의학박사 학위논문

대장암 선별검사를 위한 비침습적 바이오마커로서 대변 마이크로 RNA-221 과 Let-7a 의 유용성

Usefulness of fecal microRNA-221 and Let-7a as the potential non-invasive biomarker for colorectal cancer screening

울 산 대 학 교 대 학 원

의 학 과

박종하

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지 도 교 수　　변정식

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Abstract

Background and Aims: Fecal microRNA (miRNA) identification has been expected a non-invasive biomarker test for colorectal cancer (CRC) screening. The purpose of this study was to investigate fecal miRNAs which are altered in colorectal neoplasia. We also aimed to find out the combination of fecal miRNAs for improved performance in CRC screening.

Methods: We collected a total of 97 stool samples from 30 patients with CRCs, 35 patients with colorectal adenomas, and 32 healthy controls. We investigated the expression of 13 fecal miRNAs (miR-17, -21, -27a, -92, -106a, -145, -155, -181b, -199a, -200c, -221, -494, Let-7a) and compared their expression between CRC, colorectal adenoma, and healthy control groups.

Results: In univariate analysis, age, fecal miR-21, -145, -155, -199a, -221, -494 and Let-7a showed significant differences between the neoplastic group and healthy control. In logistic regression, age (Odds ratio (OR) 21.1, 95% confidence interval (CI) 2.2-151.7, p=0.005), miR-221 (OR 4.1, 95% CI 1.1-16.5, p=0.037) and Let-7a (OR 11.7, 95% CI 1.3-103.6,

p=0.034) were identified as independent risk factors for colorectal neoplasia. The area under the curve (AUC) of age, fecal miR-221, and Let-7a were 0.656 (95% CI, 0.552-0.750), 0.742 (95% CI, 0.643-0.827) and 0.645 (95% CI, 0.540-0.740) for the neoplastic group, respectively. The AUC of the combination of age, fecal miR-221 and Let-7a was higher than that of each variable (0.839; 95% CI, 0.749-0.907). The combination of fecal miRNAs and age showed high sensitivity (79.4%) and specificity (86.7%) for diagnosis of colorectal neoplasia.

Conclusion: Fecal miR-221 and Let-7a may be useful non-invasive biomarkers for CRC screening. The combination of these two fecal miRNAs with age can predict the presence of CRCs and colorectal adenomas with high confidence.

Contents

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List of abbreviations

AUC; area under the curve

CRC; colorectal cancer

FIT; fecal immunochemical test

FOBT; fecal occult blood test

gFOBT; Guaiac FOBT

MSI-H; microsatellite instability-high

MSS; microsatellite stable

qRT-PCR; quantitative real-time polymerase chain reaction

UTR; untranslated region

MLH1; mutL homolog 1

MSH2; mutS homolog 2

EGFR; epidermal growth factor receptor

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in males and the second in females worldwide.[1-3] The incidence rates of CRC are rapidly increasing in many countries of Eastern Asia and Eastern Europe, which have been considered low-risk areas for CRC.[4,5] These unfavorable trends are thought to be due to westernized dietary patterns, obesity, and smoking increases, etc.[4-7] However, fortunately, the mortality rates of CRC in some developed countries have been decreasing.[5] It results from significant efforts for early detection of CRC as well as improvement of treatment.[5,8-10]

There are many options for CRC screening. In general, colonoscopy is considered the most accurate method for CRC screening. However, because of limited medical resources and invasiveness of colonoscopy, the CRC screening mainly using colonoscopy in the general population has many difficulties in the real world. Fecal occult blood test (FOBT), especially fecal immunochemical test (FIT) has been recommended as one of main CRC screening methods.[11-13] FIT showed high sensitivity (81%) for CRC, but its specificity

was not satisfactory (28-94%).[14,15] Furthermore, FIT showed lower detection rates for adenoma, advanced adenoma and advanced neoplasm than those by colonoscopy (0.4% vs. 4.2%, 0.9% vs. 1.9%, 1.0% vs. 2.0%; $p<0.001$).[16] Multitarget fecal DNA test revealed higher sensitivity than FIT for CRC, advanced precancerous lesions, polyps with high-grade dysplasia and serrated polyps (92.3% vs. 73.8%, 42.4% vs. 23.8%, 69.2% vs. 46.2% and 42.4% vs. 5.1%).[17] However, this test showed lower specificity than FIT (86.6% VS 94.9%). Because of these limitations of current CRC screening methods, the development of new non-invasive methods by using reliable molecular biomarkers for effective mass screening and early diagnosis of CRC has been strongly required.

MicroRNAs (miRNA) are a class of small, non-coding RNAs (18-25 nucleotides) which regulate cell process in about 30% of mammalian genes. miRNA-related regulation is mediated by imperfect binding to the 3' untranslated region (UTR) of target messenger RNAs, which results in prevention of protein accumulation by transcription repression or by messenger RNA degradation.[18] There have been many studies about the role of miRNAs in

the development of various cancers (miR-15a /-16-1 for pituitary adenoma, Let-7a /-155/-17-92/-106a for lung cancer and miR-21/-155 for breast cancer).[19,20] Many previous studies also evaluated the role of miRNA in colorectal tumorigenesis. The majority of these studies identified miRNA from CRC tissue or blood.[21-31] Investigation of tissue miRNAs cannot be used as a screening method. Analysis of blood sample can be used as a screening tool but it is still invasive. A fecal test is a non-invasive method and can be used as a mass screening test. However, there have been only a few studies which investigated the role of fecal miRNAs for CRC.[22,23,31] Therefore, we aimed to investigate the fecal expression of miRNAs which were known to be altered in the CRC tissue and blood sample in patients with colorectal neoplasm. We also aimed to provide a CRC screening strategy using fecal miRNAs which are altered in patients with colorectal neoplasm.

Materials and Method

Study population

This study was conducted at a single university hospital from July 2014 to November 2014. We prospectively enrolled 30 consecutive patients with newly diagnosed CRCs at Asan Medical Center during the study period. All CRCs were pathologically confirmed by colonoscopic biopsy. We also prospectively enrolled 35 consecutive patients with colorectal adenomas who underwent colonoscopic polypectomy at Asan Medical Center during the same period. Finally, we enrolled 32 consecutive healthy controls who visited the Health Screening and Promotion Center at Asan Medical Center during the study period. All healthy controls underwent screening colonoscopy and showed no adenoma or CRC. The neoplastic group was defined as the sum of CRC patients and adenoma patients. Those who had following conditions were excluded: inflammatory bowel diseases, acute infectious diarrhea within one month, history of CRC, and refusal to participate in the present study. Detailed medical history was obtained from all enrolled patients and controls by structured

questionnaire and interview with physicians. All participants provided written informed consents. This study was approved by the institutional review board of our institution (No. 2014-0331).

