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Detecting miRNAs expression as the early prognostic factor for patients with colorectal cancer in Dr. Sardjito Hospital Yogyakarta : A preliminary study

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ABSTRACT

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Background: Colorectal cancer (CRC) is the third highest-ranked cancer and causes high mortality in patients with a low survival rate. The lack of sensitivity and specificity of clinical and other diagnostic modalities results in a higher mortality rate. Therefore, the exploration of potential early biomarkers for CRCs is necessary.
Objective: We aimed to evaluate the local expressions of potential tumor suppressor and oncogenic miRNAs in CRC patients in Indonesia.
Methods: This retrospective cohort study involving thirty-one colorectal carcinoma patients at Dr. Sardjito Hospital Yogyakarta from January 2014-December 2017. Total RNA was isolated, and the expressions of miR-21, miR-92a, miR-96, miR-26b, miR144, and miR-195 were measured by real-time quantitative PCR. The correlation between miRNAs and other predictors was determined by Spearman correlation, and the association of miRNA expression and other clinical parameters used logistic regression.
Results: The local expression of miR-195 decreased significantly in the tumor sites. In contrast, miR-21 activity tends to increase in the local tumor. Meanwhile, the expressions for miR-92a, miR-96, miR26b, and miR-144 in the same subjects were non-significant. MiR-195 was also significantly associated to cancer stage (r=-0.570, p=0.001) with significant odds ratio (OR=0.892, 95% CI=0.804–0.990, p=0.031).
Conclusion: Our study was the first to report aberrant expressions of miRNA-21, miRNA-195, miRNA-92a, miRNA-26b, miRNA-96, and miRNA-144 in Indonesian CRC patients. The tumor suppressor miRNA-195 expression was superior among others to serve as an early biomarker in detecting and predicting CRC disease progression.

Latar Belakang: *Kanker kolorektal (CRC) adalah kanker terbanyak ketiga dan yang terbanyak menyebabkan mortalitas pada pasien dengan angka* survival *yang rendah. Rendahnya sensitivitas dan spesifitas dari modalitas klinis dan diagnostik lainnya menyebabkan tingginya angka mortalitas. Maka dari itu, pencarian biomarker awal yang potensial untuk kolorektal dibutuhkan.*
Tujuan: *Kami bertujuan untuk mengevaluasi ekspresi lokal dari* tumor suppressor *dan onkogen miRNA pada penderita kanker kolorektal di Indonesia.*
Metode : *Studi ini merupakan studi kohort retrospektif yang melibatkan 31 pasien kanker kolorektal di RSUP Dr. Sardjito Yogyakarta pada Januari 2014-Desember 2017. RNA total dari sampel diisolasi dan ekspresi miR-21, miR-92a, miR-96, miR-26b, miR144, dan miR-195 diukur dengan* real time quantitative PCR*. Korelasi antara miRNA dan prediktor lain ditentukan dengan korelasi Spearman, sedangkan asosiasi ekspresi miRNA*

dengan parameter klinis lain diuji dengan regresi logistik.

Hasil: *Ekspresi lokal miR-195 berkurang secara signifikan pada lokasi tumor. Kebalikannya, aktivitas miR-21 cenderung meningkat pada area lokal tumor. Sedangkan, ekspresi miR-92a, miR-96, miR26b, dan miR-144 tidak signifikan pada subjek yang sama. Ekspresi miR-195 juga sangat berkaitan dengan stadium kanker (r=-0.570, p=0.001) dengan* odds ratio *yang signifikan (OR=0.892, 95% CI=0.804–0.990, p=0.031).*

Kesimpulan: *Studi kami adalah yang pertama melaporkan ekspresi miRNA-21, miRNA-195, miRNA-92a, miRNA-26b, miRNA-96, dan miRNA-144 pada pasien kanker kolorektal di Indonesia. Ekspresi* tumor suppressor *MiRNA-195 lebih superior dibanding lainnya sebagai biomarker dalam mendeteksi dan prediksi progresi kanker kolorektal.*

