Identification of MicroRNA 15b-3p as a Diagnostic Marker for Early Stage of Colorectal Cancer Through Comprehensive RNA Analysis

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Objective: Early detection is the key to treatment outcome in colorectal cancer (CRC). This study aimed to identify microRNAs (miRNAs) as a useful diagnostic marker for early-stage CRC.

Materials: We selected 7 cases without lymph node metastasis or any typical risk factors from 52 stage II CRC cases. Blood sampled before and after surgery, and cancer and normal tissue samples of the selected cases were supplied. Aside from this, a public data set of miRNA expression in the serum of patients with CRC and healthy controls obtained from Gene Expression Omnibus National Center for Biotechnology Information (GSE40247) was used.

Methods: A microarray analysis was conducted using the total RNA extracted from the serum in 4 cases. The expression levels of RNAs in the bloods of remaining 3 cases and cancer and normal tissues of all the 7 cases were evaluated using real-time quantitative polymerase chain reaction (RT-qPCR). Based on the public data, the expression level of miRNAs in the serum of patients with CRC was compared with those of healthy controls (HC).

Results: miR-15b-3p and miR-339-5p were significantly decreased after the surgery (n=4, p<0.05). Higher expression in preoperative blood was verified for both miR15b-3p (p=0.040) and miR-339-5p (p=0.046) (n=3). The cancer tissue-specific higher expression was detected for miR-15b-3p (p=0.0490) but not for miR339-5p (p=0.095). In the public data, the expression levels of miR-15b-3p in stages I, II and III CRC patients were higher than those in HC (p=0.041, 0.038, and 0.014, respectively). The expression levels of miR-339-5p were not different among CRC and HC sera (p>0.05).

Conclusions: miR-15b-3p may be a useful diagnostic marker for early-stage CRC.

Key words: colorectal neoplasm, microRNA, tumor marker, early detection, blood markers

Introduction

According to the global cancer statistics GLOBOCAN 2018, colorectal cancer (CRC) is the third most common type of cancer (10.2%) and the second most frequent cause of cancer-related death (9.2%) in both sexes11. Despite recent progress in chemotherapy and other treatments (e.g., immunotherapy, targeted therapy, and endoscopic ablations) for CRC, patients with advanced-stage CRC are associated with poor prognoses. The relative 10-year survival rates of stage I, stage II, and stage III CRC patients were relatively higher (94.3%, 83.2%, and 72.1%, respectively), whereas...
that of stage IV patients was only 15.1% (diagnosed between 2006 and 2008) in Japan; stages were classified according to the Japanese Society for Cancer of the Colon and Rectum guidelines 2013 for the treatment of colorectal cancer (JSCCR guidelines, 8th edition)\(^3\). Although early detection of CRC is the key to treatment outcome, it remains difficult. A census in the USA (2007–2013)\(^6\) indicated that only 39% of patients with CRC were diagnosed in the early stage of the disease without detectable invasions. Conventional cancer markers in the blood, such as a carcinoembryonic antigen (CEA) or carbohydrate antigen 19–9 (CA19–9), are hardly detected in early-stage CRC\(^5\). Therefore, the development of new biomarkers with high sensitivity for early-stage CRC diagnosis is strongly desired.

Mature microRNAs (miRNAs) are single-stranded non-coding RNA molecules consisting of 19–22 nucleotides; these molecules silence gene expression and regulate essential cellular processes\(^6,7\). A miRNA is complementary to a part of one or more messenger RNAs and inhibits translation\(^8\). Thus far, >2,600 mature miRNAs have been identified in humans (miRbase v22)\(^9\). The expression of miRNAs is related to some diseases\(^10-13\), including cancers\(^14\). It has been reported that numerous miRNAs act as tumor suppressor genes or oncogenes (oncomiRs)\(^15-20\).

miRNAs in blood samples (containing whole blood, blood serum, and blood plasma) are potential prognostic and diagnostic markers for numerous types of cancers\(^21-24\). In CRC, an array of miRNAs are up-regulated or down-regulated in the blood samples of patients\(^25,26\).

