Title: Differential expression of microRNAs in colorectal cancer: different patterns between isolated cancer gland and stromal cells

Running title: microRNAs dysregulated in CRC

Ayaka Sato, MD (1), Yasuko Fujita, MD (1), Koki Otsuka, MD (2), Akira Sasaki, MD (2), Hiromu Suzuki, MD (3), Takayuki Matsumoto, MD (4), Tamotsu Sugai, MD (1)

(1) Department of Molecular Diagnostic Pathology, School of Medicine, Iwate Medical University, 2-1-1 Idaidori, Yahaba-cho, Shiwa-gun, Iwate prefecture, 028-3695, Japan

(2) Department of Surgery, Iwate Medical University

(3) Department of Molecular Biology, Sapporo Medical University School of Medicine

(4) Division of Gastroenterology, Department of Internal Medicine, Iwate Medical University

Correspondence:

Tamotsu Sugai, MD

Department of Molecular Diagnostic Pathology

Iwate Medical University, 2-1-1 Idaidori, Yahaba-cho, Shiwa-gun, Iwate prefecture, 028-3695, Japan

TEL: +81-19-613-7111; FAX: +81-19-907-8145

E-mail: tsugai@iwate-med.ac.jp

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Abbreviations: miRNA, microRNA; CRC, colorectal cancer; CAF, cancer-associated fibroblast; PCR, polymerase chain reaction; 5FU, 5fuluorourasil; EMT, epithelial-mesenchymal transitions; MET, mesenchymal-epithelial transitions; SMA, smooth muscle actin

Abstract

Although microRNAs (miRNAs) play an important role in invasive tumor lesions, which involve cancer tissues mixed with stromal tissues, the differences in miRNA expression between cancer and stromal cells remain unclear. We selected 13 miRNAs and examined their differential expression patterns in cancer gland cells and surrounding stromal cells isolated from 24 colorectal cancer (CRC) specimens using a crypt isolation method. Although six miRNAs were upregulated in gland cells, only three were upregulated in the corresponding stromal cells, in the cancer compared with non-cancer specimens. Next, we examined the differences in miRNA expression between isolated cancer gland and stromal cells. Five miRNAs showed statistical differences in their cancer-related differential expression patterns between isolated cancer gland and stromal cells. We then compared these miRNA expression patterns in isolated cancer gland and stromal cells with those in fresh intact tumor tissues, consisting of cancer nests and stromal tissue, obtained from the 24 CRCs. The expression patterns of three miRNAs in the intact cancer tissue samples did not correspond with those in the isolated components. Identification of the expression patterns of

miRNAs in both the cancer gland and stromal cell components of the tumor microenvironment greatly contributes to evaluating epigenetic regulation in CRC.

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide, [1] due to its metastasis. Although many therapeutic options including the combination of chemotherapy with targeted therapy have been developed for metastatic CRC, [2] there has been little improvement in the survival rate of patients with metastatic CRC, [3] prompting investigators to evaluate the underlying mechanisms of CRC progression [2, 3] The tumor microenvironment, which promotes the growth and invasion of cancer cells, has attracted attention in the efforts to understand colorectal carcinogenesis.[4, 5]

MicroRNAs (miRNAs) are endogenous small (~22 nucleotides) noncoding RNAs that silence genes at the post-transcriptional level. [6, 7] Previous studies have shown that miRNAs play fundamental roles in colorectal carcinogenesis including invasion and metastasis. [6, 7] Aberrant expression of miRNAs has been detected in various cancers including breast, [8] lung, [9] pancreatic, [10] colorectal [11] and ovarian cancers [12]. MiRNAs regulate the expression of many known oncogenes (suppressor miRNAs) and tumor suppressor genes (onco-miRNAs)

in cancer pathogenesis. [13] These functions of miRNAs in human carcinogenesis will help identify new targets for cancer research, diagnosis, and treatment. [14]

The tumor microenvironment is composed of cancer cells and non-cancer cells, so-called stromal cells such as cancer-associated fibroblasts (CAFs), tumor-associated macrophages, pericytes, endothelial cells, and infiltrating immune cells. [4, 5] Recent accumulating evidence suggests that dysregulation of miRNAs promotes tumor invasion and metastasis. [4, 5] Although stromal cells interact with tumor cells as well as other stromal cells to stimulate tumor growth and metastasis, 14 CAFs are one of the most important factors facilitating tumor progression. [15, 16] Disrupting the interactions between cancer and stromal cells within the tumor microenvironment is a potential strategy to not only evaluate colorectal carcinogenesis and but also develop novel therapies for cancer treatment. [17] A recent report suggested that miRNAs contribute to regulation of the microenvironment. [17]

Although miRNAs closely associated with colorectal carcinogenesis are generally classified as oncogenic or tumor suppressive, [13] it is unclear whether

these miRNAs originate from cancer cells or the surrounding stromal cells in CRC. [18, 19, 20] A recent study indicated that miRNA-21 is expressed in both cancer and stromal cells in CRC, pancreatic cancer and urothelial carcinoma. [19, 20, 21] This suggests that the roles of miRNA-21 in CRC should be evaluated separately in the cancer versus non-cancer components. [19] A recent study showed that miRNA-21 expression in CAFs, but not cancer cells, was associated with a poor prognosis in lung adenocarcinoma. [22] Identification of the role of miRNAs in the invasive area (tumor microenvironment) is necessary for stratifying patients with CRC according to genetic and epigenetic alterations.

