Silencing of microRNA-122 is an early event during hepatocarcinogenesis from non-alcoholic steatohepatitis

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Non-alcoholic steatohepatitis (NASH) has emerged as a common cause of chronic liver disease and virus-independent hepatocellular carcinoma (HCC) in patients with obesity, diabetes, and metabolic syndrome. To reveal the molecular mechanism underlying hepatocarcinogenesis from NASH, microRNA (miRNA) expression profiles were analyzed in STAM mice, a NASH-HCC animal model. MicroRNA expression was also examined in 42 clinical samples of HCC tissue. Histopathological images of the liver of STAM mice at the ages of 6, 8, 12, and 18 weeks showed findings compatible with fatty liver, NASH, liver cirrhosis (LC), and HCC, respectively. Expression of miR-122 in non-tumor LC at the age of 18 weeks was significantly lower than that in LC at the age of 12 weeks. Expression of miR-122 was further decreased in HCCs relative to non-tumor LC at the age of 18 weeks. Expression of miR-122 was also decreased in clinical samples of liver tissue showing macrovesicular steatosis and HCC, being consistent with the findings in the NASH model mice. DNA methylation analysis revealed that silencing of miR-122 was not mediated by DNA hypermethylation of the promoter region. These results suggest that silencing of miR-122 is an early event during hepatocarcinogenesis from NASH, and that miR-122 could be a novel molecular marker for evaluating the risk of HCC in patients with NASH.

Hepatocellular carcinoma is the most common type of liver cancer. Most cases of HCC are secondary to either chronic hepatitis or liver cirrhosis caused by viral infection (hepatitis B or C) or alcoholism. Hepatocellular carcinoma accounts for 85–90% of all primary liver cancers and is one of the most lethal, affecting many of the world’s population. Despite improvements in the treatment of viral infections, such as interferon therapy, the incidence of HCC is still increasing in parallel with the increased incidence of obesity, diabetes mellitus, and metabolic syndrome. Recently, NAFLD has emerged as a common cause of chronic liver disease in patients with metabolic syndrome. Non-alcoholic steatohepatitis is a more severe form of NAFLD and is defined by the presence of steatosis with inflammation and progressive fibrosis, leading to LC and HCC. Non-alcoholic steatohepatitis may account for a large proportion of virus-independent HCC in developing countries. However, the molecular mechanism underlying hepatocarcinogenesis from NAFLD and NASH is poorly understood.

MicroRNAs are small non-coding RNAs that function as endogenous silencers of various target genes. MicroRNAs are expressed in a tissue-specific manner and play important roles in cell differentiation, proliferation, and metabolism. Links between miRNAs and the initiation and development of cancer and metabolic disorders are becoming increasingly apparent. We have recently reported that the important tumor suppressor miRNAs are regulated by epigenetic alterations such as DNA methylation and histone modification at their CpG island promoters. Regulation of miRNAs by chromatin-modifying drugs may be a novel therapeutic approach for malignant disorders. Despite these discoveries, little is known about the roles of miRNAs in NASH-associated hepatocarcinogenesis.

To reveal the roles of miRNAs during hepatocarcinogenesis from NAFLD and NASH, we examined miRNA expression profiles in NASH-HCC model mice. In the present study, we used STAM mice as a NASH-HCC model. Here we show that the liver-specific miR-122 gene is down-regulated at the early stage of hepatocarcinogenesis from NASH in both an animal model and samples of human tissue.

