QUANTITATION OF INTRAHEPATIC cccDNA USING DROPLET DIGITAL PCR IN PATIENTS WITH HBV-RELATED HCC AND OBI-RELATED HCC

Kanyakorn SINGSUNG[1], Pisit TANGKIJVANICH[1] and Natthaya CHUAYPEN[1*]

1 Center of Excellence in Hepatitis and Liver Cancer, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; kanyakornsingsung@gmail.com (K. S.); pisittkvn@yahoo.com (P. T.); natthaya.c@chula.ac.th (N. C.) (Corresponding author)

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ABSTRACT

Chronic infection with Hepatitis B virus (HBV) is a common health issue worldwide and cannot be completely cured. Patients are required to continuously take medication due to the high mutational rate of HBV, with the persistence of closed circular DNA (cccDNA) in the nucleus of hepatocyte. The cccDNA was difficult to eliminate and undetectable in the serum, poses a significant challenge. The standard approach to cccDNA examination requires the liver tissues and highly sensitive methods. Therefore, droplet digital PCR (dd-PCR) was performed to quantify the intrahepatic cccDNA level in liver tissues from 27 patients with HBV-related hepatocellular carcinoma (HBV-related HCC) and 8 patients with occult HBV-related HCC (OBI-related HCC). To access the performance of intrahepatic cccDNA detection from different DNA extraction methods, digested with Plasmid-safe ATP-dependent of DNase (PSAD) assay and without digestion by PSAD techniques were compared. The results showed that there was no statistically significant difference in intrahepatic cccDNA detection between the PSAD digestion after DNA extraction and without PSAD digestion (without PSAD; 6.53 copies/ug vs. PSAD; 15.50 copies/ug, P = 0.249). Then, we selected the PSAD digestion method in detecting cccDNA in liver tissues from patients. The result demonstrated that the difference of intrahepatic cccDNA level between patients with OBI-related HCC and HBV-related HCC was not statistically significant (8.5 copies/ug vs. 5.2 copies/ug, P=0.232). Digestion of DNA with PSAD before measuring cccDNA expression may be a suitable method for cccDNA quantitation. However, this study still requires more sample size to validate by this technique in further study.

Keywords: Hepatitis B Virus (HBV), Covalently Closed Circular DNA (cccDNA), Hepatocellular Carcinoma (HCC), Chronic Hepatitis B Infection (CHB), Occult Hepatitis B Virus Infection (OBI)

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INTRODUCTION

Hepatitis B virus (HBV) is a major public health problem worldwide. It has been found that up to 300 million people are infected with hepatitis B worldwide and 1.3 million died from complications such as cirrhosis and hepatocellular carcinoma (HCC). Chronic hepatitis B infection is often asymptomatic, and only 10% of cases are diagnosed and treated promptly (Mohd-Ismail et al., 2019). Hepatitis B infection can be detected by the antigen and antibody in the serum (HBsAg, HBeAg, HBcAg and Anti-HBc), indicating that the patient has been infected with hepatitis B. Although chronic hepatitis B infection is currently being treated with antiviral drugs, it is still not possible to treat chronic hepatitis B infection. However, it still cannot be cured. It is because of the persistence of covalently closed circular DNA (cccDNA) within the hepatocyte, which is the template of other hepatitis B proteins. There is no standardized method for measuring cccDNA. Therefore, this study focuses on the determination of cccDNA in the liver of patients using Droplet Digital PCR (ddPCR). ddPCR is a new technique used to quantify nucleic acids such as DNA, cDNA, and RNA by using the water-in-oil emulsion principle by dividing the reaction into droplets(Fontanelli et al., 2015).
In a previous study comparing the efficacy of cccDNA measurement between qPCR and ddPCR, it was found that ddPCR was able to detect less cccDNA than qPCR (10-100 copies/ul) (Lebossé et al., 2020). However, the infectious HBV tissue does not only contain cccDNA but also includes rcDNA. The structure of rcDNA closely resembles that of cccDNA, creating a significant challenge in distinguishing between the two (Zhong et al., 2014). To enhance specificity, a method involving the use of specific primers and probes tailored for the selective amplification of cccDNA while excluding rcDNA has been developed. This is achieved by designing primers that span the nick in rcDNA and employing a probe that hybridizes to the gap region. Another strategy to improve the accuracy of cccDNA detection involves reducing the copy number of rcDNA. One approach for achieving this is the pretreatment of HBV-DNA with Plasmid-Safe ATP-Dependent DNase (PSAD), which degrades DNA molecules with free ends. This pre-treatment can significantly enhance discriminatory power by up to 1000-fold (Huang et al., 2018). So, this study focuses on comparing methods for extracting DNA from liver tissues in liver cancer patients that promotes the efficiency of DNA analysis by the ddPCR technique.

