Identification of MicroRNA-181 by Genome-Wide Screening as a Critical Player in EpCAM–Positive Hepatic Cancer Stem Cells

Junfang Ji,[1] Taro Yamashita,[1] Anuradha Budhu,[1] Marshonna Forgues,[1] Hu-Liang Jia,[2] Cuiling Li,[3] Chuxia Deng,[3] Elaine Wauthier,[4] Lola M. Reid,[4] Qing-Hai Ye,[2] Lun-Xiu Qin,[2] Wen Yang,[5] Hong-Yang Wang,[5] Zhao-You Tang,[2] Carlo M. Croce,[6] and Xin Wei Wang[1]

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that regulate gene expression with functional links to tumorigenesis. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and it is heterogeneous in clinical outcomes and biological activities. Recently, we have identified a subset of highly invasive epithelial cell adhesion molecule (EpCAM)$^+$ HCC cells from alpha-fetoprotein (AFP)$^+$ tumors with cancer stem/progenitor cell features, that is, the abilities to self-renew, differentiate, and initiate aggressive tumors *in vivo*. Here, using a global microarray-based miRNA profiling approach followed by validation with quantitative reverse transcription polymerase chain reaction, we have demonstrated that conserved miR-181 family members were up-regulated in EpCAM$^+$AFP$^+$ HCCs and in EpCAM$^+$ HCC cells isolated from AFP$^+$ tumors. Moreover, miR-181 family members were highly expressed in embryonic livers and in isolated hepatic stem cells. Importantly, inhibition of miR-181 led to a reduction in EpCAM$^+$ HCC cell quantity and tumor initiating ability, whereas exogenous miR-181 expression in HCC cells resulted in an enrichment of EpCAM$^+$ HCC cells. We have found that miR-181 could directly target hepatic transcriptional regulators of differentiation (for example, caudal type homeobox transcription factor 2 [CDX2] and GATA binding protein 6 [GATA6]) and an inhibitor of Wnt/β-catenin signaling (nemo-like kinase [NLK]). Taken together, our results define a novel regulatory link between miR-181s and human EpCAM$^+$ liver cancer stem/progenitor cells and imply that molecular targeting of miR-181 may eradicate HCC. (HEPATOLOGY 2009;50:472-480.)

Human epithelial cell–derived cancers are highly heterogeneous, often composed of a hierarchy of mixed tumor cells with different biological properties. Tumor-initiating cells (TICs) with self-renewal and differentiation capabilities constitute a small proportion of this hierarchy and are thought to give rise to tumor heterogeneity.[1] Some TICs may be cancer stem cells (CSCs) because of their potential derivation from

Abbreviations: AFP, alpha-fetoprotein; CDX2, caudal type homeobox transcription factor 2; CSC, cancer stem cells; CYP3A4, cytochrome P450 3A4; EnSC, enrichment of stem/progenitor cell culture; EpCAM, epithelial cell adhesion molecule; FACS, fluorescence-activated cell sorting; GATA6, GATA binding protein 6; HCC, hepatocellular carcinoma; HpSC-HCC, hepatic stem cell–like hepatocellular carcinoma; MH-HCC, mature hepatocyte-like hepatocellular carcinoma; miRNA, microRNA; mRNA, messenger RNA; RC, regular culture; NLK, nemo-like kinase; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA; TACSTD1, tumor-associated calcium signal transducer 1; TIC, tumor-initiating cells; UGT2B7, uridine diphosphate-glucuronosyltransferase-2B7.

From the [1]Liver Carcinogenesis Section, Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, MD; the [2]Liver Cancer Institute and Zhongshan Hospital, Fudan University, Shanghai, China; the [3]Mammalian Genetics Section, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD; the [4]Department of Cell and Molecular Physiology, University of North Carolina School of Medicine, Chapel Hill, NC; the [5]International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute, Shanghai, China; and the [6]Comprehensive Cancer Center, Ohio State University, Columbus, OH.

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Address reprint requests to: Dr. Xin Wei Wang, Laboratory of Human Carcinogenesis, CCR, NCI, NIH, 37 Convent DR, Building 37 Room 3044A, Bethesda, MD 20892. E-mail: xw3u@nih.gov; fax: 301-496-0497.

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adult stem cells. TICs or CSCs have been associated with aggressive and metastatic cancers of the breast, brain, colon, and liver.[2-8] TIC eradication may be critical to achieve stable remission, and even a cure, of aggressive malignancies.[9] Normal stem cells and CSCs share many common cellular properties and signaling pathways (e.g. Wnt/β-catenin, transforming growth factor beta, and Notch).[9] MicroRNAs (miRNAs, miRs), a novel class of small, noncoding RNAs that post-transcriptionally regulate gene expression through complementary base pairing to messenger RNAs (mRNAs),[10] are functionally linked to stem cells.[11] The miRNA pathway affects stem cell division, and the loss of *dicer1*, a critical endonuclease for generating mature miRNAs, can reduce stem cell populations and induce embryonic lethality.[12,13] Moreover, a distinct miRNA subset is specifically expressed in pluripotent embryonic stem cells but not in adult tissues.[14] The role of miRNAs in CSCs, however, remains undetermined.[15]

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide, with observable heterogeneity and a cellular origin that has yet to be identified.[16] Recent studies indicate that epithelial cell adhesion molecule (EpCAM), a membrane-associated glycoprotein encoded by tumor-associated calcium signal transducer 1 (TACSTD1), could serve as a hepatic stem/progenitor cell-specific marker.[17-19] Similarly, alpha-fetoprotein (AFP) is one of the earliest markers detected in the liver bud.[20] Using HCC EpCAM and AFP status together with HCC transcriptome analyses, we recently identified two distinct prognostic HCC subtypes, $EpCAM^+AFP^+$ HCC (referred to as HpSC-HCC; hepatic stem cell–like HCC) with venous metastases and poor survival and $EpCAM^-AFP^-$ HCC (referred to as MH-HCC; mature hepatocyte-like HCC) with relatively good outcome, which differ significantly in their molecular profiles.[21] Furthermore, $EpCAM^+$ HCC cells isolated with an EpCAM-specific antibody by fluorescence-activated cell sorting (FACS) from AFP^+ HCC cell lines or AFP^+ HCC clinical specimens are hepatic TICs with stem/progenitor cell features.[7] Here, we identify a conserved gene family, miR-181, that is functionally critical in the maintenance of $EpCAM^+AFP^+$ HCC cells, possibly by inhibiting hepatic cell differentiation and promoting HCC stemness through targeting the transcriptional regulators caudal type homeobox transcription factor 2 (CDX2), GATA binding protein 6 (GATA6), and the Wnt signaling inhibitor nemo-like kinase (NLK). Our results suggest that miR-181 may serve as a novel biomarker and a molecular target for hepatic TICs.

Patients and Methods

Clinical Samples, Microarray, and Cells. HCC samples were obtained with informed consent from patients who underwent radical resection at the Liver Cancer Institute of Fudan University. The study was approved by the Institutional Review Board of the respective institutes. Human fetal liver RNAs, obtained from Clontech Inc. (Mountain View, CA), were isolated from a pool of 63 fetal livers from spontaneously aborted male/female fetuses between 22 and 40 weeks old. Normal liver tissue samples were obtained from eight disease-free liver donors as previously described.[22] Purified human hepatic stem cells, hHpSCs, from fetal liver were isolated as previously described.[28] The miRNA microarray profiling data, previously described,[22] are publicly available (GEO accession number: GSE6857). Other materials and methodologies are described in detail in the Supporting Text.

Results

A Unique miRNA Signature in HpSC-HCC. We searched for miRNAs unique to $EpCAM^+AFP^+$ HCC by interrogating miRNA expression profiles of 53 HpSC-HCC and 95 MH-HCC clinical specimens using the array dataset recently described.[22] We performed multivariate nearest neighbor class prediction and found 20 unique miRNAs that could significantly predict HpSC-HCC and MH-HCC cases with 78% overall accuracy (multivariate $P < 0.01$) (Fig. 1A). Noticeably, multiple miR-181 transcripts, that is, miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c, along with several miRNAs in the miR-17-92 cluster, that is, 17, 20a, 25, 92, 93, and 106b, were up-regulated in HpSC-HCC, but down-regulated in MH-HCC (Fig. 1A). The expression of miR-181s was inversely correlated with mature hepatocyte-specific genes in the same clinical specimens (Supporting Fig. 1).

Examination of the miRBase database (http://microrna.sanger.ac.uk/) showed that six human miR-181s—miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c, and miR-181d—were previously identified. Strikingly, five of these were up-regulated in HpSC-HCC by our microarray study. Genome structure analyses indicated that these six miR-181s were encoded in three independent transcripts located on three separate chromosomes (Fig. 1B) (Supporting Fig. 2A). The corresponding precursors appeared to yield four sets of mature miR-181s, that is, miR-181a, miR-181b, miR-181c, and miR-181d, based on their sequence homology, where miR-181a-1 and miR-181a-2, as well as miR-181b-1 and miR-181b-2, were identical in their mature forms (Fig.

