Serum HBV RNA Correlated with Intrahepatic cccDNA More Strongly than other HBV Markers During Peg-Interferon Treatment

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Research

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Abstract

**Background:** Serum hepatitis B virus RNA (HBV RNA) has been reported to be a surrogate marker of intrahepatic cccDNA during nucleos(t)ide analogs therapy. However, whether HBV RNA is superior to other HBV markers reflecting cccDNA profile in HBeAg-positive patients during peg-interferon (peg-IFN) treatment was still unclear.

**Methods:** Serum HBV RNA, HBcrAg, HBV DNA, and HBsAg were longitudinally assessed among 30 HBeAg-positive patients during 48-week peg-IFN treatment. The intrahepatic cccDNA was detected at baseline and week 48, respectively. The individual correlations between HBV RNA, HBcrAg, HBV DNA, HBsAg, and cccDNA were then statistically analyzed.

**Results:** HBV RNA levels in patients with HBeAg seroconversion decreased more rapidly compared with those without HBeAg seroconversion. Among all patients, cccDNA correlated better with HBV RNA than with HBcrAg, HBV DNA, and HBsAg at baseline. After 48 weeks of treatment, cccDNA still correlated more strongly with HBV RNA than other HBV markers. For subsequent analysis, cccDNA positively correlated with HBV RNA and HBcrAg whereas did not correlate with HBV DNA and HBsAg in patients with HBeAg seroconversion. However, cccDNA highly correlated with HBV RNA and HBV DNA, moderately correlated with HBcrAg, while no correlation was observed between cccDNA and HBsAg in patients without HBeAg seroconversion.

**Conclusion:** Serum HBV RNA correlated more strongly than HBcrAg, HBV DNA, and HBsAg with intrahepatic cccDNA levels before and after 48-week peg-IFN treatment. The level of serum HBV RNA may be a superior surrogate marker reflecting the intrahepatic cccDNA profile in HBeAg-positive patients during peg-IFN treatment.

**Trial registration:** ClinicalTrials, NCT03546530. Registered 1 January 2015. 
https://clinicaltrials.gov/ct2/results?cond=&term=NCT03546530&cntry=&state=&city=&dist=

Background

Hepatitis B virus (HBV) infection is a major global health problem. Chronic HBV infection greatly increases the risk of terminal liver diseases, such as cirrhosis, hepatic decompensation, and hepatocellular carcinoma, which contributes to more than 780,000 deaths every year worldwide [1, 2]. Currently, the goal for the treatment of HBV is to suppress viral replication to the lowest possible level thus halting disease progression. Nowadays, the approved agents for the treatment of chronic HBV infections mainly belong to two classes: pegylated interferon (peg-IFN) and nucleotide/nucleoside analogs (NAs)[3]. Among these agents, peg-IFN, which has the effects of suppressing HBV replication and immunomodulation, is still widely used in the clinic as the first-line therapeutic option for patients with HBeAg-positive chronic hepatitis B (CHB) [4].
Although antiviral agents can effectively reduce the serum HBV DNA level in CHB patients, complete elimination of HBV is difficult due to the existence of intrahepatic covalently closed circular DNA (cccDNA) [5]. HBV cccDNA, which serves as the template for the transcription of a 3.5-kb pregenomic RNA, can produce progeny viral DNA and proteins even in the absence of detectable HBV DNA or HBsAg in the blood [6–8]. Low levels of intrahepatic cccDNA predict a sustained virologic response after cessation of antiviral treatment. Therefore, quantitation of intrahepatic cccDNA is suggested to be a valuable marker in evaluating the cure of CHB and assessing treatment endpoints. However, the invasive nature of liver biopsy is the major obstacle for quantitation of cccDNA, which greatly limits the use of cccDNA as a marker in real-world clinical practice. Therefore, finding non-invasive surrogate markers of intrahepatic cccDNA is clinically meaningful.

Several traditional serum markers, such as quantitative HBsAg and HBV DNA, have been proposed to reflect the intrahepatic cccDNA profile [9, 10]. However, the correlations between cccDNA and those traditional markers are too weak to apply them in clinical practice [11].

