substitutions of amino acid in the HCV core region also served as predictors of early, as well as end-of treatment response [13,14]. The miRNA expression obtained from the therapeutic response, can be applied to the prediction of drug response. The advantages of using miRNA for the microarray analysis include the following; (i) It was relatively easy to analyze because fewer probes were installed compared with the usual cDNA array. (ii) The change in each manifestation of a miRNA was low, in fact, in most miRNA, standard deviation was twice or less in average value (data not shown). The expression levels of miR-34b and 422b in the early response phase and final responses to treatment were consistently and significantly high and low in non-responders, respectively. Therefore these two miRNAs may be useful markers for early-to-final drug response to the IFN treatment.

Further studies are indeed needed to clarify the connection between miRNA expression and patient response to CHC combination therapy. Because information on miRNA is regularly being updated, we are planning to performed more analysis using the latest microarray and a larger sample in the future. However, in the meantime, as we have shown in Figure 4, the bigger the size of the training set, the higher the prediction performance that is achieved. This combined with the results of our Monte Carlo cross validation provided a strong based to verify the concepts in this report. We believe that our results have three advantages (i) the prediction methods used were quite reasonable, (ii) the prediction performance can later be improved if more patients' data become available and (iii) obtaining miRNA profile (not specific miRNAs) is useful for predicting the drug response. While current therapy is based on positive selection with HCV genotype or negative selection with IL28B SNP, and is limited to only some cases, our methods are applicable to all patients [13,18].

Conclusions

Our study shows that the specific miRNA are expressed differently depending on patient's drug response. As result we feel that miRNA profiling can be useful for predicting patient drug response before the administering combination therapy thereby reducing ineffective treatments. Moreover, miRNA expression profile can facilitate the accumulation of basal information for the development of novel therapeutic strategies. This approach allows for more suitable therapeutic strategies based on clinical information of individuals.

Additional material

Additional file 1: miRNA hypothetical target genes according to in silico analysis.

Additional file 2: Real-time qPCR validation of immune-related hypothetical target genes of miRNAs. The expression levels of hypothetical target genes in HEK293 cells were compared among three groups treated with control RNA, ds miRNA, and ASO miRNA. The data shown are means+SD of three independent experiments. Asterisk indicates a significant difference of $p < 0.05$.

Additional file 3: human miRNA target on the HCV genome genotype 1b (Accession No. AF333324)

List of abbreviations

HCV: hepatitis C virus; CH: chronic hepatitis C; LC: liver cirrhosis; HCC: hepatocellular carcinoma; miRNA: microRNA; IFN, interferon; SVR: sustained virological responder; R: relapse; NR: non-responder; RVR: rapid virological responder; EVR: early virological responder.

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Author details

[1]Center for Genomic Medicine, Kyoto University Graduate School of Medicine, 53 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. [2]Department of Molecular Pathology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan. [3]Department of Gastroenterology, Ogaki Municipal Hospital, 86-4 Minaminokawa-cho, Ogaki, Gifu 503-8502, Japan. [4]DNA Chip Research Inc., 43-1-1 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. [5]Department of Molecular Life Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan. [6]Research Institute, Chiba Institute for Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan.

Authors' contributions

YM and KS conceived and designed the experiments; YM, HT and KH performed the experiments; MT and MK performed statistical analysis; YM, MT, HT and AT contributed to writing and editing the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Original Article

Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development

Masatoshi Shigoka,[1] Akihiko Tsuchida,[1] Takaaki Matsudo,[1] Yuichi Nagakawa,[1] Hitoshi Saito,[1] Yoshiaki Suzuki,[1] Tatsuya Aoki,[1] Yoshiki Murakami,[2] Hidenori Toyoda,[3] Takashi Kumada,[3] Ralf Bartenschlager,[4] Nobuyuki Kato,[5] Masanori Ikeda,[5] Tomoki Takashina,[6] Masami Tanaka,[6] Rieko Suzuki,[6] Kosuke Oikawa,[7] Masakatsu Takanashi[6] and Masahiko Kuroda[6]

[1]Third Department of Surgery and [6]Department of Molecular Pathology, Tokyo Medical University, Tokyo, Japan, [2]Center for Genomic Medicine, Kyoto University, Kyoto, Japan, [3]Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan, [4]Department of Infectious Diseases, Molecular Virology, University of Heidelbergand, Heidelberg, Germany, [5]Department of Tumor Virology, Okayama University Graduate School of Medicine, Okayama, Japan, and [7]First Department of Pathology, Wakayama Medical University, Wakayama, Japan

MicroRNAs (miRNAs) belong to a class of the endogenously expressed non-coding small RNAs which primarily function as gene regulators. Growing evidence suggests that miRNAs have a significant role in tumor development and may constitute robust biomarkers for cancer diagnosis and prognosis. The *miR-17-92* cluster especially is markedly overexpressed in several cancers, and is associated with the cancer development and progression. In this study, we have demonstrated that miR-92a is highly expressed in hepatocellular carcinoma (HCC). In addition, the proliferation of HCC-derived cell lines was enhanced by miR-92a and inhibited by the anti-miR-92a antagomir. On the other hand, we have found that the relative amount of miR-92a in the plasmas from HCC patients is decreased compared with that from the healthy donors. Interestingly, the amount of miR-92a was elevated after surgical treatment. Thus, although the physiological significance of the decrease of miR-92a in plasma is still unknown, deregulation of miR-92 expression in cells and plasma should be implicated in the development of HCC.

Key words: hepatocellular carcinoma, microRNA, miR-638, miR-92a, plasma

Correspondence: Masahiko Kuroda, MD, PhD, Department of Molecular Pathology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan. Email: kuroda@tokyo-med.ac.jp

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MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate gene expression and have a critical role in many biological and pathological processes.[1] Recent studies have shown that deregulation of miRNA expression contributes to the multistep processes of carcinogenesis, and have shown promise as tissue-based markers for cancer classification and prognostication.[2,3] However, biological roles of only a small fraction of known miRNAs have been elucidated to date.

The miR-17-92 cluster at 13q31.3 is consists of six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1, and plays an important role for development of lung cancer,[4] B-cell lymphomas,[5] chronic myeloid leukemia,[6] medulloblastomas,[7] colon cancer[8] and hepatocellular carcinoma (HCC).[9] In addition, mice deficient in the miR-17-92 cluster died shortly after birth with lung hypoplasia, and B-cell development was impaired in the mice.[10] It has been reported, however, that miR-92a increases cell proliferation by negative regulation of an isoform of the cell-cycle regulator p63.[11] Furthermore, miR-92a regulates angiogenesis.[12] Thus, it is clear that the miR-92a has some oncogenic characteristics. However, the specific biological role of miR-92a in the processes of human cancer development has remained unclear.

Here, we have revealed that miR-92a is implicated in human HCC development. Furthermore, we have demonstrated that miR-92a in human blood has the potential to be a noninvasive molecular marker for diagnosis of human HCC.

MATERIALS AND METHODS

In situ hybridaization of miR-92a

Locked nucleic acid (LNA)-modified probes for miR-92a and negative control (miRCURY-LNA detection probe, Exiqon, Vedbaek, Denmark) were used. The probe sequences were as follows; *miR-92a*, 5′-ACAGGCCGGGACAAGTGCAATA-3′; and a scrambled oligonucleotides used for negative control, 5′-GTGTAACACGTCTATACGCCCA-3′. *In situ* hybridization was performed using the RiboMap *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ, USA) on the Ventana Discovery automated *in situ* hybridization instrument (Ventana Medical Systems). The *in situ* hybridization steps were performed as previously described.[13] Staining was evaluated by two investigators and graded as follows: negative (–), no or occasional (<5%) staining of tumor cells; positive (+), mild to strong (>5%) staining of tumor cells. Paraffin–embedded tissue samples of hepatocellular carcinoma (HCC) and adjacent non-tumorous liver cirrhosis (LC) were obtained from HCC patients at Ogaki Municipal Hospital (Ogaki, Japan). Details of the clinical data are provided in Table 1.

Plasma collection, RNA isolation and quantitative RT-PCR

Whole blood samples were collected from healthy donors and the patients with HCC at Ogaki Municipal Hospital. This study was approved by the institutional review board (IRB) of Tokyo Medical University, and all subjects provided written informed consent under the institutional review board. Details of clinical data are provided in Table 1. Diagnoses were confirmed using the post-operated tissues. Blood samples of the patients (Cases 1–10) were collected one day before the operation and then properly stored. One week after operation, blood samples of the patients were collected again. Whole blood was separated into plasma and cellular fractions by centrifugation at 1600 *g* for 15 min. Total RNA in the

Table 1 Summary of clinical details of hepatocellular carcinoma (HCC)used for in situ hybridaization and serum analysis

	Year	Sex	Virus type	Histologic type	Stage	Child-Pugh	miR-92a
Case 1	53	Male	HBV	Poorly	I	A	+
Case 2	59	Male	HBV	Moderate	II	A	+
Case 3	79	Male	NBNC	Moderate	III	A	+
Case 4	73	Male	HCV	Well	I	A	+
Case 5	76	Female	HCV	Moderate	IV-A	A	+
Case 6	59	Male	HCV	Moderate	II	A	+
Case 7	69	Female	HCV	Moderate	I	A	+
Case 8	71	Male	HCV	Moderate	I	A	+
Case 9	59	Female	HBV	Well	I	A	–
Case 10	69	Male	NBNC	Moderate	IV-A	A	–
Case 11	61	Female	HBV	Poorly	IV-A	B	+
Case 12	73	Male	NBNC	Moderate	II	A	+
Case 13	67	Male	NBNC	Moderate	IV-A	A	+
Case 14	61	Male	NBNC	Moderate	III	A	+
Case 15	45	Male	HBV	Moderate	I	A	+
Case 16	68	Female	HCV	Moderate	III	A	+
Case 17	70	Male	NBNC	Poorly	II	A	+
Case 18	59	Male	HCV	Moderate	III	A	+
Case 19	43	Male	HBV	Moderate	II	A	+
Case 20	69	Male	HCV	Moderate	II	A	–
Case 21	76	Male	HCV	Moderate	III	A	–
Case 22	53	Male	HCV	Moderate	II	A	–

HCV, hepatitis C virus; HBV, hepatitis B virus; NBNC, non-B non-C virus.