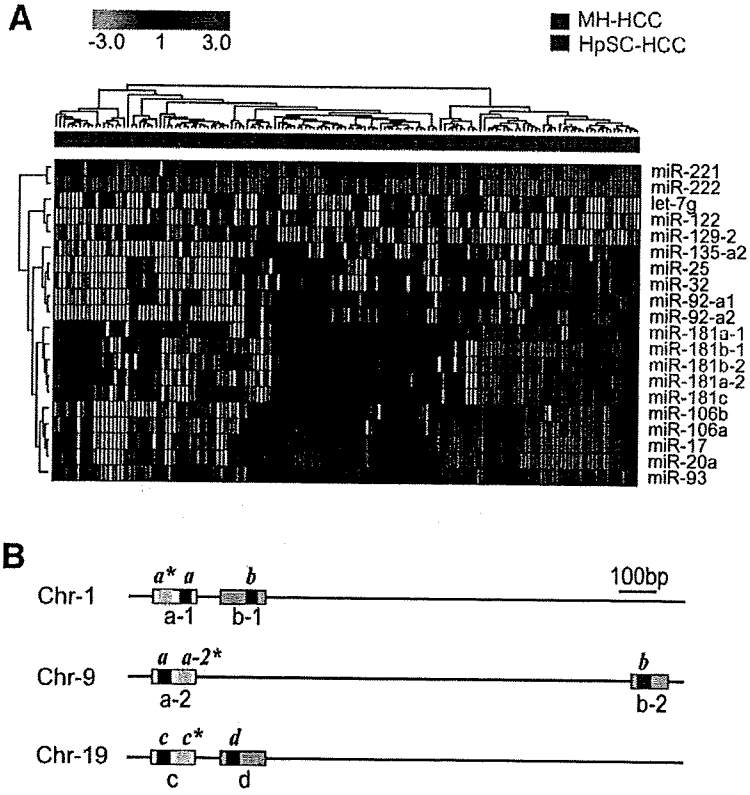

Fig. 1. The expression of miR-181 precursors is positively correlated with clinical HCC specimens with features of hepatic stem/progenitor cells. (A) Hierarchical clustering of 20 miRNAs that could significantly discriminate HpSC-HCCs samples (n = 53) from MH-HCCs samples (n = 95) (multivariate $P < 0.01$). Each row represents an individual miRNA, and each column represents an individual case. Red, black, and green pseudocolors indicate the up-regulation, unchanged expression, and down-regulation of genes in HCCs normalized to disease-free hepatic tissues from eight donors, respectively. (B) Schematic depiction of human miR-181 family. Two types of paralog groups of miR-181 precursors can be identified: miR-181a-1/a-2/c (yellow) and miR-181b-1/b-2/d (green). The names of the miR-181 precursors are denoted below the boxes. The positions of the mature miRNAs are indicated by the blue boxes with labels written above. (C) Sequence homology of all four human mature miR-181s. The blue shading highlights the conserved bases among the different miR-181s. The red letters specify the seed sequences.

1C, Supporting Fig. 2A). Their genomic organization appeared to be evolutionarily conserved from bony fishes to mammals, suggesting an important role in development (Supporting Fig. 2B). It appeared that miR-181b and miR-181d were closely linked, whereas miR-181a and miR-181c were closely linked (Supporting Fig. 2C,D). Although sequence variations were evident among the four mature miR-181s, each miR-181 was highly conserved among species (Supporting Fig. 2E-H). Each transcript contained two miR-181 paralogs in most species, whereas the same 5′ "seed" region (nucleotides 2-8) and 3′ "variable region" were found in all four miR-181s (Fig. 1B, Supporting Fig. 2E-H). Thus, the four miR-181 family members are evolutionarily conserved among the vertebrate lineage with high homology implicating their functional redundancy.

We performed quantitative reverse transcription polymerase chain reaction (RT-PCR) to validate the microarray-based miR-181 expression, in 20 HCC cases (10 HpSC-HCC and 10 MH-HCC). We found that all four mature miR-181s were significantly elevated in HpSC-HCC than MH-HCC (Fig. 2A with a significant correlation between the RT-PCR and microarray data (Supporting Fig. 3). An examination of miR-181 expression levels in several HCC cell lines with different EpCAM and AFP status[23] showed that miR-181 expression was much higher in Hep3B cells (all cells expressing EpCAM and AFP) than HuH1 or HuH7 cells (heterogeneous in EpCAM and AFP expression) (Fig. 2B,C). Furthermore, miR-181 levels were much higher in human fetal livers and isolated HpSCs from fetal liver than in adult livers or freshly isolated mature hepatocytes (Fig. 2D,E). On examination of different mouse fetal liver stages, we found that miR-181 levels were highest at E10.5, at which time the liver bud is rich in liver stem/progenitor cells, with a gradual decline in their expression between E11.5 and 15.5 to reach that of adult livers (15 weeks) (Fig. 2E). Taken together, the miR-181s are

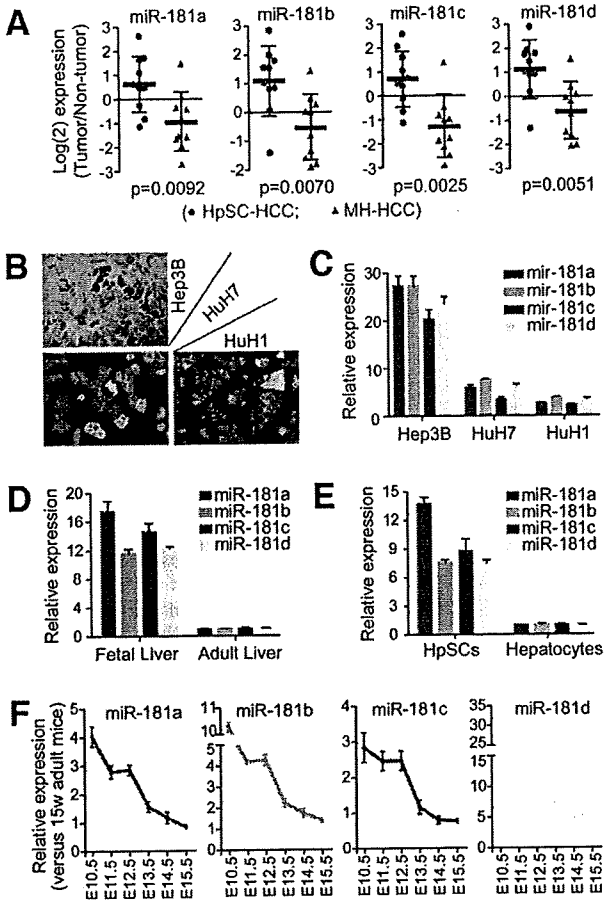

Fig. 2. The expression levels of mature miR-181s in HCC. (A) Quantitative real-time RT-PCR analysis of the four mature miR-181s in 20 pairs of HCCs and their noncancerous hepatic tissues (10 HpSC-HCC cases and 10 MH-HCC cases). The y-axis refers to the gene expression ratio (tumor versus nontumor), shown as the median ± the interquartile range in $\log_2$ scale. Gene expression was measured in triplicate, and *P* values were generated by a Student's *t* test. (B) EpCAM expression was examined by immunofluorescence in Hep3B, HuH7, and HuH1 cell lines. Green fluorescence represents EpCAM, and blue represents the cell nucleus stained by 4′,6-diamidino-2-phenylindole. (C) Quantitative RT-PCR analysis of the four mature miR-181s in Hep3B, HuH7, and HuH1 cell lines, normalized by the levels in adult primary human hepatocytes. (D) Quantitative RT-PCR analysis of mature miR-181s in human fetal and adult normal livers. (E) Quantitative RT-PCR analysis of mature miR-181s in isolated HpSC and adult primary human hepatocytes. (F) Quantitative RT-PCR analysis of mature miR-181s in mouse fetal liver at the different mouse embryonic stages, normalized to the levels in normal adult mice livers (15 weeks old). E10.5 is the stage of liver bud formation, and E12-15 is the stage of functional liver organ formation. Gene expression was measured in triplicate in C-F and is shown as the mean ± standard deviation.

highly expressed in embryonic liver tissues, pluripotent hepatic stem/progenitor cells in human livers, and EpCAM$^+$AFP$^+$ HCC samples.

Elevated Levels of miR-181s in EpCAM$^+$ HCC Cells. FACS-isolated EpCAM$^+$ from HuH1 or HuH7 cell populations had significantly higher miR-181 expression than EpCAM$^-$ cells ($P < 0.01$) (Fig. 3A-C). Consistently,[7] the EpCAM$^+$ fraction decreased in sorted EpCAM$^+$ cells after 7 days of culture to a level similar to unsorted cells (Fig. 3A,B). In parallel, mature miR-181 levels decreased with time following incubation with sorted EpCAM$^+$ HuH1 or HuH7 cells (Fig. 3D). We also compared the effects of an "enrichment of stem/pro-

