A pilot study of urinary microRNA as a biomarker for urothelial cancer

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Cite as: *Can Urol Assoc J* 2013;7:28-32. http://dx.doi.org/10.5489/cuaj.11115. Epub 2012 May 15.

Abstract

Objective: MicroRNAs (miRNAs) are part of a class of small ribonucleic acid (RNAs). They are important regulatory molecules, involved in several cell processes, such as developmental timing, stem cell division and apoptosis. Dysregulated miRNAs have been identified in several human malignancies, including bladder cancer tissue samples, and may confer a "tumour signature" that can be exploited for diagnostic purposes. We report on a prospective pilot study investigating the diagnostic capability of miRNAs in the urine of patients with urothelial cancer.

Methods: Voided urine samples were collected from patients with urothelial carcinoma just prior to bladder tumour resection, as well as age-matched healthy control patients. Pathology demonstrated both low- and high-grade cancer. Total RNA was isolated and quantitative reverse transcriptase-polymerase chain reaction was performed on the RNA extracts using primers for 4 miRNAs shown previously to be dysregulated in solid urothelial carcinomas with RNU6B as the endogenous control. Standard urine cytology was performed on all samples in a blinded fashion.

Results: Two miRNAs of interest were dysregulated in the urine from cancer patients with miR-125b showing an average 10.42-fold decrease ($p < 0.01$) and miR-126 showing an average 2.70-fold increase ($p = 0.30$) in the cancer samples compared to the normal controls. The sensitivity and specificity of the cytology on the same urine samples were 50% and 80%, respectively. Using these 2 miRNAs only, a decision-tree prediction model was generated for a validation cohort of patients yielding a specificity of 100% and a sensitivity of 80%.

Discussion: This preliminary study of candidate urinary miRNA in patients with low- and high-grade urothelial cancer demonstrated a significantly improved diagnostic accuracy over cytology. These results provide rationale for further studies on discovery and validation of candidate miRNAs in voided urine and may potentially lead to the development of a non-invasive and sensitive test for bladder cancer diagnosis and prognosis.

Introduction

Bladder cancer is the second most common urological cancer and the sixth most common cancer in Canadians, with 7100 new cases and 1850 resultant deaths in 2010.[1] Despite some advances in prevention, early diagnosis and treatment, bladder cancer remains a source of significant morbidity and mortality.[2] Given the high rates of recurrence of non-muscle invasive transition cell carcinoma (TCC), as well as the possibility of progression of higher risk disease, relatively close and long-term surveillance of the urothelium is the hallmark of bladder cancer management. Standard surveillance strategies of repetitive cystoscopy on an arbitrary schedule can be finessed through risk stratification, but are still invasive and associated with both expense and patient discomfort. Furthermore, flat lesions or carcinoma in situ (CIS) may be difficult to detect by visual inspection and the increased incidence of cancer in the upper urinary tract in these patients would highlight the need for accurate urine-based markers of TCC as an adjunct to cystoscopy.

Cytological study of exfoliated cells in the urine is beneficial for the diagnosis and surveillance of high-grade disease, but is often normal and of limited value for low-and intermediate-grade tumours.[3,4] A number of novel immunoassays for cancer-associated proteins in voided urine have been investigated as diagnostic tool to replace cytology, including BTA test, BTA Stat test, NMP22 and Immunocyt.[5-8] Most of these markers appear to have a greater sensitivity than cytology, especially for detecting lower grade tumours,[9,10] although often at a cost of specificity. Therefore, few markers have become integrated into the routine surveillance of patients at risk for recurrent TCC.[11-13] A number of chromosomal abnormalities have been reported in TCC and have facili-

tated the search for molecular-based genomic tests to determine the presence of cancer cells in urine. Several authors have reported the detection of single genetic abnormalities using fluorescent in situ hybridization[14-17] improving on the test characteristics of cytology, particularly the specificity in inflammatory conditions; however, sensitivity appears to remain an obstacle for low to intermediate disease.

