Role of microRNA 4717, its effects on programmed cell death protein-1 in hepatitis B infection, and interaction between PDCD1 and miR-4717

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Abstract
It is suggested that programmed cell death protein-1 (PD-1) is involved in hepatitis B virus (HBV) infection, the leading cause of hepatocellular carcinoma globally. This study was multi-aimed, that is, to investigate the role of microRNA (miR) 4717 and its target, PD-1 and to determine how the rs10204525 polymorphism in the 3′ untranslated region (3′UTR) of PD-1 affects its interaction with miR-4717. The expression levels of miR-4717 with various single-nucleotide polymorphisms were measured by reverse transcription–quantitative polymerase chain reaction (RT-qPCR). A total of 54 tissue samples from HBV-infected individuals were collected, genotyped, and categorized into three groups; AA (n = 32), AG (n = 18), and GG (n = 4). The expression levels of gene PDCD1 and its corresponding PD-1 protein were significantly declined in the AA group as compared to AG and GG groups. There was a negative linear association between PDCD1 and miR-4717 in the tissue samples. HEPG2 cells transfected with an miR-4717 mimic or PD-1 small interfering (si)RNA exhibited significantly reduced expression levels of PDCD1 and PD-1, whereas cells transfected with an inhibitor of miR-4717 demonstrated greater expression levels of PDCD1 and PD-1 compared with the scramble control. In addition, cell viability and apoptosis were assessed in cells transfected with an miR-4717 mimic, PD-1 siRNA, or an miR-4717 inhibitor. Results revealed that treatment with the miR-4717 mimic or PD-1 siRNA enhanced viability of cells and reduced apoptosis. The results of this study suggest that rs10204525 polymorphism interferes with the interaction between PD-1 and miR-4717 and therefore induces apoptosis in liver cancer cells.

Keywords
apoptosis, hepatitis B virus infection, liver function, rs10204525 polymorphism

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Introduction
Hepatitis B virus (HBV) is a hepatotropic, enveloped, partially double-stranded DNA virus that causes chronic and acute hepatitis B infections in humans.¹ The HBV virion consists of an outer envelope made of lipid and a nucleocapsid core that encloses circular viral DNA. Following attachment, penetration and uncoating of the virus in the host cell, viral DNA becomes fully double-stranded and is subsequently transformed into, covalently closed circular DNA, which serves as a template for the transcription of viral mRNAs. The longest mRNA is utilized to generate copies of the genome,
the capsid (core protein) and the viral DNA polymerase. Following additional processing, these transcripts form progeny virions, which are released from the cell.\textsuperscript{1,2}

Hepatocellular carcinoma (HCC) and liver fibrosis/cirrhosis may be caused by chronic HBV infection.\textsuperscript{3} Neonates and infants are susceptible to the development of chronic HBV infection, whereas the majority of adults are resistant to it, and therefore, there is an inverse association between infection status and age.\textsuperscript{4} The factors implicated in chronic HBV infection include chronic inflammation of the liver and an impaired immune response to viral antigens, which may result in the progression of liver disorders. Acute HBV infection occasionally causes liver disease and/or failure; however, the majority of cases of liver disease caused by HBV are due to chronic HBV.\textsuperscript{5} In numerous developing countries, chronic HBV is epidemic, and it affects >350 million people worldwide.\textsuperscript{6} Therapies are required to prevent infection and to treat liver disorders following infection. Chimpanzees and small animal models have been utilized to develop vaccines and treatments to prevent HBV. However, there remains a lack of robust animal models to investigate the underlying mechanism by which HBV escapes host immunity to cause chronic infection and liver disorders.\textsuperscript{7} MicroRNAs (miRNAs) play a role in the improvement of numerous species and are commonly associated with genetic disorders, including cancer.\textsuperscript{8} MicroRNAs exert their effects by targeting mRNA and downregulating gene expression.\textsuperscript{9} Mature miRNAs cause suppression of mRNA cleavage or translation by interaction with the 3′untranslated regions (UTRs), which are targeted by RNA-induced silencing complexes.\textsuperscript{10} Gene expression may be additionally post-transcriptionally regulated by miRNAs, as demonstrated in previous studies. miRNAs serve as regulators of epigenetic machinery.\textsuperscript{11} Notably, as miRNAs serve roles in biological processes and dysregulation of miRNA expression may be related with human diseases, such as hepato carcinogenesis and chronic HBV infection.\textsuperscript{12} Previous studies have demonstrated that programmed cell death protein-1 (PD-1) serves a role in apoptosis of liver cells, which is associated with deterioration of liver function in HBV-infected individuals.\textsuperscript{13} A single-nucleotide polymorphism in the 3′UTR of \textit{PDCD1} (the gene encoding PD-1; rs10204525) may disrupt the interaction between miRNA (miR)-4717 and \textit{PDCD1} and has been associated with liver disease.\textsuperscript{14} This study investigated how the rs10204525 polymorphism affects the interaction between miR-4717 and PD-1, and whether this affects liver function in individuals infected with HBV.

