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A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer☆

Merle Hanke, Ph.D.[a,d], Kai Hoefig, Ph.D.[b], Hartmut Merz, M.D.[b], Alfred C. Feller, M.D.[b], Ingo Kausch, Ph.D.[c], Dieter Jocham, M.D.[c], Jens M. Warnecke, Ph.D.[a,d], Georg Sczakiel, Ph.D.[a,d,*]

[a] *Kompetenzzentrum für Drug Design und Target Monitoring, Lübeck, Germany*
[b] *Institut für Pathologie, UK S-H and Universität zu Lübeck, Lübeck, Germany*
[c] *Klinik und Poliklinik für Urologie, UK S-H and Universität zu Lübeck, Lübeck, Germany*
[d] *Institut für Molekulare Medizin, UK S-H and Universität zu Lübeck, Lübeck, Germany*

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Abstract

Objectives: MicroRNAs have been shown to be related to specific types of malignant cell growth. In case of urothelial bladder cancer (BCa), novel noninvasive diagnosis is particularly required and it is attractive to consider, as urine is an easily available source for molecular markers including RNA. In this context, we aimed to develop a clinically applicable and sensitive protocol for the preparation and molecular analysis of low molecular weight RNA from urine samples obtained from bladder cancer patients or healthy volunteers.

Materials and methods: First, a method was developed for the preparation of low molecular weight RNA from a set of urine samples from different donor groups: (1) patients with low-grade BCa, (2) patients with high-grade BCa, (3) patients with urinary tract infections, (4) healthy donors; each n = 9. The RNA extracts were then used to monitor a number of 157 microRNA species by quantitative reverse transcriptase-polymerase chain reaction. Subsequently, those microRNAs that showed a higher abundance in urine samples from BCa patients were detected in an independent set of urine samples (n = 47).

Results: The significance and diagnostic usefulness of this methodology is reflected by the finding that the RNA ratio of microRNA-126:microRNA-152 enabled the detection of BCa from urine at a specificity of 82% and a sensitivity of 72%, with an area under the curve of 0.768 (95% confidence interval, 0.605–0.931).

Conclusions: This study describes a novel, robust, and useful technology platform that is suitable to analyze small RNAs, including novel RNA-based tumor markers, in urine samples. A detailed technical analysis of this methodology provides new insights into the characteristics of urine microRNA such as composition and the donor-dependent variability.

Keywords: Bladder cancer; Cell-free RNA; miRNA; Non-invasive diagnosis; Tumor marker; Urine

1. Introduction

Recent findings indicate that small, non-coding RNA may act as gene regulatory molecules, termed microRNA (miRNA). They are suitable for the classification of tumors because of aberrant expression of miRNAs in human cancer [1]. Lu et al. showed that the classification of poorly differentiated tumors by miRNA-profiling was even more accurate than the use of mRNA classifiers, suggesting a potential role of miRNAs in the diagnosis of cancer [2]. In case of bladder cancer (BCa), the diagnostic "gold standard" is based on cystoscopy, which is invasive and relatively expensive. As noninvasive, superior, and specific marker urine cytology is used, although this method fails to detect low-grade BCa sensitively. Thus, novel, highly sensitive, and

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* Corresponding author. Tel.: +49-451-500-2731; fax: +49-451-500-2729.
E-mail address: sczakiel@imm.uni-luebeck.de (G. Sczakiel).

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specific urine-based diagnostic tools are particularly attractive as urine is a promising and easily available source for molecular markers, including RNA. On the technical level, urine samples can be obtained at high quantities and contain less proteins than blood-based samples, which reduces the interference of proteins during the RNA preparation and subsequent analyses. On the conceptual level, it is noteworthy that the investigation of urine RNA-based tumor markers is not only of particular interest for the detection of BCa but also for the diagnosis of other tumor entities [3,4].

Recently, we reported on the feasibility of total urine as a source for the detection of the mRNA-based BCa marker ETS2:urokinase plasminogen activator [5]. Despite these findings, the use of urinary mRNA tumor markers remains challenging since urine contains a high amount of nucleases, including RNases, which could lead to degradation of unstable RNA and, consequently, could result in extremely low abundances of mRNA tumor markers. In contrast to long-chain mRNA, it is reasonable to assume that short mature miRNAs are more stable against nuclease degradation due to the smaller size of typically 19 to 25 nucleotides. In this context, and with regard to the potentially wide usefulness of urine RNA-based diagnostics, we focused on the development of a simple and robust method for the preparation of small RNAs from urine. Here, we describe the development of such a technical protocol and its use to identify novel miRNA-based molecular markers for BCa.

