

Subversion of cellular inhibitor of apoptosis protein 2 gene expression in hepatitis B virus infected peripheral blood mononuclear cells

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ABSTRACT

Members of cellular inhibitor of apoptotic protein family (cIAP) plays crucial role in regulating cellular apoptosis. Overexpression of cIAP2 gene has been implicated in promoting hepatitis B virus (HBV) mediated hepatocellular carcinoma (HCC). cIAP2 has also been shown to inhibit HBV replication by in vitro studies.

Since the expression status of cIAP2 has not been studied in HBV infected cells from patients, we tried to evaluate the same in peripheral blood mononuclear cells of HBV infected subjects.

A total of 42 HBV infected subjects were clarified for their status of infection by biochemical, serological and real time polymerase chain reaction (PCR) assay. Total RNA obtained from isolated PBMCs were subjected to reverse transcription PCR for amplification of cIAP2 mRNA. HBV pregenomic RNA (pgRNA) was also evaluated as a marker of intracellular viral replication. PBMCs from 24 healthy voluntary blood donors were used as control cells.

In comparison to controls, expression of cIAP2 was significantly ($p=0.001$) downregulated in infected PBMCs obtained from patients. Considering the role of cIAP2 expression in controlling viral infection, the present finding is indicative of HBV mediated inhibition of antiviral defense mechanism of infected host cell that favor viral persistence in the host cell.

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KEYWORDS

Gene expression;
Hepatitis B virus;
cIAP2;
Peripheral blood
mononuclear cells.

INTRODUCTION

The biological process of apoptosis plays a pivotal role in regulating homeostasis and immune defense of organisms by clearing superfluous, abnormal or infected cells. Execution of the apoptotic program upon activation by a cell death signal is being determined by a subtle balance between pro-apoptotic and anti-apoptotic

mechanisms where, pro-apoptotic proteins prop up apoptosis and anti-apoptotic proteins hinder apoptosis. Inhibitors of apoptosis proteins (IAPs) can stall the downstream caspase activation pathways, and, thus, control the progress of apoptosis^[1]. The studies have shown that the apoptotic cell death plays an important physiological role for normal cell development and tissue homeostasis whereas, dysregulation of apoptosis

has been implicated in carcinogenesis and tumor progression^[2].

The primarily hepatotropic and secondarily lymphotropic hepatitis B virus (HBV) is a major human pathogen responsible for acute and chronic liver disease where more than 350 million people are chronically infected world-wide with HBV and more than one third of these individuals develop grave liver diseases such as hepatocellular carcinoma (HCC), resulting an estimated 1 million mortality per annum^[3].

Recently, cellular inhibitor of apoptosis protein 2 (cIAP2) has been reported as a potent suppressor of apoptotic cell death as well as is involved in the tumor necrosis factor alpha (TNF- α)-induced anti-hepatitis B virus (HBV) response^[4]. Since HBV is known to produce chronic infection with the potential to remain as inactive carriers without generating overt clinical symptoms in majority of the infected subjects plus also able to trigger tumorigenesis in the liver, we are interested in whether HBV could modulate the expression of cIAP2 in virus infected cells. Henceforth, we evaluated the expression status of cIAP2 mRNA in HBV infected peripheral blood mononuclear cells (PBMCs) of HBV infected subjects who are clinically considered as inactive carriers of the disease. We relied on the detection of HBV pregenomic RNA (pgRNA) in PBMCs as marker of the presence of replicating HBV in the cells since pgRNA is the major replicating intermediate of the virus.

MATERIALS AND METHODS

Patients

Forty two HBsAg positive subjects sorted out on the basis of past history, clinical presentations, anti-HBc IgM/IgG status and imaging data were enrolled in the study. Patients coinfecting with HIV, HAV, HCV, HDV, or HEV were excluded from this study. A group of 24 healthy voluntary blood donors served as controls. Prior informed consents were taken from all the study subjects and the study protocol was approved by the institutional ethics committee

Serologic and biochemical parameters

Serum aspartate transferase (AST) and alanine transferase (ALT) levels were estimated by an automated clinical biochemistry analysis system (Randox, Oceanside, CA). The serum HBeAg and anti-

HBe status of the subjects were ascertained by commercial enzyme-linked immunosorbent assay (ELISA) kits (Amar-EASE, Taiwan) as per the manufacturer's instructions.