MicroRNAs (miRNAs) are a class of small ribonucleic acid (RNAs) (20-25 nucleotides in length) that are important regulatory molecules within the cells of plants, animals and viruses.[18,19] Since their discovery in 2001, miRNAs have been found to regulate several cell processes[18-26] by negatively regulating gene expression at the post-transcriptional level.[19] Through the use of several molecular techniques, dysregulated miRNAs have been identified in several human cancers[27] and, in some cases, may confer a "tumour signature" that can be exploited for diagnostic purposes. Several recent reports have detailed the miRNA expression profiles in bladder cancer cell lines and tissue samples.[28-31] Up-regulated or down-regulated miRNAs have been associated with tumour stage and grade, as well as prognosis, although investigations into the utility of miRNAS as a diagnostic tool are limited.[31] We report a prospective pilot study investigating the diagnostic ability of a small panel of miRNAs in the urine of bladder cancer patients.

Methods

This study was approved by the Queen's University Research Ethics Board. After receiving patient consent, we collected voided urine samples immediately prior to transurethral resection of bladder tumour from eligible patients with suspected bladder cancer based on previous cystoscopy. Healthy control patients were identified after investigation for voiding symptoms or microscopic hematuria with no evidence of TCC on cystoscopy or imaging and no history of urothelial cancer. Voided urine was collected in a similar manner as those index cases. Inclusion criteria for TCC samples included subsequent biopsy-proven urothelial carcinoma (in situ, low grade or high grade).

The urine samples were split, with a portion (about 15 mL) processed for routine cytology and the remaining used for RNA extraction. The cytology slides were screened by the cytotechnologists at the centre and any abnormal samples were authorized by one of the cytopathologists as per standard practice.

Total RNA was extracted from the urine samples using the Urine (Exfoliated Cell) RNA Purification Kit (Norgen Biotek Corp, Thorold, ON), according to the manufacturer's instructions. Total RNA was stored at -80 ºC. Prior to analysis, the quantity and quality of total RNA was verified with the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), according to the manufacturer's instructions.

Quantitative reverse transcription (RT) was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems [ABI], Foster City, CA) with ABI miRNA specific primers for miR-125b, miR-126, miR-143 and miR-200a. These 4 miRNAs were chosen based on previous reports of miRNA expression in bladder cancer specimens. Quantitative polymerase chain reaction (PCR) was run on an Eppendorf Realplex Thermal Cycler (Eppendorf, Mississauga ON) using TaqMan microRNA assay kits (ABI). Samples were detected in triplicate and relative expression levels were calculated using U6 small nuclear RNA (RNU6B, ABI) as the endogenous control.

Statistical analysis

Analyses were performed using Genespring GX software (Agilent Technologies, Santa Clara, CA). Briefly, raw threshold cycles (Ct) values were imported into Genespring and relative expression levels for each microRNA were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The average-fold change among the two groups for each microRNA was determined and an unpaired Student's *t*-test was used for comparisons. For the miR125b:miR-126 combination, the ΔCt value (Ct miR-125b – Ct miR-126) for each sample was calculated and compared between the two groups. A decision-tree prediction model was generated within Genespring using the miR-125b:miR126 ΔCt data from all of the samples included in the study.

Results

This pilot study was performed in two stages: (1) to identify miRNA of interest in the urine of bladder cancer patients and (2) to identify a second group of patients for validation studies. For the first part of the study, 8 patients identified in the bladder cancer group (7 men, 1 woman, median age 76.5 years) and 5 healthy controls (4 men, 1 woman, median age 63 years). We tallied the demographic and cancer characteristics of each group (Table 1). Importantly, there was a mix of patients with low and higher risk TCC in the cancer dataset, with 1 case of CIS, 3 cases of low-grade TCC (1973 WHO grade 2) and 4 cases of high-grade TCC (1973 WHO grade 3).

Using the expression values for each miRNA relative to RNU6B, 2 miRNAs were found to be dysregulated between the two groups. MicroRNA-125b showed an average 10.42-fold decrease in the cancer samples compared to the control samples ($p < 0.01$) and miR-126 showed an average 2.70-fold increase in the cancer samples compared to the controls ($p = 0.30$). Even though the miR-126-fold change did not reach significance, it was chosen for further analysis because it showed an average increase in the cancer samples.

