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Circular microRNA profiles as biomarkers for early stage hepatocellular carcinoma in patients infected with hepatitis B virus: a pair-matched case-control study

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Abstract

Hepatocellular carcinoma (HCC) is frequently observed in patients infected with hepatitis B virus (HBV); however, early detection of HBV-related HCC is difficult due to the lack of an optimal biomarker allowing discrimination of early stage HCC patients from non-HCC HBV carriers. Here, we identify circulating microRNAs (miRNAs) allowing identification of early stage HCC patients among HBV carriers at low to high risk of hepatocarcinogenesis. We conducted a case-control study to estimate serum microRNA levels in hepatitis B surface-antigen (HBsAg)-positive patients with early-stage HCC for comparison with controls matched according to ethnicity, age, sex, presence or absence of hepatitis B e antigen, treatment with a nucleoside/nucleotide analogue, and presenting with clinical forms of HBV infection, including inactive carrier, chronic hepatitis, or cirrhosis. We recruited and obtained sera from 20 HBsAg-positive patients [HCC group and controls (n = 10 each)] and performed microarray analysis, which identified five candidate miRNAs (miR-548a, miR-1281, miR-4634, miR-4646-3p, and miR-4659a) exhibiting significantly different levels between the two groups. These results suggest these miRNAs as potential biomarkers for predicting early stage HCC development in patients with HBV infection.

Key words : *circulating microRNA, hepatocellular carcinoma, p53, miR-1281, microarray*

I. Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus belonging to the *Hepadnaviridae* family. HBV infection is a global health problem, with over 250 million chronically infected individuals worldwide. HBV is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), with HBV-related HCCs mostly presenting in older patients with high titers of HBV DNA, positive for hepatitis B e antigen (HBeAg), or exhibiting severe hepatic fibrosis, including cirrhosis [1]. However, HBV-related HCC sometimes develops in patients following long-term follow-up as asymptomatic or inactive HBV carriers without severe hepatic fibrosis or inflammation. Integration of the HBV genome into host cells is a cause of hepatic carcinogenesis in HBV carriers

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without severe fibrosis and inflammation in the liver [2]. In many patients presenting several clinical forms of hepatitis B infection, appropriate biomarkers for the early detection of HBV-related HCC are required to establish effective treatment strategies. Currently, the most widely used serological biomarker for HCC diagnosis in this context is alpha-fetoprotein (AFP); however, the clinical availability of AFP is limited in its efficacy for early diagnosis of HCC, as AFP levels are frequently normal in early stage HCC, and patients with chronic liver disease might also show elevated AFP levels. Due to its poor sensitivity and specificity, AFP is not an ideal diagnostic marker for HCC; therefore, other biomarkers are required [3].

MicroRNAs (miRNAs) are short, noncoding RNA (ncRNA) that regulate many cellular and molecular processes involved in both maintenance of normal cellular homeostasis and promotion of various carcinogenic pathways [4]. Viral hepatitis infection affects miRNA profiles and can promote HCC development, with a previous study reporting changes in miRNA levels in blood or tissue from HCC patients and suggesting critical roles in different stages of HCC progression and prognosis [5]. This suggests that changes in serum miRNA levels in patients with early stage HCC might represent a potential diagnostic biomarker. Lin et al. [6] identified a classifier involving seven miRNAs capable of detecting HCC, with the sensitivity of this classifier for HCC prediction at 48.1% 6 months before clinical diagnosis according to an age- and sex-matched case-control study and relative to an 18.5% sensitivity for AFP. It remains unclear whether HBV infection, ethnicity, age at infection, HBV genotype, HBeAg seroconversion, and treatment with nucleoside/nucleotide analogues (NAs) affect circulating miRNA levels and their status as factors involved in hepatocarcinogenesis. Strict case-control matching is necessary to identify miRNAs efficacious for detecting preclinical HBV-related HCC.

