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**Original Article**

# CDC42-Interacting Protein 4 Gene Is Down Trans-Regulated by HBV DNA polymerase Trans Activated Protein 1

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**Abstract**

**Background:** Hepatitis B Virus (HBV) DNA polymerase transactivated protein 1 (HBVDNAPTP1) is a novel protein transactivated by HBV DNA polymerase, screened by suppression subtractive hybridization technique (GenBank accession no: AY450389). The biological function of HBVDNAPTP1 was investigated in this study.

**Methods**: We constructed a vector pcDNA3.1 (-)/myc-His A-HBVDNAPTP1 and used it to transfect acute monocytic leukemia cell line THP-1. HBVDNAPTP1 expression was detected by western blot analysis in the cells. A cDNA library of genes transactivated by HBVDNAPTP1 in THP-1 cells was made in pGEM-T Easy using suppression subtractive hybridization (SSH). The cDNAs were sequenced and analyzed with BLAST search against the sequences in GenBank.

**Results**: Some sequences, such as CIP4, might be involved in apoptosis development. mRNA and protein expression of CIP4 was identified by Real time RT-PCR and western blot in THP-1 cells. HBVDNAPTP1 could down-regulate the expression of CIP4 at both transcription and translation levels.

**Conclusion**: HBVDNAPTP1 may be involved in the positive regulation on the initiation of monocyte apoptosis. The result contribute to reveal the HBVDNAPTP1 biological functions and provide new evidences for further exploration of the regulatory mechanism of HBVDNAPTP1.

**Keywords**: Hepatitis b virus, DNA polymerase, Trans-regulation, Suppression subtractive hybridization, CDC42-interacting protein 4

## Introduction

It is well known that after the infection of HBV into the target cells, the interaction of the virus genome and proteins with genes and proteins in target cells plays important roles in determining HBV replication, immune evasion, and chronic infection (1). In recent years, it has been found that complex trans-regulation mechanism is involved in the interaction of HBV with target cells and the HBV proteins play a trans-regulative role in gene expression in target cells (2). The critical antigen components of HBV associated with trans-regulation function have been found in target cells and the specific mechanisms have been clarified, which are of great significance in confirming the pathogenic mechanisms of HBV and discovering effective prevention and treatment methods. HBV DNA polymerase transactivated protein 1 (HBVDNAPTP1) is a protein that is worth studying. Thus, suppression subtractive hybridization technology (GenBank accession

no. AY450389) has been used to study the transregulatory target genes of the HBV DNA polymerase, which was verified by dot blot hybridization. The HepG2 hepatoblastoma cell line was screened to obtain a novel gene (GenBank accession no. AY450389), which was located on the long arm of chromosome 9, region 2, band 2, sub-band 31 (9q22.31). Using Unigene database for expression analysis of tissue distribution, this gene is expressed in a variety of tissues but not in pituitary gland, tonsil, tongue, thymus, trachea and umbilical cord. A preliminary study clarified that HBVDNAPTP1 is localized in the cytoplasm (3).

In the present study, we first analyzed the expression of HBVDNAPTP1 in THP-1 cells and subsequently screened genes transactivated by HBVDNAPTP1 using suppression subtractive hybridization (SSH). SSH was designed to generate a cDNA library which is enriched in differentially expressed sequences and, more importantly, equalized for the number of individual cDNA species, thus allowing the detection of rare transcripts (4). The full-length gene from the library was searched for homologs in GenBank. Finally, the relationship of the CIP4 and the HBVDNAPTP1 was discussed.

## Materials and Methods

### *Construction of vectors*

For construction of eukaryotic expression vector pcDNA3.1 (-)/myc-His A-HBVDNAPTP1, the HBVDNAPTP1 fragment was PCR-amplified with the forward primer (5′- GGATCCATGATGTTTGTGCTGCTAAAC), containing an BamHI site and reverse primer (5′- AAGCTTATAAGTCCTCTCTAAAATTGC), containing a HindIII site. The fragment was inserted into the cloning vector pGEM-T (Promega), resulting in pGEM-T-HBVDNAPTP1. An BamHI-HindIII fragment was isolated from the vector and inserted into BamHI and HindIII digested pcDNA3.1 (-)/myc-His A (Invitrogen), giving pcDNA3.1 (-)/myc-His A-HBVDNAPTP1. The vector was sequenced and digested with corresponding restriction enzymes to confirm the sequence accuracy.