Stool sample collection

All participants had free diet without specific limitation of food for at least one week before stool collection. They did not use any laxatives or bowel preparation agents for stool collection. All stool samples were collected at least two days before colonoscopy and were collected from the naturally defecated stool. Stool samples from CRC patients who were referred from other hospitals were collected at least two weeks after the previous colonoscopy at other hospitals. A 30 ml disposable stool sample container with a screw cap was used for collection and delivery of stool samples. These stool containers were manufactured to maintain aseptic conditions to avoid any biological contamination. Different aliquots were immediately stored at 4°C and transferred and stored at -80°C for each

subsequent analysis.

RNA extraction from stool samples

The stool samples were thawed at room temperature. 200 mg (wet weight) of stool sample was added to 1 ml Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) in a 2 ml RNase-free tube. The sample was subsequently homogenized by a vortex mixer with RNase-free pestles (USA Scientific, Woodland, CA, USA) to deform completely. 200 ul of chloroform was added to the homogenized sample. Total RNA including miRNA was extracted using the miRNA Mini Kit (Qiagen, Valencia, CA, USA) as described in the product manual and quantified using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All isolated RNA samples were stored at -80°C until subsequent use.

miRNA quantitation by quantitative real-time PCR (qRT-PCR)

qRT-PCR of each miRNA was carried out with the TaqMan miRNA reverse transcription Kit (Applied Biosystems Inc., Foster City, CA, USA). Briefly, the reaction mixture was prepared in a final volume of 3 ul containing four ng of total RNA, 0.3 ul of TaqMan miRNA reverse transcription primer, 3 nM dNTP (with dTTP), ten units reverse transcriptase, 0.6 units RNase inhibitor and 0.3 ul of 10 X RT buffer. The thermal cycling conditions were as follows: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and hold at 4°C. The RT product was subsequently diluted four-fold by adding 9 ul nuclease-free water. The PCR reaction mix contains ten µl TaqMan Universal PCR Master Mix with no AmpErase Uracil-N-Glycosylase (UNG), 0.5 µl miRNA TaqMan primers, four µl diluted RT product and 5.5 µl nuclease free water. Real-time PCR was carried out using the 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA, USA). The PCR profile was as follows: 95°C for 10 min, 50 cycles of 95°C for 15 sec and 60°C for 1 min. Data collection was carried out at the 60°C step. The cycle threshold (Ct) values, which is defined as the number of cycles required for the fluorescent signal to cross the threshold in qRT–PCR, were

converted to the absolute number of copies/ng RNA based on standard curves obtained from dilution series of known input quantities of synthetic target miRNA (Integrated DNA Technologies Inc., Coralville, IA, USA). All assays were carried out in a blinded fashion. We analyzed the expression of 13 fecal miRNAs (miR-17, -21, -27a, -92, -106a, -145, -155, -181b, -199a, -200c, -221, -494, Let-7a) which were reported to be altered in CRC tissues and/or blood in previous studies.[21-31]

Fecal immunochemical test

For fecal immunochemical test (FIT), the Eiken OC-Sensor (Eiken Chemical Co. Ltd., Tokyo, Japan) was used following the manufacturer's instructions. Stool samples for FIT by the Eiken OC-Sensor were obtained from the stool samples in 30 ml disposable stool sample container with a screw cap which was collected for fecal miRNA assay as described above. The FIT positivity was defined based on the cut-off value of 100 ng Hemoglobin/g feces in this study.

Review of medical records and questionnaire survey

All clinical data were collected by review of medical records of patients with adenoma and CRC and by the questionnaire survey for healthy controls. Gender, age, anthropometric results, family history of CRC, and status of smoking and alcohol were reviewed. In CRC patients, location and pathological stage were investigated. In patients with adenoma, location, size, the presence of villous component and degree of dysplasia were assessed. Advanced adenoma was defined as adenomas with advanced features such as adenoma over 1 cm in diameter, containing villous component and/or high-grade dysplasia. The advanced neoplasm was defined as advanced adenoma and CRC.

Statistics

Continuous variables were reported as mean ± standard deviations and categorical variables as percentages. Continuous variables were compared by Student's t-test and ANOVA test.

Associations between categorical variables were evaluated by Pearson's chi-squared test and Fisher's exact test. Receiver operating characteristic (ROC) curves were regenerated based on the fecal miRNA levels of CRC patients, adenoma patients and healthy controls. Cut-off value of ROC curve of each diagnostic test was determined as the value which maximizes the Youden's index. Combination analysis was calculated by logistic regression (forward stepwise). Correlation analyses were shown by Spearman correlation coefficient. P values <0.05 were considered statistically significant. All analyses were performed with SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA) and MedCalc software version 11 (MedCalc Software bvba, Belgium).

Results

Baseline characteristics

A total of 97 participants were enrolled in this study; 30 patients with CRCs, 35 patients with

colorectal adenomas, and 32 healthy controls. The mean age of CRC group was higher than the other groups (60.7±12 vs.53.5±7.2 vs. 52.4±6.6 years, P < 0.001). Gender ratio, body mass index (BMI), family history of CRC and smoking status were not different between three groups (Table 1). We investigated clinicopathological characteristics of CRC and adenoma groups (Table 2). Most CRCs were adenocarcinomas and only two CRCs were mucinous adenocarcinomas. All of 28 CRC cases in which microsatellite instability was checked showed microsatellite stable state.

Table 1. Baseline characteristics of the study population

Characteristics	CRC* group (n=30)	Adenoma group (n=35)	Healthy control (n=32)	P value
Age, yr (range)	60.7 ± 12.0 (33-84)	53.5±7.2 (36-67)	52.4±6.6 (36-67)	<0.001
Gender, male (%)	19 (63.3)	30 (85.7)	21 (65.6)	0.080
Body mass index, kg/m^2 (range)	24.1±4.1 (18.8-39.2)	23.4±2.5 (19-28.9)	23.4±2.7 (16.5-28.9)	0.151
Family history of CRC (%)	6 (20.0)	5 (14.3)	0 (0)	0.151

Alcohol (%)	14 (46.7)	27 (77.1)	26 (81.3)	0.006
Smoking (%)	13 (43.3)	19 (54.3)	17 (53.1)	0.153

Values are mean ± standard deviation or number (%); CRC*, colorectal cancer.

Table 2. The characteristics of the CRC group and adenoma group

CRC group (n=30)				
Size (mm)	46.2±20.4 (range; 6-97)			
Location	Rectosigmoid (RS)=22		Proximal to RS = 8	
TNM staging	Ⅰ	Ⅱ	Ⅲ	Ⅳ
	3	8	15	4
Tissue genetic markers	MSI status (n=28)* (MLH1, MSH2)		EGFR (n=3)*	K-ras (n=2)*
	All positive (MSS)		All positive	All negative
Adenoma group (n=35) (Total polyp number =64)				
Size (mm)	5.14±4.2 (range; 2-20)			
Number	1.77±1.1 (range; 1-5)			
Location	Rectosigmoid (RS)=22		Proximal to RS = 13	
Advanced adenoma (n)	>10 mm		Villous component	High-grade dysplasia
	3		0	2
Paris endoscopic classification	Is	Isp	Ip	Ⅱ
	43	12	2	7

Values are expressed as mean ± standard deviation, MLH1; mutL homolog 1, MSH2; mutS homolog 2, EGFR; epidermal growth factor receptor, MSS; microsatellite stable status, Advanced adenoma; adenoma over 1 cm in diameter, containing villous component and/or high-grade dysplasia

* Checked in 28, 3, and 2 patients, respectively.

