RESEARCH
O&G Forum 2024; 34-3s: 1597-1601

URINARY MICRORNAS AS INDICATOR OF BLADDER CANCER AND INSPECTION OF THE MECHANISMS OF (MIRNA-93-5P AND MIRNA-516A-5P) IN IRAQI PATIENTS

Mundher Jabbar Al-Okhedi[1], Zubaida Adnan Al-ĵashamy[2], Duraid Taha Abdulkareem[3]

[1]Department of Medical Laboratories Techniques, Al-Maarif University College, Al-Anbar Governorate, Iraq,
[1]Ministry of Education, AL -Anbar Education Directorate, Iraq, mundher_alokhedi@yahoo.com
[2]Department of biology, College of Education of Pure and Applied Sciences, Tikrit university, Iraq, zubaida.biology@tu.edu.iq
[3]Consultant urologist / Assistant professor of urology /Anbar university/college of medicine, duraid71@uoanbar.edu.iq

Abstract
Objective: This study's objective was the possibility of using urine-derived miRNAs (miR-516a-5p and miR-93-5p) as good indicators for detecting bladder cancer.
Methods: The study was conducted from November 2022 to May 2023 in Ghazi AL_ Hariri Hospital of the Medical City (Baghdad/ Iraq). We studied a total of 45 individuals (25 BC and 20 healthy controls), using RT-q PCR tests.
Results: This study's results showed that the gene expression of miR-93-5p and miR-516a-5p was discovered by RT-q PCR, It was the miR-516a-5p and miR-93-5p were upregulated in bladder cancer patients compared to controls. The level of miR-93-5p was significantly different in the patients of BC compared to the control ($P < 0.001$)*, but the level of miR-516a-5p showed no significant differences compared to the control ($P > 0.001$). The AUC values of miR-93-5p were 0.772 (95 % CI: 0.636–0.908) and miR-516a-5p were 0.476 (95 % CI: 0.301–0.651). The sensitivity and specificity were (52 and 95%), (55 and 50%) respectively. The AUCs of miR-516a-5p and miR-93-5p did not differ significantly ($p > 0.05$).
Conclusion: Conclusion: miR-93-5p and miR-516a-5p are not good biomarkers for the diagnosis of bladder cancer. These findings imply that these urine-derived miRNAs may be play important role for the etiology and progression of BC. However, further independent more independent research is required to validate our findings and demonstrate the potential clinical utility of our miRNA.
Keywords: Bladder Cancer, miR-516a-5p and miR-93-5p.

Introduction

Three layers are characterized the bladder, the outer muscular layer, the underlying submucosa and a mucous layer of epithelial cells (urothelium). Recently, new bladder cancer was diagnosed. The type of cancer is located in the epithelial lining. The tumor cells grow from the submucosa into the muscular layer. Then, they attack the surrounding tissue to invade the lymph nodes and causes metastasis at the end (1).

One of the most prevalent urinary tract malignancy is the bladder cancer BC. Patients with BC are classified into two groups. About 75% of BC cases are non-muscle-invasive BC (NMIBC), while 25% are classed as muscle-invasive BC (MIBC). The main way of MIBC treatment is cystectomy, which has approximately 15% probability of repetition. This treatment happen a half-one and a half year after surgery (2).

Generally, BC is generated from the epithelium (urothelium)that covers the bladder's interior surface. One of the most common cancers which affects the bladder is urothelial cancer. BC with different histomorphological phenotypes was divulged (15-30% of cases) (3). These cases involve adenocarcinoma, small-cell-carcinoma and squamous cell-carcinoma. They could be diverted into glandular and squamous histologies. Different histology BCs are linked to various diseases, poor response to present therapies, and metastasis (4).

One of the small non- coding RNAs is MicroRNA (miRNAs). The RNAs are approximately 21-23 nucleotides in length. These RNAs serve as suppressors for the expression of genes via post transcriptional regulation. microRNAs contribute to different pathological and physiological processes (5), which include immune regulation, oncogenesis and embryo development. These types of RNAs were isolated from different biofluids such as urine, serum and plasma (6). The presence of miRNA withing these biofluids suggests a potential role of these miRNAs biomarkers of cancers (7).

MicroRNA-93-5p is a key miRNA involved in human cancer development. It is also referred to by different names, such as hsa-mir-93, MIR21, miR-93, MIRN93, and MIRN9. In humans, the miR-93 gene encoding premiR-93 is located on chromosome 7q22.1, and is expressed at high levels in various types of tumors (8).

