

Over- and under-expressed microRNAs in human colorectal cancer

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Abstract. MicroRNAs (miRNAs) constitute a class of small (21-23 nucleotides) noncoding RNAs that function as post-transcriptional gene regulators. It is becoming increasingly clear that altered miRNA expression correlates with the pathogenesis of cancers. The purpose of this study was to determine the up-regulated miRNAs in human colorectal cancer. Total RNA was isolated from cancer tissues and corresponding noncancerous tissues from surgically resected colorectal cancers. The expression profiles of miRNAs were determined using a miRNA microarray containing 455 human miRNA probes. The expression status of selected miRNAs in paired clinical samples was then investigated by real-time RT-PCR. Twenty-one miRNAs were identified by miRNA array analysis as overexpressed in colorectal cancer tissues compared to normal epithelial tissues. Among them, the expression of *miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* were confirmed to be significantly higher in cancer tissues than in normal tissues ($P < 0.05$). In contrast, the expression of *miR-143* and *miR-145* in cancer tissues were significantly lower than in normal tissues ($P < 0.05$). The *miR-18a* overexpression group tended to have a poorer clinical prognosis than the low expression group ($P = 0.07$). We identified miRNAs that were overexpressed or under-expressed in colorectal cancers and which may be correlated with colorectal carcinogenesis.

Introduction

MicroRNAs (miRNAs) are evolutionarily-conserved, endogenous, small, noncoding RNA molecules of ~21-23

nucleotides that function as post-transcriptional gene regulators (1-4). Mature miRNAs are integrated into a ribonucleoprotein complex called the RNA-inducing silencing complex (RISC) and associate with 3' untranslated regions (3'UTRs) of specific target messenger RNAs (mRNAs) to suppress translation and also to occasionally induce mRNA decay (5-9). It is estimated that vertebrate genomes encode up to 1,000 unique miRNAs, each of which is thought to regulate the expression level of a target gene (10). Up to 30% of human genes are thought to be regulated by miRNAs; however, most of the targets remain unknown (11). Recent evidence has shown that miRNAs are involved in regulation of cellular development, differentiation, proliferation and apoptosis (12).

More than 500 miRNAs have been identified in humans and more than half of human miRNAs are located at specific chromosomal regions, including fragile sites, as well as in regions that are frequently amplified, deleted, or rearranged in cancers (13,14). Recent evidence has shown that altered expression of miRNAs is associated with the pathogenesis of various human cancers and has indicated that some miRNAs may function as oncogenes or tumor suppressors (15-20). A number of studies were recently published that focus on the significance of miRNAs in colorectal cancer (21-26). Although assays such as Northern blots and real-time RT-PCR are important in understanding the expression status of individual miRNAs, comprehensive microarray analysis using clinical samples is needed to elucidate the clinical significance of miRNAs in colorectal cancer.

In this study, a microRNA microarray containing 455 human miRNA probes was used to determine expression profiles in colorectal cancer tissue and 21 up-regulated colorectal cancer-related miRNAs were identified. Expression of *miR-31* in cancers was significantly higher than in normal tissues on 69 clinical colorectal cancers by real-time RT-PCR, suggesting that *miR-31* may be one of the potent colorectal cancer-related miRNAs. The *MiR-17-92* cluster may also play an important role in colorectal cancer progression. Furthermore, we demonstrate that *miR-18a* expression could be used as a prognostic factor in predicting survival of colorectal cancer patients.

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Materials and methods

Patients and clinical samples. Samples of cancerous tissue and matched noncancerous tissues were obtained from 69 patients with colorectal cancer who underwent surgical resection at Kyushu University Hospital (Beppu, Japan). None of the patients received preoperative treatments, such as radiation and/or chemotherapy. The follow-up periods ranged from 0.1 to 11.3 years with a mean of 3.7 years. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board and this study was conducted under the supervision of the ethical board of Kyushu University.

The 69 tumor samples and the matched control samples taken from normal tissue located at a distance from the colorectal cancer were frozen in liquid nitrogen immediately after surgical resection and were stored at -90°C until RNA extraction.

