Reduced Expression of DNA Damage Repair Genes High Mobility Group Box1 and Poly(ADP-ribose) Polymerase1 in Inactive Carriers of Hepatitis B Virus Infection-A Possible Stage of Viral Integration

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Background: High mobility group box1 (HMGB1) and poly(ADP-ribose) polymerase1 (PARP1) proteins repair cellular DNA damage. Reduced expression of the corresponding genes can lead to an impaired DNA damage repair mechanism. Intracellular replication of hepatitis B virus (HBV) in such conditions can favor the integration of viral DNA into host genome leading to the development of hepatocellular carcinoma (HCC). **Objective:** This study was performed to assess the expression of HMGB1 and PARP1 mRNAs in conjunction with the estimation of HBV replication intermediate pregenomic RNA (PgRNA) in various phases of HBV infection. **Materials:** Eighty eight patients and 26 voluntary blood donors as controls were included in the study. Patients were grouped in to acute (AHB; $n = 15$), inactive carriers (IC; $n = 36$), cirrhosis (Cirr; $n = 25$) and hepatocellular carcinoma (HCC; $n = 12$). Serum HBV DNA was quantified by real time polymerase chain reaction (PCR) assay. Expression of HMGB1, PARP1 and PgRNA were evaluated using peripheral blood mononuclear cells (PBMCs) derived RNA by reverse transcription PCR (RT-PCR) and densitometry. **Results:** Significant reduction of HMGB1 and PARP1 gene expressions ($P < 0.05$) were observed in patients than controls with more explicit decline of PARP1 ($P = 0.0002$). Both genes were significantly downregulated ($P < 0.001$) in ICs than controls. In ICs, HMGB1 was significantly lowered than cirrhosis ($P = 0.002$) and HCC ($P = 0.0006$) while PARP1 declined significantly ($P = 0.04$) than HCC. Level of PgRNA was comparable in all the disease categories. **Conclusion:** In conclusion, our findings indicate impaired DNA damage repair mechanisms in HBV infected cells of ICs. This, along with low viral load but higher level of PgRNA in this group is suggestive of the diversion of HBV replication pathway that might facilitate viral DNA integration in to host genome. Intrusion of HBV PgRNA reverse transcription in early stage of infection might appear advantageous to thwart the development of HCC. (J CLIN EXP HEPATOL 2013;3:89-95)

Keywords: hepatitis B virus, gene expression, high mobility group box1, poly(ADP-ribose) polymerase1, pregenomic RNA

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Abbreviations: HMGB1: high mobility group box1; PARP1: poly(ADP-ribose) polymerase1; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; PgRNA: pregenomic RNA; IC: inactive carriers; PCR: polymerase chain reaction; PBMCs: peripheral blood mononuclear cells; RT-PCR: reverse transcription PCR; CHB: chronic HBV; cccDNA: covalently closed circular DNA; dsDNA: double stranded HBV DNA; rcDNA: relaxed circular DNA; NER: nucleotide excision repair; BER: base excision repair; CP: Child-Pugh; ELISA: enzyme-linked immunosorbent assay; AST: aspartate transferase; ALT: alanine transferase; DEPC: diethyl pyrocarbonate; DTT: dithiothreitol; dNTPs: deoxynucleoside triphosphates; CIRRH: cirrhosis; AHB: acute hepatitis B; ADP: adenosine diphosphate; HBsAg: hepatitis B virus surface antigen; HBeAg: hepatitis B virus e antigen; IgM: immunoglobulin M; IgG: immunoglobulin G; HIV: human immunodeficiency virus; HAV: hepatitis A virus; HDV: hepatitis delta virus; HEV: hepatitis E virus; MuLV-H: moloney murine leukemia virus Rnase H; SD: standard deviation; UIs: unique integration sites; HBx: hepatitis B virus X protein <http://dx.doi.org/10.1016/j.jceh.2013.04.003>

The human hepatitis B virus (HBV) is a small enveloped DNA virus causing acute and chronic hepatitis. In spite of the availability of an effective vaccine, near about 360 million people are chronically infected globally where about 1 million infected people die per year due to HBV-associated liver pathologies.¹ Despite the noncytotoxic nature of HBV, the spectrum of liver infection often progresses to liver cirrhosis and development of hepatocellular carcinoma (HCC).