In this study, we aimed to identify miRNAs as diagnostic markers that can detect early-stage CRC. The expression levels of miRNAs in preoperative and postoperative blood samples obtained from patients in stage II CRC (by JSCCR classification)\(^3\) without lymph node inversion were comprehensively analyzed and compared to select miRNAs marker candidates. To evaluate their effectiveness as the diagnostic marker for early-stage CRC, the expression levels of these miRNAs in the serum of CRCs and HC were examined using a dataset deposited in a public databank (GSE39833)\(^27\).

Materials and methods

[I] Selection of candidates for the diagnostic markers of early–stage CRC

Patients

Among 52 stage II CRC cases, we selected 7 cases without lymphatic invasion nor venous invasion (staging was conducted according to the JSCCR classification\(^3\)). Patients who received chemotherapy developed other types of cancer and had blood relatives with a history of cancer were also excluded. Consequently, samples of the 7 cases with stage II disease (hereafter “selected stage II cases”) were used for the following procedures. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of Juntendo University (No. 2018115).

Written informed consent was obtained from all study participants after proper counseling using documents that described the research aim and any possible risks involved.

Sample processing and miRNA extraction from bloods

Peripheral whole blood samples (hereafter blood samples) obtained from the selected stage II cases prior to primary surgery (preoperative blood samples) and 7 days after surgery (postoperative blood samples) were stored in PAXgene Blood RNA Tubes (Becton, Dickinson and Company, United Kingdom) at −80°C until use. Total RNA containing miRNAs was prepared using the PAXgene Blood miRNA Kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. RNA quantity and quality were accessed using the NanoDrop ND–1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All total RNA samples showed A260/A280 absorbance ratios ≥1.5, A260/230 ratios ≥1.0, a 28S/18S rRNA ratio ≥1.5, and an RNA integrity number ≥7.

Microarrays

RNA was labeled with cyanine 3 (Cy3) using the miRNA Complete Labeling and Hyb Kit (Agilent Technologies), following the instructions provided...
by the manufacturer. Briefly, 30 ng of RNA was dephosphorylated using a calf-intestinal alkaline phosphatase master mix in an incubator at 37°C for 30 min. Dephosphorylated RNA was denatured with dimethyl sulfoxide, incubated at 100°C for 5 min, and immediately transferred on ice for 2 min. These products were mixed with a ligation master mix for T4 RNA Ligase and Cy3-cytidine bisphosphate (Cy3-pCp) and incubated at 16°C for 2 h. Labeled RNA was dried using a vacuum concentrator at 55°C for 1.5 h. Cy3-pCp-labeled RNA was hybridized on the Agilent SurePrint G3 Human miRNA 8x60K Rel.21 (G4872A; #70156; Agilent Technologies) without a control probes array at 55°C for 20 h. There were 2,549 human miRNA probes except for the control probes on this array. After washing, the microarray was scanned using an Agilent DNA microarray scanner. Intensity values of each scanned feature were quantified using the Agilent Feature Extraction software version 11.5.1.1, which performs background subtractions using default parameters. The signal levels of probes that were below 0.1 were rounded off to 0.1. We only used features that were flagged as no errors (Detected flags) and excluded those that were not positive, not significant, not uniform, not above background, saturated, and population outliers (Not Detected flags). The expression analyses were performed with Agilent GeneSpring GX version 13.1.1. We applied a ≥1.5-fold change in signal intensity to identify significant differences in gene expression in this study.

Total RNA extraction from sectionalized tissues

Frozen cancer and normal tissues of colorectal ducts from the selected stage II cases were sectioned (thickness: 16 μm) using a cryostat (Thermo Scientific Microm HM550; Thermo Fisher Scientific) and stored at −80°C until use. The cancer tissue sections were manually micro-dissected under the guidance of a pathologist. Subsequently, the submucosal layer was removed from the tissue section, and the mucosal layer containing >95% of tumor cells was used for RNA extraction.