MicroRNA microarray analysis. Total RNAs from tumor and the matched control samples of 4 of 69 cases were analyzed by microRNA microarray. Total RNA was extracted from tissue using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Concentration and purity of the total RNAs were assessed by a spectrophotometer and RNA integrity was verified using an Agilent 2100 bioanalyzer (Agilent Technologies).

Total RNA (100 ng) was directly labeled with cyanine 3-CTP (Cy3), without fractionation or amplification, using an Agilent protocol that produces precise and accurate measurements spanning a linear dynamic range from 0.2 amol to 2 fmol of input miRNA. Each (100 ng) of 4 total RNAs from cancer tissue samples and a mixture of total RNAs (100 ng) extracted from normal epithelial tissues were competitively hybridized to a miRNA array (Agilent Microarray Design ID = 014947, Early Access version) containing 455 miRNAs, according to the manufacturer's protocol (27). A list of miRNAs contained in the array is available from version 8.2 of the Sanger miRNA database (<http://microrna.sanger.ac.uk>).

Data analysis. The intensity of each hybridization signal was evaluated using Feature extraction Software (Agilent Technologies). Feature Extraction analysis examines multiple probes and multiple features per probe and studies the measurements and errors for each miRNA. The observed values were imported into GeneSpring GX version 7.3 (Agilent Technologies). Generated miRNA profiles were normalized to the amount of input total RNA. A miRNA was designated overexpressed if expression was >2.6 -fold compared to normal epithelial rectal tissues in all 4 clinical colorectal cancer samples (Table I).

MiRNA real-time RT-PCR. *MiR-31*-, *miR-183*-, *miR-17-5p*-, *miR-18a*-, *miR-20a*-, *miR-92*- and *RNU6B* (internal control)-specific cDNAs were synthesized from total RNAs extracted from a maximum of 69 paired clinical samples using gene-specific primers according to *TaqMan* MicroRNA assays (Applied Biosystems). Reverse transcriptase reactions contained 10 ng of total RNA, 50 nM stem-loop RT primer,

Table I. Clinicopathological data in 4 colorectal cancers for microRNA microarray.

Case	1	2	3	4
Age	42	65	68	67
Gender	Female	Male	Male	Female
Tumor size (cm)	3.5x3.5	4.1x3.5	2.5x2.5	3.5x2.5
Location	S	Rb	S	S
Histological type	Well	Well	Well	Well
Lymph node metastasis	(+)	(+)	(-)	(-)
Lymphatic invasion	(+)	(-)	(-)	(-)
Venous invasion	(+)	(+)	(-)	(-)
Depth	se	se	sm	ss
Peritoneal dissemination	(-)	(-)	(-)	(-)
Liver metastasis	(-)	(-)	(-)	(-)
Stage	IIIa	IIIa	I	II
Outcome	Alive	Death	Alive	Alive

S, sigmoid colon; Rb, rectum (below the peritoneal reflection); well, well-differentiated adenocarcinoma; sm, submucosa; ss, subserosa and se, serosa.

1X RT buffer, 0.25 mM each of dNTPs, 3.33 U/ μl MultiScribe reverse transcriptase and 0.25 U/ μl RNase Inhibitor. The 7.5 μl reaction volumes were incubated in a 96-well plate in a Bio-Rad iCycler (Bio-Rad Laboratories) for 30 min at 16°C , 30 min at 42°C , 5 min at 85°C and were then held at 4°C .

Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR system. Each 10 μl PCR volume included 0.67 μl RT products, 1X *TaqMan* Universal PCR master mix and 1 μl of primers and probe mix from each *TaqMan* microRNA assay. The reactions were incubated in 96-well optical plates at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 10 min. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, with the ratio of the median expression sample among all tumor samples/all non-tumor samples being used as the calibrator. The expression level of each miRNA was normalized to *RNU6B* expression.

Statistical analysis. Biostatistical analyses were performed with JMP 5.0.1a for Windows software (SAS Institute). Possible differences between groups were analyzed using the Student's t-test. Survival curves were obtained by the Kaplan-Meier method (28); comparison between curves was made by log-rank test. A probability level of 0.05 was chosen for statistical significance.