Inactive carriers (IC) constitute the largest group among chronic HBV (CHB) infected patients and are not been considered eligible for receiving antiviral treatment as per the existing practice guidelines.² This group is diagnosed by presence of HBsAg for more than six months, HBeAg negative and anti-HBeAg positivity, serum HBV DNA less than 2000 IU per mL, persistently normal ALT and AST levels, as well as liver biopsy confirming absence of significant hepatitis.^{2,3} Even though, the risk and the predictors of liver disease progression in ICs are unclear, they are supposed to have a substantial risk of developing HCC and liver-

related death compared with individuals not infected with HBV.⁴

The unique genomic organization and replication strategy of HBV helps the virus to persist in infected hepatocytes. A hallmark of HBV infection is the formation of a stable non-integrative HBV-DNA minichromosome, the so-called covalently closed circular DNA (cccDNA) in hepatocyte nuclei, which act as template to generate all RNAs necessary for protein production and viral replication. Furthermore, integrations of linear double stranded HBV DNA (dsDNA) sequences in to host genome do occur in infected hepatocytes, particularly in the presence of DNA damage⁵ and cell turnover.^{6,7} The overlength pregenomic RNA (pgRNA) which act as a replication intermediate is reverse transcribed to form relaxed circular DNA (rcDNA), cccDNA as well as can produce truncated form of linear HBV dsDNA capable of integration.⁸ As a consequence, persistence of cccDNA and integration of viral DNA in to host genome appeared as key factors responsible for relapse of HBV infection, failure of viral clearance after completion of antiviral therapy and development of HCC in CHB patients.

The non-histone chromosomal protein high mobility group box 1 (HMGB1) belongs to the family of the high mobility group (HMG) nuclear proteins. The intrinsic capacity of HMGB1 for altering DNA structures allows it to participate in many biological processes like regulation of chromatin structure,⁹ transcription,¹⁰ DNA damage repair and recombination processes.¹¹ Recent cell biology and biochemical studies indicated that HMGB1 is actively involved in modulating the efficiency of all four major DNA repair pathways, i.e. nucleotide excision repair (NER),¹² base excision repair (BER),¹³ mismatch repair,¹⁴ double strand break repair and nonhomologous end-joining¹⁵ respectively. Hence, HMGB1 appears to accelerate the whole process of cellular DNA repair that includes lesion removal and darning as well as unpacking and restoration of chromatin structure.

Poly(ADP-ribose) polymerase1(PARP1), a well-known DNA-binding enzyme is responsible for the major poly(ADP-ribose)ylation activity observed during mending of DNA damage and has a potential role on DNA repair.¹⁶ In vitro studies have shown that inhibition of PARP1 activity resulted increased frequency of HBV integration where PARP1 has the potential to limit the occurrence of *de novo* HBV DNA integrations.⁵

Notwithstanding the reported integration of HBV DNA in HCC, acute and chronic infections¹⁷⁻¹⁹ and identification of a novel PARP1 binding sequence motif in HBV core promoter region which has potential to disrupt host DNA damage repair pathways,²⁰ no study has been done so far to evaluate the status of host DNA damage repair mechanisms in HBV mediated progressive liver diseases. Furthermore, the status of HBV pgRNA as viral replication intermediate has not been evaluated in HBV disease categories.

Based on the above fact and considering the proven role of both HMGB1 and PARP1 as protectors of integrity and stability of the host genome, we aimed to analyze the expression profile of HMGB1 and PARP1 mRNAs in relation to the HBV replicative intermediate pgRNA from peripheral blood mononuclear cells (PBMCs) of HBV infected subjects belong to different disease categories.