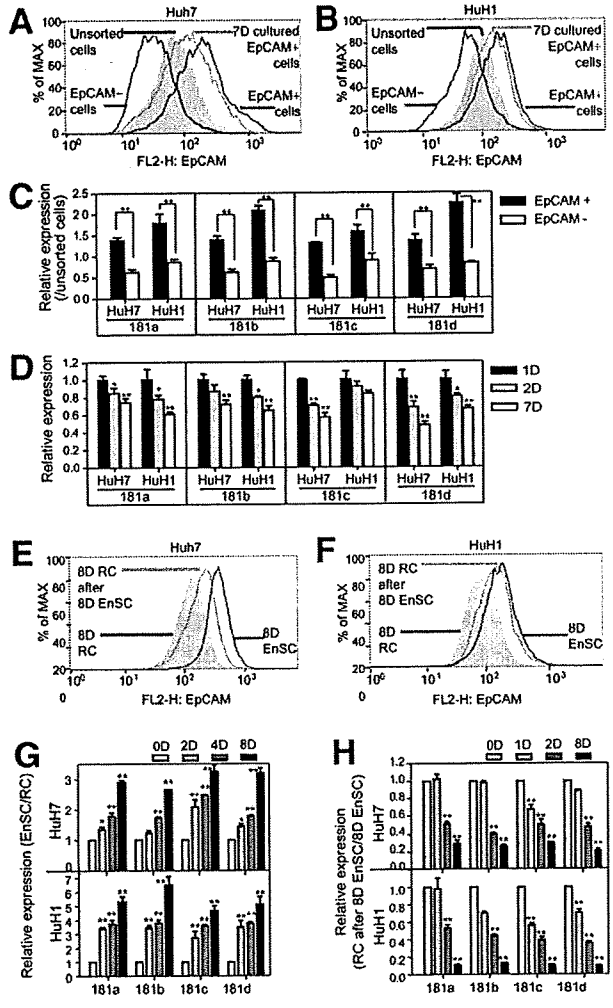

Fig. 3. The expression levels of miR-181s in EpCAM$^+$ HCC cells. EpCAM$^+$ and EpCAM$^-$ HuH7 (A) or HuH1 (B) cells were isolated by cell sorting. After 7 days in culture, the EpCAM$^+$ fraction in isolated EpCAM$^+$ HuH7 (A) or HuH1 (B) cells were analyzed by FACS. (C) miR-181 expression levels of isolated EpCAM$^+$ and EpCAM$^-$ HuH7 and HuH1 cells after 1 day in culture. The y-axis refers to relative expression levels of miR-181s normalized to unsorted HuH7 or HuH1 cells. (D) The expression levels of miR-181s in sorted EpCAM$^+$ HuH7 or HuH1 cells after 1 day, 2 days, and 7 days of culture. (E, F) EpCAM$^+$ fractions of HuH7 and HuH1 cells were analyzed by FACS after 8 days' culture in "enrichment of stem/progenitor cell culture" medium (EnSC), or a further culture in regular medium (RC) for an additional 8 days. (G H) Time-dependent miR-181 expression level in HuH7 and HuH1 cells culturing in EnSC (G) or further culturing in RC (H). The miR-181s' expression was measured in triplicate in C through G and is shown as the mean ± standard deviation. A Student's *t* test was employed ($^*P < 0.05$) ($^{**}P < 0.01$).

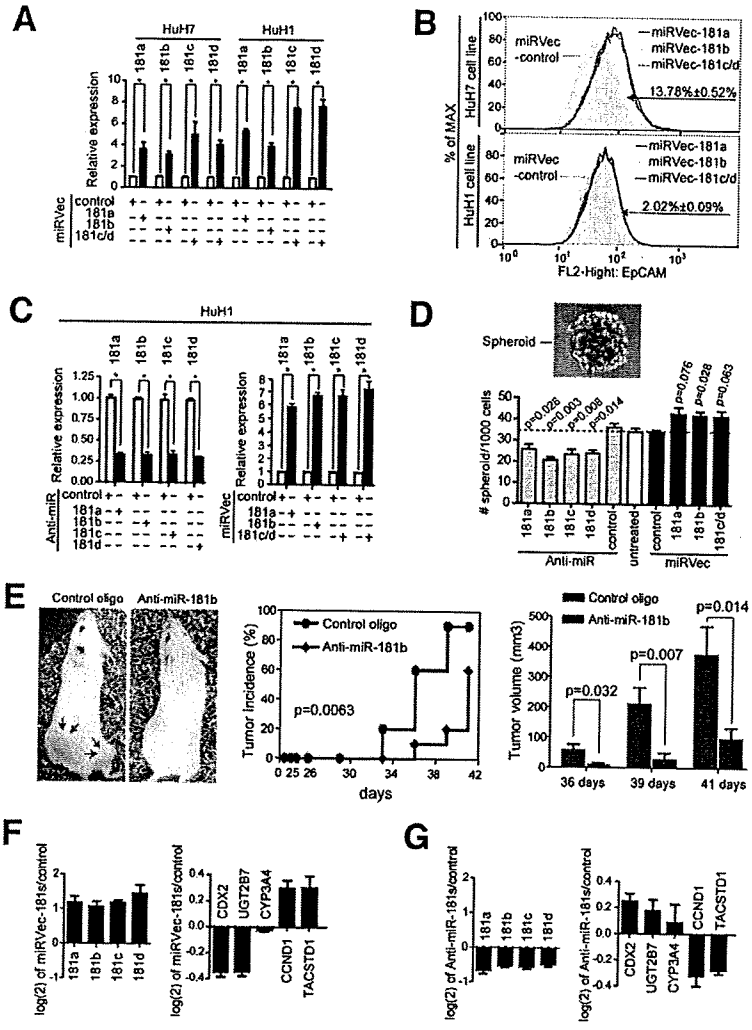

Fig. 4. The consequences of alteration of miR-181 expression in HuH7 and HuH1 cells. (A, B) HuH7 and HuH1 cells transfected with various miRVec-181s and cultured for 3 days were analyzed by quantitative RT-PCR (A) or by FACS with anti-EpCAM antibody (B). Changes in EpCAM$^+$ cell population after transfection with various miRNA vectors as compared with vector controls are indicated. (C) miR-181 levels in HuH1 cells, as determined by quantitative RT-PCR, 24 hours after transfection with various miR-181 inhibitors or miRVec-181s. (D) Cells from (C) were seeded in non-attached plates to assay spheroid formation. A representative phase-contrast image of an HCC spheroid is shown. Quantitation of HCC spheroids is also shown where the y-axis refers to the numbers of spheroids from HuH1 cells without transfection (open bar), with anti-miR-181s transfections (dash bars), or with miRVec-181s transfections (black bars). Experiments were performed in triplicate, and the number of spheroids in each group is shown as the mean ± standard deviation. (E) miR-181b blockage results in tumor repression of EpCAM$^+$ HCC cells. EpCAM$^+$ HuH1 cells were isolated by FACS with anti-EpCAM antibody and re-seeded in six-well plates. Anti-miR-181b or a control oligo was transfected 16 hours after FACS. After an additional 24 hours' incubation, 1000 cells in each group were injected subcutaneously into NOD/SCID mice. Left panel: a representative NOD/SCID mouse with two subcutaneous tumors (red arrows) after injection of 1000 cells transfected with a control oligo or a mouse after injection of 1000 cells transfected with anti-miR-181b oligo; middle panel: tumor incidence curve; right panel: tumor volumes from control and anti-miR-181b-treated groups. Data are generated from 10 injection experiments in each group. The mRNA expression levels of CDX2, UGT2B7, CYP3A4, CCND1, and TACSTD1 were examined in HuH1 cells after transfecting with mixed miRVec-181 vectors (F) or with mixed anti-miR-181 antagomers (G). All experiments were performed in triplicate, shown as the mean ± standard deviation.

genitor cell culture" (knockout medium referred to as enrichment of stem/progenitor cell culture [EnSC]) that was optimized to maintain undifferentiated embryonic stem cells while hindering the proliferation of differentiated cells, to a regular culture (RC) in an attempt to enrich hepatic CSCs after prolonged culture.[24] After 8 days in EnSC, the EpCAM$^+$ fraction of HuH7 or HuH1 cells increased, but decreased when further cultured in RC medium (Fig. 3E,F). Consistently, EpCAM$^+$ cell enrichment in HuH1 or HuH7 cells resulted in elevated miR-181 levels (Fig. 3G), which decreased after further culture in RC medium (Fig. 3H). Therefore, miR-181 expression patterns appear to correlate with an enriched EpCAM$^+$ HCC cell population.

Augmentation of EpCAM$^+$ HCC Cells by miR-181s. Our findings that four miR-181 family members, encoded by different transcripts, were highly elevated in HpSC-HCCs and EpCAM$^+$ HCC cells suggested that the miR-181s may be critical in maintaining a stem cell phenotype. To test this hypothesis, we examined the effect of miR-181 on EpCAM$^+$ cell distribution by expressing each miR-181 in unsorted HuH1 or HuH7 cells. Individual expression of miR-181s resulted in an enrichment of EpCAM$^+$ HuH7 cells compared with a control (Fig 4A,B). A similar and reproducible observation was made in HuH1 cells. However, the smaller induction could be attributable to low basal miR-181 levels and poor transfection efficiency (Supporting Fig. 4). The ability to specifically induce miR-181 expression by each miRVec-181 construct was verified (Supporting Fig. 5A).

Similar to mammary, hepatic, and neural stem cells as well as melanoma CSCs,[25-28] EpCAM$^+$ HuH1 cells could efficiently form spheroids, whereas EpCAM$^-$ cells failed to do so.[7] Inhibition of endogenous miR-181s by various anti-miRNA miR-181 inhibitors resulted in a significant reduction of spheroid formation in HuH1 cells (Fig. 4D). Meanwhile, forced expression of miR-181s led to a modest induction of spheroid formation (Fig. 4D). The spec-

ificity of each miR-181 inhibitor was also validated (Supporting Fig. 5B). We also found that the miR-181a and miR-181b inhibitors could inhibit the tumorigenicity of EpCAM$^+$ HuH1 cells (Fig. 4E and data not shown).