Total RNA extraction was performed with the miRNeasy Micro Kit (Qiagen) according to the instructions provided by the manufacturer. The concentration of RNA was estimated using the NanoDrop 1000 (Nanodrop Technologies, Wilmington, DE, USA) and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific).

miRNA quantification by real-time quantitative polymerase chain reaction (RT-qPCR)

The expression levels of miRNAs selected as candidate markers were quantified by RT-qPCR using the miScript PCR System (Qiagen), according to the instructions provided by the manufacturer. For the validation of the postoperative down-regulation of the 2 identified miRNAs, total RNA extracts from the blood samples of the 3 selected stage II cases which had not been used in the microarray analysis were applied. Total RNA extracted from cancer and normal tissues was also analyzed to ascertain that the miRNAs originated from CRC tissues.

Reverse transcription was conducted to generate cDNA from the total RNA samples using the miScript II RT Kit (Qiagen). Total RNA samples (500 ng to 2 μg RNAs) templates were mixed with 5× miScript HiSpec Buffer, 4 μl of 10× Nucleics Mix, 2 μl of miScript Reverse Transcriptase Mix, and RNase-free water to reach a final volume of 20 μl. The reaction mixture was incubated at 37°C for 60 min and 95°C for 60 min. SYBR green RT-qPCR assay was conducted for miRNA quantification with 7500 Fast Realtime PCR Systems (Thermo Fisher Scientific). Each reaction was performed in a final volume of 50 μl containing 50 ng of cDNA, 500 μl of each primer, and 25 μl of 2 × SYBR Green PCR Master Mix (Qiagen). Next, Hs_miR-15b1 miScript Primer Assay and Hs_miR-339_1 miScript Primer Assay (Qiagen) were used as primers. The amplification profile was denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s to detect fluorescence. At the end of the PCR cycles, melting curve analyses were performed. The expression levels of miRNAs were quantified using the comparative cycle threshold (Ct) method and normalized using both RNU6B and SNORD96A as endogenous controls according to the instructions provided by the manufacturer (Applied Biosystems, Waltham, MA, USA).

Statistical analysis

Values of the average signal level of all probes were obtained through log2 transformation. A
paired t-test (one-tailed) was used to compare values of miRNA levels between preoperative and postoperative samples or between cancer and normal tissues. Statistical analyses were conducted using the R software (v.3.2.5)\(^28\). P values less than 0.05 were considered statistically significant.

**[II] Evaluation of the selected marker for diagnosis of early-stage CRC**

**Analysis of miRNA expression in datasets from the Gene Expression Omnibus (GEO) database**

The differential expression levels of miR-15b-3p and miR-339-5p in sera, which were selected as the marker candidates, were determined in patients with CRC and healthy controls (HC) using datasets downloaded from the National Center for Biotechnology Information (GEO; accession code SubSeries GSE39833 in SuperSeries: GSE40247; http://www.ncbi.nlm.nih.gov/geo/\(^27\)). The expression data were analyzed by Agilent Human miRNA V3 Microarray (G4470C; Agilent Technologies) according to the instructions provided by the manufacturer. In these datasets, we used data for the miRNA levels in the sera of HC (n=11) and patients with primary CRC (n=88 in total) at various stages (stage I: n=20; stage II: n=20; stage IIIa: n=20; stage IIIb: n=16; and stage IV: n=12, according to JSCCR classification\(^3\); age range: 35–65 years old).

**Statistical analysis**

In the analysis of aforementioned public data (GSE39833 GEO), the difference between the values obtained from the sera of HC and patients with CRC (pooled data of all stages) was evaluated using Welch’s t-test (one-sided). The differences between the HC and patients with CRC of each stage were evaluated using Dunnett’s test (one-sided). Statistical analyses were conducted using the R software (v.3.2.5)\(^28\). P values less than 0.05 were considered statistically significant.