MiR-93-5p has been observed in the clinical prognosis of cancer patients and has been shown to have a critical role in the incidence and development of several cancers. Patients with (BC) that have higher levels of miR-93-5p, which is thought to be a sensitive marker in BC. It was shown that miR-93-5p played a crucial role in the development of tumors, and that the

degree of malignancy was significantly correlated with the up-expression of miR-93-5p in BC cells. The increased pathogenic grade during the development of cancer may be explained by this. For individuals with bladder cancer, the presence of miR-93-5p may be a significant clinical indication (9).

MicroRNA-516a-5p is located on chromosome q13.4219, and is also referred to by different names, such as hsa_miR-516a-5p, 516a-5p-MicroRNA, and miR-516a-5p. These microRNAs are found on chromosome 19, As members of the chromosome 19 miRNA cluster (C19MC), The longest human miRNAs cluster discovered to date, C19MC is expressed by paternal imprinting (10).

Materials and methods

Patient sample collection was recruited from Ghaze Al_Hariri Hospital of Medical City (Baghdad/Iraq) between November 2022 and May 2023 as the set of discoveries used to find miRNA suitable for markers. The sample of the study was 45 (25 patients of BC and 20 controls). Written informed permission was acquired from every individual involved. The Ghaze Al-Hariri Hospital's ethical committee gave its approval for this study.

The samples of urine from each (BC) patient and controls were collected, complying with the criteria: Urine samples were taken from individuals who had antitumor treatments, including radiation, chemotherapy, or surgery. urine samples from controls were obtained from individuals who had medical examinations and did not exhibit any disease; none of these individuals had any indications of disease in other organs. Based on histological results, BC was diagnosed. After that 250 µl of urine was added to a 2 ml Eppendorf tube pre-filled with 750 µl Trizol, mixed well, and then kept at a temperature (-20) in preparation for the miRNA extraction process.

Extraction of miRNA and qRT-PCR

Extracted miRNA from all samples by using the EasyPure® miRNA Kit Reagent (TransGen, biotech. ER601-01) in compliance with the manufacturer's guidelines. Using the EasyScript One-Step, gDNA Removal, and cDNA Synthesis Super Mix Kit (Trans Gen, biotech. AE311-02), total miRNA was reverse transcribed to (cDNA). in compliance with the manufacturer's guidelines, the running was performed in a reaction volume of 20 µl. of total miRNA.

levels of the expression for (miR 516a-5p) and (miR 93-5p) were performed by the qRT-PCR SYBR Green test to estimate the expression of the target gene.

Endogenous control gene U6 levels were amplified and utilized to normalize the miR 516a-5p and miR 93-5p genes levels. The primers used wereas follows: miR 93-5p (CAAAGTGCTGTTCGTGCAGGTAG), miR 516a-5p (TTCTCGAG GAAAGAAGCACTTT), miRNA-uniR.P.1 (GCGAGCACAGAATTAATACGA C), miRU6 F.P. (AGAGAAGATTAGCATGGCCCCT), and universal R. transcription p. (CAGGTCCAGTTTTTTTTTTTTTTVN). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression. (11).

Data Analysis

All the data were analyzed using the IBM SPSS Statistics program version 29 to determine the effect of various factors on study parameters. The Statistical analysis included a T-test and One-way ANOVA, the P-value, the mean and standard deviation (Mean ± SD) were utilized to compare means statistically. significance was chosen by using a chi-square test at probability value (< 0.001 and 0.05) was considered statistically significant. By using receiver operating characteristic (ROC) curves, potential miRNA or their combinations were estimated for their diagnostic accuracy. Estimate of CI in this study.

Results

Extracted miRNA from all samples of urine (25 patients of BC and 20 controls), and the gene expression of miR-516a-5p and miR-93-5p was discovered by real-time PCR. As shown in Tables .1and 2, It was the miR-516a-5p and miR-93-5p were upregulated in bladder cancer patients compared to controls. The level of miR-93-5p was significantly different in the patients with bladder cancer compared to the control ($P < 0.001$)*, but the level of miR-516a-5p showed no significant differences compared to bladder cancer and the control ($P > 0.001$).

Table 1: *miR-93-5p* expression of patients and control.

-Groups-	*Means ct of miR-93-5p*	*Means ct of U6*	*Δct (Means Ct of miR-93-5p)*	*2^-(ΔΔct) (Fold of gene expression)*
patient samples (urine)	18.6876	14.7528	3.9348	1.814604442
Control	19.2545	14.5875	4.667	1.115911465

Table 2: *miR-516a-5p* expression of patients and control.

- Groups-	*Means Ct of miR-516a-5p*	*Means Ct of U6*	*ΔCt (Means Ct of miR-516a-5p)*	*Fold of gene expression*
patient samples (urine)	25.2044	14.7528	10.4516	1.05370336
Control	24.9965	14.5875	10.409	1.113837375

Results showed over-expression of *miR-516a-5p* was correlated with *miR-93-5p* (r=0.328, p<0.05), which was fo by the RT-qPCR results(Fig. 1).