The "stemness" of EpCAM$^+$ HCC cells was further supported by their association with activated Wnt/β-catenin signaling, measured by target gene induction (CCND1 and TACSTD1), as well as the reduction of genes specific for differentiated hepatocytes (uridine diphosphate-glucuronosyltransferase-2B7 [UGT2B7] and cytochrome P450 3A4 [CYP3A4]).[7,21,23] If miR-181 was critical for maintaining an undifferentiated hepatic CSC phenotype, then forced miR-181expression would lead to Wnt target up-regulation, whereas inactivation of endogenous miR-181 would lead to up-regulation of differentiation markers. Consistently, the mRNA level of UGT2B7 and CYP3A4 was down-regulated whereas CCND1 and TACSTD1 were up-regulated after ectopic miR-181 expression (Fig. 4F). In contrast, inhibition of endogenous miR-181s resulted in UGT2B7 and CYP3A4 induction with CCND1 and TACSTD1 reduction (Fig. 4G). CDX2, a positive regulator of hepatocyte differentiation, had a similar expression trend as UGT2B7 and CYP3A4 (Fig. 4). Wnt/β-catenin signaling activation by miR-181expression was verified by the TOP-FLASH/FOP-FLASH reporter system (a luciferase reporter system containing wild-type or mutant beta-catenin responsive elements) (Supporting Fig. 6). Furthermore, we used the EnSC-RC strategy to alter the differentiation status of HCC cells. HuH7/HuH1 cells were grown in either RC (8D RC) or EnSC (8D EnSC) for 8 days followed by another 8 days in RC (EnSC-RC). Consistently, we found that miR-181 levels were negatively correlated with the expression of hepatocyte differentiation markers but positively correlated with β-catenin–associated genes (Supporting Fig. 7).

CDX2, GATA6, and NLK Are miR-181 Target Genes. In-silico screening with TargetScan[29] and PicTar[30] programs showed potential miR-181 target genes. Among them, CDX2 and GATA6 were previously implicated as regulators of hepatocyte gene expression[31,32] and NLK as a negative regulator of Wnt/β-catenin signaling.[33] Sequence analyses showed that the 3′ untranslated region (3′-UTR) of CDX2, GATA6, and NLK mRNA contain putative sites that are partially complementary to miR-181s (Fig. 5A) and evolutionarily conserved among human, mouse, and rat (Supporting Fig. 8A-C). To experimentally validate these targets, we measured their levels upon alteration of miR-181s (Supporting Fig. 9). Western blotting analysis showed that blockage of endogenous miR-181s in Hep3B cells, which highly express

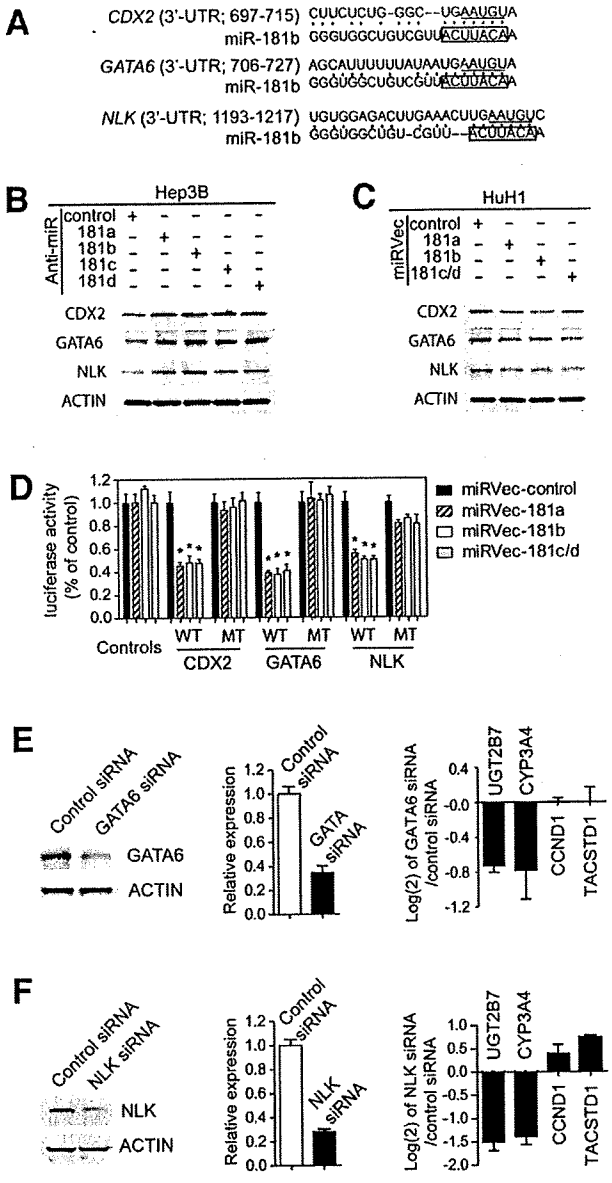

Fig. 5. CDX2, GATA6, and NLK as direct targets of miR-181s. (A) Predicted duplex formation between the 3′-UTR sequences of human CDX2, GATA6, and NLK and miR-181b. The boxes highlight the seed sequences in miR-181b. The underlined bases were mutated to UUACA in mutant 3′-UTR plasmids. (B, C) The expression levels of CDX2, GATA6, and NLK in Hep3B cells transfected with miR-181 inhibitors (B) or in HuH1 cells transfected with miRVec-181s (C), as determined by western blotting. (D) Luciferase activities of various reporter plasmids in HuH1 cells co-transfected with miRVec-control, 181a, 181b, or 181c/d. Each experiment was performed in triplicate, and the luciferase activity was shown as the mean ± standard deviation. A Student's *t* test was employed. $^*P < 0.01$. Levels of GATA6 (E) or NLK (F) in HuH1 cells transfected with control, GATA6 siRNA, or NLK siRNA were determined by western blotting (left panel) or quantitative RT-PCR (middle panel). The relative expression ratio (GATA6 siRNA or NLK siRNA versus control siRNA) of UGT2B7, CYP3A4, CCND1, and TACSTD1 genes in HuH1 cells is shown as the mean ± standard deviation in log(2) scale (right panel).

miR-181s, resulted in an induction of CDX2, GATA6, and NLK (Fig. 5B). In contrast, forced expression of miR-181s in HCC cells resulted in a reduction of CDX2, GATA6, and NLK (Fig 5C). To further determine whether CDX2, GATA6, and NLK were *bona fide* targets of miR-181–mediated small interfering RNA (siRNA) silencing, the miR-181 binding sites in the 3′-UTR of these three genes were cloned into a luciferase reporter. We found that forced expression of miR-181s resulted in decreased luciferase activity when the wild-type sequences were present (Fig. 5D). This effect was significantly reduced when the corresponding miR-181 binding sites (five nucleotides within the complementary seed sequences) were mutated (Fig. 5D). Thus, miR-181s directly target CDX2, GATA6, and NLK, and a single site within each gene is sufficient for miR-181–mediated gene silencing.

To further test whether miR-181s may maintain HCC stemness by inhibiting CDX2, GATA6, or NLK, we used RNA interference technology and successfully knocked-down GATA6 and NLK expression (Fig. 5E,F). We were unable to identify a functional siRNA specific to CDX2, and thus its functional role in HCC could not be assessed at this time. Consistently, GATA6 or NLK knock-down in HCC cells resulted in a reduction of both adult hepatocyte-specific genes UGT2B7 and CYP3A4 (Fig. 5E,F). Moreover, similar to miR-181 overexpression experiments, silencing of GATA6 and NLK resulted in a modest induction of the EpCAM$^+$ cell fraction (Supporting Fig. 10), suggesting that GATA6 and NLK may be directly involved in hepatocyte differentiation.

Discussion

Similar to observations by Lee et al.,[34] we recently used global mRNA profiling of HCC clinical specimens to identify an EpCAM$^+$ AFP$^+$ HCC subtype resembling hepatic stem/progenitor cells.[21] Furthermore, we demonstrated that EpCAM$^+$ HCC cells from AFP$^+$ tumors are a subpopulation of undifferentiated hepatic TICs with normal HpSC-like phenotypes.[7] Using miRNA expression profiling, we recently identified unique miRNAs associated with HCC metastases and patient survival.[22] With this technology, we have now identified a highly conserved miR-181 family that may contribute to the maintenance of EpCAM$^+$ hepatic TIC and EpCAM$^+$ normal HpSC activities. We presented the following evidence: First, all conserved mature miR-181 members were highly expressed in HpSC-HCCs and isolated EpCAM$^+$ HCC cells. Second, human fetal livers and early stage mouse embryonic livers, rich in HpSCs, had a high miR-181 level compared with adult livers. Consistently, isolated HpSCs expressed high miR-181 levels. Third, miR-181 levels correlated with HCC cell differentiation. Fourth, forced miR-181 expression enriched EpCAM$^+$ HCC cells with stem cell properties, whereas miR-181 blockage reduced EpCAM$^+$ HCC cells and induction of hepatic differentiation. Fifth, miR-181s could directly target CDX2, GATA6, and NLK, known regulators of hepatic cell differentiation. Collectively, our results suggest that miR-181s are important components of human EpCAM$^+$ hepatic CSCs and may maintain HCC "stemness" by inactivating critical cellular transcriptional regulators that induce hepatocyte differentiation.

