Down-regulation of fecal miR-143 and miR-145 as potential markers for colorectal cancer

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ABSTRACT

الأهداف: تحديد 4 أنواع من ميكرورنا في عينات البراز لمرضى سرطان القولون والمستقيم وذلك من أجل التحقق ما إذا يمكن اعتبار ميكرورنا من العلامات الحيوية الدالة على هذا السرطان أثناء الكشف عنه وعلاجه.

الطريقة: أُجريت هذه الدراسة الاسترجاعية في قسم جراحة المستقيم والقولون، مستشفى شينهوا، جامعة شانغهاي جيوتونغ للطب، الصين وذلك خلال الفترة من سبتمبر 2009م إلى مارس 2011م. لقد قمنا بالتحري عن 4 أنواع من ميكرورنا (ميكرورنا–143، وميكرورنا–145، وميكرورنا–21، وميكرورنا– 106a) في عينات البراز للمرض المصابين بسرطان القولون والمستقيم البالغ عددهم 38 مريض، والأفراد الأصحاء البالغ عددهم 13 شخص. لقد قمنا باستخلاص إجمالي الرنا من عينات البراز باستخدام EZNA™ stool RNA kit R6828-01، فيما قمنا بحساب كمية ميكرورنا بواسطة اختبار ميكرو رنا تاك مان، و TaqMan® Gene Expression Master Mix.

النتائج: أشارت نتائج الدراسة إلى أن مستويات ميكرورنا143–، وميكرورنا–145 في عينات البراز للمرضى المصابين بسرطان القولون والبنكرياس كانت أقل من المستويات التي لاحظناها لدى مجموعة الأفراد الأصحاء–. بالمقابل لم يكن هناك اختلاف كبير من الناحية الإحصائية في مستويات ميكرورنا21–، وميكرورنا– 106a بين مجموعة المصابين بسرطان القولون والبنكرياس ومجموعة الأفراد الأصحاء (p>0.05).

خاتمة: أثبتت هذه الدراسة بأن تحليل مستويات ميكرورنا قد يكون طريقة معتمدة أثناء تشخيص سرطان البنكرياس والقولون والكشف عنه، حيث يمكن اعتبار انخفاض مستويات ميكرورنا143–، وميكرورنا–145 من المؤشرات المشيرة للتشخيص بهذا المرض.

Objectives: To detect 4 MicroRNA (miRNA) in the stool samples of colorectal cancer (CRC) patients to determine whether these miRNAs could be biomarkers in CRC screening or treatment.

Methods: A retrospective comparison study was carried out in the Department of Colorectal Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China from September 2009 to March 2011. We detected 4 miRNAs (miR-143, miR-145, miR-21, and miR-106a) in the stool samples of 38 CRC patients and 13 healthy individuals. Total RNA from the stool samples was extracted using the EZNA™ stool RNA kit R6828-01. The miRNA quantification was carried out using TaqMan miRNA assays and the TaqMan® Gene Expression Master Mix.

Results: The expression levels of miR-143 and miR-145 in the stool of the CRC patients were lower than in those of the healthy persons (p<0.005, median of $2^{-\Delta\Delta Ct}$). No statistically significant difference was found in the expression levels of both miR-21 and miR-106a between the stool of CRC patients and those of the healthy persons (p>0.05).

Conclusion: The detection of fecal miRNAs is a potential method for CRC diagnosis or screening. Particularly, the down-regulation of fecal miR-143 and miR-145 could be a potential marker for CRC.