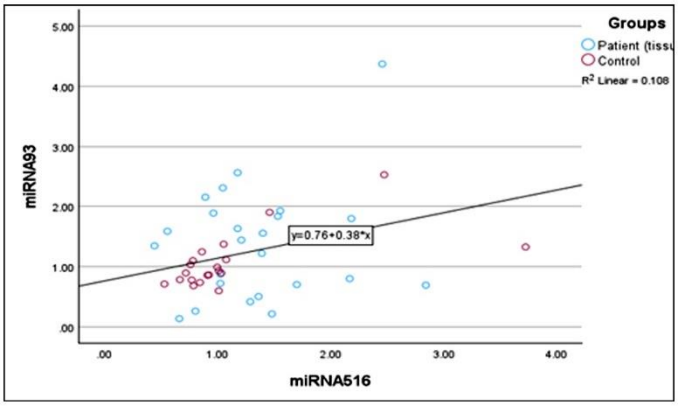


Fig. 1: Correlation between (*miR-516a-5p*) and (*miR-93-5p*) in bladder cancer group.

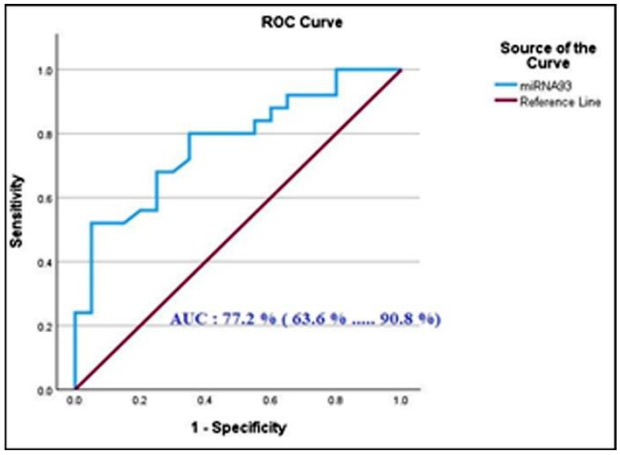
Furthermore, we found a significant differentiation in (miR-516a-5p, miR-93-5p) and age and gender (all at p<0.001) (Table -3).

Table -3: Comparison of age and gender between the patient and control groups.

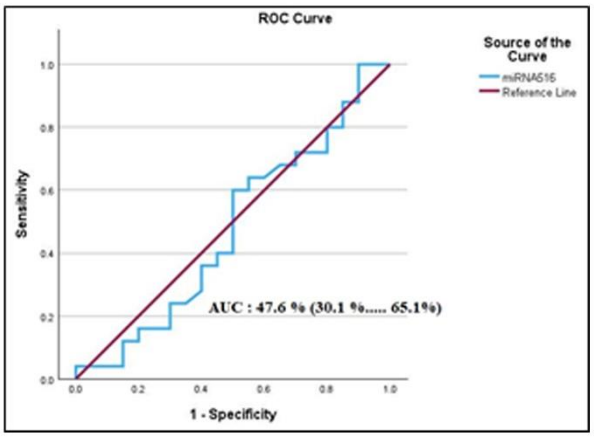
Groups	Sex	Mean	SD	SE	p-value
Patient	female	65.4444	9.96382	3.32127	0.001**
	male	62.4375	10.19783	2.54946	
Control	female	37.1429	7.66874	2.89851	
	male	39.4615	6.21310	1.72320	

Data were expressed as counts with percentages in parentheses or mean ± SD: (0.001) Statistical analyses were used by ANOVA. SD: Std. Deviation, SE: Std. Error of Mean, NS.

Receiver operating characteristic (ROC) curve analysis and AUC were performed to evaluate the expression of the potential diagnostic value of identified miRNAs.The AUC values of miR-93-5p were 0.772 (95 % CI : 0.636–0.908) and miR-516a-5p were 0.476 (95 % CI : 0.301–0.651). The sensitivity and specificity were (52 and 95%), (55 and 50%) respectively. The AUCs of miR-516a-5p and miR-93-5p did not differ significantly (p > 0.05).



(A)



(B)

Fig. 2: ROC curve analysis of (A) *miR-93-5p* (B) *miR-516a-5p*.

Discussion

Urine is a unique clinical sample that has advantages over other patient samples like blood. It can be obtained non-invasively and is readily available, making it a good source of biomarkers for a wide range of illnesses (12). Urine-derived exosomes were shown to contain substantially more enriched and persistent miRNAs than cell-free urine in several studies. Deviant expression of certain miRNAs may be indicative of altered biological processes associated with illness. Urine-extracted exosomes have been studied as potential indicators for bladder cancer(13). Nevertheless, not enough research has been done on the exosomal miRNA signature sample or how well it works in urine for BC patients' diagnosis(14).

