MicroRNA expression profiling in patients with hepatocellular carcinoma of familial aggregation and hepatitis B virus infection

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Abstract. Numerous studies have suggested that microRNAs (miRNAs) potently affect hepatocarcinogenesis. However, the miRNA expression profiling in patients with hepatocellular carcinoma (HCC) of familial aggregation and hepatitis B virus (HBV) infection has not been elucidated. In the present study, the plasma miRNA expression profiles of 3 patients with HCC with familial aggregation of HCC and HBV infection and 1 healthy volunteer were examined by microarray analysis, in order to identify relevant miRNAs involved in the pathogenesis of HCC with familial aggregation and HBV infection. The results indicated that 26 miRNAs exhibited a ≥20-fold increase or decrease in the plasma of patients with HCC, compared with the healthy control (24 upregulated and 2 downregulated). Among these altered miRNAs, 15 of them have been reported in HCC. The other 11 miRNAs have never been reported in HCC. These differentially-expressed miRNAs may be potential molecular markers for HCC pathogenesis and development.

Introduction

Hepatocellular carcinoma (HCC) remains one of the most prevalent malignant diseases in the world (1). Chronic infection with the hepatitis B virus (HBV) is the most important risk factor for HCC, accounting for 55% of global incidences and >80% of cases in Asia and sub-Saharan Africa (2). Patients infected with HBV are known to be at an increased risk of developing HCC over their lifetime (3-5). Previous studies have reported familial aggregation of HCC (6,7), and previous meta-analyses have indicated that family history of HCC increases the risk of HCC in patients with viral hepatitis, independently of hepatitis (8). A previous study examined the effect of a family history of HCC on the incidence of HCC in the entire population who were screened for HBV seromarkers, and elucidated that family history of HCC multiplied the risk of HCC at each stage of HBV infection (7). Although numerous molecular studies have revealed that the HBV-encoded X (HBx) protein performs a critical role in hepatocarcinogenesis in patients with HBV-associated HCC (9-12), the mechanisms underlying familial clustering-associated carcinogenesis remain to be fully elucidated.

MicroRNAs (miRNAs/miRs) comprise a class of highly-conserved noncoding RNAs of ~22 nucleotides in length (13). Mature miRNAs may interact with the 3'-untranslated regions (UTRs) of target mRNAs to form RNA-induced silencing complexes, resulting in the inhibition of translation or mRNA cleavage (14). Previous studies have revealed that miRNAs may regulate gene expression at the posttranscriptional level and performs a role in regulating epigenetic machinery, including DNA methylation and histone modification (15-18). Since miRNAs perform important roles in various pivotal biological processes, dysregulated miRNA expression has been implicated in a variety of human diseases, including chronic HBV infection and hepatocarcinogenesis (19). An increasing amount of evidence has indicated that dysregulation of miRNAs has important roles in HBV infection and HBV-associated HCC (9,20-22). Numerous studies have demonstrated that the HBx protein is associated with the regulation of miRNAs, which affects basic tumor processes, including cellular proliferation, differentiation and metastasis (12,23-27). In addition, a family history of HCC increases the risk of HCC, even following adjustment for other risk factors, and in patients without hepatitis serum markers (28). However, little is known regarding the association between miRNAs and family aggregation of HCC in patients with HBV infection.

In the present study, microarray analysis was performed to study the miRNA profile in the plasma of patients with HCC with familial aggregation of HCC and HBV infection, compared with the healthy control. The present study may help improve understanding of the key roles of miRNAs in the progression of HCC, and pave the way to future studies on the molecular mechanism of hepatocarcinogenesis.

Materials and methods

Patients and samples. A total of 3 patients with HCC with a history of familial aggregation of HCC and HBV infection

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Table I. Baseline information of 3 patients with HCC and 1 volunteer.

Patient	Sex	Age, years	Diagnosis	Edmondson-steiner grade	Family history of HCC	Sampling date
1	F	42	HBV/HCC	II	One sister and one brother	2011-12-24
2	M	43	HBV/HCC	I	Two brothers	2012-01-28
3	M	38	HBV/HCC	II	Father and one brother	2012-03-17
Control	M	38	Healthy	-	None	2012-03-18

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; F, female; M, male.