### *Cell culture and transient transfection*

THP-1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 100 IU of penicillin and 100 μg of streptomycin per mL, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone), at 37 ℃ in a 5% $CO_2$ and 90% relative humidity. The cells were seeded out the day prior to transfection at a density of $8\times10^5$ cells per 35 mm dish and reached 50% confluence at the time of transfection. All transfections were performed with FuGENE6 Transfection Reagent (Roche) according to the manufacturer's instructions. The medium was changed 5 h after transfection and cells were harvested 40-48 h after transfection. All transfections and assays were repeated independently three times in triplicate.

### *Detection of HBVDNAPTP1 expression*

mRNA from THP-1 cells transfected with pcDNA3.1 (-)/myc-His A-HBVDNAPTP1 and pcDNA3.1(-)/myc-His A was isolated using a micro mRNA purification kit (Amersham Biosciences), and cDNAs were reverse-transcribed from the mRNA. HBVDNAPTP1 expression was detected by Western blotting assay using lysates of the THP-1 cells. The extracts were boiled for 5 min and separated on SDS-PAGE and then were transferred to the nitrocellulose membranes. The membranes were reacted with myc monoclonal antibody and HRP-labeled goat anti-mouse IgG as the first and second antibody, respectively and then with a SuperSignal West Pico Chemiluminescent Substrate Working Solution (Pierce) according to the manufacturer's instructions. The immunoreactive bands were visualized after exposure to X-ray film.

### *Generation and analysis of a subtracted cDNA library*

SSH was performed with the PCR-Select™ cDNA subtraction kit (Clontech) according to the manufacture's protocol. In brief, 2.0 μg of poly A+ mRNA, each from the pcDNA3.1 (-)/myc-His A-HBVDNAPTP1 driver group and the pcDNA3.1 (-)/myc-His A tester group was subjected to cDNA synthesis, respectively. After restriction

with RsaI, small sizes of cDNAs were obtained. The tester cDNAs were then subdivided into two parts, ligated with the specific adaptor 1 and adaptor 2, respectively. After two subtractive hybridization reactions and two suppression PCR amplifications, differentially expressed cDNAs were selectively amplified. Then the second PCR products were used as templates for PCR amplification of G3PDH (a housekeeping gene) at 18, 23, 28, 33 cycles, respectively, to analyze subtraction efficiency. The second PCR products were directly purified using Wizard PCR-Preps DNA Purification System (Promega), and inserted into pGEM-T Easy (Promega) to construct the subtracted library. Colony PCRs were conducted to confirm the size of cDNA inserts being ranged between 200 and 1000 bp by using T7/SP6 specific primers localized in pGEM-T Easy. After DNA sequencing of the positive colonies, nucleotide homology searches were performed using the BLAST program at NCBI.

### *Detection the effect of HBVDNAPTP1 on CIP4 expression*

To detect the effect of HBVDNAPTP1 on CIP4 mRNA, total RNA was extracted from THP-1 cells transiently transfected by pcDNA3.1 (-)/myc-His A and pcDNA3.1 (-)/myc-His A-HBVDNAPTP1 using Trizol reagent (Invitrogen) according to the manufacturer's instructions and was used for real time Real time RT-PCR using the ABI 7500 Fast instrument and SYBR Green PCR master mix (Takara). The following specific primers (sense 5´- ACGCTCAATTGAACCCTGC, antisense 5´- ACGATGGTAGAAGGCACAGC) were used to amplify the CIP4 cDNA (Product size: 201bp). G3PDH specific primers (sense 5´-TGTGTCCG-TCGTGGATCTGA, antisense 5´-TTGCTGTTG-AAGTCGCAGGAG) was used as internal reference (Product size: 150bp). Cycling parameters were 95 ºC for 10 min, 40 cycles at 95 ºC for 15 s, and 60 ºC for 1 min. After PCR amplification, amelting curve was plotted to measure PCR specificity. Real-time PCR results were analyzed using the ΔΔCt method. 2−ΔΔCt represented the average relative amount of mRNA (5).