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Colorectal cancer (CRC) is the third most frequently diagnosed cancer in the world.[1,2] In the United States, 102,900 new cases of colon cancer and 39,670 new cases of rectal cancer were estimated to occur in 2010. In the same year, 51,370 people were predicted to die of CRC.[1,2] In China, morbidity from CRC increased by 19.1% in men and 17.7% in women (an increase of 120,000 cases per year).[3] The CRC mortality rates can be decreased by early diagnosis, CRC screening, and cancer prevention. However, the present CRC screening techniques (colonoscopy, fecal occult test, and carcinoembryonic antigen test) are limited by wound formation and uncertain or delayed results.[4-6] MicroRNAs (miRNAs) are small, noncoding sequences that are 17-27 nucleotides long. They regulate cell processes in ~30% of mammalian genes by imperfectly binding to the 3' untranslated region of target messenger RNAs (mRNAs). This binding prevents protein accumulation either by repressing transcription or inducing mRNA degradation.[7-11] The miRNA functions include cell development and apoptosis, as well as cell cycle progression. Therefore, specific miRNAs are thought to be critical in oncogenesis.[8-11] An increasing number of studies suggest the potential of miRNA expression analysis in tumor tissues, serum, plasma, or urine as a promising approach for tumor detection.[11-15] The expression levels of miR-143 and miR-145 were found to be severely down-regulated,[16-18] whereas those of miR-21 and miR-106a[19,20] were up-regulated in most human cancers, particularly in CRCs. However, only limited data exist on the usefulness of fecal miRNAs for CRC screening.[21,22] Ahmed et al[21] indicated that the expression of 7 miRNAs (miR-21, miR-106a, miR-96, miR-203, miR-20a, miR-326, and miR-92) increased in the stool (and also in the tissue) of CRC patients. On the other hand, the expression of 7 other miRNAs (miR-320, miR-126, miR-484-5p, miR-143, miR-145, miR-16, and miR-125b) decreased in both the stool and tissue of CRC patients. These findings seemed to be more pronounced in late Dukes stages. Link et al[22] studied 29 patients and indicated that the expression of miR-21 and miR-106a was higher in patients with adenomas and CRC than in individuals free of colorectal neoplasia. The aim of the present study was to detect 4 miRNAs (miR-143, miR-145, miR-21, and miR-106a) in the stool samples of CRC patients and healthy individuals to determine whether these miRNAs could be biomarkers in CRC screening or therapy.

Methods. *Clinical and healthy stool samples.* A retrospective comparison study was carried out in the Department of Colorectal Surgery, Xinhua Hospital, Shanghai Jiaotong University, School of Medicine, China, from September 2009 to March 2011. A total of 38 CRC stool specimens were obtained from 6 patients with ascending colon cancer, 2 with transverse colon cancer, 13 with descending or sigmoid colon cancer, and 17 with rectal cancer. These samples were randomly selected according to a table of random digits from a consecutive collection of 220 CRC fecal samples previously collected from September 2009 to June 2010 at the Department of Colorectal Surgery, Xinhua Hospital, Shanghai, Jiaotong University School of Medicine, Shanghai, China. The stool samples were kept at -80˚C. All patients provided written informed consent, and the study was approved by the ethics committee of Xinhua Hospital. The clinical and pathological data of the patients are presented in **Table 1**.

Table 1 - Clinical and pathological characteristics of 38 colorectal cancer patients presenting to the Department of Colorectal Surgery, Xinhua Hospital, Shanghai Jiaotong University, School of Medicine, China.

Items	Cases (n)
Gender	
Male	16
Female	22
Age	65.6±13.1
Location of cancer	
Ascending colon	6
Transverse colon	2
Descending/sigmoid colon	13
Rectum	17
Pathologic type	
Tubular adenocarcinoma	25
Tubular papillary adenocarcinoma	4
Villous adenocarcinoma	2
Mucinous adenocarcinoma	2
Uncertain adenocarcinoma	5
Tumor size	
≥5cm	12
3-5cm	14
≤3cm	12
Infiltration depth	
T1	0
T2	6
T3	18
T4	14
*Lymphatic metastasis**	
N0	25
N1	9
N2	4
*Distant metastasis**	
M0	31
M1	7
*Tumor, node, metastasis (TNM) staging**	
I	6
II	14
III	11
IV	7
Total	**38**

*American Joint Committee on colorectal cancer TNM staging

Stool samples were collected from 13 healthy individuals (8 men and 5 women; mean age=57.7±9.3 years). The protocol was approved by the ethics committee of the Xinhua Hospital, Shanghai, China. A written informed consent was obtained from each volunteer patient. The stool samples were stored at -80°C after collection, and RNA isolation was carried out within 2 weeks. This study was carried out according to principles of Helsinki Declaration, and the rights of patients and healthy volunteers were protected.

