

## Increasing serum miR-124-3p expression is associated with the high survival rate of a rectal cancer patient after neoadjuvant chemoradiotherapy

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### Abstrak

**Latar Belakang:** Kanker kolorektal menempati urutan ketiga penyebab kanker di dunia, dengan prevalensi kanker rektum sebanyak 30% dari total kasus. Saat ini belum ada biomarker yang efektif untuk memprediksi respon pasien terhadap terapi yang diberikan. Beberapa penelitian menggunakan potensi miRNA sebagai biomarker untuk melihat respon terapi. Salah satunya yaitu MiR-124-3p berperan sebagai tumor supresor yang mengalami penurunan ekspresi pada berbagai jenis kanker. Tujuan dari penelitian ini adalah untuk meneliti ekspresi miR-124-3p dari pasien kanker rektum yang menerima nCRT, dan menganalisis hubungannya dengan kelangsungan hidup pasien dan parameter klinis lainnya.

**Metode:** Penelitian ini melibatkan 15 orang pasien yang didiagnosis menderita kanker rektum lokal dan menjalani kemoradioterapi neoajuvan (radioterapi 45-50 Gy dengan fraksi 1,8-2 Gy selama 1-3 bulan, dan kemoterapi 5-fluorouracil secara oral). Sampel penelitian berupa darah intravena sebanyak 5 ml diambil saat sebelum dan sesudah kemoradioterapi. Selanjutnya ekspresi miR-124-3p dianalisis menggunakan qRT-PCR dan dikalkulasi menggunakan metode Livak.

**Hasil:** Terdapat hubungan signifikan antara peningkatan ekspresi miR-124-3p dengan sintasan hidup pasien ( $P=0,003$ ;  $OR=30$ , 95%  $CI=1,41-638,15$ ), serta adanya peningkatan ekspresi miR-124-3p yang signifikan ( $P<0,041$ , fold change sebelum= $1,14 \pm 1,25$ ; sesudah= $2,4 \pm 1,84$ ) setelah dilakukan kemoradioterapi.

**Kesimpulan:** Hasil ini mengindikasikan bahwa miR-124-3p berpotensi menjadi biomarker untuk memprediksi sintasan hidup pasien kanker rektum yang menerima kemoradioterapi. (*Health Science Journal of Indonesia 2019;10(2):90-5*)

**Kata kunci:** kanker rektum, kemoradioterapi, miR-124-3p, sintasan hidup

### Abstract

**Background:** Colorectal cancer is the world's third most prevalent cancer, which 30% of cases are rectal cancer. Today, the effective diagnostic marker to accurately predict clinical outcome patients response to therapy did not found yet. Several research studies have indicated that miRNA potential as a prognostic biomarker. MiR-124-3p plays as tumor suppressor that significantly down-regulated in some cancer and could radiosensitize human colorectal cancer cells. The aim of the study is to investigate the expression of miR-124-3p from rectal cancer patient who receive nCRT, and analyze its association with patient survival and others clinical parameters.

**Methods:** This research involved 15 patients with histologically confirmed locally advanced rectal cancer (LARC) and received neoadjuvant chemotherapy/nCRT (radiotherapy 45-50 Gy with 1,8-2 Gy fractions over 1 to 3 months and chemotherapy 5-fluorouracil was administered orally). Patient blood (5 ml) were collected from peripheral venous before and after neoadjuvant chemoradiotherapy. miR-124-3p expression was performed using qRT-PCR and calculate using Livak method.

**Results:** In this study, we found that increasing of miR-124 was significantly associate with high survival of rectal cancer patient ( $P=0,003$ ;  $OR=30$ , 95%  $CI=1,41-638,15$ ). Average of miR-124-3p expression increase significantly after nCRT ( $P<0,041$ , fold change before= $1,14 \pm 1,25$ ; after= $2,4 \pm 1,84$ ).

