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Non-coding MicroRNAs *hsa-let-7g* and *hsa-miR-181b* are Associated with Chemoresponse to S-1 in Colon Cancer

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Abstract. *Background: MicroRNAs (miRNAs) are small non-coding RNAs (~22 nucleotides) that regulate gene expression at a post-transcriptional level via imperfect base pairing to the 3'-UTR of their target mRNAs. Previous studies from our group identified a number of deregulated miRNAs due to the loss of p53 tumor suppressor in colon cancer cell lines. To further investigate the in vivo biological significance of these miRNAs, the expressions of hsa-let-7g, hsa-miR-143, hsa-miR-145, hsa-miR-181b and hsa-miR-200c were investigated using formalin-fixed paraffin-embedded (FFPE) colon cancer specimens to evaluate the potential relationship with chemosensitivity and tumorigenesis. Patients and Methods: Forty-six patients with recurrent or residual colon cancer lesions were treated with the 5-fluorouracil-based antimetabolite S-1. This includes twenty-one pairs of tumor and normal samples. Total RNAs were isolated and the expression level of each particular miRNA was quantified using real time qRT-PCR analysis. Results: The expression levels of hsa-let-7g, hsa-miR-181b and hsa-miR-200c were over-expressed in tumor tissues compared to normal tissues. The expression levels of hsa-let-7g ($p=0.03$; Mann-Whitney test) and hsa-miR-181b ($p=0.02$; Mann-Whitney test) were strongly associated with clinical response to S-1. Although hsa-let-7g and hsa-miR-181b are strongly associated with patient's response to S-1 treatment, they are not significant prognostic factors for predicting survival. Conclusion: hsa-let-7g, hsa-miR-181b and hsa-miR-200c may be associated with tumorigenesis in colon cancer. In addition, hsa-let-7g and hsa-miR-181b may be potential indicators for chemoresponse to S-1 based chemotherapy.*

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5-Fluorouracil (5-FU) has been one of the main anti-neoplastic drugs for treating various solid tumors for nearly a half century (1). The response rate for colorectal cancer, however, is only 14% with bolus 5-FU infusion and 22%, despite continuous infusion (2). Extensive effects have been focused on improving the response rate and survival by enhancing 5-FU cytotoxicity through various modulations. The recent standard regimen, 5-FU/leucovorin, generated higher response rate (23%) compared to 5-FU alone (11%) (3). 5-FU/leucovorin/oxaliplatin has now become one of the standard treatments for advanced colorectal cancer with improved response rate (4). S-1 (TS-1; Taiho Pharmaceutical, Tokyo, Japan) is a fourth-generation 5-FU based oral drug developed to improve the drug efficacy, to reduce side-effects and improve quality of life (5). It is the first-line therapy for treating advanced colon cancer in Japan and the administration of S-1 alone has shown better response rate of 24.3~39.5% in several phase II studies for colorectal cancer (6-8). The most recent multi-center phase II clinical trial also showed a great promise for S-1 plus cisplatin in treating advanced gastric or gastroesophageal junction adenocarcinoma (9). S-1 is composed of 5-FU prodrug tegafur (FT), a dihydropyrimidine dehydrogenase inhibitor gimestat (CDHP) and an inhibitor of orotate phosphoribosyltransferase otastat potassium (Oxo) in a molar ratio of 1:0.4:1, based on the biochemical modulation of 5-FU. Oxo has the potential to reduce 5-FU related gastrointestinal toxicity through its inhibition of orotate phosphoribosyl transferase and 5-FU phosphorylation (10). Over the past decade, extensive efforts have been focused on further improving patient response to 5-FU-based therapy using biomarkers to predict chemoresponse and disease prognosis. The expression levels of several mRNAs, such as thymidylate synthase (TS), dihydroprymidine dehydrogenase (DPD), thymidine phosphorylase (TP), were shown to be significant predictors for 5-FU-based therapy (11-13). However, due to the complexity of the disease, additional biomarkers are clearly needed to further improve the accuracy of the prediction.