LITERATURE REVIEWS

Hepatitis B virus

The hepatitis B virus is a virus in the Hepadnaviridae family that can infect both mammals and birds. The hepatitis B virus has a characteristic partially double-stranded DNA known as relaxed circular DNA (rcDNA) with a genome size of 3,200 base pairs enclosed in a capsid (Yan et al., 2012). The HBV genome contains a total of four open reading frames (ORFs), all of which partially overlap to function as start points for decoding important genes crucial for the replication and propagation of the four types of hepatitis B virus. The hepatitis B virus encompasses four significant open reading frames (ORFs). The viral polymerase is pivotal in the virus's replication and packaging (Bartenschlager & Schaller, 1992; Hirsch et al., 1991; Wei & Ploss, 2021). Hepatitis B Surface (HBs) antigens, available in three sizes, collectively constitute the viral envelope that guides the virus into liver cells and disseminates it into the bloodstream (Selzer & Zlotnick, 2015). Hepatitis B core protein (HBc) acts as the protective capsid for the viral genome, facilitating replication and governing the expression of various proteins, including HBV e antigen and pre-core protein (Inoue & Tanaka, 2019; Selzer & Zlotnick, 2015; Zlotnick et al., 2015). Meanwhile, the X Protein (HBx) is responsible for genome decryption and is essential for the virus's various functions (Belloni et al., 2009; Benhenda et al., 2009; Bouchard Michael & Schneider Robert, 2004; Slagle & Bouchard, 2018; Wei & Ploss, 2021)

Hepatitis B virus life cycle

The hepatitis B virus can enter hepatocytes through a specific receptor expressed exclusively on the surface of liver cells. The envelope of the hepatitis B virus (HBV virion) binds to the sodium taurocholate cotransporting polypeptide (NTCP receptor) on the liver cell surface and enters the hepatocyte through an endocytosis pathway. Subsequently, the outer envelope of the Hepatitis B virus, consisting of three types of Hepatitis B surface antigen (HBsAg), is released (de-envelopment), leaving only the capsid containing hepatitis B core antigen (HBcAg) that encapsulates rcDNA. The capsid then delivers the rcDNA of the hepatitis B virus into the nucleus of the liver cell, where the rcDNA is converted into cccDNA, serving as a template for decoding into important mRNA and proteins, including HBV pre-genomic RNA (pgRNA), viral polymerase, Hepatitis B surface antigen (HBsAg), Hepatitis e antigen (HBeAg), Hepatitis c antigen (HBcAg), and Hepatitis B virus x protein (HBx). The pgRNA can bind with viral polymerase and HBeAg (pgRNA encapsidation), leading to a reverse transcription process that converts pgRNA into rcDNA. The capsid is then sent back to the nucleus once more, delivering rcDNA for further replication, or it is enveloped by HBsAg and released outside the cell (Marchetti & Guo, 2020; Marchetti et al., 2022; Verrier et al., 2022).

cccDNA generation

Covalently closed circular DNA (cccDNA) is a crucial genetic structure for increasing the quantity of hepatitis B virus. This is because the fundamental DNA structure of hepatitis B virus is not like the typical DNA; it is partially double-stranded DNA known as relaxed circular DNA (rcDNA), making it unable to serve as a template for producing RNA and proteins. Therefore, there is a need to repair rcDNA to form complete DNA. The process of repairing rcDNA into cccDNA can occur due to the specific structures of rcDNA, including: 1) Positive-sense DNA on the inside with an incomplete structure and negative-sense DNA on the outside, which has an open configuration. This prevents the two DNA strands from fully binding like normal DNA. 2) RNA primers remaining from the reverse transcription process connected to the 5' end of the positive sense rcDNA strand. 3) Viral polymerase attached to the 5' end of the negative sense rcDNA strand. 4) Partially unwound DNA sections protruding from the negative sense rcDNA, resulting from a high degree of overlapping base sequences. 5) An open conformation caused by the assembly of all four components mentioned above in rcDNA, resulting in an unusual DNA structure distinct from regular DNA. When introduced into the human body with a well-organized and systematic genetic management system, the peculiar structure of rcDNA is detected, prompting proteins related to DNA damage detection. It is believed that rcDNA is the damaged DNA, signaling enzymes in the repair process to mend rcDNA into a fully closed circular DNA structure (covalently closed circular DNA; cccDNA) like normal DNA (Marchetti et al., 2022).