Recently, due to being transcribed from cccDNA, serum HBV RNA has also been reported as a potential surrogate marker for reflecting the activity of intrahepatic cccDNA in CHB patients, as well as during NAs therapy [12–14]. Serum HBV RNA was also identified as a good predictor of HBeAg seroconversion in HBeAg-positive patients during therapy with peg-IFN [4]. In addition, a mouse model experiment revealed that HBV RNA correlated positively with cccDNA before treatment, whereas no correlation between them after 6 weeks of peg-IFN therapy [12].

HBV core-related antigen (HBcrAg) is another new valuable marker of HBV infection, which consists of three species of related proteins, including hepatitis B core antigen, hepatitis B e antigen, and a truncated 22KDa precore protein[15]. Serum HBcrAg was also considered to be correlated with cccDNA activity and was a good biomarker in predicting HBeAg seroconversion in patients treated with peg-IFN[16, 17]. Despite the findings above, it still remains unclear whether the HBV RNA could offer better predictive performance than HBcrAg, HBV DNA, or HBsAg in reflecting the intrahepatic cccDNA among HBeAg-positive patients during treatment with peg-IFN.

**Materials And Methods**

**Aim**

In the present study, the correlations of HBV RNA, HBcrAg, HBV DNA, and HBsAg with cccDNA were evaluated with the aim to find a better surrogate marker of cccDNA in HBeAg-positive patients with peg-IFN treatment. At the same time, the dynamic changes in serum HBV RNA were also investigated.

**Study participants**

A total of 30 HBeAg-positive, noncirrhotic CHB patients were recruited from a multicenter, randomized, controlled clinical trial between Mar. 2015 and Dec. 2017 (ClinicalTrials.gov Identifier: NCT03546530).
The patients had completed the full course of 48 weeks treatment and had baseline and week 48 liver paired biopsy and serial serum during treatment. The patients were treated with peg-IFN (Kawin Technology, China) at a dosage of 1.5 µg/kg body weight once weekly for 48 weeks. Briefly, the inclusion criteria were as follows: 18 to 60 years old; positive HBsAg for at least 6 months; HBeAg-positive and anti-HBe negative; HBV DNA $\geq 10^5$ IU/mL; ALT $\geq 2$ and $< 10 \times$ the upper limit of normal; no treatment history. The criteria for exclusion were: positivity for antibodies against HCV, HDV, or HIV; other inflammatory diseases such as rheumatoid arthritis, diabetes, or autoimmune hepatitis; hypertension; kidney disease; or a recent history of infectious disease.

This study was conducted according to the Helsinki Declaration of 1975 and the research was approved by the Ethics Committee of the First Hospital of Jilin University and all participating institutions. Written informed consent was obtained from all patients.

**Samples**

Peripheral serum samples were obtained from all patients at baseline and at week 4, week 12, week 24, week 36, and week 48 during treatment and were stored at -80 °C for biochemical, virological analyses. Paired liver biopsies were collected at baseline and week 48 and snap frozen in liquid nitrogen until cccDNA analyses.

**Standard laboratory assessments**

Serum HBV DNA was quantified by quantitative polymerase chain reaction (qPCR) using a Roche COBAS AmpliPrep/COBAS TaqMan system (Roche Diagnostics, Mannheim, Germany) with a lower detection limit of 20 IU/ml. HBV genotypes were determined by real-time PCR with a commercial kit (Shanghai ZJ Bio-Tech, China). HBsAg, HBeAg, anti-HBs, anti-HBe, and anti-HBc were quantitated by chemiluminescence microparticle immunoassays using the Architect i2000SR platform and Abbott Architect reagents (Abbott Laboratories, Chicago, IL) according to manufactures’ instruction. Laboratory assessments were performed at baseline, week 4, week 12, week 24, week 36, and week 48.

**Serum HBV RNA quantification**

Serum HBV RNA was quantitatively detected in the period of therapy, at baseline, week 4, week 12, week 24, week 36, and week 48. All samples were measured by previously reported methods [18]. Briefly, HBV RNA was extracted from 200 µl of serum. HBV DNA was digested with DNase I at 37 °C for 30 min. cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Then, quantitative PCR was performed with 2 × RealStar Power Probe Mixture (Gen Star, Beijing, China) with a linear range of $5 \times 10^3$ copies/ml to $5 \times 10^9$ copies/ml and a lower detection limit of $1 \times 10^3$ copies/ml.