In this study, we observed significantly reduced expression of both HMGB1 and PARP1 mRNAs suggestive of impaired cellular DNA damage repair mechanisms in the IC group which has not been reported earlier. This, in conjunction with the presence of lower level of rcDNA but higher amount of pgRNA in ICs hoist the possibility of generation of linear ds HBV DNA suitable for integration. Taken together, our study provide important information focusing ICs as potentially favorable stage for HBV integration and, thus, warrant further studies for better management of HBV mediated disease progression leading to HCC.

MATERIALS AND METHODS

Study Subjects

Eighty-eight HBsAg positive subjects who attended the Asian Institute of Gastroenterology, Hyderabad, India were enrolled in this study. Patients were further categorized into acute (AHB; *n* = 15), inactive carriers (IC; *n* = 36), cirrhosis (Cirr; *n* = 25) and hepatocellular carcinoma (HCC; *n* = 12) respectively on the basis of past history, clinical presentations, anti-HBc IgM/IgG status, imaging data, Child-Pugh (CP) scores²¹ and as per AASLD practice guidelines.^{22,23} None of the subjects had decompensated liver cirrhosis. Patients coinfectd with HIV, HAV, HCV, HDV, or HEV were excluded from this study. A group of 26 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 Tests

The serum HBeAg and anti-HBe status of the subjects were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Amar-EASE, Taiwan) as per the manufacturer's instructions. Serum aspartate transferase (AST) and alanine transferase (ALT) levels were ascertained by an automated clinical biochemistry analysis system (Randox, Oceanside, CA).

Quantification of Serum Hepatitis B Virus Deoxyribonucleic Acid

Viral DNA 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 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 Ribonucleic Acid from Peripheral Blood Mononuclear Cell

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

Preparation of cDNA

A common cDNA pool was generated by reverse transcription from total RNA using random hexamers and MuLV-H reverse transcriptase (Fermentas Life Sciences, Germany). Before the 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 presence of traces of DNA was further excluded by performing control reactions without reverse transcriptase enzyme. RNA was reverse transcribed (60 min at 37 °C) with 200 U of MMuLV reverse transcriptase (Fermentas 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 (DTT), 0.5 mM deoxynucleoside triphosphates (dNTPs, Fermentas Life Sciences, Germany), 25 U ribonuclease inhibitor (Promega Corporation, Madison, WI, USA) and 200 ng random hexamers (Fermentas Life Sciences, Germany).

Polymerase Chain Reaction Amplification of High Mobility Group Box1 and Poly(ADP-Ribose) Polymerase1 Genes

After heating (95 °C, 1 min) and quick-chilling on 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 (40 ng each), 1.5 mM MgCl₂ and 2 U of Taq DNA polymerase (Fermentas Life Sciences, Germany). HMGB1 cDNA fragments were amplified by 30 cycles (95 °C–15 s, 55 °C–30 s & 72 °C–45 s per step), having an initial denaturation at 94 °C for 5 min and final extension at 72 °C for 5 min using up and downstream primers (5'-3') d(CTC AGA GAG GTG GAA GAC CAT GT), and d(GGG ATG TAG GTT TTC ATT TCT CTT TC) to yield a product of 104 bp.²⁴ PARP1 cDNA fragments were amplified by 30 cycles (94 °C–1 min, 58 °C–1 min & 72 °C–5 min per step) with an initial denaturation at 95 °C for 5 min and final extension at 72 °C for 5 min using forward and reverse primers (5'-3') d(GTG GCA CGG GTC CAG GAC CAC CAA C) and d(GCC CAA ACC TTT GAC ACT GTG CTT GCC C) generating a product of 277 bp.²⁵ Beta-actin as housekeeping 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 314 bp. Blank reactions without cDNA were included in all

experiments as negative control. Each amplified product (10 μ L) was subjected to 2% agarose gel electrophoresis (80 V, 40 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.²⁶