**Results**

**[I] Selection of miRNAs as candidates for diagnostic markers for early-stage CRC**

The characteristics of the patients are shown in Table 1. Four cases (#1–4) were chosen as the order of the amount of total RNA obtained from the preoperative blood sample of each case. Four cases (#1–4) were chosen from the 7 selected stage II cases as the order of the amount of total RNA obtained from the preoperative blood sample of each case. With microarray analysis, 366 miRNAs were detected in all examined samples (n=4, Table 1). Thirty miRNAs were differentially expressed between the postoperative and preoperative blood samples of the selected stage II cases (p<0.2, paired t-test) (Figure 1). Furthermore, the heat map (Figure 2) illustrates the expression levels of the 30 miRNAs. Among these 30 miRNAs, the expression levels of 8 miRNAs differed significantly between preoperative and postoperative blood samples (p<0.05): the levels of miR-15b-3p and miR-339-5p were decreased, whereas those of miR-29b-3p, miR-144-5p, miR-21-5p, let-7b-5p, miR-4739, and let-7c-5p were increased after the surgery (Figure 2; Table 2).

| Table 1 | Caracteristic and pathological conditions of the selectest stage cases |
|---------|---------------------------------------------------------------|
| Patient No. | Age | Sex | Location | TMN | CEA (ng/ml) | CA19-9 (U/ml) | Histopathology |
| #1 | 68 | M | S | T3N0M0 | 2.7 | 30 | mod |
| #2 | 54 | M | T | T3N0M0 | 0.4 | 6 | mod |
| #3 | 51 | F | S | T3N0M0 | 2.2 | 7 | mod |
| #4 | 72 | F | RS | T3N0M0 | 2.2 | 19 | mod |
| #5 | 88 | F | A | T3N0M0 | 1.9 | 6 | well |
| #6 | 45 | F | RS | T3N0M0 | 8.1 | 9 | well |
| #7 | 81 | M | A | T3N0M0 | 6.6 | 4 | well |

Location: location of cancer focus, S: sigmoid colon, T: transverse colon, RS: rectal sigmoid A: ascending colon. TMN, TNM (Tumor node metastasis cancer staging system) classified according to JSCCR guidelines, 8th edition\(^3\). CEA, carcinoembryonic antigen in blood. CA19-9, carbohydrate antigen 19-9 in blood. Histopathology, histopathological status of cancer tissue, mod: moderately differentiated adenocarcinoma, well, well differentiated adenocarcinoma.
Validation of the selected miRNAs in blood samples

Relative levels of miRNA expression in preoperative and postoperative blood samples of the remaining 3 selected stage II cases evaluated with RT-qPCR are indicated in Figure 3. The average levels of miR-15b-3p in postoperative samples were significantly lower than those measured in preoperative samples ($p=0.0397$, paired t-test, one tail). The expression of miR-339-5p in postoperative samples was also lower than that observed in preoperative samples ($p=0.0460$).

Expression of the selected miRNAs in cancer and normal tissues

Figure 4 illustrates the expression of miR-15b-3p and miR-339-5p in CRC and normal tissues of all 7 selected stage II cases. In the case of miR-15b-3p, on average, the relative ratio of the expression was higher in CRC tissues than normal tissues ($p=0.0490$, paired t-test). For miR-339-5p, the difference was not significant ($p=0.0952$) due to 2 cases in which the value in normal tissue was increased.
Tsukamoto R, et al: miRNA-15b-3p as a diagnostic marker for the early stage of colorectal cancer

Evaluation of the effectiveness of the marker candidate RNAs

Figure 5a,b indicates the relative expression levels of the 2 marker candidate miRNAs in serum samples of stages I–IV CRC patients and HC, the data for which were deposited in the GEO public database\(^27\). The expression levels of miR-15b-3p in all the stages of CRC were higher than those measured in HC \(^{\text{p}=0.00236}\) by one-sided Welch’s t-test. Moreover, the sera of patients with stages I, II, and IIIa CRC contained significantly higher levels of miR-15b-3p than those of HC \(^{\text{p}=0.0413, 0.0356,}\) and 0.0144, respectively, by one-sided Dunnett’s test (Figure 5a). However, significant differences were not detected between the sera of stage IIIb or stage IV CRC and that of HC \(^{\text{p}>0.05}\) by one-way analysis of variance), i.e., the progression of disease beyond stage IIIb appeared to not exert a significant effect on miR-15b-3p (Figure 5a). Notably, there was no significant difference detected in the levels of miR-339-5p between HC and all the stages of CRC \(^{\text{p}>0.05}\) by Welch’s t-test or between HC and any single stage of CRC \(^{\text{p}>0.05}\) by one-sided Dunnett’s test (Figure 5b).