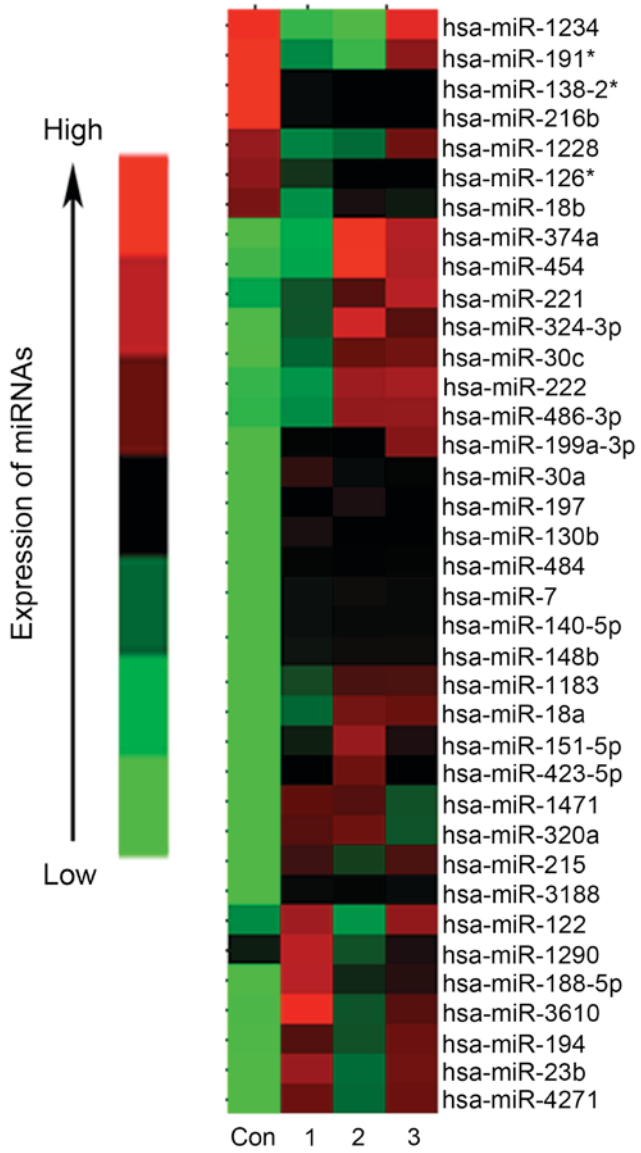

Figure 1. Heatmap of differentially-expressed miRNAs in the plasma of a healthy control and 3 patients with HCC with family history of HCC and hepatitis B virus infection using microarray analysis. miRNA/miR, microRNA; HCC, hepatocellular carcinoma; hsa, *Homo sapiens*.

underwent liver resection at the Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhonog University of Science and Technology (Wuhan, China) between December 2011 and March 2012. Blood samples were obtained from these 3 patients prior to liver resection. HCC was diagnosed based on cytohistological evidence from resected specimens. These patients were not treated with any radiochemotherapy prior to blood drawing. Blood collected from a healthy volunteer was used as a control. The baseline information of all enrolled subjects is presented in Table I. Written consent for sample collection was obtained from all the patients, and the protocol was approved by the Institutional Research Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Familial aggregation of HCC is defined as having a first degree relative (including parents, siblings and children) with HCC (7). The diagnosis of familial aggregation of HCC of these 3 patients was confirmed with the help of a standardized questionnaire that was given to all patients at the baseline visit. Other family members also received liver resection or medical treatment at Tongji Hospital with the diagnosis of HCC. Their blood samples are no longer available as they have passed away.

miRNA microarray analysis. Plasma samples were acquired by high-speed centrifugation (12,000 x g at 4˚C for 10 min) and stored at -80˚C. Agilent human miRNA V16.0 from Agilent Technologies (Santa Clara, CA, USA) was used to identify differentiated miRNAs between familial patients with HCC with HBV infection and the healthy volunteer. All protocols were performed by the professional Shanghai Biological Corporation (Shanghai, China).

Data analysis. According to the results of microarray analysis, the miRNAs that exhibited ≥20-fold increase or decrease in the plasma of patients with HCC compared with the healthy control were selected. To evaluate the list of differentially-expressed miRNAs in the context of the current literature, the names of these differentially-expressed miRNAs were used as a keyword for online searches and detailed information was extracted. The following databases were utilized: PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), Web of Science (http://www.webofknowledge.com/) and Google Scholar (http://scholar.google.com) search.