HBV DNA quantitation

HBVDNA was extracted from sera by the High Pure System Viral Nucleic Acid Kit (Roche Molecular Systems Inc, USA) as per manufacturer's protocol. Amplification and quantitation of extracted HBV DNA as viral load was performed by Cobas® TaqMan® 48 Analyzer (Roche Diagnostics, USA) using real time Cobas® TaqMan® HBV test kit (Roche Molecular System, USA) as per manufacturer's instructions.

Isolation of total RNA from PBMCs

PBMCs were immediately isolated from EDTA containing whole blood using histopaque-1077 (Sigma chemicals, USA) by recommended procedure. Total RNA was extracted from the isolated cells by Trizol (Life Technologies, USA) method and the extracted RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water.

Preparation of cDNA

Extracted RNA was subjected to reverse transcription reaction using random hexamers and MuLV-H reverse transcriptase (Fermantas Life Sciences, Germany) to generate a common cDNA pool. Prior to reverse transcription, 1 μ g of total RNA was treated with 1 U of deoxyribonuclease (DNase I amplification grade, Gibco- BRL, USA) to remove all the contaminating DNA. The possibility of presence of traces of DNA was further ruled out by employing control reactions devoid of reverse transcriptase enzyme. RNA was reverse transcribed (60 min at 37°C) with 200 U of MMuLV reverse transcriptase (Fermantas Life Sciences, Germany) in 20 μ L volume of 5 \times RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂) supplemented with 5 mM dithiothreitol (DDT), 0.5 mM deoxynucleoside triphosphates (dNTPs, Fermantas Life Sciences, Germany), 25 U ribonuclease inhibitor (Promega Corporation, Madison, WI, USA) and 200 ng random hexamers (Fermantas Life Sciences, Germany).

Polymerase chain reaction (PCR) amplification of cIAP2 gene

Upon heating (95°C, 1 min) and quick-chilling on

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ice, an aliquot of 5 μ L (0.3 μ g) of the cDNA pool was used for PCR amplification in 50 μ L of 10 \times buffer solution (100 mM Tris- HCl pH 9.3, 500 mM KCl, 1% Triton X-100) containing 0.08 mM dNTPs, forward and reverse primers (100 pM each), 1.5 mM MgCl₂ and 2 U of Taq DNA polymerase (Fermentas Life Sciences, Germany). After initial denaturation at 94°C for 5min, cIAP2 cDNA fragments were amplified by 35 cycles (94°C – 15sec, 55°C – 30sec & 72°C – 45sec per step) followed by a final extension step at 72°C for 5min. The forward and reverse primers used were (5'-3') d (GAGGAGACAGTCCTA CTGAAA) and (CATAGCATTATCCTTCGGTTC) respectively^[5] which yielded a product of 433bp. Beta-actin as house keeping gene was amplified by 30 cycles (94°C, 55°C & 72°C; 1 min per step) using forward and reverse primers (5'-3') d(TCT ACA ATG AGC TGC GTG TG) and d(GGT GAG GAT CTT CAT GAG GT) generating amplicon of 314bp^[6]. Blank reactions without cDNA template were performed in all experiments as negative reaction control. Each amplified product (10 μ l) was subjected to 2% agarose gel electrophoresis (100 V, 45 min) along with a 100 bp DNA ladder and visualized by UV fluorescence after staining with ethidium bromide. Upon capturing the gel image by Bio-Capt imager (Vilber Lourmat, France), the integrated density of DNA band was evaluated by using the software Image J 1.42 (Broken Symmetry Software, USA) in respect to the known standard marker as a control with units nanogram per milliliters (ng/ml) and further normalized against the housekeeping gene beta actin used as internal control to define the expression of respective genes by the density of the band^[6,7].