Discussion

It has been reported that miRNAs in blood-based samples (peripheral blood, sera, and plasma) are potential prognostic and diagnostic markers for numerous types of cancers\(^23\)–\(^24\), including CRC\(^29\),\(^30\). An array of miRNAs are up-regulated or down-regulated in the blood-based samples of patients with CRC\(^26\),\(^29\),\(^30\). For example, miR-885-5p\(^31\), miR145\(^32\), miR-144\(^33\), miR-203\(^34\), and numerous other miRNAs

**Table 2** Eight miRNAs of which expression level were significantly changed between pre and postoperative samples

| miRNA\(^a\) | Preoperative Signal Intensity | Postoperative Signal Intensity | Log\(_2\) FC Pre versus Post | Regulation | miRBase\(^b\) Accession no. | \(p\)-value |
|---|---|---|---|---|---|---|
| miR-15b-3p | 1.38 | 0.10 | -3.79 | down | MIMAT0004586 | 0.0012 |
| miR-339-5p | 5.12 | 2.79 | -0.88 | down | MIMAT0000764 | 0.039 |
| miR-29b-3p | 5.49 | 9.97 | 0.86 | up | MIMAT000100 | 0.010 |
| miR-144-5p | 16.80 | 29.35 | 0.80 | up | MIMAT0004600 | 0.023 |
| miR-21-2p | 50.68 | 87.25 | 0.78 | up | MIMAT0000767 | 0.028 |
| let-7b-5p | 311.77 | 758.71 | 1.28 | up | MIMAT000063 | 0.031 |
| miR-4739 | 8.32 | 13.76 | 0.73 | up | MIMAT00019868 | 0.038 |
| let-7c-5p | 96.48 | 222.01 | 1.20 | up | MIMAT0000064 | 0.042 |

LogFC, log, fold change of expression level in preoperative blood /post operative blood.

\(^a\) miRNA, microRNA.

\(^b\) miRBase, http://www.mirbase.org.

**Figure 3** Verification of postoperative down-regulation of the 2 identified miRNAs in the blood samples of selected stage II cases (\(n=3\))

Ct, cycle threshold; miRNA, microRNA; RT-qPCR, real-time quantitative polymerase chain reaction.

**Figure 4** Expression levels of miRNAs in colorectal cancer tissue and normal tissue dissected from selected stage II cases (\(n=7\))
can predict metastasis and poor prognosis in CRC\(^{35}\). High expression of miR-10b confers resistance to the chemotherapeutic agent 5-fluorouracil in CRC\(^{36}\). Some RNAs, including miR-21, miR-23a and miR-1246, are up-regulated from the early stage of CRC\(^{37,38}\), although this type of miRNAs are rather rare. miRNAs in clinical blood samples tend to be associated with short-term survival or tumor recurrence\(^{31-36}\).

We attempted to identify a new biomarker that could detect early-stage CRC in this study. We focused on miRNAs whose levels were affected by tumorigenesis itself rather than those that were affected by risk factors. Although patient in early-stage CRC (before having surgeries) has tumor lesion(s), they do not always have risk factors which concern the complication and/or the progress of the cancer at that point. Therefore, we used samples from the selected stage II CRC cases for whom no typical risk factors were detected, as listed in the guidelines of the American Society of Clinical Oncology (ASCO)\(^{39}\), National Comprehensive Cancer Network (NCCN)\(^{40}\), and/or European Society for Medical Oncology (ESMO)\(^{41}\) (i.e., lymphatic invasion, venous invasion, poorly differentiated adenocarcinoma, patients with <12 lymph nodes examined, and preoperative ileus) to choose the marker candidate in the initial part of this study.