The aim of the present study was to examine miRNA dysregulation in the microenvironment, specifically at the cancer invasion front, by assessing abnormal expression of miRNAs in cancer and stromal cells, obtained using a crypt isolation method that enables separate isolation of cancer gland and stromal cells. In addition, we attempted to identify the differences in miRNA dysregulation among intact tumor tissues containing stromal cells and each of its isolated components (i.e., cancer gland and stromal tissues).

Materials and methods

Patients

A total of 24 pairs of tumor and corresponding normal tissue samples distant from the cancer tissue samples were obtained from 24 patients with primary CRC admitted to Iwate Medical University Hospital from 2016 to 2018. The CRCs were classified into three histological groups, well differentiated, moderately differentiated, papillary adenocarcinoma and mucinous adenocarcinoma, according to the criteria of the Japanese Society for Cancer of the Colon and Rectum. [23] CRC stage was determined according to the guidelines of the Japanese Society for Cancer of the Colon and Rectum. The clinicopathological findings are shown in Table 1.

This study was approved by the Ethical Research Committee of Iwate Medical University (HG H29-29).

Colonic crypt isolation

Colonic crypt isolation from tumor and normal mucosal tissues was performed in accordance with a previously described method to obtain pure gland and stromal tissues, respectively. [24] Samples from the isolated tumor glands were obtained from the central ulcer of the tumor, which involves the invasion front. We confirmed that the samples contained the invasion front using the histological sections prepared for pathological diagnosis. In the present study, gland cells (from normal and tumor tissues) and stromal cells (from mainly fibrous tissue consisting of CAFs and normal fibroblasts) were obtained separately under a dissecting microscope. The surrounding stromal cells near the cancer glands were carefully obtained from the isolated samples. The isolated glands and stromal cells were processed routinely into paraffin-embedded histological sections for characterization. The stromal cells were immunostained with antibodies targeting smooth muscle actin (clone 1A4, Dako, Carpinteria, CA, USA) and desmin (clone D33, Dako). We confirmed the exclusive presence of fibroblasts according to a smooth muscle actin-negative and desmin-positive immunostaining pattern. Contamination of other materials (gland samples such

as interstitial cells; stromal sample, cancer glands) was not evident in the gland or stromal tissue samples examined. However, the stromal cells might have been contaminated with other non-epithelial cells, such as inflammatory and vessel cells. Although there are different types of tumor-associated stromal cells, including cancer-associated fibroblasts, inflammatory tissue and vascular rich tissue, we confirmed that the stromal cells in the present study comprised cancer-associated fibroblasts exclusively (**Supplementary Figure 1**). Representative images are shown in **Figure 1**.

Intact tumor sampling

For miRNA analysis, fresh intact tumor tissues were obtained from an area of the resected tumor adjacent to the region used for crypt isolation. Normal colonic mucosa was taken from the most distant portion of the colon. The cell population of these tumor tissue samples was confirmed to contain at least 50% neoplastic cells. Non-cancer gland and fibrous tissues (distant from the tumor) were isolated as controls. All tissue samples were stored at −80°C until RNA extraction.

RNA isolation

miRNAs were extracted using the mirVana™ miRNA Isolation kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA quantity and quality were evaluated using the DU730 spectrophotometer (Beckman Coulter, Brea, CA, USA), and RNA integrity was determined by gel electrophoresis.

Quantitative reverse-transcription PCR analysis of miRNAs

The following miRNAs expressed in CRCs were evaluated in this study: hsa-miRNA-19a-3p, [25] hsa-miRNA-21-5p, [25] hsa-miRNA-27a-3p, [25] hsa-miRNA-27b-3p, [25] hsa-miRNA-31-5p, [25] hsa-miRNA-34b-3p, [25] hsa-miRNA-130a-3p, [25, 26] hsa-miRNA-143-3p, [25] hsa-miRNA-186-5p, [25] hsa-miRNA-191-5p, [25] hsa-miRNA-195-5p, [27] hsa-miRNA-206, [28] and hsa-let-7a-5p. [26] The primer sequences used are shown in Supplementary Table 1.