Previous studies from our laboratory have identified a number of deregulated microRNAs (miRNAs) due to p53

deletion using a colon cancer cell line model (14). Over 64% of miRNA promoters contain putative p53 consensus binding site(s). A number of deregulated miRNAs were discovered due to the loss of p53 tumor suppressor in colon cancer cell lines. It was well established that the tumor suppressor p53 is one of the most frequently mutated genes in colorectal and other types of solid tumors (15, 16). It is reasonable to speculate that p53 may act through some of these miRNAs to mediate its down stream gene expression.

miRNAs have recently emerged as new players to regulate gene expression at the level of mRNA translation. miRNAs are non-coding RNAs that regulate gene expression by suppressing their target mRNAs at the post-transcriptional level. At present, over 300 mammalian miRNAs have been identified (17-23) and some of them have been shown to play important roles in the regulation of cell differentiation, proliferation and apoptosis (24-30). Each individual miRNA can potentially regulate mRNA translation for up to several hundred genes *via* imperfect base pairing based on bioinformatics analysis. We reasoned that certain miRNAs may be a better class of biomarkers because of their broad regulatory function and since they are far less noisy than mRNAs. In addition, these miRNAs are relatively stable due to its stem-loop structure in formalin-fixed paraffin-embedded (FFPE) samples for easy quantification.

To further evaluate the *in vivo* significance of deregulated miRNAs in relationship with chemosensitivity to S-1 treatment, the expression levels of *hsa-let-7g, hsa-miR-143, hsa-miR-145, hsa-miR-181b* and *hsa-miR-200c* were investigated. They were chosen because they were highly over-expressed due to the loss of p53 gene and the critical roles of their down stream mRNA targets (*e.g., Ras, cyclin-D, E2F, c-myc, cytochrome C, ECIP-1, MAPPKKK1, TEM6, E2F5, GATA6, PP2B* and *eIF5A*). Our results showed that the expressions of *hsa-let-7g, hsa-miR-181b* and *hsa-miR-200c* were over-expressed in colorectal cancer. In addition, the expressions of *hsa-let-7g* ($p=0.03$) and *hsa-miR-181b* ($p=0.02$) were significantly associated with patient response to S-1-based chemotherapy.

Patients and Methods

Patients and samples. The paraffin sections of the colorectal cancer samples were obtained from the Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Japan. Informed consent forms from all patients were obtained before conducting the treatment or study. Forty-six patients had undergone surgical removal from 1996 to 2002. Among the 46 patient samples, 21 normal samples were also obtained. Patients who had recurrence or residual colorectal cancer lesions were treated with S-1 alone or S-1 plus cisplatin (CDDP). The dose of S-1 was determined based on patient's body surface area (BSA) as follows: <1.25 m^2; 80 mg/day, ≥ 1.25 and < 1.5 m^2; 100 mg/day, >1.5 m^2; 120 mg/day. The regimen of the treatment was the following: S-1 alone: S-1 was administered twice a day orally for 28 consecutive days, followed by a two-week drug-free interval; S-1/CDDP: S-1 was administered twice-daily *via* oral administration, followed by 2-weeks rest, while CDDP (30 mg/m^2) was injected intravenously on day 1 and 8.

Table I. *Patient characteristics.*

Characteristics	Distribution
Age (years)	
range	32 ~ 78
mean	58.98
Gender	
male	25 (54.3%)
female	21 (45.7%)
Primary site	
cecum	2 (4.3%)
ascending	6 (13.0%)
transverse	2 (4.3%)
descending	4 (8.7%)
sigmoid	15 (32.6%)
rectum	17 (37.0%)
Origin of the sample	
primary	40 (87.0%)
metastasis	6 (13.0%)
Differentiation	
well	30 (65.2%)
moderate	14 (30.4%)
poor	0 (0%)
mucinous	1 (2.2%)
unknown	1 (2.2%)
Treatment	
S-1 + CDDP	14 (30.4%)
S-1 alone	32 (69.6%)

FFPE tissues were prepared using the standard method of the Department of Pathology, Tokyo Women's Medical University, Japan. The paraffin blocks were cut into 10-μm sections. The patients' characteristics are summarized in Table I.