Polymerase Chain Reaction Amplification of Hepatitis B Virus Pregenomic Ribonucleic Acid in Peripheral Blood Mononuclear Cells

Trizol extracted PBMC derived RNA (5 μ L) was reverse-transcribed to cDNA. PgRNA cDNA fragments were amplified by 40 cycles (94 °C–30 s, 58 °C–30 s & 72 °C–30 s per step), having an initial denaturation at 95 °C for 5 min and 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 earlier.²⁷ The PCR product was finally clarified upon 2% agarose gel electrophoresis to resolve 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 check specificity of PBMC derived pgRNA.

Statistical Analyses

Descriptive statistics (mean, standard deviations) and Student's *t*-tests were performed as and where applicable using GraphPad QuickCalc software, USA. A value of *P* < 0.05 was considered statistically significant.

RESULTS

All the 88 patients were HBsAg positive (Mean age $\pm$ SD = 35.8 $\pm$ 13.1) and consisted of 70 males and 18 females. The detailed demographic, biochemical and virological characteristics of 88 patients assigned to different disease categories are depicted in Table 1. Control subjects had a mean $\pm$ SD age of 29.4 $\pm$ 10.1 years and consisted of 20 males and 6 females. Except the AHB group, all patients of remaining groups were negative for HBeAg and positive for anti-HBe antibody respectively.

Expression of High Mobility Group Box1 and Poly(ADP-Ribose) Polymerase1 Genes in Control and Patients

Both HMGB1 and PARP1 expressions were significantly reduced in patients when compared to controls (Figure 1).

Table 1 Baseline characteristics of the patients.

Parameters	Patient categories			
	AHB (n = 15)	IC (n = 36)	CIRRH (n = 25)	HCC (n = 12)
Age	35.8 ± 13.1	34.5 ± 11.2	34.7 ± 10.5	46.3 ± 12.8
Sex (M:F)	12:3	30:6	19:6	9:3
ALT (IU/L)	1244 ± 1054	26.6 ± 7.96	116.9 ± 102.3	74.56 ± 34.6
HBV DNA (Log Copies/ml)	5.90 ± 1.57	3.05 ± 0.71	6.46 ± 1.79	4.82 ± 2.05

AHB = acute hepatitis B; IC = inactive carriers; CIRRH = cirrhosis; HCC = hepatocellular carcinoma.

While the expression of HMGB1 reduced to a lesser extent ($P = 0.04$) in patients (mean $\pm$ SD = 277.98 $\pm$ 73.6) than controls (mean $\pm$ SD = 308.19 $\pm$ 39.4) (Figure 1), a more significant ($P = 0.0002$) reduction was noticed in the expression of PARP1 in patients (mean $\pm$ SD = 95.135 $\pm$ 29.68) compared to control (mean $\pm$ SD = 146.81 $\pm$ 86.1) subjects (Figure 1).

Reduced Expression of High Mobility Group Box1 Gene in Inactive Carrier Group

In respect to controls, no significant change in the expression of HMGB1 mRNA was observed in patients having AHB, cirrhosis and HCC. Conversely, in comparison to the expression level of HMGB1 in controls (mean $\pm$ SD = 308.195 $\pm$ 39.485), the level of HMGB1 mRNA was significantly reduced in IC group (mean $\pm$ SD = 246.185 $\pm$ 63.07; $P < 0.0001$) (Figure 2). Expression of HMGB1 was also found to be significantly lower in IC group in comparison to cirrhosis ($P = 0.002$) and HCC ($P = 0.0006$) groups respectively.

