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Original Articles

Dysregulation of miRNA in chronic hepatitis B is associated with hepatocellular carcinoma risk after nucleos(t)ide analogue treatment

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ABSTRACT

Hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma (HCC). Nucleos(t)ide analogue (NA) therapy effectively reduces the incidence of HCC, but it does not completely prevent the disease. Here, we show that dysregulation of microRNAs (miRNAs) is involved in post-NA HCC development. We divided chronic hepatitis B (CHB) patients who received NA therapy into two groups: 1) those who did not develop HCC during the follow-up period after NA therapy (no-HCC group) and 2) those who did (HCC group). miRNA expression profiles were significantly altered in CHB tissues as compared to normal liver, and the HCC group showed greater alteration than the no-HCC group. NA treatment restored the miRNA expression profiles to near-normal in the no-HCC group, but it was less effective in the HCC group. A number of miRNAs implicated in HCC, including miR-101, miR-140, miR-152, miR-199a-3p, and let-7g, were downregulated in CHB. Moreover, we identified CDK7 and TACC2 as novel target genes of miR-199a-3p. Our results suggest that altered miRNA expression in CHB contributes to HCC development, and that improvement of miRNA expression after NA treatment is associated with reduced HCC risk.

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1. Introduction

Liver cancer is the sixth most commonly occurring cancer world wide. It is also the second most common cause of cancer death, with 782,000 new cases and 723,000 deaths annually [1]. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer in adults. Major risk factors for HCC are chronic infection with hepatitis B virus (HBV) or HCV, excess alcohol intake, non-alcoholic steatohepatitis (NASH), diabetes, and obesity [2]. In developed countries, approximately 60% of HCC cases are attributable to HCV infection, while HBV infection is the major cause of HCC in developing counties [2]. Worldwide, there are approximately 350–400 million people harboring a chronic HBV (CHB) infection, and about half of all HCC cases are attributable to that population [3]. The majority of patients with HBV-related HCC exhibit liver cirrhosis, though HBV infection leads to HCC development even without cirrhosis [4]. Nucleos(t)ide analogue (NA) therapy effectively reduces the incidence of HCC in patients with CHB, and also reduces the HCC recurrence rate [5,6]. However, a small number of CHB patients who receive NA treatment still develop HCC, despite the disappearance of HBV-DNA from their serum [7]. Elucidation of the mechanism underlying hepatocellular carcinogenesis after NA treatment is necessary to predict and prevent HCC in CHB patients.

miRNAs are 21- to 23-nucleotide non-coding RNAs that negatively regulate gene expression at the post-transcription level. This enables them to affect a wide variety of biological processes, including tumorigenesis [8]. miRNAs have been shown to act as oncogenes or tumor suppressors in various malignancies, including HCC [8]. Dysregulated miRNA expression is frequently observed in both HBV-related and HCV-related HCC, suggesting miRNAs are important mediators of disease progression from viral hepatitis to HCC [9,10]. Experiments using cell culture models have also shown that HBV infection leads to altered expression of multiple miRNAs [11]. Expression of a subset of miRNAs is reportedly associated with survival in CHB-related HCC, and serum or plasma miRNA profiles can reportedly serve as biomarkers for diagnosis of HBV-related HCC [12,13]. These results suggest miRNAs are potentially useful diagnostic markers and therapeutic targets in HBV-related HCC.

Although the available evidence strongly suggests a causal relationship between miRNA dysregulation and HCC development, its involvement in post-NA hepatocarcinogenesis remains unknown. To address that issue, we analyzed miRNA expression profiles in CHB patients before and after long-term NA therapy. We also compared the miRNA profiles of CHB patients who developed HCC during the follow-up after the start of NA treatment with those who did not. We found that miRNA profiles are significantly dysregulated in CHB, but they can be restored through NA treatment. Moreover, it appears that insufficient recovery of miRNA profiles is associated with the risk of post-NA HCC development.