Total RNA extraction. The archived colorectal cancer FFPE specimens (contained either >90% tumor or >90% normal tissues) were dissected and placed in nuclease-free microcentrifuge tubes. The deparaffinization was performed by adding 1 ml of Xylene and vortexing for 5 min at room temperature. The samples were incubated for 3 min at 60˚C. After incubation, samples were centrifuged at 14,000 rpm for 7 min at room temperature. The supernatants were removed and 1 ml of 100% ethanol was added with vortexing for 7 min at room temperature. The samples were centrifuged at 14,000 rpm for 7 min at room temperature. After the supernatants were removed, ethanol washes were repeated. After centrifugation, the samples were air dried and 180 μl of digestion buffer (30 mM Tris-HCl, 20 mM EDTA, 1% SDS and nuclease-free water) were added to the samples followed by homogenization. After homogenization, 20 μl of Proteinase K (QIAGEN Inc., Valencia, CA, USA) solution were added and the samples were incubated for additional 3 h at 56˚C. Subsequently, 500 μl of TRIzol® (Invitrogen Inc., Carlsbad,

CA, USA) were added and folowed by vortexing for 5 min at room temperature. Fifty μl of 1-bromo-3-chloropropane (BCP) solution was added and vortexed for 2 min. The samples were incubated for 3 min at room temperature. After centrifugation at 14,000 rpm for 7 min at 4˚C, the upper aqueous phase containing extracted RNA was transferred to the new microcentrifuge tube. One hundred μg of glycogen were added and mixed by vortexing. Samples were precipitated with 500 μl of 100% isopropanol and were incubated for 60 min at –20˚C. The RNA samples were centrifuged at 14,000 rpm for 7 min at 4˚C. After removing the supernatant, 1 ml of 75% ethanol was added and the samples were centrifuged at 14,000 rpm for 7 min at 4˚C. Upon removal the supernatant, the RNA pellet was air dried and resuspended with nuclease-free water.

*Reverse transcription (RT). mir*Vana™ qRT-PCR miRNA Detection Kit and *mir*Vana™ qRT-PCR Primer Sets (Ambion Inc., Austin, TX, USA) for *hsa-let-7g, hsa-miR-143, hsa-miR-145, hsa-miR-200c* and 5S ribosomal RNA as an internal control specific reverse transcription were utilized based on the manufactures' protocol. Briefly, the reaction mixture containing *mir*Vana™ 5x RT Buffer, 1x *mir*Vana™ RT Primer, ArrayScript™ Enzyme Mix and nuclease-free water were mixed with 20 ng of each total RNA. The mixture was incubated for 30 min at 37˚C and then for 10 min at 95˚C. TaqMan® MicroRNA Assay (Applied Biosystems Inc., Foster City, CA, USA) was used for *hsa-miR-181b* based on the manufacturer's protocol. Briefly, the reaction master mix containing 10x RT Buffer, 5x RT Primer for *hsa-miR-181* and *RNU6B* as an internal control, MultiScribe Reverse Transcriptase, RNase Inhibitor and nuclease-free water were mixed with 20 ng of total RNA.

Quantitative real-time PCR. Quantitative real-time PCR was carried out using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Inc.). For *hsa-let-7g, hsa-miR-143, hsa-miR-145* and *hsa-miR-200c*, the PCR master mix of a total reaction volume of 25 μl for each reaction containing *mir*Vana™ 5x PCR Buffer (with SYBR® Green I), 50x ROX, SuperTaq™ Polymerase, *mir*Vana™ PCR Primers for each target, nuclease-free water and RT products was prepared. The PCR condition was as follows: 95˚C for 3 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 35 sec. For *hsa-miR-181b*, the PCR master mix of total reaction volume of 20 μl for each reaction containing TaqMan 2x Universal PCR Master Mix (No AmpErase UNG), *hsa-miR-181b* and *RNU6B* 10x TaqMan Assay, nuclease-free water and 1.33 μl of RT products was prepared. PCR conditions were 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

