Serum miR-192-5p Levels Predict the Efficacy of Pegylated Interferon Therapy for Chronic Hepatitis B

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**Research Article**

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Abstract

We examined the association between serum miRNA (-192-5p, -122-3p, -320a and −6126-5p) levels and the efficacy of pegylated interferon (Peg-IFN) monotherapy for chronic hepatitis B (CHB) patients. We enrolled 61 CHB patients treated with Peg-IFNα-2a weekly for 48 weeks, of whom 12 had a virological response (VR) and 49 did not VR (non-VR). A VR was defined as HBV DNA < 2,000 IU/ml, hepatitis B e antigen (HBeAg)-negative, and nucleos(t)ide analogue free at 48 weeks after the end of treatment. The non-VR group showed a significantly higher HBeAg-positivity rate, ALT, HBV DNA, and serum miR-192-5p levels at baseline ($P = 0.024$, $P = 0.020$, $P = 0.007$, $P = 0.021$, respectively). Serum miR-192-5p levels at 24 weeks after the start of treatment were also significantly higher in the non-VR than the VR group ($P = 0.011$). Multivariate logistic regression analysis for predicting VR showed that miR-192-5p level at baseline was an independent factor. Serum miR-192-5p levels were significantly correlated with the levels of HBV DNA, hepatitis B core-related antigen, and hepatitis B surface antigen ($r = 0.484$, 0.384 and 0.759, respectively). The serum miR-192-5p level was useful as a biomarker for the therapeutic efficacy of Peg-IFN in CHB treatment.

Introduction

Hepatitis B virus (HBV) infection is a global public health problem, with approximately 240 million people, or 6% of the world’s population, chronically infected with HBV \(^1\). The prevalence of hepatitis B is highest in the Western Pacific and African regions, affecting 5–7% and > 8% of the adult population, respectively \(^2\).

The long-term goal of antiviral therapy for chronic hepatitis B (CHB) has been to eliminate hepatitis B surface antigen (HBsAg). However, the current standard therapies using nucleos(t)ide analogues (NAs) or pegylated interferon (Peg-IFN) are difficult to achieve the elimination of HBsAg \(^3\). Nucleos(t)ide analogues have been shown to be highly safe across a wide range of patients with CHB, including decompensated cirrhosis and pregnancy \(^4\). However, recurrence of elevated levels of HBV DNA and alanine aminotransferase (ALT) is likely when NAs treatment is discontinued, therefore long-term administration of NAs is often required \(^5\). Pegylated interferon is thought to be more effective than lamivudine based on HBV DNA suppression and seroconversion of HBsAg antibody \(^6\). Furthermore, Peg-IFN has the advantage of maintaining a drug-free therapeutic effect without additional drug administration after treatment in CHB patients who exhibit a therapeutic response \(^7\) \(^8\). However, the therapeutic effect of Peg-IFN is obtained in only 20–30% of patients who are hepatitis B e antigen (HBeAg)-positive and in 20–40% of HBeAg-negative patients \(^6\). Additionally, Peg-IFN therapy is associated with various side effects such as fever, fatigue, depression, neutropenia and thrombocytopenia \(^7\). The levels of ALT and HBV DNA, and HBV genotype at baseline, have been reported to significantly affect the response to Peg-IFN therapy after 24 weeks of treatment \(^9\). Along these lines, a reliable marker for the efficacy of Peg-IFN therapy in CHB is needed \(^10\).
MicroRNAs (miRNAs) are involved in various biological phenomena, such as cell development, differentiation, proliferation, apoptosis, and metabolism and also play roles in the pathogenesis of inflammation, fibrogenesis, and carcinogenesis in liver diseases. Several studies to date have revealed an association between serum miRNA levels and the response to IFN therapy in CHB. Brunetto et al. showed that the levels of several serum miRNAs such as miR-192-5p, miR-320a, and miR-122-3p were related to the response to IFN therapy in CHB. A study by Fujita et al. revealed that higher miR-6126-5p levels in sera during Peg-IFN therapy with or without NAs for CHB predicted the reduction of HBsAg after the completion of therapy. However, these studies analyzed only a relatively small numbers of patients. Therefore, the association between serum expression levels of these miRNAs and the response to IFN therapy in CHB should be validated in independent cohorts.