Results

Identification using miRNA array analysis of miRNAs that are overexpressed in clinical colorectal cancer. To investigate the differential expression of miRNAs in human colorectal cancers, array-based miRNA profiling of human colorectal

Table II. Twenty-one miRNAs up-regulated in clinical human colorectal cancers by miRNA array analysis.^a

MicroRNA	Cases	Fold change
<i>hsa-miR-31</i>	4/4	179.29
<i>hsa-miR-18b</i>	4/4	175.71
<i>hsa-miR-30e-3p</i>	4/4	71.82
<i>hsa-miR-220</i>	4/4	51.05
<i>hsa-miR-570</i>	4/4	35.25
<i>hsa-miR-302b</i>	4/4	31.51
<i>hsa-miR-302a</i>	4/4	27.81
<i>hsa-miR-183</i>	4/4	18.94
<i>hsa-miR-224</i>	4/4	14.46
<i>hsa-miR-18a</i>	4/4	10.49
<i>hsa-miR-95</i>	4/4	10.13
<i>hsa-miR-7</i>	4/4	7.96
<i>hsa-miR-182</i>	4/4	5.63
<i>hsa-miR-17-5p</i>	4/4	4.83
<i>hsa-miR-550</i>	4/4	4.74
<i>hsa-miR-196b</i>	4/4	4.61
<i>hsa-miR-181d</i>	4/4	4.42
<i>hsa-miR-20a</i>	4/4	4.38
<i>hsa-miR-92</i>	4/4	4.14
<i>hsa-miR-493-3p</i>	4/4	4.07
<i>hsa-miR-29a</i>	4/4	3.75

^aFold change of miRNA expression was calculated relative to normal colorectal epithelial tissues. The bolded miRNAs signify those that were analysed by real-time RT-PCR.

cancer was performed. Out of 455 human miRNAs assayed, 21 were identified that had higher expression levels in colorectal cancer tissues than in normal epithelial tissues (Table II). *MiR-31* was the most up-regulated miRNA in the colorectal cancer tissues analyzed. Among the up-regulated miRNAs, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* are included in the *miR-17-92* cluster.

Real-time RT-PCR analysis of mature miRNAs. A maximum of 69 paired clinical samples were analyzed by real-time RT-PCR to quantify the expression of six up-regulated miRNAs (*miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92*). The mean expression levels of *miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* were higher in tumor than in non-tumor tissues ($P < 0.05$, Fig. 1). The percentages of cases in which the expression levels of *miR-31*, *miR-183*, *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* were higher in tumor than in non-tumor tissues, were 63.8, 83.9, 71.6, 76.9, 77.6 and 66.7%, respectively (Table III).

We then investigated the expression levels of *miR-143* and *miR-145*, which were reported as down-regulated miRNAs in colorectal cancer (21,22,25,29). The mean expression levels of *miR-143* and *miR-145* were lower in tumor than in non-tumor tissues ($P < 0.05$, Fig. 2). The percentages of cases in which the expression levels of *miR-143* and *miR-145* were lower in tumor than in non-tumor tissues, were 72.1 and 68.2%, respectively (Table III).

High *miR-18a* expression correlates with poor prognosis. We next surveyed the relationship between the expression of *miR-18a* and prognosis in colorectal cancer patients. Based upon the mean expression level of *miR-18a*, 65 clinical cases were divided into two groups: high *miR-18a* expression ($n=21$) and low *miR-18a* expression ($n=44$). The *miR-18a* overexpression group tended to have a poorer clinical prognosis than the low expression group ($P=0.07$; Fig. 3).

Table III. The expression level of up-regulated and down-regulated miRNAs were surveyed on paired clinical samples of colorectal cancer by real-time RT-PCR.