Here, we conducted a single-center, pair-matched case-control study to identify circulating miRNAs in hepatitis B surface-antigen (HBsAg)-positive patients with early stage HCC. Control patients were matched according to ethnicity, age, the presence or absence of HBeAg, NA treatment, and clinical forms of HBV infection in order to identify and evaluate unique miRNAs as clinical confounding factors.

II. Materials and methods

1. Patients

The study design is shown in Fig. 1. We enrolled 236 HBsAg-positive patients receiving medical care at Iwate Medical University Hospital (a center for HCC treatment in northern Honshu, Japan) between January 2011 and February 2018. Patients exhibiting complications involving other cancer types, positive for anti-hepatitis C virus antibody, or positive for anti-human immunodeficiency virus antibody were excluded. The patients comprised three groups: asymptomatic or inactive HBsAg carriers, patients with chronic hepatitis, and patients with HBV-related liver cirrhosis. Asymptomatic or inactive HBsAg carriers were defined based on normal transaminase level in HBsAg-positive sera and in the absence of NA treatment. Patients with liver cirrhosis were

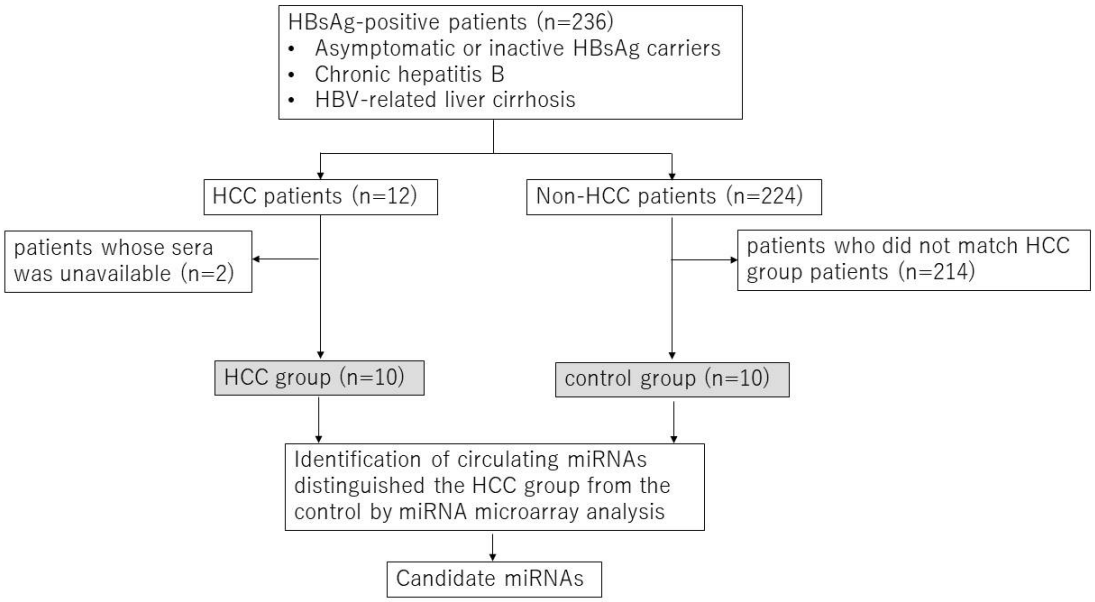

Fig. 1. Study design.
Twenty patients [HCC and control groups (n = 10 each)] were selected from 236 HBsAg-positive patients. Selected patients in the HCC group were pair-matched with those in the control group.