The ΔCt values for miR-125b and miR-126 (Ct miR-125b – Ct miR-126) were calculated for each sample (Fig. 1). A

Table 1. Clinical and pathologic characteristics of the study set

Bladder cancer patients				Healthy controls			
Age	Sex	Cytology diagnosis	Surgical diagnosis	Age	Sex	Cytology diagnosis	Surgical diagnosis
67	M	Few atypical cells suggestive of TCC	Metastatic high-grade TCC to pelvic lymph nodes	70	M	No malignant cells	N/A
81	M	Rare atypical cell – not diagnostic	Low-grade non-invasive TCC	82	M	Few atypical cells – not diagnostic	N/A
69	M	No malignant cells	High-grade non-invasive TCC	59	M	No malignant cells	N/A
72	M	No malignant cells – rare group in a voided urine	Low-grade non-invasive TCC	57	F	No malignant cells – groups in a voided urine	N/A
69	M	No malignant cells	Low-grade non-invasive TCC	63	M	No malignant cells – groups in a voided urine	N/A
83	F	Suspicious for TCC	High-grade invasive TCC				
88	M	Few atypical cells – cannot exclude neoplasm	High-grade invasive TCC				
90	M	No malignant cells	CIS, could not rule out invasive TCC				

M: male; F: female; N/A: not available; TCC: transitional cell carcinoma; CIS: carcinoma in situ.

decision-tree prediction model was generated using these data and a split value of -4.6 was calculated. Samples with a ΔCt for miR-125b and miR-126 above -4.6 are predicted to be cancer, while those with a ΔCt less than -4.6 are predicted to be non-neoplastic. Using this model yields a sensitivity of 100% and a specificity of 100%. The sensitivity and specificity of the cytology on the same samples are 50% and 80%, respectively, for this first cohort of samples.

For the validation part of the study, urine was collected from 8 patients. We tallied their histology and cytology results for the bladder cancer cases (Table 2). There were 5 patients in the bladder cancer group (4 men, 1 woman, median age 80 years), with 2 cases of low-grade carcinoma and 3 cases of high-grade carcinoma. The healthy control group consisted of 3 patients (2 men, 1 woman, median age 36 years). The DCt values for miR-125b and miR-126 (Ct miR-125b – Ct miR-126) were calculated for each sample in the validation set (Fig. 2). The sensitivity and specificity of the model were 80% and 100%, respectively. The sensitivity of cytology on the same bladder cancer cases was 20% (cytology was not performed on the normal control cases of the validation set).

Discussion

This pilot study demonstrates that miRNAs can be isolated and amplified from voided urine using standard quantitative PCR techniques. Furthermore, our results show that miRNAs are dysregulated in voided urine from bladder cancer patients and differences in miRNA expression can be used to differentiate among bladder cancer patients and normal controls. The difference in expression levels of two miRNAs (miR-125b and miR-126) appeared to accurately stratify control urines from bladder cancer urines with a higher sensitivity and specificity than routine cytology. Our model was validated on a small independent set of samples and showed a high specificity (100%) and a higher sensitivity than routine cytology (80% vs. 20%, respectively). The results of both training and validation sets suggest good sensitivity for even low-grade tumours, although this represents a very small number of samples.

Because of the nature of this pilot study, only a few miRNAs (miR-125b, miR-126, miR-143 and miR-200a) could be examined. These miRNAs were chosen based on the

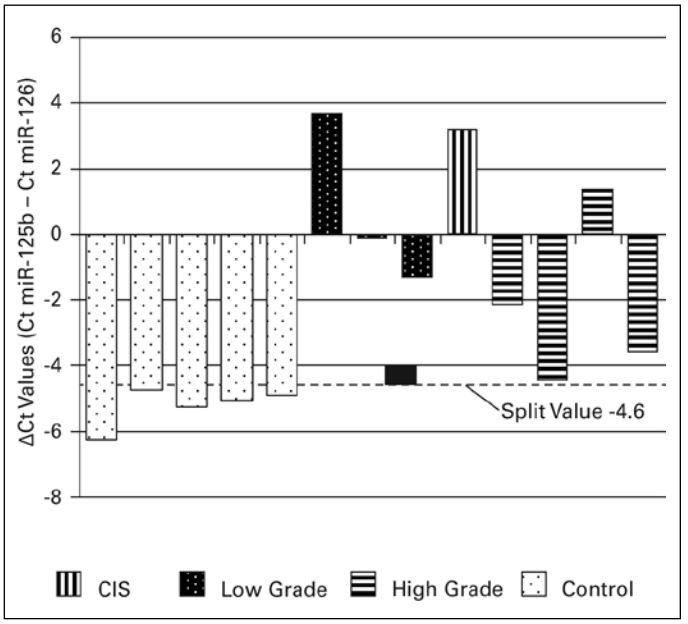

Fig. 1. Graph depicting the ΔCt values for each sample (Ct miR-125b-Ct miR-126). The dashed line at -4.6 represents the split value.