In addition to EpCAM, CD133 (PROM1) and CD90 (THY1) have also been proposed as hepatic CSC markers.[6,8] We compared EpCAM$^+$, CD133$^+$, or CD90$^+$ HCC cells and found that although EpCAM and CD133 could be detected in HuH1 and HuH7 cells, these markers appeared to overlap in HuH7 but not in HuH1 cells[7] (data not shown). Noticeably, EpCAM$^+$ HuH1 cells showed marked tumor-initiating capacity compared with CD133$^+$ HuH1 cells.[7] Our current results indicate that miR-181 is highly expressed in EpCAM$^+$ or CD133$^+$ cells when compared with EpCAM$^-$ or CD133$^-$ cells isolated from HuH1 and HuH7 cells (Supporting Fig. 11). Noticeably, miR-181 expression is much higher in EpCAM$^+$CD133$^+$ cells than double-negative or single-positive cells. However, CD90 is undetectable in these cells. Taken together, our results indicate that EpCAM is a better marker than CD133 to define HCC CSCs, and miR-181 is more closely associated with EpCAM$^+$ cells than CD133$^+$ cells, further emphasizing the role of miR-181 in HCC stemness.

The roles of miR-181 in cellular differentiation have recently been explored, with evidence as both positive and negative regulators of this phenotype, but their mechanisms remain unclear. In murine muscle, miR-181 is up-regulated during fiber regeneration, returning to basal levels at the end of the regeneration, and is poorly expressed in terminal differentiated muscle.[35] The authors suggest that miR-181 may be involved in establishing the differentiated phenotype but probably not its maintenance.[35] However, during hematopoiesis, it appears that miR-181 has opposing roles. In B-lymphocytes, miR-181a is up-regulated in differentiated cells when compared with undifferentiated progenitor cells and acts to positively regulate B-cell differentiation.[36] However, during megakaryocytic differentiation, miR-181b and miR-181c are down-regulated.[37] In addition, increased miR-181a and miR-181b levels are associated with erythroid differentiation,[38,39] whereas miR-181 expression gradually decreases after erythroid terminal differentiation.[39] In our study, we found that all members of miR-181 are up-regulated in hepatic stem cell populations and HCC

cells with progenitor cell features, implying that miR-181 functions in maintaining an undifferentiated state of hepatic progenitor cells. The apparent opposing roles of miR-181 in cellular differentiation may be attributable to repression of different target genes with tissue/cell type specificity. Of note, skeletal muscle and blood cells are derived from mesoderm, whereas liver is derived from endoderm during embryonic development.

Several signaling pathways are involved in regulating stem cell functions (for example, Wnt, transforming growth factor-beta, Notch). MicroRNAs are also involved in this process, possibly by regulating cell cycle progression.[12] Our results suggest that miR-181s may be activators of hepatic progenitor cells and HCCs through alterations in at least two cellular signaling pathways (Supporting Fig. 12). One pathway is the blockage of HCC cell differentiation through inhibition of GATA6 or CDX2, two transcriptional activators regulating hepatocyte differentiation.[31,32] The second may be to activate the Wnt/β-catenin pathway by down-regulating NLK, a Wnt/β-catenin signaling inhibitor. Our results indicated that miR-181, EpCAM$^+$ cell populations, and Wnt/β-catein signaling were elevated in HuH1 or HuH7 cells after a prolonged EnSC culture, whereas a down-regulation of differentiated genes was observed. Thus, miR-181s may bolster progenitor cell functions by affecting both pathways to ensure hepatic "stemness" (Supporting Fig. 12).

Our results indicate that all four independently encoded miR-181 family members are elevated in hepatic stem cells and HCC cells with features of hepatic CSCs and are similarly activated to maintain "stemness." This is intriguing and implies that a common cellular signaling pathway may converge to activate miR-181s. One candidate "master key" activator is the Wnt signaling pathway, because it is involved in virtually every aspect of embryonic development and also controls homeostatic self-renewal of adult stem cells.[40] Consistently, conditioned media derived from WNT10B-overexpressing cells could enhance the levels of all four mature miR-181s in HCC cells (data not shown). Examination of the genome database revealed several classical TCF4 binding sites located within the three putative promoter regions of miR-181s on chromosomes 1, 9, and 19 (data not shown). Thus, it is plausible that Wnt/β-catenin may induce miR-181s to regulate hepatic stem cells during early-stage embryogenesis and to ensure a positive feedback mechanism to maintain stemness. These could be achieved by inactivating Wnt/β-catenin inhibitors such as NLK and by blocking genes such as GATA6 or CDX2 to prevent hepatic cell differentiation. Whether miR-181s are transcriptional targets of Wnt/β-catenin signaling is currently being investigated in our laboratory using the same strategy previously described.[23] A future challenge is to explore miR-181 as a molecular target for eradicating hepatic TICs.

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Over- and under-expressed microRNAs in human colorectal cancer

KAZUO MOTOYAMA[1,2], HIROSHI INOUE[1], YASUSHI TAKATSUNO[1,2], FUMIAKI TANAKA[1], KOSHI MIMORI[1], HIROYUKI UETAKE[2], KENICHI SUGIHARA[2] and MASAKI MORI[1,3]

[1]Department of Molecular and Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumibaru, Beppu 874-0838; [2]Department of Surgical Oncology, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519; [3]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 1-1, Yamadaoka, Suita 565-0871, Japan

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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.

Correspondence to: Professor Masaki Mori, Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, 1-1, Yamadaoka, Suita 565-0871, Japan
E-mail: mmori@gesurg.med.osaka-u.ac.jp

Key words: microRNA, human colorectal cancer, microRNA microarray

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 *Taq*Man MicroRNA assays (Applied Biosystems). Reverse transcriptase reactions contained 10 ng of total RNA, 50 nM stem-loop RT primer, 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.

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.

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 *Taq*Man Universal PCR master mix and 1 μl of primers and probe mix from each *Taq*Man 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 $2^{-\Delta\Delta Ct}$ 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
hsa-miR-31	4/4	179.29
hsa-miR-18b	4/4	175.71
hsa-miR-30e-3p	4/4	71.82
hsa-miR-220	4/4	51.05
hsa-miR-570	4/4	35.25
hsa-miR-302b	4/4	31.51
hsa-miR-302a	4/4	27.81
hsa-miR-183	4/4	18.94
hsa-miR-224	4/4	14.46
hsa-miR-18a	4/4	10.49
hsa-miR-95	4/4	10.13
hsa-miR-7	4/4	7.96
hsa-miR-182	4/4	5.63
hsa-miR-17-5p	4/4	4.83
hsa-miR-550	4/4	4.74
hsa-miR-196b	4/4	4.61
hsa-miR-181d	4/4	4.42
hsa-miR-20a	4/4	4.38
hsa-miR-92	4/4	4.14
hsa-miR-493-3p	4/4	4.07
hsa-miR-29a	4/4	3.75

[a]Fold 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	*miR-31*	*miR-183*	*miR-17-5p*	*miR-18a*	*miR-20a*	*miR-92*
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 ± SD)	3.80±8.50	2.08±2.92	2.34±3.12	1.97±3.08	1.38±1.59	1.84±2.07
Mean expression level in non-tumor (mean ± SD)	0.70±0.8	0.39±0.38	1.01±1.27	0.86±1.23	0.48±0.55	1.03±0.83
Down-regulated miRNAs	*miR-143*	*miR-145*				
Down-regulated/all cases (%)	31/43 (72.1)	30/44 (68.2)				
Mean expression level in tumor (mean ± SD)	0.87±1.06	0.96±1.30				
Mean expression level in non-tumor (mean ± SD)	1.58±1.74	1.70±1.75				