The mature miRNAs were detected and quantified by quantitative reverse-transcription PCR in conjunction with TaqMan miRNA assays (Applied

Biosystems, Foster City, CA, USA) as described previously. [29] Triplicate RNA samples were used. RNA was reverse transcribed into cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), and reactions were run on the Gene Amp PCR system 9700 thermal cycler (Applied Biosystems). Samples were incubated at 16°C for 30 min, followed by 42°C for 30 min and 85°C for 5 min. We included a negative control lacking reverse transcriptase in each set of reactions. The reaction mixture (20 μL final volume) consisted of the cDNA product, TaqMan 2X Universal PCR Master Mix II, and the appropriate 20X MicroRNA Assay Mix containing the specific probe targeting the miRNA of interest. PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) under the following reaction conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Inter-assay controls and calibrators were included in each 96-well plate. All TaqMan assays were run in triplicate using the AB StepOnePlus Real-Time PCR System. *RNU6B* (assay ID: 001093) was amplified as an endogenous control for normalization of the miRNA expression levels. Ct values were generated using StepOne Software v2.2.2 with

the automatic baseline settings, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the expression level of each miRNA in the tumor tissues relative (fold-change) to the non-tumor tissues. The expression ratio was logarithmically transformed (base 10) to obtain the fold change (>0, upregulation; <0, downregulation).

Statistical analysis

The expression levels of each miRNA in each sample (isolated cancer gland cells, isolated stromal cells, and intact tumor tissues) were compared using the Wilcoxon matched-pairs signed-rank test conducted in GraphPad Prism version 6.0d (GraphPad Software, San Diego, CA, USA). A *P*-value < 0.05 was considered to indicate significance.

Results

Differential miRNA expression between matched CRC and non-cancer samples

To confirm and validate the differential expression of each miRNA examined in CRC, we first measured miRNA expression levels in isolated gland or stromal

cells from the 24 CRC specimens compared with the corresponding paired normal samples (isolated normal gland cells or normal fibroblasts, respectively).

In isolated CRC gland cells, the expression levels of six miRNAs (hsa-miRNA-130a-3p, hsa-miRNA-143-3p, hsa-miRNA-206, hsa-miRNA-31-5p, hsa-miRNA-27b-3p, and hsa-miRNA-27a-3p) were upregulated, whereas those of seven miRNAs (hsa-miRNA-21-5p, hsa-miRNA-195-5p, hsa-miRNA-19a-3p, hsa-miRNA-34b-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, and hsa-let-7a-5p) were downregulated, compared with the corresponding isolated normal gland specimens. In the isolated CRC stromal cells, the expression levels of three miRNAs (hsa-miRNA-21-5p, hsa-miRNA-31-5p, and hsa-miRNA-27b-3p) were upregulated and those of 10 miRNAs (hsa-miRNA-130a-3p, hsa-miRNA-143-3p, hsa-miRNA-206, hsa-miRNA-195-5p, hsa-miRNA-19a-3p, hsa-miRNA-34b-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, hsa-let-7a-5p, and hsa-miRNA-27a-3p) were downregulated compared with the corresponding normal fibroblasts. Finally, in the intact tumor mucosa samples, the expression levels of three miRNAs (hsa-miRNA-31-5p, hsa-miRNA-19a-3p, and hsa-let-7a-5p) were upregulated, and

those of 10 miRNAs (hsa-miRNA-130a-3p, hsa-miRNA-21-5p, hsa-miRNA-143-3p, hsa-miRNA-206, hsa-miRNA-195-5p, hsa-miRNA-27b-3p, hsa-miRNA-34b-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, and hsa-miRNA-27a-3p) were downregulated, compared with normal mucosa specimens.

Comparison of cancer-associated differential miRNA expression patterns between isolated tumor gland cells and the surrounding stromal cells

We compared the differential miRNA expression in CRC between the isolated cancer glands and isolated stromal cells. The expression levels of hsa-miRNA-130a-3p ($P<0.05$), hsa-miRNA-143-3p ($P<0.01$), and hsa-miRNA-206 ($P<0.05$) were significantly upregulated in CRC gland cells but downregulated in CRC stromal cells, respectively. On the other hand, hsa-miRNA-21-5p showed the opposite pattern, being significantly downregulated in CRC gland cells but upregulated in CRC stromal cells ($P<0.05$), respectively. hsa-miRNA-195-5p was significantly downregulated in both isolated gland and stromal cells from CRC, but the degree of downregulation was significantly greater in the stromal than

cancer gland cells (hsa-miR-195-5p) (P<0.05). These associations are depicted in **Figure 2**.

Comparison of cancer-associated differential miRNA expression patterns between isolated cancer gland cells and intact tumor tissues

The expression levels of hsa-miRNA-130a-3p (P<0.01), hsa-miRNA-143-3p (P<0.01), hsa-miRNA-206 (P<0.05), hsa-miRNA-27b-3p (P<0.01), and hsa-miRNA-27a-3p (P<0.01) were significantly upregulated in the isolated CRC gland cells compared with their non-cancer counterparts but significantly downregulated in intact tumor tissues compared with non-tumor tissues. The degree of hsa-miRNA-195-5p (P<0.01) and hsa-miRNA-34b-3p (P<0.05) downregulation in cancer compared with non-cancer samples was significantly different between the isolated cancer gland cells and intact tumor tissues. None of the other miRNAs (hsa-miRNA-21-5p, hsa-miRNA-31-5p, hsa-miRNA-19a-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, and hsa-let-7a-5p) showed significant differences in their differential expression patterns between isolated cancer gland

and intact tumor tissues. These associations are shown in **Figure 3**.

