Original Article

Molecular cystoscopy: Micro-RNAs could be a marker for identifying genotypic changes for transitional cell carcinoma of the urinary bladder

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ABSTRACT

Introduction: Normal-looking mucosa may harbor genetic changes preceding a visible tumor. This study was aimed at exploring the role of the quantitative expression of micro-RNAs (miRNAs) in bladder cancer tissue in comparison with normal mucosa and healthy controls (HCs) as a molecular marker.

Materials and Methods: Between October 2011 to December 2012, tissue from the bladder tumor of 21 patients (cases tumor, CT), normal mucosa (case control, CC) of the same patients (n-21) and normal bladder mucosa from 10 HCs were obtained. miRNAs of angiogenesis, endothelial mesenchymal transition and apoptosis were quantified using stem–loop RT Taq Man polymerase chain reaction. Statistical analysis was performed using the Chi square and independent sample T tests by using SPSS version 16.

Results: The mean age of the patients and controls were 55.41 ± 11.03 and 52.14 ± 13.04 years. miR-21, miR-205, miR-126, miR-10b and miR-200a were highly expressed in CT ($P < 0.027$, <0.048, <0.025, <0.029 and < 0.005) as compared with HC. Expression of miR-21 and miR-129 were both correlated with grade and stage ($P = 0.001$ and < 0.009, respectively) and the level of expression was different in the same grade of non-muscle invasive tumors. The fold change of miR129, miR205 and miR200a was significantly higher in the normal-looking mucosa of bladder tumor patients than the HC ($P < 0.005$).

Conclusion: Expression of miR129, miR205 and miR200a in the normal-looking mucosa of bladder cancer patients was significantly higher than the normal mucosa of a HC. This may help in predicting recurrence and formulating the follow-up strategy.

Key words: Bladder cancer, micro-RNA, muscle invasive, non-muscle invasive

INTRODUCTION

Bladder cancer is the fourth most common cancer in men and the 10th most common cancer in women in North America.[1] The clinical significance of the tumor stage is paramount. Broadly, bladder tumors are classified into two major groups, non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), based on the treatment approach.[2] In clinical practice, about 70–75% of the patients present with NIMBC and have two specific features that are different from other genitourinary tumors, i.e. multifocality (tumor arising at different sites and times, suggesting polyclonal etiology) and higher rate of recurrence. Because of the varying nature of this cancer, patients have to be followed-up regularly after initial endoscopic resection and adjuvant treatment.[3]

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Pathological characteristics like tumor stage and grades are not absolute predictors of disease recurrence and there is inter-observer and intra-observer variation among pathologists in reporting grades.[4] There is a need to have a molecular marker that could portray a true character of the tumor beyond the existing parameters on histopathology. Micro-RNA (miRNA) controls majority of the protein-coding genes by RNA interference or RNA silencing and are found to be dys-regulated in human cancer.[5,6]

Recent studies have documented a link between the expression of miRNAs and cancer pathogenesis for other types of cancer, such as glioblastoma, colorectal, lung, breast, hepatic and pancreatic cancer. However, only few reports have described miRNA expression in bladder cancer.[7-11]

The aim of this study was to analyze the expressions of various miRNAs in bladder tumor and normal-looking mucosa of the same patients and healthy controls (HCs) to characterize its significance in terms of tumor aggressiveness and possible role of understanding the recurrence and progression.

MATERIALS AND METHODS

Between October 2011 to December 2012, bladder tumor tissue from 21 patients (cases tumor, CT) and normal mucosa (case control, CC) of the same patients (N = 21) were obtained through trans-uretheral resection of bladder tumor (TURBT) and radical cystectomy. Ten samples of normal mucosa as HCs were obtained from benign prostate hyperplasia patients during their transurethral surgery. After taking ethical (A-02:PGI/IMP/IEC/57/21.10.2011) clearance from the institute and consent from every patient, we included 21 confirmed cases of bladder cancer and 10 cases of HCs.

All samples were immediately dipped in RNA and stored in -80ºC, until RNA extraction. Samples were homogenized in sterile conditions before total RNA isolation. Total RNA isolation and small RNA enrichment procedures were performed using the mir-Vana miRNA isolation kit (Ambion, USA) according to the manufacturer's instructions.