Herein, we aimed to validate the association between the levels of serum miR-192-5p, 320a, 122-3p, and 6126-5p with the efficacy of Peg-IFN monotherapy for CHB patients.

Results

Comparison of clinical characteristics at baseline according to the efficacy of Peg-IFN therapy

The clinical characteristics between the Virological response (VR) and the non-VR groups at baseline are shown in Table 1. A Virological response was defined as HBV DNA < 2,000 IU/mL, HBeAg-negative, and no need for administration of NAs until the end of the observation period (48 weeks after the end of treatment (EOT)). At the end of the observation period, 12 patients had a VR and 49 had non-VR. Compared with the VR group, the non-VR group had a significantly higher HBeAg-positive rate and levels of ALT, HBV DNA, and serum miR-192-5p ($P = 0.024$, $P = 0.020$, $P = 0.007$, $P = 0.021$, respectively). It has been reported that HBeAg-negative patients at baseline were successfully treated with Peg-IFN compared to HBeAg-positive patients at baseline. Therefore, we also compared clinical characteristics between the VR and non-VR groups among HBeAg-negative patients, which showed that there was no significant difference in ALT and HBV DNA levels, while miR-192-5p levels tended to be higher in the non-VR group (Table S1). As most patients had HBV genotype C in this study, there was no significant difference of HBV genotypes between the VR groups and non-VR groups. It is difficult to discuss the therapeutic effect of the HBV genotype in this study.
Table 1
Clinical characteristics of Chronic Hepatitis B patients and comparison between VR group and non-VR group at baseline.

| Factor                  | Total (n = 61) | VR group (n = 12) | non-VR group (n = 49) | P-value |
|-------------------------|---------------|------------------|-----------------------|---------|
| Age, years              | 35 (31 – 42)  | 42 (31 – 46)     | 35 (31 – 39)         | N.S.    |
| Male, n (%)             | 35 (57)       | 5 (42)           | 30 (61)               | N.S.    |
| HBV genotype A/B/C      | 4 / 6 / 51    | 2 / 1 / 9        | 2 / 5 / 42            | N.S.    |
| AST (U/L)               | 47 (28 – 102) | 38 (22 – 69)     | 49 (31 – 103)        | N.S.    |
| ALT (U/L)               | 79 (38 – 171) | 32 (26 – 94)     | 85 (40 – 182)        | 0.020   |
| Platelet counts (×10^9/L) | 198 (166 – 224) | 215 (182 – 220) | 197 (166 – 226)      | N.S.    |
| HBeAg-positive, n (%)   | 33 (54)       | 3 (25)           | 30 (61)               | 0.024   |
| HBV DNA (log IU/mL)     | 6.2 (4.7 – 8.0) | 4.3 (3.5 – 6.2) | 7.1 (5.0 – 8.2)      | 0.007   |
| HBsAg (IU/mL)           | 7,989 (2,290 – 15,940) | 3,444 (1,665 – 9,912) | 10,470 (2,868 – 23,493) | N.S.    |
| HBcrAg (log U/mL)       | 5.7 (4.0 – 6.9) | 4.2 (2.9 – 6.8) | 6.0 (4.4 – 6.9)      | N.S.    |
| miR-192-5p              | 0.032 (0.016 – 0.086) | 0.016 (0.007 – 0.032) | 0.048 (0.020 – 0.123) | 0.021   |
| miR-320a                | 0.229 (0.148 – 0.296) | 0.185 (0.128 – 0.209) | 0.249 (0.156 – 0.309) | N.S.    |
| miR-122-3p              | 0.002 (< 0.001 – 0.007) | 0.003 (< 0.001 – 0.007) | 0.002 (0.001 – 0.006) | N.S.    |
| miR-6126-5p             | 0.112 (0.053 – 0.205) | 0.057 (0.036 – 0.182) | 0.119 (0.067 – 0.205) | N.S.    |

Data from all patients were expressed as numbers for categorical data and medians (first–third quartiles) for noncategorical data.

Categorical variables were compared between groups by the chi-square test, and noncategorical variables were compared using the Mann-Whitney U test.