Fecal miRNA expression

Of 13 fecal miRNAs we investigated, miR-17, -92,-106a, 145, -221 and Let-7a showed

significantly different fecal expression among 3 groups (Table 3, Figure 1).

In comparison of fecal miRNA expression between the neoplastic group and healthy control, seven miRNAs (miR-21, -145, -155, -199a, -221, -494 and Let-7a) showed significantly different fecal expression (Table 4).

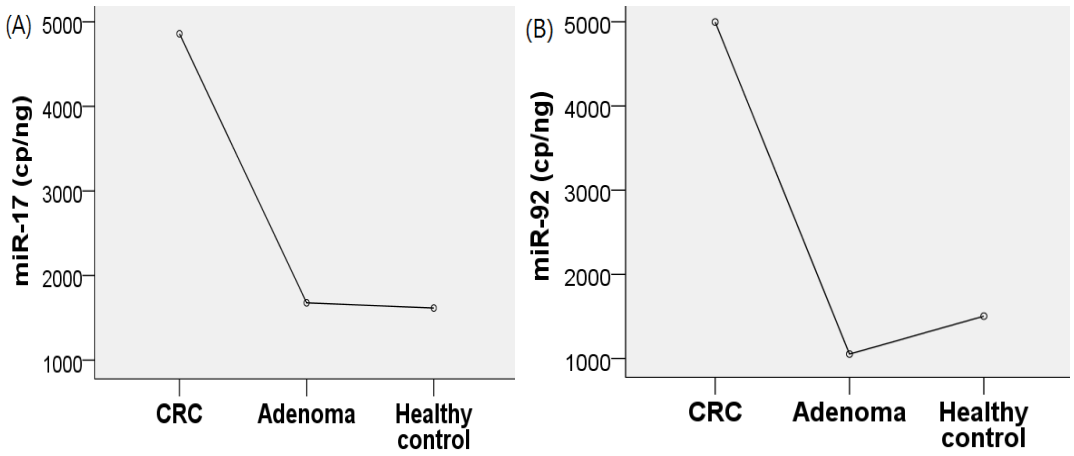
Table 3. Difference in fecal miRNA expression between CRC patients, adenoma patients, and healthy controls

Fecal miRNA (cp/ng)	CRC group (n=30)	Adenoma group (n=35)	Healthy control (n=32)	P value
miR-17	4929.9±7233.2	1800.6±1714.8	1615.8±2831.2	0.006
miR-21	254433.1±2.7x10^5	199083±2.0x10^5	118727.5±1.9x10^5	0.063
miR-27a	53302.2±1.5x10^5	15943.1±14883.4	9465.6±10119.2	0.132
miR 92	5107.4±9950.5	1183.9±1134.7	1503.5±5245.4	0.029
miR 106a	11816.5±15497.3	4644.1±4049.3	4010.8±8042.3	0.005
miR 145	9264.9±6479.4	10946.5±5971.6	7596.6±1929	0.038
miR 155	88445.2±64822.3	92743.7±1.2x10^5	55921±62358	0.188

miR 181b	27786.6±25931.2	30175.8±43720.6	17052.2±21591.5	0.234
miR 199a	990.0±1385.8	2282.6±4508.3	644.1±929.1	0.057
miR 200c	$1.2x10^5±1.6x10^6$	$1.2x10^6±1.3x10^6$	$750340±1.3x10^6$	0.355
miR 221	456.9±671.9	210.5±227.1	89.2±97.9	0.002
miR 494	1065.2±764.7	1269.4±1102	901.6±558.6	0.230
Let-7a	$303287.9±3.8x10^5$	$253323.2±2.1x10^5$	130309.7±98594.9	0.027

Values are expressed as mean ± standard deviation. CRC, colorectal cancer

Figure 1. Different expression of 6 fecal miRNAs between CRC, adenoma and healthy controls



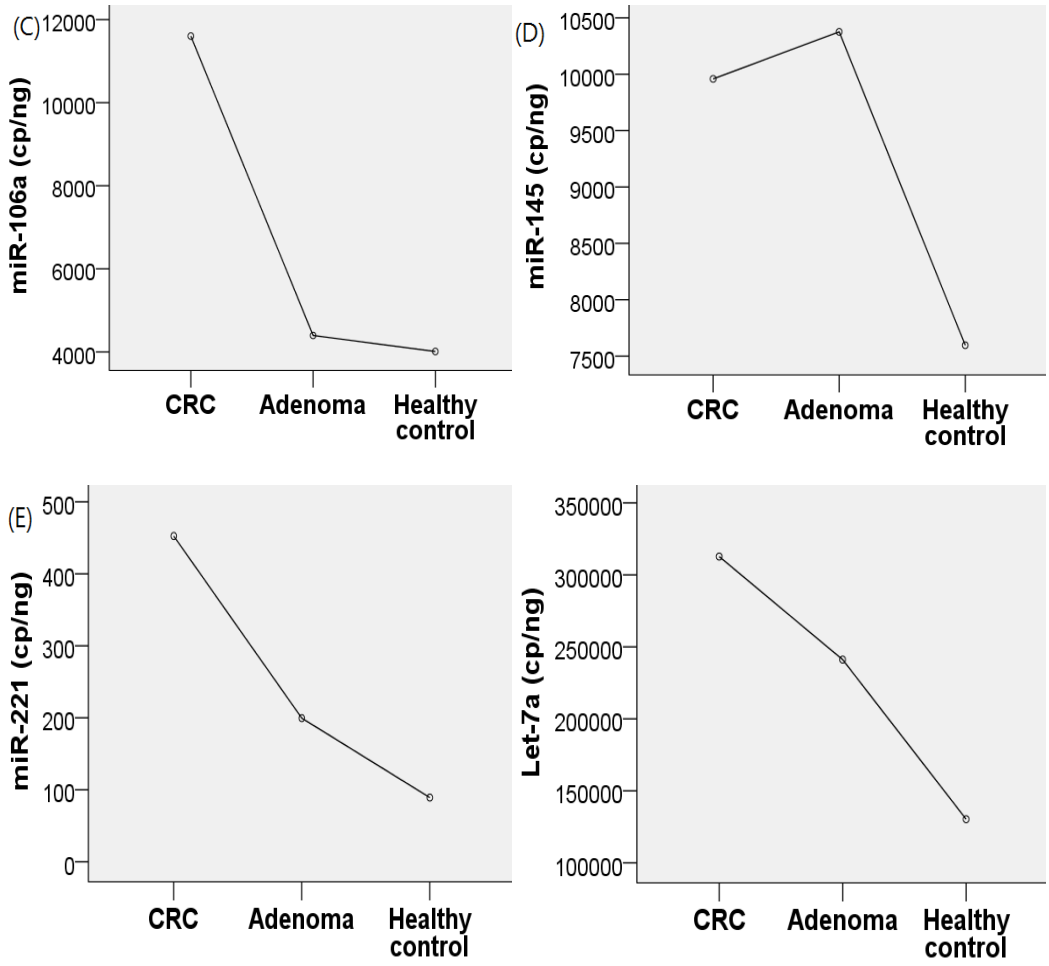


Table 4. Difference in fecal miRNA expression between the neoplastic group and healthy controls