Materials and Methods

Animals and experimental design. STAM mice (Stelic Institute & Co., Tokyo, Japan) were used as a NASH-HCC model in the present study. STAM mice were established as described previously. In brief, 2-day-old male C57BL/6J pups were injected with streptozotocin (200 μg per mouse) and fed...
a high-fat diet (HFD-32; Clea, Tokyo, Japan) from the age of 4 weeks. This mouse model shows progression of NAFLD to NASH at 8 weeks of age, and to HCC at 18 weeks of age. A total of 14 STAM mice were purchased, and those used as a model for NAFLD, NASH, LC, and HCC were killed and dissected at the ages of 6 \( (n = 4) \), 8 \( (n = 4) \), 12 \( (n = 2) \), and 18 \( (n = 4) \) weeks, respectively. C57BL/6J mice were used as controls. Tissue samples and blood were obtained from both control and STAM mice. Histological and biochemical examinations were carried out as described previously.\(^{(16)}\) All animals had free access to water and food and were maintained in a temperature-controlled specific pathogen-free animal facility. All experiments and procedures were approved by the Keio University Animal Research Committee.

**Patients and tissue specimens.** A total of 42 clinical samples of HCC were examined. Tissue specimens from HCCs and their surrounding non-tumor liver tissues were obtained from materials surgically resected from 42 patients (HCV-positive, 22; HBV-positive, 6; non-B/non-C, 14) at the National Cancer Center Hospital (Tokyo, Japan). Non-B/non-C HCC patients were further divided into two groups according to the grade of macrovesicular steatosis in the non-tumor liver tissue, as reported previously (grade 0, 5; grade 1–3, 9).\(^{(17)}\) This study was approved by the Ethics Committees of the National Cancer Center Hospital and Keio University. Written informed consent was obtained from all of the patients.

**Cell lines and drug treatment.** The human liver cancer cell lines HepG2 and HuH7 were used in this study. HepG2 was obtained from Riken Cell Bank (Tsukuba, Japan), and HuH7 from the ATCC (Rockville, MD, USA). Both cell lines were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10\% FBS and seeded at 1 \( \times \) 10\(^6\) cells per 100-mm dish 24 h prior to treatment with 1 or 3 \( \mu \)M 5-Aza-CdR (Sigma-Aldrich St Louis, MO, USA). The 5-Aza-CdR was removed from the culture medium at 24 h, and regular medium was used thereafter.

**RNA extraction and microarray analyses.** Total RNAs from liver tissues of STAM mice and liver cancer cell lines were extracted using the mirVana miRNA isolation kit (Life Technologies). Total RNAs from clinical HCC samples and matched non-tumor liver tissues were extracted using TRIzol reagent (Life Technologies). MicroRNA microarray analyses were carried out by Toray Industries (Tokyo, Japan). The microarray chips used contained probe regions that detected 1,135 (Toray Industries) miRNA transcripts listed in Sanger microarray chips used contained probe regions that detected 1,135 (Toray Industries) miRNA transcripts listed in Sanger.

**Quantitative RT-PCR of miRNA.** Levels of miRNA expression were analyzed by quantitative RT-PCR using the TaqMan miRNA assay for human/mouse miR-122 (Life Technologies) in accordance with the manufacturer’s instructions. Expression levels were normalized to U6 RNA.

**DNA methylation assay.** Genomic DNA was extracted with a QIAamp DNA Mini Kit (Qiagen Hilden, Germany) and bisulfite conversion was carried out with an Epitect Bisulfite Kit (Qiagen). DNA methylation levels were analyzed by pyrosequencing using PyroMark Q24 (Qiagen) in accordance with the manufacturer’s instructions. The sequences of the primers used are shown in Table S1. As controls for human methylated and unmethylated DNAs, EpiTect methylated and unmethylated control DNAs were purchased from Qiagen. DNA extracted from normal mouse liver tissue was treated with Sss I methylase (in vitro methylated DNA: IVD), which was used as a control for mouse methylated DNA.

**Luciferase promoter assay.** A miR-122 promoter assay was carried out using a Dual Luciferase Reporter Assay System (Promega Madison, WI, USA). Fragments of the human miR-122 promoter with or without the DR-1 and DR-2 elements were inserted between SacI and HindIII sites within pGL4.10 (Promega). Plasmids (475 ng) with or without Ss I CpG methylase (New England Biolabs, Ipswich, MA, USA) treatment were cotransfected with a Renilla luciferase expression vector (pRL-CMV, 25 ng) into HepG2 cells using Lipofectamine 3000 (Life Technologies). Forty-eight hours after transfection, luciferase activities were measured.