Table 2 Summary of clinical details of hepatocellular carcinoma (HCC) used for qPCR analysis

Code no.	Year	Sex	Virus type	Histologic type	Non-tumorous tissue	AFP	PIVKA-II
91	53	Male	HCV	Moderate	LC	5	0.06
160	59	Male	HCV	Moderate	LC	NI	NI
O89	68	Male	HCV	Moderate	LC	8	25
O90	70	Male	HCV	Moderate	LC	686	962
K89	51	Male	HCV	Moderate	LC	NI	NI

LC, liver cirrhosis; HCV, hepatitis C virus; NI, no information.

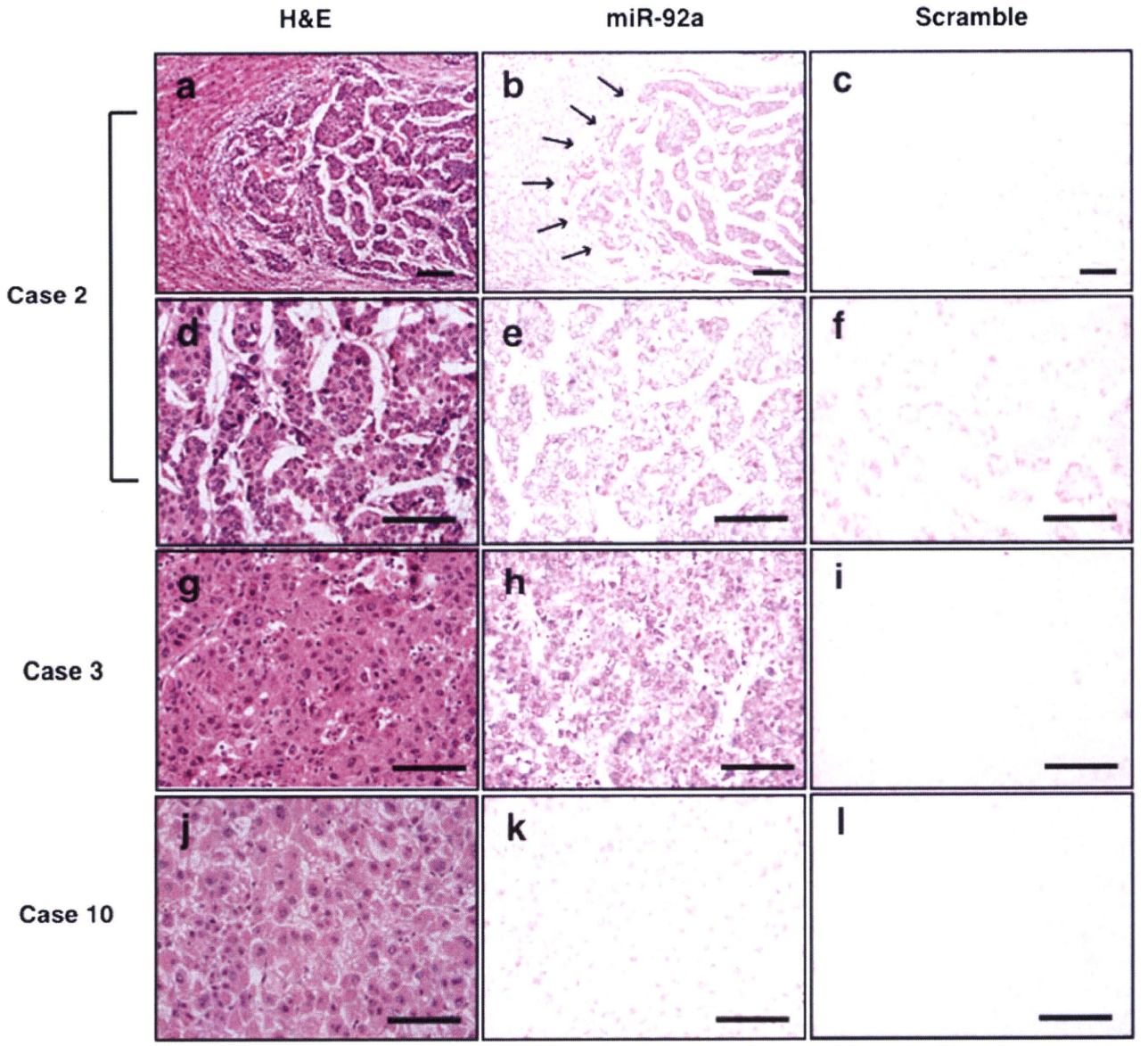

Figure 1 MiRNA expression in hepatocellular carcinoma (HCC). *In situ* hybridization was performed using Locked nucleic acid (LNA)-modified probes for miR-92a and negative control. Case 2 and Case 3 were positive cases for miR-92a. Case 10 was a negative case for miR-92a. (**a–c**) Low power field of boundary of HCC and non-tumor lesion. Arrowheads indicated a border. Only HCC regions were positive for miR-92a. (**d–l**) High power field of HCC. Blue signals represent positive for miR-92a. Bars indicate 100 μm.

plasma was isolated using Isogen-LS (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. The RNA sample was suspended in 20 μL of nuclease free water. In general, we obtained 400 ng of RNA from 1 mL of plasma. MiRNAs were quantified using TaqMan MiRNA Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) as previously described.[13]

For miR-92a quantification in tissue samples, five pairs of fresh HCC and non-tumorous LC samples were surgically resected from HCC patients (Table 2). All the patients or their guardians provided written informed consent, and the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine approved all aspects of this study. The amounts of miR-92a were normalized to RNU48 that is one of rRNAs (Applied Biosystems).

Cell culture and transfection

Hepatocellular carcinoma (HCC) cell lines HepG2, OR6 and SN1a were cultured in Dulbecco's modified Eagle's medium

(DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). OR6 and SN1a are derived from the Huh7 HCC cell line and maintain hepatitis C virus (HCV) replicon.[14–16] The miR-92a oligonucleotide used in the transfection experiments is a synthetic double-strand 19 nucleotide RNA oligonucleotide (5′-UUGCACUUGUCCC GGCCUG-3′) purchased from B-Bridge International (Tokyo, Japan). The scrambled oligonucleotide represents a mix of two different frames of the miR-92 sequence (5′-UAUUGC ACUUGUCCCGGCCUGUCCCGGCC-3′ and 5′-AUUGCAC UUGUCCCGGCCUTT-3′). Locked nucleic acid (LNA) oligonucleotide miR-92 knockdown (antagomir) was obtained from Exiqon (Vedbaek, Denmark, http://www.exiqon.com). The oligonucleotides were individually transfected by Hiper-Fect (QIAGEN K. K., Tokyo, Japan) into the cells at a final concentration of 100 nM.

In vitro proliferation assays

The effects of miR-92a and the anti-miR-92a antagomir on the growth of HepG2, OR6 and SN1a were evaluated using the MTT cell growth assay kit (Cell Count Reagent SF, Nacalai tesque, Kyoto, Japan). The cells were transfected with miR-92a or the antagomir. The cell numbers were then assessed with MTT assay at 48 or 72 h after the transfection. The MTT assay was performed according to the manufacturer's recommendation. The reagents were added to each well and incubated at 37°C for 4 h. The MTT reduced by living cells into a formazan product was assayed with a multiwell scanning spectrophotometer at 450 nm.

RESULTS

Highly expression of miR-92a in HCC cells

We first examined whether or not miR-92a is expressed in hepatocellular carcinoma (HCC). We performed *in situ* hybridization using locked nucleic acid (LNA)-modified probes digoxigenin (DIG) labelled. We found that miR-92a was strongly expressed in cancer cells of 17 out of 22 HCC cases (Table 1 and Fig. 1). No significant differences were observed in age, sex, virus type, clinical stage and tumor differentiation of the clinical samples. In contrast, we did not detect miR-92a expression in non-cancerous hepatocytes around the HCCs.

Furthermore, we quantified miR-92a levels in HCC sections (n = 5) and their adjacent non-tumorous liver cirrhosis (LC) sections (n = 5) by *Taq*Man qRT-PCR (Table 2 and Fig. 2). The levels of miR-92a expression in HCC sections were higher than that in adjacent LC sections (Fig. 2).

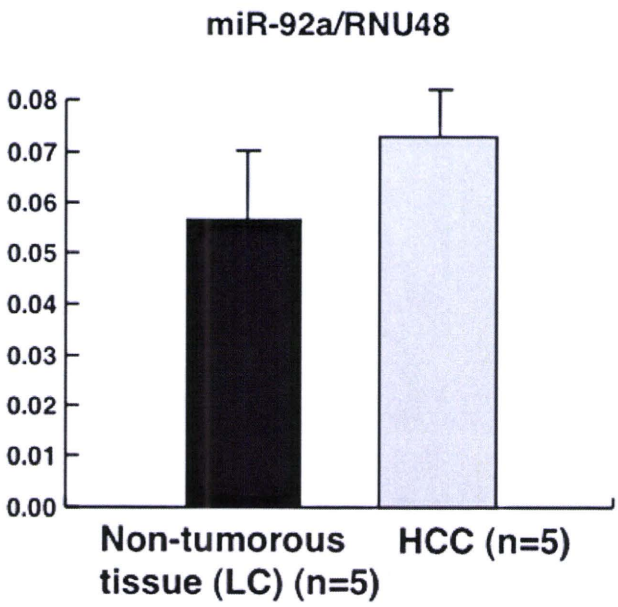

Figure 2 Quantification of miR-92a expression in hepatocellular carcinoma (HCC) tissue samples. The ratios of miR-92a to RNU48 in HCC tissues and their adjacent non-tumorous liver cirrhosis (LC) tissues were analyzed by *Taq*Man qRT-PCR. *Bars*, s.d.