**Materials and methods**

**Patients and sample collection**

In a case–control study, a total of 650 patients (with chronic HBV infection) were recruited from Xintai Hospital Affiliated to Tai’shan Medical University (Xintai, China) between December 2013 and December 2014. Diagnosis of all patients was determined by laboratory tests. Individuals with other liver diseases, including drug-induced liver injury, Wilson’s disease, viral hepatitis A, alcoholic hepatitis, hepatitis C, hepatitis E, steatohepatitis and autoimmune hepatitis were excluded from the study. In addition, individuals suffered from metabolic and endocrine diseases such as hyperthyroidism or human immunodeficiency virus infection were excluded, as were individuals with diseases unrelated to HBV infection, including respiratory, cardiovascular, or renal dysfunction. Peripheral blood samples were collected from 2,158 patients and liver tissue samples were available from 54 patients.

The Ethics and Research Committees of Xintai Hospital Affiliated to Tai’shan Medical University approved this study. Written informed consent was obtained from patients prior to sample collection, and the study was performed according to the Declaration of Helsinki.

Sample size was determined by the power analysis and the calculation was made by the software G Power (Faul, Erdfelder, Lang and Buchner, 2007) for sample size calculation.

TaqMan genotyping. The TaqMan methodology (Roche Diagnostics GmbH, Mannheim, Germany) was utilized to analyze single-nucleotide polymorphisms in patient tissue samples, in accordance with the manufacturer’s protocol.

RNA extraction and reverse transcription-quantitative polymerase chain reaction. Red blood cell lysis buffer (Tiangen Biotech Co, Ltd, Beijing, China) was used for the extraction of RNA from blood cells and TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc, Waltham, MA, USA) was utilized.
in the extraction of total RNA from HEPG2 cells and tissue samples, according to the manufacturer’s protocol. The quality and concentration of RNA was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), at wavelengths of 260 and 280 nm. The generation of cDNA was done by RT, using the high-capacity cDNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc), in accordance with the manufacturer’s protocol. The reaction contained 200 ng total RNA, 1 μL reverse transcription primer, 1 μL RNase inhibitor, 4 μL buffer, 1 μL Moloney murine leukemia virus reverse transcriptase and 2 μL dNTP. The volume in each sample was adjusted to 20 μL with water. cDNA was stored at –20°C until further use. To perform the reverse transcription of the U6 (control), 0.5 μg/μL Oligo (dT) (Sangon Biotech Co, Ltd, Shanghai, China) was utilized. qPCR was subsequently performed using the iQ5 Multicolor Real-Time PCR Detector system (Bio-Rad Laboratories, Inc, Hercules, CA, USA) on samples containing 3 μL RT product, 1 μL forward primer, 1 μL reverse primer, 12.5 μL SYBR® Green I Master mix (Thermo Fisher Scientific, Inc, Waltham, MA, USA) and 7.5 μL water. Cycling conditions were as follows: a very first pre-denaturation step at 95°C for 20 s that are followed by 40 cycles of denaturation at 95°C for 3 s and annealing at 60°C for 30 s. The quantitation cycle (C_q) value was calculated using the 2–ΔΔC_q method for gene expression analysis of PDCD1 and miR-4717 relative to GAPDH, which served as an internal standard. The experiment was repeated at least three times.