**Intrahepatic HBV cccDNA quantification**

Intrahepatic HBV cccDNA was quantified by fluorescent probe quantitative PCR assays (SUPBIO Biotechnology, Guangzhou, China). The detailed information for intrahepatic cccDNA measurement is as
our previous reported study [19].

**Serum HBcrAg quantification**

HBcrAg was measured using a fully automated Lumipulse chemiluminescence enzyme immunoassay (CLEIA) analyzer (Fujirebio Inc., Tokyo, Japan), according to the manufacturer’s instructions. Since the general analytic measurement range of this assay is from 1000 U/ml (3 log10 U/ml) to 10,000,000 U/ml (7 log10 U/ml), serial dilutions of the serum samples are required when the serum qHBcrAg level is above the detection limit of the assay.

**Statistical analysis**

The characteristics of the study participants are presented as the proportions, means or medians. Comparisons between the patients with HBeAg seroconversion and without HBeAg seroconversion were performed using the chi-squared test for categorical data and the Mann-Whitney U-test for continuous data. Serum HBV RNA, HBcrAg, HBV DNA, HBsAg, and intrahepatic cccDNA were expressed in the logarithm. Differences in mean log-transformed values were calculated using Student’s t-test or one-way ANOVA where it is appropriate. The Pearson’s correlation analysis was applied to the log-transformed values of intrahepatic and serum markers. *P* values of < 0.05 were considered significant. Analyses were performed using the statistical software SPSS version 18 for Windows PC (SPSS, Chicago, IL, USA).

**Results**

**Patient characteristics at baseline**

Our study cohort consisted of 30 patients (18 males and 12 females). The mean age was 26.57 ± 4.17 years. After 48 weeks of peg-IFN treatment, 9 of the 30 patients achieved HBeAg seroconversion. For further comparison, the patients were categorized into two groups: SR group (patients achieving HBeAg seroconversion response, *n* = 9), NSR group (patients not achieving HBeAg seroconversion response, *n* = 21).

As shown in Table 1, the comparison of the baseline values between the SR and NSR groups are listed as follows: HBV RNA (7.72 ± 1.42 log10 copies/mL vs. 7.75 ± 1.09 log10 copies/mL, *P* = 0.959), cccDNA (median 30.0 copies/cell vs. median 34.4 copies/cell, *P* = 0.946), HBcrAg (5.24 ± 1.45 log10 KU/mL vs. 5.48 ± 1.16 log10 KU/mL, *P* = 0.636), HBV DNA (7.52 ± 1.05 log10 IU/mL vs. 7.59 ± 1.15 log10 IU/mL, *P* = 0.878), and HBsAg (3.59 ± 0.49 log10 IU/mL vs. 3.86 ± 0.69 log10 IU/mL, *P* = 0.310). From the above results, it could be seen that there were no significant differences existed in HBV RNA, cccDNA, HBcrAg, HBV DNA, and HBsAg at baseline between the SR and NSR groups. In addition, no significant differences in age, HBV genotype, or ALT (all *P* > 0.05) were observed between the two groups.
Table 1
Baseline clinical characteristics and HBV RNA, HBV cccDNA, HBV DNA, HBsAg, and HBeAg levels in the SR and NSR groups.

| Characteristic                  | All (N = 30) | SR group (N = 9) | NSR group (N = 21) | P value^a (SR vs. NSR) |
|--------------------------------|--------------|------------------|--------------------|------------------------|
| Age, yr (median)               | 26.57 ± 4.17 | 27.00 ± 3.08     | 26.38 ± 4.62       | 0.216                  |
| Gender (male/female)           | 18/12        | 8/1              | 10/11              | 0.03                   |
| HBV genotype (%)               |              |                  |                    | 0.614                  |
| B                              | 10 (33.3%)   | 3 (33.3%)        | 7 (33.3%)          |                        |
| C                              | 20 (66.7%)   | 6 (66.7%)        | 14 (66.7%)         |                        |
| ALT (U/L, median (range))      | 120 (66.8–174.0) | 131.0 (96.7–181.0) | 115.0 (59.0–174.0) | 0.380                  |
| HBV DNA (log_{10} IU/mL)       | 7.57 ± 1.10  | 7.52 ± 1.05      | 7.59 ± 1.15        | 0.878                  |
| HBsAg (log_{10} IU/mL)         | 3.78 ± 0.64  | 3.59 ± 0.49      | 3.86 ± 0.69        | 0.310                  |
| HBcrAg (log_{10} KU/mL)        | 5.41 ± 1.23  | 5.24 ± 1.45      | 5.48 ± 1.16        | 0.636                  |
| HBV RNA (log_{10} copies/mL)   | 7.73 ± 1.18  | 7.72 ± 1.42      | 7.75 ± 1.09        | 0.959                  |
| HBV cccDNA (copies/cell, median (range)) | 33.8 (15.4–76.4) | 30.0 (13.2–100.8) | 34.4 (13.7–66.7) | 0.946                  |