Data analysis. The threshold cycle (C_T) value for each target was determined by SDS software v1.2 (Applied Biosystems Inc.). Expression levels of each miRNAs were normalized by calculating the ΔC_T values based on subtracting the C_T value of target miRNA from the C_T value of the internal control 5S ribosomal RNA or *RNU6B*. The sample with the highest expression levels of miRNAs was used as 100% to generate relative expression values. The statistical studies were performed using MedCalc® for Windows, version 8.1.1.0 (MedCalc software, Mariakerke, Belgium). The statistical differences of the expression level between tumor and normal tissues for each target were calculated by Wilcoxon test. The Mann-Whitney test was performed for comparison of chemoresponse data for *hsa-let-7g* and *hsa-miR-181b*. The Log-rank test for Kaplan-Meier curve was generated to evaluate the association between the expression level of each miRNA and survival rate. Statistical significance was set as a $p \leq 0.05$.

Results

Median miRNA expression levels of normal and tumor samples. The tumor tissues from 46 patients were divided into the response group (n=27, including the patients evaluated as complete remission, partial response and stable disease after treatment with S-1) and the disease progression patient group (n=19) according to their response to S-1 treatment. The expression levels of five mature miRNAs in these samples were screened *via* miRNA specific real time qRT-PCR analysis. Based on the real time qRT-PCR analysis, there was no significant difference in expression levels of *hsa-miR-143* and *hsa-miR-145* between tumor tissues and corresponding normal samples ($p=0.1219$; $p=0.0853$, respectively) (Figure 1). In contrast, the expression level of *hsa-miR-200c* was significantly over-expressed by nearly 6-fold in tumor tissues compared to the corresponding normal samples ($p=0.0001$; Figure 2). *Hsa-let-7g* was also significantly over-expressed in tumor tissues compared to corresponding normal colorectal samples ($p=0.0037$; Figure 3). The expression level of *hsa-miR-181b* was elevated in tumors compared to the corresponding normal samples ($p=0.0005$; Figure 4).

Correlation of patient pathological responses with the level of miRNA expression. To evaluate the potential association of certain miRNAs with patient clinical responses, the expression levels of each miRNAs in the response group was compared to the levels in patients with disease progression. Patients who responded to S-1 treatment tend to have lower expression level of *hsa-let-7g* compared to the disease progression group ($p=0.0305$; Figure 5). However, the expression of *hsa-let-7g* was not associated with better survival based on the Kaplan-Meier survival analysis ($p=0.9166$; Figure 6). On the other hand, the expression of *hsa-miR-181b* was strongly associated with patient response (Figure 7). Patients who responded to S-1 treatment also displayed a lower expression level of *hsa-miR-181b* compared to the disease progression group ($p=0.0209$; Figure 7). However, this parameter was not associated with patient survival based on Kaplan-Meier survival analysis ($p=0.1203$; Figure 8).

Discussion

We evaluated the *in vivo* significance of several mature miRNAs using FFPE colorectal cancer patient specimens treated with the oral 5-FU drug S-1. S-1 has been widely used as an oral 5-FU-based drug in Japan for treating