Comparison of cancer-associated differential miRNA expression patterns between isolated stromal cells and intact tumor tissues

The expression levels of hsa-miRNA-130a-3p ($P<0.05$), hsa-miRNA-143-3p ($P<0.01$), and hsa-miRNA-195-5p ($P<0.01$) were significantly downregulated in isolated stromal cells, but the degree of downregulation was greater in the intact tissues than isolated stromal cells from CRC. The expression of hsa-miRNA-27b-3p was significantly upregulated, compared with intact tumor tissue ($P<0.05$). No statistical differences were detected in the cancer-associated differential expression patterns of the other miRNAs between stromal cells and intact tumor tissues. These associations are depicted in **Figure 4.**

miRNA classification based on cancer-associated expression patterns between isolated cancer gland and stromal cells

The miRNAs evaluated were classified into two types according to their CRC-

associated differential expression patterns in isolated cancer gland and stromal cells: different (defined as a statistically differential expression between isolated cancer glands and stromal cells) and non-different (no statistical difference in differential expression between isolated cancer glands and stromal cells) types. The five miRNAs of the different type (hsa-miRNA-130a-3p, hsa-miRNA-206, hsa-miRNA-21-5p, hsa-miRNA-143-3p, and hsa-miRNA-195-5p) were further sub-classified into two subgroups: gland cell dominant and stromal cell dominant. Gland cell dominant miRNAs were those with a greater magnitude of CRC differential expression in the isolated cancer gland cells than in the stromal cells; only hsa-miRNA-130a-3p was classified into this category, showing upregulation in gland cells and downregulation in stromal cells from CRC. The stromal cell dominant miRNAs were those with a greater magnitude of differential expression in the isolated stromal cells than in the cancer gland cells; of these miRNAs, hsa-miRNA-21-5p was downregulated in cancer gland cells, but upregulated in stromal cells, hsa-miRNA-206 and hsa-miRNA-143-3p were upregulated in gland but downregulated in stromal cells, and hsa-miRNA-195-5p was downregulated

in both gland and stromal cells in CRC.

The miRNAs classified as the non-different type were further divided into upregulated (i.e., upregulated in both isolated gland and stromal cells from CRC: hsa-miRNA-27b-3p and hsa-miRNA-31-5p), downregulated (i.e., downregulated in both isolated gland and stromal cells from CRC: hsa-miRNA-19a-3p, hsa-miRNA-34b-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, and hsa-let-7a-5p), and up/downregulated (i.e., upregulated in gland cells and downregulated in stromal cells from CRC: hsa-miRNA-27a-3p). These classifications are summarized in **Table 2**.

Associations of the cancer-associated miRNA expression patterns between the isolated and intact tumor samples

The cancer-associated differential expression patterns of the 13 miRNAs in isolated cancer gland versus stromal cells were classified into three categories: up/downregulated in the gland and stromal cells (hsa-miRNA-130a-3p, hsa-miRNA-21-5p, hsa-miRNA-143-3p, hsa-miRNA-206, and hsa-miRNA-27a-3p),

upregulated in both the gland and stromal cells (hsa-miRNA-31-5p and hsa-miRNA-27b-3p), and downregulated in both the gland and stromal cells (hsa-miRNA-195-5p, hsa-miRNA-19a-3p, hsa-miRNA-34b-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, and hsa-let-7a-5p).

We then compared the miRNA expression patterns between the isolated cells and intact tumor tissue samples. Among the 13 miRNAs, hsa-miRNA-27b-3p exhibited upregulated CRC expression in both the isolated cancer gland and stromal cells but downregulated expression in the intact tumor tissues. Furthermore, hsa-miRNA-19a-3p and hsa-let-7a-5p showed downregulated CRC expression in both the isolated cancer gland and stromal cells but upregulated CRC expression in the intact tumor tissues. These associations are shown in **Table 3**.

Discussion

A total of 13 miRNAs that are closely associated with colorectal carcinogenesis were identified in CRC patients at different stages of the disease. These miRNAs

were examined extensively to evaluate their expression in CRC progression. [25-28] In the present study, hsa-miRNA-130a-3p, hsa-miRNA-143-3p, hsa-miR-206, hsa-miRNA-31-5p, hsa-miRNA-27a-3p, and hsa-miRNA-27b-3p were upregulated, whereas hsa-miRNA-21-5p, hsa-miRNA-195-5p, hsa-miRNA-19a-3p, hsa-miRNA-34b-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, and hsa-let-7a-5p were downregulated, in isolated gland cells from CRC compared with non-cancer specimens. These 13 miRNAs showed different expression patterns in the present study compared with those in previous studies. [13, 14, 15] For example, has-miRNA-21-5p was downregulated in the present study but was reported to be upregulated in CRC in previous studies. [13, 19] This discrepancy may urge investigators to reconsider the role of miRNA-21 in CRC. In addition, the expression of only 5 of the 13 miRNAs showed parallel changes between the isolated cancer gland and intact tumor tissue samples. This finding suggests that the overall expression of a miRNA in a tumor is determined by its expression in the individual components (gland and surrounding stromal tissues).