Effects of miR-92a on a Hepatoma cell lines HepG2, OR6 and SN1a

Next, we investigated whether miR-92a affects cell proliferation of human HCC cell lines, HepG2, OR6 and SN1a. We transiently transfected either miR-92a or the anti-miR-92a antagomir into the cells. Antagomirs are single-stranded RNAs that are complementary to a specific miRNA and cause the depletion of the miRNA.[17] After the transfection, we found that all of the cells transfected with the anti-miR-92a antagomir showed lower proliferation rate than the cells transfected with a control RNA oligonucleotide (Fig. 3a). In contrast, the cells except for HepG2 showed increased proliferation rate when miR-92a was transfected (Fig. 3a). We also confirmed the amounts of miR-92a in the cells by quantitative real time PCR (Fig. 3b).

The ratio of miR-92a to miR-638 serves as a biomarker for HCC

Finally, we sought to determine whether the expression level of miR-92a in blood sera could discriminate HCC patients from healthy individuals. Previously, we have revealed that miR-92a is dramatically reduced in the plasmas of acute leukemia patients although in leukemic cells it is strongly expressed.[13] We analyzed the miR-92a levels in the plasma samples from normal individuals (n = 10) and HCC patients

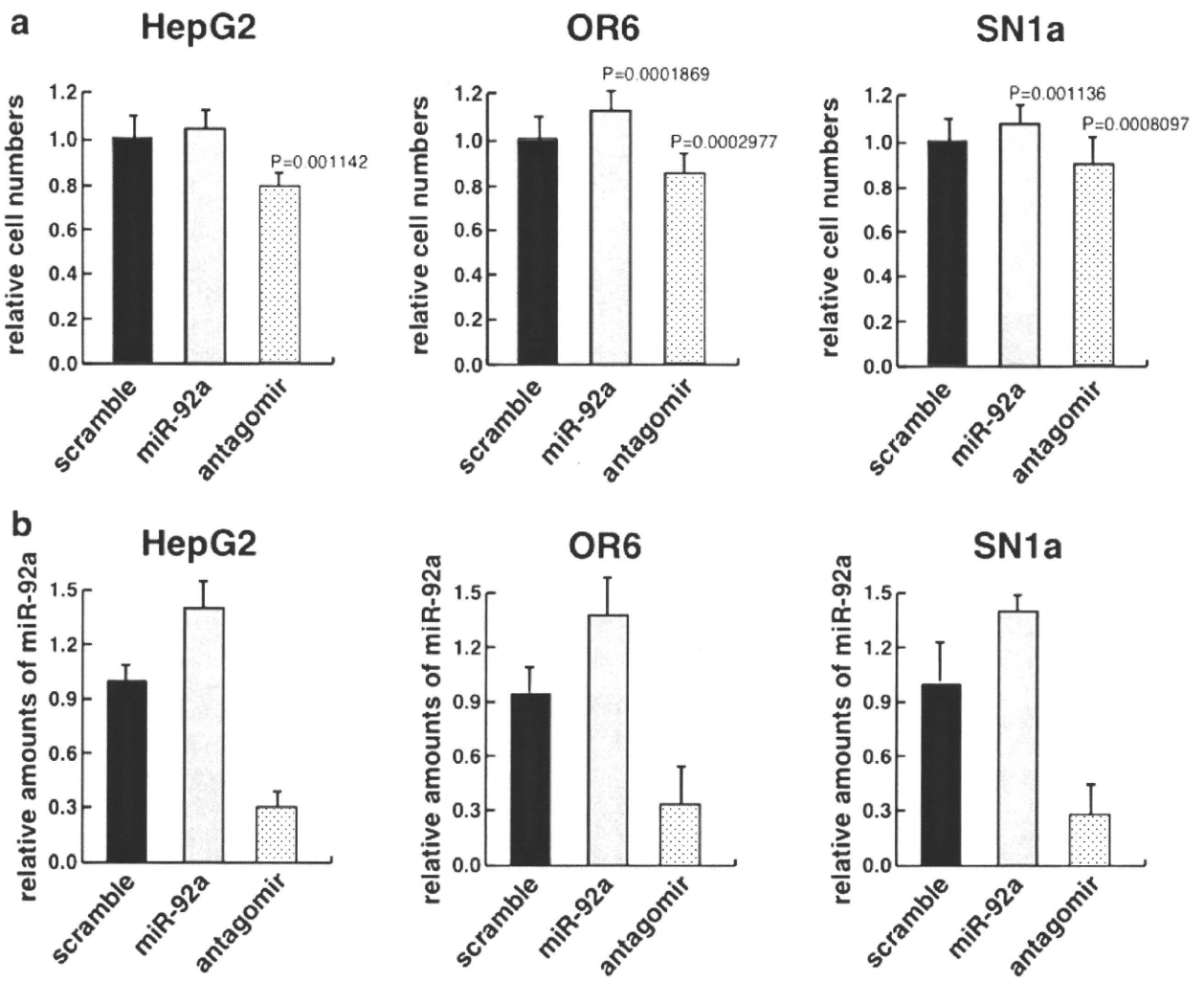

Figure 3 miR-92a modulates proliferation of HepG2, OR6 and SN1a cells. (**a**) Cell numbers of the HepG2, OR6 and SN1a cells transfected with synthetic miR-92a, anti-miR-92a antagomir, or scrambled control oligonucleotide were analyzed by MTT assays at 48 h for OR6 and SN1a and 72 h for HepG2 after transfection. *Bars*, s.d. (**b**) qRT-PCR analysis of miR-92a amounts in the cells transfected with miR-92a, anti-miR-92a antagomir or scrambled control at 48 h for OR6 and SN1a and 72 h for HepG2 after the transfection.

(n = 10) by *Taq*Man qRT-PCR. Because miR-638 is stably present in human plasmas,[13] we used miR-638 as the standard to improve the precision of the data. The ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). Then, we further examined the ratio from the patients after surgical resection. Interestingly, the miR-92a/miR-638 levels were significantly higher than that in the plasmas from the patients before surgical resection (Fig. 4b).

DISCUSSION

In this study, we found that miR-92a was highly expressed in HCC (Figs 1,2). In addition, we demonstrated that the expression level of miR-92a affects the proliferation of hepatoma cell lines, HepG2, OR6 and SN1a (Fig. 3). These results suggest that miR-92a may play an important role in tumor progression of hepatocyte. We do not know why, but addition of miR-92a did not significantly increase the proliferation of HepG2 cells. It may be possible that HepG2 cells themselves already contain enough miR-92a to promote cancer cell proliferation. In addition, miR-92a is a part of the miR-17-92 cluster, which is actively involved in the development and progression of various cancers.[4–10] However, the molecular function of miR-92a is still unknown, and its mRNA targets have not been identified. Recently, it has been shown that one of the molecular mechanisms through which miR-92a increases cell proliferation is by negative regulation of an isoform of the cell-cycle regulator p63.[11] Thus, we examined

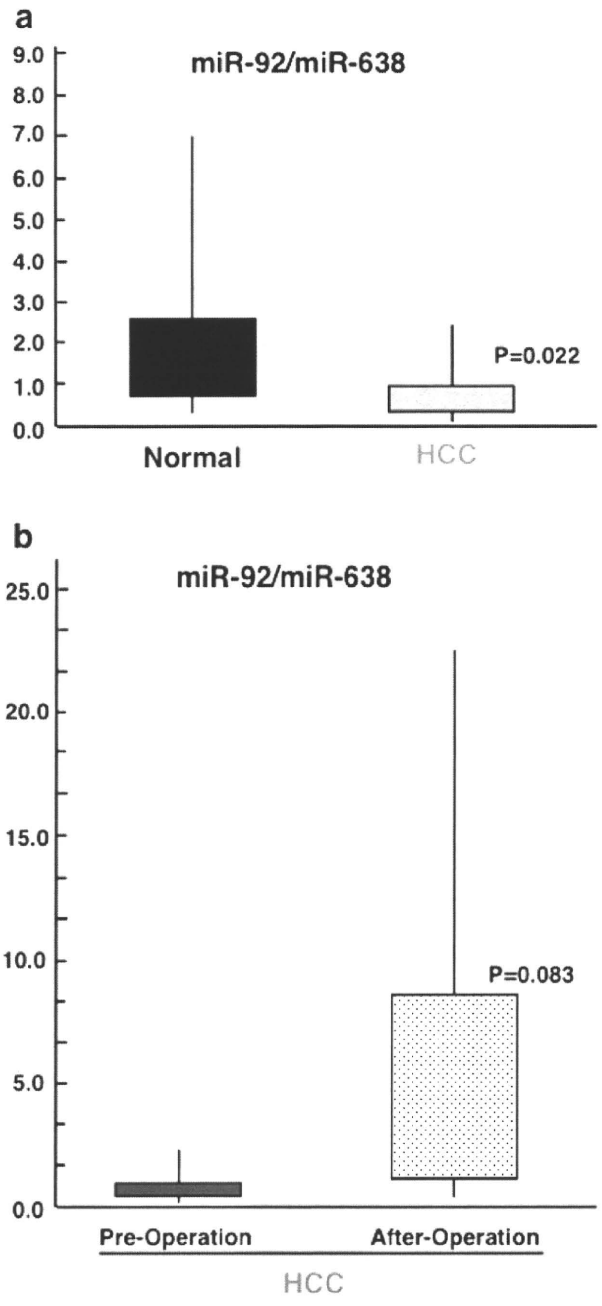

Figure 4 Comparison of miR-92a levels in the plasmas from normal individuals and hepatocellular carcinoma (HCC) patients. (**a**) The ratios of miR-92a to miR-638 in the plasmas from normal donors and HCC patients were analyzed by *Taq*Man qRT-PCR. Student's *t*-test was used to determine statistical significance. (**b**) The ratios of miR-92a to miR-638 in the plasmas from HCC patients before and after tumor resection were analyzed by *Taq*Man qRT-PCR.

the expression of p63 in HCC by immunohistochemistry. However, we could not find the positive nuclear staining both in HCC and normal hepatocyte (data not shown). On the other hand, the miRanda software found 300 different genes that have putative miR-92a binding sites conserved among *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus* at the 3′-UTR regions of their transcripts. Therefore, at least in HCC, there may be novel miR-92a targets that are involved in cancer cell proliferation.