Reduced Expression of Poly(ADP-Ribose) Polymerase1 Gene in Inactive Carrier Group

When compared against controls, expression of PARP1 gene showed a less significant decline ($P = 0.04$) in patients having cirrhosis without any notifiable change in AHB and

HCC patients. Strikingly, the IC group showed a highly significant ($P = 0.0004$) decline (mean $\pm$ SD = 89.04 $\pm$ 18.55) in the expression of PARP1 with respect to control (mean $\pm$ SD = 146.81 $\pm$ 86.125) subjects followed by cirrhosis group, although, to a much lesser extent ($P = 0.04$; Figure 3). The IC group also depicted significantly lower expression of PARP1 than HCC ($P = 0.04$).

Expression of Hepatitis B Virus Pregenomic Ribonucleic Acid in Peripheral Blood Mononuclear Cells

Unlike serum HBV DNA, which had lowest concentration in IC group (Table 1), no significant change in the PgRNA level was observed among ICs in comparison to other HBV disease categories (Figure 4). The observed mean $\pm$ SD value of PgRNA in different patient categories were 160.06 $\pm$ 60.95 in AHB, 128.525 $\pm$ 53 in IC, 121.105 $\pm$ 39.75 in cirrhosis and 136.74 $\pm$ 47.7 in HCC group respectively (Figure 4). PgRNA was not detected in any of the isolated plasma samples of respective patients.

DISCUSSION

The repair of DNA strand breaks and maintenance of genomic integrity is crucial for cell survival. Impairment of

Figure 1 Expression of HMGB1 and PARP1 genes are significantly reduced in HBV infected subjects. The HMGB1 and PARP1 mRNA expression is reduced in HBV infected patients ($n = 88$) than healthy control ($n = 26$). For gene expression, HMGB1 and PARP1 mRNAs were PCR amplified from the cDNAs prepared from total RNA isolated from PBMC. (A) Representative pattern of PARP1 and HMGB1 PCR products separated by 2% agarose gel. Lanes: 1 and 2 – beta actin (314 bp) control and patient, 4 and 5 – PARP1 (277 bp) control and patient, lanes 7 and 8 are HMGB1 (104 bp) control and patient respectively, lanes 3, 6, 9 – negative controls, lane 10 – 100 bp DNA marker; (B) The gene expression was normalized against beta-actin gene, quantified by densitometry analysis and expressed as ng/mL. HMGB1: high mobility group box 1; PARP1: poly(ADP-ribose) polymerase1; PBMC: peripheral blood mononuclear cells; RT-PCR: reverse transcription polymerase chain reaction; vertical bar: SD.

Figure 2 HMGB1 gene expression is significantly reduced in IC group. The HMGB1 gene expression was compared among healthy controls ($n = 26$) and HBV patients categorized into different clinical phases of the disease that includes AHB ($n = 15$), IC ($n = 36$), CIRRH ($n = 25$), HCC ($n = 12$). (A) PCR products of HMGB1 were run on 2% agarose gel, lane 1 represent control, lanes 2–5 are patient disease categories i.e. AHB, IC, CIRRH and HCC respectively. Lane 6 is negative control and lane 7 is 100 bp DNA marker. (B) The HMGB1 gene expression was reduced significantly in inactive carrier group ($P = 0.0001$) than other groups. Relative gene expression was analyzed by densitometry analysis after normalization with beta actin signal. AHB: acute hepatitis B, IC: inactive carrier, CIRRH: cirrhosis, HCC: hepatocellular carcinoma.

DNA damage repair mechanisms increases the susceptibility of cells to DNA damaging agents, possibly by preventing DNA strand break rejoining and by affecting several DNA damage signaling pathways. It has been shown that HBV DNA integration occurs preferentially at sites of cell DNA damage and may indicate the presence of more widespread genetic changes caused by viral DNA integration itself.²⁸ Unlike retroviruses, in which insertion of viral DNA into host DNA is integral to the replication cycle, integration of HBV DNA take place as a result of an illegitimate recombination through in situ priming to form linear dsDNA which serve as the primary substrate for integration.^{29,30} Existence of such form of HBV genome in an

integrated form has been demonstrated in PBMCs of CHB patients and it has been postulated that unrelenting replication of HBV is associated with a high frequency of integration of viral sequences into the human genome while a lower frequency is observed during acute hepatitis B infections.¹⁹ A recent study employing next generation sequencing technique has identified 286 unique HBV integration sites (UISs) with precise HBV–human DNA junctions suggesting a clonal expansion process during HCC development.³¹ It is also known that HBV encodes HBX and preS2/S truncated proteins which may have transforming activity³² and may interfere with DNA repair.³³ Despite the reported evidence of HBV

