive association between changes in DNA methylation and miRNA expression in all three cell lines examined (Fig. 2C). Demethylation confirmed results in untreated cells which showed a weak association between both parameters. For example, the demethylation experiment revealed a strong methylation-dependency of miRNA expression for miR-127-3p in 5637 in terms of decreasing DNA methylation with increasing miRNA expression. Similar associations were also observed for miR-410 in RT4 (Fig. S5).

Reviewing the results obtained for untreated cells and demethylation experiments collected in the present study, it becomes clear that a comprehensive analysis of both untreated and Aza-treated cells is the most appropriate way to evaluate the methylation-dependency of miRNA expression. As previously mentioned, the percentage of unmethylated DNA sufficing for miRNA expression might differ between cell lines (e.g., compare results for miR-10a in RT4 *vs* J82). Thus, a comparison of methylation and expression levels in untreated cells does not necessarily indicate a methylation-dependent regulation, even if the latter becomes evident from the demethylation-approach (e.g., see results for miR-10a in 5637).

According to our results, methylation-dependency is not necessarily conserved among cell lines (e.g., miR-152 appears to show methylation-dependent regulation in 5637 but not in J82 cells), and can be explained by a disparity in the level of differentiation. However, cell lines represent the individual situation of their donors, whose molecular constitutions are not fully known, e.g., polymorphisms that might impact the regulatory processes investigated. Further studies investigating clinical samples will elucidate the cell line that best models the majority of common bladder tumors.

