MIRNA-92A PROFILING IN MUSCLE INVASIVE AND NON-MUSCLE INVASIVE BLADDER CANCERS IN RSUP DR SARDJITO YOGYAKARTA

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ABSTRACT

Objective: *The purpose of this study was to determine the expression of miRNA-92A in Bladder Cancer can be used as a marker of tumor.* ***Material & Methods:*** *This study used a total of 30 samples. 15 samples of non-invasive bladder cancer and 15 samples of muscle invasive bladder cancer. The sample was obtained in the Anatomical Pathology laboratory from January 2016 to December 2016. Tumor tissue then extracted miRNA-92A with a RT-PCR examination with Total miRNA Mini Kit FavorPrepTM. The result is done by Mann Whitney U test.* ***Results:*** *The mean expression of miRNA-92A in non-muscle invasive bladder cancer is 12.75 while in muscle invasive bladder cancer had an average value of 31.79. The Mann Whitney U test was used to evaluate the median difference between these groups. There was a significant difference in the expression of miRNA-92A in both groups with P = 0.000 (<0.005).* ***Conclusion:*** *There is a significant difference between the expression of miRNA-92A in non-muscle invasive bladder cancer compared to muscle invasive bladder cancer with more dominant expression on muscle invasive bladder cancer.*

Keywords: *Muscle invasive, bladder cancer, miRNA,*

ABSTRAK

Tujuan: *Tujuan dari penelitian ini adalah untuk mengetahui ekspresi miRNA-92A pada kanker kandung kemih yang dapat digunakan sebagai marker tumor.* ***Bahan & Cara:*** *Penelitian ini menggunakan total 30 sampel. 15 sampel kanker kandung kemih non-invasif dan 15 sampel kanker kandung kemih invasi otot. Sampel diperoleh di laboratorium Patologi Anatomi pada bulan Januari 2016 sampai Desember 2016. Kemudian jaringan tumor diekstraksi miRNA-92A dengan pemeriksaan RT-PCR dengan total miRNA Mini Kit FavorPrepTM. Hasilnya dilakukan dengan uji Mann Whitney U.* ***Hasil:*** *Rerata ekspresi miRNA-92A pada kanker kandung kemih non-muscle invasif adalah 12.75 sedangkan pada kanker kandung kemih invasif otot memiliki nilai rerata 31.79. Uji Mann Whitney U digunakan untuk mengevaluasi perbedaan median antara kelompok-kelompok ini. Terdapat perbedaan ekspresi miRNA-92A yang bermakna pada kedua kelompok dengan P=0.000 (<0.005).* ***Simpulan:*** *Terdapat perbedaan yang siginifikan antara ekspresi miRNA-92A pada kanker kandung kemih invasif non-otot dibandingkan dengan kanker kandung kemih invasif otot dengan ekspresi lebih dominan pada kanker kandung kemih invasif otot.*

Kata Kunci: *Invasi otot, kanker kandung kemih, miRNA.*

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INTRODUCTION

Bladder malignancy has emerged as the 5th most common cancer all of malignancy in the United States, with an estimated 74.690 new cases and 15.580 deaths in 2014. Bladder cancer is included in the 10 most often malignancies among Indonesian males, with an increase in incidence rates as much as 15% each year in the last decades. In Indonesia, the majority of bladder cancer type is Transitional cell carcinoma (TCC), which accounted for 78.8 % of all bladder cancer cases.

Bladder malignancy is divided into two types, based on their invasion of the bladder muscles; Non-Muscle Invasive Bladder Cancer (NMIBC) and Muscle Invasive Bladder Cancer (MIBC). The diagnosis of NMIBC can be established by pathology examination, among the NMIBC patient who underwent cystectomy and was analyzed pathologically, 50% of them were found to be MIBC.[1] This can be challenging for many urologists, as we need more additional diagnostic modality in NMIBC cases to analyze whether it has progressivity to develop as MIBC.

The necessity for accurate and less-invasive diagnostic tool for diagnosing bladder malignancy and enhancement in genetical therapy is very much expected for managing bladder cancer in the future. Along with the growing knowledge of genetics, mainly regarding microRNA (miRNA) in the development of normal cell or malignancy, profiling miRNA expression shows the characteristics related to the classification, diagnosis, and natural history of illness. This gives us hope to develop miRNA utilization in managing bladder malignancy. One of the miRNAs that is recently investigated regarding its correlation with bladder cancer is miRNA-92A, and some previous studies showed that there was an increase in miRNA-92A expression among bladder cancer patients.