Figure 3 Expression of PARP1 gene is significantly reduced in IC group. Comparative expression profile of PARP1 gene in healthy controls and different disease categories of HBV infection. (A) PCR amplicon of PARP1 with product size 277 bp were run on 2% agarose gel. Lane 1—control, lanes 2–5 are AHB, IC, CIRRH and HCC respectively, lane 6—negative control, lane 7—100 bp DNA ladder. (B) The PARP1 gene expression was reduced significantly ($P = 0.0004$) in IC groups than AHB, CIRRH, HCC patient groups and healthy control. Expression of β -actin gene was used as an internal control for gene expression normalization in densitometry analysis. AHB: acute hepatitis B; IC: inactive carriers; CIRRH: cirrhosis; HCC: hepatocellular carcinoma; PARP1: poly(ADP-ribose) polymerase1; vertical bar: SD.

Figure 4 Expression of HBV pregenomic RNA among different disease groups. The HBV pregenomic RNAs in PBMCs were PCR amplified from the cDNAs prepared from the total RNA and compared among the HBV disease categories. (A) Representative agarose gel picture of PgRNA bands belong to different disease categories. Lanes 1–4 depicted AHB, IC, CIRRH and HCC respectively, lane 5—negative control, lane 6—100 bp DNA ladder; (B) PCR derived amplicon signals were quantified by densitometry analysis after normalization against β -actin and expressed as ng/mL. No significant change in HBV pregenomic RNA expression was observed among various disease groups. AHB: acute hepatitis B; IC: inactive carriers; CIRRH: cirrhosis; HCC: hepatocellular carcinoma; PBMC: peripheral blood mononuclear cells; PgRNA: pregenomic ribonucleic acid.

integrants in almost all the human chromosomes favored by host cellular DNA damage and the potential role of HBV integration in development of HCC,¹⁷ status of host DNA damage repair mechanisms has not been elucidated in HBV mediated disease.

In this study, we evaluated and compared the expression status of two major DNA damage repair genes i.e. HMGB1 and PARP1 in different stages of HBV infection. We found that both the genes are downregulated in PBMCs of HBV patients compared to uninfected controls. Moreover, in comparison to HMGB1, downregulation of PARP1 was more pronounced in HBV patients. Interestingly, expression of both HMGB1 and PARP1 genes were significantly reduced in IC group than controls. While the mechanism of HBV mediated repression of HMGB1 is still not known, the present observations might have bearing with the identification of a novel octamer motif in the HBV core promoter region that binds PARP1 and has the ability to inhibit the DNA repair capacity of PARP1.²⁰ On top, our finding is indicative of inadequacy of DNA damage repair mechanism vis-a-vis increased damage of cellular DNA in IC group than the AHB, cirrhosis and HCC categories. Since prevailing DNA strand breakage for prolonged duration can allow more 'hot-spots' for integration of foreign DNA, it can be envisaged that the ICs are likely to be more vulnerable to HBV DNA integration.

In order to assess the replication status of HBV in our study group, we analyzed the level of PgRNA as viral replication intermediate which upon reverse transcription, is able to generate rcDNA measured as viral load, the episomal cccDNA and linear dsDNA meant for integration. Strikingly enough, our data showed that besides having much lower viral load (rcDNA), the IC group possess comparable level of pgRNA which has not been deviated mark-

edly from other disease categories. This raises the stipulation whether a major portion of pgRNA being reverse transcribed to either cccDNA or linear dsDNA instead of rcDNA in the IC group and warrant further study. This possibility can also explain at least, partly, the occurrence of lower viral load (rcDNA) in ICs where immune function restrict the extracellular spread of the virus. Since ICs are known to possess lower concentration of HBsAg³⁴ which has been further correlated with lower amount of cccDNA,³⁵ it can be tacit to speculate that as a replacement for the generation of cccDNA, increased diversion of pgRNA reverse transcription process toward formation of linear dsDNA might be a likely event in ICs.