Droplet digital polymerase chain reaction (ddPCR)

Droplet digital PCR (dd-PCR) is a technique used to quantify nucleic acids, such as DNA, cDNA, and RNA. It employs the principle of water-in-oil emulsion by partitioning the reaction into numerous droplets, enabling multiple reactions to occur simultaneously in a single sample. This technology calculates the positive droplets (those containing the target nucleic acid) and negative droplets (those without the target nucleic acid) according to Poisson's law. It determines the quantity of the desired nucleic acid without the need for a standard curve or reference samples, providing both qualitative and quantitative data in a single analysis (Fontanelli et al., 2015). This method differs from traditional PCR, which can only conduct one reaction at a time per sample and requires a standard curve for analysis. In a study comparing the efficiency of detecting cccDNA in the liver tissues of chronic hepatitis B virus-infected patients using both dd-PCR and qPCR techniques on 27 samples, it was found that dd-PCR could detect cccDNA in all 27 samples, whereas qPCR could only detect it in 11 out of the 27 samples. Additionally, dd-PCR was able to detect cccDNA in samples that were negative

or undetectable using the qPCR technique. The study also revealed that samples with cccDNA expression in both techniques had a significantly higher cccDNA concentration compared to samples where cccDNA expression was not detected, as measured by qPCR (40.0 [5.7-331.6] vs. 7.5 [2.3-15.5] copies/105 cells). When comparing the ability to measure cccDNA expression between dd-PCR and qPCR, both methods showed only moderate correlation (R2=0.6037) (Caviglia et al., 2018).

RESEARCH METHODOLOGY

Patients

Thirty-five patients were diagnosed with HCC at King Chulalongkorn Memorial Hospital. The patients were separated into 2 groups. The first group was hepatitis B virus infection related hepatocellular carcinoma (HBV-related HCC, n=27). The second group was occult hepatitis B virus infection related hepatocellular carcinoma (OBI-related HCC, n=8). Non-adjacent tumor tissues were collected from patients between surgery at King Chulalongkorn Memorial hospital. The expression of the serological markers was obtained. This study was approved by Institutional Review Board from the Ethics Committee of the faculty of Medicine, Chulalongkorn University (IRB no.0638/66).

DNA Extraction

DNA extraction was performed by using phenol: chloroform method following the previous studied (Hayashi et al., 2021). In details, 50 mg of tissue was lysed in 5 ml of lysis buffer and 0.025 µl of Proteinase K at 37˚C overnight. Following this, 5 ml of tris-saturated phenol was added and rotated at room temperature for 30 min, then centrifuged at 2,500 rpm, 4 ˚C for 10 min. Subsequently, 2 ml of tris-saturated phenol and 1 ml of chloroform: isoamyl alcohol (49:1) were added, followed by centrifugation for 30 min. The supernatant was transferred to a new tube, and 0.1 volume of 3M NaOAC and 2.5 volumes of absolute ethanol were added to the supernatant. This mixture was incubated at -80˚C for 1 hour and then centrifuged at 3,000 rpm for 15 minutes. The resulting precipitate was washed with 1 ml of 70% Ethanol, centrifuged at 3,000 rpm for 15 minutes, and the DNA was subsequently diluted with 300 µl of TE buffer and store at -80°C.

PSAD digestion

The DNA was treated with 6 µl of PureLink RNase A and digested with 5 µl of *HindIII* per 1 µg of DNA, incubated at 37˚C for 30 minutes**.** Then it was digested with 1 µl of PSAD at 37˚C for 30 minutes and inactivated at 70˚C for 1 hour and store at -80°C

cccDNA detection with Droplet Digital PCR (ddPCR)

The master mix was prepared following table 1. Then dropped 20 µl of the mixture in the middle well of the cartridges droplet generator and added 70 µl of droplet generation oil for probes at the lower well. Subsequently, droplets were generated by QX200^{TM} Droplet generator. Transferred the droplets to 96 well plate PCR and sealed plate with PX1^{TM} PCR Plate Sealer at 180˚C. After that, cccDNA was amplified using a T100^{TM} Thermal Cycler following the condition in tables 2. Following amplification, the droplets were analyzed using a QX200 Droplet Digital PCR reader. Finally, the data obtained were analyzed using QuantaSoft™. (Forward primer: 5’-ACGGGGCGCACCTCTCTTTACGCGG-3’, Reverse: 5’-CAAGGCACAGCTTGGAGGCTTGAAC-3’, Probe; 5’-FAM-AACGACCGACCTTGA GGCAT-MGB-3)

Statistical Analysis

The correlation between the serological markers and intrahepatic cccDNA level were analyzed by Spearman’s rank correlation. Other statistical analyses were performed by using Wilcoxon signed rank test.