diagnosed by pathological examination (F4 according to METAVIR score). If patients had not undergone pathological examination, they were diagnosed as having liver cirrhosis by the presence of typical features via imaging techniques (ultrasound, computed tomography, or magnetic resonance imaging), or a history of major cirrhotic complications, including splenomegaly, ascites, jaundice, hepatoencephalopathy, or esophagogastric varix [7]. HBsAg-positive patients, except those that were asymptomatic, inactive HBsAg carriers, or with cirrhosis, were regarded as having contracted a chronic hepatitis B infection, with the majority of these patients having received NA treatment. Occurrence of HCC was diagnosed and confirmed as early stage contamination and outflow by CT or MRI. HCC stage was determined according to Barcelona Clinic Liver Cancer (BCLC) criteria, with BCLC stage 0 and A tumors classified as early stage HCC. Among the enrolled patients, 12 developed HCC during the observation period. Ten of these 12 patients comprised the HCC group based on sera collection and confirmation at a time point upon initial diagnosis of HCC. Among enrolled HBsAg carriers without HCC, 10 patients were selected as pair-matched controls according to ethnicity (Japanese), age ± 5 years, sex, clinical form of HBV infection (asymptomatic or inactive HBsAg carriers, chronic hepatitis, or liver cirrhosis), HBV genotype, presence or absence of HBeAg, and NA treatment. Serum samples from a total of 20 patients were prepared for analyses. This study was approved by the ethics committee of Iwate Medical University School of Medicine (HG2018-502), and all procedures were performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. The need

for informed consent was waived.

2. Blood sampling

Serum samples from the 20 patients (10 HCC, 10 controls) were maintained at − 30° C. Total RNA was extracted from sera using an miRNeasy serum/plasma advanced kit (Qiagen, Hilden, Germany) according to manufacturer instructions.

3. Microarray analysis

Total RNA was analyzed using a GeneChip miRNA 4.0 array (Affymetrix; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions. Total RNA was labeled with biotin using a Flashtag biotin HSR RNA labeling kit (Thermo Fisher Scientific). Samples on the microarray were hybridized using a Genechip hybridization, wash, and stain kit (Thermo Fisher Scientific), stained with phycoerythrin, and then scanned using a GeneChip Scanner 3000 7G (Thermo Fisher Scientific). Raw data of the fluorescent signals were analyzed with GeneChip Command Console (Thermo Fisher Scientific).

4. Statistical analysis

Patient characteristics were compared between the HCC and the control groups using the Mann-Whitney *U*-test. Affymetrix Expression Console and Affymetrix Transcriptome Analysis console (Thermo Fisher Scientific) were used for data analysis. Results of ncRNA expression were $\log_2$ transformed, and one-way analysis of variance was performed to detect differential miRNA expression. Candidate miRNAs were selected according to significantly different expression levels showing absolute fold changes exceeding the mean and two standard deviations. A $p < 0.05$ was considered statistically significant.

III. Results

1. Patient characteristics

Patient characteristics are shown in Table 1. None of the 20 patients (12 men and 8 women) had a history of HCC before enrollment and showed a median age of 60 years (range: 41–77 years), with eight patients aged ≧65 years and 12 having developed HCC prior to age 65. Two patients were inactive HBsAg carriers, and 10 and eight presented with chronic hepatitis and liver cirrhosis, respectively. Six patients harbored HBV genotype B, eight harbored genotype C, and six patients had undetermined HBV genotype. Two patients were HBeAg positive and had chronic hepatitis, and 18 were HBeAg negative. Eighteen patients had undergone NA treatment, including lamivudine, entecavir, and adefovir, and two inactive HBsAg carriers had not undergone NA treatment. Two patients presenting with HCC complications underwent pathological examination of a surgical specimen, resulting in identification of moderate-to-poorly differentiated HCC in one patient and well-to-moderately differentiated HCC in the other patient. Assessment of liver fibrosis in three patients presenting with HCC complications was performed at the time of HCC occurrence and resulted in METAVIR scores of F2, F3, and F4, respectively, whereas no patients in the control group underwent pathological examination of the liver. The median body mass index (BMI) of the patients was 23 kg/m^2 (18–27 kg/m^2), with six patients displaying a BMI ≧25 kg/m^2. Two patients had hepatorenal echo contrast data, and none of the patients reported alcohol consumption ≧100 g/day. There were no differences in age, sex, clinical forms of HBV infection, HBV