Abbreviations: VR, virological response; N.S., not significant; HBV, hepatitis B virus; AST, aspartate transaminase; ALT, alanine transaminase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; miR, microRNA.

Predictive factors for the efficacy of Peg-IFN therapy during treatment
The clinical characteristics of the VR and non-VR groups during 24-week treatment (24W) are shown in Table 2. The proportion of HBeAg-positive patients and the levels of HBV DNA and serum miR-192-5p were significantly higher in the non-VR versus the VR group ($P = 0.020, P < 0.001, P = 0.011$, respectively).

### Table 2
Comparison of clinical data between VR and non-VR Groups at 24 weeks during Peg-IFN therapy.

| Factor                  | Total (n = 61) | VR group (n = 12) | non-VR group (n = 49) | $P$-value |
|-------------------------|----------------|-------------------|-----------------------|-----------|
| AST (IU/L)              | 34 (27 – 50)   | 32 (22 – 43)      | 36 (27 – 51)          | N.S.      |
| ALT (IU/L)              | 43 (27 – 64)   | 32 (20 – 45)      | 45 (29 – 67)          | N.S.      |
| HBV DNA (log IU/mL)     | 2.9 (< 1.3 – 5.0) | < 1.3 (ND – 1.3)  | 3.7 (1.6 – 5.9)       | < 0.001   |
| HBsAg (IU/mL)           | 2,800 (957 – 8,490) | 1,668 (60 – 3,035) | 3,392 (1,019 – 9,977) | N.S.      |
| HBeAg-positive, n (%)   | 30 (49)        | 2 (17)            | 28 (57)               | 0.020     |
| HBcrAg (log U/mL)       | 5.4 (3.3 – 6.6) | 3.8 (3.2 – 5.1)   | 6.0 (3.5 – 6.7)       | N.S.      |
| miR-192-5p              | 0.024 (0.011 – 0.045) | 0.012 (0.005 – 0.025) | 0.027 (0.013 – 0.061) | 0.011     |
| miR-320a                | 0.198 (0.118 – 0.267) | 0.123 (0.090 – 0.216) | 0.210 (0.140 – 0.269) | N.S.      |
| miR-122-3p              | 0.001 (< 0.001 – 0.004) | 0.001 (< 0.001 – 0.001) | 0.001 (< 0.001 – 0.005) | N.S.      |
| miR-6126-5p             | 0.056 (0.034 – 0.102) | 0.045 (0.018 – 0.092) | 0.061 (0.034 – 0.104) | N.S.      |

Data from all patients were expressed as numbers for categorical data and medians (first–third quartiles) for noncategorical data.

Categorical variables were compared between groups by the chi-square test, and noncategorical variables were compared using the Mann-Whitney $U$ test. Abbreviations: VR, virological response; Peg-IFN, pegylated interferon; AST, aspartate transaminase; N.S., not significant; ALT, alanine transaminase; HBV, hepatitis B virus; ND, not detected; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBcrAg, hepatitis B core-related antigen; miR, microRNA.

### Cutoff values of variables for predicting virological response to Peg-IFN therapy

The receiver operating characteristic (ROC) curves were created using factors that showed significant differences in the comparison between the 2 groups at baseline and 24W, and the cutoff value and area under the curve (AUC) were calculated (Fig. 1). The cutoff values for miR-192-5p levels were 0.016 at both baseline and 24W, and that for HBV DNA at baseline was 4.74 log IU/ml. The AUCs for miR-192-5p levels at baseline and 24W, and HBV DNA levels at baseline were 0.72, 0.74, and 0.75, respectively, indicating that they were equivalent in predicting the response to Peg-IFN therapy (Table 3).
Table 3
Area under the curve and cutoff values for predicting virological response to Peg-IFN therapy.

| Factor                  | Area under the curve | lower | higher | \( P \)-value | cutoff value |
|-------------------------|----------------------|-------|--------|---------------|--------------|
| HBV DNA at baseline     | 0.75                 | 0.58  | 0.92   | 0.004         | 4.74         |
| miR-192 at baseline     | 0.72                 | 0.56  | 0.88   | 0.007         | 0.016        |
| miR-192 at 24 weeks     | 0.74                 | 0.59  | 0.89   | 0.002         | 0.016        |

Area under the curve was calculated from the receiver operating characteristic (ROC) curve as shown in Fig. 1.

