

# Tumor necrosis factor- $\alpha$ -induced protein 1 and immunity to hepatitis B virus

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## Abstract

**AIM:** To compare the gene expression profile in a pair of HBV-infected twins.

**METHODS:** The gene expression profile was compared in a pair of HBV-infected twins.

**RESULTS:** The twins displayed different disease outcomes. One acquired natural immunity against HBV, whereas the other became a chronic HBV carrier. Eighty-eight and forty-six genes were found to be up- or down-regulated in their PBMCs, respectively. Tumor necrosis factor- $\alpha$ -induced protein 1 (TNF- $\alpha$ IP1) that expressed at a higher level in the HBV-immune twins was identified and four pairs of siblings with HBV immunity by RT-PCR. However, upon HBV core antigen stimulation, TNF- $\alpha$ IP1 was downregulated in PBMCs from subjects with immunity, whereas it was slightly upregulated in HBV carriers. Bioinformatics analysis revealed a K+ channel tetramerization domain in TNF- $\alpha$ IP1 that shares a significant homology with some human, mouse, and *C elegans* proteins.

**CONCLUSION:** TNF- $\alpha$ IP1 may play a role in the innate immunity against HBV.

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**Key words:** TNF- $\alpha$ ; HBV; Immunity

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## INTRODUCTION

Though substantial advances have been made in understanding of the pathogenesis of hepatitis B virus (HBV), HBV infection remains a global health threat with currently more than 350 million carriers and causes about one million deaths worldwide. The situation is particularly severe in high-endemic areas like southeastern Asia. For instance, a study of 16 334 subjects in 1978-1979 in Hong Kong suggested that 43% of the local population have evidence of past infection and 10% are HBs Ag carriers<sup>[1]</sup>.

The major spread mode of HBV in most high endemic areas, such as Hong Kong and China, is perinatal transmission, which accounts for 40%-50% of chronic HBV infection<sup>[2,3]</sup>. The reason for the preponderance of perinatal transmission among Orientals is at least in part related to the high prevalence of HBV infection among the Asian carriers of reproductive age<sup>[2]</sup>. Though the incidence of perinatal transmission is high in China, not all siblings in the same family with HBV-infected mothers remain persistently infected with HBV as some acquired natural immunity against the virus. We are among the first group to demonstrate that the use of HLA-matched donor marrow from siblings with natural immunity could enable serological clearance of HBV in their HBsAg positive recipient siblings<sup>[4-7]</sup>. This indicates that the transfer of certain molecules from the donor's immune system is sufficient to confer the recipient immunity against HBV. However, the identities of these molecules are unknown.

In this study, Affymetrix cDNA microarray was employed to investigate the differential gene expression patterns in PBMCs in an identical twin pair who had different outcomes to HBV infection. Use of the twin pair could eliminate the potential confounding factors like age, gender, and living environment, etc. A novel cytokine signaling-related gene, TNF- $\alpha$ IP1, is significantly downregulated in chronic HBV carriers. Moreover, we have confirmed that this gene is indeed differentially expressed in several groups of HBV-infected siblings who display different disease outcomes by reverse transcription-polymerase chain reaction (RT-PCR). We also found that upon HBcAg stimulation, TNF- $\alpha$ IP1 exhibited different

responses between PBMC isolated from subjects with immunity and chronic infection. These results suggest that TNF- $\alpha$ IP1 may be involved in immunity against HBV infection. An understanding of the immunological and genetic differences in PBMCs of these sibling pairs should provide insight into the molecular mechanisms of the protection and enable a more rationale design of therapeutic regimen for chronic HBV infection in Chinese.

## MATERIALS AND METHODS

### Patient sample information

Eight groups of HLA-A, B and DR identical Chinese siblings (including pair of identical twins) who differed in their outcomes to HBV infection were used in this study. RNA was isolated from PBMC of the siblings with spontaneous recovery (anti-HBs and anti-HBc positive) and their corresponding HLA-matched HBV-infected siblings (HBsAg positive).

### PBMC preparation, RNA extraction, and labeling

Heparinized venous blood was collected and PBMC was separated by density-gradient centrifugation over Ficoll-Hypaque. Total RNA was extracted from PBMC using RNeasy kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. For each sample, 60 mg of total RNA was reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

### DNA microarray

Generation of antisense RNA (aRNA) probe for cDNA microarray analysis was carried out as described previously<sup>[8]</sup>. Briefly, mRNA was amplified using two or three rounds of cDNA synthesis followed by aRNA synthesis. T7-promoter-oligo-dT [5'-GCCAGTGAATTGTAATAC GACTCACTATAGGGAGGCGG-(dT)<sub>24</sub>-3'] was used for cDNA synthesis and T7 RNA polymerase was used for aRNA synthesis. Ten micrograms of starting total RNA was used to obtain 10 mg of final aRNA. During the process of the final aRNA synthesis, biotinylated UTP and CTP were used for labeling purpose (Enzo, Farmingdale, NY, USA). Detection of the hybridized probe using streptavidin-phycoerythrin fluorescent conjugate was done according to manufacturer's protocol (Molecular Probes, Eugene, OR, USA).