**Cell proliferation assay**

Cell Counting kit 8 (Dojindo Molecular Technologies, Inc, Kumamoto, Japan) was utilized to analyze the proliferation of HEPG2 cells 48 h following transfection, in accordance to the manufacturer’s protocol. Synergy HT microplate spectrophotometer (BioTek Instruments, Inc, Winooski, VT, USA) was used to measure the absorbance of each sample at a wavelength of 450 nm. Experiments were performed at least three times.

**Vector construction, mutagenesis, and luciferase assay**

Taq polymerase, and Taq PCR mix (Xi’an Runde Biotechnology Co, Ltd, Xi’an, China) were used to amplify the 3′ UTR of PDCD1 that contained the miR-4717 binding site. The wild-type PDCD1 sequence was subsequently put in into the HindIII and SpeI sites of the pMIR-REPORT™ luciferase vector. A mutant PDCD1 sequence was generated using QuikChange® Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Inc, Santa Clara, CA, USA) and inserted at the same restriction sites of a control vector (Ambion; Thermo Fisher Scientific, Inc). Cells were transfected with the wild-type or mutant PDCD1 vector using lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc). Analysis of luciferase activity was done, 48 h post-transfection via the Dual-Luciferase Reporter assay system (Promega Corporation, Madison, WI, USA), by following the manufacturers protocol. The Renilla luciferase plasmid served as an endogenous positive control. Each test was repeated in triplicate.

**Western blot analysis**

HEPG2 cells or liver tissue samples were lysed with lysis buffer containing protease inhibitors
Cellular lysates were subsequently centrifuged at 4°C for 10 min at 15,000 r/min and the Bradford assay (Beyotime Institute of Biotechnology, Beijing, China) was utilized to evaluate the concentration of protein. The samples were mixed with 4 mL loading buffer (Promega Corporation), loaded onto 12% gels and exposed to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (PerkinElmer, Inc, Waltham, MA, USA) by the process of electrophoresis at 180 mA, for 60 min with transfer buffer (0.2 M glycine, 20% methanol and 25 mM Tris; Promega Corporation). Subsequently, the membranes were washed twice with phosphate-buffered saline (PBS) containing 0.1% Tween-20 and blocked with 5% non-fat milk for 60 min to avoid nonspecific binding. Membranes were probed with monoclonal antibodies against PD-1 or β-actin (1:1,000; Wuhan Boster Biological Technology Ltd, Wuhan, China) overnight at 4°C, followed by a horseradish peroxidase-conjugated secondary antibody (1:5,000; Wuhan Boster Biological Technology Ltd) for 60 min at room temperature. An Enhanced Chemiluminescence Detection reagent (Beyotime Institute of Biotechnology) was utilized to detect protein bands. Relative protein expression levels of PD-1 to β-actin were obtained by densitometry. Each test was performed for three times.

Apoptosis analysis
The Annexin V-Fluorescein Isothiocyanate/Propidium Iodide Apoptosis Detection kit (Nanjing KeyGen Biotech Co, Ltd, Nanjing, China) was used to stain HEPG2 cells, by following the manufacturer’s protocol. Flow cytometry was performed to measure apoptosis, following incubation for 15 min at room temperature in the dark. Three independent experiments were performed.