To detect the effect of HBVDNAPTP1 on CIP4 protein, total soluble proteins were extracted from the transfected THP-1 cells and separated on 12.5% SDS-PAGE for immunoblotting assay. The expression of CIP4 was probed by monoclonal antibody, and β-actin antibody (Santa Cruz) was used as internal reference. The immunoreactive bands were visualized in an UVP Biospectrum Imaging System.

## Results

Digestion of recombinant vector pcDNA3.1 (-)/myc-His A-HBVDNAPTP1 with BamHI/HindIII, Bgl Ⅱ yielded expected bands (Fig. 1). DNA sequencing results indicated that the recombinant vector contains the HBVDNAPTP1 sequence was completely correct. HBVDNAPTP1 protein in THP-1 cells were successfully detected by Western blot (Fig. 2).

To gain a general view of genes which might be involved in pathogenesis of HBV, we identified down-regulated genes in THP-1 cells expressing HBVDNAPTP1 by the generation of a subtracted cDNA library.

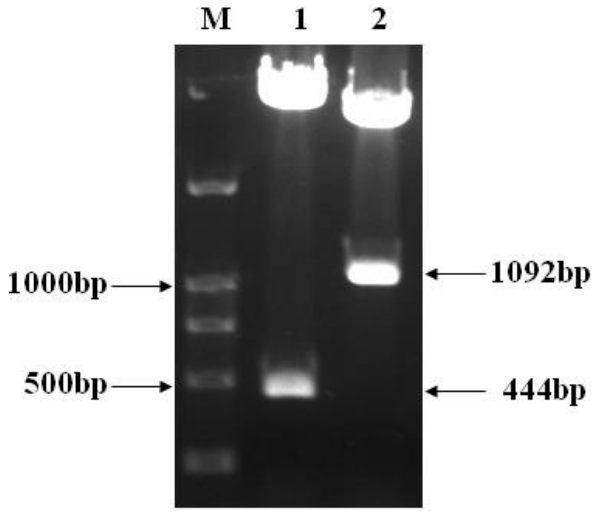



**Fig. 1:** Digestion Identification of pcDNA3.1(-)/myc-His A-HBVDNAPTP1. DNA Marker DL2,000 (lane M). pcDNA3.1(-)/myc-His A-HBVDNAPTP1 Digested by *BamH*I and *Hind*III (lane 1). pcDNA3.1(-)/myc-His A-HBVDNAPTP1 Digested by *Bgl*II (lane 2)

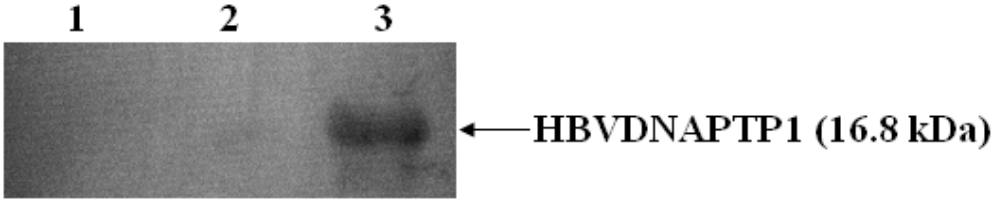



**Fig. 2:** Western blot analysis of transient expression of HBVDNAPTP1 in THP-1 cells. lysates from THP-1 cells (lane 1). lysates from THP-1 cells transfected with pcDNA3.1(-)/myc-His A (lane 2). lysates from THP-1 cells transfected with pcDNA3.1(-)/myc-His A-HBVDNAPTP1 (lane 3)

Subtraction efficiency analysis showed that PCR products of the housekeeping gene G3PDH in unsubtracted library were obviously visible after 18 cycles, however, 28 cycles were required in the subtracted one (Fig. 3), indicating that the abundance of non-differentially expressed gene was effectively reduced and the subtraction method had high subtraction efficiency. Using SSH, a total of one hundred positive clones were obtained.