Recent studies have shown dysregulation of miRNAs in stromal cells in

various cancers. [17, 22, 30] High miRNA-21 expression in CAFs was associated with a poor prognosis in lung adenocarcinoma, [22] suggesting that at least some miRNAs are associated with cancer progression. [17, 31, 32] In the present study, 10 of the 13 miRNAs evaluated were downregulated (hsa-miRNA-130a-3p, hsa-miRNA-143-3p, hsa-miRNA-206, hsa-miRNA-195-5p, hsa-miRNA-27a-3p, hsa-miRNA-19a-3p, hsa-miRNA-34b-3p, hsa-miRNA-186-5p, hsa-miRNA-191-5p, and hsa-let-7a-5p), and 3 miRNAs (hsa-miRNA-21-5p, hsa-miRNA-31-5p, and hsa-miRNA-27b-3p) were upregulated, in stromal cells from CRC compared with non-cancer specimens. Thus, these findings suggest that miRNAs downregulated in the stroma may play a central role in cancer invasion. However, Nishida et al. showed upregulation of two oncogenic clusters, 17 suggesting that oncogenic miRNAs in stromal cells are crucially involved in cancer progression. [17] The discrepant findings may be due to different sample collection methods (microdissection vs. crypt isolation), detection methods (miRNA array vs. real time PCR), stage (early vs. late stage), sample type (fresh sample vs. paraffin-embedded tissue), or tumor heterogeneity. The present study suggests that

dysregulation of miRNA expression in the cancer-associated stroma is important. This is supported by the finding that CAFs affect the sensitivity of CRC cells to chemotherapies, such as oxaliplatin and 5FU, [31] and the role of CAFs as a relevant contributor to cancer progression and development of de novo resistance to targeted therapies is becoming increasingly apparent. [31]

In the present study, we examined differential expression of miRNAs between isolated cancer gland and stromal cells. Due to reciprocal crosstalk between cancer cells and fibroblasts, CAFs undergo various morphological and biological transitions in response to tumor progression. [4, 5] Epithelial–mesenchymal (EMT) and mesenchymal–epithelial (MET) transitions that occur during colorectal pathogenesis are processes allowing the tumor to adapt to the changing microenvironment. [32] In the present study, the different expression patterns of miRNAs observed between isolated cancer gland and stromal cell samples may be a result of EMT or MET in the invasive area of the tumor. [16, 32] In addition, we found various expression patterns of miRNAs in the isolated cancer gland and stromal cells. Our classification of miRNA expression patterns in cancer gland

and stromal cells (Table 3) may be helpful for evaluating the role of miRNAs in the tumor microenvironment. According to this classification, not only tumor cells but also stromal cells play major roles in miRNA regulation in the microenvironment. We suggest that multiple miRNAs act as key epigenetic regulators in cancer and stromal cells to regulate tumor invasion and metastasis.

Many investigations of miRNAs for evaluating tumorigenesis have been reported using tumor tissue samples containing stromal tissue. [7, 8] Analysis of miRNAs in tumor tissues is influenced by the presence of stromal cells, which may confound the results. It is thought that miRNA expression in tumor tissue is determined by the relative proportions of cancer cells and surrounding stromal cells. [4] In the present study, three conflicting differential expression patterns of miRNAs between cancer and non-cancer tissues were observed, suggesting that the miRNA expression status in cancer should not be based only on expression in isolated samples (e.g., cancer gland and stromal tissues). Although it remains unclear why a discrepancy was found between the isolated samples (isolated

cancer gland sample and stromal tissue) and tissue samples, one reason may be contamination of stromal cells other than fibroblasts, such as inflammatory cells, pericytes, and endothelial cells.

There are some limitations to the present study. First, although we carefully obtained the isolated stromal tissue, there was still the possibility of contamination of some cancer cells. Although in situ hybridization can be used to confirm the absence of contamination of cancer cells in a stromal cell population, this method is difficult to perform reproducibly and reliably and thus was not attempted. However, we believe that such a low degree of contaminating cancer cells did not affect our results. Second, we examined the expression patterns of select miRNAs that are frequently reported in CRC. Although high-throughput screening has been performed to determine miRNA expression profiles in various cancers, [17, 33] real-time PCR may be more appropriate for comparing miRNA expression between isolated cancer gland and stromal tissues.