Table 1 Show the amount of substance used to prepare master mixed for ddPCR

Reagent	Volume/ reaction
2x ddPCR supermix for Probe (No dUTP)	10 µl
10 µM Forward primer	1.8 µl
10 µM Reverse primer	1.8 µl
10 µM Probe	0.5 µl
5 U restriction (HindIII)	1 µl
Deionized water	3.9 µl
DNA template	1 µl
Total	20 µl

Table 2 Show the condition for amplified cccDNA

Conditions	Temperature	Time	
Enzyme activation	95˚C	10 min	
Denaturation	94˚C	30 sec	40 Cycles
Annealing/Extension	61.2˚C	1 min	
Enzyme deactivation	98˚C	10 min	
Hold	4˚C	α	

RESEARCH RESULTS

Baseline characteristics of donors

The patients in HBV-related HCC showed that 27 of 27 (100%) were HBsAg positive, serum HBsAg level was 1146.19 (0.4-4,915) lU/mL, Anti-HBs level was 76.62 (0-272), Anti-HBc was 0.01 (0-0.01), HBV viral load 34,235.73(<10-337,391) lU/ml, intrahepatic cccDNA level non-PSAD was 18.55 (0-248) copies/ug and intrahepatic cccDNA level PSAD 5.95 (0-16.6) copies/ug. In OBI-related HCC patients, showed that 0 of 8 (0%) were HBsAg positive, serum HBsAg level was 0.39 (0.25-0.87) lU/mL, Anti-HBs level was 44.66 (0.01-269), Anti-HBc was 0.09 (0-0.81), HBV viral load 10 (0-10) lU/ml, intrahepatic cccDNA level non-PSAD was 5.2 (0-22.8) copies/ug and intrahepatic cccDNA level PSAD 8.5 (2.2-21.4) copies/ug. The HBcrAg levels in each group was 2.84 (0-5.6) LogU/mL (9/35) (Table 3.).

Table 3 Demographic and virologic features of the donors

Variables	HBV related HCC (n = 27)	OBI related HCC (n=8)
Age, years	64 (47-87)	72(66-81)
Gender, M/F	18/9	7/1
HBsAg positive, n (%)	27 (100%)	0 (100%)
Serum HBsAg level (lU/mL)	1146.19 (0.4-4,915)	0.39 (0.25-0.87)
Anti-HBs	76.62 (0-272)	44.66 (0.01-269)
Anti-HBc	0.01 (0-0.01)	0.09 (0.01-0.81)
HBcrAg (LogU/mL)	2.84 (0-5.6)	2.84 (0-5.6)
HBV viral load (lU/mL)	34235.73 (<10-337391)	10 (0-10)
Intrahepatic cccDNA level non-PSAD (copies/ug)	18.55 (0-248)	5.2 (0-22.8)
Intrahepatic cccDNA level PSAD (copies/ug)	5.95 (0-16.6)	8.5 (2.2-21.4)

*P < 0.05

Sensitivity, accuracy, and specificity of the ddPCR-based HBV cccDNA assay

The performance of ddPCR method for cccDNA quantitation was assessed using the of plasmid pGEM-T Easy vector containing cccDNA sequence. Serial 10-fold dilutions, ranging from 10^9-10 copies/ul were used to test accuracy of ddPCR method (Fig.1A). To assess the specificity of the cccDNA assay, plasmid pGEM-T Easy vector containing cccDNA sequence was digested with PSAD. As shown (Fig. 1B), amplification of plasmid without PSAD treatment showed no difference in comparison to plasmid digested with PSAD, confirming the specificity of PSAD digestion for linear DNA molecules.

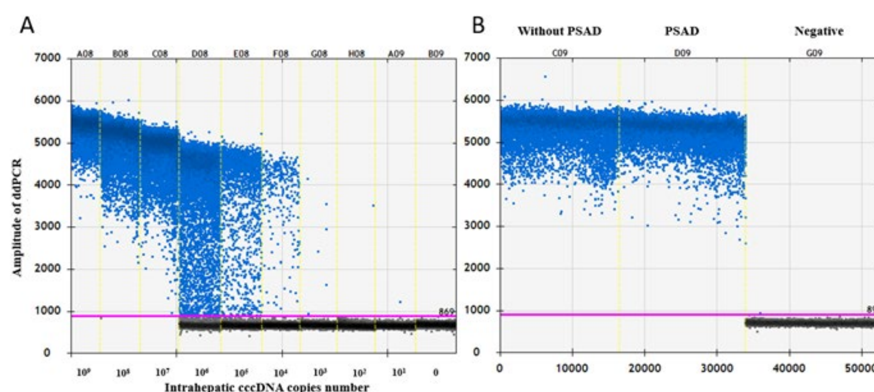

Figure 1 Sensitivity and accuracy of the ddPCR-based HBV cccDNA assay. (A) Amplification of a 10-fold dilution of plasmid pGEM-T Easy vector containing cccDNA sequence by ddPCR. (B) Amplification by ddPCR of plasmid pGEM-T Easy vector containing cccDNA sequence with PSAD and without PSAD.

Comparison of intrahepatic cccDNA quantitation in liver tissue between treated with PSAD and without PSAD by ddPCR

The study of cccDNA expression in the tissues of patients with HBV-related HCC and OBI-related HCC group. The figure 2A shown example of droplet cccDNA between treat with PSAD and without PSAD in same patients. The comparison of intrahepatic cccDNA levels between the tested with PSAD group (15.50 copies/ug) and the without PSAD group (6.53 copies/ug), it was observed that there was no significant difference in cccDNA quantities between both groups (P = 0.2486) (Fig. 2B.).