Statistical analysis
SPSS software version 16.0 (SPSS, Inc, Chicago, IL, USA) was utilized to perform statistical analyses. Results are presented as the mean ± standard deviation. A Student’s t-test was utilized to compare variable data, a paired t-test was utilized to make comparisons between transfection groups and a two-group test was utilized for other experiments. Pearson’s correlation coefficient was utilized to determine the correlation between two parameters. A two-way analysis of variance followed by the Dunnett’s post hoc test was utilized to perform comparisons between genotype groups and demographic or clinicopathological characteristics of patients. \( P < 0.05 \) was considered to specify a statistically significant difference.

Results

Genotype status and patient characteristics
Of the 650 individuals enrolled in this study, 352 exhibited the \( \text{PDCD1} \) rs10204525 AA genotype, 252 exhibited the AG genotype, and 46 exhibited the GG genotype. The demographic and clinicopathological characteristics of the patients, including age, gender, levels of aspartate transaminase (AST), alanine transaminase (ALT), bilirubin and HBV DNA, and the presence or absence of the HBV antigen HBeAg, are presented in Table 1. No significant differences were identified between genotype status and age \( (P=0.796) \), gender \( (P=0.896) \), bilirubin \( (P=0.030) \), HBV DNA \( (P=0.441) \), or HBeAg \( (P=0.870) \). However, it was observed that significant differences exist between genotype status and ALT \( (P<0.001) \) and AST \( (P<0.001) \) levels.

### Table 1. Association of the rs10204525 polymorphism with clinicopathological characteristics of HBV-infected individuals.

| Parameter                  | AA (n = 352) | AG (n = 252) | GG (n = 46) | \( P \) value |
|----------------------------|--------------|--------------|-------------|--------------|
| Age (years)                | 35.21 ± 9.14 | 36.19 ± 10.63| 37.22 ± 12.64| 0.796        |
| Gender (M/F)               | 280/72       | 189/63       | 37/9        | 0.986        |
| ALT (U/L)                  | 102.54 ± 48.14| 115.63 ± 65.21| 180.21 ± 77.24| <0.01       |
| AST (U/L)                  | 76.31 ± 18.25| 142.32 ± 51.84| 164.32 ± 39.25| <0.01       |
| Bilirubin (μmol/L)         | 226.45 ± 138.84| 263.24 ± 142.23| 374.21 ± 262.14| 0.030       |
| HBV DNA (log₁₀ copies/mL) | 5.21 ± 1.94  | 6.25 ± 3.74  | 5.57 ± 1.62  | 0.441       |
| HBeAg (±)                  | 212/140      | 178/74       | 32/14        | 0.870        |

M: male; F: female; ALT: alanine transaminase; ASP: aspartate transaminase; HBV: hepatitis B virus; HBeAg: hepatitis B antigen.
PDCD1 is a target of miR-4717

Based on the computational analysis of an online target prediction tool (www.targetscan.org), miR-4717 was predicted to bind to the 3′UTR of PDCD1 mRNA, which suggested that PDCD1 may be a potential target of miR-4717 (Figure 1). To confirm this, luciferase reporter plasmids were constructed containing wild-type or mutant PDCD1 3′UTR segments (Figure 1). The luciferase activity of cells transfected with wild-type PDCD1 3′UTR was significantly decreased as compared to the blank control, scramble control, and cells transfected with the mutant PDCD1 3′UTR (Figure 2). The luciferase activity of cells transfected with the mutant PDCD1 3′UTR was comparable to that of cells treated with the scramble control (Figure 2), suggesting that PDCD1 was a target gene of miR-4717 and the position of binding site was situated at the 3′UTR of PDCD1.

miR-4717 expression levels in patient tissue and genotype status

The number of patient tissue samples that exhibited the AA, AG, and GG genotypes were 32, 18, and 4, respectively. The expression levels of miR-4717 in patients with the AA, AG, and GG genotypes were not significantly different (Figure 3). This proposed that there was no link between the rs10204525 polymorphism and the expression of miR-4717.