These clones were prescreened by using PCR amplification to ensure that they had different inserts before sequencing. Among these clones, 79 contained inserts of 200 to 1000 bp. A total of 32 clones from the cDNA library were randomly chosen and sequenced, and their nucleotide sequence homology searches were performed using the BLAST program at NCBI. The analysis results showed that there were 18 coding sequences, of them, 16 were known and 2 unknown genes.

These genes with known functions can be divided into 3 groups, namely genes related to cell signal transduction and apoptosis, cell energy and substance metabolism, cell transcription and protein synthesis. Interestingly, the cell CIP4 was down-regulated by HBVDNAPTP1. Summary of the data is presented in Table 1.

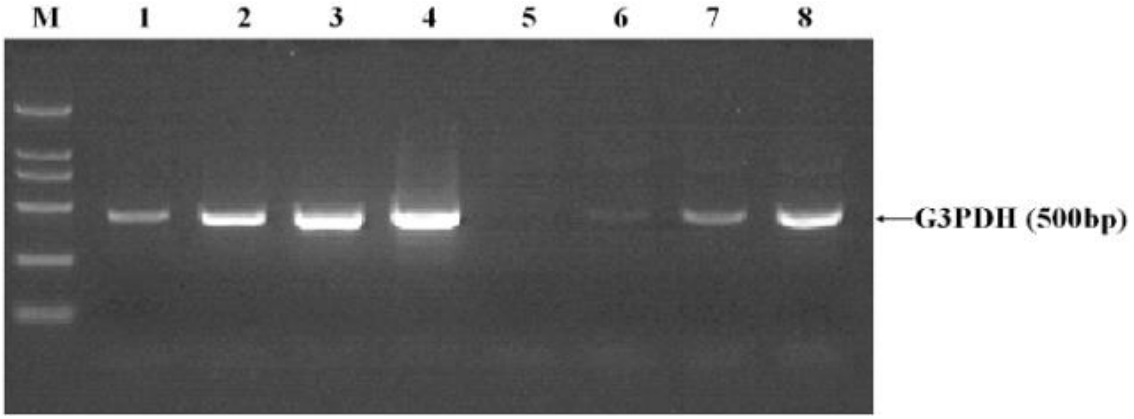
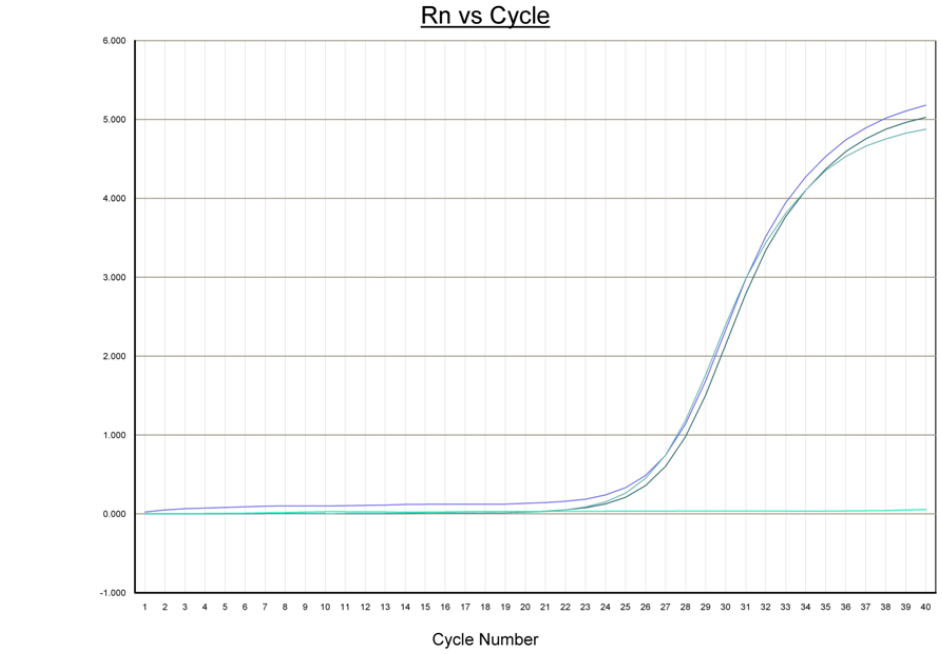