Polymerase chain reaction amplification of HBV pregenomic RNA (pg RNA)

In line to make certain the presence of replicating HBV through presence of pgRNA in PBMCs, cDNA fragments obtained from Trizol extracted PBMC derived total RNA were further amplified using an amplification protocol of 40 cycles (94 °C–30 s, 58 °C–30 s & 72 °C–30 s per step), after an initial denaturation at 95°C for 5 min and later a final extension at 72 °C for 10 min using up and downstream primers (5'-3') d(GCC TTA GAG TCT CCT GAG CA), and d(GAG GGA GTT CTT CTT CTA GG) as mentioned ear-

lier^[7]. The PCR product thus obtained was finally elucidated upon 2% agarose gel electrophoresis resolving an amplicon of 364 bp after staining with ethidium bromide followed by densitometric evaluation. Corresponding plasma of respective PBMC samples were subjected to identical extraction and amplification procedures as controls to rule out the possibility of contamination from plasma.

Statistical analyses

Descriptive statistics (mean, median, standard deviations, Inter Quartile Range [IQR]) and Student's *t*-test were performed using the GraphPad Prism 3.0 software (GraphPad, San Diego, CA, USA). A value of *p*<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

All the patients were adults (Mean age \pm SD=36.4 \pm 9.1 years), and consisted of 31 males and 11 females respectively. The detailed demographic, biochemical and virological characteristics of 42 patients are shown in TABLE 1. Control subjects had a mean \pm SD age of 32.7 \pm 10.2 years and consisted of 15 males and 9 females.

TABLE 1 : Demographic and clinical features of the study subjects

| Parameters | Value-Mean \pm SD |
|-------------------------|---------------------|
| Age | 36.4 \pm 9.1 |
| M:F | 31:11 |
| AST (IU/L) | 41.7 \pm 19.5 |
| ALT (U/L) | 31.2 \pm 11.7 |
| HBV DNA (Log Copies/mL) | 3.28 \pm 0.71 |
| HBv pg RNA (ng/ml) | 0.58 \pm 0.18 |

In comparison to control, the expression of cIAP2 gene was noted to be reduced in HBV infected PBMCs of patients (Figure 1,a,b). Semiquantification of the PCR product by densitometry upon normalization against the house keeping gene beta actin further revealed the downregulation of cIAP2 in HBV infected PBMCs. cIAP2 gene expression was significantly downgraded (*p*=0.001) in HBV infected cells having median value of 166.7 ng/ml (mean \pm SD=158.5 \pm 57.8, IQR=108.8) against the median value of 256.2 ng/ml (mean \pm SD=219 \pm 93.2, IQR=164.5) in non infected cells obtained from healthy controls (Figure 2).

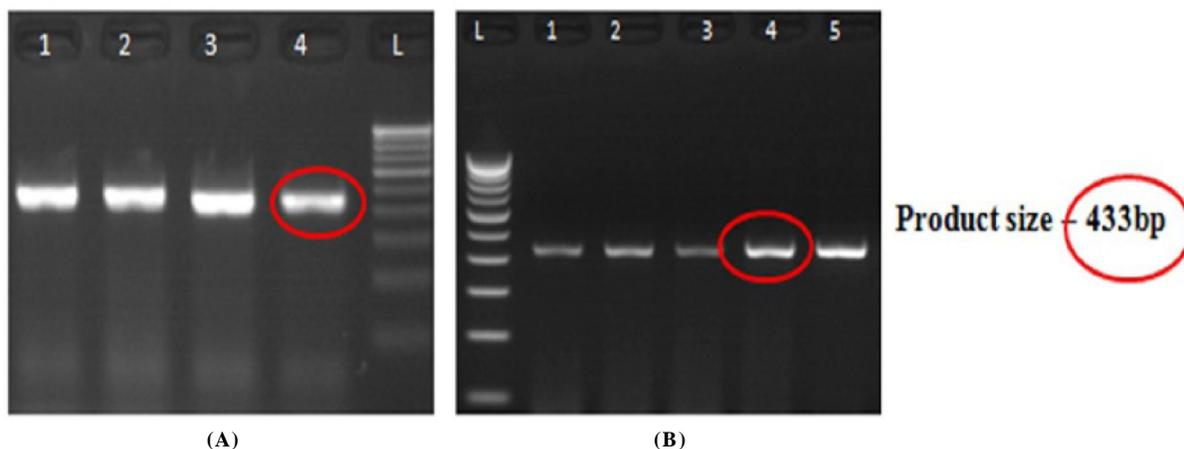


Figure 1 : (A) Representative pattern of cIAP2 PCR products (433bp) obtained from PBMCs separated by 2% agarose gel. (A) cells from controls and (B) cells from patients; L=100bp DNA Ladder.

