Association between Tissue miR-141, miR-200c and miR-30b and Bladder Cancer: A Matched Case-Control Study

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Purpose: To evaluate the expression of microRNAs in tissue samples from patients with bladder cancer and to compare it with healthy adjacent tissue samples as controls.

Materials and Methods: Thirty five tissue samples from patients with newly diagnosed untreated bladder transitional cell carcinoma and 35 adjacent normal urothelium were collected during 2013 to 2014. TRIzol reagent was used to isolate total RNA including microRNAs. RNA concentration and purity were determined using a nanodrop spectrophotometer. Also 1% agarose gel electrophoresis was used to assess integrity of RNA. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) method was performed using the PARSGENOME microRNA RT-PCR system. Data was analyzed by STATA 11.

Results: A couple of patients were female the remainder were male. Mean age of patients were 71.06 ± 11.43 years. The expression level of miR-30b, miR-141 and miR-200c in case group were significantly higher than that of control normal tissue samples. miR-141 had higher expression rate in malignant tissue than two other miRNAs ($P < .001$).

Conclusion: There was a more expression rate of miR-200c, miR-141 and miR-30b in bladder cancer tissues than healthy adjacent control tissues. Further studies are needed to draw final conclusion.

Keywords: carcinoma, transitional cell; gene expression regulation; microRNAs; genetics; urinary bladder neoplasms.

INTRODUCTION

Bladder cancer (BC) is the ninth prevalent and the second most common genitourinary tract malignant tumor with high mortality and 70% recurrence rate worldwide.[1,2] It is the fourth most common cancer in Western industrialized countries[2,3] and is among the top ten leading causes of cancer death.[4] In 2008 and 2010, 386,300 and 70,530 new cases and 150,200 and 14,680 deaths from BC was estimated worldwide, respectively.[5-7] Although the exact pathogenesis of BC is unknown, however, a range of irreversible genetic and reversible epigenetic changes, chromosomal anomalies and genetic polymorphisms involve in tumorigenesis and progression of BC. Genetic alterations are important in BC prognosis and treatment.[8,9]

micoRNAs (miRNAs) are a class of small non-coding RNA molecules, almost 22 (18-25 nt) nucleotides, which bind to the 3′ untranslated region of target mRNAs to control protein synthesis or degradation of the mRNAs. More than 1000 human miRNAs are known until now. miRNAs regulate protein expression, negatively regulate gene expression and modulate biological processes such as cell differentiation, proliferation, death, apoptosis, metabolism, tumorigenesis, immune response and viral infection. miRNAs and changes in their expression may play an important role in initiation, differentiation, suppression and development of various malignant tumors.[1,3,8,10,11]

miRNAs are obviously expressed in human cancers and affect carcinogenesis and cancer progression. miRNAs may be tumor suppressors or oncogenes.[3] Different miRNAs expression between tumor and normal tissues can identify miRNAs involved in carcinogenesis which can be used as novel therapeutic, diagnostic and prognostic markers.[11]

Altered miRNAs expression and function have also been reported as an important modulators in most urologic cancers.[8] Higher number of miRNAs species have been detected in the samples from patients with urothelial cancers.[12] Alteration in miRNAs expression may occur early in BC and affect carcinogenesis and tumor behavior.[13] Altered miRNAs expression in BC first reported in 2007 by Gottardo and colleagues.[14] They

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reported upregulation of 10 miRNAs. To date, several large-scale profiling experiments have been described about different miRNAs expression in BC.[8,13] Dyrskjot and colleagues analyzed 117 samples and found altered expression of many miRNAs.[15] Therefore changing in miRNAs expression play an important role in bladder tumorigenesis.

Some researchers have reported up-regulation of miR-200c, miR-141 cluster,[16] miR-141,[17] miR-200c,[18] miR 200c, hsa-miR-14[19] and miRNA 141[20] in BC. Other researchers have reported down regulation of miR-200 family,[21] miR-141, miR-200c and miR-30b[22] in BC. Among these miRNAs, in a study, three miRNAs panel including miR-200c, miR-141 and miR-30b had a sensitivity of 100% and a specificity of 96.2% to differentiate invasive BC from noninvasive BC.[22]

According to sensitivity of miR-200c, miR-30b and miR-141 in tissue samples of patients with BC and controversy among different studies, we aimed to evaluate the level of these miRNAs in tissue samples of patients with BC and healthy adjacent tissue samples.

MATERIALS AND METHODS

Demographic Characteristics of Participants

In this study 2 females and 33 male were included. Twenty-three of them lived in urban and the other 12 were from rural areas. Twenty-three patients were smoker and 12 were nonsmoker. Thirty subjects had no history of carcinogen exposure and 5 had history of carcinogen exposure. The study protocol was approved by the Medical Ethics Committee of the Hamadan University of Medical Science (Hamadan, Iran) and written informed consent was obtained from all patients after explaining the purpose of the study.