**Statistics.** Data were analyzed using the spss statistical software package version 21.0. Differences at \( P < 0.05 \) were considered significant. All data are represented as average ± SD.

**Results**

**Clinicopathological findings in NASH-HCC model mice.** To investigate the molecular mechanism underlying hepatocarcinogenesis from NASH, we used the STAM mouse, which is a simple model system using C57BL/6J mice for inducing NASH-HCC in a diabetic background through a combination of chemical and dietary interventions.\(^{(14,15)}\) Figure 1(a) shows age-dependent changes in body and liver weight in STAM and control mice. Control mice showed age-dependent body weight gain, whereas STAM mice showed no body weight gain. The average body weight of control mice was significantly higher than that of STAM mice (\( P < 0.01 \)). In contrast, the average liver weight in STAM mice was significantly higher than in control mice (\( P < 0.05 \)). STAM mice at the age of 18 weeks, which developed HCC, showed especially prominent hepatomegaly. Serum AST and ALT levels were markedly increased in STAM mice at the age of 18 weeks (Fig. 1b), suggesting that hepatocytes had been severely damaged by the development of HCC. The average levels of serum total cholesterol and triglyceride in STAM mice were significantly higher than those in control mice (Fig. 1b; \( P < 0.001 \) and \( P < 0.05 \), respectively). Hematoxylin–eosin, Azan, and Sirius red staining showed fatty liver with moderate inflammation including neutrophils, lymphocytes, and monocytes, and ballooning degeneration of hepatocytes at the age of 8 weeks (Fig. 2a). Azan and Sirius red staining indicated liver fibrosis at the age of 12 weeks (Fig. 2a). At the age of 18 weeks, the liver of NASH–HCC model mice macroscopically showed a granular surface and tumor protrusion (Fig. 2b). We confirmed that these tumors were pathologically compatible with HCC (Fig. 2c).

**MicroRNA-122 is downregulated in HCC derived from NASH model mice.** To determine aberrantly expressed miRNAs in HCC derived from NASH, we carried out microarray analyses using HCCs and non-tumor liver tissues in STAM mice. As shown in Figure 3(a), the results of microarray analysis indicated that some miRNAs including miR-31, miR-122, and miR-203 were downregulated in HCCs relative to non-tumor liver tissues. Among these miRNAs, we focused on miR-122, because recent studies have reported that miR-122 is the liver-specific miRNA that modulates HCV replication and is downregulated in HCCs with modulation of its target gene, cyclin G1.\(^{(18–20)}\)

To confirm the microarray data, we carried out a TaqMan quantitative RT-PCR analysis of miR-122 expression in the liver tissues of STAM mice at the ages of 6, 8, and 12 weeks, as well as HCCs and non-tumor LC tissues at the age of
18 weeks (Fig. 3b). There was no significant difference in miR-122 expression among normal liver tissues of control mice and fatty liver (6 weeks), NASH (8 weeks), and LC (12 weeks) tissues in STAM mice. In contrast, miR-122 expression in non-tumor LC at the age of 18 weeks was significantly lower than that in LC at the age of 12 weeks in STAM mice. MicroRNA-122 expression was further decreased in HCCs relative to non-tumor LC tissues at the age of 18 weeks in STAM mice (P < 0.05, Fig. 3b). This result was consistent with the microarray data.