**Conclusion:** Our finding suggests that miR-124-3p expression in blood serum was potential as biomarkers to predict rectal cancer patient survival after neoadjuvant chemoradiotherapy. (*Health Science Journal of Indonesia 2019;10(2):90-5*)

**Keywords :** rectal cancer, chemoradiotherapy, miR-124-3p, survival

Colorectal cancer is the world's third most prevalent cancer and causing death due to cancer in the second rank. Rectal cancer occurs approximately 30% of all colorectal cancer cases and has higher recurrence and resistance to therapy than colon cancer.<sup>1,2</sup> The standard treatment rectal cancer is called trimodality therapy, consist of neoadjuvant chemoradiotherapy (nCRT), then followed by a total mesorectal excision and chemotherapy. Organization of nCRT can essentially increment patient survival, however local recurrence is still common due to resistance. Approximately 40–60% of rectal cancer patients who treated with nCRT achieve improvement in the pathological reaction. Although about 40% of patients still recur and some of them were unable to survive.<sup>2,3</sup> The overall 5-years survival rate for people with rectal cancer is 67%. However, colorectal cancer survival rates may differ based on several factors.<sup>3</sup> Today, many molecular mechanisms underlying sensitivity or resistance to chemoradiotherapy have been discovered and developed to accurately predict clinical result and response to rectal cancer treatment. MicroRNA (miRNA) is one of molecular factor that closely associates to tumor chemoradiotherapy sensitivity and appears great prospects of research and clinical application.

MiRNA is small endogenous non-coding RNA (19-25 nucleotides) that regulates post-transcription gene expression by partly binds complementary sequences of its target messenger RNA (mRNA). This binding results in degradation and/or translation inhibition and leads to reduced protein expression. miRNA can function as an oncogenic (onco-miR) or tumor-suppressive (tumor-suppressor miR).<sup>4</sup> During the growth of cancer, miRNA expression is frequently deregulated in many types of cancer and cause abnormal level of expression that related with cancer clinicopathological characteristics. Therefore, the expression modifications of cancer-associated miRNA emerge as promising diagnostic markers that correlate with cancer development of cancer, patient survival, sensitivity and resistance to therapy.<sup>5</sup>

MiR-124-3p or called miR-124 acts as a tumor suppressor that significantly down-regulated in many human malignant tumors including breast cancer, malignant glioma, gastric cancer, and colorectal cancer.<sup>6-9</sup> Recent studies have shown that miR-124-3p can radio sensitizes human colorectal cancer cells through PRRX1 targetting.<sup>9</sup> MiR-124-3p may also be a prospective marker for gastric cancer and associated with poor prognosis in colorectal cancer. Increasing

of miR-124-3p expression in colorectal cancer could inhibit colorectal cancer cells development.<sup>8,10</sup>

MiRNA can be secreted by cells and circulate in a stable phase in the human blood. Blood samples are easy to acquire and suitable for clinical use. Circulating miRNA are a new class of non-invasive biomarkers that show excellent stability under a multitude of physical and chemical circumstances.<sup>11</sup> The aim of this study is investigating the expression of miR-124-3p from blood serum in a rectal cancer patients who receive nCRT, and analyze its association with patient survival and other clinical parameters. Serum samples are much easier to obtain and simpler to access than cancer tissues. Serum based miRNAs can provide clues in rectal cancer therapy surveillance.

## METHODS

### Patients and sample collection

This Cohort study involved 15 patients with histologically confirmed locally advanced rectal cancer (LARC) with clinical TNM stage II and III, no history of previous malignancy based on MRI and histopathology examination. All patients received neoadjuvant chemoradiotherapy in Kariadi Hospital, Central Java, Indonesia, between 2017-early 2018. All patients received neoadjuvant chemoradiotherapy with 1,8-2 Gy fractions over 1 to 3 months depending on radiotherapy 45-50 Gy and 5-fluorouracil chemotherapy orally.

Patient blood (5 ml) were collected from peripheral venous, before and after neoadjuvant chemoradiation. By centrifugation at 3000 rpm for 15 minutes, serum was isolated from the blood. Serum samples were separated into 3 aliquots and stored at -80°C until further analysis. The characteristics of patients are shown in Table 1. This research was approved by the Medical Faculty of Diponegoro University and Kariadi Hospital Ethics Committee number 14/EC/FK-RSDK/I/2017, then continued by the Medical, Public Health and Nursing Faculty Ethics Committee, Universitas Gadjah Mada (Ref : KE/FK/1008/EC/2018), in accordance with the Declaration of Helsinki. All respondents acquired informed consent prior to enrollment in the research.