Isolation of total RNA from stool samples. Total RNA (including miRNAs) from the stool samples was extracted using the EZNA.™ stool RNA kit R6828-01 (Omega Bio-Tek, Norcross, USA) according to the instructions of the manufacturer with some modifications. Approximately 200 mg of stool sample was mixed with 200 mg of glass beads in a 2 mL centrifuge tube, which was placed on ice. Approximately 500 µL of buffer RPL was added and the tube was vortexed to mix well. Approximately 500 µL of water-saturated phenol was added and the tube was again vortexed to mix well. Incubation at 65°C for 10 minutes followed. Approximately 500 µL of chloroform was added and the sample was thoroughly by vortexing the tube for 30 seconds. The sample was incubated on ice for 5 minutes, and was centrifuged at full speed (<13 000×g) in a microcentrifuge for 5 minutes. Careful aspiration of 500 µL of supernatant to a new 2 mL microfuge tube (not supplied) was performed to ensure that the pellet was not disturbed and no debris was transferred. Approximately 500 µL of buffer RB and 500 µL of absolute ethanol were added, and the tube was vortexed to mix well. Approximately 750 µL of the sample from the previous step was applied to a HiBind® (Omega Bio-Tek, Norcross, Georgia, USA) RNA column assembled in a 2 mL collecting tube (supplied). Centrifugation at full speed (>10,000 × g) for 30 seconds at room temperature followed. The flow-through liquid was discarded and the collection tube was reused in the next step. The previous step was repeated to load the remaining samples to the column. After centrifugation as above, the flow-through and the collection tube were discarded. Approximately 500 µL of Rideal Walker Coefficient wash buffer was pipetted into the column. After re-centrifugation as above, the flow-through was discarded and the collection tube was reused in the next step. The column was placed in a new collection tube (provided). Approximately 500 µL of RWB wash buffer (prediluted with absolute ethanol) was pipetted into the column. After centrifugation at 10,000 × g for 30 seconds, the flow-through was discarded and the collection tube was reused. Another 500 µL of RWB wash buffer was added to the column and the mixture was centrifuged at 10,000×g for one minute. The flow-through was discarded and the collection tube was reused. The HiBind™ RNA column was placed in the same 2 mL collection tube, which was centrifuged at full speed (≥12,000×g) for 2 minutes to completely dry the membrane. For the RNA elution, the HiBind® RNA column was placed in a 1.5 mL RNase-free microtube. Approximately 30-50 µL of DEPC water was added directly onto the center of the HiBind™ RNA column silica membrane. The column was allowed to sit at room temperature for 2 minutes and was centrifuged at 10 000×g for one minute to elute the RNA. The concentration of the extracted RNA from the stool samples was measured using NanoDrop 2000 (Wilmington, Delaware, USA).

MiRNA quantification by real-time reverse transcriptase polymerase chain reaction (RT-PCR). The miRNAs were quantified using TaqMan miRNA Assays (Applied Biosystems, Foster, California, USA) and the TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster, California, USA). The sequences of miR-143, miR-145, miR-21, miR-106a, and miR-16 (internal control)[22] for the RT-PCR assays are listed in **Table 2**. The primer sequences used were from Applied Biosystems.

The first step of the protocol involved preparing a reverse transcriptase (RT) master mix. Each RT mix required the following components and volumes (which were scaled up to suit the required number of reverse transcriptions): 0.15 µL of dNTPs, one µL of RT, 1.5 µL of RT buffer, 0.19 µL of RNase inhibitor, and 4.16 µL of nuclease-free water. The master mix

Table 2 - Sequences of miR-143, -145, -21, -106a and -16 used in the present study.