Expression levels of miR-122 in clinical samples of HCC tissue. We examined levels of miR-122 expression in 42 clinical samples of HCC. Specimens of HCC tissue and the surrounding non-tumor liver tissues were obtained from materials surgically resected from 42 HCC patients (HCV-positive, 22; HBV-positive, 6; non-B/non-C, 14). Histological diagnosis of NASH in the liver of HCC patients is difficult, because it is considered that liver steatosis is decreased after progression to LC and HCC. In addition, HCC patients usually require food restriction before surgery, which may reduce their liver steatosis. A previous report has graded macrovesicular steatosis from 0 to 3 based on the percentage of hepatocytes showing steatosis (0, none; 1, <33%; 2, 33-66%; 3, >66%).

We divided the non-B/non-C group into two according to the grade of macrovesicular steatosis in the non-tumor liver tissues. We considered grades 1–3 to be steatosis(+) (n = 9) and grade 0 to be steatosis(−) (n = 5). Figure 4(a) shows the histological appearance of non-tumor liver tissue showing steatosis in non-B/non-C patients. The liver steatosis in this case was considered to be grade 3 (macrovesicular steatosis >66%), and portal chronic inflammation and pericellular fibrosis were confirmed.

As shown in Figure 4(b), the average levels of miR-122 expression were lower in HCC tissues than in non-tumor liver tissues in patients with HBV and HCV infection, and in non-B/non-C patients without liver steatosis. In particular, the average level of miR-122 expression in HCCs was significantly reduced relative to the non-tumor liver tissues in HCV-positive patients (P < 0.05). On the other hand, miR-122 expression in non-tumor liver tissues with steatosis in non-B/non-C patients was significantly reduced in comparison to that without steatosis (P < 0.05). Thus, miR-122 expression is reduced in liver showing macrovesicular steatosis and HCCs, consistent with the findings in NASH model mice. These results suggest that miR-122 is downregulated at the early stage during hepatocarcinogenesis from NASH.

DNA methylation status of the miR-122 promoter region in liver cancer cell lines and HCC tissues. To reveal the molecular mechanism underlying regulation of miR-122, we analyzed the DNA methylation status of the miR-122 promoter region, which contains a TATA-box, a CCAAT-box, and DR-1 and DR-2 elements (Fig. 5a). We carried out the promoter assay using fragments of the human miR-122 promoter with or without the DR-1 and DR-2 elements (Fig. 5b). Plasmids with or without Sss I CpG methylase treatment were used to cotransfect HepG2 cells with the Renilla luciferase expression vector. Forty-eight hours after transfection, luciferase activities were measured. The relative luciferase activity of the construct containing the DR-1 and DR-2 elements was significantly higher
than that of the construct lacking these elements (*P < 0.01, Fig. 5b). After treatment with CpG methylase, the relative luciferase activities were significantly decreased in the constructs both with and without the DR-1 and DR-2 elements (**P < 0.005, Fig. 5b). These results indicate that the DR-1 and DR-2 elements in the miR-122 promoter are essential for regulation of miR-122 expression and that DNA methylation around the DR-1 and DR-2 elements suppress miR-122 expression.

We analyzed the DNA methylation status of human HCC samples by bisulfite pyrosequencing. For this, we designed three sets of primers (P1, P2, and P3) to determine DNA methylation status at the CpG sites, indicated by asterisks in Figure 5(a). As shown in Figure 6(a), the levels of DNA methylation in the P1 and P2 regions were very high in both HepG2 and HuH7 cells, and were decreased to ~70% after treatment with 5-Aza-CdR. The P3 region was not methylated in HuH7 cells, whereas it was highly methylated in HepG2 cells, and was decreased to ~60% after 5-Aza-CdR treatment. The expression level of miR-122 was much higher in HuH7 cells than in HepG2 cells (Fig. 6b). The expression of miR-122 was significantly increased after 5-Aza-CdR treatment in HepG2 cells, whereas there was no significant difference after 5-Aza-CdR treatment in HuH7 cells (Fig. 6b). These findings indicate that DNA methylation of the P3 region is critical for regulation of miR-122 expression.