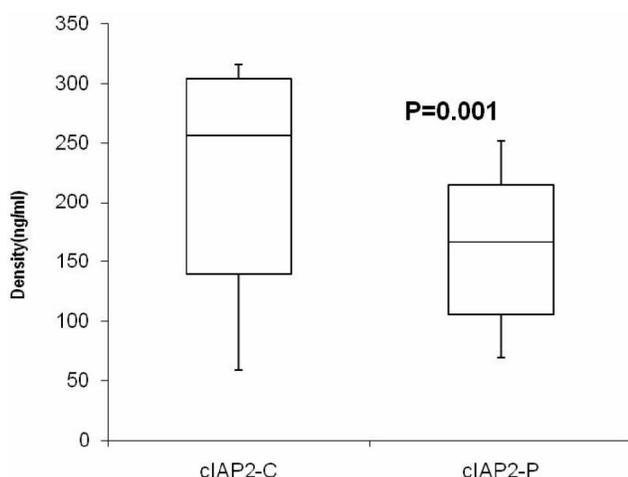


Figure 2 : Expression of cIAP2 gene is significantly reduced in infected PBMCs of patient (P; n=42) than control (C; n=24) groups. The boxes represent the interquartile ranges, the whiskers indicate the ranges and the center line in the box indicate the medians.

Members of the anti-apoptotic family of proteins, inhibitors of apoptosis proteins (IAPs) acts as endogenous inhibitor of caspases, the main executioners of apoptosis^[8]. Many studies have established a circumstantial association between IAPs and cancer where overexpression of several IAP family members has been detected in several classes of human cancers^[9,10]. Overexpression of cIAP2 has been observed in pancreatic ductal adenocarcinomas^[11] apart from its role in cellular apoptotic mechanism, cIAP2 inducible by TNF α was found to inhibit HBV protein synthesis, viral replication, and transcription through activation of the NF- κ B signaling pathway^[12].

Noncytopathic inhibition of HBV replication has been reported which has been shown to be mediated through TNF- α secreted by HBV specific cytotoxic T

lymphocytes (CTLs)^[13]. Furthermore, involvement of cIAP2 in the tumor necrosis factor alpha (TNF- α)-induced anti-hepatitis B virus (HBV) response has been attributed to the cIAP2 interceded acceleration of the ubiquitin-proteasome-mediated decay of viral polymerase and reduction of the encapsidation of HBV pg RNA^[4].

Since, the studies regarding expression of cIAP2 in HBV infection are scanty and mostly performed in cell lines, we wanted to assess cIAP2 expression in human cells infected with HBV. We clarified the presence of replicating HBV in PBMCs by detection of HBV pgRNA in those cells for measuring the expression status of cIAP2 in the same cell types. To our knowledge, this is the first study of its kind demonstrating expression of cIAP2 mRNA in HBV infected cells obtained from chronically infected patients without overt symptoms. Our data showed significant downregulation of cIAP2 gene in HBV infected PBMCs than the expression pattern observed in non infected cells obtained from healthy control subjects. This findings might have implication related to the survival strategy of the virus for long term persistence in chronic infection.

Lu et al^[5] demonstrated marked increase of cIAP1 and cIAP2 in HBV expressing hepatoma cells. Considering HBV as a tumor trigger, they suggested that the long time stimulation of HBV viral proteins changes the expression of apoptosis profile of host cells through modulation of cIAP1 and cIAP2, which probably results in the carcinogenesis of liver cells. On the otherhand, Wang et al^[4] observed that cIAP2 promoted the degradation of the viral polymerase through a proteasome-dependent pathway which established a

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strong role of cIAP2 as antiviral mediator to prevent viral replication. Considering the antiviral property of cIAP2 as suggested by Wang et al^[4], our observations that replicating HBV significantly suppressed expression of cIAP2 in virus infected cells indicate the virus induced modulation of host intracellular protein to thwart anti viral defense mechanism. Creation of the intracellular state through downregulation of cIAP2 might appear advantageous for HBV to persist within the cell which further help to establish a chronic infection, and, thus, the mechanism of cIAP2 mediated viral inhibition can be considered as a novel strategy for HBV therapy.