In this report, we have revealed that the value of miR-92a/miR-638 in plasma has potential as a very sensitive marker for HCC. We found that the ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). We did not find any differences in the values of the ratios between hepatitis B virus (HBV) infection and hepatitis C virus (HCV) infection (data not shown). On the other hand, we recently observed decrease of miR-92a in plasma samples of acute leukemia.[13] These results suggest that the decrease of the miR-92a/miR-638 level in human plasma may serve as a valuable diagnostic marker for not only acute leukemia but also solid tumors such as HCC. Moreover, we observed increase of miR-92a/miR-638 levels in the plasmas from the HCC patients after tumor resection (Fig. 4b). Thus, the miR-92a/miR-638 levels in human plasmas may also be a potential noninvasive follow up marker of HCC. To confirm this notion, a large number of plasma samples should be examined. Nevertheless, the levels of miR-92a/miR-638 promise to be an effective biomarker for malignant tumors. The physiological significance of the decrease of miR-92a in plasma is still unknown.

In summary, we have shown that miR-92a may be involved in HCC development. In addition, we have demonstrated that the ratio of miR-92a/miR-638 in blood is expected to be useful for diagnosis of HCC patients. This study may also provide useful information for further investigations of functional association between miRNAs and HCC.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health, Labour and Welfare of Japan, Japan Health Sciences Foundation and a grant of Yamaguchi Endocrine Research Association and the grant of 'University-Industry Joint Research Project' for private universities as well as a matching fund subsidy from the MEXT (Ministry of Education, Culture, Sports, Science and Technology, 2007–2009). We thank Koji Fujita for his technical assistance and Satoko Aochi for her outstanding editorial assistance.

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The Progression of Liver Fibrosis Is Related with Overexpression of the miR-199 and 200 Families

Yoshiki Murakami[1]*, Hidenori Toyoda[2], Masami Tanaka[3], Masahiko Kuroda[3], Yoshinori Harada[4], Fumihiko Matsuda[1], Atsushi Tajima[5¤], Nobuyoshi Kosaka[6], Takahiro Ochiya[6], Kunitada Shimotohno[7]

1 Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **2** Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan, **3** Department of Molecular Pathology, Tokyo Medical University, Tokyo, Japan, **4** Department of Pathology and Cell Regulation, Kyoto Prefectural University of Medicine, Kyoto, Japan, **5** Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Japan, **6** Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan, **7** Research Institute, Chiba Institute of Technology, Narashino, Japan

Abstract

Background: Chronic hepatitis C (CH) can develop into liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Liver fibrosis and HCC development are strongly correlated, but there is no effective treatment against fibrosis because the critical mechanism of progression of liver fibrosis is not fully understood. microRNAs (miRNAs) are now essential to the molecular mechanisms of several biological processes. In order to clarify how the aberrant expression of miRNAs participates in development of the liver fibrosis, we analyzed the liver fibrosis in mouse liver fibrosis model and human clinical samples.

Methodology: In a CCL_4-induced mouse liver fibrosis model, we compared the miRNA expression profile from CCL_4 and olive oil administrated liver specimens on 4, 6, and 8 weeks. We also measured expression profiles of human miRNAs in the liver biopsy specimens from 105 CH type C patients without a history of anti-viral therapy.

Principle Findings: Eleven mouse miRNAs were significantly elevated in progressed liver fibrosis relative to control. By using a large amount of human material in CH analysis, we determined the miRNA expression pattern according to the grade of liver fibrosis. We detected several human miRNAs whose expression levels were correlated with the degree of progression of liver fibrosis. In both the mouse and human studies, the expression levels of miR-199a, 199a*, 200a, and 200b were positively and significantly correlated to the progressed liver fibrosis. The expression level of fibrosis related genes in hepatic stellate cells (HSC), were significantly increased by overexpression of these miRNAs.

Conclusion: Four miRNAs are tightly related to the grade of liver fibrosis in both human and mouse was shown. This information may uncover the critical mechanism of progression of liver fibrosis. miRNA expression profiling has potential for diagnostic and therapeutic applications.

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* E-mail: ymurakami@genome.med.kyoto-u.ac.jp

¤ Current address: Department of Human Genetics and Public Health, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

Introduction

Chronic viral hepatitis is a major risk factor for hepatocellular carcinoma (HCC) [1]. Worldwide 120–170 million persons are currently chronically Hepatitis C Virus (HCV) infected [2]. Due to repetitive and continuous inflammation, these patients are at increased risk of developing cirrhosis, subsequent liver decompensation and/or hepatocellular carcinoma. However, the current standard of care; pegylated interferon and rivabirin combination therapy is unsatisfied in the patients with high titre of HCVRNA and genotype 1b. Activated human liver stellate cells (HSC) with chronic viral infection, can play a pivotal role in the progression of liver fibrosis [3]. Activated HSC produce a number of profibrotic cytokines and growth factors that perpetuate the fibrotic process through paracrine and autocrine effects.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that control gene expression by degrading target mRNA or suppressing their translation [4]. There are currently 940 identifiable human miRNAs (The miRBase Sequence Database - Release ver. 15.0). miRNAs can recognize hundreds of target genes with incomplete complementary; over one third of human genes appear to be conserved miRNA targets [5][6]. miRNA is associated several pathophysiologic events as well as fundamental cellular processes such as cell proliferation and differentiation. Aberrant expression of miRNA can be associated with the liver diseases [7][8][9][10]. Recently reported miRNAs can regulate the activation of HSCs and thereby regulate liver fibrosis. miR-29b, a negative regulator for the type I collagen and SP1, is a key regulator of liver fibrosis [11]. miR-27a and 27b allowed culture-activated rat HSCs to switch to a more quiescent HSC phenotype,

with restored cytoplasmic lipid droplets and decreased cell proliferation [12].

In this study, we aimed to reveal the association between miRNA expression patterns and the progression of liver fibrosis by using a chronic liver inflammation model in mouse. We also sought to identify the miRNA expression profile in chronic hepatitis (CH) C patients according to the degree of liver fibrosis, and to clarify how miRNAs contribute to the progression of liver fibrosis. We observed a characteristic miRNA expression profile common to both human liver biopsy specimens and mouse CCL_4 specimens, comprising the key miRNAs which are associated with the liver fibrosis. This information is expected to uncover the mechanism of liver fibrosis and to provide a clearer biomarker for diagnosis of liver fibrosis as well as to aid in the development of more effective and safer therapeutic strategies for liver fibrosis.

Results

The expression level of several mouse miRNAs was increased by introducing mouse liver fibrosis

In order to identify changes in the miRNA expression profile between advanced liver fibrosis and non-fibrotic liver, we intraperitoneally administered CCL_4 in olive oil or olive oil alone twice a week for 4 weeks and then once a week for the next 4 weeks. Mice were sacrificed at 4, 6, or 8 weeks and then the degree of mouse liver fibrosis was determined by microscopy (Figure S1). miRNA expression analysis was performed from the liver tissue collected at the same time. Histological examination revealed that the degree of liver fibrosis progressed in mice that received CCL_4 relative to mice receiving olive oil alone (Figure 1A). Microarray analysis revealed that in CCL_4 mice, the expression level of 11 miRNAs was consistently higher than that in control mice (Figure 1B).

miRNA expression profile in each human liver fibrosis grade

We then established human miRNAs expression profile by using 105 fresh-frozen human chronic hepatitis (CH) C liver tissues without a history of anti-viral therapy, classified according to the grade of the liver fibrosis (F0, F1, F2, and F3 referred to METAVIR fibrosis stages)(Figure 2, Table S2). Fibrosis grade F0 was considered to be the negative control because these samples were derived from patients with no finding of liver fibrosis. In zebrafish, most highly tissue-specific miRNAs are expressed during embryonic development; approximately 30% of all miRNAs are expressed at a given time point in a given tissue [13]. In mammals, the 20–30% miRNA call rate has recently been validated [14]. Such analysis revealed that the diversity of miRNA expression level among specimens was small. Therefore, we focused on miRNAs with a fold change in mean expression level greater than 1.5 ($p<0.05$) in the two arbitrary groups of liver fibrosis.

Expression of several miRNAs was dramatically different among grades of fibrosis. In the mice study 11 miRNAs were related to the progression of liver fibrosis (mmu-let-7e, miR-125-5p, 199a-5p, 199b, 199b*, 200a, 200b, 31, 34a, 497, and 802). In the human study 10 miRNAs were extracted, and the change in their expression level varied significantly between F0 and F3 (F0<F3: hsa-miR-146b, 199a, 199a*, 200a, 200b, 34a, and 34b, F0>F3: hsa-miR-212, 23b, and 422b). The expression level of 6 miRNAs was significantly different between F0 and F2 (F0<F2: hsa-miR-146b, 200a, 34a, and 34b, F0>F2: hsa-miR-122 and 23b). 5 extracted miRNAs had an expression level that was significantly different between F1 and F2 (F1<F2: hsa-miR-146b, F1>F2: hsa-miR-122, 197, 574, and 768-5p). The expression level of 9 miRNAs changed significantly between F1 and F3 (F1<F3: hsa-miR-146b, 150, 199a, 199a*, 200a, and 200b, F1>F3: hsa-miR-378, 422b, and 768-5p). The miRNAs related to liver fibrosis were extracted using two criteria: similar expression pattern in both the human and the mice specimens and shared sequence between human and mouse. We compared the sequences of mouse miRNAs as described on the Agilent Mouse MiRNA array Version 1.0 (miRbase Version 10.1) and human miRNAs as described on the Agilent Human MiRNA array Version 1.5 (miRbase Version 9.1). The sequences of mmu-miR-199a-5p, mmu-miR-199b, mmu-miR-199b, mmu-miR-200a, and mmu-miR-200b in mouse miRNA corresponded to the sequences of hsa-miR-199a, hsa-miR-199a*, hsa-miR-199a, hsa-miR-200a, and hsa-miR-200b in human miRNA, respectively (Table S3).