2. Materials and methods

2.1. Clinical samples

The study was approved by the local research ethics committee. The tumor grading was determined by urinary bladder cystoscopy. In addition to biopsy, urine cytology was performed. All BCa tumors were identified, completely resected and, furthermore, pathologically evaluated according to their T-stage and grading on the basis of the World Health Organization 1973 grading system. All spontaneously voided urine samples investigated in this study were obtained with written informed consents of the participants. For the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) array experiment, whole urine was collected from 4 different groups (each $n = 9$). The first group consisted of healthy donors (5 men, 4 women; median age 69 years), the second group consisted of patients with infections of the urinary tract (1 man, 8 women; median age 76 years), the third group consisted of patients with G1 BCa (7 men, 2 women; median age 67 years), and the fourth group consisted of patients with G2 or G3 BCa (6 men, 3 women; median age 72 years). For the investigation of specific miRNAs, which were selected out of the array experiment, whole urine samples from 47 donors were analyzed: 11 healthy donors, median age 69 years; 7 patients with infections of the urinary tract, median age 73 years (microbial status: *Escherichia coli* (2×), *Klebsiella oxytoca* (2×), *Klebsiella ornithinolytica* (1×); not significant (2×), no data available (1×); 11 patients with a G1 BCa, median age 71 years; 18 patients with a G2 or G3 BCa, median age 72 years).

2.2. Processing of urine samples

For the monovette-based collection and storage of urine samples, we transferred 3.54 g of guanidinium thiocyanate (GTC) into a 10-mL urine monovette (Sarstedt, Nuembrecht, Germany) and adjusted the stamp to its 7-ml position. Further handling of the monovette was performed according to manufacturer's instructions. After GTC was dissolved in spontaneously voided urine and salt solutions were added (final concentrations: 6 mol/l GTC, 0.025 mol/l sodium acetate, 0.25% N-lauroylsarcosin, 0.5 mol/l HEPES pH 7 in a final volume of 10 ml), the sample was transported to the analytic laboratory where stabilized urine samples were frozen in liquid nitrogen and stored at −80°C until RNA preparation.

2.3. Preparation of RNA

The preparation of miRNAs was performed with a modified protocol for the miRNeasy kit (Qiagen, Hilden, Germany). The stabilized urine samples were thawed and the pH value was adjusted to pH 7.0 with 1 mol/l HEPES (pH 7.0). Thereafter, urinary RNA was extracted with an equal volume of acidic phenol:chloroform. The aqueous phase was loaded onto the silica membrane after the addition of 1.5 volume 100% (vol/vol) ethanol. The further preparation was performed according to the manufacturer's instructions and resulted in 40 μl RNA extract.

2.4. Protocols for the detection of urinary miRNAs

To investigate the robustness of the miRNA preparation and detection system, the inter-assay and intra-assay coefficients of variation (CVs) had to be determined. Therefore, RNA was prepared in triplicate from urine of two patients with BCa as described above. The miRNAs miR-16, miR-17-5p, miR-21, and the small nuclear RNA U6 (RNU6B, U6) were detected in duplicate via qRT-PCR using the human TaqMan MicroRNA Assay Kits (Applied Biosystems, Darmstadt, Germany). The reverse transcription reaction of 5 μl RNA extract was carried out with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) and contained 5 μl RNA, 1 mmol/l of each deoxyribonucleotide triphosphate, 50 units of Multiscribe Reverse Transcriptase, 1× reaction buffer, 4 units RNase inhibitor, and 1× gene-specific primer. Nuclease-free H_2O was added to a final volume of 15 μl. For the synthesis of cDNA, the reaction mixture was incubated at 16°C for 30 minutes, followed by an incubation step at 42°C for 30 minutes. The enzyme was inactivated at 85°C for 5 minutes. Afterwards, 1.33 μl of the cDNA solution was amplified using 1× TaqMan Universal PCR Master Mix