The labeled aRNA probe from PBMC was hybridized to a HU 95A gene chip representing 12 000 full-length human genes (Affymetrix, Santa Clara, CA, USA). The threshold for significant up- or down-regulation was 2.0- and 0.5-fold, respectively. Hybridizations were typically carried out for 16 h at 45 °C followed by washing, staining and using Affymetrix fluidic stations. Stained arrays were scanned in the G2500 A Hewlett-Packard Gene Array Scanner (Hewlett-Packard, Palo Alto, CA, USA) at the excitation wavelength of 488 nm. The amount of emitted light was proportional to the bound target at each location on the probe arrays.

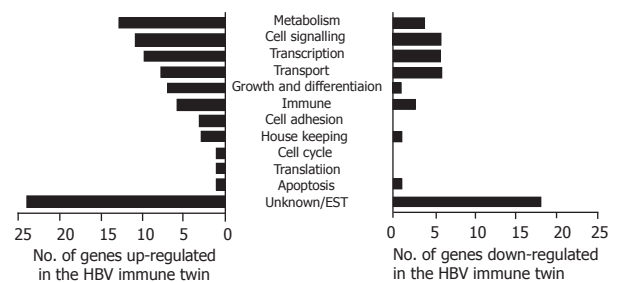
### RT-PCR

Total RNA was reverse transcribed using the Superscript II (Invitrogen) according to the manufacturer's instructions. PCR conditions for pre-B-cell colony-enhancing factor (PBEF), IL-18 receptor accessory protein (IL-18 RAP), TNF- $\alpha$ IP1 and GAPDH were as follows: at 94 °C for 30 s, at 57 °C for 30 s, at 72 °C for 1 min for 35, 35, 35, and 32 cycles, respectively. The primer sequences of the genes tested were PBEF (sense: 5'-AAAAGCTGTTCCTGAGG GCTTTG-3'; anti-sense: 5'-TGACCACAGATACAGGCA CTGATG-3'); IL-18RAP (sense 5'-CCAGAGCCACAGA AATCACATTTTC-3'; anti-sense 5'-CAAGAAATAGAGCC AGTGCTCCCA-3'); TNF- $\alpha$ IP1 (sense: 5'- TTACCTCCG AGATGACACCATCAC-3'; anti-sense: 5'-TCCTCATCTT CACTGGGGGAA-3').

## RESULTS AND DISCUSSION

Perinatal transmission is the most common spread mode of hepatitis B infection in Chinese. Yet not all the siblings infected with HBV from mothers become chronic HBV carriers. It appears that different disease outcomes of these siblings represent success and failure of their immune systems in controlling HBV infection. In this study, we attempted to explore this phenomenon by investigating gene expression profiles in PBMCs of two identical twins who displayed diverse disease outcomes.

The differences in PBMC gene expression between two identical twins were analyzed using a DNA microarray chip containing 12 000 human expressed genes. Eighty-eight genes were expressed at a higher level and 46 genes were expressed at a lower level in twins with immunity (HBV resistant) compared to the HBV carrier twins [HBV susceptible). These genes were grouped on the basis of their predicted functions into 12 major groups (metabolism, cell signaling, transcription, transport physiology, growth, and differentiation, immune system, cell adhesion, house keeping, cell cycle, translation, apoptosis, and unknown genes (ESTs)) (Figure 1). We found that genes with unknown functions represented the major portion of genes with altered expression levels in the HBV immune twin.



**Figure 1** Number of genes upregulated or downregulated in the immune twin compared to the HBV carrier twin. PBMCs from two HBV infected twins with different disease outcomes were analyzed for gene expression using DNA microarray. Eighty-eight genes were identified as upregulated and 46 downregulated. They were further divided into 12 groups based on their presumed functions.