### RNA isolation and cDNA synthesis

Total RNA was isolated from 200 µl of blood serum using miRCURY™ RNA Isolation Kit – Biofluids

(Qiagen), following the manufacture's guidelines and specific application instruction. RNA concentration and purity were controlled by UV spectrophotometry using Nanodrop (NanoVue Plus, GE Healthcare, Life Science). Total RNA was eluted in RNase-free water and stored at  $-80^{\circ}\text{C}$  until use. cDNA was synthesized using the reverse transcription reaction miRCURY LNA RT Kit (Exiqon) and performed using thermal cycler (Applied Biosystems™ A24811) following the manufacture's instructions and stored at  $-20^{\circ}\text{C}$  until use.

### Quantitative real-time PCR (qRT-PCR)

Analysis of qRT-PCR was performed using a SYBR-green-containing PCR (miRCURY LNA SYBR Green PCR Kit, Qiagen) and samples were run on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) in a total volume 10  $\mu\text{l}$ . U6 small non-coding RNA was used as a reference gene. The primer sequence for miR-124-3p was 5'-UAAGGCACGCGGUGAAUGCC-3' and U6 was 5'-CGCTTCGGCAGCACATACTA-3' (miRCURY LNA miRNA PCR Assay, Qiagen). All these samples were standardized to reference gene and fold changes were calculated using the Livak's method relative quantification ( $2^{-\Delta\Delta\text{Ct}}$ ).

### Statistical methods

Categorical data are shown as the frequency counts and percentages, while continuous variables are shown as the mean plus or minus standard deviation. The association between miR-124-3p expression and patient's characteristic was assessed using  $\chi^2$  test (Chi-square, Fisher's exact test), while Odds Ratio (OR) equivalent to the 95% CI was used to evaluate the power of the associations. Significant differences in miR-124-3p expression before and after neoadjuvant chemoradiation were determined using Wilcoxon's test. A statistically significant difference was regarded to show P-value  $< 0,05$ .

## RESULTS

### Patient clinicopathological characteristics

Table 1 shows the main clinicopathological characteristics of 15 patients included in the study. Their median age was 46 years (range 26-65 years) and dominated by male. More than half of patients had well differentiated histopathology and had clinical stage III. Early CEA level average is 33,9 ng/mL, higher than the recommended normal level (5 ng/mL). Eleven patient from 2017 until early 2018 who still alive until early 2019, were grouped as the patient who has high survival.

Table 1. Patient clinicopathological characteristic

Patient Characteristics	
<b>Sex, n (%)</b>	
Male	10 (67)
Female	5 (33)
<b>Age (years)</b>	
Mean $\pm$ SD (min-max)	46,1 $\pm$ 12,1 (26-65)
<b>Differentiation, n (%)</b>	
Well	11 (74)
Moderate	2 (13)
Poor	2 (13)
<b>Early T Stages, n (%)</b>	
T1	1 (7)
T2	2 (13)
T3	6 (40)
T4	6 (40)
<b>Early N Stages, n (%)</b>	
N0	2 (13)
N1	9 (60)
N2	4 (27)
<b>Early Clinical TNM Stage, n (%)</b>	
II	2 (13)
III	13 (87)
<b>Early CEA level (ng/mL)</b>	
Mean $\pm$ SD (min-max)	33,9 $\pm$ 68,4 (0,04 – 200)

SD=standard deviation; n=amount of subject; T=tumor; N=node; TNM=Tumor size, Node status, Metastasis; CEA=carcino embryonic antigen; min=minimal; max=maximum

### Relationship between miR-124-3p expression and patient clinicopathological characteristics

The relationship between miR-124-3p expression and clinicopathological characteristics is shown in Table 2. Increasing of miR-124-3p expression during nCRT were significantly associated with patient survival ( $P < 0,05$ ; OR =30, 95% CI = 1,41-638,15). No significant relationship was observed between the miR-124-3p and sex, age, differentiation, early T stage, early N stage, early clinical TNM stage, down staging and early CEA level.