The cutoff values were calculated from the point on the ROC curve closest to top left of HBV DNA at baseline and miR-192-5p levels at baseline and 24 weeks during Peg-IFN therapy.

Abbreviations: Peg-IFN, pegylated interferon; CI, confidence interval; HBV, hepatitis B virus; miR, microRNA.

Table 3

Prediction of VR to Peg-IFN therapy using miR-192-5p and HBV DNA levels

We calculated the sensitivity, specificity, and positive and negative predictive values (PPV and NPV) for predicting VR, using the above-mentioned cutoff values for miR-192-5p levels at baseline and 24W (Table 4). The PPVs were 44% and 38%, and the NPVs were 89% and 90%, using the cutoff values for miR-192-5p levels at baseline and 24W respectively, indicating that they were equivalent in predicting the response to IFN therapy. The PPV and NPV using the cutoff value for HBV DNA levels were 43% and 87%, respectively.

Table 4
Prediction for virological response to Peg-IFN therapy.

|                  | PPV        | NPV        | sensitivity | specificity |
|------------------|------------|------------|-------------|-------------|
| miR-192-5p < 0.016 at baseline | 7 / 16 (44%) | 40 / 45 (89%) | 7 / 12 (58%) | 40 / 49 (82%) |
| miR-192-5p < 0.016 at 24 weeks   | 8 / 21 (38%) | 36 / 40 (90%) | 8 / 12 (67%) | 36 / 49 (73%) |
| HBV DNA < 4.74 at baseline       | 6 / 14 (43%) | 41 / 47 (87%) | 6 / 12 (50%) | 41 / 49 (84%) |

* The cutoff values of HBV DNA level at baseline and the miR-192 levels at baseline and 24 weeks during the Peg-IFN therapy for predicting virological response were determined by the receiver operating characteristic analyses.

Abbreviations: Peg-IFN, pegylated interferon; PPV, positive predictive value; NPV, negative predictive value; HBV, hepatitis B virus; miR, microRNA.

Multivariate logistic regression analysis for predicting virological response to Peg-IFN therapy
Next, we conducted multivariate logistic regression analysis to determine predictive factors for discriminating VR. Based on previous findings regarding predictive factors for the response to IFN therapy in CHB patients\textsuperscript{15}, and our results as shown in Tables 1–4, we included the following variables as covariates: HBeAg, HBV DNA level $\geq 4.74$ log IU/ml and miR-192-5p level $\geq 0.016$ at baseline. As a result, miR-192-5p level $\geq 0.016$ was identified an independent predictive factor VR (odds ratio = 0.2; $P = 0.041$) (Table 5).

| Table 5 | Multivariate logistic regression analysis of factors at baseline associated with virological response to Peg-IFN therapy. |
|---------|---------------------------------------------------------------------------------------------------------------|
| Factor  | Odds  | Lower | Upper  | $P$ value |
| HBeAg-positive | 0.3 | 0.07 | 1.74 | 0.198 |
| HBV DNA $\geq 4.74^*$ | 0.4 | 0.09 | 2.15 | 0.311 |
| miR-192-5p $\geq 0.016^*$ | 0.2 | 0.05 | 0.94 | 0.041 |

Abbreviations: Peg-IFN, pegylated interferon; CI, confidence interval; HBeAg, hepatitis B e antigen; miR, microRNA; HBV, hepatitis B virus

* The cutoff values of HBV DNA and miR-192 levels at baseline for predicting virological response was determined by the receiver operating characteristic analysis.