ALT, alanine aminotransferase; HBV, hepatitis B virus; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; cccDNA, covalently closed circular DNA.

Dynamic changes in HBV RNA, HBV DNA, and cccDNA levels during 48 weeks of peg-IFN treatment

Among all patients, all levels of serum HBV RNA, HBV DNA and intrahepatic cccDNA showed a rapid decline from baseline to week 48 (from 7.73 ± 1.18 log_{10} copies/mL to 4.66 ± 1.56 log_{10} copies/mL for HBV RNA; from 7.57 ± 1.10 log_{10} IU/mL to 4.22 ± 2.45 log_{10} IU/mL for HBV DNA; from median 33.8 copies/cell to 2.53 copies/cell for cccDNA). In patients with HBeAg seroconversion, the HBV RNA levels decreased more rapidly than those without HBeAg seroconversion. At week 48, serum HBV RNA levels were significantly lower in the SR group than the NSR group (3.56 ± 0.64 log_{10} copies/mL vs. 5.00 ± 1.60 log_{10} copies/mL, P = 0.002) (Fig. 1A). In the SR group, serum HBV RNA levels decreased more slowly than serum HBV DNA levels, whereas, both HBV RNA and HBV DNA levels decreased similarly in the NSR group (Fig. 1A). During the 48-week peg-IFN treatment period, the cccDNA levels decreased significantly...
from a median value of 30.2 copies/cell to 1.94 copies/cell in the SR group and from a median value of 34.4 copies/cell to 3.23 copies/cell in the NSR group. No difference in cccDNA decline was observed between the SR and NSR groups (Fig. 1B).

**Correlations of cccDNA with HBV RNA, HBcrAg, HBV DNA, and HBsAg before and after 48-week IFN treatment**

To further evaluate the performance of HBV RNA, HBcrAg, HBV DNA, HBsAg in reflecting cccDNA, the correlation values of each parameter were calculated. Among all patients, intrahepatic cccDNA was positively correlated with serum HBV RNA, HBcrAg, HBV DNA, and HBsAg at baseline. However, cccDNA correlated better with HBV RNA ($r = 0.781, P < 0.001$) than with HBcrAg ($r = 0.741, P < 0.001$), HBV DNA ($r = 0.664, P < 0.001$), and HBsAg ($r = 0.484, P = 0.005$) (Fig. 2A-2D). At 48 weeks after peg-IFN treatment, intrahepatic cccDNA was still significantly correlated with serum HBV RNA, HBV DNA and HBcrAg ($r = 0.728, P < 0.001$; $r = 0.655, P < 0.001$; $r = 0.721, P < 0.001$). Figure 3A, 3B, 3C, respectively), while no correlations were observed between cccDNA and HBsAg levels ($r = 0.361, P = 0.064$. Figure 3D).

After analyzing the different groups of CHB patients separately at week 48, we found that significant positive correlations of intrahepatic cccDNA with serum HBV RNA ($r = 0.709, P = 0.020$. Figure 4A) and HBcrAg ($r = 0.735, P = 0.014$. Figure 4B) rather than HBV DNA ($r = 0.335, P = 0.102$, Fig. 4C) or HBsAg ($r = 0.354, P = 0.349$, Fig. 4D) were observed in the SR group. Different from the SR group, cccDNA correlated strongly with HBV RNA ($r = 0.766, P < 0.001$. Figure 5A) and HBV DNA ($r = 0.820, P < 0.001$. Figure 5C), moderately with HBcrAg ($r = 0.558, P = 0.008$. Fig. 5B), but not with HBsAg (Fig. 5D) in the NSR group.