Inclusion and Exclusion Criteria

We included all eligible patients irrespective of sex and age. All patients had pathologically confirmed bladder transitional cell carcinoma (TCC). The exclusion criteria were patients with other organ cancers, genitourinary infection and history of radiotherapy and chemotherapy.

Data Collection Tools

A predetermined questionnaire including two parts was used for data-gathering. The first part includes the demographic characteristics of the participants such as sex, age, smoking history, history of exposure to carcinogens and urban/rural residence. The second part of the questionnaire encompasses imaging and pathological findings such as tumor size, grade, muscle invasion and tumor type.

Tissue Sample Collection

Thirty five tissue samples were collected from newly diagnosed and untreated BC. Control samples were matched adjacent normal urothelium resected about 10 cm far from the neoplastic lesions. Both samples from each subject were washed with RNase-free cold saline solution, snap-frozen in liquid nitrogen and stored at -80ºC until pathologic examination and further analysis.[20]

Pathological Examinations

All samples were examined by two pathologists. Tumor staging and histological grading were done according to the International Union Against Cancer and World Health Organization/International Society of Urological Pathology criteria of 2004, respectively.

RNA Extraction

TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) was used to isolate total RNA including miRNAs from about 50 mg tissue samples according to the manufacturer's instructions. The isolated RNA was dissolved in 20-50 µL RNase-free water depending on the amount of precipitation. RNA concentration and purity were determined by optical density measurement using a nanodrop spectrophotometer (BioTeK, Winooski, Vermont, USA). One percent agarose gel electrophoresis was used to assess integrity of RNA.

Reverse Transcription and Real-Time Polymerase Chain Reaction Analysis

Reverse transcription and real-time polymerase chain reaction (qRT-PCR) steps were completed by the PARSGENOME microRNA RT-PCR system (Tehran, Iran) according to the manufacturer's instructions. Briefly this system is a three-step protocol. First, Poly A enzyme step added a polyA tail to 3' end of RNA, second, first-strand cDNA synthesis produced specific miR cDNA product using specific primers and finally real-time qRT-PCR amplification with SYBR green master mix and miR specific primers with thermal cycling was done as follows: polymerase activation/denaturation at 95º, 5 min, 35 amplification cycle including denaturation at 95º, 5 s, annealing at 62º, 20 s, extension at 72º, 30 s and melting curve analysis. To analyze microRNA expression by qRT-PCR, 2 µg of RNA was reversely transcribed and 50 ng of synthesized cDNA used in the PARSGENOME microRNA RT-PCR system in a CFX96 real- time PCR detection system (Bio-Rad, Biosystems, Foster City, California, USA). The CT values were normalized using 5s rRNA as reference gene. All reactions were run in duplicate and to assess contamination, no template control (NTC) included in each PCR Run. All NTC were negative. The $2^{(-\Delta\Delta\ CT)}$ method was used to calculate Relative quantification of miRNA expression.[23,24] miRNA $\Delta\Delta CT$ formula is as follow: $\Delta\Delta CT = \Delta CT_1 - \Delta CT_2$, which ΔCT_1 = CT of the miRNA target (tumor sample) – CT of the reference gene (tumor sample) and ΔCT_2 = CT of miRNA target (normal tissue sample) – CT of reference gene (normal tissue sample).

Statistical Analysis

Statistical analysis was performed using STATA- 11 (StataCorp, College Station, TX, USA) software. All values were reported as mean ± SD. The differences in expression levels between case and control was estimated by paired *t*-test and *P* value less than .05 was considered as significant.

RESULTS

In this study 70 tissue samples from 35 patients with BC were studied (35 from malignant site and 35 from adjacent normal urothelium). In processing stage two RNA samples from each group were damaged; therefore, 66 specimens were studied. All of the cancers were TCC. Two (6%) of patients were female and 33 (94%) were male. Mean age of the patients were 71.06 ± 11.43 (range, 44-91) years. There was no difference between mean CT values of 5s rRNA in two groups (case 14.93 ± 2.05 and control 16.07 ± 3.19, $P = .084$). Therefore, it was suitable as a reference gene to normalize gene expression between malignant and healthy tissues. A single peak was observed on melting curve analysis, confirmed specificity of primers.

Table. Relative expression of the three miRNAs in bladder cancer and control tissue.