MicroRNAs	Sequence (5'-3')	Kit part number*	Lot number*
miR-143	UGAGAUGAAGCACUGUAGCUC	4395360	1007082
miR-145	GUCCAGUUUUCCCAGGAAUCCCU	4395389	1007110
miR-21	UAGCUUAUCAGACUGAUGUUGA	4373090	1007626
miR-106a	AAAAGUGCUUACAGUGCAGGUAG	4395280	1006056
miR-16	UAGCAGCACGUAAAUAUUGGCG	4373121	1007702

* TaqMan miRNA assays (Applied Biosystems, Foster, California, USA)

was gently mixed and centrifuged to bring the mix to the tube bottom, and was then placed on ice. Each RT reaction was prepared at a ratio of 7 uL of RT master mix to 5 uL of total RNA. Finally, the mix was subjected to 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and was held at 4°C.

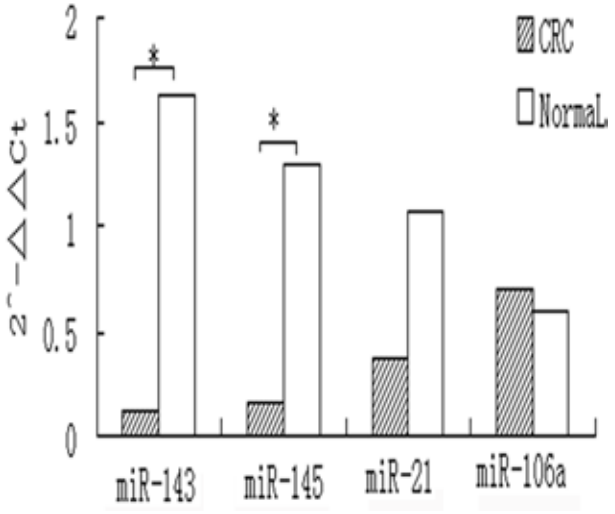

Figure 1 - Expression levels of miR-143, miR-145, miR-21, and miR-106a in the stool of colorectal cancer (CRC) patients (n=38) compared with healthy persons (normal, n=13) (*p<0.05). The MiR-143, and -145 expression in CRC patients were lower than those in healthy persons (median of $2^{-\Delta\Delta Ct}$, 0.1199 versus 1.6305, p=0.004, and 0.1507 versus 1.3048, p=0.001). However, there is no statistical difference between the CRC patients and healthy controls in MiR-21, and -106a expressions.

For the real-time PCR step, amplification was carried out on an applied Biosystems Steponeplus real-time PCR system. The 20 µL PCR reaction included 1.00 µL of TaqMan small RNA assay reagent (20×), 1.33 µL of RT reaction product, 10.00 µL of TaqMan Universal PCR Master Mix II (2×), and 7.67 µL of nuclease-free water. The reactions were incubated in a 96-well optical plate at 95˚C for 10 minutes, followed by 55 cycles at 95˚C for 15 seconds and 60˚C for one minute. The real-time PCRs for each assay was run in triplicate. Small RNA detection, internal control detection (miR-16), and blank control detection were included. The data were analyzed, and $2^{-\Delta\Delta Ct}$ was obtained.

Statistical analysis. Normal distribution data were described by mean ± standard deviation (SD), and were analyzed using one-way analysis of variance. Non-normal distribution data were described by the median and range, which were analyzed through the rank-sum test. All statistical analyses were performed using Statistical Package for the Social Sciences 18.0. A p-value <0.05 (95% confidence interval) was considered significant for overall analyses, and p<0.005 (95% confidence interval) was considered significant for multiple comparisons in the rank-sum test.