Results

Microarray results of the differentially-expressed miRNAs in the plasma of patients with HCC. Microarray results identified 37 miRNAs with a significant difference in expression between the patients with HCC and the healthy control, including 30 upregulated and 7 downregulated (Fig. 1) (27). Among

Table II. miRNAs have been reported in HCC, which exhibited at least a 20-fold increase or decrease in plasma expression of patients with HCC compared with the healthy control.

miRNA name	Sequence	Fold-change	Targets	Published year	Chr	HBx regulation
hsa-miR-215	GTCTGTCAATTCATAGGTCAT	277.3	PTPRT	2014	chr1	Yes
hsa-miR-3188	CCCCGTATCCGCA	124.5	mTOR	2016	chr19	No
hsa-miR-23b	GGTAATCCCTGGCAATG	102.9	c-Met	2009	chr9	No
hsa-miR-18a	CTATCTGCACTAGATGCA	97.3	ER-α	2009	chr13	Yes/no
hsa-miR-320a	TCGCCCTCTCAAC	88.3	GNAI1	2012	chr8	No
hsa-miR-199a-3p	TAACCAATGTGCAGACTACT	63.7	mTOR/c-Met	2010	chr1	No
hsa-miR-151-5p	ACTAGACTGTGAGCTCC	61.2	RhoGDIA	2010	chr8	No
hsa-miR-140-5p	CTACCATAGGGTAAAACCACT	38.2	TGF-β	2013	chr16	No
hsa-miR-130b	ATGCCCTTTCATCATTGC	34.9	PPAR-γ	2014	chr22	No
hsa-miR-197	GCTGGGTGGAGAAGGTG	33.8	CD82	2014	chr1	No
hsa-miR-148b	ACAAAGTTCTGTGATGCAC	33.4	NA	2014	chr12	No
hsa-miR-30a	CTTCCAGTCGAGGATG	31.1	SNAI1	2014	chr6	No
hsa-miR-7	ACAACAAAATCACTAGTCTTCC	24.5	CCNE1	2013	chr9	Yes
hsa-miR-30c	GCTGAGAGTGTAGGATGT	24.4	NA	2014	chr1	No
hsa-miR-194	TCCACATGGAGTTGCT	22.2	NA	2014	chr1	No
hsa-miR-138-2*	AACCCTGGTGTCGTGA	-106.3	Cyclin D3	2012	chr16	Yes/no

Yes/no means these miRNAs have been reported differently between HBV-associated HCC and HCC with no HBV infection. miRNA/miR, microRNA; HCC, hepatocellular carcinoma; chr, chromosome; HBx, hepatitis B virus-encoded X protein; hsa, *Homo sapiens*; PTPRT, protein tyrosine phosphatase receptor type T; ER-α, estrogen receptor-α; GNAI1, guanine nucleotide-binding protein (G protein); mTOR, mechanistic target of rapamycin; TGF-β, transforming growth factor β; PPAR-γ, peroxisome proliferator-activated receptor γ; CD82, cluster of differentiation 82; NA, not available; SNAI1, snail homolog 1; CCNE1, cyclin E1.

Table III. miRNAs have never been reported in HCC, which exhibited at least a 20-fold increase or decrease in expression of patients with HCC compared with the healthy control.

miRNA name	Sequence	Fold-change	Chr
hsa-miR-374a	CACTTATCAGGTTGTATTATAA	121.4	chrX
hsa-miR-4271	CCCCACCTTTTCTTCC	96.8	chr3
hsa-miR-1471	ACACCTGGCTCCACA	96.3	chr2
hsa-miR-188-5p	CCCTCCACCATGC	82.1	chrX
hsa-miR-1183	TGCCCACTCTCACCA	81.9	chr7
hsa-miR-3610	CGGCGCCTCCTT	64.8	chr8
hsa-miR-454	ACCCTATAAGCAATATTGCAC	62.9	chr17
hsa-miR-324-3p	CCAGCAGCACCTGGGG	48.7	chr17
hsa-miR-484	ATCGGGAGGGGACTGA	29.4	chr16
hsa-miR-216b	TCACATTTGCCTGCAG	-127.6	chr2

miRNA/miR, microRNA; HCC, hepatocellular carcinoma; chr, chromosome; hsa, *Homo sapiens*.

the differentially expressed miRNAs, 26 miRNAs exhibited a ≥20-fold increase or decrease in the plasma of patients with HCC, compared with the healthy control.

By searching the databases of PubMed, Web of Science and Google Scholar, 15 of these differentially-expressed miRNAs have been extensively reported in HCC, and 12 of them have been fully elucidated for their roles in the progression of hepatocarcinogenesis (Table II). Notably, the remaining 11 miRNAs (10 upregulated and 1 downregulated) have never been reported in liver cancer (Table III). Among them, the levels of plasma miR-3188, miR-374a, miR-4271, miR-1471, miR-188-5p, miR-1183, miR-3610, miR-454, miR-324-3p and miR-484 were significantly increased in patients with HCC compared with the healthy control, corresponding to a fold-change of 124.5, 121.4, 96.8, 96.3, 82.1, 81.9, 64.8, 62.9, 48.7 and 29.4, respectively ($P<0.05$). Comparing the patients with HCC with the healthy control, only plasma miR-216b level was significantly downregulated with a fold-change of 127.6

(P<0.01). The expression status of miR-216b was investigated in 150 HCC tissues, and it was observed that miR-216b expression was downregulated in 90 patients compared with normal liver tissues. The 5-year overall survival and disease-free survival rates were significantly improved in HCC patients with increased miR-216b expression in comparison with the downregulated miR-216b expression group (P<0.001) (27).