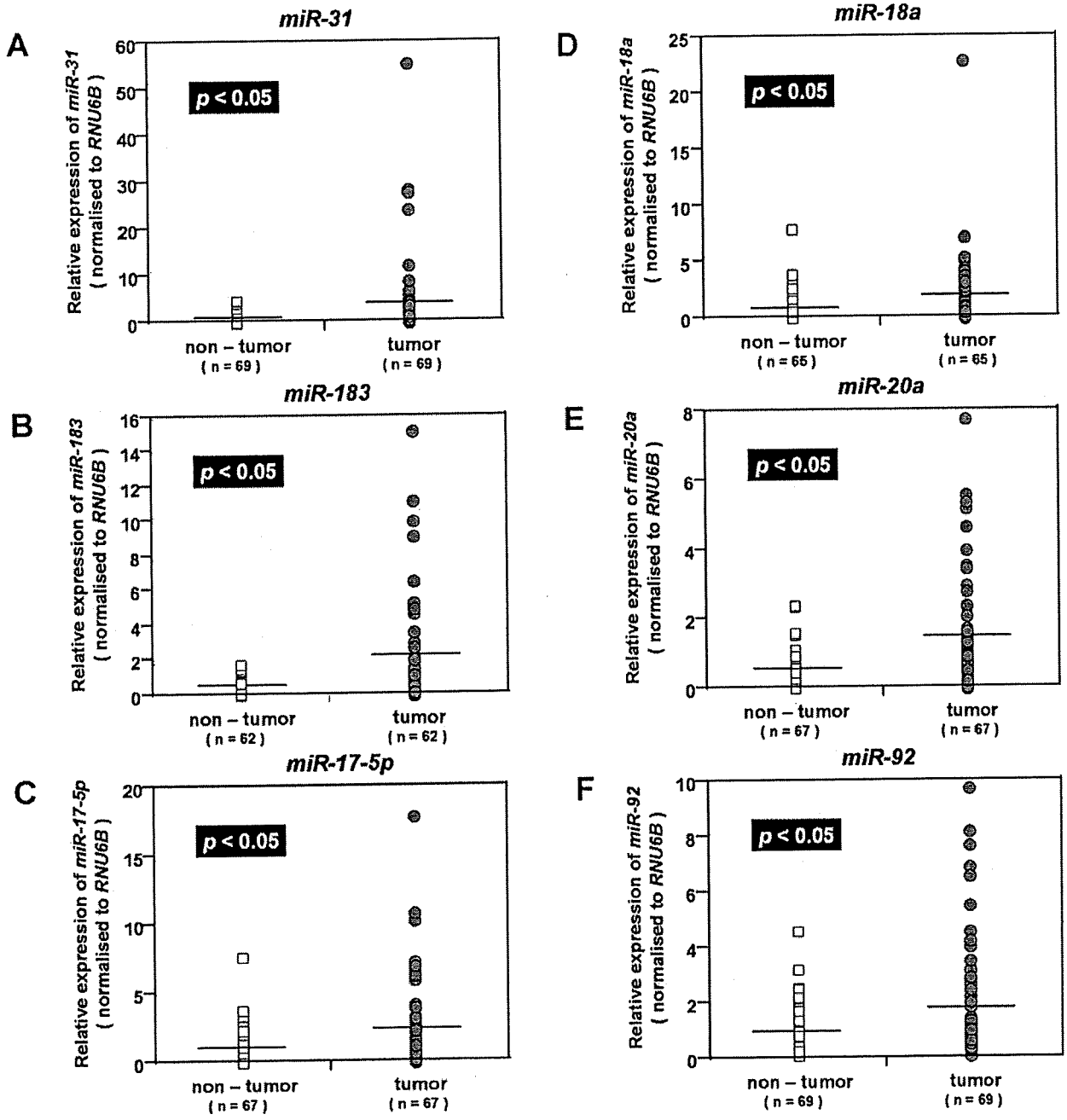

Figure 1. Part 1.

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

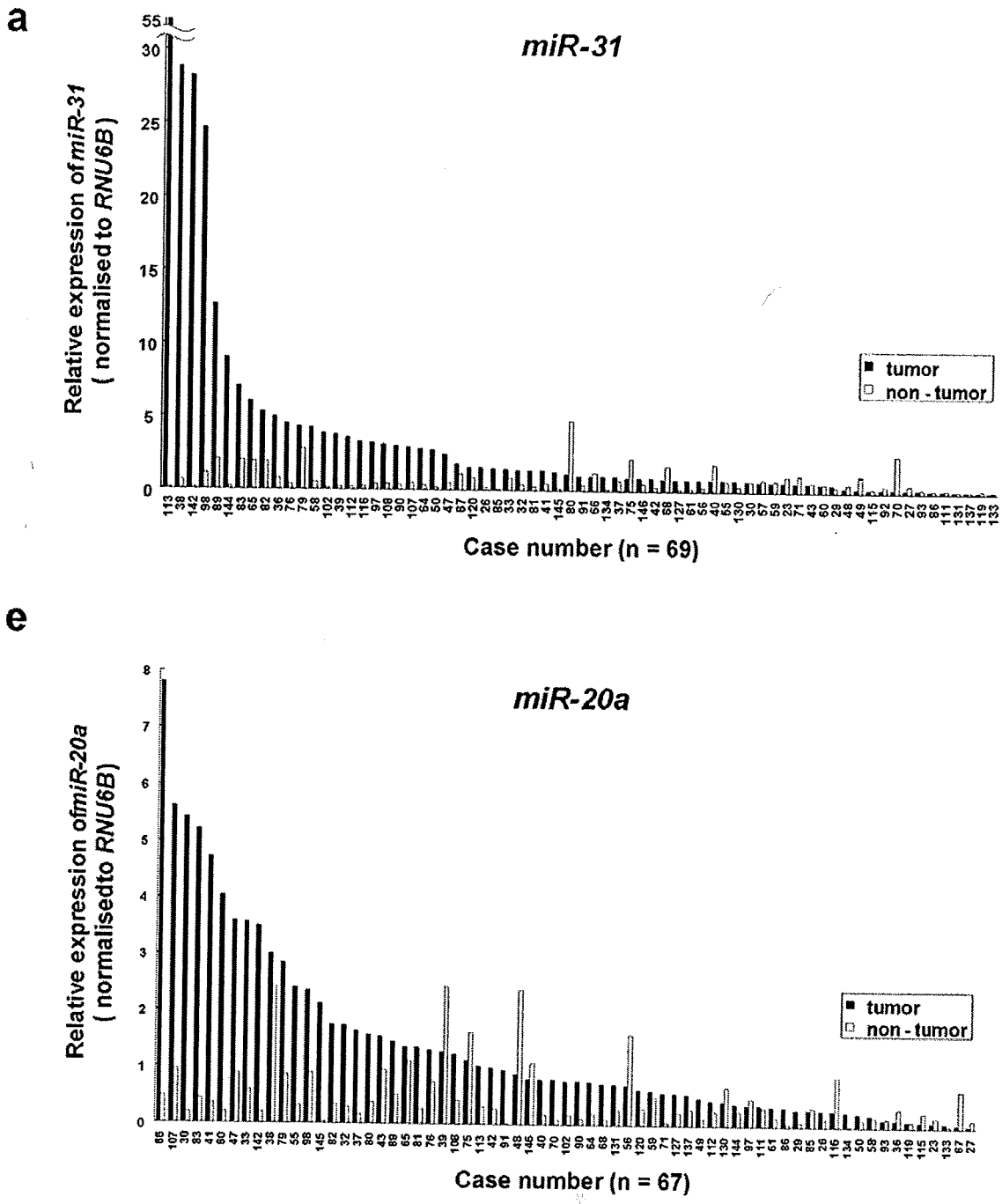

Figure 1. Part 2. Real-time RT-PCR analysis of 6 up-regulated miRNAs in tumor and non-tumor samples from colorectal cancer cases. Expression values of miRNAs were shown as the expression ratio of each miRNA to *RNU6B*. (A, a) *miR-31* expression; (B) *miR-183* expression; (C) *miR-17-5p* expression;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

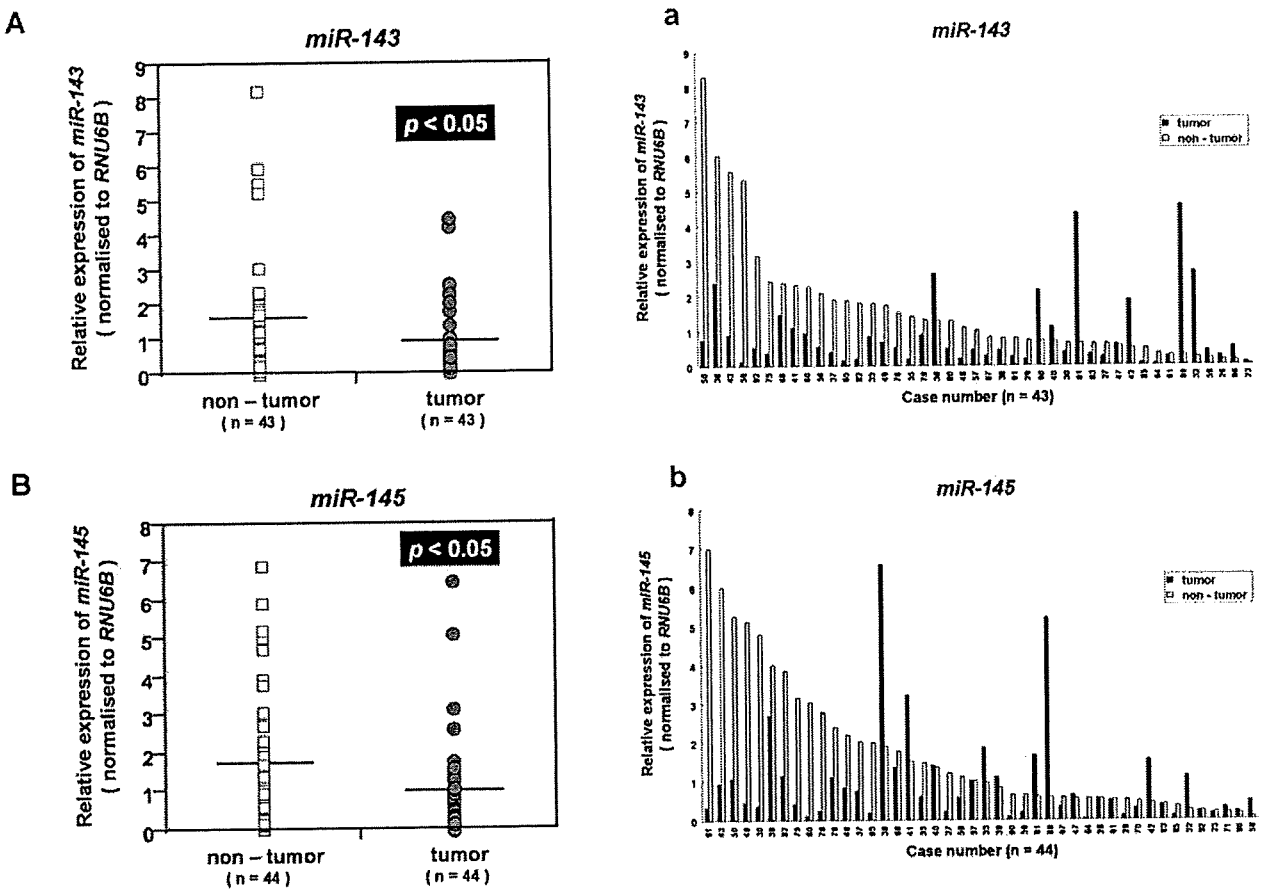

Figure 2. Real-time RT-PCR analysis of 2 down-regulated miRNAs in tumor and non-tumor samples from colorectal cancer cases. (A, a) *miR-143* expression; (B, b) *miR-145* expression. Horizontal lines indicate the mean values of tumor and non-tumor samples.