Results. A total of 38 CRC stool specimens were detected (**Table 1**). The specimens were from patients at different cancer stages (6 at stage I, 14 at stage II, 11 at stage III, and 7 at stage IV) (AJCC TNM staging).[23]

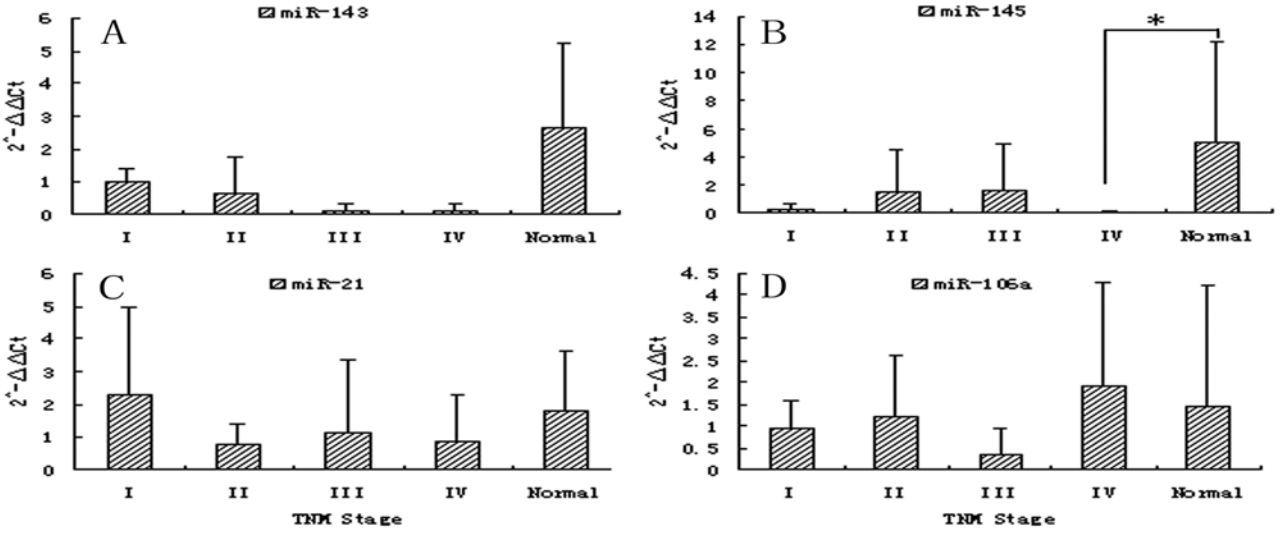

Figure 2 - Expression levels of **a**) miR-143, **b**) miR-145, **c**) miR-21, and **d**) miR-106a in the stool of patients with CRC TNM stage (American Joint Committee on colorectal Cancer TNM Staging)[23] I (n=6), II (n=14), III (n=11), and IV (n=7) and of healthy persons (normal, n=13). *p=0.001. The MiR-145 expression in stage IV CRC patients was statistically different from that in healthy persons (p=0.001). There was no statistical difference among other multiple comparisons. CRC - colorectal cancer

These cancerous specimens were compared with stool samples from 13 healthy individuals.

Expression of miR-143 and miR-145 in the stool of CRC patients. The expression levels of miR-143 and miR-145 in the stool of CRC patients were lower than in those of healthy persons (0.1199 versus 1.6305, p=0.004, median of $2^{-\Delta\Delta Ct}$; and 0.1507 versus 1.3048, p=0.001, median of $2^{-\Delta\Delta Ct}$) (**Figure 1**).

The expression levels of miR-143 in the stool of stage I-IV CRC patients and of healthy persons were 1.0265±0.3772, 0.6665±1.0706, 0.1228±0.2059, 0.1182±0.2029, and 2.6165±2.6259, (mean of $2^{-\Delta\Delta Ct}$). There was no statistical difference among them (p=0.005–0.699 ≥ 0.005; **Figure 2a**).