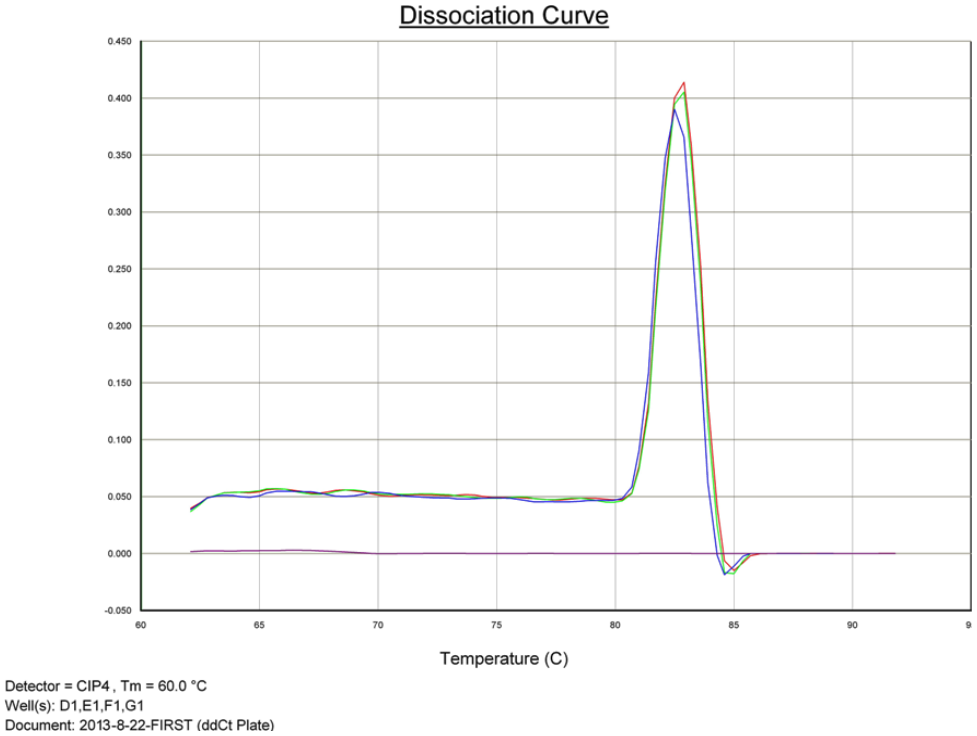

**Fig. 3:** Analysis of subtracted cDNA library (Reduction of G3PDH abundance showed high subtraction efficiency). Unsubtracted secondary PCR product (lanes 1-4) and subtracted (lanes 5-8). DNA Marker DL2,000 (lane M). 18 cycles (lanes 1, 5). 23 cycles (lanes 2, 6). 28 cycles (lanes 3, 7). 33 cycles (lanes 4, 8)

To further elucidate the mechanisms of HBVDNAPTP1 on CIP4 expression at transcription and translation levels, we investigated the effect of HBVDNAPTP1 on expression of the gene. The signals were normalized using the housekeeping gene G3PDH. As shown in Fig. 4, CIP4 mRNA level significantly decreased after transient transfection with pcDNA3.1 (-)/myc-His A-HBVDNAPTP1 (Student-Neumann-Keuls' test, n=3, $*P<0.05$). The Western blot analysis indi-