2. Materials and methods

2.1. Tissue samples and cell lines

A total of 52 CHB patients who received NA (entecavir, ETV) treatment at St. Marianna University School of Medicine Hospital, Kawasaki Municipal Tama Hospital, St. Marianna University School of Medicine, Yokohama City Seibu Hospital, Shonan Fujisawa Tokushukai Hospital, Kiyokawa Hospital, or Teine-Keijinkai Hospital were enrolled in this study. Biopsy specimens of CHB tissues were collected from 42 patients before administration of NA therapy. Post-treatment CHB tissues were obtained from 28 patients through biopsy or surgical resection. In patients who developed post-NA HCC, tumor tissues and adjacent non-tumorous tissues were collected. Normal liver tissues were obtained from five liver hemangioma patients who underwent surgical resection at St. Marianna University School of Medicine Hospital. Specimens were stored at −80 °C until RNA extraction. Written informed consent was obtained from all patients, and this study was approved by the institutional review board of St. Marianna University. HCC cell lines (HLE, HLF, HepG2, Hep3B, huH-1, HuH-7, Li-7, PLC/PRF/5, HT-17) were obtained and cultured as described previously [14]. HepG2-hNTCP-C4 cells were infected with HBV as described previously [15]. Total RNA was extracted using TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, USA) or a RNeasy Mini Kit (Qiagen, Hilden, Germany).

2.2. miRNA expression analysis

miRNA microarray analysis was carried out as described previously [16]. Briefly, 100 ng of total RNA from fresh-frozen tissues were labeled using miRNA Labeling Reagent (Agilent Technologies, Santa Clara, CA, USA), after which the labeled RNA was hybridized to a Human miRNA Microarray V3 (Rel 12.0, G4470C; Agilent Technologies). The microarray data were analyzed using GeneSpring GX ver. 13 (Agilent Technologies). The Gene Expression Omnibus accession number for the miRNA microarray data is GSE110217. Expression of selected miRNAs was analyzed using TaqMan microRNA Assays (Thermo Fisher Scientific). The PCR was run in triplicate using a model 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) and SDS v1.4 software (Applied Biosystems). U6 snRNA (RNU6B; Thermo Fisher Scientific) served as an endogenous control.

2.3. Transfection of miRNA mimics and siRNA

HCC cells (5×10^5 cells/well in 6-well plates) were transfected with 25 pmol of mirVana miRNA mimics (Thermo Fisher Scientific) or mirVana miRNA mimic Negative Control #1 (Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. For RNA interference-mediated knockdown of miRNA target genes, cells were transfected with 25 pmol of Silencer Select siRNAs (Thermo Fisher Scientific) or Silencer Select Negative Control (Applied Biosystems) using Lipofectamine RNAiMAX (Thermo Fisher Scientific).

2.4. Cell viability assay

Cells were transfected with miRNA mimics or siRNAs as described above, after which they were seeded into 96-well plates to a density of 5000 cells per well. After incubation for 72 h, cell viability was assessed using a Cell Counting kit-8 (Dojindo) according to the manufacturer's instructions.

2.5. Cell invasion and migration assays

For Matrigel invasion assays, cells were transfected with miRNA mimics or siRNAs as described above, after which 5×10^4 transfectant cells were suspended in 500 μL of serum-free Dulbecco's Modified Eagle medium (DMEM) (Sigma-Aldrich). Aliquots of the suspension were then added to the tops of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) prehydrated with phosphate-buffered saline (PBS). At the same time, 750 μL of DMEM supplemented with 10% fetal bovine serum (FBS) were added to the lower wells of the plate. For migration assays, a control insert (BD Biosciences) was used instead of a Matrigel Invasion Chamber. After incubation for 22 h, invading or migrating cells were stained and counted in five randomly selected microscope fields per membrane.

3. Results

3.1. Effects of NA therapy on miRNA expression profiles in CHB

To clarify whether miRNA dysregulation is associated with post-NA HCC development in CHB, we analyzed the miRNA expression profile in