OBJECTIVE

The purpose of this study was to determine the expression of miRNA-92A in Bladder Cancer can be used as a marker of tumor.

MATERIAL & METHODS

This is a pilot study research. Under Institutional Review Board approval and patient's informed consent, we prospectively collected freshly bladder tissue between July 2017 and August 2018 at Sardjito General Hospital, these included 15 NMIBC subjects and 15 MIBC subjects. The inclusion criteria was NMIBC and MIBC tissue samples from the Pathology Anatomy Department of FK-UGM/ RSUP Dr. Sardjito. The exclusion criteria was defined as (1) broken sample or can't be used for qRT-PCR examination, (2) non-bladder cancer patient.

The outline of this study is shown in Figure 1. The stages of DNA extraction from bladder cancer tissue samples. Isolation using Total RNA Mini Kit FavorPrepTM with the following stages: (i) Sample preparation: A total of 15 mg (5 pieces 7-8 µ) of tissue, transferred into a microcentrifuge tube. Added 1 ml xylol to the tube, then mixed using a vortex tool then incubated at room temperature for 10 minutes. The tube is centrifuged at a speed of 14,000 rpm, for 3 minutes, then discard the supernatant. Add 0.5 ml of xylol to the tube, then mixed, incubated at room temperature for 3 min, then centrifuged at 14.000 rpm for 3 min, discard the supernatant. (Repeated 2 times).

Add 1 ml of absolute ethanol to the tube for tissue depurization, then mixed, incubated at room temperature for 3 minutes. The tube is centrifuged at 14.000 rpm for 3 minutes, then discard the supernatant. (Repeated 2 times). The tube was opened and incubated at 37ºC for 15 minutes to evaporate ethanol. (ii) Cell lysis: Add as many as 350 µl FARB buffers and 3.5 µl β-mercaptoethanol into the tube. Homogenized the tissue using micropestle several times.

Tubes were incubated at room temperature for 5 minutes. Filter column placed on 2 ml collection tube, then mix the sample moved to filter column. The tube is centrifuged at 1.000 rpm for 2 minutes, then remove the filter column. The filtrate is transferred to a new micro centrifugation tube. (iii) RNA binding: A total of 400 µl of ethanol 70% (RNase-free) is added to the tube, then mixed with the vortex. FARB mini column placed on collection tube 2ml, then move the mixture to FARBmini column. The tube was centrifuged at a speed of 14.000 rpm for 1 minute. The bottom was removed, then place the RB column back to the 2 ml collection tube. (iv) Wash: A total of 500 µl of Wash buffer1 was added to the FARB mini column, then centrifuged at 14.000 rpm for 1 minute.

The bottom was removed, then place the FARB mini column back to the 2 ml collection tube. As many as 750 µl Wash buffer2 was added to the FARB mini column, then centrifuged at 14.000 rpm for 1 minute, then the bottom was discarded, and the FARB mini column was put back on 2 ml collection tube (Repeated 2 times). The tube was centrifuged at a speed of 14.000 rpm for 3 minutes to dry the column matrix. (v) RNA elution: FARB mini column that has been dried was put on a 1.5 ml micro centrifugation tube. Add a total of 50 µl RNase-free water to the center of the column. The tube is left standing for 2 minutes to ensure that RNase-free water was absorbed appropriately in the column matrix. The tube was centrifuged at a speed of 14.000 rpm for 1 minute to extract a pure RNA. The RNA was stored at -70ºC.

The qPCR stages are as follows: (i) Sample maps were created and programmed in PCR machines (including negative controls). (ii) a mixture was created with the following composition: PCR-grade water = 6.4 µl, KAPA SYBR® = 10 µl, Forward Primer (PD-1 / PDL-1) = 0.4 µl, Reverse Primer (PD-1 / PDL-1) = 0.4 µl, Deoxyuridine Triphosphate (dUTP) = 0.4 µl, KAPA RT MIX = 0.4 µl, Template RNA = 2 µl. (iii) The mixture was incorporated into 48 well plates per primer.