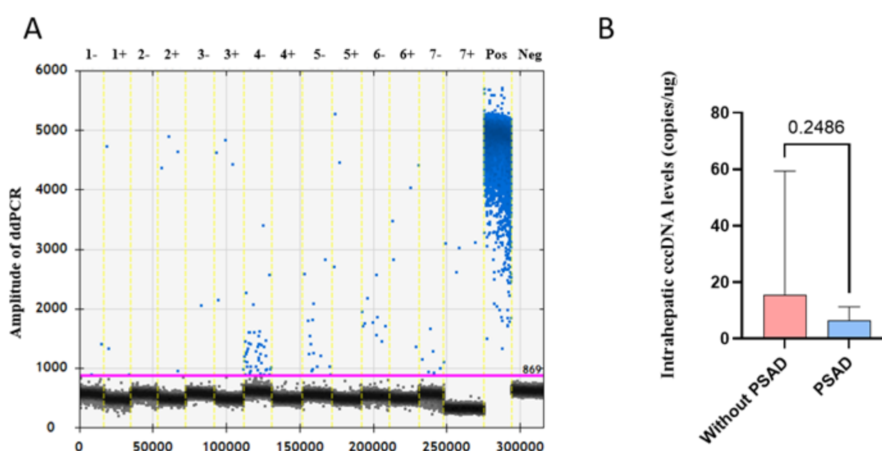

Figure 2 Intrahepatic cccDNA levels in HBV and OBI related HCC liver biopsy. (A)Droplet of cccDNA between treat with PSAD and without PSAD. (B) The comparison of intrahepatic cccDNA levels between the tested with PSAD group and the without PSAD group (number-= without PSAD, number + = PSAD)

Comparison of intrahepatic cccDNA quantitation in liver tissue between HBV-related HCC and OBI-related HCC

Intrahepatic cccDNA was detected in 35 liver biopsies. HBV-related HCC 27 patients, the average cccDNA expression was 5.2 copies/ug. Conversely, patients with OBI-related HCC exhibited an average cccDNA expression of 8.5 copies/ug. This comparison highlights that the group with OBI related HCC had higher cccDNA expression levels in their tissues compared to the group with HBV-related HCC, but not significantly (P = 0.2317) (Fig. 3.).

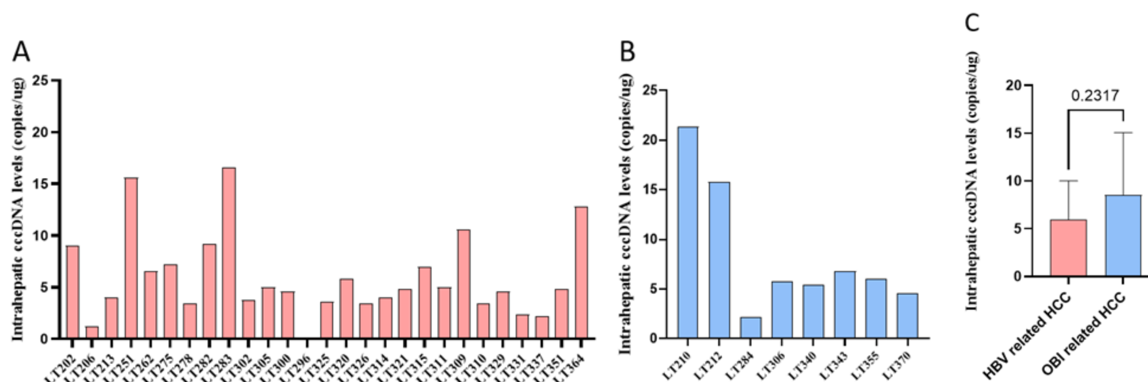

Figure 3 Intrahepatic cccDNA levels in HBV-related HCC and OBI-related HCC group. (A) Intrahepatic cccDNA levels in HBV-related HCC group. (B) Intrahepatic cccDNA levels in OBI-related HCC group. (C) Comparison of intrahepatic cccDNA levels between HBV-related HCC group and OBI-related HCC group when treated with PSAD.

Relationship between intrahepatic cccDNA level and serological markers

From the study of the relationship between the expression of cccDNA in liver tissue tested with PSAD and the expression of serological markers in the blood of patients in the HBV-related HCC and OBI-related HCC group, it was found that the levels of cccDNA expression were not correlated with HBV viral load and serum HBsAg levels in both group at -0.104 and 0.268, respectively (Fig. 4A, 4B). Accordingly, the levels of cccDNA expression were not correlated with HBV viral load in HBV-related HCC and OBI-related HCC group at 0.036 and 0.327, respectively (Figure 4C, 4D). The levels of cccDNA expression in HBV-related HCC and OBI-related HCC group no had relationship with HBsAg at 0.245 and 0.595, respectively (Figure 4E, 4F). It was also found that the levels of cccDNA expression were not correlated with HBcrAg in 9 samples from both group, with a correlation of 0.637, as shown in Figure 4G.

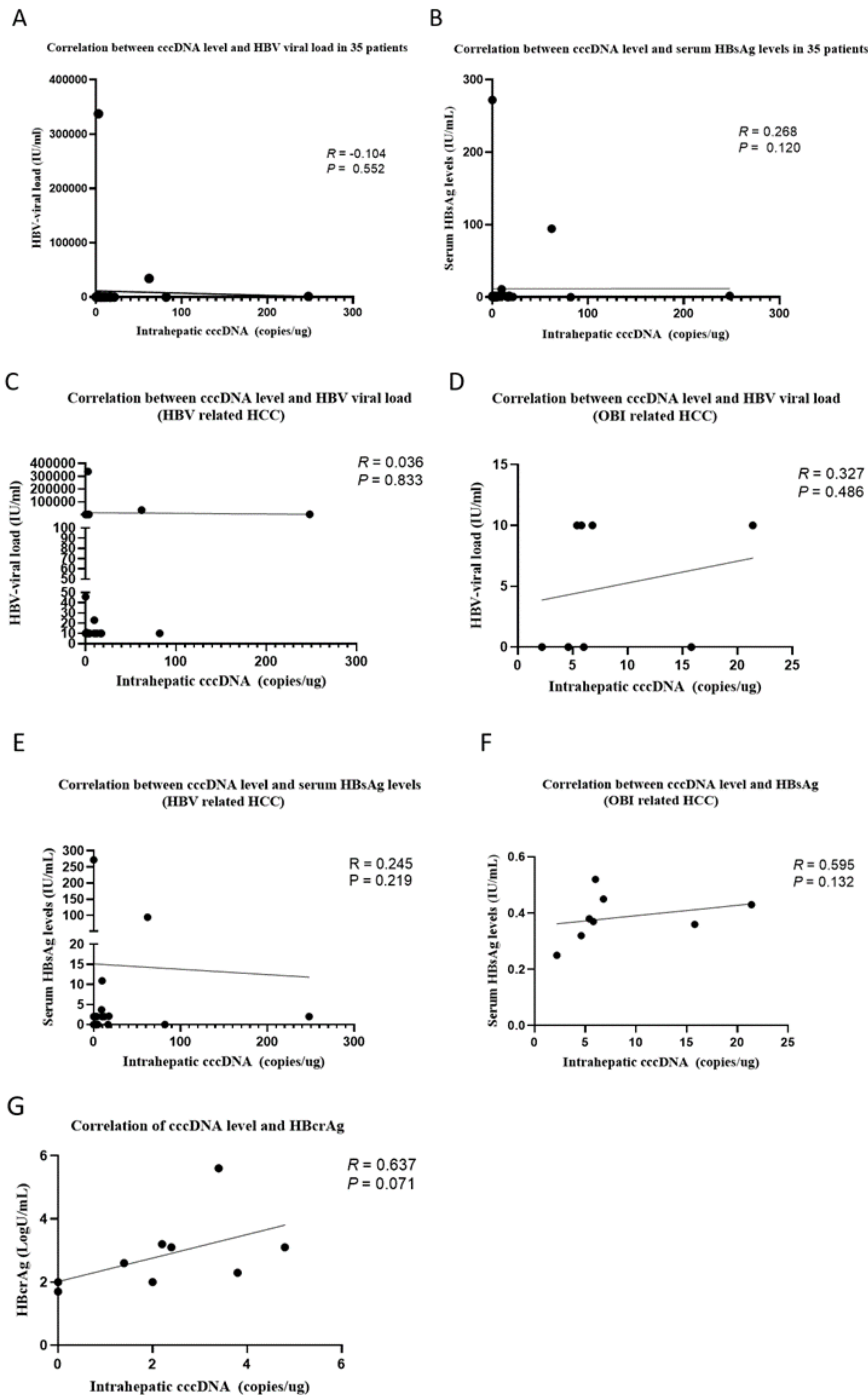

Figure 4 Correlation between the serological markers and intrahepatic cccDNA level. (A) Correlation between cccDNA level in HBV-related HCC and OBI-related HCC group with HBV viral load. (B) Correlation between cccDNA level in HBV-related HCC and OBI-related HCC group with serum HBsAg levels. (C) Correlation between cccDNA level and HBV viral load in HBV-related HCC group. (D) Correlation between cccDNA level and HBV viral load in OBI-related HCC group (E) Correlation between cccDNA level and serum HBsAg levels in HBV-related HCC group. (F) Correlation between cccDNA level and serum HBsAg levels in OBI-related HCC group. (G) Correlation between cccDNA level and HBcrAg

DISCUSSION & CONCLUSION

In our research, we formulated a ddPCR method for measuring intrahepatic cccDNA that offers great sensitivity and specificity. From the comparative study of DNA extraction methods from the tissues of patients in the CHB group and the OBI group, they found a higher expression of cccDNA in CHB compared to the OBI groups. Due to CHB had more active viral replication inside the liver cells than patients with OBI (Caviglia et al., 2018). However, our study showed no significant difference in intrahepatic cccDNA level between patients with OBI-HCC and HBV-HCC. In term of DNA extraction assays, using PSAD treated DNA is more suitable than without PSAD treated. There was observed that the expression of DNA that did not undergo PSAD was higher than that of DNA which did undergo PSAD, because it usually includes additional DNA types that can bind with cccDNA primers, resulting in non-specific DNA detection. In contrast, DNA that treated with PSAD degraded other DNA strands and leave only target cccDNA (Mu et al., 2015). Furthermore, it was found that specific enzyme cleavage with HindIII before PSAD testing of DNA could enhance the efficiency of degrading non-cccDNA (Caviglia et al., 2018). Subsequently, a study was conducted to analyze the correlation between the levels of intrahepatic cccDNA from liver tissues and clinical blood parameters. It was found that the cccDNA quantity in the OBI group correlated moderately with HBsAg in the blood and correlated with HBcrAg in all 9 cases. This corresponds with the previous research by (Hayashi et al., 2021), which suggests that HBcrAg could potentially represent the cccDNA quantity in liver cells in the future. However, it was found that the cccDNA quantity in both groups had relatively low correlation with HBV viral load. This could be due to the small sample size, and further studies might be needed with a larger population for clearer results.

REFERENCES

Bartenschlager, R., & Schaller, H. (1992). Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. *Embo j, 11*(9), 3413-3420. https://doi.org/10.1002/j.1460-2075.1992.tb05420.x .

Belloni, L., Pollicino, T., De Nicola, F., Guerrieri, F., Raffa, G., Fanciulli, M., Raimondo, G., & Levrero, M. (2009). Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. *Proceedings of the National Academy of Sciences, 106*(47), 19975-19979. https://doi.org/10.1073/pnas.0908365106.

Benhenda, S., Cougot, D., Buendia, M.-A., & Neuveut, C. (2009). Chapter 4 Hepatitis B Virus X Protein: Molecular Functions and Its Role in Virus Life Cycle and Pathogenesis. In *Advances in Cancer Research* (Vol. 103, pp. 75-109). Academic Press. https://doi.org/10.1016/S0065-230X(09)03004-8.

Bouchard Michael, J., & Schneider Robert, J. (2004). The Enigmatic X Gene of Hepatitis B Virus. *Journal of Virology, 78*(23), 12725-12734. https://doi.org/10.1128/JVI.78.23.12725-12734.2004.

Caviglia, G. P., Abate, M. L., Tandoi, F., Ciancio, A., Amoroso, A., Salizzoni, M., Saracco, G. M., Rizzetto, M., Romagnoli, R., & Smedile, A. (2018). Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: A new tool to detect occult infection. *J Hepatol, 69*(2), 301-307. https://doi.org/10.1016/j.jhep.2018.03.021.

Fontanelli, G., Baratè, C., Ciabatti, E., Guerrini, F., Grassi, S., Del Re, M., Morganti, R., Petrini, I., Arici, R., Barsotti, S., Metelli, M. R., Danesi, R., & Galimberti, S. (2015). Real-Time PCR and Droplet Digital PCR: two techniques for detection of the JAK2(V617F) mutation in Philadelphia-negative chronic myeloproliferative neoplasms. *Int J Lab Hematol, 37*(6), 766-773. https://doi.org/10.1111/ijlh.12404.

Hayashi, S., Isogawa, M., Kawashima, K., Ito, K., Chuaypen, N., Morine, Y., Shimada, M., Higashi-Kuwata, N., Tangkijvanich, P., Mitsuya, H., & Tanaka, Y. (2021). *Highly*

Accurate, Specific and Sensitive Quantitation By Droplet Digital PCR Reveals The Stability of Intrahepatic Hepatitis B Virus cccDNA. https://doi.org/10.21203/rs.3.rs-583058/v1.

Hirsch, R. C., Loeb, D. D., Pollack, J. R., & Ganem, D. (1991). cis-acting sequences required for encapsidation of duck hepatitis B virus pregenomic RNA. *J Virol, 65*(6), 3309-3316. https://doi.org/10.1128/jvi.65.6.3309-3316.1991.