INTRODUCTION

Colorectal cancer is the third most prevalent cancer in the world and is the leading cause of cancer deaths for men and women.[1] In 2014, in the United States, it is estimated that more than 130,000 new CRC cases were diagnosed, with more than 50,000 deaths caused by this disease.[2] Nonetheless, the incidence of CRC has decreased slowly, given the growing development of diagnostic and therapeutic choices. The five-year survival rate of colorectal cancer is around 64.9%, suggesting that early detection and innovative treatment are needed.[3]

The incidence of CRC in Asia is increasing and reflects an increased prevalence of risk factors from CRC, such as unhealthy diets, obesity, and smoking.[4] In Indonesia, based on the data from the Ministry of Health in 2018, CRC cases are estimated at 12.[8] per 100,000 adult populations.[5] Early detection of colorectal precancerous lesions is crucial in increasing the 5-year survival rate.[6]

However, CRC diagnostic facilities in Indonesia is facing limitation to low sensitivity and specificity.[7] Although in the last decade, the identification of biomarker molecules and the small non-coding RNA (microRNA/ miRNA), has shown a strong correlation with the CRC rate.[8]

Changes in potential tumor inducers miRNAs such as miR-21, miR-92a, and miR-96 have been reported to increase significantly in the plasma of patients with CRC, correlated with mortality, and can distinguish advanced adenocarcinomas compared to healthy subjects.[7,9,10] On the contrary, gradual changes or lower tumor-suppressor miRNAs level, namely miR-26b, miR-144, and miR-195, were reported to be associated with CRC progression in both in vitro and in-vivo studies.[11-13,19] However, several studies have been conducted to study the crucial role of tumor-suppressor and oncogenic miRNAs in the development of colorectal cancer. But it is still obscure whether the potential microRNA expression is similar to that in the Indonesian population. Therefore, we aim to evaluate and trace the basic profile of miRNAs in patients with CRC in Indonesia.

METHODS

Study populations

The population study was an ongoing clinical-based case study under the collaboration between our faculty and our hospital, conducted from January 2014 to December 2017 with the Institutional Review Board of our faculty approved the research protocols (KE/FK/1242/EC/2017). Written informed consent was obtained from all participants, with demographic information collected from patient records and registries. The sample consisted of thirty-one colorectal cancer patients with four different stages.

Patients were excluded from the study if they had received preoperative chemotherapy or radiation therapy or had a previous history of malignancy. All patients underwent surgical resection at the Digestive Surgery Division, Dr. Sardjito Hospital, from January 2014–December 2017, with a final pathological diagnosis of colorectal cancer. The tumor specimens were histologically classified and staged according to the seventh edition of the tumor-node-metastasis (TNM) staging system. The samples used in this research were formalin-fixed paraffin-embedded (FFPE) tissue which contains more than 70% of adenocarcinoma. The samples were stored at -8°C until nucleic acids were extracted. The survival parameters used were a five-year survival rate obtained from the medical record and phone confirmation from 2018 until 2019.

RNA isolation

The tissues were resected during surgery and placed in a paraffin block, with no need to freeze the tissue at -8°C. Briefly, FFPE tissue was sliced into 25 mg, then transferred to a 1.5 ml microcentrifuge tube with the addition of 1

ml xylene. It was vortexed vigorously and then incubated at room temperature for 10 minutes. It was then centrifuged at 16,000× G for 3 minutes and the supernatant was removed. Then 1 ml of absolute ethanol was added to wash the sample pellet by inverting the tube. This process of centrifugation and washing was done three times. After that, the tube lid was opened and the sample was incubated at 37°C for 15 minutes to evaporate ethanol residue. RNA purification was done immediately to preserve contents inside the sample including miRNA. This process used Hybrid-RTM miRNA (Cat No. 325-150, GeneAll, Korea). To homogenize the sample, 50 mg of FFPE was mixed with 500 µL RiboExTM. The mixture was incubated for 5 minutes at room temperature. Then we added 100 µL chloroform, vortexed it for 15 s, and stored it for 2 min at room temperature. Next, it was centrifuged at 12,000 x g for 15 min at 4°C, and the aqueous phase was transferred to a fresh tube. A volume of 50% ethanol was added to the sample and mixed thoroughly by inverting the tube. Then 700 µL of the mixture was transferred to a mini spin column, and centrifuged at 10,000 x g for 30 s at room temperature. The passed-through was transferred, and one volume of 100% ethanol was added, then it was mixed well by pipetting. Then, 650 µL of the mixture was transferred to the mini spin column, and centrifuged at 10,000 ×g for 30 s at room temperature. Onto the column was added 500 µL buffer RBW, centrifuged at 10,000 ×g for 30 s at room temperature, the passed-through was discarded, and the column was re-inserted. This process was repeated three times. Centrifugation was done once more at 10,000 ×g for 1 min at room temperature to remove residual wash buffer. The column was transferred to a new 1.5 ml collection tube then 50 µL RNase-free water was added to the center of the membrane in the column. Finally, the last centrifugation was done at 10,000 ×g for 1 minute at room temperature.