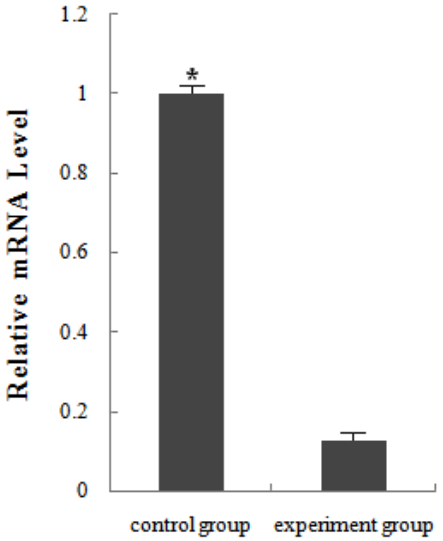
cated that the expression of the gene was high in the control group, whereas in the experiment group, its expression was significantly weakened (Fig. 5). The result indicated that HBVDNAPTP1 could down trans-regulate the expression of CIP4 at both transcription and translation levels.




4A




4B




4C

**Fig. 4:** Quantitative Real time RT-PCR analysis of CIP4 gene in THP-1 cells. The amplification plot of CIP4 gene (Fig 4A). The dissociation curve of CIP4 gene (Fig 4B). The mRNA level of CIP4 in the experiment group (transfected with pcDNA3.1(-)/myc-His A-HBVDNAPTP1) compared with the control group (transfected with pcDNA3.1(-)/myc-His A) was detected using quantitative Real time RT-PCR. Data is expressed as relative quantity (Fig 4C).

**Table 1:** Sequence analysis of 32 clones isolated from subtracted cDNA library down trans-regulated by HBVDN-APTP1

| Gene name | GenBank Accession | Homology (%) |
|---|---|---|
| **cell signal transduction and apoptosis** | | |
| **CDC42-interacting protein 4 (*TRIP10*)** | NM_004240 | 100 |
| **FGFR1 oncogene partner 2 (*FGFR1OP2*)** | NM_015633 | 99 |
| **Guanine nucleotide binding protein, beta polypeptide 2 (*GNB2*)** | NM_005273 | 100 |
| **Insulin-like growth factor binding protein 3 (*IGFBP3*)** | NM_001013398 | 100 |
| **Prothymosin, alpha (*PTMA*)** | NM_002823 | 100 |
| **Thymosin beta 4, X-linked (*TMSB4X*)** | NM_021109 | 99 |
| **cell energy and substance metabolism** | | |
| **ATPase, Na+/K+ transporting, beta 3 polypeptide (*ATP1B3*)** | NM_001679 | 99 |
| **ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F6 (*ATP5J*)** | NM_001003701 | 99 |
| **DnaJ (Hsp40) homolog, subfamily C, member 8 (*DNAJC8*)** | NM_014280 | 99 |
| **Lactate dehydrogenase B (*LDHB*)** | NM_001174097 | 98 |
| **NADH dehydrogenase subunit 5 (mitochondrion)** | NC_012920 | 100 |
| **Transferrin receptor (*TFRC*)** | NM_003234 | 99 |
| **cell transcription and protein synthesis** | | |
| **Eukaryotic translation initiation factor 3, subunit K (*EIF3K*)** | NM_013234 | 88 |
| **H2A histone family, member Z (*H2AFZ*)** | NM_002106 | 100 |
| **NADH dehydrogenase 1 alpha subcomplex, 4, 9kDa (*NDUFA4*)** | NM_002489 | 98 |
| **Polymerase (RNA) II (DNA directed) polypeptide L (*POLR2L*)** | NM_021128 | 100 |

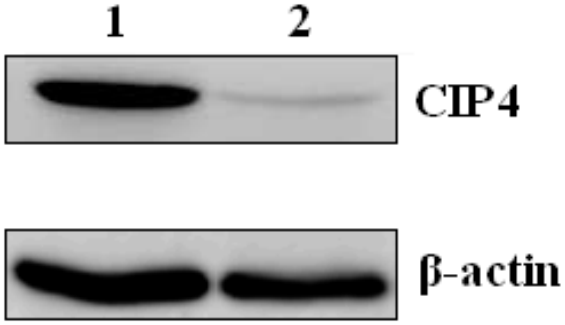



**Fig. 5:** Western blot analysis of CIP4 expression in THP-1 cells. THP-1 cells transfected with pcDNA3.1(-)/myc-His A (lane 1). THP-1 cells transfected with pcDNA3.1(-)/myc-His A-HBVDNAPTP1 (lane 2)

