ORIGINAL ARTICLE

Evaluation of miR-146a Urinary Level as a Marker for Recurrence of Superficial Bladder Cancer after Transurethral Resection of Bladder Tumor (TUR-BT)

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ABSTRACT

Background: The most prevalent malignancy affecting the urinary system is bladder cancer which exhibits a markedly high recurrence rate. The level of urinary microRNA-146a (miR-146a) was significantly higher in superficial bladder cancer patients, and were decreased to normal range after transurethral resection of the bladder tumor (TUR-BT). Its level was increased again if there is recurrence of the superficial bladder tumor diagnosed by follow up cystoscopy, miR-146 may be a clinically important marker for diagnosis and recurrence of superficial bladder cancer and is used instead of follow up operations as follow up cystoscopy for follow up of recurrence. Objective: our work aims to detect a simple, reliable and noninvasive diagnostic and surveillance methods for follow up of superficial bladder cancer. Methodology: The level of miR-146a in urine of 30 superficial bladder cancer patients was evaluated by quantitative reverse transcription polymerase chain reaction assay using voided urine samples before, after TUR-BT and after 3, 6, 9, 12 months of resection and compared with the result of follow up cystoscopy. Results: miR-146a was significantly increased in urine samples from patients with superficial cancer bladder than in those from the normal individuals (P <.000). Elevated urinary miR-146a levels in patients with bladder cancer were lowered to the normal level after TUR-BT and increased again in those who have tumor recurrence and remain in a normal level in those who have no recurrence after follow up for 3, 6, 9, 12 months after surgery(P=. 007, P=.000 respectively). Conclusion: Our study concluded that urinary miR-146a may be useful as a novel noninvasive diagnostic and follow up marker, anticancer agent or therapeutic target for superficial cancer bladder, also for increasing our knowledge of cancer biology.

INTRODUCTION

Bladder cancer is the commonest malignancy affecting the urinary system,1,2. As a result of its high recurrence rate,3,4 effective surveillance and diagnostic methods are required. Cystoscopy is the standard method for diagnosis of cancer bladder;5,6 however, it involves an uncomfortable and painful procedure. Although cytology of urine was used for diagnosing bladder cancer and was very specific, its sensitivity is still low.7,8 Therefore, another noninvasive method that has high sensitivity and specificity is required. For these reasons, various biomarkers for detecting bladder tumors, including UroVysion and NMP-22, BTA, have been developed9,12, although no urinary biomarkers exhibit sufficient specificity and sensitivity to allow them to replace urine cytology and cystoscopy.

The percentage of superficial type of bladder tumors have been reported to be approximately 75%. For such superficial (none muscle invasive) disease, intravesical instillation of chemotherapeutic agents has been already used as a treatment after transurethral resection of bladder tumor (TUR-BT)3,4. MiRNAs are single-stranded RNA molecules of 19-25 nucleotides in length that mediate post-transcriptional gene silencing of target genes and are expressed in a variety of cells. MiRNAs were initially discovered in Caenorhabditis elegans in 1993 as silencers of genes that regulate developmental timing 13. Subsequently, miRNAs play important roles in regulating a variety of biological processes, such as development, proliferation, apoptosis, and carcinogenesis.14-16 MiRNAs directly suppress gene expression through base pairing to the 3’ untranslated region (UTR) of target mRNA. A single miRNA can target high number of genes, and any gene can be regulated by multiple miRNAs. MiRNAs can do also effects on gene expression by either affecting epigenetic mechanisms, as acetylation of histone, or targeting transcription factors or DNA methylation17. Numbers of miRNAs have been known recently as candidate diagnostic and
prognostic markers for various types of malignancies, including bladder cancer as miRNA-146a\(^\text{18}\).

Recently, the miRNA level could be detected in body fluids and serum. In urine, miRNA could be measured in both the urinary supernatant and the sediment after centrifugation\(^\text{19}\).

**The aim of the work**

The aim of this study was to measure the level of miR-146a in urine of patients with superficial bladder cancer and after TUR-BT then after 3, 6, 9, 12 months for follow up of recurrence and comparing it with the results of follow up cystoscopy.