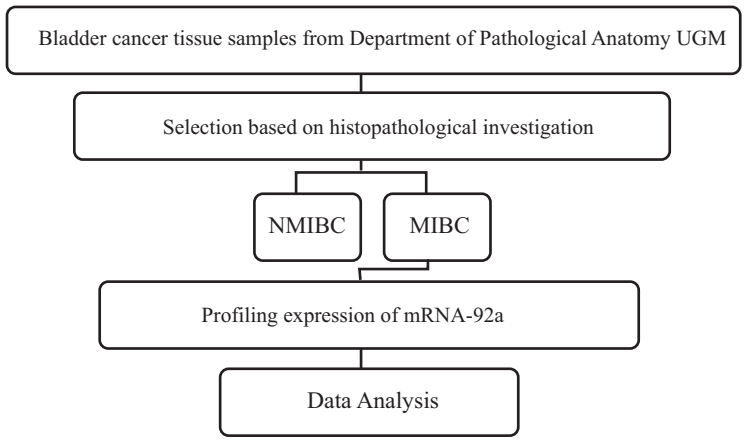

Figure 1. Study outline. Flow chart of the procedures in this study.

RESULTS

There were 30 subjects who met the inclusion criteria in this study with the study subjects divided into groups of muscle invasion and non-invasion muscles, respectively 15. The mean age of bladder cancer patients was 59.2 years. Bladder cancer patients were more commonly encountered in men than in women (Table 1).

The data obtained from the study were tabulated first. The mean expression of miRNA-92a in the muscle invasion group was 31.79 times bigger

Table 1. Characteristics of bladder cancer patients

Characteristics	Total (%)	Muscle invasive (%)	Non muscle invasive(%)
Gender			
Male	24 (80)	14 (93.3)	10 (66.7)
Female	6 (20)	1 (6.7)	5 (33.3)
Total	30	15	15

Table 2. mRNA miRNA-92a expression on muscle-invasive and non-muscle invasive bladder cancer.

N	Non Invasive	Invasive
1	14.92	29.85
2	10.55	39.39
3	9.18	22.62
4	18.37	13.92
5	13.92	36.75
6	19.69	32
7	6.96	21.11
8	9.18	19.69
9	14.92	22.62
10	13.92	27.85
11	12.99	59.71
12	16	55.71
13	10.55	21.11
14	12.12	48.5
15	8	25.99
Mean	12.75	31.79

Table 3. Data distribution of value of expression miRNA-92a on muscle-invasive and non-muscle invasive bladder cancer.

N	Non Invasive	Invasive
1	14.92	29.85
2	10.55	39.39
3	9.18	22.62
4	18.37	13.92
5	13.92	36.75
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7	6.96	21.11
8	9.18	19.69
9	14.92	22.62
10	13.92	27.85
11	12.99	59.71
12	16	55.71
13	10.55	21.11
14	12.12	48.5
15	8	25.99
Mean	12.75	31.79

Table 4. Comparison of miRNA-92a Expression values between muscle-invasive and non-invasive muscle.

Groups	Mirna-92a Expression		
	Total patient (%)	Median	*Asymp.Sig.**
Muscle invasive	15 (50)	22.57	0.000
Non muscle invasive	15 (50)	8.43	

than the normal expression of the bladder, while the mean of miRNA-92a mRNA expression in the non-invasive muscle group was 12.75 (Table 2).

The data were first tested by using the Saphiro-Wilk test (Table 3). Normality test results showed normal distributed data in both the muscle invasion group (p = 0.099) and the non-invasive muscle group (p = 0.872).

The results of the data distribution test showed normal results but due to unmet parametric assumptions in this study then used non parametric test with Mann Whitney U test. The Mann Whitney U test was used to test the median difference between the two groups, not to test the mean difference (mean of the two groups) Table 4 shows that there was a significant difference between the tumor group of muscle invasion and the non-invasive muscle tumor, Asymp.Sig. 0.000 (<0.005).

DISCUSSION

This is a pilot study, conducted on patients diagnosed with bladder cancer in the Urology Department of Sardjito General Hospital. The sample used for this study amounted to 30 samples, consisting of 15 samples of NMIBC and 15 samples of MIBC. The sample of this research was taken from patient of RSUP Sardjito between 2015-2017. There were a total of 24 males (80%) and 6 women (20%), this is concordant with the literature that bladder cancer cases in men are more common than women in most countries in the world. Bladder cancer occupies number four as the most common malignancy in males and number eight in women. It has extensive clinical manifestations, beginning with superficial lesions and having good differentiation, up to malignant tumors that have a poor prognosis. Transitional cell carcinoma is a major type of bladder cancer in the United States, which develops into in situ carcinoma or invasive cancer.[2]