**Correlations of miR-192-5p expression levels in serum with clinical parameters**

We examined the correlations between serum miR-192-5p levels and other clinical parameters, which showed significant correlations with the levels of HBV DNA, hepatitis B core-related antigen (HBcrAg), and especially HBsAg, at baseline and 24W, whereas there was no correlation with the levels of AST, ALT, and platelet counts (Table 6). In addition, serum miR-192-5p levels at baseline were significantly higher in HBeAg-positive versus than HBeAg-negative patients (0.063 vs. 0.022, $P = 0.0058$), but there was no difference at 24W between the 2 groups.
Table 6
Correlations of serum miR-192-5p levels with clinical parameters at baseline and 24 weeks during Peg-IFN therapy.

| miR-192-5p levels | r* | P-value |
|-------------------|----|---------|
| Factor            | baseline | 24 weeks | baseline | 24 weeks |
| AST               | -0.055 | -0.080 | N.S. | N.S. |
| ALT               | 0.082 | 0.092 | N.S. | N.S. |
| PLT               | -0.096 | N.A. | N.S. | N.A. |
| HBV DNA           | 0.484 | 0.655 | < 0.001 | < 0.001 |
| HBsAg             | 0.759 | 0.730 | < 0.001 | < 0.001 |
| HBcrAg            | 0.384 | 0.551 | 0.005 | < 0.001 |

* The correlation coefficient (r) is calculated using the Pearson correlation test.

Abbreviations: Peg-IFN, pegylated interferon; AST, aspartate transaminase; ALT, alanine transaminase; PLT, platelet counts; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; miR, microRNA.; N.S., not significant; N.A., not available.

Discussion

The present study validated that serum miR-192-5p levels were associated with response to IFN therapy in CHB patients, whereas serum miR-320a, miR-122-3p, and miR-6129-5p levels were not validated. Multivariate analysis showed that a lower miR-192-5p level at baseline was an independent predictor of VR. Furthermore, the NPV for VR using the cutoff value of miR-192-5p level at baseline was high (89%). To date, several factors have been reported to be associated with VR in the treatment of CHB, for instance patients with HBeAg-negative CHB who show decreased HBcrAg levels during treatment with Peg-IFN combination therapy with or without NAs were more likely to succeed with anti-viral treatment. Another study in HBeAg-negative CHB patients mostly with genotype D, showed that no reduction in HBsAg levels and a lack of decrease in HBV DNA levels below 1.2 log_{10} IU/ml during 12-week treatment has a NPV of nearly 100% of untreated persistent VR, and this was therefore recommended as a stopping rule for early discontinuation of ineffective Peg-IFN. In combination with these predictors, higher levels of serum miR-192-5p may be useful as a factor for the decision to discontinue treatment.

MicroRNA-192-5p is associated with hypertension, diabetes, and various cancers, such as lung, gastric, pancreatic, and liver cancer, and its potential as a disease marker has been suggested. It has been reported that miR-192-5p is abundantly expressed in hepatic tissues, as well as in serum and urine, and their exosome. Several studies on liver diseases have identified serum miR-192-5p level as a potential early biomarker for detecting hepatocellular carcinoma, and revealed that miR-192-5p plays
an important role in the pathophysiology of non-alcoholic fatty liver disease and liver injury\(^{28}\). As for HBV-related diseases, the replication of HBV was shown to be correlated with the in vitro expression of miR-192-5p in a HepG2 cell model system, and overexpression of miR-192-5p by mimics reduces the protein level of pro-apoptotic BIM (Bcl-2-like protein 11)\(^{29}\). In addition, miR-192-5p was shown to be overexpressed in both the sera and HBsAg particles of CHB patients\(^{13}\). Subsequent research revealed that miR-192-5p is present in hepatoma-derived extracellular vesicles and abundantly expressed in HBeAg-positive patients compared with HBeAg-negative patients\(^{30}\). Our study showed a strong correlation between HBsAg and miR-192-5p levels in serum (Table 6), and miR-192-5p levels were higher in HBeAg-positive patients than in HBeAg-negative patients, while the VR rate tended to be lower in HBeAg-positive versus HBeAg-negative patients (3/33 vs. 9/28, \(P = 0.053\)). Thus, HBV replication might influence serum miR-192-5p levels, which accounts for serum miR-192-5p levels being associated with the response to IFN therapy. However, serum miR-192-5p level at baseline was an independent predictor for VR in our study; therefore, there might be a further mechanism by which miR-192-5p influences or predicts IFN efficacy. Intriguingly, previous studies demonstrated induction of miR-192 by IFN-\(\alpha\) in Huh7.5 cells and downregulation upon hepatitis C virus infection\(^{31}\), indicating that miR-192-5p upregulation in serum could be an independent variable for non-response to Peg-IFN and ribavirin treatment in chronic hepatitis C\(^{32}\). These findings led us to speculate that miR-192-5p might be associated with antiviral immunity. Further studies are necessary to elucidate the functional roles of miR-192-5p in CHB.