Validation of the microarray result by real-time qPCR

The 4 human miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) with the largest difference in fold change between the F1 and F3 groups were chosen to validate the microarray results using stem-loop based real-time qPCR. The result of real-time qPCR supported the result of that microarray analysis. The expression level of these 4 miRNAs was significantly different between F0 and F3 and spearman correlation analysis also showed that the expressions of these miRNAs were strongly and positively correlated with fibrosis grade ($n = 105$, $r = 0.498$(miR-199a), 0.607(miR-199a*), 0.639(miR-200a), 0.618(miR-200b), p-values<0.0001) (Figure 3).

Over expression of miR-199a, 199a*, 200a, and 200b was associated with the progression of liver fibrosis

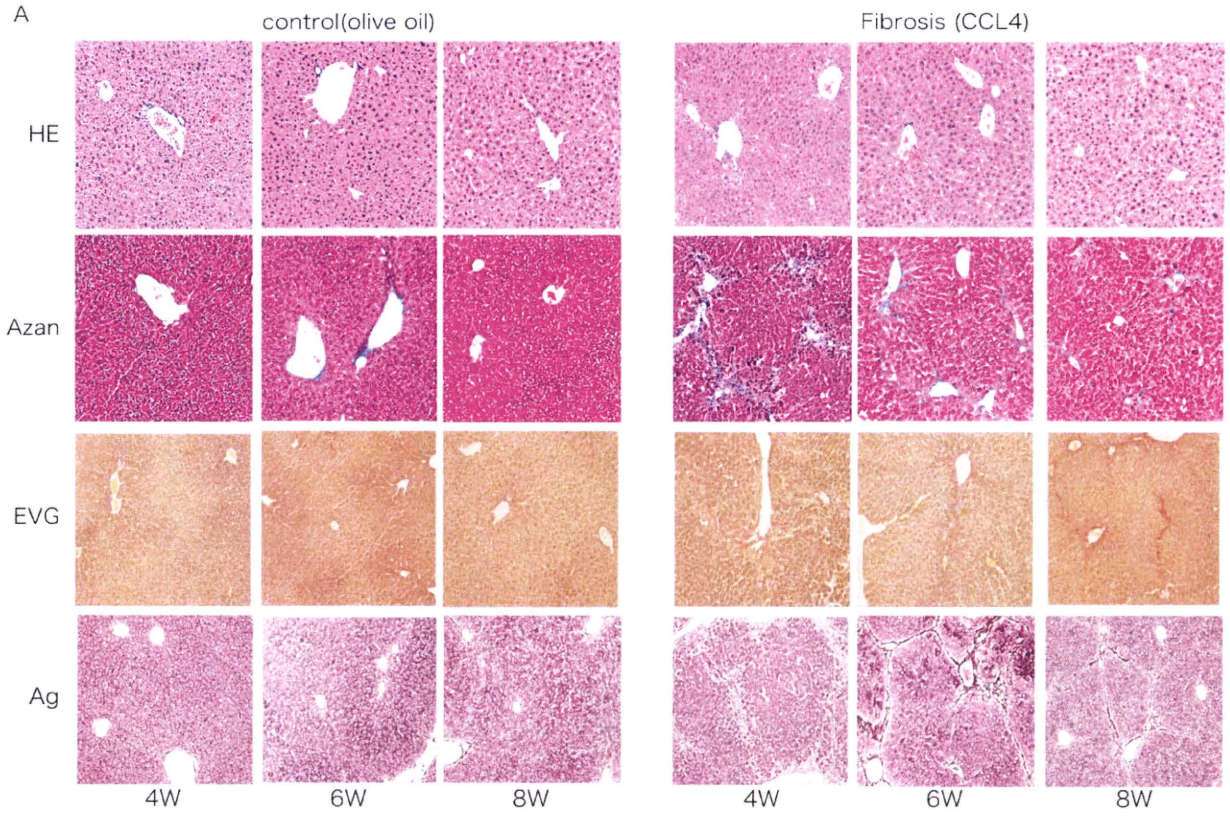
In order to reveal the function of miR-199a, miR-199a*, miR-200a, and miR-200b, we investigated the involvement of these miRNAs in the modulation of fibrosis-related gene in LX-2 cells. The endogenous expression level of these 4 miRNAs in LX2 and normal liver was low according to the microarray study (Figure S2). Transforming growth factor (TGF)β is one of the critical factors for the activation of HSC during chronic inflammation [15] and TGFβ strongly induced expression of three fibrosis-related genes include a matrix degrading complex comprised of α1 procollagen, matrix remodeling complex, comprised of metalloproteinases-13 (MMP-13), tissue inhibitors of metalloproteinases-1 (TIMP-1) in LX-2 cells (Figure 4A). Furthermore, overexpression of miR-199a, miR-199a*, miR-200a and miR-200b in LX-2 cells resulted significant induction of above fibrosis-related genes compared with control miRNA (Figure 4B). Finally we validated the involvement of TGFβ in the modulation of these miRNAs. In LX-2 cells treated with TGFβ, the expression levels of miR-199a and miR-199a* were significantly higher than in untreated cells; the expression levels of miR-200a and miR-200b were significantly lower than in untreated cells. Thus, our in vitro analysis suggested a possible involvement of miR-199a, 199a*, 200a, and 200b in the progression of liver fibrosis.

Discussion

Our comprehensive analysis showed that the aberrant expression of miRNAs was associated with the progression of liver fibrosis. We identified that 4 highly expressed miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) that were significantly associated with the progression of liver fibrosis both human and mouse. Coordination of aberrant expression of these miRNAs may contribute to the progression of liver fibrosis.

Prior studies have discussed the expression pattern of miRNA found in liver fibrosis samples between previous and present study. In this report and prior mouse studies and the expression pattern of

A



B

miRNA	Fold change CCL4 mouse/control		
	4week	6week	8week
mmu-let-7e	2.92	2.09	2.64
mmu-miR-125a-5p	4.29	2.35	2.67
mmu-miR-199a-5p	2.71	2.12	2.38
mmu-miR-199b	3.25	2.55	2.49
mmu-miR-199b*	5.13	4.47	3.94
mmu-miR-200a	2.23	1.63	2.02
mmu-miR-200b	2.38	2.06	2.20
mmu-miR-31	3.4	2.35	3.19
mmu-miR-34a	3.89	3.22	3.5
mmu-miR-497	2.25	2.00	2.55
mmu-miR-802	2.02	2.87	2.74

Figure 1. The change of liver fibrosis in mouse model. A. Representative H&E-stained, Azan-stained, Ag-stained, and EVG-stained histological sections of liver from mice receiving olive oil alone or CCL_4 in olive oil. Magnification is ×10. B. The expression level of mmu-miRNA in mouse liver with olive oil or CCL_4 at 4W, 6W, and 8W respectively, by microarray analysis.
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3 miRNAs (miR-199a-5p, 199b*, 125-5p) was found to be similar while the expression pattern of 11 miRNAs (miR-223, 221, 24, 877, 29b, 29a, 29c, 30c, 365, 148a, and 193) was partially consistent with fibrosis grade [16]. In low graded liver fibrosis, the low expression pattern of 3 miRNAs (miR-140, 27a, and 27b) and the high expression pattern of 6 miRNAs in rat miRNAs (miR-29c*, 143, 872, 193, 122, and 146) in rat miRNA was also similar to our mouse study (GEO Series accession number GSE19865) [11] [12] [17].

The results in this study and previously completed human studies reveal that the expression level of miR-195, 222, 200c, 21, and let-7d was higher in high graded fibrotic liver tissue than in low graded fibrotic liver tissue. Additionally, the expression level of miR-301, 194, and 122 was lower in the high graded fibrotic liver tissue than in low graded fibrotic liver tissue [18] [19] [20](GEO Series accession number GSE16922). This difference in miRNA expression pattern may be contributed to (1) the difference of microarray platform, (2) difference of analytic procedure, and (3) the difference of the species (rat, mouse, and human).

The miR-199 and miR-200 families have are circumstantially related to liver fibrosis. TGFβ-induced factor (TGIF) and SMAD