Fecal miRNA (cp/ng)	Neoplastic group (n=65)	Healthy group (n=32)	P value
miR 17	3218.5±5225.5	1615.8±2831.2	0.084
miR 21	224629.2±2.4x10^5	118727.5±1.9x10^5	0.037

miR 27a	33185.8±1.0x10^5	9465.6±10119.2	0.215
miR 92	2994.7±7030.9	1503.5±5245.4	0.365
miR 106a	7883.2±11319.1	4010.8±8042.3	0.149
miR 145	10170.4±6219.4	7596.6±1929	0.030
miR 155	90795±95730	55921±62358	0.046
miR 181b	29073.1±36354	17052.2±21591.5	0.112
miR 199a	1696.9±3498	644.1±929.1	0.010
miR 200c	1.2x10^5±1.4x10^6	750340±1.3x10^6	0.140
miR 221	326±500.2	89.2±97.9	0.005
miR 494	1175.4±959.7	901.6±558.6	0.016
Let-7a	276383.8±3.0x10^5	130309.7±98594.9	<0.001

Values are expressed as mean ± standard deviation.

Fecal immunochemical test (FIT)

FIT was positive in 73.3% (22/30) of CRC group, in 0% (0/35) of adenoma group, and 3.1% (1/32) of the healthy control. FIT positivity was significantly higher in the neoplastic group than in the healthy control (22/65, 33.8% vs. 1/32, 3.1%; P=0.001). The sensitivity and

specificity of FIT for diagnosis of colorectal neoplasm was 34.9% and 96.9%.The AUC of FIT was 0.659 (95% CI 0.551-0.767).

Independent risk factors for the presence of colorectal neoplasia

In the linear regression analysis for significant risk factors of colorectal neoplasia, the age, miR-221, and Let-7a showed independent significance (Table 5). When analyzed together with fecal miR-221 and Let-7a, FIT did not show statistically independent significance (P=0.143).

We performed the ROC curve analyses for these risk factors. The AUC of age was 0.656 (95% CI 0.552-0.750). The sensitivity and specificity of > 61 years of age for colorectal neoplasia were 34.4% and 96.9%. The AUC of miR-221 was 0.742 (95% CI 0.643-0.827). The sensitivity and specificity of fecal miR-221 > 156.2 cp/ng were 56.2% and 87.1%. The AUC of Let-7a was 0.645 (95% CI 0.540-0.740).The sensitivity and specificity of Let-7a > 264434.5 cp/ng were 36.9% and 96.8% (Figure 2).

Table 5. Multivariate analysis of independent risk factors for presence of colorectal neoplasia*

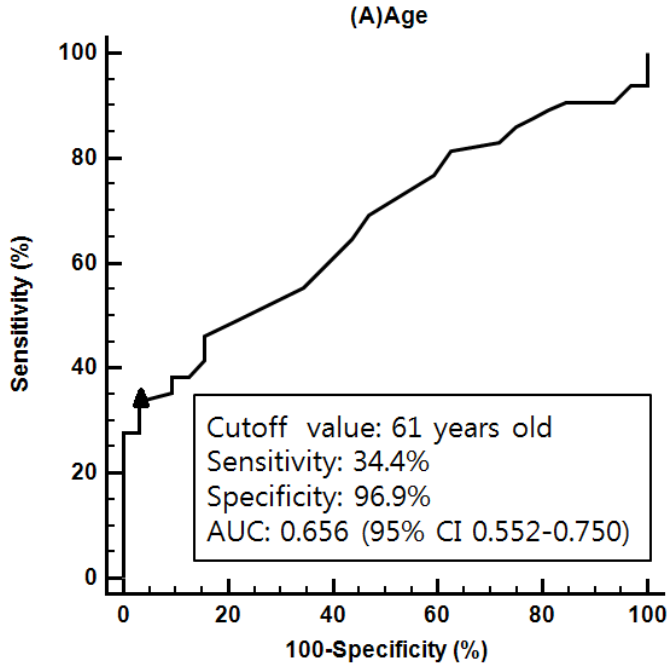
Variables	Odds ratio	[95% CI†]	P value
Age	21.1	2.2-151.7	0.005
miR-221‡	4.1	1.1-16.5	0.037
Let-7a	11.7	1.3-103.6	0.034

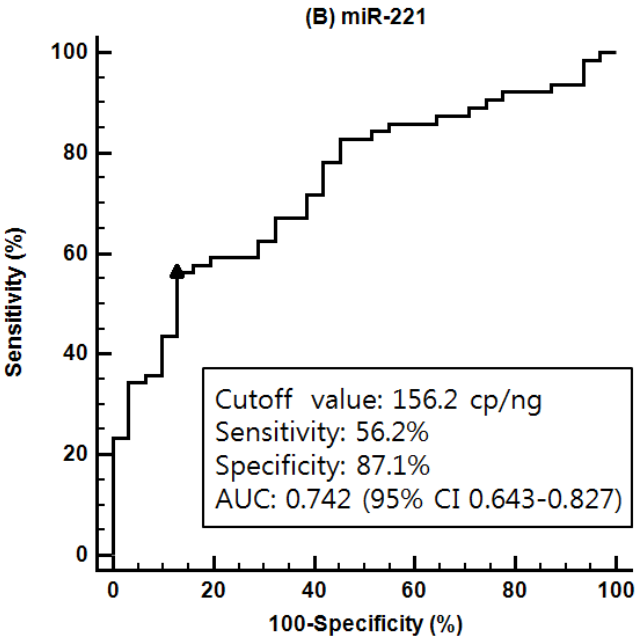
*Colorectal neoplasia means colorectal adenoma plus colorectal cancer.