Real-time quantification of miRNAs by stem-loop reverse transcriptase-polymerase chain reaction

Complementary DNAs (cDNAs) were synthesized from total RNA using gene-specific looped primers according to the TaqMan MicroRNA assay protocol (PE Applied Biosystems, Foster City, CA, USA). RT reactions utilized 10 ng of small RNA sample (<200 ntds), 50 nM of stemloop RT primer, 1 × RT buffer and 0.25 mM each of dNTPs, 3.33 U/μL MultiScribe RT and 0.25 U/μL RNase inhibitor (all from the TaqMan MicroRNA Reverse Transcription kit of Applied Biosystems; 4366597). Real-time PCR was performed using the Applied Biosystems 7800 Sequence Detection System. The 20-μL PCR reaction mixture included 1.3 μL of RT product, 1 × TaqMan (NoUmpErase UNG) Universal PCR Master Mix and 1 μL of primer and probe mix of the TaqMan MicroRNA Assay protocol (PE Applied Biosystems). Reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 10 min. The threshold cycle data were determined using the default threshold settings. All real-time PCR reactions were run in triplicate and average threshold cycle (CT) and SD values were calculated. Nine miRNAs were quantified using stem–loop RT Taq Man PCR. RNU-48 was used as the endogenous control.

Data normalization and statistical analysis

Expression data were normalized according to expression of the RNU48 endogenous control (Applied Biosystem). miRNA expression was calculated using the $2^{-\Delta\Delta ct}$ method.[12] Statistical analysis was performed using the Chi square and independent sample T tests by using SPSS version 16.

RESULTS

Nine miRNAs were quantified in 21 pairs of bladder cancer tissues, their adjacent non-cancerous tissues and HC tissues using real-time RT-PCR, and their expression was normalized with endogenous control RNU48 expression and by using the relative quantification approach. The mean age of patients and controls were 55.41 ± 11.03 and 52.14 ± 13.04 years [Table 1].

miRNA-21, miR-126 of the apoptosis pathway, miR-205, miR-10b and miR-200a of the EMT pathway were highly expressed in CT ($P < 0.027$, <0.048, <0.025, <0.029 and <0.005 respectively) as compared with the mucosa of HCs [Figure 1].

Table 1: Clinico-pathologic characteristics of 21 patients with bladder cancer

Clinical features	Frequency (%)
Mean age (years)	
Patient	55.41±11.384
Control	52.14±13.04
Stage	
Ta	8 (40.9)
T1	7 (31.8)
T2	4 (18.2)
T3	2 (9.1)
Grade	
High grade	14 (68.2)
Low grade	7 (31.8)
Surgical procedure	
TURBT	10 (48%)
Radical cystectomy	11 (52%)

TURBT=Trans-uretheral resection of bladder tumor

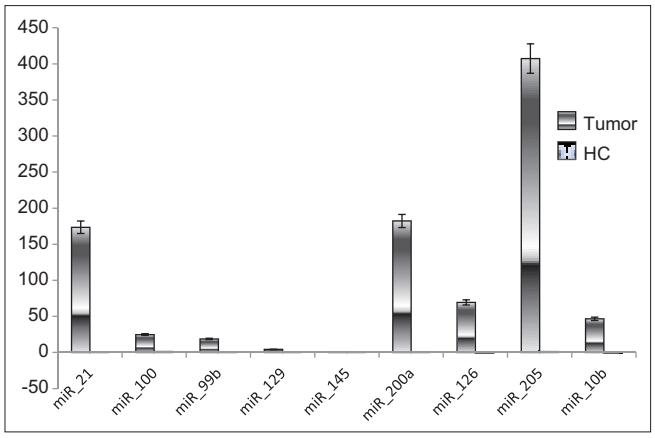

Figure 1: Graphical representation of miRNA expression of tumor versus healthy controls