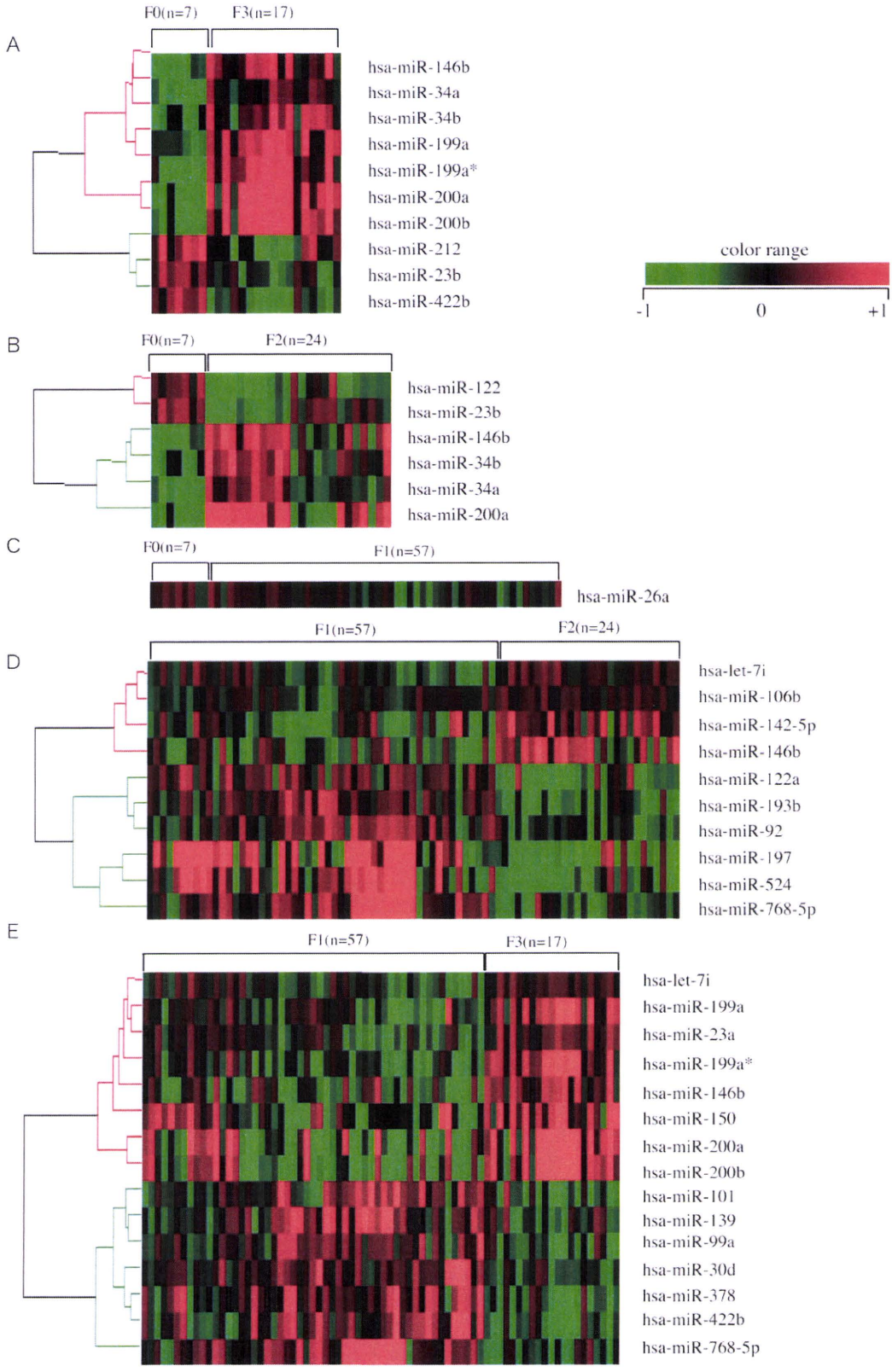
A
F0(n=7)
F3(n=17)
hsa-miR-146b
hsa-miR-34a
hsa-miR-34b
hsa-miR-199a
hsa-miR-199a*
hsa-miR-200a
hsa-miR-200b
hsa-miR-212
hsa-miR-23b
hsa-miR-422b
color range
-1
0
+1
B
F0(n=7)
F2(n=24)
hsa-miR-122
hsa-miR-23b
hsa-miR-146b
hsa-miR-34b
hsa-miR-34a
hsa-miR-200a
C
F0(n=7)
F1(n=57)
hsa-miR-26a
D
F1(n=57)
F2(n=24)
hsa-let-7i
hsa-miR-106b
hsa-miR-142-5p
hsa-miR-146b
hsa-miR-122a
hsa-miR-193b
hsa-miR-92
hsa-miR-197
hsa-miR-524
hsa-miR-768-5p
E
F1(n=57)
F3(n=17)
hsa-let-7i
hsa-miR-199a
hsa-miR-23a
hsa-miR-199a*
hsa-miR-146b
hsa-miR-150
hsa-miR-200a
hsa-miR-200b
hsa-miR-101
hsa-miR-139
hsa-miR-99a
hsa-miR-30d
hsa-miR-378
hsa-miR-422b
hsa-miR-768-5p

Figure 2. Liver fibrosis in human liver biopsy specimen. A. B. C. D. and E. miRNAs whose expression differs significantly between F0 and F3, F0 and F1, F0 and F2, F1 and F2, and F1 and F3, respectively. Relative expression level of each miRNA in human liver biopsy specimen by microarray. Data from microarray were also statistically analyzed using Welch's test and the Bonferroni correction for multiple hypotheses testing. Fold change, p-value are listed in Table S2.
doi:10.1371/journal.pone.0016081.g002

specific E3 ubiquitin protein ligase 2 (SMURF2), both of which play roles in the TGFβ signaling pathway, are candidate targets of miR-199a* and miR-200b, respectively, as determined by the Targetscan algorithm. The expression of miR-199a* was silenced in several proliferating cell lines excluding fibroblasts [21]. Down regulation of miR-199a, miR-199a* and 200a in chronic liver injury tissue was associated with the hepatocarcinogenesis [9]. miR-199a* is also one of the negative regulators of the HCV replication [22]. According to three target search algorithms (Pictar, miRanda, and Targetscan), the miRNAs that may be associated with the liver fibrosis can regulate several fibrosis-related genes (Table S4). Aberrant expression of these miRNAs may be closely related to the progress of the chronic liver disease.

Epithelial-mesenchymal transition (EMT) describes a reversible series of events during which an epithelial cell loses cell-cell contacts and acquires mesenchymal characteristics [23]. Although EMT is not a common event in adults, this process has been implicated in such instances as wound healing and fibrosis. Recent reports showed that the miR-200 family regulated EMT by targeting EMT accelerator ZEB1 and SIP1 [24]. From our observations, overexpression of miR-200a and miR-200b can be connected to the progression of liver fibrosis.

The diagnosis and quantification of fibrosis have traditionally relied on liver biopsy, and this is still true at present. However, there are a number of drawbacks to biopsy, including the invasive nature of the procedure and inter-observer variability. A number of staging systems have been developed to reduce both the inter-observer variability and intra-observer variability, including the METAVIR, the Knodell fibrosis score, and the Scheuer score. However, the reproducibility of hepatic fibrosis and inflammatory activity is not as consistent [25]. In fact, in our study, the degree of fibrosis of the two arbitrary fibrosis groups was classified using the miRNA expression profile with 80% or greater accuracy (data not shown). Thus, miRNA expression can be used for diagnosis of liver fibrosis.

In this study we investigated whether common miRNAs in human and mouse could influence the progression of the liver fibrosis. The signature of miRNAs expression can also serves as a tool for understanding and investigating the mechanism of the onset and progression of liver fibrosis. The miRNA expression profile has the potential to be a novel biomarker of liver fibrosis.

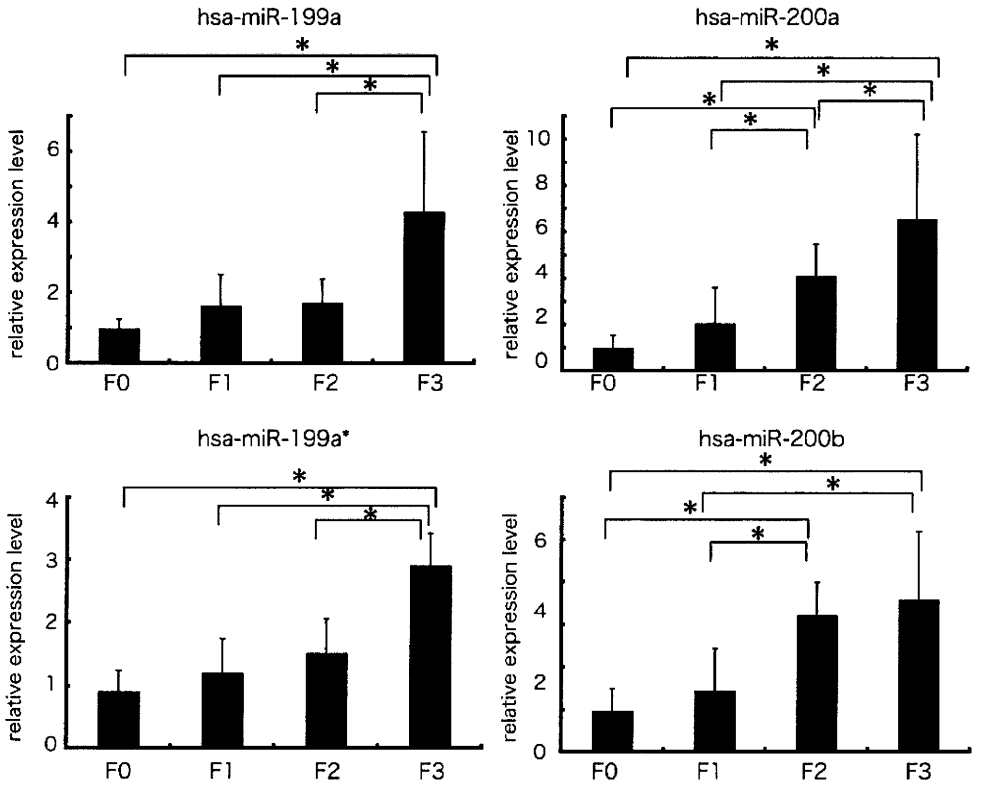

Figure 3. The expression level of miR-199 and 200 families in human liver biopsy specimen by real-time qPCR. Real-time qPCR validation of the 4 miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b). Each column represents the relative amount of miRNAs normalized to the expression level of U18. The data shown are the means+SD of three independent experiments. Asterisks indicates to a significant difference of $p<0.05$ (two-tailed Student-t test), respectively.
doi:10.1371/journal.pone.0016081.g003