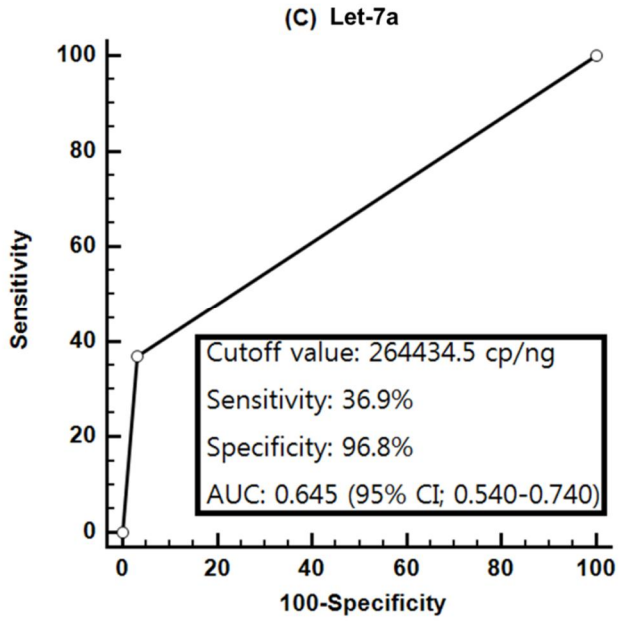
†CI, confidence interval; ‡miR, microRNA

Figure 2. ROC curves of independent risk factors for colorectal neoplasia

(A) AUC of age was 0.656 (95% CI 0.552-0.750). (B) AUC of fecal miR-221 was 0.742 (95% CI 0.643-0.827). (C) AUC of Let-7a was 0.645 (95% CI 0.540-0.740). AUC, area under the ROC curve


(A)Age
Sensitivity (%)
100-Specificity (%)
Cutoff value: 61 years old
Sensitivity: 34.4%
Specificity: 96.9%
AUC: 0.656 (95% CI 0.552-0.750)


(B) miR-221
Sensitivity (%)
100-Specificity (%)
Cutoff value: 156.2 cp/ng
Sensitivity: 56.2%
Specificity: 87.1%
AUC: 0.742 (95% CI 0.643-0.827)



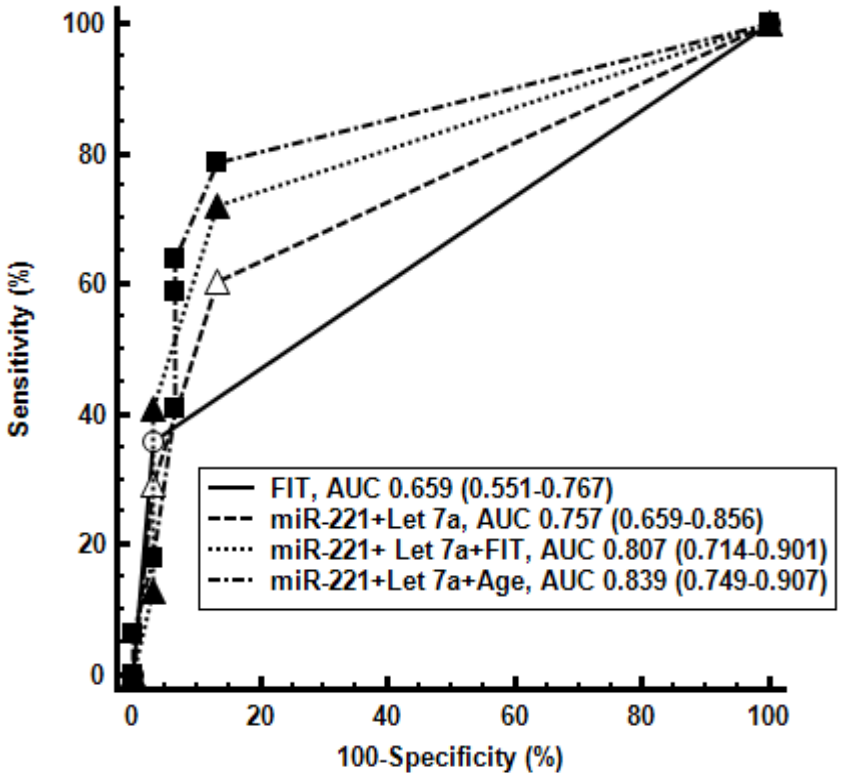
Combination of risk factors for improved screening of colorectal neoplasia

Combination of fecal miR-221 > 156.2 cp/ng , Let-7 a > 264434.5 cp/ng and age over 61 years showed higher sensitivity (79.4%) and specificity (86.7%) than those of each variable. The AUC of the combination of age, fecal miR-221 and fecal Let-7a was 0.839 (95% CI 0.749-0.907), which was higher than the AUC of each variable. The AUC of this combination was also higher than the AUC of FIT (AUC 0.659, 95% CI 0.551-0.767) (P <0.001) (Figure 3).

The AUC of combination of fecal miR-221, Let-7a and FIT was 0.807 (95% CI 0.714-0.901). The AUC of combined fecal miR-221 and Let-7a was 0.757 (95% CI 0.659-0.856). All these

combinations showed higher screening performance than FIT alone (Figure 3).

Figure 3. The ROC curves of FIT, the combinations of fecal miR-221, Let-7a, age for differentiation between the neoplastic group and healthy control



Fecal miRNA expression according to stages and locations of CRC in the CRC group

In the CRC group, the level of fecal miR-221 and Let-7a did not show any significant differences according to TNM stages and CRC locations (Table 6). There were no significant differences in the level of fecal miR-221 and Let-7a between metastatic CRC patients and non-metastatic patients (187.0±108.8 cp/ng vs 498.4±713.3 cp/ng, P=0.208;

264760.4±1.6x10^5 cp/ng vs 309215.2±4.1x10^5 cp/ng, P=0.285).

Table 6. Fecal miRNA expression according to TNM stages and location of CRC

Fecal miRNA (cp/ng)	TNM stage I & II (n=11)	TNM stage III & IV (n=19)	P-value
miR-221	471.9±547.4	448.3±748. 8	0.922
Let-7a	342249.1±4.2x10^5	280731.4±3.7x10^5	0.678
	Rectum – sigmoid colon (n=22)	**Descending colon – cecum (n=8)**	
miR-221	516.1±751. 3	294.1±367.9	0.433
Let-7a	310896.7±3.6x10^5	282363.7±4.6x10^5	0.860

Values are a mean ± standard deviation.

Fecal miRNA expression according to the number, location, and advanced features of adenoma in the colorectal adenoma group

Fecal miR-221 expression showed weak positive linear correlation with the number of colorectal adenomas (Spearman correlation coefficient, r=0.030). However, fecal Let-7a showed negligible linear correlation (r=-0.086) (Figure 4).

Fecal miRNA expression was not different according to locations of colorectal adenomas (Table 6). Fecal miRNA expression was not different between advanced adenoma group and non-advanced adenoma (Table 7).