**Table 1** Differential expression of immunity-related genes in PBMCs of HBV identical twin patients

UniGene ID	Gene name	Known function(s)	Fold change
Hs.244613	Signal transducer and activator of transcription 5b (STAT 5b)	Signal transducer of IL-2, IL-4, CSF1, and different growth hormones. Important for TCR signaling, apoptosis, adult mammary gland development, and sexual dimorphism of liver gene expression	-4.2
Hs.239138	Pre-B-cell colony-enhancing factor (PBEF)	A cytokine that increases the expression of IL-6 and IL-8 in fetal membrane and may be important in both normal spontaneous labor and infection-induced preterm labor	2.2
Hs.158315	Interleukin 18 receptor accessory protein (IL-18RAP)	An accessory subunit of the heterodimeric receptor for IL-18 enhances the IL-18 binding a ligand binding subunit of IL-18 receptor. The coexpression of IL-18R1 and this protein is required for the activation of NF- $\kappa$ B and MAPK8 (JNK) in response to IL-18	3.0
Hs.76090	Tumor necrosis factor-alpha-induced protein 1 (endothelial) (TNF- $\alpha$ IP1)	Unknown	2.3
Hs.225948	Chemokine (C-C motif) ligand 27 (CCL27)	Cytokine that plays a role in mediating homing of lymphocytes to cutaneous sites. It specifically binds to chemokine receptor 10 (CCR10). Studies of murine protein indicate that these protein-receptor interactions have a pivotal role in T-cell-mediated skin inflammation	-2.5
Hs.301921	C-C chemokine receptor type 1 (C-C CKR-1)	Cytokine receptor important for host protection from inflammatory response and susceptibility to virus and parasite	-2.7
Hs.57735	Scavenger receptor class F, member 1 (SCARF1)	Scavenger receptor that has roles in the binding and degradation of acetylated low density lipoprotein and may be involved in atherosclerosis	-2.2
Hs.4930	Low density lipoprotein receptor-related protein 4 (LRP4)	A membrane protein which may be involved in calcium ion binding	-2.8

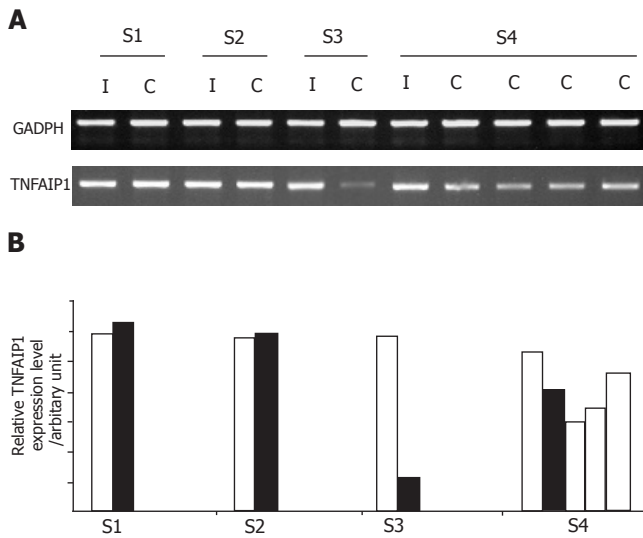
<sup>1</sup>Fold changes between the HBV immune twin pair. Positive number indicates upregulation, whereas negative number denotes downregulation.

We are interested in the immune system-related genes that are putative candidates for conferring HBV immunity. Among the up- or down-regulated genes, eight genes were related to immune responses including several cytokine/chemokine signaling-related genes (Table 1). Three genes, namely cytokine PBEF, interleukin IL-18 receptor (R) accessory protein and TNF- $\alpha$ IP1 were upregulated in the immunity twin. PBEF is a cytokine transcribed in human bone marrow, liver tissue and muscle<sup>[9,10]</sup> and synergized with stem cell factor (SCF) and IL-7 activating pre-B-cell colony formation<sup>[11]</sup>. IL-18R accessory protein enhances the IL-18 binding activity of IL-18R1 and is required for the activation of nuclear factor-kappa B (NF- $\kappa$ B) and mitogen-activated protein kinase 8 (MAPK 8) in response to IL-18. TNF- $\alpha$ IP1 is first identified as a primary response gene in human umbilical vein endothelial cells towards TNF- $\alpha$  stimulation<sup>[12]</sup>, but yet with no known function.

Five genes were downregulated in the HBV-immuned twins. They are the signal transducer and activator of transcription 5b (Stat5b), which is crucial for normal immune function and T-cell-mediated mitogenic signals

and is a key signal transducer of T-cell receptor<sup>[13]</sup>. The chemokine (C-C motif) ligand 27 (CCL27) and C-C chemokine receptor type 1 (C-C CKR-1) are important for host inflammatory response. We also observed the downregulation of the scavenger receptor class F member 1 (SCARF1) and low-density lipoprotein receptor-related protein 4. Scavenger receptors encompass a broad range of molecules involved in receptor-mediated endocytosis of selected polyanionic ligands, including modified low-density lipoproteins (LDL)<sup>[14]</sup>. Further investigating the role of these genes and the molecular differences between immune siblings and HBV chronic carrier siblings may help us in designing therapeutic measures that modulate the immune system of patients in controlling HBV infection.