In addition, we also analyzed the correlation of HBV RNA with other serum markers among all patients. The results showed that serum HBV RNA levels strongly correlated with HBcrAg ($r = 0.793, P < 0.001$. Figure 6A) and HBV DNA($r = 0.594, P < 0.001$, Fig. 6B) at baseline, and the correlations of HBV RNA with HBcrAg ($r = 0.673, P < 0.001$. Figure 6D) and HBV DNA ($r = 0.907, P < 0.001$. Figure 6E) were still positive after 48 weeks peg-IFN treatment. However, HBV RNA only moderately correlated with HBsAg both at baseline and after 48 weeks of treatment ($r = 0.457, P = 0.010$ at baseline; $r = 0.314, P = 0.090$ at week 48; Fig. 6C, 6F).

**Discussion**

In previous studies, both serum HBV RNA and HBcrAg were reported to be potential surrogate biomarkers of cccDNA in predicting the outcomes of peg-IFN or NAs treatments [4, 20, 21]. The correlation of cccDNA with HBV RNA has also been studied before and after nucleos(t)ide analog treatment [5, 21, 22]. However, it is still unclear whether serum HBV RNA or HBcrAg is better as the surrogate marker of intrahepatic cccDNA.

In the present study, we analyzed the correlations of intrahepatic cccDNA with serum HBV RNA, HBcrAg, HBV DNA, and HBsAg before and after peg-IFN treatment in HBeAg-positive CHB patients, to determine
which factor has the strongest correlation with intrahepatic cccDNA and could be a better surrogate marker of intrahepatic cccDNA. Furthermore, the kinetics of HBV RNA and intrahepatic cccDNA among all patients were also analyzed.

The results showed that the baseline levels of serum HBV RNA or HBcrAg were similar in patients with and without HBeAg seroconversion (all \( P > 0.05 \)), which indicated that HBV RNA and HBcrAg at baseline may be not associated with HBeAg-seroconversion in HBeAg-positive patients treated with peg-IFN. During peg-IFN treatment, HBV RNA declined more rapidly in patients with HBeAg seroconversion than those without HBeAg seroconversion. After 48-week peg-IFN treatment, a smaller decrease of serum HBV RNA than serum HBV DNA was observed in the SR group, whereas HBV RNA and HBV DNA decreased parallel in the NSR group. Previous studies have reported that cccDNA reduced rapidly in the first year of antiviral treatment \([23, 24]\). Our study also showed that peg-IFN could profoundly reduce the load of cccDNA after 1 year of treatment. In addition, we found that the reduction of cccDNA in the SR group was greater than that in the NSR group. The possible reason was that Peg-IFN can suppress the activity and transcription of cccDNA, which may result in increased clearance rates of HBV RNA, HBcrAg, and cccDNA \([25]\).

Our study also showed that intrahepatic cccDNA levels correlated best with serum HBV RNA \((r = 0.781)\) than HBcrAg \((r = 0.741)\), HBV DNA \((r = 0.664)\), and HBsAg \((r = 0.484)\) at baseline, and the correlation between cccDNA and HBV RNA was still strong \((r = 0.728, P < 0.001)\) after 48 weeks of peg-IFN treatment. While HBsAg did not correlate with cccDNA \((r = 0.361, P = 0.064)\). These data indicated that serum HBV RNA might be a superior serological marker for the persistence of active cccDNA molecules during peg-IFN treatment. Our results were different from Gao's study, which identified that serum HBV RNA only had a weak correlation with cccDNA in a 96-week NAs treatment \([5]\). The difference could be attributed to the different antiviral mechanisms of NAs and peg-IFN. Interestingly, an animal experiment in HBV-infected mice found that there was no correlation between serum HBV RNA and intrahepatic cccDNA after 6 weeks of peg-IFN therapy \([12]\). The discrepancies might because the mouse model could not fully reflect the real conditions in clinical patients.