Fig. 4 Correlation of fecal miRNA expression and the number of adenomas in the adenoma group

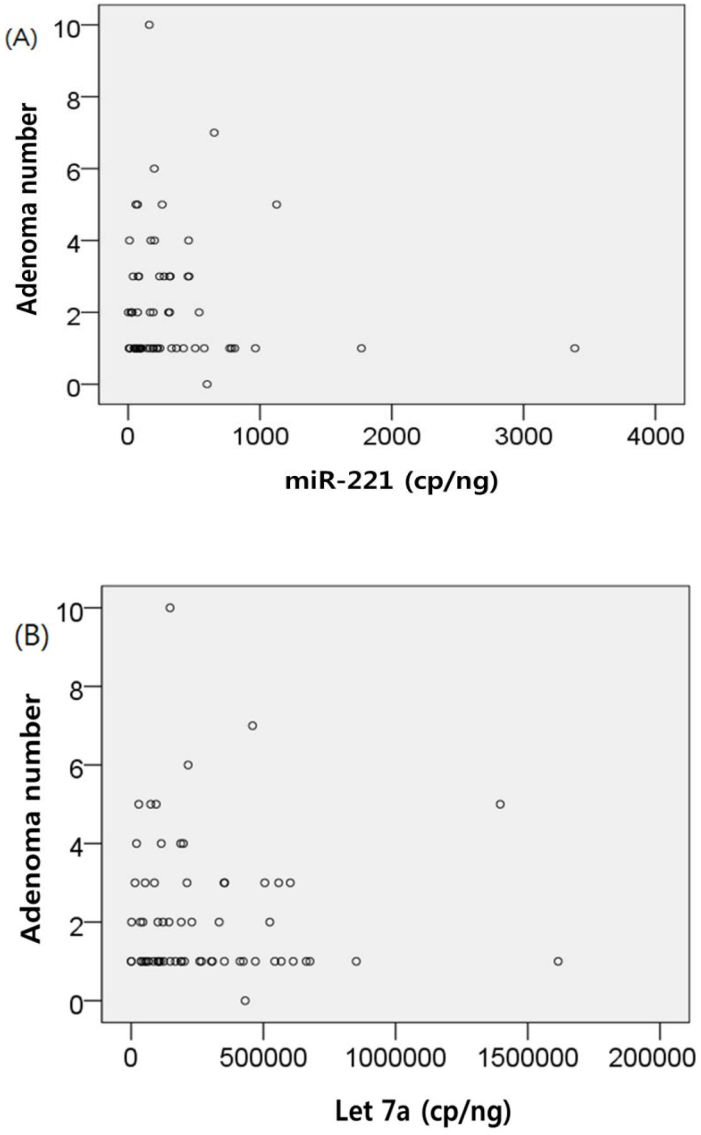


Table 7. Fecal miRNA expression according to the location and advancement of adenomas.

Fecal miRNA	Rectosigmoid(RS) (n=18)	Proximal to RS (n=17)	P-value

miR 221	228.9±275.2	189.9±163.6	0.092
Let-7a	272701.2±2.5x10^5	232805±2.8x10^5	0.066
	Advanced adenoma (AA) (n=4)	**Non AA(n=31)**	
miR 221	279.4±158.5	201.4±235.3	0.427
Let-7a	298400.6±2.1x10^5	247506.7±2.2x10^5	0.669

Values are expressed as mean ± standard deviation,

Discussion

In the present study, we showed increased expression of fecal miR-221 and Let-7a in patients with colorectal neoplasia including adenoma and colorectal cancer. The sensitivity of combined use of fecal miR-221 > 156.2 cp/ng, Let-7a > 264434.5 cp/ng, and age > 61 was 79.4% for the screening of colorectal neoplasia, which was higher than 34.9% by FIT. Based on these findings, we suggest that fecal miR-221 and Let-7a can be used as new fecal screening biomarkers for CRC.

Although there are multiple options for CRC screening such as FOBT, stool DNA test, CT colonography, and colonoscopy, need for new modalities still exists because current CRC screening tests do not satisfy all the features required for ideal CRC screening. For mass screening for CRC, the screening test has to fulfill the good accuracy, less invasiveness, appropriate test interval, and low cost. Annual and/or biennial FOBT, especially, FIT is less expensive, less invasive, and convenient. In addition, FIT has high sensitivity (50-87%) and specificity (92- 95%) for CRC.[32,33] However, the sensitivity of FIT for colorectal adenoma is unsatisfactory and the sensitivity for adenomas over 10 mm in diameter was under 50%, which implies limited usefulness in the purpose of early detection of colorectal neoplasm.[32] Stool DNA test combined with FIT has been recently approved by the Food and Drug Administration for CRC screening.[34,35] This multitarget stool DNA test showed higher

sensitivity for CRC than FIT (92.3% vs. 73.8%). However, its specificity was lower than that of FIT (86.6% vs. 94.9%).[34] Furthermore, the cost for stool DNA test is more expensive than FIT. Although CT colonography showed similar performance to colonoscopy in the detection of colorectal neoplasia, it has the risk of radiation hazard if repeated, and the mortality reduction from CRC by CT colonography is unclear.[36] In general, colonoscopy is considered the best modality for CRC screening because it not only detects colorectal neoplasia but also can remove most colorectal adenomas and some early cancers during the examination. In addition, colonoscopy showed about 68% mortality reduction from CRC.[37] Nonetheless, colonoscopy is invasive, expensive, and cumbersome, which leads to poor adherence to colonoscopy screening. Moreover, although high-quality colonoscopy is essential for the effective mass screening of CRC, qualified colonoscopists are not sufficient in many regions.

Till now, many studies reported the possibility of miRNAs as candidate biomarkers for colorectal neoplasia including CRCs. Kanaan et al. reported several plasma miRNAs such as miR-431, miR-15b, and miR-139-3p could distinguish colonic adenoma from healthy mucosae.[38] Yong et al. identified a panel of 3 miRNAs (miR-193-3p, miR-23a, and miR-338-5p) from tissue samples had a significant correlation with CRC.[39] Other studies also showed different miRNAs related to CRCs. Interestingly, in most of these studies, these miRNAs have been obtained from CRC tissues or blood samples. Investigations of miRNAs in fecal samples were lacking. In our present study, we showed fecal miR-221, and Let-7a could be useful biomarkers for screening of colorectal neoplasia. Fecal miRNA may have several strengths as a biomarker. First, miRNA has been considered to be stable and can be detected technically easily and effectively compared to mRNA which lacks stability.[40,41] Second, because colonocytes including miRNAs are exfoliated continuously into the colonic lumen, fecal miRNAs can be present relatively constantly, which makes the probability of their detection high. Third, fecal sampling is completely non-invasive. Putting together these advantages of fecal miRNA tests and the positive results in our present study, we suggest fecal miR-221 and Let-7a could be used as a useful screening modality in CRC screening.

Further, large scale, confirmative studies are warranted.

Overexpression of miR-221 has been shown in a variety of cancers such as pancreatic cancer,[42] papillary thyroid cancer,[43] glioblastoma,[44,45] breast cancer,[46] melanoma,[47] and prostate cancer.[48] MiR-221 has been reported to be associated with carcinogenesis and chemotherapy resistance through the regulation of the cell cycle. The target molecules of miR-221 were suggested to be c-KIT, p27, and p57.[47] The association between CRC and miR-221 was also reported in a recent study which showed the high level of circulating plasma miR-221 was an independent poor prognostic factor in patients with CRC. The authors proposed that miR-221 might be related to the prognosis of CRC through its association with p27 expression which is a regulator of cell cycle progression via the G1/S transition.[49] Let-7 was discovered in the early 1990s. The Let-7 family consists of 12 genes encoding 9 distinct miRNAs (Let-7a to Let-7i).[50] Several studies showed the relation between epigenetic activation and/or overexpression of Let-7a and CRC in the investigation of CRC-derived cells and human CRC tissues.[51] In our study, we demonstrated the increase of miR-221 and Let-7a in the stool of CRC patients, which suggested the possible usefulness of fecal miR-221 and Let-7a as CRC screening biomarkers.