Table 1. Patient characteristics

Variables	HCC group (n = 10)	Controls (n = 10)	p-value
Age [y]#	60 [41-77]	60 [45–77]	0.80
Male, n (%)	6 (60)	6 (60)	1.00
Clinical phenotypes, n			
Inactive carrier/CH/LC	1/ 5/ 4	1/ 5/ 4	1.00
HBeAg-positive, n (%)	1 (10)	1 (10)	1.00
HBV genotypes, n			
Genotype B/C/Undetermined	3/ 3/ 4	3/ 5/ 2	0.56
Antiviral therapy, n			
ETV/LAM+ADV/Free	8/ 1/ 1	8/ 1/ 1	1.00
Alpha-fetoprotein [ng/mL]#	3.2 [1.1–22.9]	2.2 [1.3–19.2]	0.78
BMI [kg/m^2]#	23 [19–29]	23 [18–27]	0.41
Hepatorenal echo contrast, n (%)	1 (10)	1 (10)	1.00
Tumor size [mm]#	13 [7–40]	N/A	
Single tumor, n (%)	10 (100)	N/A	
BCLC stage 0 or A, n (%)	10 (100)	N/A	

Presented as median and range.
ADV, adefovir dipivoxil; CH, chronic hepatitis; ETV, entecavir; LAM, lamivudine; LC, liver cirrhosis; N/A, not applicable.

genotype, presence or absence of HBeAg, NA treatment, AFP level, BMI, or hepatosteatosis between the HCC and the control groups (Table 1).

2. Identification of differentially expressed miRNAs in HBV-related HCC patients

A total of 6599 ncRNA clusters were validated by GeneChip microarray analysis. We identified five serum miRNAs, including miR-548a, miR-1281, miR-4634, miR-4646-3p, and miR-4659a, showing significant differential expression between the groups ($p < 0.05$) and with absolute fold changes exceeding the cut-off threshold (Table 2), with two (miR-548a and miR-4646-3p) upregulated and three downregulated (miR-1281, miR-4634, and miR-4659a) in the HCC group relative to their levels in the control group. These were selected as candidates for subsequent analysis of their efficacy as biomarkers of early stage HCC development in patients with HBV infection.

Table 2. Candidate miRNAs identified by a microarray analysis

Variable		Fold change	p-value
miR-548a	Upregulated	1.36	0.01
miR-1281	Downregulated	− 1.69	0.03
miR-4634	Downregulated	− 1.65	0.03
miR-4646-3p	Upregulated	1.27	0.02
miR-4659a	Downregulated	− 1.33	0.01

IV. Discussion

We identified five serum miRNAs (miR-548a, miR-1281, miR-4634, miR-4646-3p, and miR-4659a) associated with early stage HBV-related HCC. Because miRNAs are differentially expressed in specific organs,

they represent potential organ- and disease-specific biomarkers. Previous studies demonstrated the efficacy of multiple miRNAs for detecting early or advanced stages of HCC, estimating HCC recurrence, and/or predicting prognosis [8]. Previous studies reported the use of single or multiple miRNAs to distinguish early stage HCC from control patients or HBsAg carriers [6], and Zhang et al. [9] constructed an miRNA panel comprising three miRNAs (miR-92-3p, miR-107, and miR-3126-5p) identified by microarray analysis using HCC cell-culture supernatant. There have been different results reported in the efficacy of serum miRNA expression as diagnostic biomarkers for early stage HCC according to findings from clinical and basic research. Among the possible reasons for variations in these results, studies differ in the source and measurement of circulating miRNAs [10]. Initial screening of candidate miRNAs depends upon microarrays that contain a limited number of probes and might not include recently identified miRNAs. Moreover, quantifications of miRNA candidates using real-time quantitative polymerase chain reaction (PCR) [11] often involve different normalization procedures, making direct comparison of expression patterns between studies difficult. Additionally, patient diversity can alter the identification of circulating miRNAs. Although miRNA dysregulation occurs in HCC, background miRNA expression can also differ according to the specific type of associated liver disease. Serum miR-34a and miR-29a levels are upregulated and downregulated according to fibrosis stage in patients with liver fibrosis [12], and serum miRNA levels are upregulated in HBeAg-positive patients as compared with those in HBeAg-negative patients [13].