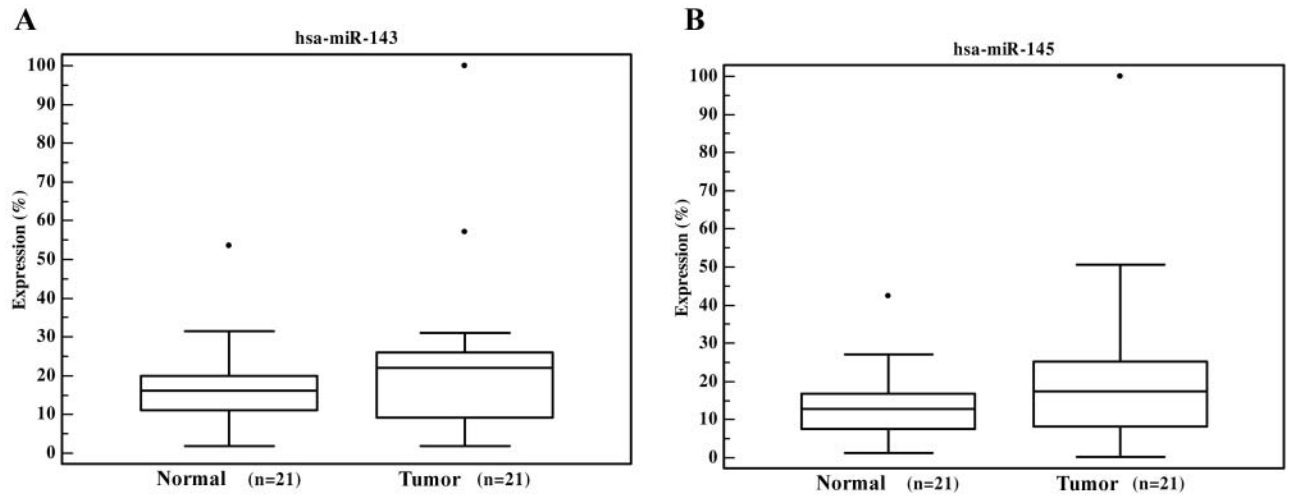

Figure 1. *Hsa-miR-143 (A) and hsa-miR-145 (B) expressions in colorectal cancer and normal tissue specimens. Relative gene expression values were calculated using samples with the highest expression level of miRNA as 100% (see Materials and Methods). Hsa-miR-143 (p=0.1219; Wilcoxon test) (A) and hsa-miR-145 (p=0.0853; Wilcoxon test) (B).*

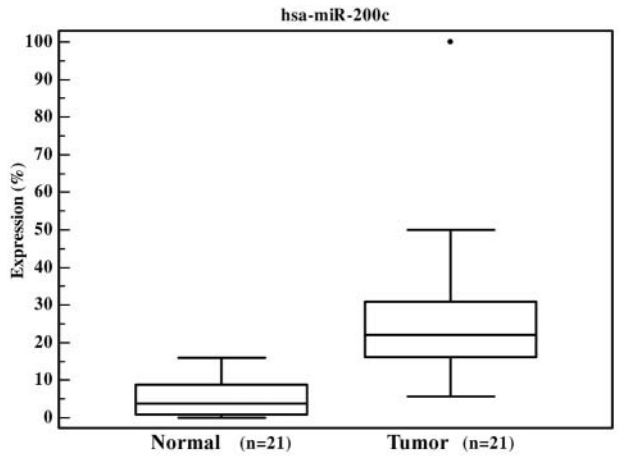

Figure 2. *Hsa-miR-200c expression in colorectal cancer and normal tissue specimens. Relative gene expression values were calculated using sample with the highest expression level of miRNA as 100% (see Materials and Methods). P=0.0001; Wilcoxon test.*

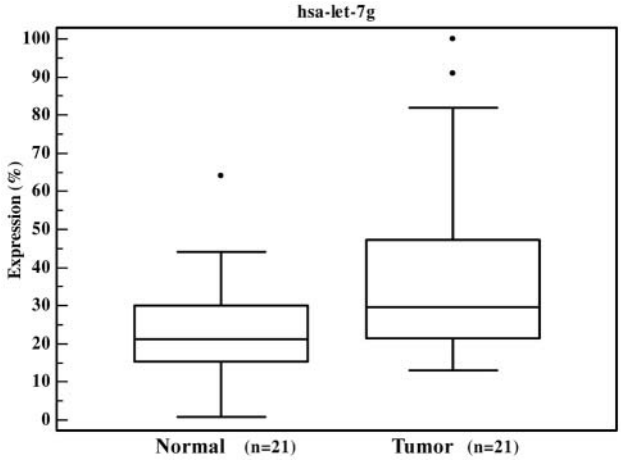

Figure 3. *Hsa-let-7g expression in colorectal cancer and normal tissue specimens. Relative gene expression values were calculated using sample with the highest expression level of miRNA as 100% (see Materials and Methods). P=0.0037; Wilcoxon test.*