In our analysis, fecal miR-221 and Let-7a showed not only simply different level of expression between the neoplastic group and healthy control (Table 5) but also linearly increasing pattern from the healthy control through the adenoma group to the CRC group (Fig. 1 (E) and (F)). In comparison, the expression level was not significantly different according to the stages of CRCs. These findings suggest that expression of miR-221 and Let-7a may increase during the early period of colorectal adenoma-carcinoma pathway and may be stabilized at the late stage of CRC carcinogenesis. However, this hypothesis should be investigated further in future studies because several recent studies showed a different level of miRNA expression between different CRC stages. Wang et al. reported the expression of miR-31 in the CRC tissue was associated with nodal metastasis.[52] Arndt et al. reported the tissue expression of miR-31, miR-7, miR-99b, miR-378, miR-133a, and miR-125a showed significant difference between early and late stages of CRC.[27]

We found the performance of single fecal miRNA for the screening of colorectal neoplasia, especially for screening of colorectal adenomas, was not satisfactorily high. Therefore we analyzed and designed a combination model for high performance of fecal miRNAs as a screening biomarker for colorectal neoplasia. The AUC of the combination of age, fecal miR-221, and fecal Let-7a was 0.839, which is acceptably good performance as an initial screening modality. Because the combination of several markers may increase the cost, future studies should investigate the usefulness of further combination with clinical risk factors which can be added without additional cost.[53]

Our study has some limitations. First, the number of enrolled participants was small, and the conclusion of this study should be confirmed by large-scale studies. Second, it is a single center study in which the study population was only Asian. Considering the possible different genetic predispositions and different fecal miRNA signatures between different ethnicities, multi-national multi-center studies are warranted. Third, we did not conduct functional studies about miR-221 and Let-7a in detail which could support the relation between CRC and these miRNAs. Finally, we did not investigate the expression of miR-221 and Let-7a in CRC and adenoma tissues. If we had investigated the correlation of miR-221 and Let-7a between the tissues and feces, we could have confirmed the direct association between these miRNAs and CRC and the clear role of fecal miRNAs in CRC screening. Despite these limitations, we believe our study was meaningful in the viewpoint that we showed the usefulness of fecal miRNAs as a non-invasive CRC screening modality.

Conclusion

In conclusion, fecal miR-221 and Let-7a may be useful non-invasive biomarkers for CRC screening. The combination of these two fecal miRNAs with clinical risk factors such as age could predict the presence of colorectal neoplasia with high confidence. Further larger studies are warranted for determination of best cut-off values of these fecal miRNAs and best model which combines several biomarkers and clinical risk factors.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA: a cancer journal for clinicians*. 2011;61(2):69-90.

2. Ahmed FE. Gene-gene, gene-environment & multiple interactions in colorectal cancer. *Journal of Environmental Science and Health Part C*. 2006;24(1):1-101.

3. World Health Organization. *Basic documents.* World Health Organization; 2014.

4. Center MM, Jemal A, Smith RA, Ward E. Worldwide variations in colorectal cancer. *CA: a cancer journal for clinicians*. 2009;59(6):366-378.

5. Center MM, Jemal A, Ward E. International trends in colorectal cancer incidence rates. *Cancer Epidemiol Biomarkers Prev*. 2009;18(6):1688-1694.

6. de Kok IM, Wong CS, Chia KS, et al. Gender differences in the trend of colorectal cancer incidence in singapore, 1968–2002. *Int J Colorectal Dis*. 2008;23(5):461-467.

7. García-Álvarez A, Serra-Majem L, Ribas-Barba L, et al. Obesity and overweight trends in catalonia, spain (1992–2003): Gender and socio-economic determinants. *Public Health Nutr*. 2007;10(11A):1368-1378.

8. Edwards BK, Ward E, Kohler BA, et al. Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. *Cancer*. 2010;116(3):544-573.

9. Mitry E, Bouvier A, Esteve J, Faivre J. Benefit of operative mortality reduction on colorectal cancer survival. *Br J Surg*. 2002;89(12):1557-1562.

10. Walsh JM, Terdiman JP. Colorectal cancer screening: Scientific review. *JAMA*. 2003;289(10):1288-1296.

11. Allison JE, Sakoda LC, Levin TR, et al. Screening for colorectal neoplasms with new fecal occult blood tests: Update on performance characteristics. *J Natl Cancer Inst*. 2007;99(19):1462-1470.

12. Benson VS, Atkin WS, Green J, et al. Toward standardizing and reporting colorectal cancer screening indicators on an international level: The international colorectal cancer screening network. *International journal of cancer*. 2012;130(12):2961-2973.

13. Sung JJ, Ng SC, Chan FK, et al. An updated asia pacific consensus recommendations on colorectal cancer screening. *Gut*. 2015;64(1):121-132.

14. Health Quality Ontario. Fecal occult blood test for colorectal cancer screening: An evidence-based analysis. *Ont Health Technol Assess Ser*. 2009;9(10):1-40.

15. Lee JK, Liles EG, Bent S, Levin TR, Corley DA. Accuracy of fecal immunochemical tests for colorectal CancerSystematic review and meta-analysis. *Ann Intern Med*. 2014;160(3):171-181.

16. Quintero E, Castells A, Bujanda L, et al. Colonoscopy versus fecal immunochemical testing in colorectal-cancer screening. *N Engl J Med*. 2012;366(8):697-706.

17. Imperiale TF, Ransohoff DF, Itzkowitz SH, et al. Multitarget stool DNA testing for colorectal-cancer screening. *N Engl J Med*. 2014;370(14):1287-1297.

18. Ahmed FE. Role of miRNA in carcinogenesis and biomarker selection: A methodological view. *Expert Rev Mol Diagn*. 2007;7(5):569-603.

19. Kent O, Mendell J. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. *Oncogene*. 2006;25(46):6188-6196.

20. Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM. MicroRNA expression and function in cancer. *Trends Mol Med*. 2006;12(12):580-587.

21. Monzo M, Navarro A, Bandres E, et al. Overlapping expression of microRNAs in human embryonic colon and colorectal cancer. *Cell Res*. 2008;18(8):823-833.

22. Ahmed FE, Ahmed NC, Vos PW, et al. Diagnostic microRNA markers to screen for sporadic human colon cancer in stool: I. proof of principle. *Cancer Genomics Proteomics*. 2013;10(3):93-113.

23. Earle JS, Luthra R, Romans A, et al. Association of microRNA expression with microsatellite instability status in colorectal adenocarcinoma. *The Journal of Molecular Diagnostics*. 2010;12(4):433-440.

24. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*. 2006;103(7):2257-2261.

25. Sarver AL, French AJ, Borralho PM, et al. Human colon cancer profiles show differential microRNA expression depending on mismatch repair status and are characteristic of undifferentiated proliferative states. *BMC Cancer*. 2009;9:401-2407-9-401.