Investigations that can be performed to help diagnose bladder cancer are laboratory, radiological, and cystourethroscopy. In laboratory tests, routine blood tests, urine cytology, and tumor markers, such as Bladder Tumor Antigen (BTA) stat test, BTA TRAK assay, NMP22 assay, NMP22 Bladderchek test, ImmunoCyt, and UroVysion are examined. These tumor marker checks can detect protein specific to bladder tumors (AFB / NMP22) or by detecting specific markers of malignant cell nuclei (UroVysion and ImmunoCyt). On radiology examination, intravenous urography is generally performed for the evaluation of hematuria. However, the examination has been replaced by more accurate Computed Tomography (CT) urography in the evaluation of abdominal cavity, renal parenchyma, ureter, and bladder. For superficial cancer, TUR can be performed and to assess the degree of invasion, CT and Magnetic Resonance Imaging (MRI) can also be done with the accuracy for CT and MRI being 40-85% and 50-90%, respectively.[3]

Although laboratory and radiological examinations provide much useful information in the assessment of urinary tract organs, cystoscopy is still the gold standard for assessing bladder and urethra. During the process of examination with cystoscopy, biopsies of bladder cancer tissue that is considered to be abnormal will be examined microscopically.[2]

This study was aimed to analyze the microarray of mRNA expression in bladder cancer. Under normal conditions, human cells express an RNA (mRNA) which is a template for translating genes into proteins. Various studies have shown that RNA cells (mRNAs) that encode proteins also express thousands of functional RNA molecules which does not encode protein (non-coding RNA).

This non-coding RNA is transcribed from RNA sequences (which are small molecules referred to as microRNA or miRNA) and are shown to have regulatory functions in normal cells. Although only a small part of the miRNA is known for its biological role, from identified miRNAs it is known that the molecules play a role in regulating important processes in growth, differentiation, apoptosis, adhesion, and other cellular processes. Therefore, it was alleged to play a role in the mechanism of cancer occurrence. From various studies, it is evident that the abnormal expression of miRNA can promote

tumorigenesis, metastasis, and various other cancer properties. Bioinformatic and microarray studies reveal that a single miRNA can bind to 200 target genes and that this target gene can be either a transcription factor, a receptor, a secreted factor, or a transporter.[4-5]

A study conducted by Tores et al in the urinary cell microRNA-based prognostic classifier for non-muscle invasive bladder cancer showed that regression analysis showed miR-92a in urine as an independent predictor of NMIBC tumor progression. This study's results in concordance with the previous studies that there was a statistically significant difference in miRNA-92a expression between muscle-invasive tumor groups and non-invasive muscle tumors ($p < 0.005$). Expression of miRNA-92a increased 31.79-fold in the muscle-invasive group and 12.75-fold in the non-invasive muscle group compared with a non-cancerous bladder.[4,6]

The results of this study open the possibility of examination miRNA-92a as a tool to see the progressivity of the bladder malignancy process. In bladder malignancy, there is an increase in miRNA-92a expression and it appears that in cases of malignancy with muscle invasion there is an higher increased expression of miRNA-92a compare to non-invasive muscle. An abnormal expression profile of miRNA was found in cancer cell specimens when compared with normal tissue control. MicroRNA binds specifically to its target, so single nucleotide polymorphism (SNP) in the miRNA sequence or target mRNA may lead to disease, including cancer. In many different types of cancer, an erroneous set of miRNAs indicates that miRNA affects the target genes involved in cell proliferation, apoptosis, differentiation, invasion, and motility that are important for cancer progression.[7-8]

As a biomarker, miRNA can be measured and evaluated as an indicator of normal or pathogenic biological processes and pharmacologic responses to therapy. For cancer, miRNA may act as a biomarker for early cancer detection or diagnosis, allowing predictions of patient prognosis and therapeutic efficacy. The abnormal expression of miRNA is a phenomenon that occurs in various cancers in humans. The ability of miRNA to differentiate tumor origin, subtype, oncogenic mutation, and cancer predisposition, and regulate important processes in the cells make miRNA potentially a diagnostic biomarker, prognosis, and response specific to therapy. miRNA plays an important role in cancer, therefore the identification of fundamental pathways in cells is important to provide a more complete understanding of the function and regulation of miRNA in pathogenesis and cancer progression so that it can be applied clinically for future cancer therapies.[6,9]

CONCLUSION

There is a significant difference between the expression of miRNA-92A in non-muscle invasive bladder cancer compared to muscle-invasive bladder cancer with more dominant expression on muscle-invasive bladder cancer.

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