Up-regulated miRNAs	<i>miR-31</i>	<i>miR-183</i>	<i>miR-17-5p</i>	<i>miR-18a</i>	<i>miR-20a</i>	<i>miR-92</i>
Up-regulated/all cases (%)	44/69 (63.8)	52/62 (83.9)	48/67 (71.6)	50/65 (76.9)	52/67 (77.6)	46/69 (66.7)
Mean expression level in tumor (mean \pm SD)	3.80 \pm 8.50	2.08 \pm 2.92	2.34 \pm 3.12	1.97 \pm 3.08	1.38 \pm 1.59	1.84 \pm 2.07
Mean expression level in non-tumor (mean \pm SD)	0.70 \pm 0.8	0.39 \pm 0.38	1.01 \pm 1.27	0.86 \pm 1.23	0.48 \pm 0.55	1.03 \pm 0.83
Down-regulated miRNAs	<i>miR-143</i>	<i>miR-145</i>				
Down-regulated/all cases (%)	31/43 (72.1)	30/44 (68.2)				
Mean expression level in tumor (mean \pm SD)	0.87 \pm 1.06	0.96 \pm 1.30				
Mean expression level in non-tumor (mean \pm SD)	1.58 \pm 1.74	1.70 \pm 1.75				

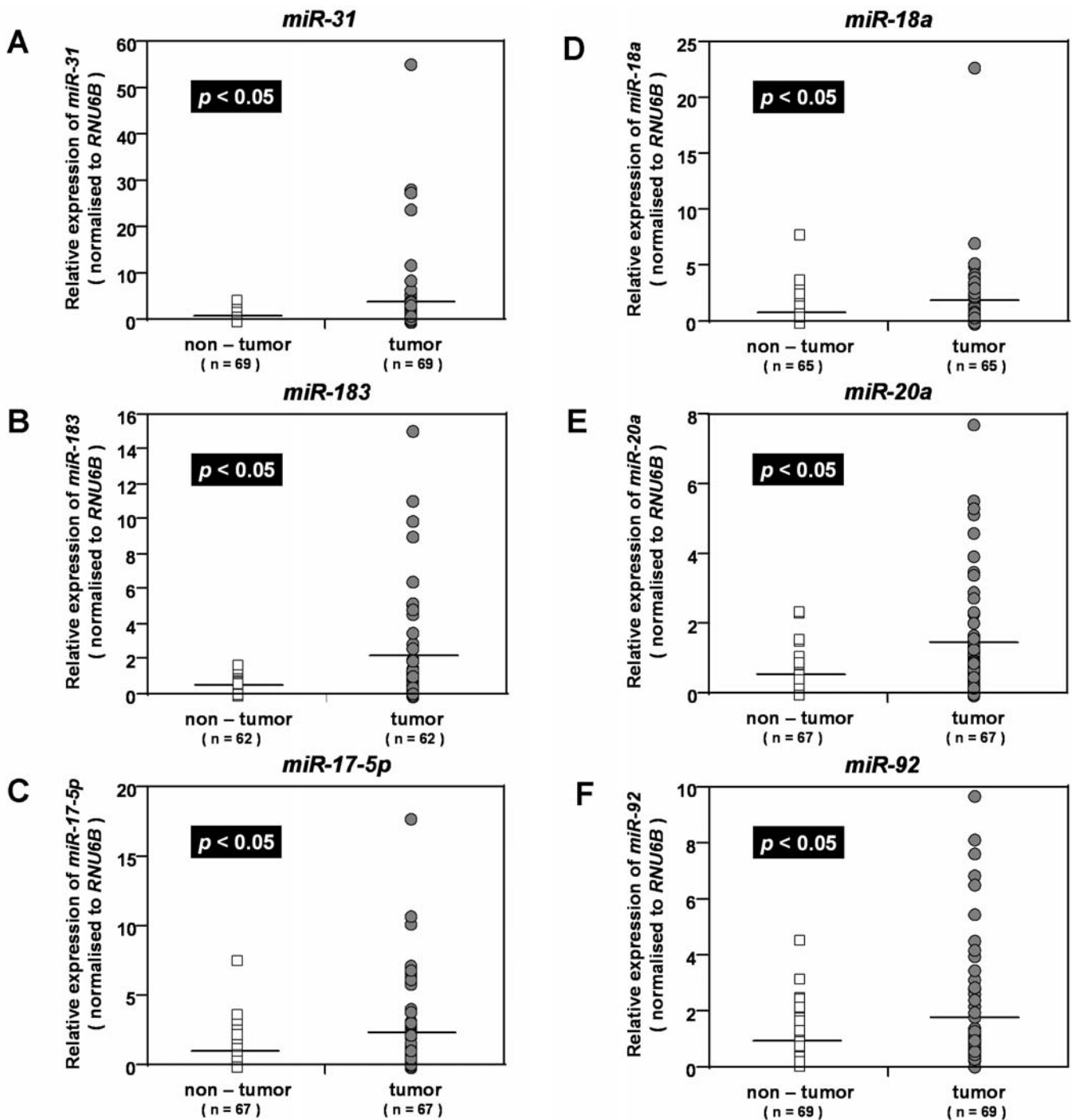


Figure 1. Part 1.

Discussion

Because of the large amount of evidence indicating that miRNAs are involved in carcinogenesis (15-20), it is important to identify colorectal cancer-related miRNA profiles by comprehensive analysis to increase understanding of colorectal cancer biology. In this study, a miRNA array with 455 known human miRNAs was applied to clinical samples of colorectal cancers to identify cancer-associated miRNAs. As a result, 21 cancer-related miRNAs were identified that were overexpressed in colorectal cancer tissues compared to normal colorectal epithelial tissues (Table II).

MiRNA expression profiling demonstrated that *miR-31* expression was the highest among the 21 overexpressed miRNAs seen in the colorectal cancer samples that were assayed (Table II). In addition, real-time RT-PCR analysis of 69 clinical colorectal cancers showed that *miR-31* expression was significantly higher in cancer than in normal tissues (Fig. 1A and a). Bandres *et al* surveyed the expression of 156 mature miRNAs in 16 colorectal cancer cell lines and 12 matched pairs of tumor and non-tumor tissues by real-time PCR and reported that *miR-31* was one of the seven overexpressed miRNAs and was associated with tumor stages of colorectal cancer (24). Slaby *et al* reported that the