Taken together, the reduced expression of DNA damage repair genes, presence of substantial amount of HBV PgRNA along with low viral load, the IC group appears to be the preferably potential stage for HBV DNA integration. Above and beyond the existing practice guidelines, there are notions to consider earlier treatment of chronic HBV infections.³⁶ Consequently, in view of the fact that integrated HBV DNA has been perceived as a major cause of development of HCC, therapeutic intervention of PgRNA reverse transcription process in an early stage like ICs might appear beneficial for better management of HBV infections.

In conclusion, our data for the first time, demonstrated considerably reduced expression of HMGB1 and PARP1 genes in inactive carriers of HBV infection signifying a flawed DNA strand breakage repair mechanism in this group. Higher level of PgRNA and lower viral load in this group further indicate the possible diversion of the HBV replication pathways toward the formation of integration compatible linear HBV DNA. This conjecture which might have insinuation favoring integration of HBV DNA into host cell genome, thus, heave the admonition that the

inactive carriers need more attention regarding viral persistence and long term severity of HBV infection.

CONFLICTS OF INTEREST

All authors have none to declare.

REFERENCES

- Block TM, Guo H, Guo JT. Molecular virology of hepatitis B virus for clinicians. *Clin Liver Dis*. 2007;11:685–706.
- Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000 – summary of a workshop. *Gastroenterology*. 2001;120:1828–1853.
- Lok ASF, McMahon BJ. Chronic hepatitis B. *Hepatology*. 2001;34:1225–1241.
- Chen JD, Yang HI, Iloeje UH, et al. Carriers of inactive hepatitis B virus are still at risk for hepatocellular carcinoma and liver-related death. *Gastroenterology*. 2010;138:1747–1754.
- Dandri M, Burda MR, Burkle A, et al. Increase in de novo HBV DNA integrations in response to oxidative DNA damage or inhibition of poly(ADP-ribosylation). *Hepatology*. 2002;35:217–223.
- Petersen J, Dandri M, Gupta S, et al. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. *Proc Natl Acad Sci USA*. 1998;95:310–315.
- Mason WS, Jilbert AR, Summers J. Clonal expansion of hepatocytes during chronic woodchuck hepatitis virus infection. *Proc Natl Acad Sci USA*. 2005;102:1139–1144.
- Nassal M. Hepatitis B viruses: reverse transcription a different way. *Virus Res*. 2008;134:235–249.
- Catez F, Yang H, Tracey KJ, Reeves R, Misteli T, Bustin M. Network of dynamic interactions between histone H1 and high-mobility-group proteins in chromatin. *Mol Cell Biol*. 2004;24:4321–4328.
- Calogero S, Grassi F, Aguzzi A, et al. The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat Genet*. 1999;22:276–280.
- Lange SS, Vasquez KM. HMGB1: the jack-of-all-trades protein is a master DNA repair mechanic. *Mol Carcinog*. 2009;48:571–580.
- Lange SS, Mitchell DL, Vasquez KM. High mobility group protein B1 enhances DNA repair and chromatin modification after DNA damage. *Proc Natl Acad Sci USA*. 2008;105:10320–10325.
- Prasad R, Liu Y, Deterding LJ, et al. HMGB1 is a cofactor in mammalian base excision repair. *Mol Cell*. 2007;27:829–841.
- Yuan F, Gu L, Guo S, Wang C, Li GM. Evidence for involvement of HMGB1 protein in human DNA mismatch repair. *J Biol Chem*. 2004;279:20935–20940.