It should be noted that the detection of low-abundance miRNAs might be reduced in FFPE tissue blocks if stored for more than seven years. The extent of RNA degradation in older tissue blocks probably reduces the qRT-PCR efficiency in miRNA analysis, resulting in a higher technical variability.[14]

miRNA profiling

A NEXproTM qRT-PCR Master Mix system (Cat. No. NexQ-7000, Genes Laboratories, Korea) was applied for miRNA profiling using two pooled tissue samples, i.e., 31 tumors and 31 paired normal controls. The assay for the profiling included a universal reverse transcriptase (RT) and sequential qPCR amplification with special primers using SYBR Green.

Quantification of miRNAs by qRT-PCR

The PCR reaction was performed in a 48-well PCR plate, with each well containing 20 µL of the reaction system, including 10 µL qRT-PCR Master (SYBR), 2 µL Forward Primer, 2 µL Reverse Primer, and 1 µL Template RNA. The reaction was performed under 5°C for 30 min. The conditions of real-time PCR were: hot-start denaturation at 95°C for 10 minutes; 40 cycles of amplification with denaturation at 95°C for 10 s, annealing at 65°C for 40 s, and extension at 72°C for 60 s then the final extension for 5 min.

Data analysis

After clinical data collection, the statistical analyses were performed using SPSS version 16.0 (IBM, Chicago). Data are presented as mean ± standard deviation (SD) for continuous variables. Overall, to compare differences between groups, unpaired t-test analysis was used. The correlation between miRNAs and other predictors was determined by Spearman correlation, while logistic regression tested the association of miRNA expression and other clinical parameters. A p-value less than 0.05 was considered statistically significant.

RESULTS

Baseline characteristics of study participants

In this study, the population included 31 subjects with colorectal cancer. All subjects were divided into four groups based on cancer stages, as shown in Table 1. There were three, ten, fourteen, and four patients in stages I, II, III, and IV, respectively. Female patients are more common than males, with almost 70-80% of tumor stages at T3 and T4. Furthermore, thirteen patients were identified at the N0 stage, while eighteen subjects developed to N1 to N2 stages. A similar pattern was observed in the M stages, where almost 60% (18

patients) were already in M1 stages, particularly for patients with stages III and IV based on the preliminary clinical screening.

Gene expression for microRNAs at tumor site of CRCs patients

We obtained total RNA to investigate changes in the expressions of several microRNA levels in tumor tissue, using U6 expression as the control/housekeeping gene. Previous studies have shown the potential tumor suppressor and inducer microRNAs, therefore, we choose them in our clinical case study. Figure 1 presents the general feature of target miRNA expression. The expressions of tumor inducer miRNA-21 were increased significantly along with the severity of cancer stage in all patients (p=0.003).

Similarly, the linear pattern was observed in other tumor inducer microRNAs. The local expressions of miR-92a (p=0.952) and miR-96 (p=0.144) tended to increase in both microRNAs but were non-significant. In contrast, we found that the tumor suppressor microRNA, miR-195 expression, was significantly decreased in the four stages of CRCs (p=0.024). However, we could not find a significant result for the other tumor suppressor microRNAs, miR-26b (p=0.872) and miR-144 (p=0.378) among groups.