Scores associated with Japanese risk estimations of HBV-related HCC account for patient age, sex, the presence of pre-existing cirrhosis, alanine aminotransferase level, AFP level, platelet count, HBeAg status, and HBV DNA show good discrimination ability in predicting HBV-related HCC development [1]. Importantly, NA treatment is associated with inhibited hepatocarcinogenesis; therefore, these factors should not be ignored in the screening process used to evaluate miRNA levels associated with hepatocarcinogenesis. In the present study, our pair-matched case-control analysis adjusted for age, sex, ethnicity, HBV genotype, the presence of HBeAg, NA treatment, and HBV genotype to assess miRNAs as potential confounding factors in HBV-related HCC development.

Jiang et al. [14] revealed changes in miR-1281 level and function through its induction by endoplasmic reticulum stress via binding of the tumor suppressor p53 to the miR-1281 promoter region and promotion of apoptosis in osteosarcoma cells. Previous studies reported *TP53* mutations as drivers of hepatocarcinogenesis and associated with HBV-related HCC [15]. Additionally, miR-1281 levels differ according to cancer type, with downregulated levels identified in bladder cancer tissue [16] and upregulated levels in patients with cholangiocarcinoma and malignant pleural mesothelioma [17,18]. In the present study, we identified downregulated levels of serum miR-1281 in patients with early stage HCC. Notably, previous studies identifying elevated circulating miR-1281 levels in patients with advanced or metastatic cancer

did not observe this in association with early stage cancer. The potential p53-related role of miR-1281 in promoting cancer-cell apoptosis requires verification in future studies.

Although miR-4634 function is currently unknown, previous studies reported elevated miR-4634 levels in patients with rheumatoid arthritis and decreased levels in patients with breast cancer [19, 20]. Bioinformatics prediction of miR-4634 targets identified *bromodomain and PHD finger containing-1, cycling-dependent kinase inhibitor 2A, myelin basic protein, and Toll-like receptor 8* [21]. Additionally, a report suggested a potential relationship between hypomethylation of the miR-4634 promoter and adolescent depression, although a role in carcinogenesis was not determined [22].

One limitation of the study was the small number of enrolled patients with HBV-related HCC and pair-matched controls (n = 20), which might explain the low fold changes in differential miRNA expression between cases and controls. Although we plan to conduct a validation study to determine the sensitivity and specificity of the candidate miRNAs, we need to ensure that an adequate number of patients are available in order to ensure the significance of our findings.

In summary, we conducted a case-control study adjusted strictly to identify unique circulating miRNAs involved in early stage HBV-related HCC and identified five candidate miRNAs potentially capable of distinguishing patients with early stage HBV-related HCC from those with only HBV infection. Although further validation is required, these findings provide critical insight into potential biomarkers associated with hepatocarcinogenesis in patients infected with HBV.

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Conflicts of interest: The authors have no conflict of interest to declare.

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B 型肝炎関連肝癌の早期診断バイオマーカーとしての circular microRNA の検討

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要旨

B 型肝炎ウイルス持続感染者における肝発癌の早期診断・発癌予測は困難である．そこで，マイクロアレイを用い，B 型肝炎関連肝癌の早期診断に有用なバイオマーカーの候補となる末梢血中の microRNA を探索した．B 型肝炎のため通院中に肝発癌を来した患者 10 人（発癌群）の肝発癌時の血清とマッチングさせた非発癌患者 10 人（対照群）の血清から microRNA を抽出し，2 群間で有意な差異を認めた 5 種類の microRNA（miR-548a および miR-1281，miR-4634，miR-4646-3p，miR-4659a）を早期診断のバイオマーカー候補として見出した．