In conclusion, we examined the expression patterns of 13 miRNAs that are closely associated with colorectal carcinogenesis in isolated CRC glands and

stromal tissue consisting of CAFs. Our results indicate an important role of miRNAs expressed in stromal cells in colorectal pathogenesis. We largely classified the 24 CRCs into two categories according to their differential expression pattern between cancer and non-cancer specimens. This classification might be helpful for understanding the roles of these miRNAs in CRC. We compared the cancer-associated differential expression patterns of the miRNAs between the isolated cancer gland/stromal samples and intact tumor tissues, consisting of both cancer and stromal cells. Although there were a few conflicting findings between the isolated and intact tissue samples in the present study, the overall association of the miRNA expression patterns between the two sample types was good. Assessing miRNA expression in tumor tissues that contain stromal tissue may need careful re-consideration for accurate evaluation of colorectal carcinogenesis. These findings require further evaluation in future studies.

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Human rights statement and informed consent

All procedures were performed in accordance with the ethical standards of the Iwate Medical University and with the Declaration of Helsinki. Substitute for informed consent (approval by the institutional review board of Iwate Medical University) was obtained from all patients included in the study.

Conflict of interest statement: We declare that we have no conflicts of interest.

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Author contributions and disclosure statement

AS (first author) performed all data collection and analyses. YF generated the

figures and tables. KO and AS assisted with the clinical data collection. TM provided input regarding the preparation of the manuscript. HS provided assistance with the molecular technologies used. TS (corresponding first author) contributed to the preparation of the manuscript.

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Figure legends

Figure 1. Representative figure of isolated cancer glands and stromal cells. Colorectal cancer glands observed under a dissecting microscope (tumor crypt: large crypts or sheets of epithelium, thick epithelial clusters of various sizes). b. Isolated adenocarcinoma gland identified by hematoxylin and eosin staining of tissue sections. c. Colorectal stromal cells observed under a dissecting microscope (large sheet resembling to fibrous tissue showing an irregular contour). d. Histology of cancer-associated fibroblasts according to hematoxylin and eosin staining of tissue sections. e. Positive immunostaining of smooth muscle actin. f. Negative immunostaining of desmin. According to these results, the fibrous tissue was considered to comprise activated fibroblasts (cancer-associated fibroblasts).

Figure 2. Differential expression of the 13 examined miRNAs between isolated cancer gland and stromal cells of colorectal cancer. a. hsa-miRNA-130a-3p. b. hsa-miRNA-21-5p. c. hsa-miRNA-143-3p. d. hsa-miRNA-206. e. hsa-miRNA-195-5p. f. hsa-miRNA-31-5p. g. hsa-miRNA-27b-3p. h. hsa-miRNA-19a-3p. i.

hsa-miRNA-34b-3p. j. hsa-miRNA-186-5p. k. hsa-miRNA-191-5p. l. hsa-let-7a-5p. m. hsa-miRNA-27a-3p. *: $P<0.05$; **: $P<0.01$.

Figure 3. Differential expression of the 13 examined miRNAs between isolated cancer gland cells and fresh intact tumor tissues of colorectal cancer. a. hsa-miRNA-130a-3p. b. hsa-miRNA-21-5p. c. hsa-miRNA-143-3p. d. hsa-miRNA-206. e. hsa-miRNA-195-5p. f. hsa-miRNA-31-5p. g. hsa-miRNA-27b-3p. h. hsa-miRNA-19a-3p. i. hsa-miRNA-34b-3p. j. hsa-miRNA-186-5p. k. hsa-miRNA-191-5p. l. hsa-let-7a-5p. m. hsa-miRNA-27a-3p. *: $P<0.05$; **: $P<0.01$.

Figure 4. Differential expression of the 13 examined miRNAs between isolated cancer stromal cells and fresh intact tumor tissues of colorectal cancer. a. hsa-miRNA-130a-3p. b. hsa-miRNA-21-5p. c. hsa-miRNA-143-3p. d. hsa-miRNA-206. e. hsa-miRNA-195-5p. f. hsa-miRNA-31-5p. g. hsa-miRNA-27b-3p. h. hsa-miRNA-19a-3p. i. hsa-miRNA-34b-3p. j. hsa-miRNA-186-5p. k. hsa-miRNA-191-5p. l. hsa-let-7a-5p. m. hsa-miRNA-27a-3p. *: $P<0.05$; **: $P<0.01$.

Supplementary Figure 1

Histological features (hematoxylin and eosin staining) and immunohistochemical staining of smooth muscle actin (SMA) and desmin. a. Hematoxylin and eosin staining; b. SMA immunostaining; c. desmin immunostaining. 1–24 indicate the case numbers. Histological features include fibrous tissue with positive immunostaining of SMA and negative immunostaining of desmin, suggesting cancer-associated fibroblasts (myofibroblasts).

Supplementary Table

Primer sequences for the microRNAs examined.