Variables	Cases			Controls			Difference	Paired *t*-Test	Fold Change in BC vs. Normal
	Number	Mean	SD	Number	Mean	SD	Mean	*P* Value	
miR-30b	33	5.16	1.87	33	6.44	1.67	1.28	.005	2.42
miR-141	33	1.44	2.56	33	5.74	2.64	4.30	< .001	19.7
miR-200c	33	-.44	2.46	33	1.16	2.73	1.60	.005	3.03

Abbreviation: BC, bladder cancer.

The higher ΔCT means the lower expression; therefore the expression level of miR-30b, miR-141 and miR-200c in case group showed a statistically significant increase compared to control normal tissue samples. Overall expression of these miRNAs was noticeably upregulated in BC tissue samples than control tissues, but we observed individually that miR-141, miR-200-c and miR-30b were down regulated or unchanged in 3, 7 and 12 of malignant tissue samples, respectively. Mean, standard deviation and *P* value of all miRNA are shown in **Table**. As shown in Table, miR-141expression level had a closer relationship with malignancy than two other miRNAs ($P < .001$ vs. $P = .005$). In case group the expression of miR-30b and miR-200c was significantly higher than that of control group. Mean ΔΔCT of miR-30b, miR-141 and miR-200c were -1.28,-4.30 and -1.60, respectively. Average relative quantification of miRNAs calculated by applying $2^{(-\Delta\Delta CT)}$. **Table** demonstrates average fold change of miR-30b, miR-141 and miR-200c in cancer group compared to control group. Mean relative expression of miR-141 was remarkable (mean fold change = 19.7).

DISCUSSION

miRNAs play an important role in cancer initiation, progression and metastasis.[25] One systematic review study has concluded that the deregulated miRNAs are common in BC.[26] Limited studies have investigated the exact function of miRNAs and their roles in BC. The results of this study were similar to Han and colleagues, Scheffer and colleagues, Xie and colleagues and Ratert and colleagues studies.[16,17,19,20] Han and colleagues reported that miR-200c and miR-141 are upregulated in BC compared to healthy control group.[16] In Scheffer and colleagues' research miR-141 level has increased in BC patients compared to control.[17] Ratert and colleagues investigated miRNA profile for BC diagnosis and clinical outcome. miR-141 was one of the seven upregulated miRNAs.[20] Hsa-miR -200c and miR-141 were also upregulated in infiltrating BC compared to non-infiltrating cancer.[19] The other research identified low expression of miR-30b, miR-31, miR-141, miR-200a, miR-200b and miR-200c in invasive BC cell lines as well as miR-21 and miR-99a, which showed high expression in the same cell lines.[22] In contrast to our study results, Wszolek and colleagues demonstrated that expression of miR-30b, miR-141 and miR-200c was reduced in invasive BC.[22] This may be due to different study design which they investigated invasive and non-invasive BC but we compared BC tissue with normal tissue.

miR-200 family in Wang and colleagues' study was down regulated in urine of BC patients.[21] In advanced cancer, miR-200 family (miR-200a,-200b, -200c, -141 and -429) are frequently silenced and play a role in epithelial to mesenchymal transition.[27] In a research, miRNA expression was correlated with tumor grade, size and presence of carcinoma in situ for miR-222, recurrence (miR-222 and miR-143), progression (miR-222 and miR-143), disease specific survival (miR-222), and overall survival rate (miR-222).[13] In this study we did not evaluate clinical and histological parameter of BC and miRNA expression. Further longitudinal studies are needed to determine the association between miRNA expression with different stage, grade and other clinicopathologic features of BC.

miR-200c, miR-141 and miR-30b are best classifier of invasive from noninvasive BC, and poor prognosis is linked to low levels of these three miRNAs.[22] Different results may be explained by different methods, regions, genetics and epigenetics alterations, lifestyles, study population, sample size, tumor grade and stage. Follow up of BC patients demonstrated that down regulation of miR-200c expression is related to progression of cancer to muscle and is associated with poor prognosis. Therefore miR-200c expression can be helpful in prediction of BC progression and treatment decisions.[27] Song and colleagues demonstrated that, miR-200c, miR-141 and miR-30b potentially can be used to diagnose invasive bladder tumors that were misdiagnosed in pathologic assessment of bladder biopsy specimens.[11] According to another study some of miRNAs were down regulated in low grade and upregulated in high grade BC.[8] Certain miRNAs may have therapeutic effects on BC.[5] Epithelial to mesenchymal transition is regulated by miR-200 expression in the BC cells and helps treatment with epidermal growth factor receptor.[28]

CONCLUSION

We demonstrated a more expression of miR-200c, miR-141 and miR-30b in BC tissues compared to normal healthy adjacent tissues. Further studies should be performed about miRNA alterations in BC to better understand the role of miRNAs in tumor initiation, progression, metastasis and treatment response.

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CONFLICT OF INTEREST

None declared.

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