**METHODOLOGY**

This case-control study was conducted on 50 individuals, (30 superficial cancer bladder patients and 20 healthy control subjects) attending the Urology Department, Benha University Hospital in the period between March 2018 and December 2019. The study was approved by the Clinical Research Ethical Committee of Benha University Hospital.

All the patients provided informed consent. Subjects were divided into 2 groups:

**Group 1**: 30 patients with superficial bladder cancer (10 females and 20 males, their age ranges were from 50-65 years).

**Group 2**: 20 apparently healthy individuals as a control group (5 females and 15 males, their age ranges from 45-67).

Inclusion criteria for group I:
- Superficial cancer bladder patients newly diagnosed and not receiving any treatment

Exclusion criteria for group I:
- Patients receiving chemotherapy or radiotherapy.
- Patients known to have systemic inflammatory diseases.
- Patients with UTI and other inflammatory diseases which increase urinary miR-146a.

All subjects were subjected to the followings:
- History taking and clinical examination.
- Ct scan on urinary system
- Cystoscope and biopsy to diagnose the tumor and its grade
- Quantification of miR-146a in urine samples by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis:

Patients with untreated newly diagnosed by biopsy as superficial bladder cancer were involved in the study (Study group). Early-morning urine sample was collected from normal patients (Control group) and from the patients before transurethral resection of bladder tumor (TUR-BT) and additional urine specimens were also collected at (3, 6, 9, 12) months after surgery (TUR-BT) and follow up cystoscopy was done at the same time.

**Sample Preparation:**

Urine samples were centrifuged at 3000g for 30 minutes and at 13,000 g for 5 minutes at 4\( ^\circ\)C. The urinary cell pellets were lysed by RNAlysis buffer (Qiagen, Germany). All the specimens were then stored at –80\( ^\circ\)C until use.

**Measurement of miR-146a Level:**

RNA was extracted by use of miRN easy Mini Kit (QIAGEN, Germany) from urinary sediment according to the protocol of manufacturer. For each subject, 400 \(\mu\)L of urine supernatant was used for miRNA extraction.

cDNA was synthesized from miRNA by using miScript II RT Kit (QIAGEN, Germany) in a total volume of 25 \(\mu\)L. The real time PCR was done following the SYBR Green PCR protocol by use of QuantiTect SYBR Green PCR Kit (QIAGEN, Germany) with the Step One real-time PCR. Each reaction mix contained 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Primer Assay specific for miR-146a. Template cDNA, RNase- free water in a total volume of 25 \(\mu\)L. The real time PCR instrument Rotor-Gene Q (QIAGEN, Germany) was used with the following cycling conditions: enzyme activation at 95 \( ^\circ\)C for 15 min, followed by 45 cycles of denaturation at 94 \( ^\circ\)C for 15 s., and extension at 70 \( ^\circ\)C for 30 s.

The primer sequences of miR-146a and GAPDH which was used as an internal reference are presented in Table 1.

**Relative quantification was performed according to the \(\Delta\Delta CT\) method, and results were expressed in the linear form by using the formula \(2^{-\Delta\Delta CT}\) and expressed as relative units (RU) using Step One software (Applied Bio systems, USA)\(^\text{20}\).**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| miR-146a     | Forward 5'-TGAGAAGCTGAATTCCATGGTT-3'  
               2. Reverse 5'-CTGAAGAAGCTGAATTCCAGG-3' |
| GAPDH       | Forward: 5'GAAATCCCATCACCATCTCCAGG-3'  
               - Reverse: 5'GAGCCCGAGCCTTCTCCATG-3' |
Statistical analysis

The data were presented as means ±SD. Student’s t test was used for comparisons between cases and control groups. One-way ANOVA (F-test) was used to determine the significance of the differences in miRNA levels between groups and within the same group (pre-operative, post-operative and follow up later). A p-value of < .05 was considered statistically significant &p <.01 was considered highly significant. Receiver operating characteristic (ROC) curve was calculated to determine the role of miR-146a to discriminate between cancer bladder and normal individuals. Statistical analyses were performed by the Statistical Package for Social Science (SPSS) version 22

RESULTS

Bladder Cancer Patients have Increased Urinary miR-146a Levels:

The levels of miR-146a in urine samples from 30 superficial bladder cancer patients and 20 normal individuals were analyzed using qRT-PCR. Characteristics of the superficial bladder cancer patients are summarized in table 2.