There were 2 limitations to this study. First, ALT levels were low in the VR cases, although previous studies have reported that ALT levels were high in responders to IFN therapy for CHB. In the non-VR group, higher HBV DNA levels and HBeAg-positive rate, which were considered to be predictors for non-response to IFN therapy in CHB, were associated with active hepatic inflammation, namely higher ALT levels. Second, we examined only a small number of patients, especially in the VR group. This resulted from difficulty in collecting VR cases, because few CHB patients being treated with Peg-IFN and the low VR rate. Future study is needed to evaluate serum miR-192-5p level as a marker for the response to Peg-IFN therapy in a large group of CHB patients, who should be divided by HBeAg status.

In conclusion, serum miR-192-5p levels might be a predictive biomarker for the response to Peg-IFN therapy in CHB patients.

**Methods**

**Patients and study design**

The study design is shown in Fig. 2. We enrolled 61 CHB patients from 2012 to 2016 in 8 hospitals (Nagoya City University Hospital, Shinshu University Hospital, Hyogo College of Medicine Hospital, Osaka City University Hospital, Chiba University Hospital, St. Marianna Medical University Hospital, Hiroshima University Hospital, and Nippon Medical School Chiba Hokusoh Hospital). All patients were chronically mono-infected with HBV and confirmed to be HBsAg-positive for at least 6 months. Hepatitis B e antigen
was positive in 33 patients and negative in 28. None of the patients received NAs within 48 weeks prior to Peg-IFN treatment. Patients were treated with Peg-IFNα-2a weekly for 48 weeks and were observed for 48 weeks after the EOT with monitoring at monthly intervals. At each follow-up, data for biochemical markers, virological markers, blood counts, and clinical status were recorded. Following data collection, the patients were divided into VR and non-VR groups.

Written informed consent was obtained from all individual participants. The study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee of Nagoya City University Hospital (60-08-0024).

**Laboratory Tests And Serological And Virological Assays**

Hematologic and blood chemistry tests were carried out using standard assays. Serum HBV DNA levels were measured using COBAS TaqMan HBV 2.0 (Roche Diagnostics K. K., Tokyo, Japan [lower limit of detection, 20 IU/mL]) \(^3\). Positive results (signals) below the quantitative HBV DNA concentrations were referred to as “detected”, which was defined as < 1.3 log IU/mL, and negative signals, as “not detected”. Hepatitis B e antigen was determined using an HISCL HBeAg kit (Sysmex, Kobe, Japan) and HBsAg was determined using an HISCL HBsAg (Sysmex, Kobe, Japan) (detection range, 30 to 2,500,000 mIU/mL). Hepatitis B core-related antigen was determined by Lumipulse HBcrAg assay (Fujirebio.K.K., Tokyo, Japan) (detection range, 3.0 to 6.7 log U/mL). The genotypes of HBV were determined serologically by enzyme immunoassay using commercial kits, HBV GENOTYPE EIA (Institutes of Immunology Co., LTD, Tokyo, Japan).

**Sampling Serum and Isolation of RNA**

We followed the protocols of sampling serum and isolation of RNA as we described previously \(^3\). Peripheral blood was collected from each participant at baseline and 24W, and was centrifuged at 1,500 g for 5 minutes at room temperature. After serum separation, the samples were stored at -80°C until use. Total RNAs including miRNAs in serum were purified with miRNeasy Serum/Plasma kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. Specifically, we extracted total RNA from 200 µL of serum from each subject, to which 5.6 × 10^8 copies of *Caenorhabditis elegans* cel-miR-39-3p (cel-miR-39-3p) were added as spike-in RNA for later normalization; then total RNA was eluted from each column with 30 µL of nuclease-free water. The concentration of total RNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