Expression levels of PDCD1 and its PD-1 protein product and genotype status

The expression levels of PDCD1 (Figure 4(a)) and its PD-1 protein product (Figure 4(b)) were measured in the 54 tissue samples, and were greater in the AG and GG groups compared with the AA
group, as determined by RT-qPCR and Western blotting, respectively.

**mRNA expression levels of PDCD1 and miR-4717 had a negative linear correlation**

The association between the expression levels of *PDCD1* and miR-4717 was inverse, in the liver tissue samples, as demonstrated by a negative correlation coefficient ($r = -0.4217; P < 0.05$; Figure 5).

**Expression levels of PDCD1, miR-4717, and PD-1 following transfection**

To further confirm the negative linear association between *PDCD1* and miR-4717, HEPG2 cells were transfected with an miR-4717 mimic, miR-4717 inhibitor, or scramble control. It was revealed by Western blot analysis that PD-1 protein expression increased following treatment with the miR-4717 inhibitor compared with the scramble control; however, the intensity of the PD-1 band was reduced following treatment with the miR-4717 mimic compared with the scramble control (Figure 6(a)). RT-qPCR analysis revealed that the mRNA expression levels of *PDCD1* were significantly improved in cells treated with the miR-4717 inhibitor, and reduced in the miR-4717 mimic treatment group, compared to the scramble control (Figure 6(b)). This supported our conclusion that there was a negative linear association between the expression levels of *PDCD1* and miR-4717.

**Effect of miR-4717 on cell viability and apoptosis**

PD-1 is a regulator of T cell function and is a primary factor influencing cell viability and apoptosis in HBV infection. MTT assays and flow cytometric analysis were performed to determine cell viability and apoptosis. The viability of cells transfected with an miR-4717 mimic or PD-1 siRNA was greater compared with the blank or scramble control, whereas cells treated with the miR-4717 inhibitor were less viable (Figure 7(a)). In addition, the viability of cells transfected with
PD-1 siRNA was greater, as compared to the cells transfected with the miR-4717 mimic, which suggested that overexpression of PD-1 inhibited the viability mediated by miR-4717 (Figure 7(a)). The result of the apoptosis analysis is presented in Figure 7(b).

The fraction of transfected, apoptotic cells with an miR-4717 mimic or PD-1 siRNA was reduced as compared to the scramble control, whereas the apoptotic fraction of cells treated with an miR-4717 inhibitor was greater compared with the scramble control. This suggested that overexpression of PD-1 increased apoptosis.

**Discussion**

An association between enhanced expression levels of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), and inhibition of PD-1 has been observed in lymphocytes. Notably, there may be an association between enhanced expression levels of TNF-α and IFN-γ, and individuals that exhibit the rs10204525 GG genotype who are persistently infected with HBV. miR-4717 serves as an inhibitor of PD-1 that subsequently results in enhanced secretion of TNF-α and IFN-γ, via interaction with mRNA of a specific polymorphic genotype. This suggests that miR-4717 may be at least partially associated with the protection provided by the rs10204525 GG genotype to HBV infection. A number of reports have suggested that antiviral immune responses are downregulated by PD-1 and that IFN-γ and TNF-α serve a role in the control of HBV replication. This study demonstrated that individuals exhibiting the rs10204525 AA genotype exhibited the absence of the HBV protection since miR-4717 failed to inhibit PD-1. There was an association with the rs10204525 genotype AA and chronic HBV infection and liver function deterioration. Therefore, the virus life cycle of HBV in infected individuals that exhibit the rs10204525 AA genotype may be non-responsive to miR-4717. Bioinformatics analysis predicted that miR-4717 would bind to the 3’UTR of PDCD1, suggesting that this gene may be a possible target for miR-4717, which was confirmed by performing a luciferase assay. Low levels of PD-1 are present on naïve T-cells; however, they are increased within 24 h of activation. Phosphatases antagonize proximal kinases involved in the T-cell receptor