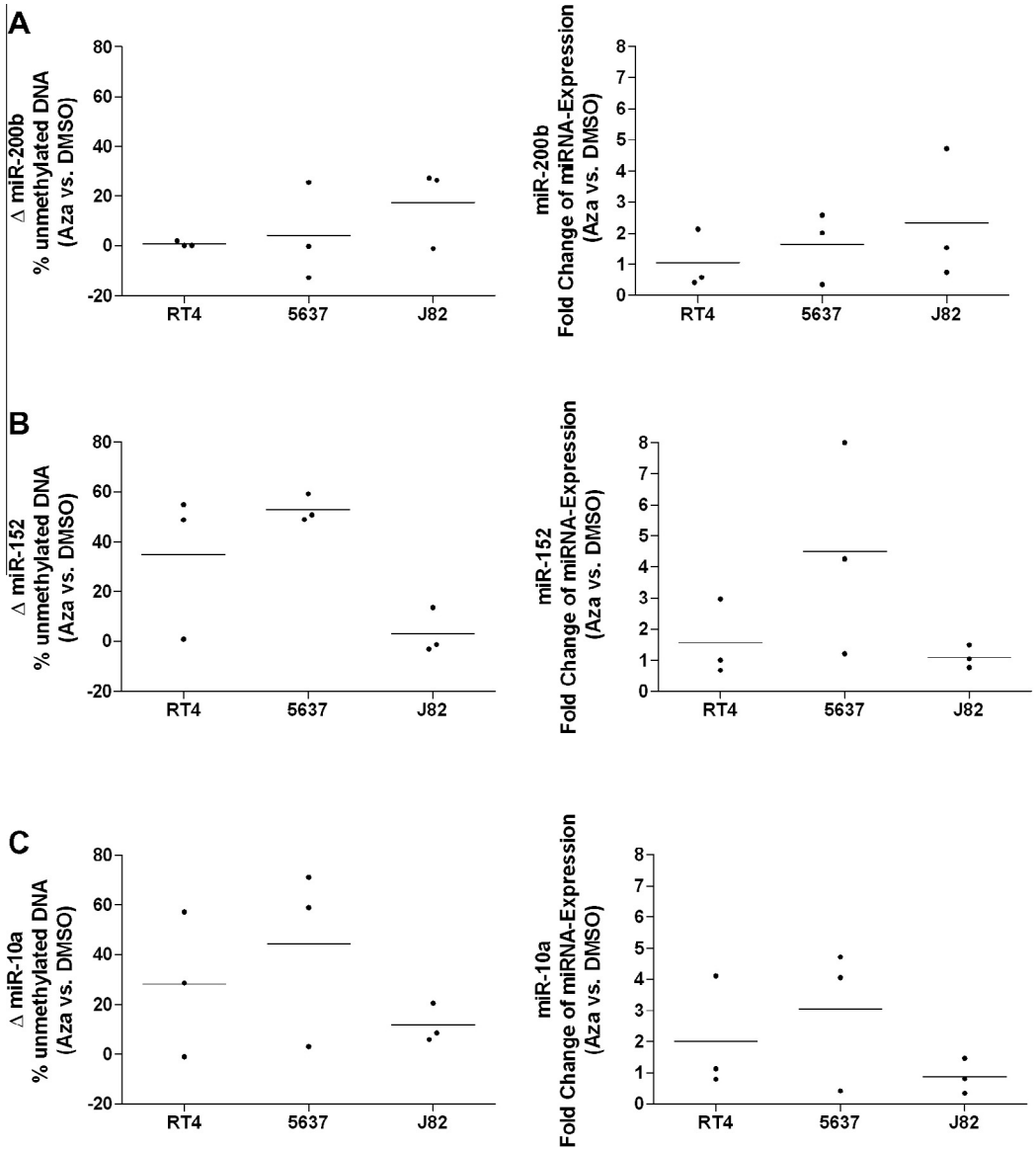

Fig. 2. Aza-induced changes in DNA methylation (left diagrams) and miRNA expression (right diagrams) in cancer cell lines. Individual values and medians of three independent experiments are shown for the differences in DNA methylation and fold-change values of miRNA expression for miR-200b (A), miR-152 (B) and miR-10a (C).

3.4. miRNA-regulative regions as methylation-biomarkers

An optimal clinical bladder cancer marker will indicate a single cancer cell within a complex mixture of cells, such as tissue or, ideally, urine sediment. Thus, downregulated miRNAs in bladder cancer cell lines compared to HUEPC (e.g., miR-200b, miR-152 and miR-10a) can be shortlisted as potential candidates for methylation-markers because due to strongly elevated corresponding levels of DNA methylation. Although increased expression is not a crucial prerequisite for a biomarker with an exclusively indicative function, the additional incidence of methylation-dependency suggests that increased methylation is echoed at downstream-levels. This may result in possible interference with regulative pathways leading to carcinogenesis or metastasis. Our results provide strong evidence for the methylation-dependent regulation of miR-152 in 5637 and miR-200b in J82. The results also suggest that regulation of miR-10a expression is methylation-dependent in RT4 and 5637 cells. Therefore, both criteria are fulfilled for the same three miRNAs investigated. Furthermore, this miRNA panel comprises differential methylation and methylation-dependent regulation in all three cancer cell lines, with the increased probability of detecting a broad range of cancer-subtypes.

An association between miRNA expression and the methylation of miRNA-regulative DNA-sequences has already been reported for miR-152 [24]. The authors showed that miRNA-152 expression was higher in urinary samples from controls than in patients suffering from bladder cancer. In addition, miRNA-152 expression was reduced in tumor compared to control tissue. Concordantly, hypermethylation was significantly more common for miRNA-152 in malignant than in normal urothelium. In accordance with our findings, the authors concluded that miR-152-methylation is a promising diagnostic marker. Our data confirm this suggestion and add the insight of a causal relationship between miRNA expression and the respective DNA methylation in at least one human bladder cancer cell line, as shown using the Aza-approach in 5637 cells.

miR-200b is a popular target in bladder-cancer research, as this miRNA has been shown to play an important role in epithelial-mesenchymal transition [25–27]. In accordance with our methylation- and expression-data in untreated cells, former studies revealed that the expression of miRNA-200b was repressed in

invasive compared to non-invasive urothelial carcinoma [28,29]. Moreover, the level of DNA methylation was reported to be significantly higher in muscle-invasive compared with superficial bladder tumors [28]. Taken together, present and former data on miR-200b thus support the hypothesis of a causal relationship between both DNA methylation and miRNA expression and support miRNA-200b as a potential methylation-biomarker.

Unlike miRNA-152 and miR-200b, there is no current literature on the methylation of miR-10a regulative sequences. However, Zhu and co-workers found that miR-10a expression was significantly upregulated in bladder cancer compared to the matched normal urothelium [30]. In another study, miR-10a was described to significantly discriminate Ta-cases from T1/2/3 tumors, demonstrating a 2.5-fold higher expression in superficial carcinomas compared to the latter [31]. This finding is in line with the low expression of miRNA-10a observed in J82 compared to an approximately 3.5-fold higher expression in 5637 cells observed in the present study. Both studies suggest that 5637 is the best cell line model for tumors investigated in the studies discussed. The strong hypermethylation observed for miRNA-10a in RT4 cells compared to HUEPC, together with the trend for a methylation-dependent regulation upon Aza-treatment in 5637 strongly suggests miR-10a as a candidate methylation-biomarker for bladder cancer.

In conclusion, our studies in multiple bladder cancer cell lines and primary urothelial epithelial cells suggest that the methylation of the regulative DNA sequences of miR-152, miR-10a and miR-200b might serve as epigenetic bladder cancer biomarkers. Future studies in urine sediment of bladder cancer patients and healthy controls should thus evaluate its diagnostic performance in clinical samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.021.

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