Between superficial bladder cancer patients and normal individuals, the mean ages (57.96±5.4 and 56.15±6.4) and sex ratio (male/female, 20/10 and 15/5) were not statistically significant (P>0.05). Urinary miR-146a levels were significantly increased more in bladder cancer patients than in normal individuals (P<.000) (Table 2)

Table 2: Age, sex and level of Pre-operativemiR-146a of the studied groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control(n=20)</th>
<th>Cases(n=30)</th>
<th>Test of significance</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>45</td>
<td>50</td>
<td>t-test =1.08</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Maximum</td>
<td>66</td>
<td>69</td>
<td></td>
<td>(.284)</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>56.15±6.4</td>
<td>57.96±5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15(75%)</td>
<td>20(66.7%)</td>
<td>Chi² test=.397</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>Female</td>
<td>5(25%)</td>
<td>10(33.3%)</td>
<td>Odds ratio</td>
<td>(Male/Female=1.5)</td>
</tr>
<tr>
<td>Pre-operative MicroRNA level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>.01</td>
<td>1.3</td>
<td>t-test =4.73</td>
<td>&lt;.000**</td>
</tr>
<tr>
<td>Maximum</td>
<td>3.9</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>1.52±1.09</td>
<td>3.48±1.62</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Urinary Level of miR-146a was decreased After TUR. Postoperative voided urine was collected from 30 patients after TUR and after 3.6.9.12 months for follow up of recurrence. We divided the 30 patients into 2 groups diagnosed by follow up cystoscopy, the miR-146a positive group who had tumor recurrence (urinary miR-146a level of 3.91±1.4) and the miR-146a negative group who had no recurrence (urinary miR-146a level of1.0± .6). The urinary miR-146a levels of miR-146a positive group fell significantly after TUR and significantly increased again after recurrence (P=. 007).Whereas those of the negative group miR-146a level were not changed after TUR and after follow up cystoscopy indicating no recurrence of the tumor. (P=.000) (Table 3, fig. 2)

These results indicated that the urinary miR-146a levels of the patients that have elevated miR-146a levels before TUR declined to the normal range after TUR. Then increased again in positive group but remain low in negative group.

Table 3: Mean Urinary Level of miR-146a Before, After and follow up after TUR-BT

<table>
<thead>
<tr>
<th>Groups diagnosed by cystoscopy</th>
<th>Micro RNA level(Mean ±SD)</th>
<th>F-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before TUR-BT</td>
<td>After TUR-BT</td>
<td>Follow up after TUR-BT</td>
</tr>
<tr>
<td>Positive(8)</td>
<td>3.96±1.8</td>
<td>.95±.8</td>
<td>3.91 ± 1.4</td>
</tr>
<tr>
<td>Negative(22)</td>
<td>3.31±1.5</td>
<td>.96±.7</td>
<td>1.0 ± .6</td>
</tr>
</tbody>
</table>
Fig 1: Comparison between patients of cancer bladder regarding level of miR-146a (positive group)

Fig 2: Comparison between patients of cancer bladder regarding level of miR-146a (negative group)

ROC curve analysis shows that urinary levels of miR-146a have an area under the ROC curve value of 0.773 in distinguishing superficial cancer bladder patients from normal individuals (table 4, fig.3). At the cutoff value of 1.5, the sensitivity was 100% and the specificity 77.3%. Therefore, a subsequent analysis was performed on miR-146a.

Table 4: ROC curve for specificity and sensitivity of miR-146a

<table>
<thead>
<tr>
<th>ROC</th>
<th>miR-146a</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>986</td>
</tr>
<tr>
<td>P value</td>
<td>&lt; 0.000**</td>
</tr>
<tr>
<td>CI 95% Lower</td>
<td>0.953</td>
</tr>
<tr>
<td>CI 95% Upper</td>
<td>1.00</td>
</tr>
<tr>
<td>Cut off point (equal or more than)</td>
<td>1.5</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>77.3%</td>
</tr>
</tbody>
</table>

Fig 3: ROC curve for specificity and sensitivity of miR-146a as a diagnostic marker for recurrence of superficial ladder cancer
DISCUSSION