**Measurement Of Serum Mirmas**

The levels of miRNA levels were determined using by quantitative real-time polymerase chain reaction (qRT-PCR) with Step One Plus (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and TaqMan MicroRNA Assay: hsa-miR-192-5p (assay ID 000491), has-miR-122-3p (assay ID 002130), has-miR-320a
(assay ID 002277), hsa-miR-6126-5p (assay ID 475618), and cel-miR-39-3p (assay ID 000200) (Thermo Fisher Scientific). One microliter of total RNA extracted from serum were subjected to reverse transcription with a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and the respective TaqMan MicroRNA Assay reagents for the target molecules, in a total volume of 15 µL, followed by qRT-PCR in a total volume of 20 µL, according to the manufacturer’s protocol. Amplification was carried out as follows: 95°C for 10 min, 45 cycles at 95°C for 15 s and 60°C for 60 s. All reactions were carried out in duplicate. Cycle threshold (Ct) values were calculated using Step One Software v2.3 (Thermo Fisher Scientific). Expression levels of miRNAs were normalized to those of the spike-in cel-miR-39-3p. The expression levels were determined by the $2^{-\Delta Ct}$ method, in which $\Delta Ct$ was calculated as: $\Delta Ct = Ct (\text{miRNA (miR-192-5p, miR-122-3p, miR-320a and miR-6126-5p)}) - Ct (\text{cel-miR-39-3p})$.

**Statistical Analysis**

Categorical variables were compared between groups by chi-square test, and non-categorical variables were analyzed by Mann–Whitney $U$ test. Changes in serum miRNA levels from baseline to 24W were compared by two-way analysis of variance. Receiver operating characteristic curve analyses were carried out and the AUC was calculated to evaluate the feasibility of using the miRNA levels as markers for discriminating VR. Multivariate logistic regression analyses were performed to determine whether several covariates were independently associated with VR. A $P$ value $< 0.05$ was considered significant in all tests was set as the target variables in the multiple regression formula for in multivariate analysis. Correlation coefficients were calculated using Pearson’s correlation test. Statistical analyses were performed using BellCurve Excel statistics (SSRI Inc., Tokyo, Japan).

**Declarations**

**Author Contributions**

Y.N., K.M. and Y.T. designed this study, Y.N. and K.M. performed the statistical analyses, and Y.N. and K.M. wrote the manuscript. K.M., E.I., T.I., K.F., A.M., E.T., S.N., J.K., T.M., M.E., H.I., T.W., C.O., M.T., M.A., M.T., H.K., and Y.T. collected the data. Y.T. revised the manuscript for important intellectual content. All authors reviewed the manuscript.

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**Conflict of Interest**
Yasuhito Tanaka received lecture fees from Gilead Sciences, Inc., Fujirebio Inc., and Sysmex Corp. Yasuhito Tanaka received research fees from Fujifilm Corp., Janssen Pharmaceutical K.K, Gilead Sciences, GlaxoSmithKline Pharmaceuticals Ltd, and Leland Stanford Junior University. The other authors declare no competing interests.

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**Figures**
Figure 1

ROC curves for HBV DNA and miR-192-5p. The receiver operating characteristic (ROC) curves were created using, miR-192-5p at baseline and 24W, and HBV DNA at baseline as factors. Cutoff values were calculated from the point on the ROC curve closest to the top left of HBV DNA at baseline, miR-192-5p at baseline, and 24W. Abbreviations: TPF, true positive fraction; FPF, false positive fraction
Study design. We collected serum from 61 patients who received Peg-IFN monotherapy for untreated CHB. Patients were treated with Peg-IFNα-2a weekly for 48 weeks and observed up to 48 weeks after therapy. Serum was collected at baseline, 24W, the end of treatment (EOT), and 48 weeks after the EOT. Virological response (VR) was defined as HBV DNA < 2,000 IU/mL, HBeAg-negative, NAs free at 48 weeks after the EOT. There were 12 VR cases and 49 non-VR cases in this study, and comparisons were made between VR group and non-VR group. Abbreviations: CHB, chronic hepatitis B; HBeAg, hepatitis B e antigen; Peg-IFN, pegylated interferon; EOT, end of treatment.

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