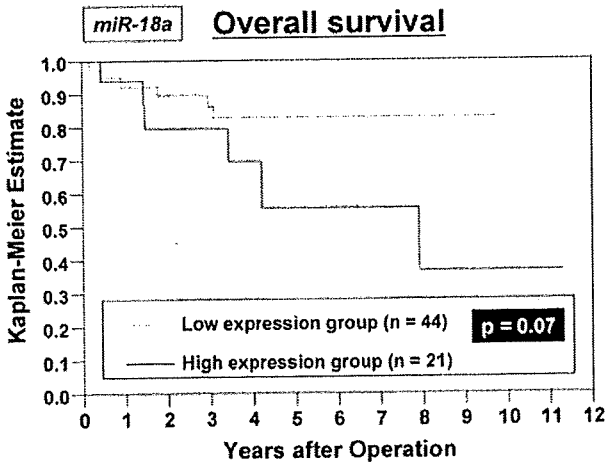

Figure 3. Survival curves of colorectal cancer patients according to *miR-18a* expression status. High expression group (n=21): *miR-18a* > mean value; low expression group (n=44): *miR-18a* < mean value. By the log-rank test, patients with high *miR-18a* expression tended to have poorer prognoses than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

Screening of potential molecular targets for colorectal cancer therapy

Kimi Honma[1]
Ichiro Takemasa[2]
Ryo Matoba[3]
Yusuke Yamamoto[1]
Fumitaka Takeshita[1]
Masaki Mori[2]
Morito Monden[2]
Kenichi Matsubara[3]
Takahiro Ochiya[1]

[1]Section for Studies on Metastasis, National Cancer Center Research Institute, Tokyo, Japan; [2]Graduate School of Medicine, Osaka University, Osaka, Japan; [3]DNA Chip Research Inc., Yokohama, Japan

Abstract: Colorectal cancer is a leading cause of cancer death worldwide. To identify molecular targets for colorectal cancer therapy, we tested small interfering RNAs (siRNAs) against 97 genes whose expression was elevated in human colorectal cancer tissues for the ability to promote apoptosis of human colorectal cancer cells (HT-29 cells). The results indicate that the downregulation of *PSMA7* (proteasome subunit, α-type, 7) and *RAN* (ras-related nuclear protein) most efficiently induced apoptosis of HT-29 cells. *PSMA7* and *RAN* were highly expressed in colorectal cancer cell lines compared with normal colon tissues. Furthermore, *PSMA7* and *RAN* were overexpressed in not only colon tumor tissues but also the other tumor tissues. Moreover, *in vivo* delivery of *PSMA7* siRNA and *RAN* siRNA markedly induced apoptosis in HT-29 xenograft tumors in mice. Thus, silencing of *PSMA7* and *RAN* induces cancer cells to undergo apoptosis, and *PSMA7* and *RAN* might be promising new molecular targets for drug and RNA interference-based therapeutics against colorectal cancer.

Keywords: colorectal cancer, molecular target, RNAi, *PSMA7*, *RAN*

Introduction

Colorectal cancer is one of the most common cancers in women and men worldwide. Nearly 1.2 million cases of colorectal cancer were expected to occur in 2007.[1] The highest incidence rates are found in Japan, North America, parts of Europe, New Zealand, and Australia.[1] Worldwide, some 630,000 people die from colorectal cancer per year, accounting for 8% of all cancer deaths.[1] The five-year survival for persons with colorectal cancer is about 65% in Japan; however, when this cancer is detected at advanced stages, the five-year survival rate decreases to 10%,[2] necessitating effective therapeutic targets.

A tumor is characterized by uncontrolled growth and spread of abnormal cells, which invade adjacent normal tissue and spread to other organs, a process that causes death.[3] Multiple molecular alterations are involved in a transformation from a normal cell into a cancerous cell and a progression from a pre-cancerous lesion to malignant tumors.

Angiogenesis is critical in tumor growth and survival. Its inhibition is a promising target for cancer therapy.[4–6] Vascular endothelial growth factor (VEGF) plays a key role in angiogenesis in cancer. In many human tumors including colorectal cancer, VEGF amd VEGF receptors (VEGFR) are overexpressed. Regulating the VEGF/VEGFR pathway is an effective approach to treat cancer.[6,7] A humanized anti-VEGF monoclonal antibody, Bevacizumab, is the first approved biological inhibitor against VEGF for the treatment of metastatic colorectal cancer.[4–6,8] Small-molecule tyrosine

Correspondence: Takahiro Ochiya
Head, Section for Studies on Metastasis, National Cancer Center Research Institute, 1-1, Tsukiji, 5-chome, Chuo-ku, Tokyo 104-0045, Japan
Tel +81 3 3542 2511 Ext. 4800
Fax +81 3 5565 0727
Email tochiya@ncc.go.jp

kinase inhibitors against VEGFRs are also being developed for cancer therapy.[4,6,9,10]

Furthermore, the survival of abnormal cells is a characteristic feature of cancer. In colorectal tumors, some signal transduction pathways drive abnormal cell growth. The most important factor promoting cell survival is epidermal growth factor (EGF).[11,12] Its signaling is a potential target for cancer therapy.[4,5] In treating colorectal cancer, a monoclonal antibody against EGF receptor (EGFR) such as a cetuximab is active,[4,5,12–14] and small-molecular tyrosine kinase inhibitors of EGFRs have been shown to be effective.[4,12]

Additionally, one of the hallmarks of human carcinogenesis is the breakdown of cell apoptotic machinery.[15] Overexpression of anti-apoptotic Bcl-2 family members frequently relates to decreased sensitivity to anticancer drugs and radiotherapy in many types of cancer.[16] The antisense oligonucleotide drug targeting Bcl-2 mRNA expression such as an oblimersen is being investgated in some cancers.[17] The BH3-domain of anti-apoptotic Bcl-2 family proteins is required for the antiapoptotic function. BH-3 mimic peptides that interfere with Bcl-2 signaling are currently under development.[16] Moreover, therapies based on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which induces programmed cell death, have been studied.[18] Monoclonal antibodies against TRAIL receptors with an agonistic effect on the TRAIL pathway have been generated.[18,19] Thus, inducing apoptosis is a promising approach in the development of a molecular targeted therapy for cancer.

In this paper, we focused on apoptosis induction to identify molecular targets for colorectal cancer therapy. We tested siRNAs against 97 genes whose expression was elevated in human colorectal cancer tissues for the ability to promote apoptosis of human colon cancer cells (HT-29 cells). The results showed that the downregulation of proteasome subunit, α-type, 7 (PSMA7) and ras-related nuclear protein (RAN) strongly caused apoptosis of HT-29 cells. PSMA7 siRNA and RAN siRNA markedly induced apoptosis in HT-29 xenograft tumor tissues in mice. This silencing of PSMA7 and RAN that induces cancer cells to undergo apoptosis suggests that PSMA7 and RAN are potential key targets for future RNA interference (RNAi)-based therapeutics against colorectal cancer.

Materials and methods

Cell culture

Five colorectal cancer cell lines were obtained from the American Type Culture Collection (ATCC), and maintained at 37 °C under 5% CO_2 in a humidified incubator. Caco-2 (human colorectal adenocarcinoma) cells were cultured in Eagle's minimum essential medium (EMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and nonessential amino acids (Invitrogen). Human colorectal carcinoma (HCT116) and human colorectal adenocarcinoma (HT-29) cells were cultured in McCoy's 5A medium (Invitrogen) containing 10% FBS. LoVo (human colorectal adenocarcinoma, derived from supraclavicular lymph node metastatic site) cells were cultured in Ham's F12 medium (Invitrogen) containing 10% FBS. T84 (human colorectal carcinoma, derived from lung metastatic site) cells were cultured in DMEM/Ham's F-12 medium (Invitrogen) containing 10% FBS. To obtain total RNA from these cell lines we plated cells at 1×10^5 cells per well (6-well plate) and culture for three days.

Design and synthesis of siRNAs

We designed siRNAs and synthesized them with four siRNA duplexes for each gene target (Dharmacon, Chicago, IL, USA). The siRNA sequences were described in Table 1.

Cell transfection array

For RNAi-based functional screening of genes, we used a reverse transfection based-cell transfection array.[20] HT-29 cells were plated into the cell transfection array in a 96-well format and transfected with siRNA. We evaluated the effects of the downregulation of genes on promotion of apoptosis, as mentioned below.