- Stros M, Cherny D, Jovin TM. HMG1 protein stimulates DNA end joining by promoting association of DNA molecules via their ends. *Eur J Biochem*. 2000;267:4088–4097.
- Bouchard VJ, le Rouleau M, Poirier GG. PARP-1, a determinant of cell survival in response to DNA damage. *Exp Hematol*. 2003;31:446–454.
- Murakami Y, Saigo K, Takashima H, et al. Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. *Gut*. 2005;54:1162–1168.
- Kimbi GC, Kramvis A, Kew MC. Integration of hepatitis B virus DNA into chromosomal DNA during acute hepatitis B. *World J Gastroenterol*. 2005;11:6416–6421.
- Murakami Y, Minami M, Daimon Y, Okanoue T. Hepatitis B virus DNA in liver, serum, and peripheral blood mononuclear cells after the clearance of serum hepatitis B virus surface antigen. *J Med Virol*. 2004;72:203–214.
- Ko HL, Ren EC. Novel poly (ADP-ribose) polymerase 1 binding motif in hepatitis B virus core promoter impairs DNA damage repair. *Hepatology*. 2011;54:1190–1198.
- Durand F, Valla D. Assessment of the prognosis of cirrhosis: Child–Pugh versus MELD. *J Hepatol*. 2005;42:s100–s107.
- Lok ASF, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology*. 2009;50:1–3.
- Bruix J, Sherman M. Management of hepatocellular carcinoma. *Hepatology*. 2005;42:1208–1236.
- Andersson Å, Covacu R, Sunnemark D, et al. Pivotal advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis. *J Leukoc Biol*. 2008;84:1248–1255.
- Zaniolo K, Rufiange A, Leclerc S, Desnoyers S, Guerin SL. Regulation of the poly(ADP-ribose) polymerase-1 gene expression by the transcription factors Sp1 and Sp3 is under the influence of cell density in primary cultured cells. *Biochem J*. 2005;389:423–433.
- Mukherjee RM, Bansode B, Gangwal P, et al. Human interferon regulatory factor 2 gene expression is induced in chronic hepatitis C virus infection – a possible mode of viral persistence. *J Clin Exp Hepatol*. 2012;2:27–34.
- Belloni L, Allweiss L, Guerrieri F, et al. IFN- α inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *J Clin Invest*. 2012;122:529–537.
- Bill CA, Summers J. Genomic DNA double-strand breaks are targets for hepadnaviral DNA integration. *Proc Natl Acad Sci USA*. 2004;101:11135–11140.
- Yang W, Summers J. Integration of hepadnavirus DNA in infected liver: evidence for a linear precursor. *J Virol*. 1999;73:9710–9717.
- Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev*. 2000;64:51–68.
- Ding D, Lou X, Hua D, et al. Recurrent targeted genes of hepatitis B virus in the liver cancer genomes identified by a next-generation sequencing based approach. *PLoS Genet*. 2012;8(12): e1003065. <http://dx.doi.org/10.1371/journal.pgen.1003065>.
- Brechot C, Gozuacik D, Murakami Y, et al. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). *Semin Cancer Biol*. 2000;10:211–231.
- Grisham JW. Molecular genetic alterations in primary hepatocellular neoplasms: hepatocellular adenoma, hepatocellular carcinoma, and hepatoblastoma. In: Coleman WB, Tsongalis GJ, eds. *The Molecular Basis of Human Cancer*. Totowa, New Jersey: Humana Press; 2001:269–346.
- Brunetto MR, Oliveri F, Colombatto P, et al. Use of HBsAg serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. *Gastroenterology*. 2010;139:483–490.
- Chan HL, Wong VW, Tse AM, et al. Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol*. 2007;5:1462–1468.
- Zoulim F, Mason WS. Reasons to consider earlier treatment of chronic HBV infections. *Gut*. 2012;61:333–336.