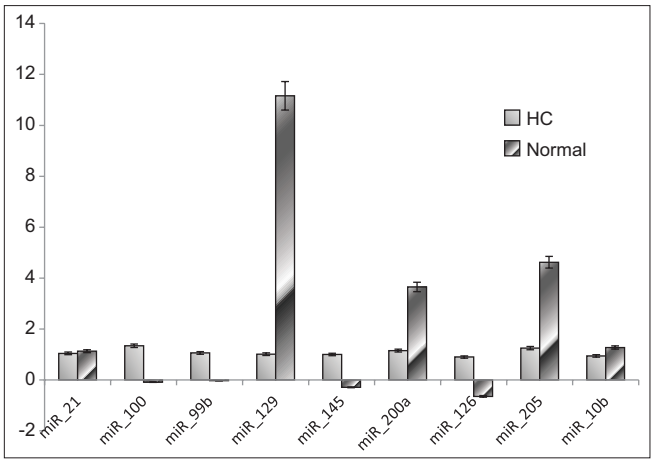

Figure 2: Graphical representation of miRNA expression of normal mucosa of bladder cancer patients versus healthy controls

Although not significant, miR-21, miR-100, miR-126, miR-10b, miR-205 and miR-200a were also elevated in CT as compared with the mucosa of the same patients (CC)[Table 2]. Expression of only miR-21 and miR-129 were both correlated with grade and stage ($P = 0.001$ and <0.009, respectively). But, miR-100 and miR-99 correlated with grade only ($P =$ <0.0049 and <0.063, respectively). Similarly, non-muscle invasive tumors of the same grade had significantly varied levels of expression, which could be seen in the context of follow-up. Interestingly, miR-129, miR-200a and miR-205 were up-regulated in normal-looking mucosa of the patients with tumor (CC) as compared with those without tumor (HC) ($P < 0.05$) [Figure 2].

Table 2: Median values of fold change of various miRNAs in various tissue samples

miRNA	CT*	CC**	HC***
miR-21	173.43	1.13	1.04
miR-100	24.69	−0.09	1.34
miR-99b	18.68	−0.03	1.06
miR-129	4.31	11.16	1.01
miR-145	1.13	−0.29	1.00
miR-200a	182.32	3.65	1.15
miR-126	69.37	−0.65	−0.90
miR-205	407.36	4.62	1.25
miR-10b	46.63	1.27	−0.94

*Case tumor, **Case control, ***Healthy control. miRNA=Micro-RNAs

DISCUSSION

Prediction of recurrence and progression of NMIBC is currently based upon clinical and pathological factors such as grade, stage, number of lesions, size, prior recurrence rate and presence of concomitant carcinoma *in situ*. But, the ability to assess prognosis of bladder cancer is not always satisfactory as the grading and staging of bladder cancer are subjected to inter- and intra-observer variability. Similarly, two different tumors of same stage and grade may behave differently. Therefore, there should be something more than the pathological characteristics that should predict the outcome of treatment in a better way. The follow-up of patients is expensive and requires invasive procedure in the form of cystoscopy, adding further trauma to the patient. There is a need for more accurate criteria to increase the predictive values of risk groups so that a suitable follow-up strategy can be offered to such patients.

Molecular differentiation characterizing the nature of transitional cell carcinoma has been studied and research is ongoing to type and stage TCC based on molecular profiling of various genetic disorders to reclassify the tumor. The most relevant research done so far is on allelic loss on chromosome 9, which is found to be associated with well-differentiated tumors, whereas in the aggressive subtype of TCC, alterations on chromosome 17p, which is the site for the p53gene, has been reported.[13]

Recently, there has been an upsurge in research in the field of miRNAs in various cancers. MiRNAs control around one-third of all protein-coding genes associated with the pathophysiology of many diseases and different biological processes such as glucose and fat metabolism, neuronal activity, cell cycle regulation, apoptosis and inflammatory process.[14,15] Growing evidences suggest that expression of miRNAs genes are dys-regulated in human cancer, and several studies indicated the existence of a functional miRNAs network that interacts with well-recognized proto-oncogenes and tumor suppressor genes.[16,17]

The majority of the studies reported that quantification of miRNA may help to predict the future of various cancers. The present study analyzed nine miRNAs by using stem–loop RT-PCR. One interesting finding that has emerged from this pilot study is that the expression profile of miRNAs was significantly different between bladder urothelial carcinoma tissue and adjacent normal bladder as compared with HCs.