It has been reported that serum HBcrAg was better than HBV RNA in reflecting intrahepatic cccDNA levels in treatment naive patients \([17]\). However, our study showed that although HBcrAg and HBV RNA have a similar correlation with cccDNA at baseline, the correlation of cccDNA with HBcrAg became weaker than with HBV RNA after 48 weeks of peg-IFN therapy.

We further compared the correlations of cccDNA with HBV RNA, HBV DNA, and HBsAg between patients with and without HBeAg seroconversion after 48 weeks of peg-IFN treatment. The results showed that HBV RNA and HBcrAg were positively correlated with cccDNA both in the SR and NSR groups at week 48 (HBV RNA: \(r = 0.709, P = 0.002\) in the SR group; \(r = 0.766, P < 0.001\) in the NSR group. HBcrAg: \(r = 0.735, P = 0.014\) in the SR group; \(r = 0.662, P = 0.008\) in the NSR group). HBV DNA was strongly correlated with cccDNA in the NSR group \((r = 0.818, P < 0.001)\), but no positive correlation was observed in the SR group \((r = 0.335, P = 0.102)\). The correlation between HBsAg and cccDNA was not found in either the SR group \((r = 0.335, P = 0.102)\).
= 0.354, \( P = 0.349 \) or the NSR group (\( r = 0.374, \ P = 0.094 \)). The results indicated that HBV RNA and HBcrAg were better markers than HBV DNA and HBsAg in reflecting cccDNA no matter the results of antiviral treatment.

Although previous studies have shown that serum HBsAg quantification may reflect the level of intrahepatic cccDNA both in HBeAg-positive and HBeAg-negative CHB patients [26, 27], our study showed that cccDNA weakly correlated with HBsAg before treatment and did not correlate with HBsAg after 48 weeks of peg-IFN therapy. This pattern possibly because HBsAg could be produced not only from cccDNA in infected hepatocytes but also from viral sequences integrated into the host genome [28]. These data indicated that serum HBsAg level could not accurately reflect the level of intrahepatic cccDNA in the cohort of our study.

Our results also showed that serum HBV RNA correlated well with HBcrAg at baseline and at week 48 with peg-IFN treatment, most likely because HBcrAg was translated from preC mRNA which is also transcribed from cccDNA just like HBV RNA [17]. In contrast, the HBV RNA level decreased significantly, whereas the HBsAg level remained stable after 48 weeks of treatment. As a result, no significant correlation between HBV RNA and HBsAg (\( r = 0.314, \ P = 0.090 \)) was observed after 48 weeks of IFN treatment.

Due to the difficulty of acquiring liver samples both at baseline and after 48 weeks of peg-IFN therapy, the sample size in our study was small and the patients enrolled were mainly those infected with HBV genotype B or C. The selection bias might have affected the internal validity of this study. Future studies are required to recruit more patients and other HBV genotypes.

**Conclusions**

Both Serum HBV RNA and HBcrAg correlated significantly with intrahepatic cccDNA before and after 48-week peg-IFN treatment in HBeAg-positive patients. However, serum HBV RNA correlated with cccDNA more strongly than HBcrAg, HBV DNA, and HBsAg, irrespective of treatment results. HBV RNA also correlated well with HBcrAg before and after peg-IFN therapy. The study indicated that serum HBV RNA may be a better surrogate non-invasive marker in reflecting the intrahepatic cccDNA profile during peg-IFN treatment.

**Abbreviations**

HBV: Hepatitis B virus; HBV RNA: hepatitis B virus RNA; HBcrAg: HBV core-related antigen; ALT: alanine aminotransferase; cccDNA: Covalently closed circular DNA; CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; Peg-IFN; Pegylated interferon; NAs: nucleotide/nucleoside analogs.

**Declarations**

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee of the First Hospital of Jilin University and all participating institutions. All patients gave written informed consent for their participation in the study.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data analyzed in the current study is available from the corresponding author on reasonable request.

**Conflicts of Interest**

The authors declare no conflicts of interest.

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**Author’s contributions**

Xiaomei Wang performed the research. Xiumei Chi, Xiuzhu Gao, Lei Yu, Longgen Liu, Mingxiang Zhang, and Youwen Tan acquired the data. Ruihong Wu and Hongqin Xu analyzed the data. Junqi Niu and Qinglong Jin edited, reviewed, and approved the final manuscript.

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