Expression of miR-29a in whole Blood of Patients with Colorectal Neoplasm

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Colorectal cancer (CRC) is major cancer with high incidence and mortality worldwide. It is known that most CRCs arise from precursor adenomatous polyps (APs). Recently, microRNA (miRNA) has been proposed as a biomarker for various cancers including CRC. In this study, the expression patterns of miR-29a in the whole blood (WB) of CRC, AP, and control groups were analyzed by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) to evaluate the expression level of miR-29a in patients with colorectal neoplasm (CRN) including CRC and AP. As a result, the relative expression of miR-29a was significantly decreased in the patients with CRN compared to the control group ($P<0.001$). The results were in agreement with previous *in vitro* cell studies and studies that used tissue and feces samples, suggesting that miR-29a in WB may be useful in demonstrating the status of colorectal tissue. Additionally, we divided the control group into healthy control (HC) without any colorectal symptoms and non-tumor control (NTC) with colorectal symptoms but without any CRN. And then the relative expression of miR-29a was also significantly decreased in the NTC group compared to the HC group ($P<0.001$). Therefore, our study revealed that miR-29a can differentiate patients with CRN from HC group, but they are also involved in the early stage of inflammatory response and cannot be specific biomarkers for CRN.

Key Words: Colorectal cancer, Adenomatous polyp, Colorectal neoplasm, MicroRNA, MicroRNA-29a, Whole blood, Biomarker

INTRODUCTION

Colorectal cancer (CRC) is important cancer with the third highest incidence rate and the second highest mortality rate among all cancers worldwide (Sung et al., 2021). At a local stage, the 5-year survival rate of CRC is 90%, but the survival rate declines to 14% for patients with the distant-stage disease. Since most symptoms do not appear in the early stages of CRC, only 37% of patients are diagnosed at

a local stage (Siegel et al., 2020). Therefore, Regular examination can decrease the mortality rate of CRC. Most CRCs are known to originate from adenomatous polyps (APs), then many studies have reported that it is possible to prevent the occurrence of CRC by removing the AP in advance when it is detected (Winawer et al., 1993; Citarda et al., 2001). It represents that detecting and removal of AP are important to decrease the incidence rate of CRC.

MicroRNA (miRNA) is a short non-coding RNA of 18~22 nucleotides that regulate gene expression by binding to

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the specific regions of target genes (Bartel, 2004). It is known that miRNA exists in not only cells but also diverse body fluids and is involved in various life phenomena such as cell division, cell differentiation, immunity, metabolism, and carcinogenesis (Pritchard et al., 2012; Dong et al., 2013). Depending on the function of the target genes, miRNA can exert either a tumor-suppressing or oncogenic role in cancer-related mechanisms (Bartel, 2004). Considering these characteristics, miRNAs have been suggested as blood-based biomarkers that can be used for the diagnosis of cancers (Schetter et al., 2008; Wang et al., 2016).

MiR-29a belongs to the miR-29 family and acts as a tumor suppressor in various cancers including cervical cancer (Gong et al., 2019), lung cancer (Liu et al., 2018b), nasopharyngeal cancer (Shi et al., 2019), and gastric cancer (Bai et al., 2018). By *in vitro* cell studies, miR-29a was also known to play a tumor suppressor role in CRC, such as attenuation of cell proliferation (Zheng et al., 2019) and induction of cell cycle arrest and apoptosis (Han et al., 2018). In addition to *in vitro* cell studies, many studies also have been conducted on circulating miR-29a in blood of CRC patients. But the expression of circulating miR-29a in CRC was controversial, showing increased expression of miR-29a in CRC (Yamada et al., 2015; Liu et al., 2018a) and decreased expression in CRC (Han et al., 2018; Orosz et al., 2018) compared to control. Moreover, most of the studies evaluated the expression of circulating miR-29a in CRC but have not conducted much validation on that in AP, which is an important target as a precursor lesion of CRC. Furthermore, plasma or serum samples were mainly used for evaluating the expression of miR-29a, and the expression of circulating miR-29a in whole blood (WB) has not been evaluated.