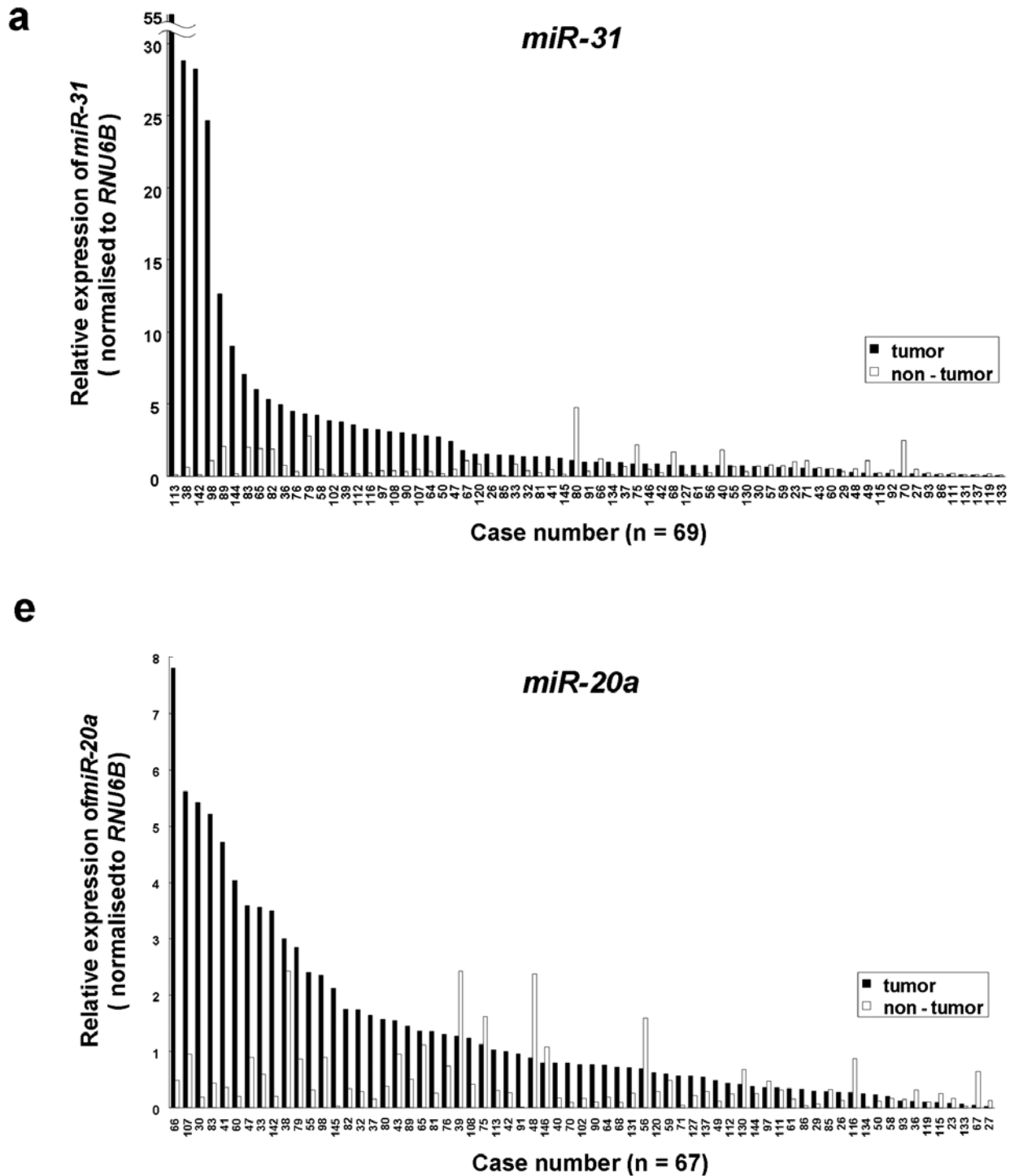


Figure 1. Part 2. Real-time RT-PCR analysis of 6 up-regulated miRNAs in tumor and non-tumor samples from colorectal cancer cases. Expression values of miRNAs were shown as the expression ratio of each miRNA to *RNU6B*. (A, a) *miR-31* expression; (B) *miR-183* expression; (C) *miR-17-5p* expression; (D) *miR-18a* expression; (E, e) *miR-20a* expression; (F) *miR-92* expression. Horizontal lines indicate the mean values of tumor and non-tumor samples.

expression of *miR-31* was up-regulated in 29 primary colorectal cancers (29). These studies suggest that *miR-31* is one of the important colorectal cancer-related miRNAs. Up-regulated miRNAs listed by Bandres *et al* included *miR-183* and *miR-20a*, which is consistent with our data that *miR-183* and *miR-20a* were overexpressed in our colorectal cancer samples by both miRNA array and real-time RT-PCR analysis (Table II, Fig. 1B, E and e).

We demonstrated that expression of *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92*, which are cleavage products of the

miR-17-92 cluster, were individually up-regulated in our miRNA expression profiles and that by real-time RT-PCR analysis, their expression in clinical sample cancer tissues were also significantly higher than in normal tissues (Table II, Fig. 1C-F). The *miR-17-92* cluster is comprised of 6 miRNAs (*miR-17-5p*, *miR-18a*, *miR-19a*, *miR-19b-1*, *miR-20a* and *miR-92*) (30). The *miR-17-92* cluster is overexpressed in malignant lymphoma cell lines and lung cancers (31,32) and cooperates with c-MYC to accelerate tumor development (31). The introduction of *miR-17-92* can enhance the growth