Table 1. Clinicopathological findings of the colorectal cancer cases examined

Factors	Cases (%)
Total	24 (100)
Sex	
Male	10 (41.7)
Female	14 (58.3)
Age (years)	
Median [range]	69 [45–90]
Tumor size (mm)	
Median [range]	50 [30–100]
Locus	
Right	15 (62.5)
Left	9 (37.5)
Macroscopic type	
Type 1	3 (12.5)
Type 2	20 (83.3)
Type 3	1 (4.2)
Predominant histological subtype	
Well differentiated adenocarcinoma	8 (33.3)
Moderately differentiated adenocarcinoma	12 (50.0)
Papillary adenocarcinoma	2 (8.3)
Mucinous adenocarcinoma	2 (8.3)
Tumor extension	
pT2	1 (4.2)
pT3	16 (66.7)
pT4 [pT4a/ pT4b]	7 (29.1) [7 (29.1)/ 0 (0.0)]
Lymphatic invasion	
Present/ absent	11 (45.8)/ 13 (54.2)
Venous invasion	
Present/ absent	14 (58.3)/ 10 (41.7)
Lymph node metastasis	
Present/ absent	15 (62.5)/ 9 (37.5)

Distant metastasis	
Present/ absent	6 (25.0)/ 18 (75.0)

Table 2. Classification of the miRNAs according to their differential expression between in cancer gland and stromal cells.

	Different type			**Non-different type**		
Subtype	**Gland cell dominant**	**Stromal cell dominant**		**Up regulated**	**Down regulated**	**Up/down regulated**
	Up regulated	**Up regulated**	**Down regulated**			
Glandular miRNA	**↑**	↓	↑↓	↑	↓	↑
Stromal miRNA	↓	**↑**	**↓**	↑	↓	↓
Classification of miRNAs	hsa-miRNA -130a-3p	hsa-miRNA -21-5p	hsa-miRNA -206, -143-3p, -195-5p	hsa-miRNA -27b-3p, -31-5p	hsa-miRNA -19a-3p, -34b-3p, -186-5p, -191-5p, hsa-let-7a-5p	hsa-miRNA -27a-3p

Bold up arrow: greater magnitude of CRC upregulation in the indicated cell type. Bold down arrow: greater magnitude of CRC downregulation in the indicated cell type. Thin up arrow: non-significant upregulation of the miRNA. Thin down arrow: non-significant downregulation of the miRNA.

Table 3. Associations of the cancer-associated miRNA expression patterns between the isolated and intact tumor samples

Up/downregulated in CRC cells	Intact CRC tissue expression	Upregulated in CRC cells	Intact CRC tissue expression	Downregulated in CRC cells	Intact CRC tissue expression
hsa-miRNA-130a-3p	↓	hsa-miRNA-31-5p	↑	hsa-miRNA-195-5p	↓
hsa-miRNA-21-5p	↓	hsa-miRNA-27b-3p	↓	hsa-miRNA-19a-3p	↑
hsa-miRNA-143-3p	↓			hsa-miRNA-34b-3p	↓
hsa-miRNA-206	↓			hsa-miRNA-186-5p	↓
hsa-miRNA-27a-3p	↓			hsa-miRNA-191-5p	↓
				hsa-let-7a-5p	↑

Up/downregulated: miRNAs up/downregulated in isolated glandular/stromal, or down/upregulated in isolated gland/stromal cells compared with paired non-cancer samples. CRC, colorectal cancer.

Upregulated: miRNAs upregulated in both isolated gland and stromal cells compared with paired non-cancer samples.

Downregulated: miRNAs downregulated in both isolated gland and stroma cells compared with paired non-cancer samples.

↓, lower expression in CRC compared with paired non-cancer intact tissues; ↑, higher expression in CRC compared with paired non-cancer intact tissues.

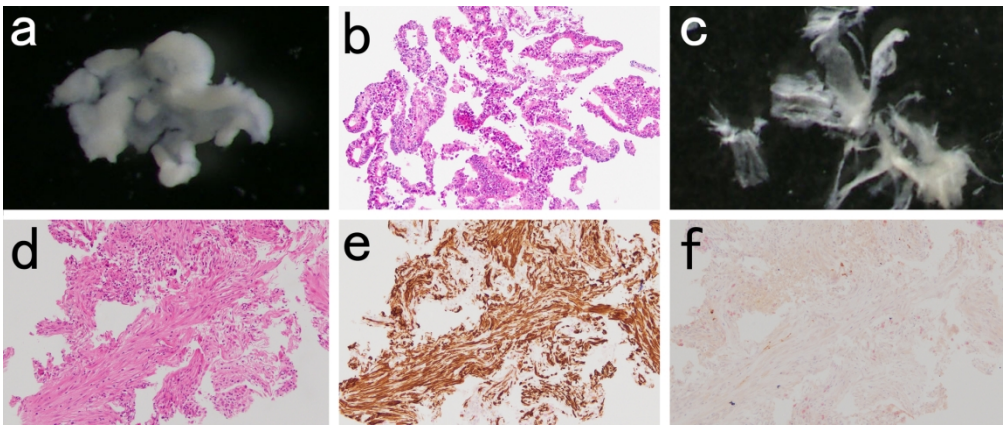

Figure 1

169x71mm (300 x 300 DPI)