Discussion

Dysregulation of miRNAs is involved in the initiation and progression of various cancers, including HCC (19). Previous studies revealed that common and specific mechanisms exist at the miRNA level during HBV-induced hepatocarcinogenesis (29), and miRNAs may function as potential biomarkers for HBV-associated HCC (20,30). In addition, family history of HCC may improve the risk of HCC at each stage of HBV infection (7). The present study aimed to identify the plasma miRNAs that are differentially expressed in patients with HCC with familial aggregation of HCC and HBV infection.

In the present study, by virtue of microarray analysis, specific groups of miRNAs were identified, whose expression is significantly altered in the plasma of patients with HCC with familial aggregation of HCC and HBV infection. A total of 37 differently-expressed miRNAs were identified, and among them, 26 miRNAs exhibited ≥20-fold-changes in the plasma of patients with HCC compared with the healthy controls. By reviewing the available literature published in previous years, among these 37 differentially expressed miRNAs, 15 miRNAs have been reported in HCC, and 12 miRNAs of them have been elaborated for the detailed molecular mechanism in the progression of HCC (Table II). The remaining 11 miRNAs have never been studied in liver cancer (Table III). Among the 11 differentially dysregulated miRNAs, the function of miR-374a, miR-188-5p, miR-1183, miR-454, miR-324-3p, miR-484, miR-3188, and miR-216b have been studied in human cancers (27,31-43); however, the exact function of miR-4271, miR-1471 and miR-3610 remains unclear.

Of these 11 miRNAs, miR-216b exhibited a 127.6-fold decrease in expression in the plasma of patients with HCC compared with that of the healthy control. Deng *et al* (44) firstly observed miR-216b expression in nasopharyngeal carcinoma and indicated that miR-216b was downregulated in nasopharyngeal carcinoma cell lines and specimens. miR-216b may suppress tumor growth and invasion by targeting KRAS, which performs an important role in the initiation and progression of HBV-associated HCC in mice models (45). Other studies have also revealed that miR-216b functions as a tumor-suppressor gene in colorectal and pancreatic cancers (46,47). The function of miR-216b in the pathogenesis of HCC was then studied. The present results indicated that miR-216b expression was significantly lower (P<0.001) in tumor tissues compared with adjacent liver tissues, and its expression was associated with tumor size, HBV infection, HBV-DNA quantity and incidence of portal vein tumor thrombosis (27). Prognostic analysis revealed that the 5-year overall survival and disease-free survival rates were significantly improved in patients with HCC with increased miR-216b expression in comparison with the downregulated miR-216b expression group (P<0.001). In an *in vitro* study, miR-216b inhibited cellular proliferation, migration and invasion of HCC by directly targeting insulin-like growth factor mRNA binding protein 2, and was downregulated by HBx (27). When a miR-216b-specific inhibitor was used to block miR-216b expression in SMMC-7721 cells, *in vitro* and *in vivo* assays revealed a significant increase in proliferation, migration and invasion, compared with control SMMC-7721 cells (27).

Cai *et al* (31) demonstrated that miR-374a may activate Wnt/β-catenin signaling to promote breast cancer metastasis and may perform as a therapeutic target for early metastatic breast cancer. Wnt/β-catenin signaling has been demonstrated to perform an important role in the development and promotion of liver cancer metastasis (48), and the HBx protein is essential for the activation of Wnt/β-catenin signaling in hepatoma cells (49). The expression of miR-374a was investigated in tumor tissues and adjacent normal tissues in the present study, and it was identified that no difference was observed between HCC and para-HCC tissue. The function of miR-374a in HBV-associated HCC requires additional study. A previous study indicated that miR-1183 and miR-188-5p may represent specific predictors of response to chemoradiotherapy in rectal cancer (32). miR-188-5p is involved in the process of prostate cancer by regulating lysosomal protein transmembrane 4 β (LAPTM4B) (33). The downregulation of miR-188-5p is an independent prognostic factor for poor overall and biochemical recurrence-free survival rates, restoration of miR-188-5p in prostate cancer (PCa) cells significantly suppresses proliferation, migration and invasion *in vitro* and inhibits tumor growth and metastasis *in vivo* (33); Overexpression of miR-188-5p in PC-3 cells may significantly enhance the chemosensitivity of the cells to adriamycin (33). The miR-188-5p/LAPTM4B/phosphoinositide-3 kinase/protein kinase B regulatory network performs an important role in PCa progression and chemotherapeutic drug sensitivity (33). Recently, Fang *et al* (50) indicated that miR-188-5p was significantly decreased in HCC tissue, and that overexpression of miR-188-5p suppresses tumor cellular proliferation and metastasis by directly targeting fibroblastic growth factor 5 in HCC. However, in the present study, miR-188-5p exhibited an 82.1-fold increase in the plasma of patients with HCC compared with the healthy control. This demonstrated that the expression of miRNAs in the serum cannot completely reflect its expression in the tissue.