Of the eight immune system-related genes, TNF- $\alpha$ IP1 demonstrated the highest frequency of differential expression among the other groups of HBV-infected HLA-matched siblings with different disease outcomes as determined by semi-quantitative RT-PCR (Figure 2A). As shown in Figure 2B, the expression level of TNF- $\alpha$ IP1 was

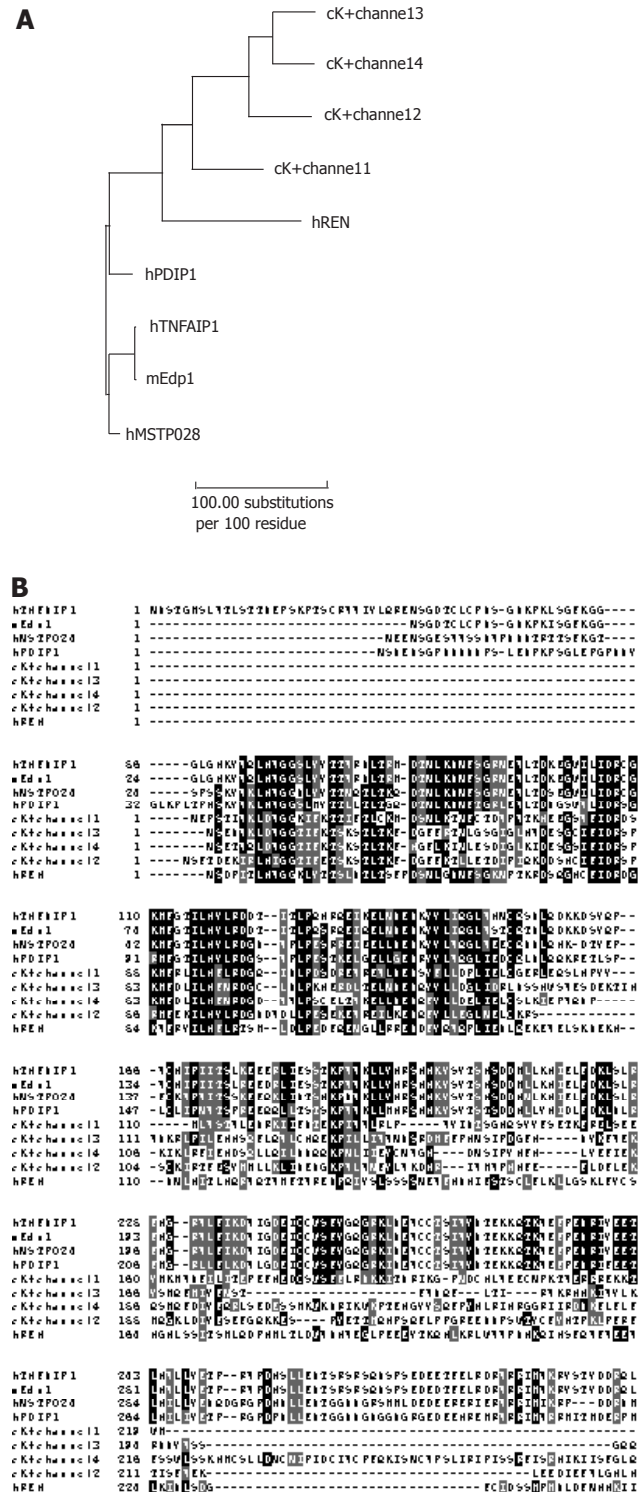


**Figure 2** Expression levels of TNF- $\alpha$ IP1 gene in HBV-infected siblings with different disease outcomes. **A:** RT-PCR analysis of TNF- $\alpha$ IP1 in four sibling pairs; **B:** Fold changes of band intensity of TNF- $\alpha$ IP1 in different sibling groups. Open bar: HBV immune patients; black or patterned bars: HBV carrier; S1: sibling group 1; S2: sibling group 2; S3: sibling group 3; S4: sibling group 4.

lower in HBV carriers than in their HBV immune siblings in two siblings groups. Noticeably, the expression of TNF- $\alpha$ IP1 was significantly downregulated in HBV carrier siblings of group 3 and all four carrier siblings displayed down-regulation of TNF- $\alpha$ IP1. Suggesting that TNF- $\alpha$ IP1 may be a gene differentially expressed in PBMCs of HBV immune and carrier patients.