Univariate correlation and multivariate regression analysis

Further analysis showed the correlation between target microRNAs and other parameters (Table 2). We found that miR-195 is strongly associated with the stage (r=-0.570; p =0.001) and survival rate (r=-0.359; p=0.047) in CRC patients. Linear to miR-195 expression, the level of miR-144 was significantly correlated to stage and survival rate (r=-0.363; p=0.048 and r=-0.588; p=0.001), respectively. The alteration of miR-92a and miR-26b expressions was only significantly associated with the survival time of patients. However, miR-96 expression did not differ between the group.

The individual correlations between miRNAs and other parameters have shown that miR-21 was significantly associated with primary tumor/T stages (r=0.433; p=0.015) and distant metastasis/M stages (r=0.570; p=0.001), while miR-195 showed a significant negative correlation with M stages (r=0.599; p=0.000). Matched with the previous data, we could not find a significant individual correlation between miR-92a, miR-96, miR-26b, and miR-144 to T, N, and M stages, respectively. We did multiple ordinal logistic regression to verify the independent association between miRNAs and cancer stage. Among all potential microRNAs, the tumor suppressor miR-195 was significantly associated with and independently can predict the progression of cancer stage in CRC patients (OR=0.892, 95% CI: 0.804-0.990, p= 0.031) compared to other microRNAs (Table 3).

Table 1. Baseline characteristics of the study population

	Stadium I	Stadium II	Stadium III	Stadium IV
N	3	10	14	4
Sex (male/female)	(3/0)	(5/5)	(5/9)	(1/3)
T stages				
T2	3	1	0	0
T3	0	4	4	0
T4	0	5	10	4
N stages				
N0	3	10	0	0
N1	0	0	4	3
N2	0	0	10	1
M stages				
M0	3	10	13	0
M1	0	0	1	4

TNM classification based on the International Union against Cancer (UICC, 2002).

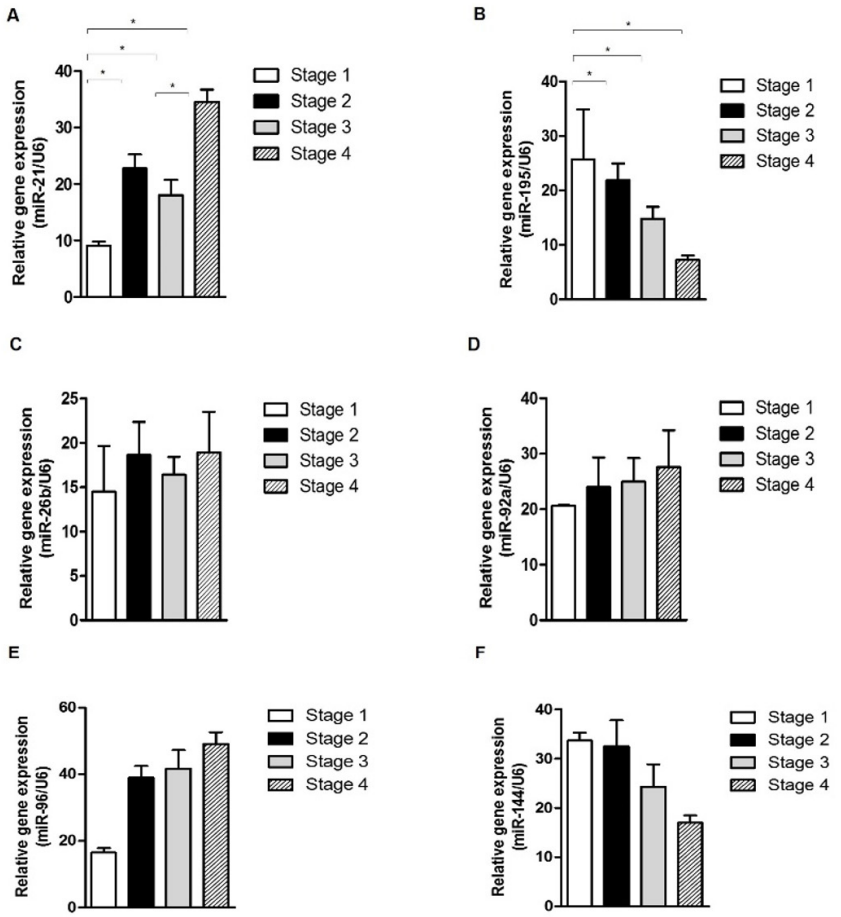

Figure 1. MicroRNAs expression in colorectal cancer. (A-F). Quantitative RT-PCR assay for miRNAs in CRCs patients from Java ethnic (n=31). The data showed as mean ± SD with p-value<0.05.