In the present study, miR-146a urinary level was significantly increased in superficial bladder cancer patients. To our understanding, this is the first evidence on urinary miR-146a as a new marker for detecting the presence of superficial bladder cancer. miR-146a has been reported to be aberrantly expressed in many types of cancer, such as prostate, thyroid, and gastric cancer. and increased miR-146a expression was shown to inhibit cell proliferation, migration, and invasion in in-vitro experimental models. In patients with cancer prostate, metastatic lesions have lower miR-146a expression levels than primary lesions, and patients with Gleason scores of >7 displayed significantly higher miR-146a expression levels than those with Gleason scores of 8. Moreover, patients after radical prostatectomy with miR-146alow level in plasma showed a low rate of recurrence and low miR-146a expression levels were known to be a high risk factor for blood invasion in patients with gastric cancer.

Accordingly, miR-146a is generally considered to act as a tumor suppressor in many different types of cancer. In patients with cancer bladder, a genetic variation of miR-146a was found to be linked with cancer bladder risk and recurrence.

In bladder cancer patients with the C allele, the level of miR-146a was higher than in those with the G allele, and C allele was associated with a lowered risk of cancer bladder and decrease the risk of recurrence. These results suggest that miR-146a also acts as a suppressor of the tumor in patients with cancer bladder.

In this study, the superficial bladder cancer patients have increased urinary miR-146a levels, as reported by Sasaki et al., that urinary miR-146a level was found to be linked with tumor histopathology status. This suggests that miR-146a could be high in response to some morbid conditions associated with cancer bladder and play a suppressor role in the progression of the tumor. It is interesting that a miRNA which suppresses the tumor as miR-146a, was increased in cancer bladder patients because many tumor-suppressive miRNAs are known to be decreased in various tumor cells. However, many other researches are needed to document this issue. In our work urinary miR-146a levels were significantly increased in superficial bladder cancer bladder patients than normal individuals. Twenty one of 30 cancer bladder patients had increased levels of urinary miR-146a. In the remaining 9 with miR-146a negative expression levels, urine samples might have been collected during the period in which the tumor cells did not have the reactive increase in miR-146a.

The result that not all patients with cancer bladder displayed increased miR-146a in urine, might reflect differences in each allelic variants of miR-146a. In the future, we have to study the relation between miR-146a expression and cancer progression and prognosis.

In the miR-146a positive group, the patients that showed elevated urinary miR-146a lowered to normal level after the TUR, and increased again if there is recurrence indicating that miR-146a is secreted from bladder cancer. This is consistent, that miR-146a was known as a differentially expressed miRNA during comparisons between normal ureteral epithelial tissue and human cancer bladder cell lines as reported by Sasaki et al.

Other studies have extracted miRNA that approved to be valuable biomarkers of bladder cancer from urinary supernatants. Thus, we thought we might use whole urine samples for our analysis.

miR-146a was reported to act as an important regulator of inflammation suggesting that the elevated miR-146a levels observed in our study might have been due to the presence of UTI. Therefore, we excluded the patients with UTI and routine urinary testing should be performed prior to sample collection.

Although our results were made on a small number of subjects, our findings suggest that the miR-146a present in urine is secreted from bladder tumor and is possibly involved in biological changes of the bladder tumor. It was noting that themiR-146a urinary levels in all the normal individuals were under the convincing cutoff value obtained from ROC curve analysis. This indicates that miR-146a level in urine is a possible marker for diagnosis of superficial bladder tumor and for follow up of its recurrence. Because no detailed functional analyses of this molecule have been performed till now, further in vitro and in-vivo functional analyses of miR-146a are recommended. Assessing the biological function of miR-146a might lead to the development of novel treatment options for superficial cancer bladder. Moreover, follow-up studies on a higher number of the cancer bladder patients should be made for understanding the relationship between miR-146a levels in urine in cancer bladder patients and their recurrence progression, and mortality.

CONCLUSION

This study suggested that urinary miR-146a might be useful as a new noninvasive, cheap diagnostic marker for follow up of recurrence, therapeutic target, or anticancer agent for superficial bladder cancer, as well as for increasing our knowledge of cancer biology.

Acknowledgments

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Conflicts of interest:
The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

REFERENCES