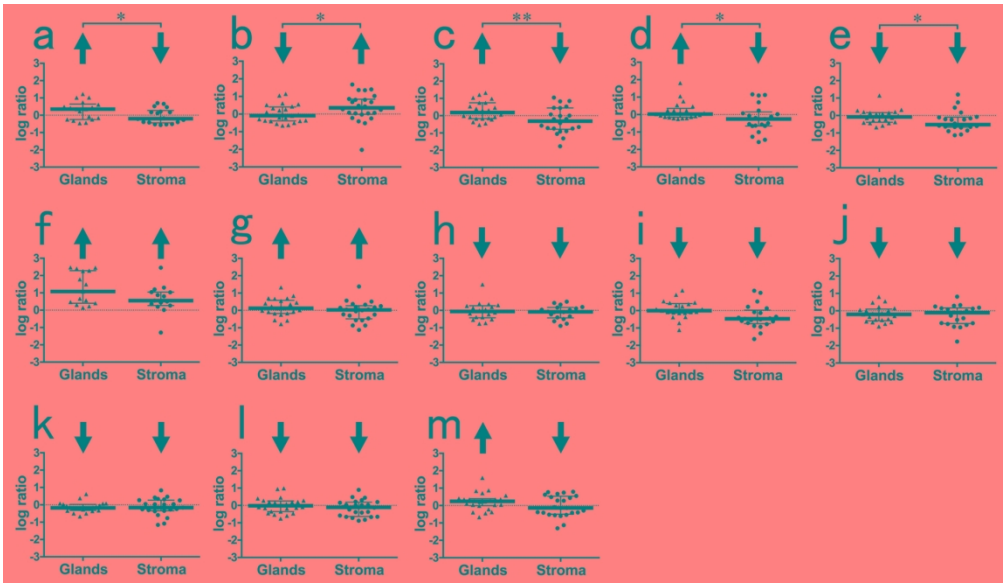

Figure 2

170x98mm (300 x 300 DPI)

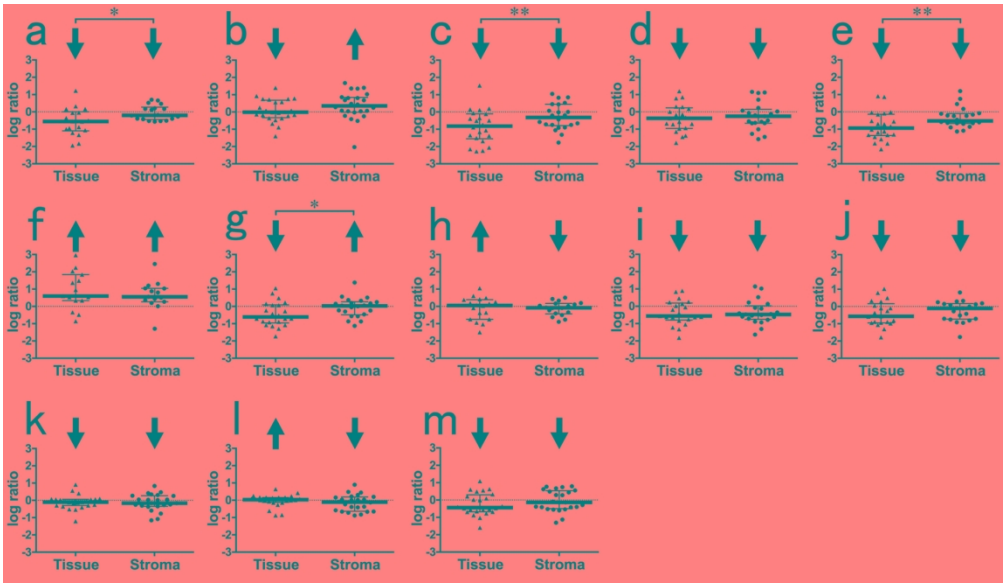

Figure 4

170x98mm (300 x 300 DPI)

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Supplementary Table: Primer sequences for the microRNAs examined

MicroRNA target	**Sequence (5' - 3')**
hsa-miRNA-19a-3p	UGUGCAAAUCUAUGCAAAACUGA
hsa-miRNA-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miRNA-27a-3p	UUCACAGUGGCUAAGUUCCGC
hsa-miRNA-27b-3p	UUCACAGUGGCUAAGUUCUGC
hsa-miRNA-31-5p	AGGCAAGAUGCUGGCAUAGCU
hsa-miRNA-34b-3p	CAAUCACUAACUCCACUGCCAU
hsa-miRNA-130a-3p	CAGUGCAAUGUUAAAAGGGCAU
hsa-miRNA-143-3p	UGAGAUGAAGCACUGUAGCUC
hsa-miRNA-186-5p	CAAAGAAUUCUCCUUUUGGGCU
hsa-miRNA-191-5p	CAACGGAAUCCCAAAAGCAGCUG
hsa-miRNA-195-5p	UAGCAGCACAGAAAUAUUGGC
hsa-miRNA-206	UGGAAUGUAAGGAAGUGUGUGG
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU