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The Significant Impact of Programmed Cell Death-1 Gene Polymorphism on HBV Infection and Viral Load

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Abstract

Background: Hepatitis B virus (HBV) is a common and international problem associated with severe liver diseases. Programmed cell death-1 (PCD-1) is an immunosuppressive molecule that negatively regulates T-cell activity. Genetic variation in PCD-1 gene could impact the encoded protein, and, thus HBV infection.

Objective: To assess the single nucleotide polymorphisms (SNPs) in the PCD-1 gene's promotor area effect of viral load and HBV infection.

Patients and Methods: This case-control study recruited 117 subjects (67 patients with HBV and 50 apparently healthy persons as control). From June 2024 to November 2024, all subjects had been selected at the Gastroenterology and Liver Hospital-Medical City (Baghdad, Iraq). Every participant donates about 5 mL of blood extracted from a vein and stored at -80 °C until it was needed. Blood samples were used to obtain genomic DNA, and gene fragment corresponding the rs38084323 polymorphism in PCD-1 gene was amplified and genotyping by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: The patients' and controls' mean ages did not differ significantly, out of the 67 patients, 39 (43.28%) had an acute infection, and 38 (56.7%) had a chronic infection. Merely 34.33% of the patients were receiving HBV-specific therapy. Patients having the mutant homozygous genotype (GG) of rs38084323 were significantly more likely to have it (28.36% vs. 14%) (OR= 3.34, 95%CI= 1.07-10.38, p= 0.037). On the level with allele, the G allele was more often found in patients than in healthy subjects (54.48% vs. 41%), demonstrating an important disparity (OR=1.72, 95% CI=1.02-2.91, p=0.042). The various genotypes did not substantially affect the progression of infection to a chronic status. Although, 30.77% of patients carrying the AA genotype had viral ≥ 200000 IU/ml compared with 5.71% for AG carriers and 15.79% for GG carries with such a viral load, and these results were shown significant difference.

Conclusion: The homozygous mutant genotype (GG) and G allele may be regarded as an indicator of risk for HBV infection. However, their impact on viral load is negligible.

Keywords: Hepatitis B virus, Programmed cell death-1, Promoter, Single nucleotide polymorphism, Apoptosis.

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Introduction

The World Health Organization (WHO) projected that by 2024, there will be 254 million people with a chronic HBV infection, a 3.2% global seroprevalence of hepatitis B surface antigen (HBsAg), and around 820,000 deaths from HBV-related causes (1). Hepatitis B virus is recognized as a source of several viral diseases, including acute hepatitis B, which may resolve naturally, and chronic infection, which increases the risk of fatalities from hepatocellular carcinoma (2). The clinical consequences of HBV infection rely on the intricate interplay between the host immune response and viral replication. Innate immunity has a significant influence in the initial stages of the disease. However, HBV evades identification by the host innate immune system through stealth (3). The complete elimination of the virus during acute infection is contingent upon an individual's immune system. Generally, individuals infected with HBV can eradicate the virus; nevertheless, acute infection may progress to chronic infection in cases of immunological inadequacy (4). Adaptive immunity, comprising a variety of cell types, has both preventive and detrimental functions in generating an antiviral acquired response. Numerous strong arguments demonstrate that the human cellular immune response which is enabled by CD8+ and CD4+T cells, predominantly HBV-specific CD8+T cells is essential for HBV pathogenesis (5-7). If the virus is not completely eradicated from the body, HBV persists.

A dynamic equilibrium between the host's immune response and viral replication is established, ultimately leading to immunological tolerance and T cell dysfunction in the long term. The depletion of HBV-specific CD8+ T cells predominantly occurs through the stimulation the immunosuppressive checkpoint PCD-1/PCD-L1 signaling cascade. Genetic factors donated in advancement of diversity of HBV disease extension including single nucleotide polymorphism of diversity genes such as Programmed cell death -1 (PCD-1) (8). The PCD-1 gene is situated on chromosome 2 at position q37.3 areas, and over thirty single nucleotide polymorphism (SNPs) sites are in various areas of the gene comprising exon, promoter, intron and 3´UTR area (9-10). PD-1(also known CD 279) is 55-KD protein and a kind of the immunoglobulin subfamily which are immunological sensor revert to the CD28/CTLA-4 that recognized to be restraint of receptor (11,12). PCD-1 offered at the level on trigger B lymphocytes, natural killer cells, T cells, myeloid dendritic cells, and can found in elevated amount at T lymphocytes that get in the repose phase (13). Studies have announced that there are newly genetic interrelation reports had examined genetic variant of (PCD-1) and its ligand (PCDL-L1) with cancer, autoimmune and certain disease in various world people (14,15). Given the significant function of PCD-1 in T-cell activity within the immune response specific to HBV, it is essential to consider the potential effects of gene polymorphism regarding the expression or functional alteration of PCD-1 and its implications for immunological reaction. In this condition, it we conducted a case-control research with the objective of defining the correlation between HBV infection and the SNP rs36084323. Therefore, the objective of this study was to assess the single nucleotide polymorphism (SNPs) in the gene's promotor area effect of viral load and HBV infection.

Patients and Methods

The study design: One Hundred and seventeen subjects registered in this case- control research, encompass 67 patients with HBV and compared with 50 healthy persons. All subjects enlisted at Gastroenterology and liver Hospital-Medical City (Baghdad, Iraq) from June 2024 to November 2024. Diagnosis was executed for HBs Ag by laboratory tests involving enzyme-

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linked immunosorbent assays (ELISAs) and asserts these tests by viral load specified by real time-PCR.

Data and sample collection: Written agreement was acquired from all group's previous enrollment in the research project. Data on gender, age, body mass index (BMI), residence, family history of HBV and comorbidities was taken from each subject through direct interview in a preformed formula. Type of infection and treatment assorted under clinical features. Approximately 5 mL of blood taken from a vein gained from everyone who participated, this volume divided into two part 3 µl utilized for serological analysis and 2 µl utilized for molecular assay and both of these kept at −80 °C til be utilized.

Serum preparation and storage: To ensure full clotting, the tubes were centrifuged for 10 minutes at 1900 x g at 4 °C after being kept at room temperature (15–25 °C) for 20 minutes. Ultimately, the supernatant was cautiously moved to a fresh tube. For HBs Ag.

Determination of Hepatitis B surface antigen (HBs Ag) by ELISA: All 117 samples were examined for detection of (HBs Ag) by ELISA kit (Cat. No: BXEO741C ,CAMP/Romania). The procedure according to the manufacture, Once the necessary number of strips had been removed, they were secured into the micro well. The ELISA working sheet was followed when adding the specimens. 100 µl of samples were added to the test well, and 100 µl of HBs Ag positive, negative control was added to the control well. To every well, one hundred microliters of conjugate were added. After 50 minutes of incubation at 37 °C, the well was rinsed four times with wash buffer. After 50 minutes of incubation at 37 °C, the well was rinsed four times with wash buffer. After adding 90 µl of TMB substrates, the mixture was incubated for 20 minutes at 37 °C in a dark environment. The well becomes blue. Lastly, 50 µl of stop solution was carefully added, convert the color to yellow. The result was measured at 450 nm in 15 minutes.

Isolation DNA and Polymerase Chain Reaction and restriction fragment length polymorphism (RFLP): A commercial kit (Gsync TM DNA extraction kit quick organizer, Cat.No: GS100, Geneaid, Taiwan) was utilized for Genomic DNA extraction as stated by steps by the manufacturer. Biospec Nano spectrophotometer identified extracted DNA concentration the at 260 nm/280 nm (A260/A280). Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) was employed to inspect the -538 locus (rs3608423). For this purpose, two primers utilized (16):

Forward (F): 5'-CTCAACCCCACTCCCATTCT-3' and reverse for above F (R) 5'-TTCTAGCCTCGCTTCGGTTA-3' with an expected fragment length of 552 bp. The ABI 9600 (Hybaid / England) was employed in PCR in entire volume of 25 µl comprising 50 ng of genomic DNA, 1.5 µl of 10×PCR buffer, 0.3 µl of 10mMdNTPs, 0.25 µl of 10pmol/µl of every primer, and 1.25 U of Taq DNA polymerase (Cat. No: K-2012, Bioneer, Korea). The cycling parameters included preliminary denaturation at 94 °C for 5 minutes, subsequently 30 cycles of 1 min. at 94 °C, 1 min. at 56 °C, 1 min. at 72 °C and finally extension step at 72 °C for 5 min. The PCR products underwent enzymatic digestion with 5 U MspI (Cat.No: E091, Sib Enzyme Russia) adhering to the manufacturer's guidelines. Individuals' genotypes were identified based on the length of digested parts subsequent 2% agarose gel electrophoresis, which was colored with ethidium bromide. Allele differentiation was determined by fragment size post- digestion which was 227 bp for G allele and 282 bp for A allele.

Quantitation of HBV DNA: For the purpose of

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quantifying HBV DNA in all 117 samples, we employed the COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Diagnostics, Laval, QC, Canada), a nucleic acid amplification test, in conjunction with an automated real-time PCR assay. An HBV DNA fragment from the S gene served as the primer for the HBV DNA quantification.

Statistical analysis

The analyses were executed employed SPSS 25.0 (SPSS, Chicago). The mean and standard deviation of Continual information were presented, and the student t-test was employed for analysis. The Chi-square test assessed variables with categories, noted as numbers and percentages. The relationship between HBV infection and rs36084323, positioned in the promoter area of the PCD-1 gene, was assessed through binary logistic regression analysis. The odds ratio (OR) and in line with 95% confidence interval (CI) were determined from this test. Any change regarded statistically significant had a p-value of less than 0.05.

Results

Demographic features of the patients: There was no substantial disparity in the mean age of the patients and controls, the previous processing range was 14–75 years and the last one possessing a range of 44.98±9.5 29 years. Despite being less common in patients than in controls (47.76% vs. 56%), the variation was not substantial significant. Similarly, there were no appreciable variations between the two groups' BMIs or places of residence. However, there existed a notable disparity between the 19.4% of patients and the 6% of control group who had a family history of HBV. Diabetes mellitus (DM) and hypertension were prevalent co-occurring conditions in both the patient and control groups, with no discernible variations in the occurrence of these conditions between them as shown in Table 1.

Table 1. Presents the demographic features of the study population.

Variables	Patients (n=67)	Controls (n=50)	p-value
Age, years			0.363
Mean±SD	42.72±15.47	44.98±9.5	
Range	14-75	17-62	
Gender			0.378
Male	35(52.24%)	22(44%)	
Female	32(47.76%)	28(56%)	
BMI, kg/m^2			0.101
Mean±SD	26.2± 3.76	25.26±3.6	
Range	17.42-35.8	21.67-28.73	
Residence			0.697
Rural	53(79.1%)	41(82%)	
Urban	14(20.9%)	9(18%)	
Family history			0.037
No	54(80.6%)	47(94%)	
Yes	13(19.4%)	3(6%)	
Comorbidity			0.614
No	51(76.12%)	36(72%)	0.937
DM	7(10.45%)	5(10%)	0.741
Hypertension	8(11.94%)	5(10%)	0.131
Others	3(4.48%)	6(12%)	
SD: standard deviation, BMI: body mass index, DM			

Clinical characteristics of the patients: Acute infection was reported in 39 patients out of 67 (43.28%), while chronic infection was found in 38 patients (56.7%). Only 34.33% of the patients were under specific treatment for HBV. Viral load was ≥ 200000 IU/ml in 9 patients (13.43%) as shown in Table 2.

Table 2. Patient clinical features.

Variables	Values
Type of infection	
Acute	29(43.28%)
Chronic	38(56.72%)
Treatment	
No	44(65.67%)
Yes	23(34.33%)
Viral load, IU/ml	
<200000	56(83.58%)
≥200000	9(13.43%)

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Molecular assay: This study examined the relationship between HBV infection and the SNP rs36084323, which is situated in the promoter region of the PD-1 gene. RFLP was used for the genotyping process, as shown in Figure 1, display the PCR products prior to digestion, Lane M is 1:100 bp ladder, primarily all samples explained in 1 to14 lanes gave positive result for PD-1.1 primers with product length 552bp before utilized the restriction enzyme. Following digestion with the MspI restriction enzyme, three genotypes for this SNP were identified: AA, AG, and GG, as illustrated in Figure 2. Current study announced about 13 patients and 16 controls carrying AA genotype, 35 patients and 27 controls carrying AG genotype and 19 patients and 7controls carrying GG genotyping.

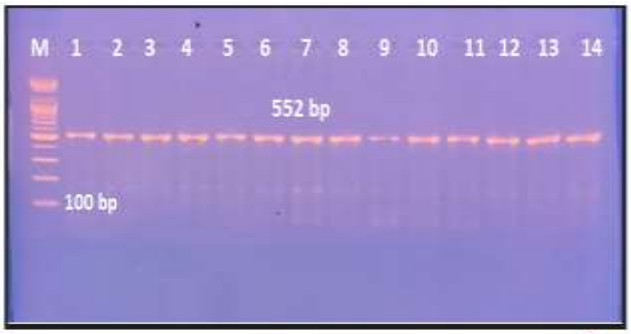

Figure 1. Gel electrophoresis of the PD-1-606 G/A gene polymorphism amplified with an identifiable primer pair via conventional PCR. The PCR result was dyed with ethidium bromide. The fragment's size measured 552 base pairs.

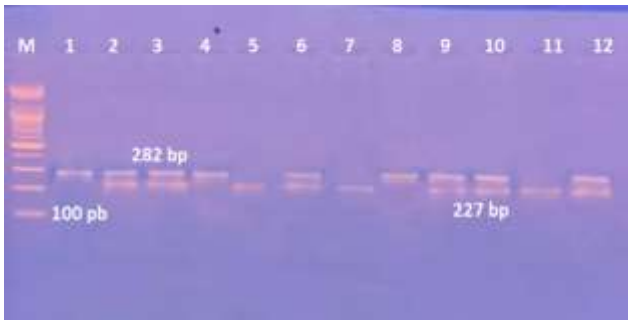

Figure 2. Genotype variations in PD-1-606 G/A in patients with HBV following digestion with the MpsI restriction enzyme, shown under U.V light after colored with ethidium bromide. Lanes M is 1:100 bp ladder, Lanes 1, 4, and 8: AA genotype; lanes 2, 3, 6, 9, 10, and 12: GA genotype; lanes 5, 7, and 11: GG genotype; M: 100 bp molecular ladder.

Association of rs36084323 with HBV Infection: Both wild type homozygous and heterozygous genotypes were less frequent in patients (19.4% and 52.24%, respectively) than controls (32% and 54%, respectively) although the differences were not significant. Conversely, the mutant homozygous genotype (GG) was increased frequency in patients than (28.36% vs. 14%) with a substantial disparity (OR= 3.34, 95%CI= 1.07-10.38, p= 0.037). In dominant approach, the rate of GG genotype was higher in patients compared to controls (28.36% vs. 14%) with no substantial disparity. At the allelic level, the G allele was greater prevalent in patients compared to controls (54.48% vs. 41%), exhibiting an important distinction (OR=1.72, 95%CI=1.02-2.91, p=0.042), as illustrated in Table 3.

Table 3. The prevalence of various genotypes and alleles of the rs36084323 polymorphism in HBV patients and healthy subjects.

rs36084323 Polymorphism	Patients (n=67)	Controls (n=50)	*P*-value	OR (95%CI)
Genotypes				
AA	13(19.4%)	16(32%)	0.113	1.0
AG	35(52.24%)	27(54%)	0.148	2.1(0.77-5.7)
GG	19(28.36%)	7(14%)	0.037	3.34(1.07-10.38)
HWE	0.663	0.411		
Dominant model		43(86%)		1.0
AA+AG	48(71.64%)	7(14%)	0.069	2.43(0.93-6.35)
GG	19(28.36%)			
Recessive model		16(32%)		1.0
AA	13(19.4%)	34(68%)	0.121	1.96(0.84-4.57)
AG+GG	54(80.6%)			
Alleles	61(45.52%)	59(59%)		1.0
A	73(54.48%)	41(41%)	0.042	1.72(1.02-2.91)
G				

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Correlation of various genotypes of rs36084323 with the clinical attributes of patients: There was no discernible effect of different genotypes of rs36084323 polymorphism on the development of infection into chronic status. Although, 30.77% of patients carrying AA genotype had viral load ≥200000 IU/ml compared with 5.71% for AG carriers and 15.79% for GG carries with such a viral load, the change was not substantial as shown in Table 4.

Table 4. Correlation of various genotypes of rs36084323 with the clinical attributes of patients.

Variables	AA (n=13)	AG (n=35)	GG (n=19)	p-value
Type of infection Acute Chronic	 3(3.08%) 10(76.9%)	 16(45.71%) 19(54.9%)	 10(52.63%) 9(47.37%)	0.232
Viral load, IU/ml <200000 ≥200000	 9(69.23%) 4(30.77%)	 32(91.43%) 2(5.71%)	 14(73.68%) 3(15.79%)	0.072

Discussion

This study found no variation in impact related to age between patients and controls, also the findings contradict previous studies conducted in Iraq, Iran, and China, which indicated that age was related with a raised risk of infection. (17-19). The study found no variation in impact based on gender, BMI, or residence; however, a positive association was observed between family history and HBV prevalence. This result compared with previous study from Iraq showed that the proportion of family members of infected people who received the HBV vaccine was much lower than those who did not (20). This finding aligns with the report from Ethiopia. (21), Vietnam, (22) in Northeast China (23), and Africa Uganda. (24) In contrast, this is in consistent with other reports (25,26). This result may from insufficient understanding of HBV transmission methods, inadequate caution when sharing sharp objects, traditional practices, or unsafe sexual practices. The transmission of viruses occurs through various methods, including blood transfusion. Additionally, individuals within the same household may be exposed to similar external risk factors. For instance, family members engaging in the same conventional medical practices may significantly elevate the risk of infection through various means, including intravenous drug use, unsafe healthcare-related injections, blood transfusions, and dialysis. Additionally, exposure to infected blood via razors and nail clippers can contribute to infection, as can the use of contaminated piercing instruments, tattoos, and other cosmetic procedures. New sexual discharge and an additional fluid, such as saliva (26-28). Additionally, HBV infection can be transmitted from a mother with HBsAg at birth or through postnatal blood exposure. Research indicates that children of HBsAg positive individuals face a heightened risk of HBV infection through the exchange of items containing infected bodily secretions, such as chewing gum or toothbrushes (29-30). Several factors contribute to the variation in HBV infection outcomes, encompassing host, viral, and environmental elements. Genetic polymorphism and specific comorbidities, such as diabetes are recognized as host factors influencing on clinical outcomes of HBV infection. The viral determinants encompass HBV viral load. This study indicated no variation in impact concerning specific comorbidities and viral load between patients and controls, which contradicts previous research suggesting a positive association

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between specific comorbidities and viral load with the outcomes of HBV infection (31). This study examines the host genetic polymorphism associated with HBV infection outcomes, particularly detecting the SNPs in the promoter area of PCD-1.1 (-538G/A) (rs36084323). A multitude of studies has examined the identified connection between PCD.1 gene polymorphisms and diverse disorders (32-33). In this context, additional studies examined the connection between PD-1.1 gene polymorphism and colorectal cancer in Iranian patients, they showed that A and G alleles are correlated with raised risk of colon cancer (34). The prior report detailed the risk of various PD-1 variants in myocardial infarction (MI), no association was identified between PCD-1.1 alleles or genotypes and MI. They noted a limited protective effect of the PD-1.3A allele regarding myocardial infarction (35). A recent study assessed PCD-1 mRNA expression levels in Peripheral Blood Mononuclear Cells (PMCs)and examined its correlation with clinical characteristics of hepatitis B infection and the genotypes kinds of PCD-1 rs10204525, which includes three kinds genotypes: AA, GA, and GG. The findings indicated whom patients with chronic HBV infection displayed a notable higher PCD-1 mRNA expression compared to the healthy control group (36). Currently, there limited number of published research demonstrating a relationship between the PCD-1.1 (-538G/A) gene polymorphism and vulnerability to HBV infection. This research is confirmed and indicated a significantly higher prevalence of the GG genotype and G allele at position -538G/A of PCD.1 in HBV patients compared to the normal group. Conversely, the results indicated a lower frequency of the AA genotype and A allele in HBV patients compared to the normal group. Our research and prior studies indicated a strong correlation between elevated PD-1 expression and heightened HBV viral transcription levels (2,37). Furthermore, efficient viral transcription of HBV contributes to the advance of disease in patients, characterized by elevated expression of PCD-1 on HBV-specific CD8+ T cells, CD4+ T cells, and CD25+ regulatory T cells. This is attributed to increased RNA polymerase activity at the promoter area of the gene, resulting in impaired T cell functionality and persistent HBV infection (38). This polymorphism may serve as a target for designing immunotherapy strategies and assessment and selecting patients with HBV based on specific genetic immunological profiles, potentially enhancing future immunotherapy approaches for hepatitis B virus disease (39).

Conclusions

The mutant homozygous mutant genotype (GG) and G allele may be regarded as an indicator of risk for HBV infection. The detection of HBs Ag and employed real time PCR is the best way that assert the infection with virus. However, their impact on viral load is negligible. Expanding screening methods should be the main focus of future work since it is crucial to better therapy.

Recommendations

recommended for using another primer in another region of gene for obtain the additional risk factors for HBV infection and recommended for diagnose another gene.

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Conflict of interest: None.

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التأثير الكبير لتعدد اشكال جين موت الخلية المبرمج -١ على عدوى فيروس التهاب الكبد نوع ب والحمل الفيروسي

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الملخص

الخلفية: فيروس التهاب الكبد نوع ب هو مشكلة عامة شائعة مرتبطة بالتهاب الكبد الشديد. موت الخلايا المبرمج ١ هو جزئ مثبط ينظم نشاط الخلايا التائية بشكل سلبي يمكن ان يؤثر الاختلاف الجيني في جين موت الخلية المبرمج النوع ١ على البروتين المشفر وبالتالي على عدوى فيروس التهاب الكبد نوع ب.

الأهداف: تقييم دور تعدد الاشكال النيوكلوتيدات المفردة في منطقة المحفز لجين (موت الخلايا المبرمج-١) في عدوى فيروس التهاب الكبد نوع ب والتركيز الفيروسي.

المرضى والطرق: شملت الدراسة الحالية ١١٧شخصا (٦٧مريضا بالتهاب الكبد الفيروسي نوع ب و٥٠ شخصا سليما ظاهريا كمجموعة تحكم) تم استخراج الحمض النووي الجينومي من عينات الدم ومن ثم تم تضخيم جزء الجين المقابل لتعدد اشكال ٣٨٠٨٤٣٢٣في جين موت الخلايا المبرمج-١ والنمط الجيني عن طريق تفاعل البلمرة المتسلسل – تعدد الاشكال- القطع باستخدام الانزيم القاطع).

النتائج: كان النمط الجيني المتماثل المتحور نوع (ج ج) ل ٣٨٠٨٤٣٢٣ اكثر شيوعا لدى المرضى (٢٨,٣٦٪ مقابل ١٤ على المستوى الاليلي كان الاليل (ج) اكثر شيوعا لدى المرضى مقارنة بمجموعة الاصحاء مع وجود فرق كبير . لم يكن هناك تأثير كبير للأنماط الجينية المختلفة على تطور العدوى الفايروسية الى عدوى مزمنة وعلى الرغم من ان ٣٠,٧٧٪ المرضى الحاملين للنمط الجيني (أ أ) كان لديهم حمولة فيروسية ≤٢٠٠٠٠٠ وحدة دولية مقارنة ب ٥,٧١ لحاملي النمط الجيني (أ ج) و١٥,٧٩ لحاملي النمط الجيني (ج ج) مع مثل هذا الحمل الفيروسي , الا ان الفرق لم يكن كبيرا.

الاستنتاج: يمكن اعتبار النمط الجيني المتماثل المتحور (ج ج) والاليل (ج) من العوامل الخطورة للإصابة بفيروس التهاب الكبد الفيروسي نوع ب ومع ذلك فان تأثيرها على الحمل الفيروسي لا يذكر.

الكلمات المفتاحية: فيروس التهاب الكبد نوع ب , موت الخلايا المبرمج-١, منطقة المحفز, تعدد الاشكال للنيوكلوتيد المفرد.

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