![Figure 6. Effect of miR-4717 on PD-1 and PDCD1 expression levels: (a) Western blotting and (b) reverse transcription-quantitative polymerase chain reaction were performed to determine PD-1 and PDCD1 expression levels, respectively. The expression levels of PD-1 and PDCD1 were reduced following transfection with an miR-4717 mimic and PD-1 siRNA, compared with the controls. Treatment of cells with an miR-4717 inhibitor caused an increase in PD-1 and PDCD1 expression levels compared with the controls. PDCD1, programmed cell death protein-1; PD-1, programmed cell death protein-1; miR-4717, microRNA-4717; siRNA, short interfering RNA.](image-url)
signaling cascade following binding of PD-1 to its ligands, programmed death ligand-1 and -2, and this prevents cluster of differentiation (CD)3-dependent gene transcription. PD-1 is a multipotent protein that regulates T-cell homeostasis, and the PD-1 signaling pathway serves a role in limiting activated antigen-specific T-cell expansion. Negative regulation of T-cells may be associated with the level of PD-1 secreted on their surface. Negative signaling via PD-1 is associated with regulation of homeostatic T-cell proliferation in lymphopenic conditions and prevention of autoimmunity during peripheral tolerance.

Functionally impaired HBV-specific CD8+ T-cell responses occur in individuals with chronic HBV infection, and these T-cells may express PD-1. Therefore, the exhausted CD8+ T-cell responses during chronic HBV infection may be explained by the expansion of functionally defective CD8+ T-cells due to hepatocellular priming. However, an effective HBV-specific CD8+ T-cell response may be observed in ~95% of adults with acute onset HBV infection. In HBV-infected chimpanzees, early priming of HBV-specific CD4+ T-cells prior to or during viral dissemination may be critical for the initiation of an efficient CD8+ T-cell response. In addition, CD4+ T-cell reduction prior to HBV infection may prevent functional T-cell priming and result in chronic infection. Ongoing experiments are investigating whether HBV-specific T-cell rescue may stimulate fully functional CD8+ T-cell responses in a transgenic mouse model. In this study, the viability of cells transfected with an miR-4717 mimic or PD-1 siRNA were greater compared with the scramble control, whereas the viability of cells treated with an miR-4717 inhibitor was reduced. In addition, the viability of cells treated with PD-1 siRNA was greater than the viability of cells treated with the miR-4717 mimic, indicating that overexpression of PD-1 inhibited the viability mediated by miR-4717. The degree of apoptosis in cells treated with the miR-4717 mimic and PD-1 siRNA was reduced compared with the scramble control; however, apoptosis was greater in cells treated with the miR-4717 inhibitor.

The PDCD1 rs10204525 polymorphism that is present on the 3′UTR may modulate polyadenylation and subsequently modulate the expression levels of inflammatory cytokines. The risk of developing esophageal squamous cell carcinoma may be reduced by the presence of a mutation in PD-1 rs10204525 region, as described in a previous study. Li et al. demonstrated that the onset of HCC may be caused by the PD-1 rs10204525 A > G polymorphism in combination with chronic HBV infection. This study determined the effect of the rs10204525 polymorphism on the mRNA expression levels of PDCD1 and protein expression levels of its PD-1 product, in liver tissues that were divided into three genotype groups. The expression levels of PD-1 in the AG and GG groups were greater compared with the AA group. However, the expression levels of
miR-4717 were not significantly different between the three genotype groups.

In this study, we were unable to determine the relation of regulatory T-cells (Tregs) and PD1, the regulatory T-cells (Tregs) and chronic hepatitis B infection and the regulatory T-cells (Tregs) and miR-4717.

**Conclusion**

From the findings of this project, it can be concluded that, the data of this study demonstrated that rs10204525 polymorphism interfered with the interaction between PDCD1 and miR-4717 and is associated with liver function deterioration in HBV-infected patients, possibly by altering the degree of apoptosis in liver cells. rs10204525 may be a prognostic biomarker for liver function in these patients.

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**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical approval**

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**Informed consent**

Written informed consent was obtained from all subjects before the study.

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