Dyrskjot *et al.* profiled the expression of 290 miRNAs in bladder tumors, 11 normal tissue and various immortalized

and cancerous bladder cell lines, and found that miRNA-21 was the most up-regulated in cancer.[18] Our study also support these findings, showing that miRNA-21 was found to be up-regulated in bladder cancer. Up-regulation of miRNA-21 has been described in several other solid cancers as well.[19] Interestingly, miRNA-21 has been reported to promote cell transformation in breast cancer cells[20] and it is involved in invasion in colorectal cancer cells.[21] Depletion of miR-21 in breast cancer cell lines, furthermore, was shown to suppress cell growth and promote apoptosis.[22]

MiRNAs of the miRNA-200 family have a tight association with epithelial phenotype, and sensitivity to epidermal growth factor receptor (EGFR) inhibitor-induced growth inhibition in bladder carcinoma cell lines. The miRNA-200 family appears to control the epithelial-to-mesenchymal transition (EMT) process and sensitivity to epidermal growth factor receptor (EGFR) therapy in bladder cancer cells.[23] The present study justifies the mechanism – that the expression levels of miR-200a, miR-205 and 129 were significantly up-regulated in the normal-looking mucosa of patients with tumor as compared with HCs.

Therefore, the creation of lists of miRNAs differentially expressed between tumor and normal tissues gives the chance to identify the miRNAs most likely to be involved in cancer and to identify new diagnostic and prognostic markers. It is also worth mentioning that, because bladder cancer is multifocal, the normal-looking mucosa in a cancer patient may not be genetically normal and may predict the future recurrences as it would be desirable to know the likelihood of recurrence to tailor the plan of follow-up in such patients.

MiRNAs-21 and miRNA-129 showed higher expression in high-grade tumors as compared with low-grade tumors. Despite the high expression of these miRNAs, varied expression among the tumors with similar high grades may help in understanding the different behaviors of the same grade of the tumors. As this study is ongoing, further follow-up would help in characterizing tumors of the same grade for predicting the outcome. This would help in taking decisions of radical cystectomy in favor of BCG and vice versa in lamina-invasive high-grade bladder cancer.

Another interesting down-regulated miRNA was miR-126 located at chromosome 9q34, a region commonly lost in the early stages of bladder cancer, and we found that the down-regulation of miR-126 in HC as compared with CT might indicate the protective effect of this miRNA. Ma *et al.* found that miR-10b initiates invasion and metastasis in breast cancer.[24] Findings from the present study also support that miR-10b was up-regulated in bladder cancer and down-regulated in HC.

In summary, the present study has shown that miRNAs may play an important role in the patho-physiology of bladder urothelial carcinoma and could be a possible diagnostic and therapeutic target as well. Although our results are promising, these preliminary findings require more investigation to substantiate the conclusions. A larger, multi-institutional study would be needed to determine the validity and reliability of quantitative PCR with miRNAs. Furthermore, microarray techniques could be used to measure the expression of miRNAs in bladder cancer patients to identify the "tumour signature" in these patients. Pending further studies and validations, we suggest that miRNA-200a, miR-205 and miR-129 might be used for predicting early metastasis in clinical practice. This may represent a next step to better decision making regarding treatment strategy and, eventually, improvement in survival in bladder cancer patients. As genetic changes precede phenotypic changes, knowledge of altered expression of miRNAs could predict the recurrence of progression according to the site of altered expression. This may open a new dimension of research in this cancer.

CONCLUSION

There was a highly significant difference in the expression of miR-129, miR-200a and miR-205 between normal-looking mucosa of a bladder cancer patient and that of a healthy person. Thus, the normal mucosa of patients with bladder tumor may not be genetically normal. Genetic changes precede phenotypic changes, which could be the reason of recurrence of tumor in different zones of bladder epithelium. Quantification of varied expression of these miRNAs may help in predicting recurrence or progression in NMIBC and derive a follow-up strategy as well. Varied expression of miRNAs in the same grade of non-muscle invasive tumors may help in characterizing tumors for outcome prediction and making a correct clinical decision. The subsequent logical step of functional analysis of the target proteins of these miRNA may help in finding therapeutic means in the form of restoration of their expression or inhibition of an abnormal expression.

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Conflicts of interest

There are no conflicts of interest.

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