advanced colorectal cancer. S-1 was also evaluated in both Europe and the United States recently in clinical trials for treatment of advanced gastric and colorectal cancer with promising results (8, 9, 31, 32). Therefore, it is important to discover new biomarkers that can be used for disease prognosis with S-1-based chemotherapy. Here we focused on small non-coding miRNAs that may be associated with chemoresponse and disease progression. Recent studies from our group identified a number of miRNAs that were found to be deregulated in colon cancer due to the loss of p53 function (14). As mentioned above, there are several advantages of using miRNAs as biomarkers instead of mRNAs. First, there are only over 300 known mammalian miRNAs discovered to date and they can potentially regulate up to 30% of the total genes at the translational level. It is relatively easier to discover reliable biomarkers from approximately 300 miRNA candidates than from over 50,000 genes. As a translational regulator, miRNAs can mediate a number of genes in response to acute cellular stress caused by drug treatment. Another advantage is that due to their small size and stem-loop structure, they are less subjected to degradation, fixation and sample processing.

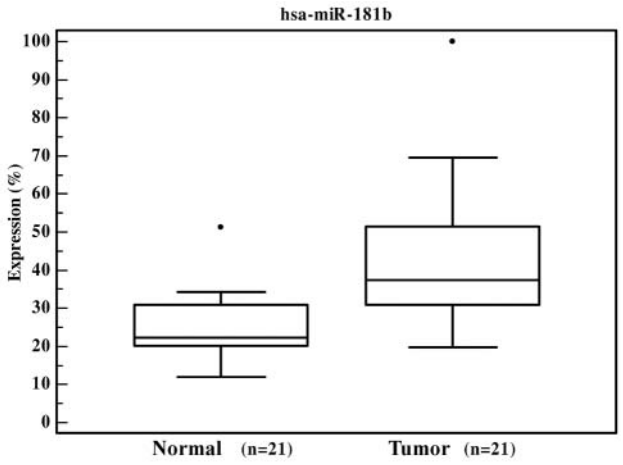

Figure 4. *hsa-miR-181b expression in colorectal cancer and normal tissue specimens. Relative gene expression values were calculated using sample with the highest expression level of miRNA as 100% (Details see Materials and Methods). (p=0.0005; Wilcoxon test).*

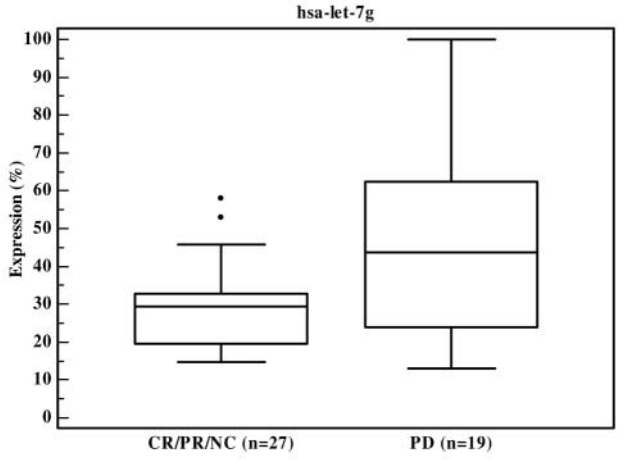

Figure 5. *Response based on hsa-let-7g expression (p=0.0305; Mann-Whitney test). CR=complete response; PR=partial response; NC=no change; PD=progressive disease.*