REFERENCES

- [1] Y.Weil, T.Fan, M. Yu; Inhibitor of apoptosis proteins and apoptosis. *Acta Biochim.Biophys.Sin.*, **40**, 278-288 (2008).
- [2] U.Ramp, T.Krieg, E.Caliskan, C.Mahotka, T.Ebert, R.Willers, H.Gabbert, C.Gerharz; XIAP expression is an independent prognostic marker in clear-cell renal carcinomas. *Human Pathology*, **35**, 1022-1028 (2004).
- [3] R.P.Beasley; The Hepatitis B virus: The major etiology of hepatocellular carcinoma. *Cancer*, **61**, 1942-1956 (1980).
- [4] Z.Wang, J.Ni, J.Li, B.Shi, Y.Xu, Z.Yuan; Inhibition of Hepatitis B virus replication by cIAP2 involves accelerating the Ubiquitin-Proteasome-Mediated destruction of polymerase. *J.Virol.*, **85**, 11457-11467 (2011).
- [5] X.Lu, M.Lee, T.Tran, T.Block; High level expression of apoptosis inhibitor in hepatoma cell line expressing Hepatitis B virus. *Int.J.Med.Sci.*, **2**, 30-35 (2005).
- [6] G.V.Shravanthi, R.M.Mukherjee, A.Jakkampudi, P.B.Reddy, P.N.Rao, R.Gupta, D.N.Reddy; Human RNA methylating enzyme DNMT2 gene expression is down regulated in chronic hepatitis B virus infection. *Exp.Clin.Hepatol.*, **8**, 53-59 (2012).
- [7] R.M.Mukherjee, G.V.Shravanti, A.Jakkampudi, R.Kota, A.L.Jangala, P.B.Reddy, P.N.Rao, R.Gupta, D.N.Reddy; Reduced expression of DNA damage repair genes high mobility group box 1 and Poly(ADP-ribose) polymerase 1 in inactive carriers of Hepatitis B virus infection-A possible stage of viral integration. *J.Clin.Exp.Hepatol.*, **3**, 89-95 (2013).
- [8] T.J.Fan, L.H.Han, R.S.Cong, J.Liang; Caspase family proteases and apoptosis. *Acta Biochem. Biophys.Sin.*, **37**, 719-727 (2005).
- [9] R.W.Watson, J.M.Fitzpatrick; Targeting apoptosis in prostate cancer: Focus on caspases and inhibitors of apoptosis proteins. *BJU Int.*, **96**, 30-34 (2005).
- [10] X.H.Peng, P.Karna, R.M.O'Regan, X.Liu, R.Naithani, R.M.Moriarty, W.C.Wood et al; Down-regulation of inhibitor of apoptosis proteins by deguelin selectively induces apoptosis in breast cancer cells. *Mol.Pharmacol.*, **71**, 101-111 (2007).
- [11] I.Esposito, J.Kleeff, I.Abiatari, X.Shi, N.Giese, F.Bergmann, W.Roth et al; Overexpression of cellular inhibitor of apoptosis protein 2 is an early event in the progression of pancreatic cancer. *J.Clin.Pathol.*, **60**, 885-895 (2007).
- [12] X.Liu, J.Shao, W.Xiong, S.Yu, Y.Hu, J.Liu, X.Wang, L.Xiang, Z.Yuan; Cellular cIAP2 gene expression associated with Anti-HBV activity of TNF- α in hepatoblastoma cells. *J.Interferon & Cytokine Res.*, **25**, 617-626 (2005).
- [13] L.G.Guidotti, F.V.Chisari; Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu.Rev.Immunol.*, **19**, 65-91 (2001).