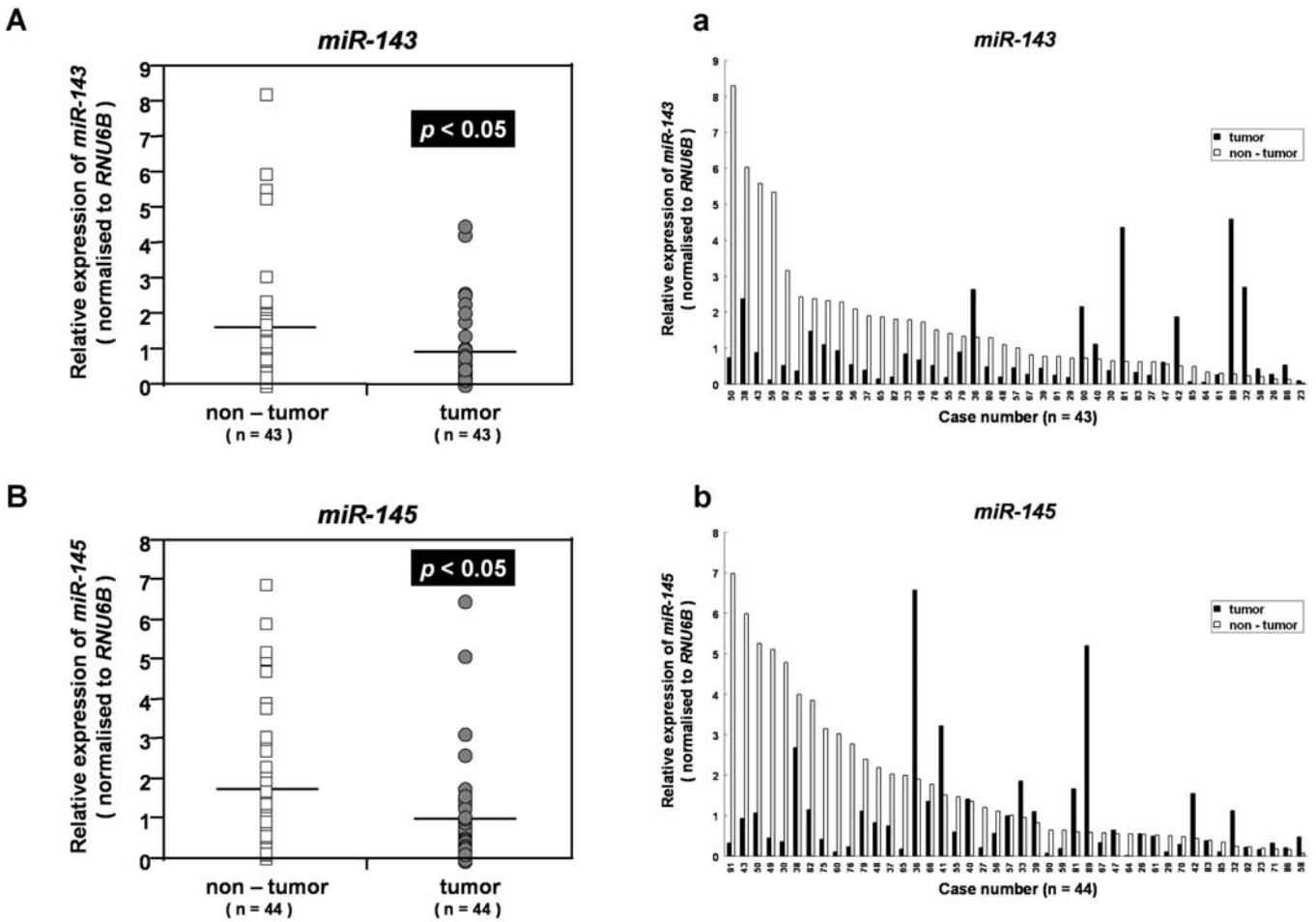


Figure 2. Real-time RT-PCR analysis of 2 down-regulated miRNAs in tumor and non-tumor samples from colorectal cancer cases. (A, a) *miR-143* expression; (B, b) *miR-145* expression. Horizontal lines indicate the mean values of tumor and non-tumor samples.