## Discussion

Based on previous research, HBVDNAPTP1 interacts with PILRA intracellular domain. PILRA is a transmembrane receptor with the effect of inhibitory regulation, which is not expressed in lymphocytes but mostly in monocytes (6). As PILRA binds with its ligand (primary cytokines), it triggers PILRA to terminate cell proliferation signal or induce apoptosis signal (7, 8). It is worth mentioning that PILRA in normal PBMCs binds with cytokines but not terminates cell proliferation or triggers apoptosis (9). Due to the interaction of HBVDNAPTP1 and PILRA, it is easy to speculate that HBVDNAPTP1 is very likely to bind with PILRA to mediate the negative regulation of JAK/STAT signaling pathway, and thus activate monocyte apoptosis signal. This study indicated that the expression of CIP4 at both transcription and translation levels in experiment group that stably expresses HBVDNAPTP1 was lower than the controls. Therefore, in a way, it might disprove the pro-apoptotic activity of HBVDNAPTP1.

Chronic infection with HBV is still a big healthy problem worldwide. The liver is the main target organ of HBV infection, but many extrahepatic tissues and organs including the heart, spleen, lung, kidney etc are also infected by HBV (10-12). In recent years, various forms of HBV DNA, replicative RNA intermediates and antigen components have been detected in the PBMC of chronic HBV-infected individuals (13). The major clinical effect of HBV infection of PBMC is to cause host immune dysfunction, which leads to chronic HBV infection and causes latent infection, chronic infection and mother-to-child transmission. Moreover, re-infection may occur after liver transplantation or using drugs to clear viruses from serum (14, 15). Since HBV DNA can be replicated and transcribed by integration into the PBMC genome, the PBMCs have become an important replication sites and propagation vector for HBV (16). PBMCs are mainly composed of lymphocytes, monocytes, granulocytes and other immunocompetent cells, which are supposed to play a crucial role in the body's immune response against HBV infection. However, HBV infection with PBMCs leads to cell dysfunction and a decline in the number of PBMCs, which indicate that HBV DNA replication and transcription in PBMCs cause cell apoptosis and inhibition of cell proliferation. Due to the inadequate HBV-specific cellular immune response, the removal of virus is difficult (16, 17).

To gain further insight on information about the genes trans-regulated by HBVDNAPTP1, SSH was used to clone the genes down trans-regulated by HBVDNAPTP1. Excitingly, down-regulated expression of CIP4 was observed. CIP4 serves as a scaffolding protein for CDC42, a member of the F-BAR family that regulates membrane deformation and endocytosis, playing a key role in ECM deposition and cell migration (18). In vitro studies confirmed that inhibiting CIP4 expression significantly reduced cells invasiveness and migration (19). Low expression of CIP4 has been implicated in the PBMCs behavior of patients with chronic hepatitis B. Above results coincided with the results of previous research. The biologic significance of CIP4 gene down-regulation by HBVDNAPTP1, however, has not been unconfirmed.

To further elucidate the regulatory mechanisms of HBVDNAPTP1 on CIP4 expression, the down-regulated expression of CIP4 in THP-1 cells has been confirmed by cell transient transfection at mRNA level and protein level. It is reasonable to believe that the transformation effect of

HBVDNAPTP1 is involved in the down-regulation of the expression of CIP4.

## Conclusion

The transactivator function of HBVDNAPTP1 was tested and constructed a subtracted cDNA library of genes transactivated by HBVDNAPTP1. Furthermore, HBVDNAPTP1 could down-regulate the expression of CIP4 at both transcription and translation levels. Therefore, HBVDNAPTP1 may be involved in the positive regulation on the initiation of monocyte apoptosis. These findings provide new insight on the biological functions of HBVDNAPTP1 and new directions to elucidate the mechanisms of chronic infection with HBV.

## Ethical Considerations

All ethical issues including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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