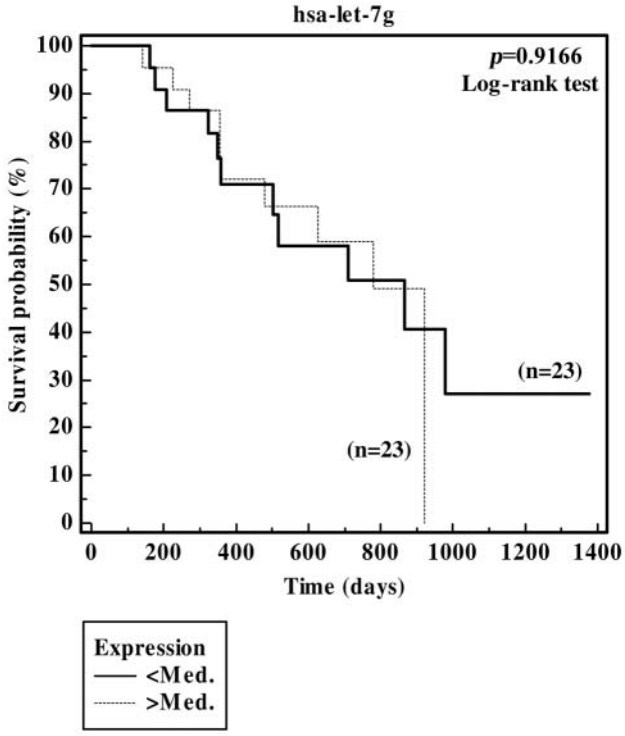

Figure 6. *Kaplan-Meier overall survival curve based on hsa-let-7g expression (p=0.9166; Log-rank test).*

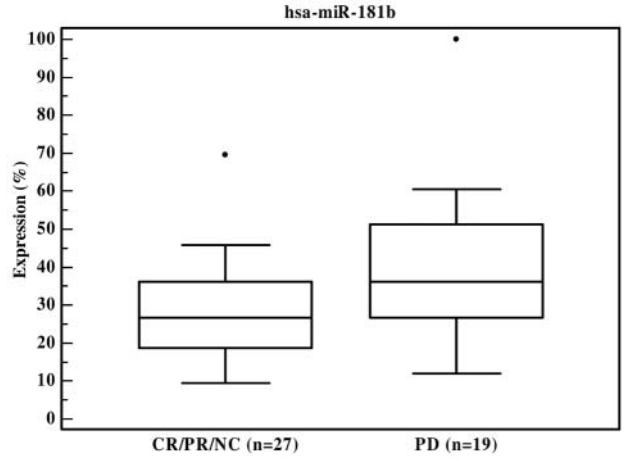

Figure 7. *Response based on hsa-miR-181b expression (p=0.0209; Mann-Whitney test). CR=complete response; PR=partial response; NC=no change; PD=progressive disease.*

This will be a big advantage for archived FFPE patient samples. The real-time miRNA-specific qRT-PCR assays are very sensitive and require only 10～25 ng of total RNA from archived FFPE patient samples.

We profiled 5 mature miRNAs utilizing real time qRT-PCR analysis on 46 FFPE colorectal cancer patient samples that were treated with S-1. The rationale in part, is that the mRNA targets (*e.g., RAS, cyclin D, c-myc, cytochrome C, ECIP-1, MAPPKKK1, TEM6, E2F5, GATA6, PP2B, eIF5A* and *E2F*) regulated by some of these miRNAs have been shown to play critical roles in cell cycle check point control and apoptosis. Because one miRNA can potentially regulate several hundred mRNA targets at the post-transcriptional level, even a small expression change of key miRNA may have a large impact on cell cycle control and chemosensitivity.

The expression levels of *hsa-miR-143* and *hsa-miR-145* were not significantly different in tumor samples compared to their