We next examined DNA methylation levels in samples of human HCC with different types of etiology, as shown in Figure 6(c). DNA methylation levels in samples of human HCC tissue were lower (~30%) than those in non-tumor liver tissues in all regions of the miR-122 promoter. In particular, the average levels of DNA methylation in the miR-122 promoter region were significantly reduced in HCC tissues relative to non-tumor liver tissues in non-B/non-C patients without liver steatosis and in HCV-positive patients (Fig. 6c). We also examined levels of DNA methylation in the miR-122 promoter region in non-tumor LC tissues and HCCs of STAM mice, as well as normal liver tissues obtained from C57BL/6J mice. As shown in Figure 6(d), there was no significant difference in the level of DNA methylation between non-tumor LC and HCC. These findings suggest that silencing of miR-122 expression during hepatocarcinogenesis is not mediated by DNA hypermethylation in the promoter region.

Discussion

MicroRNA-122 is the most abundant miRNA in the liver and is implicated in several important aspects of liver pathogenesis, including HCV replication, lipid metabolism, and development of HCC. MicroRNA-122 is known to bind to the 5'UTR of the HCV genome and promotes HCV replication. The inhibitor
of miR-122 decreases HCV RNA levels in patients with chronic HCV genotype 1 infection. Downregulation of miR-122 expression has been reported in patients with HCC. Downregulation of expression has been reported in patients with HCC. Expression of miR-122 expression during progression from normal liver to hepatosteatosis, hepatitis, and the development of HCCs. Expression of miR-122 expression was further decreased in HCCs relative to non-tumor LC tissues. These results strongly suggested that suppression of miR-122 is critical for the initiation and development of NASH-derived HCC. We also confirmed miR-122 expression in clinical samples obtained from patients with HCC. Expression of miR-122 was reduced in liver showing macrovesicular steatosis and HCC, consistent with the findings in the NASH model mice. These results suggest that silencing of miR-122 is an early event during hepatocarcinogenesis from NASH.

Recent studies have shown that peroxisome proliferator activated receptor-gamma (PPAR-γ) is associated with the DR-1 and DR-2 consensus sites in the miR-122 promoter, and that epigenetic alterations in the promoter region play important roles in the regulation of miR-122 expression. In general, DNA hypermethylation at the CpG island promoter leads to silencing of tumor suppressor genes. The miR-122 promoter region is CpG-poor, but the results of our promoter assay indicated that DNA methylation around the DR-1 and DR-2 elements suppressed miR-122 expression. Unexpectedly, our analyses revealed that DNA methylation levels in human HCC tissue samples were lower (~30%) than in non-tumor liver tissues in all regions of the miR-122 promoter, although miR-122 expression was downregulated. We also examined the DNA methylation status of the miR-122 promoter region in HCCs and non-tumor LC tissues in STAM mice. The levels of miR-122 methylation in HCCs and non-tumor LC tissues in STAM mice were around 30%, and there was no significant difference between them. These findings suggest that silencing of miR-122 during hepatocarcinogenesis is not mediated by DNA hypermethylation in the promoter region. Further studies are necessary to identify the factors that inhibit miR-122 expression during hepatocarcinogenesis from NASH.

**Table 1.** Expression of miRNAs in the liver of STAM mice

| miRNA     | Fold change T/N |
|-----------|-----------------|
| miR-31    | 0.09            |
| miR-203   | 0.24            |
| miR-122   | 0.27            |
| miR-290-5p| 0.33            |
| miR-10a   | 0.35            |
| miR-122*  | 0.35            |
| miR-101a  | 0.36            |
| miR-126-5p| 0.37            |
| miR-26b   | 0.38            |
| miR-10b   | 0.39            |

**Fig. 3.** Expression profiles of microRNAs (miRNAs) in the liver of STAM mice. (a) Microarray analyses of miRNA expression profile in hepatocellular carcinoma (HCC) tissues (T) compared with non-tumor liver tissues (N) in two STAM mice at the age of 12 weeks (HCC1 and HCC2). Expression of miR-122 expression normalized with U6 is represented as average ±SD. Downregulation of miR-122 in the liver of STAM mice from the age of 12–18 weeks for non-tumor (N) and HCC (T) was significant (*P < 0.05).