Measurement of cell proliferation

We plated HT-29 cells into a cell transfection array at a density of 5×10^3 cells per well and cultured. Three days after, we measured cell proliferation by resazurin reduction assay using CellTiter-Blue Reagent (Promega, Madison, WI, USA). Cells were incubated with CellTiter-Blue Reagent for one hour at 37 °C, and the fluorescence was then measured at 560Ex/590Em. After that, we subjected the same cell transfection array to a caspase-3/7 assay, Hoechst staining, and a cell-direct real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

Measurement of caspase activity *in vitro*

Cells were incubated with the Caspase-Glo 3/7 Reagent (Promega) for one hour at room temperature, and the luminescence was then measured.

Hoechst staining

Cells on a cell transfection array were washed with phosphate-buffered saline (PBS), and a fixative and staining solution was

Table 1 The sequences of siRNAs

	Sense strand	Antisense strand
PSMA7		
#1	5′-GAAGUAUGUUGCUGAAAUUUU-3′	5′-AAUUUCAGCAACAUACUUCUU-3′
#2	5′-GAAGAGACAUUGUUGUUCUUU-3′	5′-AGAACAACAAUGUCUCUUCUU-3′
#3	5′-GAAGAUCUGUGCUUUGGAUUU-3′	5′-AUCCAAAGCACAGAUCUUCUU-3′
#4	5′-CAUCGUGGGUUUCGACUUUUU-3′	5′-AAAGUCGAAACCCACGAUGUU-3′
RAN		
#1	5′-AGAAGAAUCUUCAGUACUAUU-3′	5′-UAGUACUGAAGAUUCUUCUUU-3′
#2	5′-GUGAAUUUGAGAAGAAGUAUU-3′	5′-UACUUCUUCUCAAAUUCACUU-3′
#3	5′-CCUAUUAAGUUCAAUGUAUUU-3′	5′-AUACAUUGAACUUAAUAGGUU-3′
#4	5′-ACAGGAAAGUGAAGGCGAAUU-3′	5′-UUCGCCUUCACUUUCCUGUUU-3′

Abbreviation: siRNA, small interfering RNAs.

added (4% paraformaldehyde, 0.1% Triton X-100, 1 μg/mL Hoechst 33342 in PBS). Twenty minutes after incubation, cells were washed with PBS. We determined the number of apoptotic cells was in three microscopic fields of each well by fluorescence microscopy.

Transfection of siRNA

We carried out transfection of HT-29 cells with siRNA using Liopfectamine 2000 (Invitrogen), according to the manufacturer's protocol. We plated HT-29 cells 24 hours before transfection, and we then transfected the cells, which were grown to 50% confluence, with 40 nM siRNAs.

Real-time RT-PCR

We purified total RNA from cells and tumor tissues with an RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN, Hilden, Germany), and produced cDNAs with an ExScript RT reagent Kit (Takara Bio, Shiga, Japan). We then subjected cDNA samples to real-time PCR using SYBR Premix Ex Taq (Takara) and specific primers as follows: for *PSMA7*, forward, 5′-CAAGTGGAGTACGCGCAGGA-3′; reverse, 5′-CTGCAGTTTGGCCACTGACTTC-3′; for *RAN*, forward, 5′-AAGTTGTCATGGACCCAGCTTTG-3′; reverse, 5′-GCTGGGCTCCAGCTTCATTC-3′. We carried out the reactions using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). We normalized gene-expression levels by 18S rRNA or *GAPDH*.

Cell-direct real-time RT-PCR

We used FastLane Cell cDNA Kit and QuantiTect SYBR Green PCR Kit (QIAGEN). We lysed cells in a well of cell transfection array and synthesized the first-strand cDNA. The cDNA template was then directly subjected to real-time RT-PCR using specific primers.

siRNA treatment *in vivo*

We performed animal experiments in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute of Japan. We subcutaneously injected 5×10^6 HT-29 cells into athymic nude mice (six-week-old females; CLEA Japan, Tokyo, Japan). When the HT-29 tumor grew to approximately 5–6 mm in diameter, we injected mice with 1 nmol siRNA by intratumoral injection. We harvested tumor tissues for analysis of mRNA and apoptosis at 24 and 72 hours after treatment, respectively.

TUNEL technique

We harvested tumor tissues 72 hours after administration of siRNA and prepared frozen sections. We then performed TUNEL (TdT-mediated dUTP nick-end labeling) staining using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. DNA strand breaks in apoptotic cells can be labeled by the addition of fluorescein dUTP using terminal deoxynucleotidyl transferase (TdT). The nuclei were stained with DAPI. We determined the number of fluorescein-positive cells in three microscopic fields of each section by fluorescence microscopy.

Human samples

The study protocol for clinical samples was approved by the Institutional Review Board of Osaka University Medical School (Osaka, Japan), and written informed consent was

obtained from each patient. We obtained total RNA from tumor tissues and normal adjacent tissues (FirstChoice Human Tumor/Normal Adjacent Tissue RNA) from Ambion (Austin, TX, USA).

cDNA micro-array analysis

We performed cDNA micro-array analysis using AceGene (DNA Chip Research Inc., Yokohama, Japan) according to the manufacturer's instructions (http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf) to obtain an expression profile of human colorectal cancer.[21] As a standard normal control reference, a mixture of total RNA extracted from normal colorectal tissues was used. We synthesized cDNA from total RNA of normal colorectal tissues and colorectal tumor tissues, and labeled cDNA with Cy3 for normal colorectal tissue, and Cy5 for colorectal tumor tissues, respectively. After hybridization of cDNA and array, the array was scanned, and Cy5/Cy3 ratios were $\log_2$-transformed to compare levels of mRNA expression in tumor and normal tissues.

Statistical analysis

We conducted statistical analysis using the analysis of variance with Student's *t*-test. We considered a *P* value of 0.05 or less as a significant difference.

Results

RNAi-based screening for identification of molecular targets

To identify molecular targets for therapy, we conducted a study of RNAi-induced gene knockdown in HT-29 human colon cancer cells. The strategy for target identification is schematically shown in Figure 1. We performed gene expression profiling of 191 subjects with colorectal tumors and selected 97 genes whose expression was elevated in human colorectal cancer tissues by rank order of mRNA expression (Table 2). We used the siRNAs specific to these genes which are siRNA pools composed of four distinct siRNA species targeting different sequences of the same target transcript for RNAi-based screening. We transfected HT-29 cells with the

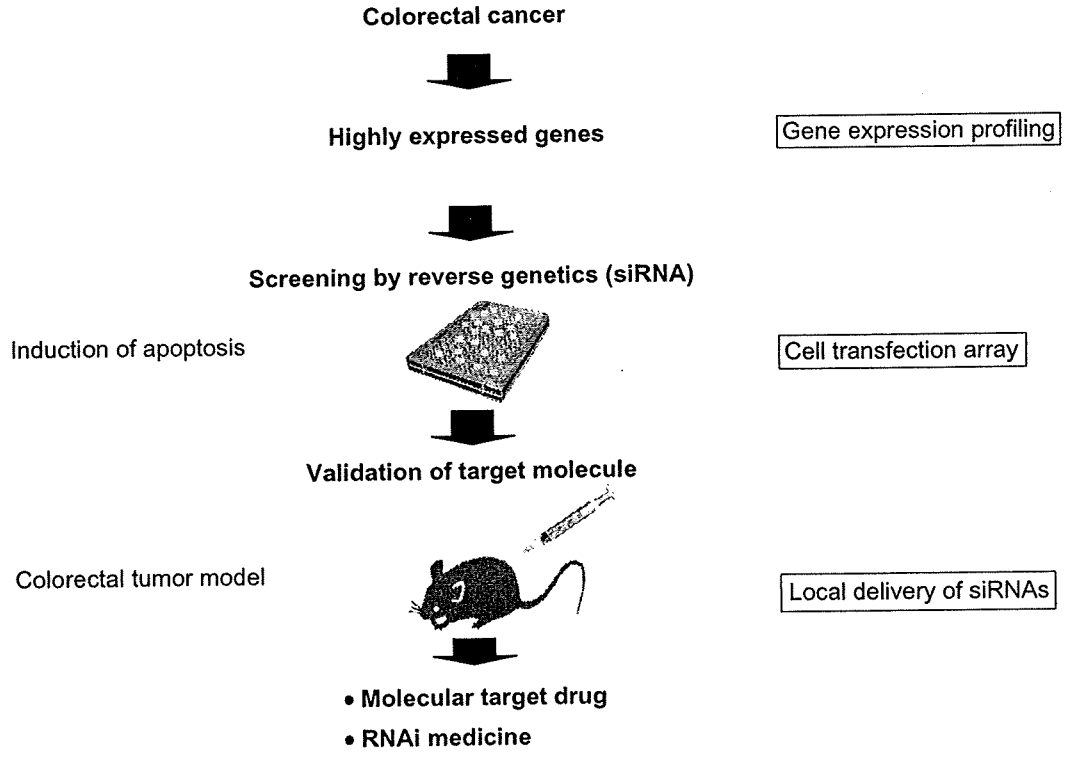

Figure 1 Schematic representation of the strategy for targets identification by RNAi-based reverse genetics *in vitro* and *in vivo*. First, we carried out a gene expression profiling of human colorectal tumor tissues and selected genes whose expression was elevated. We performed a functional screening of genes by a cell transfection array to test the efficacy of a specific siRNA related to apoptosis induction in human colorectal cancer. Subsequently, the siRNAs against candidate genes were applied to an *in vivo* animal tumor model. Finally, we identified new molecular targets for drug and RNAi-based colorectal cancer therapy.
Abbreviation: siRNA, small interfering RNAs.