Huang, J.-T., Yang, Y., Hu, Y.-M., Liu, X.-H., Liao, M.-Y., Morgan, R., Yuan, E.-F., Li, X., & Liu, S.-M. (2018). A Highly Sensitive and Robust Method for Hepatitis B Virus Covalently Closed Circular DNA Detection in Single Cells and Serum. *The Journal of Molecular Diagnostics, 20*(3), 334-343. https://doi.org/https://doi.org/10.1016/j.jmoldx.2018.01.010.

Inoue, T., & Tanaka, Y. (2019). The Role of Hepatitis B Core-Related Antigen. *Genes, 10*(5).

Lebossé, F., Inchauspé, A., Locatelli, M., Miaglia, C., Diederichs, A., Fresquet, J., Chapus, F., Hamed, K., Testoni, B., & Zoulim, F. (2020). Quantification and epigenetic evaluation of the residual pool of hepatitis B covalently closed circular DNA in long-term nucleoside analogue-treated patients. *Scientific Reports, 10*(1), 21097. https://doi.org/10.1038/s41598-020-78001-1.

Marchetti, A. L., & Guo, H. (2020). New Insights on Molecular Mechanism of Hepatitis B Virus Covalently Closed Circular DNA Formation. *Cells, 9*(11).

Marchetti, A. L., Zhang, H., Kim, E. S., Yu, X., Jang, S., Wang, M., & Guo, H. (2022). Proteomic Analysis of Nuclear Hepatitis B Virus Relaxed Circular DNA-Associated Proteins Identifies UV-Damaged DNA Binding Protein as a Host Factor Involved in Covalently Closed Circular DNA Formation. *J Virol, 96*(2), e0136021. https://doi.org/10.1128/jvi.01360-21.

Mohd-Ismail, N. K., Lim, Z., Gunaratne, J., & Tan, Y. J. (2019). Mapping the Interactions of HBV cccDNA with Host Factors. *Int J Mol Sci, 20*(17). https://doi.org/10.3390/ijms20174276.

Mu, D., Yan, L., Tang, H., & Liao, Y. (2015). A sensitive and accurate quantification method for the detection of hepatitis B virus covalently closed circular DNA by the application of a droplet digital polymerase chain reaction amplification system. *Biotechnol Lett, 37*(10), 2063-2073. https://doi.org/10.1007/s10529-015-1890-5.

Selzer, L., & Zlotnick, A. (2015). Assembly and Release of Hepatitis B Virus. *Cold Spring Harb Perspect Med, 5*(12). https://doi.org/10.1101/cshperspect.a021394.

Slagle, B. L., & Bouchard, M. J. (2018). Role of HBx in hepatitis B virus persistence and its therapeutic implications. *Current Opinion in Virology, 30*, 32-38. https://doi.org/https://doi.org/10.1016/j.coviro.2018.01.007.

Verrier, E. R., Ligat, G., Heydmann, L., Doernbrack, K., Miller, J., Maglott-Roth, A., Jühling, F., El Saghire, H., Heuschkel, M. J., Fujiwara, N., Hsieh, S. Y., Hoshida, Y., Root, D. E., Felli, E., Pessaux, P., Mukherji, A., Mailly, L., Schuster, C., Brino, L., Nassal, M., & Baumert, T. F. (2022). Cell-based cccDNA reporter assay combined with functional genomics identifies YBX1 as HBV cccDNA host factor and antiviral candidate target. *Gut, 72*(9), 1745-1757. https://doi.org/10.1136/gutjnl-2020-323665.

Wei, L., & Ploss, A. (2021). Hepatitis B virus cccDNA is formed through distinct repair processes of each strand. *Nat Commun, 12*(1), 1591. https://doi.org/10.1038/s41467-021-21850-9.

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., Fu, L., Song, M., Chen, P., Gao, W., Ren, B., Sun, Y., Cai, T., Feng, X., Sui, J., & Li, W. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife, 1*, e00049. https://doi.org/10.7554/eLife.00049.

Zhong, Y., Hu, S., Xu, C., Zhao, Y., Xu, D., Zhao, Y., Zhao, J., Li, Z., Zhang, X., Zhang, H., & Li, J. (2014). A novel method for detection of HBVcccDNA in hepatocytes using rolling circle amplification combined with in situ PCR. *BMC Infect Dis, 14*, 608. https://doi.org/10.1186/s12879-014-0608-y.

Zlotnick, A., Venkatakrishnan, B., Tan, Z., Lewellyn, E., Turner, W., & Francis, S. (2015). Core protein: A pleiotropic keystone in the HBV lifecycle. *Antiviral Research, 121*, 82-93. https://doi.org/https://doi.org/10.1016/j.antiviral.2015.06.020.

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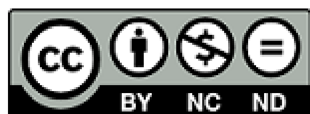

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