Table 2. Univariate correlation with stadium and survival in CRCs patients

Parameter	Stadium		Survival	
	r	p	r	p
Sex	0.301	0.099	0.128	0.491
Age	0.139	0.454	0.124	0.507
Primary tumor (T)	0.562	0.001*	-0.171	0.357
Regional lymph node (N)	0.767	<0.001*	0.200	0.290
Distant metastasis (M)	0.614	<0.001*	-0.058	0.756
miR-21	0.353	0.051	-0.041	0.826
miR-195	-0.570	0.001*	-0.359	0.047*
miR-92a	0.157	0.398	0.731	<0.001*
miR-26b	0.021	0.909	0.739	<0.001*
miR-96	0.284	0.122	-0.099	0.596
miR-144	-0.363	0.048*	-0.588	0.001*

Linear correlation by Spearman's test. *p< 0.05.

Furthermore, we used receiver operating curve (ROC) analysis to examine the sensitivity and specificity of miR-195 compared to other miRNAs (Figure 2). The area under the curve (AUC) value of miR-195 (0.716) was higher than miR-21 (0.117), miR-92a (0.401), miR-26b (0.457), miR-96 (0.136) and miR-144 (0.549).

Table 3. Multivariate regression analysis with cancer stages

Predictor	Odds-ratio (OR)	95% CI	p-value
miR-21	1.068	(0.974 - 1.169)	0.159
miR-195	0.892	(0.804 - 0.990)	0.031*
miR-92a	1.035	(0.927 - 1.154)	0.535
miR-26b	0.945	(0.788 - 1.134)	0.544
miR-96	1.003	(0.992 - 1.074)	0.111
miR-144	0.965	(0.905 - 1.028)	0.268

Ordinal logistic regression between stadium (dependent variable) and predictors (independent variable). p-values and OR (95%CI) are given.

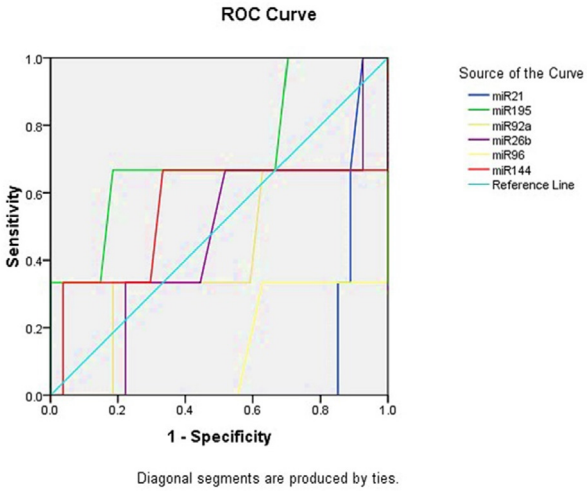

Figure 2. Receiver Operating Curve (ROC) analysis for miRNAs in colorectal cancer

DISCUSSION

This study describes novel data on the expressions of miRNA-21, miRNA-195, miRNA-92a, miRNA-26b, miRNA-96, and miRNA-144 in Indonesian CRC patients. The miRNA-21 and miRNA-195 expressions were significantly increasing and decreasing, respectively, along with the progression of the CRC. The other miRNAs, such as miRNA-92a, miRNA-26b, miRNA-96, and miRNA-144, have shown their up-regulated and down-regulated expression but without significance in the different stages of the disease. Other results showed us that according to the univariate correlation analysis, miRNA-195 was better in predicting CRC stage and survival rate than other miRNAs. It also was negatively correlated to the metastatic stage. Moreover, miRNA-21 showed significance in determining primary tumor stages and distant metastasis. In multivariate regression analysis, the miRNA-195 could independently predict the progression of CRC stages.