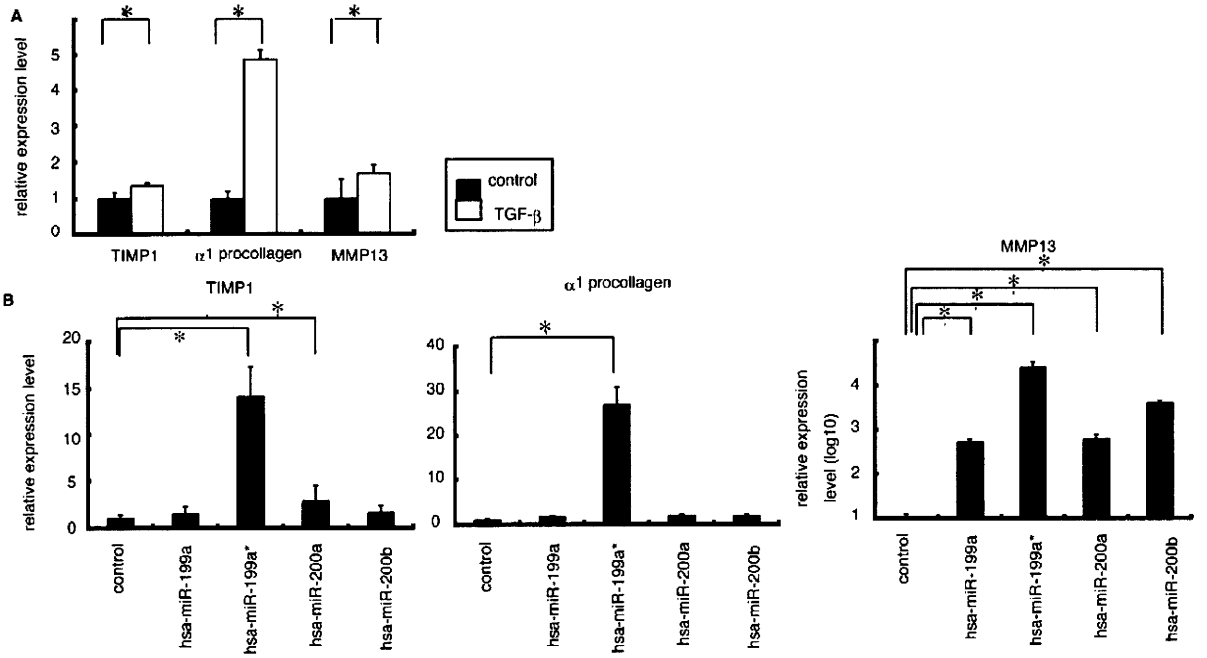

Figure 4. The relationship between expression level of miR-199 and 200 families and expression level of three fibrosis related genes. A. Administration of TGFβ in LX2 cells showed that the expression level of three fibrosis related genes were higher than that in non-treated cells. The data shown are the means+SD of three independent experiments. Asterisk was indicated to the significant difference of $p<0.05$ (two-tailed Student-t test). B. The expression levels of 3 fibrosis related genes in LX2 cells with overexpressing miR-199a, 199a*, 200a, or 200b, respectively were significantly higher than that in cells transfected with control miRNA ($p<0.05$; two-tailed Student t-test).
doi:10.1371/journal.pone.0016081.g004

Moreover miRNA expression profiling has further applications in novel anti-fibrosis therapy in CH.

Materials and Methods

Sample preparation

105 liver tissues samples from chronic hepatitis C patients (genotype 1b) were obtained by fine needle biopsy (Table S1). METAVIR fibrosis stages were F0 in 7 patients, F1 in 57, F2 in 24 and F3 in 17. Patients with autoimmune hepatitis or alcoholic liver injury were excluded. None of the patients were positive for hepatitis B virus associated antigen/ antibody or anti human immunodeficiency virus antibody. No patient received interferon therapy or immunomodulatory therapy prior to the enrollment in this study. We also obtained normal liver tissue from the Liver Transplantation Unit of Kyoto University. All of the patients or their guardians provided written informed consent, and Kyoto University Graduate School and Faculty of Medicine's Ethics Committee approved all aspects of this study in accordance with the Helsinki Declaration.

RNA preparation and miRNA microarray

Total RNA from cell lines or tissue samples was prepared using a *mir*Vana miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. miRNA microarrays were manufactured by Agilent Technologies (Santa Clara, CA, USA) and 100 ng of total RNA was labeled and hybridized using the Human microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.5 and Mouse microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.0. Hybridization signals were detected with a DNA microarray scanner G2505B (Agilent Technologies) and the scanned images were analyzed using Agilent feature extraction software (v9.5.3.1). Data were analyzed using GeneSpring GX 7.3.1 software (Agilent Technologies) and normalized as follows: (i) Values below 0.01 were set to 0.01. (ii) In order to compare between one-color expression profile, each measurement was divided by the 75th percentile of all measurements from the same species. The data presented in this manuscript have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16922 (human) and accession number GSE19865 (mouse).

Real-time qPCR for human miRNA

For detection of the miRNA level by real-time qPCR, TaqMan® microRNA assay (Applied Biosystems) was used to quantify the relative expression level of miR-199a (assay ID. 002304), miR-199a* (assay ID. 000499), miR-200a (assay ID. 000502), miR-200b (assay ID. 002251), and U18 (assay ID. 001204) was used as an internal control. cDNA was synthesized using the Taqman miRNA RT Kit (Applied Biosystems). Total RNA (10 ng/ml) in 5ml of nuclease free water was added to 3 ml of 5× RT primer, 10× 1.5μl of reverse transcriptase buffer, 0.15 μl of 100 mM dNTP, 0.19 μl of RNase inhibitor, 4.16 μl of nuclease free water, and 50U of reverse transcriptase in a total volume of 15 μl. The reaction was performed for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. All reactions were run in triplicate. Chromo 4 detector (BIO-RAD) was used to detect miRNA expression.

Animal and Chronic Mouse Liver Injury Model

Each 5 adult (8-week-old) male C57BL/6J mice were given a biweekly intra-peritoneal dose of a 10% solution of CCL_4 in olive oil (0.02 ml/g/ mouse) for the first 4 weeks and then once a week

for the next 4 weeks. At week 4, 6 or 8, the mice were sacrificed. Partial livers were fixed, embedded in paraffin, and processed for histology. Serial liver sections were stained with hematoxylin-eosin, Azan staining, Silver (Ag) staining, and Elastica van Gieson (EVG) staining, respectively. Total RNA from mice liver tissue was prepared as described previously. All animal procedures concerning the analysis of liver injury were performed in following the guidelines of the Kyoto University Animal Research Committee and were approved by the Ethical Committee of the Faculty of Medicine, Kyoto University.

Cell lines and Cell preparation

The human stellate cell lines LX-2, was provided by Scott L. Friedman. LX-2 cells, which viable in serum free media and have high transfectability, were established from human HSC lines [26]. LX-2 cells were maintained in D-MEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, plated in 60 mm diameter dishes and cultured to 70% confluence. Huh-7 and Hela cells were also maintained in D-MEM with 10% fetal bovine serum. HuS-E/2 immortalized hepatocytes were cultured as described previously [27]. LX-2 cells were then cultured in D-MEM without serum with 0.2% BSA for 48 hours prior to TGFβ1 (Sigma-Aldrich, Suffolk, UK) treatment (2.5 ng/ml for 20 hours). Control cells were cultured in D-MEM without fetal bovine serum.

miRNA transfection

LX-2 cells were plated in 6-well plates the day before transfection and grown to 70% confluence. Cells were transfected with 50 pmol of Silencer® negative control siRNA (Ambion) or double-stranded mature miRNA (Hokkaido System Science, Sapporo, Japan) using lipofectamine RNAiMAX (Invitrogen). Cells were harvested 2 days after transfection.

Real-time qPCR

cDNA was synthesized using the Transcriptor High Fiderity cDNA synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 μg) in 10.4 μl of nuclease free water was added to 1 μl of 50mM random hexamer. The denaturing reaction was performed for 10min at 65°C. The denatured RNA mixture was added to 4 μl of 5× reverse transcriptase buffer, 2 μl of 10 mM dNTP, 0.5 μl of 40U/μl RNase inhibitor, and 1.1 μl of reverse transcriptase (FastStart Universal SYBR Green Master (Roche) in a total volume of 20 μl. The reaction ran for 30 min at 50°C (cDNA synthesis), and five min at 85°C (enzyme denaturation). All reactions were run in triplicate. Chromo 4 detector (BIO-RAD, Hercules, CA, USA) was used to detect mRNA expression. The primer sequences are follows; MMP13 s; 5′-gaggctccgagaaatgcagt-3′, as; 5′-atgccatcgtgaagtctggt-3′, TIMP1 s; 5′-cttggcttctgcactgatgg-3′, as; 5′-acgctggtataaggtggtct-3′, α1-procollagen s; 5′-aacatgaccaaaaaccaaaagtg-3′, as; 5′-catt-gtttcctgtgtcttctgg-3′, and β-actin s; 5′-ccactggcatcgtgatggac-3′, as; 5′-tcattgccaatggtgatgacct-3′. Assays were performed in triplicate, and the expression levels of target genes were normalized to expression of the β-actin gene, as quantified using real-time qPCR as internal controls.

Statistical analyses

Statistical analyses were performed using Student's *t*-test; *p* values less than 0.05 were considered statistically significant. Microarray data were also statistically analyzed using Welch's test and Bonferroni correction for multiple hypotheses testing.

Supporting Information

Figure S1 Time line of the induction of chronic liver fibrosis. Upward arrow indicated administration of olive oil or CCl_4. Downward arrow indicates when mice were sacrificed.
(TIF)

Figure S2 Comparison of the expression level of miR-199 and 200 familes in several cell lines and human liver tissue. Endogenous expression level of miR-199a, 199a*, 200a, and 200b in normal liver and LX2 cell as determined by microarray analysis (Agilent Technologies). Endogenous expression level of same miRNAs in Hela, Huh-7 and, immortalized hepatocyte: HuS-E/2 by previously analyzed data [9].
(TIF)

Table S1 Clinical characteristics of patients by the grade of fibrosis.
(DOCX)

Table S2 Extracted human miRNAs related to liver fibrosis.
(DOCX)

Table S3 Corresponding human and mouse miRNAs.
(DOCX)

Table S4 Hypothetical miRNA target genes according to in silico analysis.
(DOCX)

Author Contributions

Conceived and designed the experiments: YM KS. Performed the experiments: YM HT YH NK. Analyzed the data: MT MK. Contributed reagents/materials/analysis tools: YM HT YH NK. Wrote the paper: YM MT AT FM NK TO.