Recent studies suggest that cytokine response plays an important role in successful host defense against HBV infection. In transgenic mouse and chimpanzee model, cytotoxic T lymphocytes do not directly kill HBV-infected hepatocytes, but inhibit the viral replication through the actions of TNF- $\alpha$  and interferon (IFN- $\gamma$ )<sup>[15,16]</sup>. Romero and Lavine<sup>[17]</sup> demonstrated that TNF- $\alpha$  and IFNs can downregulate the activity of HBV core/pregenomic (C/P) promoter, hence contributing to viral clearance. Moreover, activated intrahepatic antigen-presenting cells can inhibit liver HBV replication by secreting IL-12 and TNF- $\alpha$ <sup>[18]</sup>. One of the downstream mechanisms of how TNF- $\alpha$  modulates HBV infection is via the activation of NF- $\kappa$ B, which can inhibit HBV replication<sup>[19]</sup>. Interestingly, we have shown that TNF- $\alpha$ IP1 (a TNF- $\alpha$  inducible gene) expresses at a higher level in HBV immunized twin. TNF- $\alpha$ IP1 was first identified as an endothelial primary response gene towards TNF- $\alpha$  (then named B12), which induces TNF- $\alpha$ IP1 expression rapidly and transiently. Biochemical characterization suggests that TNF- $\alpha$ IP1 is a 36 ku protein, which may play a regulatory role and locate intracellularly<sup>[12]</sup>. The role of TNF- $\alpha$ IP1 in TNF- $\alpha$ -mediated NF- $\kappa$ B activation and HBV replication inhibition is of interest for further study.

Phylogenetic analysis showed that TNF- $\alpha$ IP1 could evolve into human and mouse endothelial protein 1 (Edp1), reflecting the fact that Edp1 is likely the mouse ortholog of TNF- $\alpha$ IP1 (Figure 3A). Predicted from the



**Figure 3** TNF- $\alpha$ IP1 shares significant homology with some human, mouse, and *C. elegans* proteins, having common K+ channel tetramerization/POZ domain. **A:** Phylogenetic analysis of evolutionary distance of the protein sequences. The protein sequences were analyzed by the Growtree program provided by the GCG package. Kimura distance was adopted as the distance correction method. **B:** Amino acid sequence alignment of TNF- $\alpha$ IP1 (A41784) with mouse Edp1 protein (AAC78826), human MSTP028 protein (NP\_114160), PDIP1 (AAL14962), human REN protein (XP\_208568), and four *C. elegans* K+ channel family members and with highest homology to TNF- $\alpha$ IP1 (NP\_499312, NP\_494320, NP\_494476 and NP\_494315). Black line: K+ channel tetramerization/POZ domain.

amino acid sequence of TNF- $\alpha$ IP1 (A41784), there is a K<sup>+</sup> channel tetramerization domain in the middle of the sequence. This N-terminal, cytoplasmic tetramerization domain (T1) of voltage-gated K<sup>+</sup> channels encodes molecular determinants for subfamily-specific assembly of alpha-subunits into functional tetrameric channels<sup>[20]</sup>. It is distantly related to the BTB/POZ domain which plays an oligomerization role in the function of this protein. Interestingly, the K<sup>+</sup> channel tetramerization domain is the common domain feature shared by many TNF- $\alpha$  inducible proteins. For instance, mouse TNF- $\alpha$  inducible proteins such as Edp1 share high homology with the entire amino acid sequence of TNF- $\alpha$ IP1 (97% identity) (Figures 3A and B). It is also noticed that there was a group of C elegans proteins containing the K<sup>+</sup> channel tetramerization domain. However, the function of this group of proteins is unknown.

Among the proteins containing K<sup>+</sup> channel tetramerization domain, polymerase delta-interacting protein 1 (PDIP1) is best characterized<sup>[21]</sup>. PDIP1 is a TNF- $\alpha$  inducible protein and plays a role in linking cytokine activation and DNA replication in the liver as well as in other tissues. It localizes inside the nuclei and interacts with DNA polymerase  $\delta$  small subunit (p50) and proliferating cell nuclear antigen (PCNA). In addition, it modulates DNA polymerase  $\delta$  activity in a PCNA dependent manner. Importantly, it shares high homology (62% identity) with TNF- $\alpha$ IP1 (B12). PDIP1 is involved in TNF- $\alpha$ -mediated hepatocyte regeneration and stimulates hepatocyte proliferation upon TNF- $\alpha$  signal<sup>[21]</sup>. It is of interest to know whether this is also true for TNF- $\alpha$ IP1 using cell proliferation assay.

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