By analyzing the blood samples of the selected stage II cases, we first selected 2 miRNAs, miR-15b-3p and miR-339-5p, which were significantly up-regulated prior to surgery using microarray analysis of 2,549 mature miRNAs. The postoperative down-regulation of these two miRNAs was validated by RT-qPCR. Therefore, we selected miR-15b-3p and miR-339-5p as the marker candidates. Cancer tissue-specific higher expression was significant in miR-15b-3p, but not in miR-339-5p. As a result, miR-15b-3p was assumed to have originated from tumor cells of CRC.

The effectiveness of miR-15b-3p and miR-339-5p as diagnostic markers for early-stage CRC was examined using a data set available in the GEO database\(^{27}\). The expression levels of miR-15b-3p were significantly higher in the sera of patients with CRC than in those of HC (\(p=0.0016\) by Welch’s \(t\)-test) (Figure 5a). Moreover, miR-15b-3p was conspicuously up-regulated from the early stage of the disease. Its expression levels were significantly
higher in the sera of patients with stages I, II and IIIa CRC than in those of HC. Consequently, we assumed that miR-15b-3p might be useful as a diagnostic marker for the detection of early-stage CRC. In contrast, miR339-5p was not considered a useful marker for CRC, owing to the lack of difference in its expression between the sera of patients with CRC (whole stages) and those of HC ($p>0.5$) in the deposited data (Figure 5b).

Although the magnitude of the signal level of miR-15b-3p was not higher among the 30 miRNAs, the difference in the expression levels of miR-15b-3p between preoperative and postoperative blood samples was conspicuous (Figure 2). The results of this study indicate that miR-15b-3p is detectable by both microarrays and RT-qPCR analysis in clinical blood and serum samples. To evaluate the levels of miRNAs, in this work, we applied a simple protocol that does not involve centrifugation or other troublesome procedures. This might be an advantage for clinical use.

Human miR-15b-3p is a 22-nt non-coding RNA (sequence: cgaaucaauaaucugcucua; miRbase9). miR-15b-3p and miR-15b-5p are mature products of miR-15b. In general, a pre-miRNA is cleaved with the RNase III enzyme Dicer, generating a miRNA-5p and a miRNA-3p. The miR-15 miRNA precursor family includes the related miRNAs miR-15a and miR-15b, as well as miR-16-1, miR-16-2, miR-195, and miR-49719, 42, 43). In humans, miRNA-15b is located on chromosome 3, while miR-15a and miR-16 are on 13q14 9, 44). miR-15 families suppress the expression of genes, including several oncogenes, and the downregulation of miR-15 and miR-16 has been related to cancer metastasis in several cancers43.

Both miR-15a-3p and miR-15b-3p are related to immune responses in bovine45), and miR-15b is related to sensitivity to chemotherapy in humans46). Recently, Wei et al46) reported the action mechanism of exosomal miR-15b-3p in the metastasis and malignancy of gastric cancer (GC). They also suggested the potential of miR-15b-3p in serum as a diagnostic and prognostic biomarker in GC46. In this disease, miR-15b-3p was increased with the progression of stages, lymph and vascular invasion, and poor differentiation of cancer. The function of miR-15b-3p in GC does not appear to directly explain the results of this work in CRC. However, this miRNA might play an important role in CRC. Therefore, the action mechanism of miR-15b-3p in the tumorigenesis and development of CRC is worthy of study in detail.

This study revealed a higher expression of miR-15b-3p in the peripheral blood samples of stage II CRC patients and in the serum samples of stage I and II CRC patients (by the public data). These findings suggest the high potential of miR-15b-3p as a diagnostic marker for early–stage CRC.

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Authors’ contributions

RT and HK designed the study and provided overall guidance with the advice of KS. RT analyzed the clinical data with the assistance and advice of MT and KS. NY conducted the microdissection of tissues and extraction of RNA. RT and MH mainly performed microarray and RT-qPCR analyses. MH conducted the statistical data analyses, and RT and MF prepared the tables and figures. RT and MF wrote the manuscript, which was critically reviewed by NY, KS, SK and HK. All authors have read and approved the final version of the manuscript.

Conflicting interest

The authors declare that there is no conflict of interest.

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