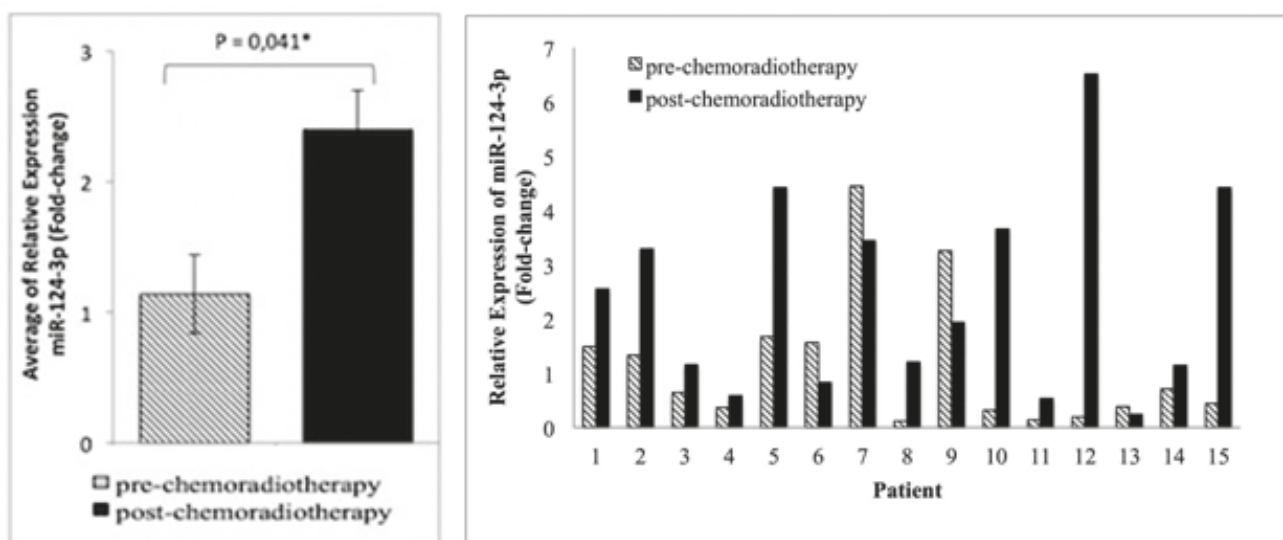
### Average difference of miR-124-3p expression

Average difference of miR-124-3p expression and each patient are shown in Figure 1. MiR-124-3p expression increase significantly after nCRT ( $P < 0,05$ , before= $1,14 \pm 1,25$ ; after= $2,4 \pm 1,84$ ; delta= $1,26$  fold). MiR-124-3p expression increase in 11 patients (73%), which 10 patients could survive. During follow-up, 4 patients died of rectal cancer because of worse prognosis (patient no. 5, 6, 9, 13). Three of them have decrease miR-124-3p expression.

Table 2. Association of miR-124-3p and patient characteristic

Patient Characteristic	Mir-124-3p Expression After Chemoradiation, n(%)		OR (95% CI)	P
	Increase	Decrease		
<b>Sex</b>				
Male	7 (63,6)	3 (75)	0,58 (0,04 - 7,6)	0,593
Female	4 (36,4)	1 (25)		
<b>Age</b>				
< 50	7 (63,6)	2 (50)	1,75 (0,17 - 17,69)	0,538
≥ 50	4 (36,4)	2 (50)		
<b>Differentiation</b>				
Well	8 (72,7)	2 (50)	2,67(0,25 - 28,44)	0,407
Moderate - Poor	3 (27,3)	2 (50)		
<b>Early T Stages</b>				
T1 - T2	3 (27,3)	0 (0)	1,5 (1 - 2,24)	0,363
T3 - T4	8 (72,7)	4 (100)		
<b>Early N Stages</b>				
N0	1 (9,1)	1 (25)	0,3 (0,14 - 6,38)	0,476
N1 - N2	10 (90,9)	3 (75)		
<b>Early Clinical TNM Stage</b>				
II	1 (9,1)	1 (25)	0,3 (0,01 - 6,38)	0,476
III	10 (90,9)	3 (75)		
<b>Down staging</b>				
Yes	9 (81,8)	4 (100)	0,69 (0,48 - 0,99)	0,542
No	2 (18,2)	0 (0)		
<b>Early CEA level</b>				
Normal (≤ 5 ng/mL)	3 (42,9)	1 (33,3)	1,5 (0,09 - 25,39)	0,667
Elevated (> 5 ng/mL)	4 (57,1)	2 (66,7)		
<b>Survival</b>				
Survive	10 (90,9)	1 (25)	30 (1,41 - 638,15)	0,033*
Died	1 (9,1)	3 (75)		

TNM=Tumor size, Node status, Metastasis; CEA=carcino embryonic antigen  
Chi-square test (Fisher's exact test) \*P-value significant < 0,05



Wilcoxon's test, P significant < 0,05

Figure 1. Difference of average miR-124-3p expression before and after nCRT (left); difference of miR-124-3p expression in each patients (right).

## DISCUSSION

In rectal cancer clinical management, a recurrence and resistance after therapy is a challenging obstacle. Cancer generally more heterogeneous during carcinogenesis influenced by complex mechanism and affect to varies therapy result. Independent variables in clinical practice, such as pathological subtype, histological type, clinical stage, lymph node status and interval, may affect the prognosis of LARC patients receiving chemoradiotherapy. Some molecular factors can also influence the prognosis and sensitivity LARC patients to chemoradiotherapy due to genetic, transcriptomic, epigenetic and/or phenotypic changes.<sup>12</sup> This study focuses mainly on epigenetic, particularly microRNA.

Chemoradiotherapy resistance is an important factor that influences the prognosis of LARC. Therefore, predictive biomarkers capable of predicting sensitivity to chemoradiotherapy urgently need to help identify patients who would actually benefit from chemoradiotherapy. Chemoradio-resistance is a complicated process, involving cancer stem cell or tumor-initiating cells (TICs), angiogenesis which promotes by tumor-associated macrophages (TAMs), overexpression of DNA repair protein and autophagy.<sup>13-15</sup> Some earlier study has shown that miRNAs are associated with tumor cell chemoradio-sensitivity. MiRNA overexpression in tumor cells may increase or decrease chemoradio-sensitivity. Some miRNA expression has been down-regulated in resistant patients, but after up-regulation or administration, it may improve the impact of chemoradiotherapy.<sup>16,17</sup>

In this research, we examine the difference of miR-124-3p expression in the LARC patient serum before and after neoadjuvant chemoradiotherapy, and focusing on its relationship with patient survival for 1-2 years after treatment. Notably, more than half of patients who can survive after neoadjuvant chemoradiotherapy have an increase miR-124-3p expression and both variable significantly associated ( $P < 0,05$ ; OR =30, 95% CI = 1,41 – 638,15). Although interesting, the limitation of this study obtained in small sample size of patients with cancer treated, so we suppose that a more precise result will be determined by a massive measurement of subjects for the next study.

In earlier study, some radiation resistance model of cell line was developed and miRNA-mediated molecular mechanism was investigated. MiR-124-3p in the radiation-resistant cell line was found to be considerably down-regulated. Down-regulation of miR-124-3p is linked with metastasis and poor

prognosis in colorectal cancer.<sup>18</sup> Whereas miR-124-3p over-expression has been able to sensitize radiation-resistant cells by reducing the fraction of cell survival and viability<sup>19,20</sup>. It can be used as independent prognostic factor in colorectal cancer patient.<sup>21</sup>

MiR-124-3p has been involved as modulator of colorectal cancer carcinogenesis in many past studies. Some targets of miR-124-3p were identified in the regulation of colorectal cancer. MiR-124-3p regulates colorectal cancer growth by targeting PKM (pyruvate kinase muscle) gene that plays a role inhibit glycolysis rate in Warburg effect and also targets PRPS1 and RPIA gene that reduces pentose phosphate pathway and proliferation.<sup>22,23</sup> MiR-124-3p enhance radiotherapy sensitivity by targeting PRRX1, which act as epithelial-mesenchymal transition (EMT) inducer and stemness regulator and directly targets DNA methyltransferase 3B and DNMT1 in a colorectal cancer cell. miR-124-3p have multiple effects and targets in the development of colorectal cancer, become very potential for therapeutic target in the future.<sup>9,24</sup>

In conclusion, we identified that the average of miR-124-3p expression in rectal cancer patient with good prognosis was increased after neoadjuvant chemoradiotherapy and associate with high survival rate of patient. MiR-124-3p was found high-abundance and easy to identify in serum. Our study showed that miR-124-3p expression in blood serum was potential as biomarkers to predict the survival of rectal cancer patients after neoadjuvant chemoradiotherapy.

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