Hu *et al* (34) detected the serum miRNA profiling in patients with breast cancer. Normalized by the two endogenous control miRNAs, the authors revealed that the serum miR-324-3p expression may act as a non-invasive prediction biomarker for breast cancer. Macconi *et al* (35) indicated that miR-324-3p promotes renal fibrosis by targeting prolyl endopeptidase. In addition, a previous study revealed that miR-324-3p targets the promoter of RelA, commonly known as p65, a subunit of nuclear factor-kB, and significantly induced the endogenous RelA mRNA and protein expression in PC12 cells (36). The present results also demonstrated that miR-324-3p exhibited a 48.7-fold increase in the plasma of patients with HCC compared with the control, which may be considered as a biomarker in HCC.

miR-484 has been identified to be increased in the serum of patients with breast and pancreatic cancer (37). The present results revealed that miR-484 exhibited a 29.7-fold-change in expression in patients with HCC compared with the

healthy control. These findings indicated that miR-484 may be produced as a result of processes common to these three cancers. The study by Wang *et al* (38) also indicated that miR-484 may function as an oncogene, suppress translation of mitochondrial fission protein (Fis1) and inhibit Fis1-mediated fission and apoptosis.

Previous studies have demonstrated that miR-454 acts as an oncogene or tumor suppressor in cancer (39-41). miR-454 has been reported to be decreased in tissue of patients with esophageal cancer compared with normal tissue, which may perform as novel molecular markers of esophageal cancer (39). Niu *et al* (40) demonstrated that miR-454 expression was downregulated in osteosarcoma tissues, acting as a tumor suppressor gene in osteosarcoma. The miR-454 expression was increased in colorectal cancer (CRC) tissues and CRC cells. Overexpression of miR-454 promoted the proliferation and anchorage-independent growth of CRC cells and its oncogenic effect was mediated chiefly through direct suppression of cylindromatosis expression (41). In the present study, miR-454 exhibited a 62.9-fold increase in the plasma of patients with HCC in comparison with the healthy control, demonstrating that miR-454 may function as an oncogene. A total of 110 pairs of HCC tissues and para-cancer tissues were compared, and the results revealed that miR-454 was significantly increased in 83 HCC tissues compared with para-cancer tissues. The 5-year overall survival and disease-free survival rates were significantly improved in patients with low expression of miR-454 (data unpublished). Until recently, Yu *et al* (42) indicated that miR-454 expression was upregulated in HCC cell lines and tissues. Knockdown of miR-454 inhibited HCC cellular proliferation, invasion and epithelial mesenchymal transition (EMT), whereas overexpression of miR-454 promoted HCC cellular proliferation and invasion and EMT, which was consistent with the present results. miR-3188 directly targets mTOR to stimulate its own expression, and participates in FOXO1-mediated repression of cell growth, tumorigenesis and nasopharyngeal carcinoma chemotherapy resistance (43). However, miR-3188 has never been studied in HCC, and the aim of the present study was to investigate the function of miR-3188 in the pathogenesis of HCC.

In conclusion, the present study (in a small sample size) identified a list of differentially-expressed miRNA candidates in the plasma of patients with familial HCC with HBV infection compared with the healthy control. A total of 11 of these miRNAs have not previously been reported in the molecular pathogenesis of hepatocarcinogenesis. These differentially expressed miRNAs in the plasma of patients with HCC with familial aggregation of HCC and HBV infection may lay the foundation for additional studies on the role of miRNAs in the pathogenesis of HCC, and provide the basal work for future study of the molecular mechanisms of HCC. Additional studies are necessary in order to understand the regulatory mechanisms of these miRNAs in the process of hepatocarcinogenesis.

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