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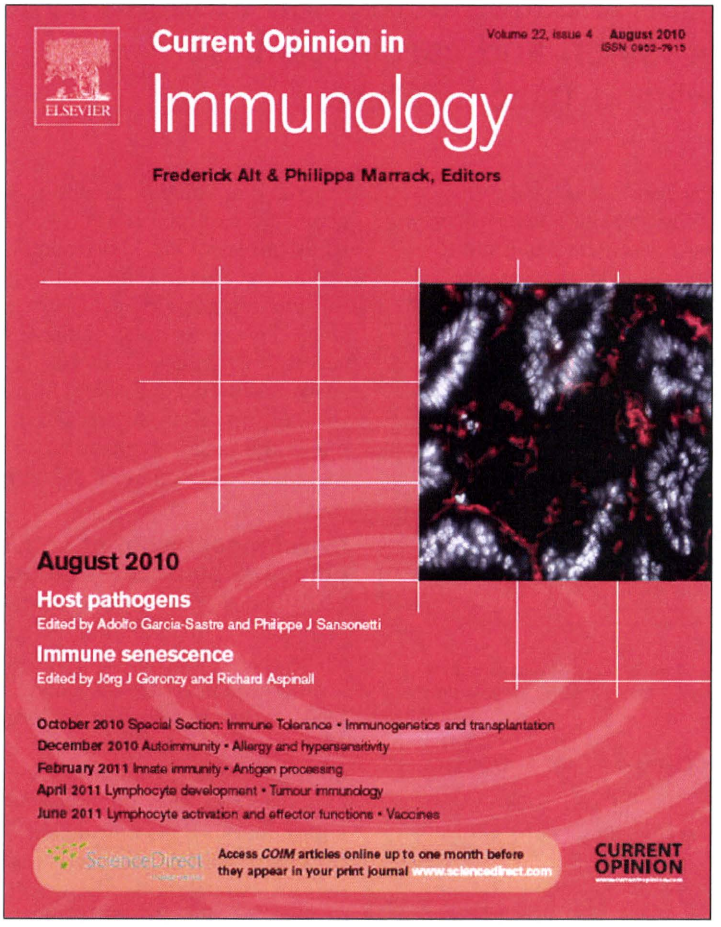

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Helicobacter pylori-induced activation-induced cytidine deaminase expression and carcinogenesis

Hiroyuki Marusawa and Tsutomu Chiba

Tumorigenesis is a multistep process in which the accumulation of genetic alterations drives the transformation of normal cells into malignant derivatives. Activation-induced cytidine deaminase (AID) contributes to immune system diversity by inducing somatic hypermutations and class-switch recombinations of human immunoglobulin genes. The mutagenic activity of AID, however, can also induce genetic changes in various genes and may lead to the development of cancer. *Helicobacter pylori*, a class 1 carcinogen for human gastric cancer, affects AID expression by two different mechanisms, introduction of bacterial virulence factors into host cells and induction of inflammatory responses, thereby contributing to the accumulation of mutations in tumor-related genes. Aberrant AID activity may therefore be a novel link between infection and carcinogenesis.

Address
Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Corresponding author: Marusawa, Hiroyuki (maru@kuhp.kyoto-u.ac.jp)

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Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral-shaped bacterium colonized in human populations for more than 58,000 years [1]. *H. pylori* infection is involved in the development of several human diseases, including gastro-duodenal ulcers, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach. *H. pylori* strains exhibit a high level of genetic diversity, and a striking difference among strains is the presence or absence of a 40-kb DNA segment, termed the cag pathogenicity island (PAI). The risk for developing *H. pylori* infection-mediated gastric disorders is closely associated with the strain [2]. The risk of developing gastric cancer is higher in patients infected with cagPAI-positive *H. pylori* compared with cagPAI-negative *H. pylori* [3,4], but how *H. pylori* infection contributes to gastric carcinogenesis remained unknown. Genetic changes in tumor-related genes are essential in the malignant transformation that leads to cancer cell development. How the intra-gastric residential bacteria induce the genetic changes required for tumorigenesis in host gastric epithelial cells is unclear, since the extracellular habitant *H. pylori* cannot directly access host genomic DNA located in the nucleus of gastric epithelial cells. Recent studies, however, revealed that cagPAI-positive *H. pylori* manipulates the host nucleotide editing enzymes to induce mutagenesis in human DNA sequences of the gastric epithelium [5].

Novel mechanism of active mutagenesis achieved by nucleotide editing enzymes

Genetic changes in tumor-related genes are essential for malignant transformation in cancer cell development. Mechanisms that account for genetic changes required for tumorigenesis are unknown, except for defects in the DNA repair system that are observed in certain human cancers. Several enzymes that induce nucleotide alterations were recently identified, providing a new avenue for understanding the mutagenesis mechanism. The apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC) family comprises nucleotide editing enzymes that insert nucleotide alterations in target DNA or RNA through cytidine deamination [6]. The human APOBEC family consists of APOBEC1, APOBEC2, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3DE, APOBEC3F, APOBEC3G, APOBEC3H, APOBEC4, and activation-induced cytidine deaminase (AID), and contributes to producing various favorable physiologic outcomes by modifying target gene sequences. For example, APOBEC1 participates in lipid metabolism through deaminating a specific cytidine to uridine in the Apo-B mRNA, resulting in the formation of a termination codon, which leads to the production of a half-length genomically encoded Apo-B100. APOBEC3G is an anti-viral molecule that induces hypermutation in viral DNA sequences and acts as a host defense factor against viruses such as HIV-1. Although the majority of APOBEC family members exhibit mutagenic activity against human RNA or exogenous viral genomes, only AID has the ability to induce nucleotide alterations and double-strand DNA breaks in human genomic sequences. Under physiologic conditions, AID is expressed in germinal center B cells and induces somatic hypermutation and class-switch recombination of immunoglobulin genes, thereby amplifying immune system diversity [7]. In sharp contrast to the favorable role of AID in the immune system, excessive AID activity might affect non-immunoglobulin

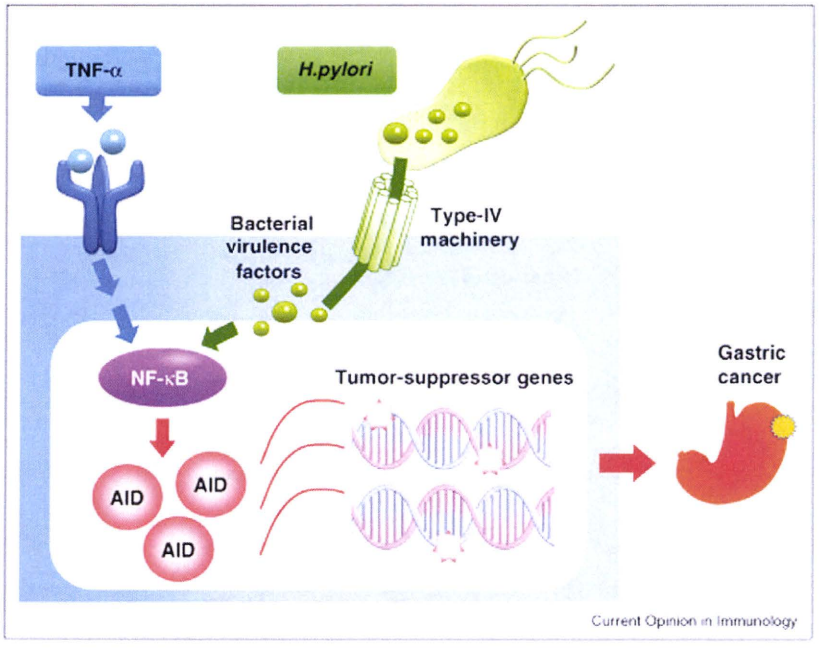
genes, including tumor-related genes in non-lymphoid cells [8].

CagPAI-positive *H. pylori* induces aberrant AID expression in gastric epithelial cells

AID can alter host genomic information, but there are safeguard mechanisms that restrict its potential tumorigenic activity, including post-transcriptional regulation by microRNA [9,10,11•], post-translational modification by protein phosphorylation or ubuiquitination [12–14], and regulation of subcellular localization [15–18]. Restriction of AID expression is also an important regulatory system that minimizes the aberrant mutagenic activity of AID. AID gene transcription is restricted mainly to activated germinal center B lymphocytes where editing of the immunoglobulin gene is required [19,20], while AID expression is not detected in normal epithelial cells under physiologic conditions. How then is AID expressed in epithelial cells under pathologic conditions, especially where the tumorigenic risk is unusually high? Strikingly, endogenous AID is expressed in the epithelial cells of *H. pylori*-infected stomach. Gastric epithelial cells and some infiltrating lymphocytes are immunoreactive for AID protein expression in the majority of chronic gastritis tissues infected with cagPAI-positive *H. pylori* [21]. Moreover, eradication of *H. pylori* infection by antibiotics substantially decreases AID protein expression in gastric mucosa. These findings suggest that cagPAI-positive *H. pylori* somehow upregulates AID protein in the gastric epithelium of the infected host.

CagPAI contain approximately 30 putative genes encoding various bacterial proteins such as cytotoxin-associated gene A (cagA) [22]. CagPAI-positive *H. pylori* introduces several bacterial virulence factors into gastric epithelial cells through a type-IV secretion apparatus, and cagPAI-positive *H. pylori*-derived peptidoglycans introduced into the host cells have been shown to be responsible for activating the transcription factor NF-κB [23]. The AID promoter region also includes sites for several transcription factors, such as NF-κB, STAT6, HoxC4, Sp1, Sp3, and Pax5 [24–27], and AID expression in B lymphocytes is induced in response to NF-κB activation through CD40 ligand signaling [28]. Together, these findings suggest that cagPAI-positive *H. pylori* induces AID expression via NF-κB activation by introducing bacterial virulence factors, and that the proinflammatory response caused by *H. pylori* infection also triggers AID expression via the activation of NF-κB in gastric epithelium, because proinflammatory cytokines such as tumor necrosis factor

Figure 1



Helicobacter pylori infection triggers AID expression in gastric epithelial cells via two distinct pathways. AID acts as a cytidine deaminase that is capable of inducing nucleotide alterations in human DNA sequences. Under physiologic conditions, no AID expression is detectable in normal gastric epithelium. *Helicobacter pylori* (*H. pylori*) infection, however, can induce AID expression in gastric epithelial cells via two distinct pathways. CagPAI-positive *H. pylori* strains possess type-IV machinery and can inject bacterial virulence factors into gastric epithelial cells, leading to the activation of the host transcriptional factor NF-κB. The host inflammatory response triggered by *H. pylori* infection also activates NF-κB in gastric epithelium. As a result, AID is transcriptionally upregulated in gastric epithelial cells, and can contribute to the production of unfavorable genetic changes in tumor-related genes, leading to gastric carcinogenesis.