**Fig. 4.** Levels of microRNA-122 (miR-122) expression in clinical samples obtained from hepatocellular carcinoma (HCC) patients. Expression of miR-122 expression normalized with U6 is represented as average ±SD. Downregulation of miR-122 in the liver of STAM mice from the age of 12–18 weeks for non-tumor (N) and HCC (T) was significant (*P < 0.05).
Fig. 5. Promoter assay of microRNA-122 (miR-122) expression. (a) The promoter region of miR-122, which contains a TATA-box, a CCAAT-box, and DR-1 and DR-2 elements. DNA methylation status was determined by bisulfite pyrosequencing at the CpG sites indicated by asterisks. Arrow indicates the transcription start site (TSS), as described previously (21). (b) Promoter assay of miR-122 expression using a Dual Luciferase Reporter Assay System. Fragments of the human miR-122 promoter with or without the DR-1 and DR-2 elements were inserted between the SacI and HindIII sites within pGL4.10. Plasmids with or without SssI CpG methylase treatment were cotransfected with Renilla luciferase expression vector into HepG2 cells. Forty-eight hours after transfection, luciferase activities were measured. *P < 0.01; **P < 0.005.

Fig. 6. DNA methylation status of the promoter region of microRNA-122 (miR-122) in liver cancer cell lines and hepatocellular carcinoma (HCC) tissues. (a) DNA methylation levels of the miR-122 promoter region in HepG2 and HuH7 cells treated with 1 or 3 µM 5-aza-2-deoxycytidine (5AZA). Methylated (MC) and unmethylated (UC) control DNAs were used as controls. (b) Average levels of miR-122 expression in HepG2 and HuH7 cells treated with 5AZA. miR-122 expression is normalized with U6. *P < 0.05. (c) Levels of DNA methylation in the miR-122 promoter region in HCC tissues (T, white columns) and non-tumor liver tissues (N, filled columns). Tissue specimens of HCC and the surrounding non-tumor liver were obtained from patients with hepatitis B virus-negative/hepatitis C virus-negative (NBNC) HCC with or without liver steatosis, as well as from patients with hepatitis C virus-positive and hepatitis B virus-positive HCC. Methylated (MC) and unmethylated (UC) control DNAs were used as controls. *P < 0.005. (d) Levels of DNA methylation in the miR-122 promoter region in non-tumor liver tissues (NT) and HCCs of STAM mice. Normal liver tissues obtained from C57BL/6J mice were used as a control (Cont). DNA from normal mouse liver treated with SssI methylase (in vitro methylated DNA [IVD]) was used as a control for methylated DNA.
In summary, our present results indicate that the tumor suppressor miR-122 is downregulated at an early stage of hepatocarcinogenesis from NASH in both an animal model and samples of human tissue. Silencing of miR-122 in the steatotic liver may play an important role in the initiation of HCC through modulation of its target genes. These novel findings suggest that miR-122 could be used as a molecular marker for evaluating the risk of HCC, and as a therapeutic target for HCC, in patients with NASH.

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Disclosure Statement
The authors have no conflict of interest.

Abbreviations
5-Aza-CdR 5-aza-2’-deoxycytidine
HBV hepatitis B virus
HCC hepatocellular carcinoma
HCV hepatitis C virus
LC liver cirrhosis
miRNA microRNA
NASH non-alcoholic steatohepatitis
NAFLD non-alcoholic fatty liver disease
NBNC hepatitis B virus-negative/hepatitis C virus-negative

Supporting Information
Additional supporting information may be found in the online version of this article:
Table S1. Sequences of primers used for pyrosequencing of the miR-122 promoter region.

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