Table 2. Clinical and pathologic characteristics of the cancer cases in the validation set

Age	Sex	Cytology diagnosis	Surgical diagnosis
66	M	Few atypical cells, not well preserved	Invasive high grade TCC
82	M	No malignant cells	Low grade non-invasive TCC
77	M	No malignant cells	Invasive high grade TCC
80	F	No malignant cells, rare group in a voided urine	Low grade non-invasive TCC
91	M	No malignant cells	High grade non-invasive TCC

TCC: transitional cell carcinoma; urothelial carcinoma.

published literature on miRNA expression profiles in solid bladder tumours.[28,29] A large-scale, microarray-based study of dysregulated miRNAs in urine has yet to be published. Screening for dysregulated miRNAs in urine using a microarray-based platform may yield more informative miRNAs and could increase the sensitivity of the test.

Hanke and colleagues published the first study on how miRNAs isolated from voided urine can be used for cancer diagnostics.[32] In this study, the expression of 157 miRNAs were compared from voided urine of patients with low-grade bladder cancer, high-grade bladder cancer, urinary tract infections,and healthy controls (n=9 for each group) using multiplex quantitative PCR.[32] MicroRNAs of interest were subsequently validated on an independent set of samples (n = 47). Their results show that the microRNA-126:microRNA-152 ratio enabled the detection of bladder cancer from urine with a specificity of 82% and a sensitivity of 72%. These results support our hypothesis that urine miRNAs hold promise for bladder cancer detection. Further studies are needed in this area of cancer diagnostics.

Conclusion

Although our results are promising, the preliminary findings require more investigation. A larger, multi-institutional study would be needed to determine the validity and reliability of quantitative PCR with primers for miR-125b and miR-126. Furthermore, microarray techniques could be used to measure the expression of over 700 human miRNAs in the urine of bladder cancer patients and better identify a miRNA "tumour signature" in these patients. Although much more work is required prior to suggesting that urinary miRNA may play an integrative role in bladder cancer management, these results do support the recent literature suggesting miRNA profiles may allow non-invasive, highly sensitive and specific detection of TCC of all grades and stages. Future work should focus on answering specific clinical questions, including comparing miRNA diagnostic test characteristics to other urine-based tests. Studies should also question its ability to improve on clinico-pathologic factors to predict progression or treatment response.

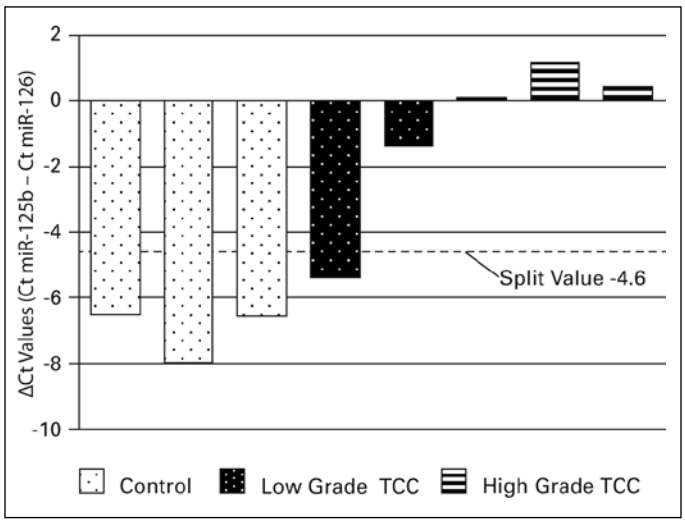

Fig. 2. Graph depicting the ΔCt values for each sample in the validation set. The dashed line at -4.6 represents the split value.

Competing interests: None declared.

This paper has been peer-reviewed.

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