26. Lanza G, Ferracin M, Gafa R, et al. mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. *Mol Cancer*. 2007;6:54.

27. Arndt GM, Dossey L, Cullen LM, et al. Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. *BMC Cancer*. 2009;9:374-2407-9-374.

28. Wang YX, Zhang XY, Zhang BF, Yang CQ, Chen XM, Gao HJ. Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis. *J Dig Dis*. 2010;11(1):50-54.

29. Schepeler T, Reinert JT, Ostenfeld MS, et al. Diagnostic and prognostic microRNAs in stage II colon cancer. *Cancer Res*. 2008;68(15):6416-6424.

30. Sun H, Chen X, Ji H, et al. miR-494 is an independent prognostic factor and promotes cell migration and invasion in colorectal cancer by directly targeting PTEN. *Int J Oncol*. 2014;45(6):2486-2494.

31. Diosdado B, van de Wiel MA, Terhaar Sive Droste JS, et al. MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. *Br J Cancer*. 2009;101(4):707-714.

32. Zauber AG, Lansdorp-Vogelaar I, Knudsen AB, Wilschut J, van Ballegooijen M, Kuntz KM. Evaluating test strategies for colorectal cancer screening: A decision analysis for the US preventive services task ForceTest strategies for colorectal cancer screening. *Ann Intern Med*. 2008;149(9):659-669.

33. Lee JK, Liles EG, Bent S, Levin TR, Corley DA. Accuracy of fecal immunochemical tests for colorectal CancerSystematic review and meta-analysis. *Ann Intern Med*. 2014;160(3):171-181.

34. Imperiale TF, Ransohoff DF, Itzkowitz SH, et al. Multitarget stool DNA testing for colorectal-cancer screening. *N Engl J Med*. 2014;370(14):1287-1297.

35. Johnson DH, Kisiel JB, Burger KN, et al. Multitarget stool DNA test: Clinical performance and impact on yield and quality of colonoscopy for colorectal cancer screening. *Gastrointest Endosc*. 2017;85(3):657-665. e1.

36. Inadomi JM. Screening for colorectal neoplasia. *N Engl J Med*. 2017;376(2):149-156.

37. Nishihara R, Wu K, Lochhead P, et al. Long-term colorectal-cancer incidence and mortality after lower endoscopy. *N Engl J Med*. 2013;369(12):1095-1105.

38. Kanaan Z, Roberts H, Eichenberger MR, et al. A plasma microRNA panel for detection of colorectal adenomas: A step toward more precise screening for colorectal cancer. *Ann Surg*. 2013;258(3):400-408.

39. Yong FL, Law CW, Wang CW. Potentiality of a triple microRNA classifier: miR-193a-3p, miR-23a and miR-338-5p for early detection of colorectal cancer. *BMC Cancer*. 2013;13(1):280.

40. Wu CW, Ng SS, Dong YJ, et al. Detection of miR-92a and miR-21 in stool samples as potential screening biomarkers for colorectal cancer and polyps. *Gut*. 2012;61(5):739-745.

41. Yu Z, Hecht NB. The DNA/RNA-Binding protein, translin, binds microRNA122a and increases its in vivo stability. *J Androl*. 2008;29(5):572-579.

42. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *International journal of cancer*. 2007;120(5):1046-1054.

43. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A*. 2005;102(52):19075-19080.

44. Ciafre S, Galardi S, Mangiola A, et al. Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem Biophys Res Commun*. 2005;334(4):1351-1358.

45. le Sage C, Nagel R, Egan DA, et al. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *EMBO J*. 2007;26(15):3699-3708.

46. Miller TE, Ghoshal K, Ramaswamy B, et al. MicroRNA-221/222 confers tamoxifen resistance in breast cancer by targeting p27Kip1. *J Biol Chem*. 2008;283(44):29897-29903.

47. Felicetti F, Errico MC, Bottero L, et al. The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. *Cancer Res*. 2008;68(8):2745-2754.

48. Galardi S, Mercatelli N, Giorda E, et al. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. *J Biol Chem*. 2007;282(32):23716-23724.

49. Li W, Sanki A, Karim RZ, et al. The role of cell cycle regulatory proteins in the pathogenesis of melanoma. *Pathology*. 2006;38(4):287-301.

50. Johnson SM, Lin S, Slack FJ. The time of appearance of the C. elegans let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. *Dev Biol*. 2003;259(2):364-379.

51. Torrisani J, Parmentier L, Buscail L, Cordelier P. Enjoy the silence: The story of let-7 microRNA and cancer. *Curr Genomics*. 2007;8(4):229-233.

52. Wang CJ, Zhou ZG, Wang L, et al. Clinicopathological significance of microRNA-31, -143 and -145 expression in colorectal cancer. *Dis Markers*. 2009;26(1):27-34.

53. Yeoh KG, Ho KY, Chiu HM, et al. The asia-pacific colorectal screening score: A validated tool that stratifies risk for colorectal advanced neoplasia in asymptomatic asian subjects. *Gut*. 2011;60(9):1236-1241.

한글 요약

연구 배경 및 목적: 대변 마이크로 RNA (miRNA) 분석은 대장암 선별검진을 위한 비침습성 바이오마커 검사의 하나로 기대되어왔다. 이 연구에서는 대장선종 및 대장암을 포함하는 대장종양 환자에서 발현 변화를 보이는 대변 miRNA를 찾아내고, 효율적 대장암 선별검진을 위한 다양한 대변 miRNA의 조합을 찾고자 하였다.

방법: 대장암 환자 30명, 대장선종 환자 35명, 건강 대조군 32명으로부터 대변 검체를 채취하여 13 가지 miRNA (miR-17, -21, -27a, -92, -106a, -145, -155, -181b, -199a, -200c, -221, 494, Let-7a) 발현을 정량적 실시간 PCR 방법으로 측정, 비교 분석하였다.

결과: 단변량 분석에서 연령, 대변 miR-21, -145, -155, -199a, -221, -494 및 Let-7a가 대장종양군과 건강 대조군 사이에 유의한 차이를 보였다. 다변량 분석에서 연령 (Odds ratio (OR) 21.1, 95% 신뢰구간 (CI) 2.2-151.7, p=0.005), miR-221 (OR 4.1, 95% CI 1.1-16.5, p=0.037) 및 Let-7a (OR 11.7, 95% CI 1.3-103.6, p=0.034)가 대장종양의 독립적 위험인자로 확인되었다. 연령, 대변 miR-221 및 Let-7a의 area under the curve (AUC)는 각각 0.656 (95% CI, 0.552-0.750), 0.742 (95% CI, 0.643-0.827) 및 0.645 (95% CI, 0.540-0.740)이었다. 연령, 대변 miR-221 및 Let-7a 조합의 AUC는 0.839 (95% CI, 0.749-0.907)로 각각의 AUC에 비해 우수하였고, 민감도(79.4%)와 특이도(86.7%)도 높았다.

결론: 대변 miR-221 및 Let-7a는 대장암 선별검진을 위한 유용한 비침습성 바이오마커였으며, 이들을 연령과 조합하여 분석하면 대장종양 진단의 신뢰도를 높일 수 있을 것으로 생각된다.