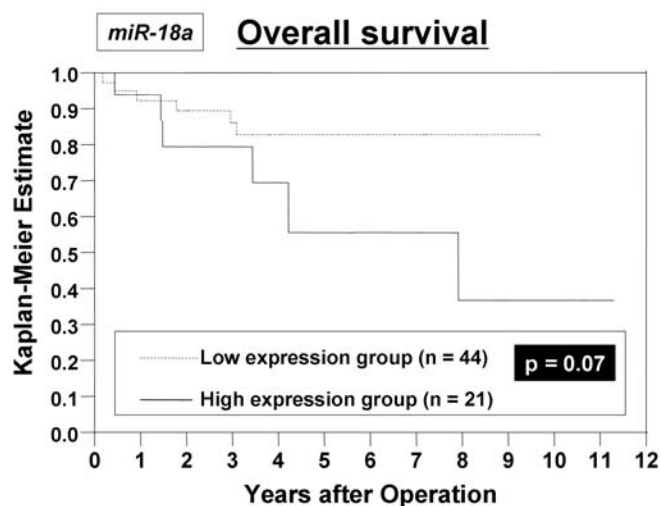


Figure 3. Survival curves of colorectal cancer patients according to *miR-18a* expression status. High expression group (n=21): *miR-18a* > mean value; low expression group (n=44): *miR-18a* < mean value. By the log-rank test, patients with high *miR-18a* expression tended to have poorer prognoses than those with low *miR-18a* expression (P=0.07).

property of lung cancer cells *in vitro* (32). Additional evidence indicates that *miR-17-92* can be a tumor angiogenesis mediator (33) and affects the expression of the members of the E2F

family of oncogenic transcription factors (34-36). These studies suggest that the *miR-17-92* polycistron may be the most prominent oncogenic miRNA cluster. Most recently, Ventura *et al* reported a link between the oncogenic properties of *miR-17-92* and its functions during B cell lymphopoiesis and lung development (37).

In clinical colorectal cancers, He *et al* reported that the level of *miR-17-92* pri-miRNA was up-regulated in 15% of tested samples and showed >5-fold up-regulation compared to corresponding normal tissues by real-time quantitative PCR (31). In this study, the percentages of cases with >5-fold expression levels of *miR-17-5p*, *miR-18a*, *miR-20a* and *miR-92* in clinical colorectal cancer tissues were 29.2, 19.4, 31.3 and 11.6%, respectively. Volinia *et al*, using prediction analysis of microarrays (PAM), reported that 21 miRNAs were overexpressed in 46 colorectal cancer samples compared to 8 normal colorectal tissues (23) and that elevated expression levels of *miR-17-5p* and *miR-20a* were found by miRNA expression profiling. Schetter *et al* reported that 27 miRNAs were overexpressed in miRNA array expression profiling of 84 colorectal tumor and paired non-tumorous tissues, which included *miR-17-5p*, *miR-20a* and *miR-92* (26). Matsubara *et al* showed that inhibition of *miR-17-5p* and *miR-20a* expression by antisense oligonucleotides could selectively induce apoptosis in lung cancer cells that overexpressed *miR-17-92* (38). To our knowledge, there have been no studies

published that elucidate the biology of *miR-17-5p* and *miR-20a* in human colorectal cancer.

Recently, *miR-200c* (39) and *miR-21* (26) expression were reported to be associated with poor survival in colorectal cancer patients. In this study, we demonstrated that the *miR-18a* high expression group tended to have a poorer prognosis than the low expression group ($P=0.07$; Fig. 3) and we believe that *miR-18a* expression can be used as a prognostic factor in predicting survival of postoperative colorectal cancer patients.

The expression of *miR-143* and *miR-145* are down-regulated in colorectal tumors and their *in vitro* transfection into human colon cancer cell lines (DLD-1, SW480) led to growth inhibition (21,22,25). In our study, real-time RT-PCR analysis showed that expression of *miR-143* and *miR-145* were significantly lower in 43 and 44 clinical colorectal cancers, respectively, than in normal tissues ($P<0.05$; Fig. 2).

In conclusion, this study identified 21 up-regulated miRNAs in human colorectal cancers. *MiR-31*, *miR-183*, *miR-18a*, *miR-17-5p*, *miR-20a* and *miR-92* were significantly overexpressed in cancer compared to normal tissues. The advent of miRNA research may lead to possible applications to molecular diagnostics and prognostics in colorectal cancer. More study is required to clarify the precise contributions of miRNAs to colorectal cancer progression.

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