Therefore, in this study, to evaluate the expression of miR-29a in WB of patients with colorectal neoplasm (CRN) including CRC and AP, we estimated and compared the expression level of miR-29a in patients with CRN and control.

MATERIALS AND METHODS

Clinical samples

This study included analysis of WB samples that were

obtained from 117 healthy volunteers and 235 people who visited gastroenterology in a tertiary hospital and got a colonoscopy. According to the results of colonoscopy, a total of 235 people who visited the tertiary hospital were divided into 3 groups. As a result, Subjects with CRC were classified into CRC group (n=48), subjects with AP into AP group (n=137), and subjects without colorectal lesions into non-tumor control (NTC) group (n=50). 117 Healthy volunteers and 50 NTCs are classified as a control group. But considering the difference that healthy volunteers didn't show any colorectal symptoms and NTC showed colorectal symptoms and visited the tertiary hospital but there had no colorectal lesions on colonoscopy, healthy volunteers were separated from NTCs into healthy control (HC) group and used in additional analysis.

Ethics statement

This study was approved by the Institutional Ethics Committee at each institution. Informed written consent was obtained from all the study participants. All 235 blood samples from people who visit the tertiary hospital were collected at the Department of gastroenterology, Yonsei University Sinchon Severance Hospital, Gangnam Severance Hospital, Kangbuk Samsung Hospital, Seoul, the Republic of Korea between 2017 and 2019 (approval no. 4-2017-0148, 3-2017-002, 2017-02-022-009). All 117 blood samples from healthy volunteers were collected at Yonsei University Sinchon Severance Hospital, Seoul, the Republic of Korea, and Yonsei University Wonju Campus, Wonju, the Republic of Korea between 2013 and 2014 (approval no. 4-2011-0011, 1041849-201311-BM020-02).

Total RNA extraction

Total RNA in EDTA blood tube was isolated using Isol-RNA Lysis Reagent (5 Prime, USA) and total RNA in Tempus blood tube was isolated using Tempus spin RNA isolation kit (ThermoFisher Scientific, USA) according to the manufacturer's protocol, respectively. The purity and concentration of total RNA were determined by measuring the absorbance at 260 nm and 280 nm using the Infinite 2000 (Tecan, Switzerland). All steps in the preparation and handling of total RNA were performed in a laminar flow

hood under RNase-free conditions. The isolated total RNA was stored at -80°C until use.

cDNA synthesis

Complementary DNA (cDNA) was synthesized using a Taqman microRNA Reverse Transcriptase kit (Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, 2~20 ng of total RNA was used for cDNA synthesis. The reverse transcriptase (RT) reaction mixture contained 0.15 µL of 100 mM dNTP mix (100 mM each dATP, dGTP, dCTP, and dTTP at a neutral pH), 1 µL of 50 U/µL reverse transcriptase, 1.5 µL of 10x reverse transcriptase buffer, 0.19 µL of 20 U/µL RNase inhibitor, and adjusted the total reaction volume to 15 µL with nuclease-free water. The cDNA synthesis reaction was performed as follows: 16°C for 30 min followed by 42°C for 30 min, and 85°C for 5 min. RT reactions were performed using a Veriti 96 Well Thermal Cycler (Applied Biosystems, USA).

miRNA analysis using qPCR

TaqMan miRNA small RNA assay (Applied Biosystems, USA) was used to detect and quantify miRNA expression with miRNA-specific primers according to the manufacturer's instructions. Briefly, 2 µL of cDNA was added to 11 µL of probe qPCR mix and 7 µL of nuclease water. The following TaqMan small RNA assay (Applied Biosystems, USA) primers were used: hsa-miR-29a-3p and hsa-miR-16-5p. All analyzed miRNAs are of human (*Homo sapiens*) origin and therefore, the prefix "hsa" is omitted throughout the text. qPCR reactions were performed using a CFX96 Real-Time PCR System Detector (Bio-Rad, USA). Samples were run in duplicate for each experiment. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method using the has-miR-16-5p, as an endogenous control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 7.0 (GraphPad, USA). One-way ANOVA tests were used to determine statistical significance between each group. Receiver operating characteristic (ROC) curves were generated to assess the diagnostic accuracy of each miRNA, and the area under the ROC curve (AUC) was

calculated to measure discriminatory capacity. The maximum value of (sensitivity+specificity-1) was set as a cutoff value in the ROC curve according to the Youden index (Youden, 1950).

RESULTS

Expression levels of miR-29a in WB of CRC, AP, and control group

To analyze the expression pattern of miR-29a in WB of patients with CRN compared to control, the relative expression level of miR-29a was determined in 167 Controls, 137 patients with AP, and 48 patients with CRC. Clinical

Table 1. Clinicopathological characteristics of CRC and AP patients, and control

Characteristics	CRC (n=48)	AP (n=137)	Control (n=167)
Age, y (SD)	64.3 (11.0)	59.6 (9.9)	42.8 (17.5)
Gender, n (%)			
Male	27 (56.3)	84 (61.3)	65 (38.9)
Female	21 (43.8)	53 (38.7)	102 (61.1)
AP feature			
Size, n (%)			
> 1 cm		90 (65.7)	
< 1 cm		47 (34.3)	
CRC feature			
Tumor stage, n (%)			
I-II	25 (52.1)		
III-IV	18 (37.5)		
Unknown	5 (10.4)		
Metastasis, n (%)			
M0	38 (79.2)		
M1	9 (18.8)		
Unknown	1 (2.0)		
Histology, n (%)			
Well differentiated	10 (20.8)		
Moderately differentiated	26 (54.2)		
Poorly differentiated	1 (2.1)		
Unknown	11 (22.9)		

CRC, Colorectal cancer; AP, Adenomatous polyp; SD, Standard deviation

Fig. 1. Relative expression of miR-29a in whole blood from control (n=167), patients with AP (n=137), and CRC (n=48). Expression of miR-29a was normalized by miR-16. Data are provided as a mean $\pm$ SEM. *P*-values were determined by one-way ANOVA test. SEM, Standard error of mean; ****P* < 0.001; AP, Adenomatous polyp; CRC, Colorectal cancer.

characteristics of the CRC, AP, and control are summarized in Table 1. The data showed that miR-29a in WB was significantly decreased in patients with AP and CRC compared to controls (both *P* < 0.001, Fig. 1).

Diagnostic value of miR-29a in CRC and AP

To estimate the diagnostic value of miR-29a in CRC and AP, the ROC curve was established. The analysis suggested that the WB level of miR-29a was a potential biomarker in differentiating patients with CRC and AP from controls, with an AUC of 0.8288 (95% CI: 0.7764~0.8813) and 0.8255 (95% CI: 0.7768~0.8742), respectively (Fig. 2). According to the Youden index (Youden, 1950), we set the optimal cut-off to 0.008478 for CRC classification, and as a result sensitivity was 100% and the specificity was 71.26%. As a result of setting the optimal cut-off to 0.02685 for AP classification, the sensitivity was 100% and the specificity was 70.66%.

Expression levels of miR-29a in CRC, AA, NA, NTC, and HC

In the above result, specificity was low compared to the sensitivity, suggesting that a detailed analysis of the control group was necessary. Therefore, control group was divided

Fig. 2. Diagnostic performance for CRC and AP of miR-29a. ROC curve was plotted to discriminate (A) CRC and (B) AP patients from control group. CRC, Colorectal cancer; AP, Adenomatous polyp; ROC, Receiver operating characteristic; AUC, Area under curve; CI, Confidence Interval.

into healthy control (HC) without any colorectal symptoms and NTC with colorectal symptoms. As a result, compared to healthy control, the expression level of miR-29a was also significantly decreased in NTC with AP and CRC groups (*P* < 0.001, Fig. 3).

DISCUSSION

CRC accounts for about 1 in 10 cancer cases and cancer-

Fig. 3. Relative expression of miR-29a in whole blood from HC (n=117), NTC (n=50), and patients with AP (n=137), and CRC (n=48). Data are provided as a mean $\pm$ SEM. Expression of miR-29a was normalized by miR-16. *P*-values were determined by one-way ANOVA test. SEM, Standard error of mean; ****P* < 0.001; HC, Healthy control; NTC, Non-tumor control; AP, adenomatous polyp; CRC, Colorectal cancer.

related deaths worldwide (Sung et al., 2021). The 5-year survival rate of CRC is 90% at a local stage, but it declines to 14% at a distant stage (Siegel et al., 2020). Besides, the fact is known that most colorectal cancers develop from AP. Therefore, regular examination for screening CRC or AP early can significantly reduce the incidence and mortality of CRC. The confirmative test for CRC diagnostics is colonoscopy, but this method is invasive to be a regular examination for the population (Yeol, 2013). A screening test that is carried out regularly in most countries is stool-based. But it has limited sensitivity for detection of adenomas and early-stage CRC and subjects undergoing the test feel uncomfortable in using stool samples (Yeol, 2013; Niedermaier et al., 2017). Thus, a screening test for CRC and AP based on a blood sample can be effectively used in the regular examination.

MiR-29a was reported to play a tumor suppressor role in many cancers (Bai et al., 2018; Liu et al., 2018b; Gong et al., 2019; Shi et al., 2019) including CRC (Han et al., 2018; Zheng et al., 2019). Zheng, et al. (Zheng et al., 2019), showed that miR-29a was at the lower expression in CRC cell lines and that miR-29a attenuated cell proliferation and induced

cell cycle arrest and apoptosis by targeting ribosomal protein S15A (RPS15A) in CRC cells. Han et al. (Han et al., 2018), represented that miR-29a suppressed cell viability and induced apoptosis by suppressing the PTEN/Akt/GSK3 β and Wnt/ β -catenin signaling pathway in CRC cells. The result of this study in which the expression of miR-29a was lowered in CRN compared to HC can be explained by the role of miR-29a as a tumor suppressor reported from *in vitro* cell studies. In addition, the expression of miR-29a was also decreased in studies that used tissue (Zheng et al., 2019; Wang et al., 2021) and feces (Zhu et al., 2016) of CRC patients. Therefore, it seems that expression of miR-29a in WB can be useful for representing the state of colorectal tissue

However, in addition to CRN, the expression of miR-29a was lowered even in NTC without any CRN in the colorectum, suggesting that miR-29a may play a role in the inflammatory response that causes colorectal symptoms as well as in the tumorigenesis process. Indeed, in acute pancreatitis, miR-29a was reported to promote inflammation (Dey et al., 2021). In addition, it has been reported that miR-29a promotes lipopolysaccharide (LPS)-induced inflammatory response in macrophage via the Akt1/NF- κ B pathway (Tang et al., 2017), which is expected to be related to the functional defect of the gut barrier caused by the change of gut microbiome in the colorectum. The composition of gut microbiota can be changed by disruption of homeostasis due to environmental or physical stress or psychological stimuli (Lobionda et al., 2019). And changes in gut microbiota induce intestinal inflammatory responses and cause defects in the function of the gut barrier (Balfour Sartor, 1997; Sellon et al., 1998; Frank et al., 2007). When the gut barrier is defective, bacterial molecules such as LPS can leak into the blood (Fukui, 2016; Mu et al., 2017). Therefore, miR-29a is expected to be associated with the inflammatory response that causes symptoms in the colorectum.

In conclusion, the expression of miR-29a in WB of patients with CRN was significantly decreased and it was in line with tumor suppressor role of miR-29a in CRC cells and results from previous studies conducted using tissue samples. However, miR-29a was not specific for CRC and AP and was also significantly decreased in NTC compared

to HC, representing that miR-29a may participate in the early stage of inflammatory reaction.

Abbreviations

APs, adenomatous polyps; AUC, area under the ROC curve; CI, Confidence Interval; CRC, colorectal cancer; CRN, colorectal neoplasm; HC, healthy control; LPS, lipopolysaccharide; miRNA, microRNA; NTC, non-tumor control; p, *P*-value; ROC, receiver operating characteristic; RPS15A, ribosomal protein S15A; RT, reverse transcriptase; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction; SEM, standard error of mean; WB, whole blood.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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