The expression levels of miR-145 in the stool of stage I-IV CRC patients and healthy persons were 0.3055±0.3531, 1.4938±3.0440, 1.6199±3.3255, 0.1085±0.0606, and 4.9947±7.1707, (mean of $2^{-\Delta\Delta Ct}$). miR-145 expression in stage IV CRC patients was statistically different from that in healthy persons (p=0.001, **Figure 2b**).

Expression levels of miR-21 and miR-106a in the stool of CRC patients. No statistically significant difference was found in the expression levels of both miR-21 and miR-106a in the stool between CRC patients and healthy persons (0.3754 versus 1.0735, p=0.051, median of $2^{-\Delta\Delta Ct}$, and 0.7042 versus 0.5955, p=0.763, median of $2^{-\Delta\Delta Ct}$) (**Figure 1**). Specifically, the expression levels of miR-21 in the stool of stage I-IV CRC patients and of normal persons were 2.3302±2.6651, 0.7738±0.6089, 1.1243±2.2403, 0.8336±1.4396, and 1.7689±1.8932 (mean of $2^{-\Delta\Delta Ct}$). For miR-106a, the expression levels were 0.9540±0.6154, 1.2312±1.3872, 0.3799±0.5543, 1.9198±2.3617, and 1.4574±2.7575 (mean of $2^{-\Delta\Delta Ct}$) (**Figures 2c & 2d**).

Discussion. Numerous reports have indicated the down-regulation of miR-143 and miR-145 in tumor tissues, serum, or plasma, as well as the up-regulation of miR-21 and miR-106a.[16–20] In the current study, the expression levels of miR-143 and miR-145 in the stool of CRC patients (n=38) were lower than in those of healthy persons (n=13). However, the statistical difference between these expression levels in CRC patients and healthy persons was not significant.

For multiple comparisons, previous reports[21,24,25] have revealed that changes in the miRNAs of CRC patients seemed to be more pronounced in late stages. In the current study, miR-145 expression significantly differed only between stage IV CRC patients and healthy persons.

Two similar studies[21,22] on CRC fecal miRNAs have been previously reported. Ahmed et al,[21] selected 5 healthy persons and 15 patients with various stages of colon adenocarcinoma (Dukes stages 0–3). Their results indicated that miR-21 and miR-106a expression increased in the stool of CRC patients, whereas miR-143 and miR-145 expression decreased. These findings seemed to be more pronounced in late Dukes stages. However, the p-value was not indicated. Link et al[22] selected freshly collected stool samples from 8 healthy volunteers, as well as 29 samples collected via fecal occult blood testing from subjects with normal colonoscopies (n=10), colon adenomas (n=9), and CRCs (n=10). The miRNA expression analyses were performed using TaqMan quantitative RT-PCR for a subset of miRNAs. The results indicated higher expression levels of miR-21 and miR-106a in patients with adenomas and CRCs than in individuals free of colorectal neoplasia. However, the expression levels of both miRNAs decreased at higher tumor stages.

The discrepancies between the results of the present and previous studies may be attributed to the following reasons. First, fecal samples contain various impurities, unlike tissues, serum, or plasma. Hence, although an internal control was used, the SD values were larger and many p-values were not significant in the present study. These results mean that more precise isolation and quantification kits or methods for stool sampling are needed. Second, the sample sizes in previous studies were too small, particularly for each TNM stage. In the present study, more cases (38 CRC and 13 healthy stool samples) were detected using real-time RT-PCR. Serious statistical analyses were also performed, which conveyed the significant points of the present research. However, there are some limitations in this study. Firstly, the study was a retrospective comparison research; secondly, the sample sizes were still too small, particularly for each TNM stage; thirdly, only 4 objective microRNAs were included in the study. For these reasons, a large-scale prospective study including 300 cases and 8 objective microRNAs will be carried out in our center.

In conclusion, the detection of fecal miRNAs is a promising method for CRC diagnosis or screening. Particularly, the down-regulation of fecal miR-143 and miR-145 is a potential marker for CRC detection.

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