This study's results showed that the gene expression of miR-93-5p and miR-516a-5p was discovered by RT-q PCR, It was the miR-516a-5p and miR-93-5p were upregulated in bladder cancer patients compared to controls. The level of miR-93-5p was significantly different in the patients of BC compared to the control (P < 0.001)*, but the level of miR-516a-5p showed no significant differences compared to the control (P > 0.001). The AUC values of miR-93-5p were 0.772 (95 % CI: 0.636–0.908) and miR-516a-5p were 0.476 (95 % CI: 0.301–0.651). The sensitivity and specificity were (52 and 95%), (55 and 50%) respectively. The AUCs of miR-516a-5p and miR-93-5p did not differ significantly (p > 0.05).

miR-93-5p has been identified as being in several of tumor types (15, 16) and primarily involved in the carcinogenic growth of malignant neoplasms, such as BC. According to research by Sun (15) and colleagues, miR-93-5p, is upregulated in cervical neoplasm cells and accelerates the growth of the malignancy by suppressing the THBS2/MMPS signal pathway. The results somewhat supported findings that miR-93-5p plays an oncogenic function in bladder cancer by suppressing PEDF expression to encourage cancer cell proliferation and invasion(17).

According to new research, miRNAs are abnormally expressed and have a significant impact on the onset, progression, and metastasis of cancer. Demonstrated that expression of miR-93 was correlated with lymph and node metastasis tumor stage, and it was elevated in tissues of BC compared to healthy samples. According to our findings, miR-93 has a role in BC cell proliferation as an onco-miRNA (22). Numerous cancer types have dysregulated miR-93–5p (18).

Some studies found that *miR-93-5p* is high regulated in BC tissues compared with healthy bladder tissues. This regulation has been associated with promoting tumor development, invasion, and metastasis by attacking various tumor-suppressor genes. For example, *miR-93* has been reportedly to target PTEN (phosphatase and tensin homolog), a known tumor suppressor gene include in the regulation of cell reproduction, apoptosis, and invasion (19). On the other hand, there are studies suggesting a tumor-suppressor role for *miR-93-5p* in BC. These studies showed that the down-regulation of *miR-93-5p* is the relationship between tumor aggressiveness and poor diagnosis in BC patients. In addition, miR-93-5p plays to inhibit migration, cell proliferation, and invasion by targeting oncogenes involved in BC development (20). Overall, the role *of miR-93-5p* in BC appears to be context-dependent, and its precise function may vary depending on the specific molecular pathways and cellular contexts involved in bladder cancer development. Furthermore, several studies are needed to elucidate the precise mechanisms of the involvement of *miR-93-5p* in the development of BC, which may have implications for the progression of new diagnostic and therapeutic strategies for this disease (21).

The majority of miRNAs genes are found in genomic regions or risk locations linked with cancer, and their dysregulated expression encourages the growth of cancer. *miR-516a* is a member of the C19 MC and has been identified in certain investigations as an oncogene. It also encourages the growth of aneurysms(22), If when *miR-516a-5p* is expressed, neuroblastoma patients' risk stratification is enhanced, indicating that *miR-516a-5p* may have carcinogenic potential. These miRNAs are categorized as "context-dependent" because their activity is influenced by the cellular environment, resulting in their dual roles as tumor suppressors and oncogenes. Several study's findings suggested that BC cell proliferation was enhanced by *miR-516a* overexpression. Because *miR-516a* acted as an oncogene in bladder cancer, it is possible that this miRNA might be used as a therapeutic agent as well as a biomarker for prognosis and diagnosis (23).

According to another study, miR-516a targets the 3′-untranslated region (3′-UTR) of leucine-rich PH domain-containing protein phosphatase 2 (PHLPP2), which lowers the amount of PHLPP2 protein and may have an impact on BC cell proliferation. This study identified a new mechanism that contributes to PHLLP2 down-regulation in BC cells. Furthermore, the study's conclusions expanded on our knowledge of PHLLP2's function in cancer metastasis (24).

In summary, we conducted a thorough and comprehensive examination of the urine miRNA profile in BC patients and found alterations in the expression levels of miR-93-5p and miR-516a-5p as compared to healthy controls. In particular, miR-93-5p and miR-516a-5p are not suitable markers for BC diagnosis. These findings imply that these urine-derived miRNAs may be important for the etiology and progression of BC and call for more research. Nevertheless, more independent research is required to validate our findings and demonstrate the possible therapeutic use of our miRNA.

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