These results were significant yet different from the previous study. Abundant evidence reported that miRNA-92a, miRNA-26b, miRNA-96, and miRNA-144 have remarkable aberrant expressions throughout CRC progression. A meta-analysis of six articles, a total of 695 patients from China, Japan, Taiwan, and Norway, showed a distinct association of miR-92a with a depth of tumor invasion, TNM stage, distant metastasis, and lymph node metastasis. Another study measured miR-26b and proved it was a potent inhibitor of CRC proliferation through repressing LEF-1 activation of c-Myc and Cyclin D1 expression.[11] The study of miR-96 done on 20 patients in China showed its upregulation expressions in CRC tissues and was proposed to have an oncogenic role by promoting cell proliferation. In contrast, miR-96 inhibition resulted in the suppression of cell proliferation.[10]

However, some of our results were also similar to previous studies. Through univariate correlation analysis, our study showed that the miR-144 level was significantly associated with the stage and survival rate of the disease. In-vitro assays in 137 patient samples in Japan showed

miR-144 downregulation was associated with the proliferation of CRC cell lines through mTOR signaling pathway activation.[12,13] The miR-92a and miR-26b levels were significant but only in the disease survival rate.

Growing evidence confirmed miR-21 and miR-195 aberrant expressions and their significant role in the progression of CRC. The previous study on more than 1600 patients assessed in East Asia and Europe stated that patients with higher expression of tissue miR-21 had significantly inferior overall survival and disease-free survival. High tissue miR-21 level is associated with adverse colorectal cancer prognosis.[9] The last miRNA, miR-195, was reported to be a tumor suppressor. Downregulation of miR-195 correlates with lymph node metastasis and poor prognosis in CRC. It downregulated decreased Cyclin B1, Cyclin D1, and CDK2. Together, these conditions resulted in cell growth repression, colony formation, invasion, and migration.[15] In contrast, inhibition of miR-195 function contributed to aberrant cell proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT).[16,20]

Finally, our ROC analysis showed that miR-195 outperformed the others in sensitivity and specificity by an AUC value of 0.716, the highest value of diagnostic performance among other miRNAs. On the contrary, previous studies reported other miRNAs to have better diagnostic performance. A study examining the diagnostic value of miR-92a reported an AUC of 0.838 and 0.749, respectively, in discriminating CRC and advanced adenomas from controls. A total of 157 CRC patients in Chine with advanced adenomas in China were included in the study.[7] On the other side, the meta-analysis of miR-21 demonstrated a promising biomarker for early detection and prognosis of colorectal cancer. In diagnosing colorectal cancer, the pooled sensitivity, specificity, and AUC of circulating miR-21 were 0.76, 0.81, and 0.81, respectively.

These significant findings regarding tumor inducer and suppressor miRNAs shed some light towards a better understanding of miRNAs mechanism in CRCs regulation and their capabilities to serve a diagnostic and prognostic function, especially miR-195, in which its role in CRC still needs to be elucidated. The significant presence and miR-195 role in this study as the biomarker for cancer diagnosis and therapy was consistent with findings in previous research.11,17,18 These oncogene and tumor suppressor miRNAs have been recognized to have a high correlation with the progression of diseases. However, these findings only were applied to Caucasian and East Asian populations. However, this study should be interpreted with caution as our main study weakness is the small sample size, which suggests that a larger sample size needs to be involved to clarify and confirm our results. Further investigation into other miRNAs is required to elucidate their primary role in the pathogenesis of CRC.

CONCLUSION

In conclusion, our study was the first to report aberrant expressions of miRNA-21, miRNA-195, miRNA-92a, miRNA-26b, miRNA-96, and miRNA-144 in Indonesian CRC patients. The tumor suppressor miR-195 expression was superior among others to serve as an early biomarker in detecting and predicting CRC disease progression.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest concerning the research, authorship, and, or publication of this article.

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