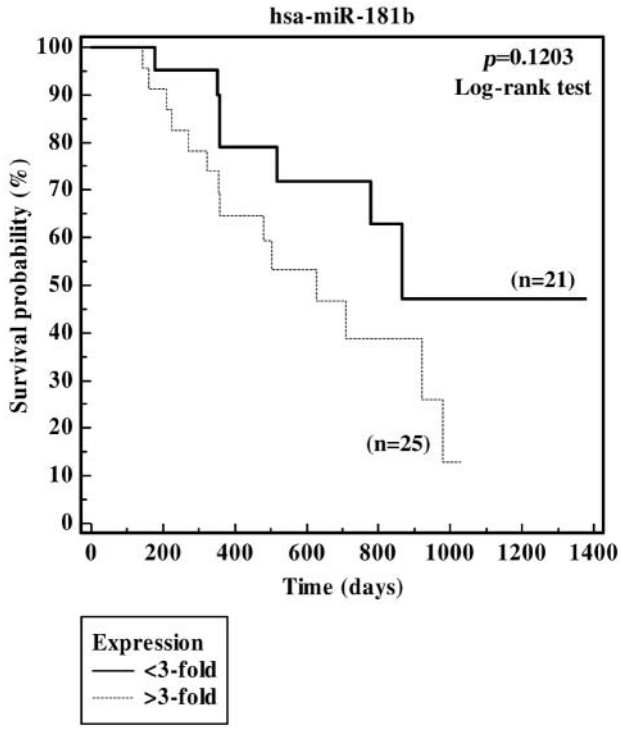

Figure 8. *Kaplan-Meier overall survival curve based on hsa-miR-181b expression (p=0.1203; Log-rank test).*

corresponding normal samples. This was a bit surprising since it had been reported by Michael *et al.* that the expression of *hsa-miR-143* and *hsa-miR-145* in human colorectal cancers decreased compared to normal samples (33). Based on our results, the reduction of the expression level in tumor tissue compared to corresponding normal sample was observed in only seven (33%) for *hsa-miR-143* and eight (38%) for *hsa-miR-145* out of twenty-one pairs (Figure 1). These observetions could be due to the genetic differences between the Japanese patients and European patients.

The expression level of *hsa-miR-200c* was significantly over-expressed in our colorectal tumor samples compared to the corresponding normal samples (Figure 2), in corroboration with the report from the Michael *et al.* study in colorectal tumor cells (33). The expression of *hsa-let-7g* was highly over-expressed in colorectal cancer samples compared to the corresponding normal samples (Figure 3). Based on the bioinformatics analysis, *hsa-let-7g* can potentially interact with more than 200 mRNA targets. This includes several critical cell cycle control genes such as *RAS, cyclin D, c-myc* and *E2F* family members. It's known that *E2F* family proteins are key transcription factors for regulating the expression of enzymes involved in DNA synthesis, such as TS and TK (34).

Based on our knowledge, this is the first report to demonstrate that *hsa-let-7g* is associated with chemo-sensitivity to S-1 based chemotherapy (Figure 5). Our results were consistent with the finding from a recent report by Johnson SM that the *let-7* family regulates the expression of the oncogene RAS in *C. elegans* and human lung tumors (35). *Let-7* interacts with multiple interaction sites at the 3'-UTR of *RAS* mRNA to regulate its translation. Previous reports have shown that there is a strong link between the chemosensitivity *vs. RAS* status (36, 37). We speculate that chemotherapeutic sensitivity of S-1 is potentially affected by the *hsa-let-7g*-mediated target transcripts such as *E2F, cyclin D* and *c-myc* (38-40). Although the expression level of *hsa-let-7g* was significantly associated with patient response to S-1 (Figure 5), there was no discernible benefit in predicting patient survival (Figure 6). This is not surprising because the response and survival markers are two different entities and often there is no connection between the two. Nonetheless, it is still important to be able to predict which patient will respond to a particular therapy. The expression of *hsa-miR-181b* was strongly associated with patient response to S-1 (Figure 7). Many genes, such as cytochrome *C, ECIP-1, MAPPKKK1, TEM6, E2F5, GATA6, PP2B* and *eIF5A*, are predicted to be regulated by *hsa-miR-181b*. These genes have been shown to be important for cell signaling, cell cycle control and chemosensitivity. We speculate that the miRNAs can modulate the expression of a number of genes at the translational level in a quick and energy-efficient control process. Clearly the detailed molecular and cellular mechanisms of miRNA-mediated translational control will be required in future studies to establish the direct link of *hsa-miR-181b* and chemosensitivity to fluoropyrimidine based drugs. Although *hsa-miR-181b* was associated with patient clinical response, the expression of *hsa-miR-181b* was not significant for patient survival (Figure 8).

In conclusion, the *in vivo* significance of 5 mature miRNAs was evaluated in colorectal cancer patient samples treated with S-1. H*sa-let-7g, hsa-miR-181b* and *hsa-miR-200c* were over-expressed in colorectal cancer patients and may be useful as tumor markers. *Hsa-let-7g* and *hsa-miR-181b* may be significant indicators for chemoresponse to S-1-based chemotherapy.

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