Estimating High Mobility Group Box Protein 1 (HMGB1) Single Nucleotide Polymorphisms Among Hepatitis B Virus Infected Patients of Pakistan Origin

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Short report

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Abstract

HMGB1 is nuclear non-histone protein and unique member of cytokines. In viral hepatitis infection HMGB1 serum level increases and translocates towards cytoplasm and extracellular spaces where it activates single stimulating hepatic stellate cell proliferation which inducesfibrogenic protein expression and causes hepatocellular carcinoma. In this study, total 150 subjects were recruited to assess the association between HMGB1 SNPs and HBV. Three types of genotypes were found visible in rs3742305 of HMGB1; wild type homozygous GG with 65%, homozygous minor type CC with 6% and heterozygous minor type GC with 26% frequency distribution. High prevalence of GG genotype in the selected population presenting that GG genotype may have higher risk for susceptibility to HBV infection. Our resultsshowedsignificant correlationof HMGB1 polymorphism with HBV infection in the selected Pakistani population.

Introduction

Hepatitis B virus may cause either acute or chronic liver infection. It may also involve in liver cirrhosis and hepatic-cellular carcinoma. Chronic infection with HBV is a major worldwide health problem despite the availability of vaccine (Wiegand et al.,2010). The HBV infection prevalence is highly changeable as estimated more than 10% in some Western Pacific countries and Asian's to under 0.5% in the northern European countries and United States. Viral infections are increasing day by day (Saeed U et al., 2021). Globally the estimated HBV infected individuals are 350 million (Piracha et al., 2018; Saeed et al., 2019; Piracha et al., 2020). Approximately 7–9 million carriers of HBV are found in Pakistan with an increase in carrier rate of 3–5 (Ali et al., 2009). The lifecycle of HBV has been studies exclusively however, the host factors involved in HBV replication and HBV associated HCC mechanisms are not fully understood.

The high mobility group (HMG) proteins binds to nucleosomes in non-sequence specific manner and have the ability to bring structural changes in chromatin (Reeves, 2010). HMG proteins belonged to three families: HMGA, HMGB, and HMGN. These members are structurally divergent but share functional similarity (Reeves, 2010). The most abundant protein of this family is HMGB (high-mobility group box) which is further divided into four groups (HMGB1, HMGB2, HMGB3 and HMGB4) (Reeves, 2010). HMG-box family showcases diversity of characters including chromatin remodeling, transcription, replication, repair, recombination and genomic stability, moreover extracellular HMGB1 involves in cell growth and mitotic activity, receptor signaling(Reeves, 2010). This emphasis that HMGB1 plays a critical role in the pathogenesis and treatment of liver diseases.

A single nucleotide polymorphism (SNP) is the stable replacement of single base in human gene. It is considered the most common genetic mutation in humans appearing in more than 1% of population. SNP is not identified as a disease causing variant, but genetic variation may impact suggestively on the disease susceptibility (Day, 2005; Karlsen et al., 2010). Common and rare SNPs and structural genomic changes may influence or restrict HCC development. Early identification of moderate to high genetic variants associated with HBV-related HCC will help to determine susceptibility of infection better

outcomes (Thomas et al., 2009; Brennan et al., 2011; Michailidou et al., 2015). For instance, mutational screening for BRCA1 and BRCA2 is used to identify women at risk for breast and ovarian cancer and is routinely used in accuracy of treatment protocols (Brennan et al., 2011; Michailidou et al., 2015). It is already a known fact that HBV infection is closely related with cytokines and polymorphisms of cytokine gene (ESR1) is reported to be associated with HBV infection (Yan et al., 2012; Deng et al., 2005). One of the SNP rs3742305 was identified in HMGB1 in HBV infected Chinese patients (Deng et al, 2013). Since there is no evidence of reported SNP in Pakistani population, identifying the association of HMGB1 and HBV. We first time reported the study based on polymorphism of HMGB1 gene in HBV patients identified in population of Pakistan origin.

Materials And Methods

Patients Data

Total 150 patients were randomly selected from the Nuclear Medicine, Oncology and Radiotherapy Institute, Islamabad. 5 ml blood samples from patients were collected along with singed consent forms. The diagnostic criteria for HBV infected patient were positive patients of HBsAg, HBeAg and anti- HBc and the patients have following symptoms of jaundice, fatty liver, abdominal pain and dark urine. Acute hepatitis B, Chronic hepatitis B and severe hepatitis B were selected. Patients having HBV were inducted with micro complications along with age, sex-matched, jaundice, fatty liver, abdominal pain, dark urine. HBV patients were assessed for AHB, CHB and SHB. The features/parameters recorded for the HBV patients are summarized in Tables 1 and 2.

Table 1
Mean values of different parameters of HBV

Characteristics	Mean ± Standard deviation
Age (Years)	2.52 ± 1.068
Jaundice	1.69 ± 0.465
Fatty liver	1.29 ± 0.465
Abdominal pain	1.33 ± 0.473
Dark urine	1.45 ± 0.500
HBsAg	1.02 ± 0.141
HBeAg	1.05 ± 0.219
Anti HBc	1.67 ± 0.473
Viral load	3.217 ± 2.183

Table 2
Demography and anthropometric parameters

Parameters	HBV N = 100	Frequency (%age)
Male	57	57%
Female	43	43%
Married	70	70%
Un-married	30	30%
Family History	12	12%
AHB	29	29%
CHB	54	54%
SHB	17	17%

DNA extraction method

The leukocytes genomic DNA from 5 mL whole blood was isolated using organic phenol chloroform extraction method (Köchl et al., 2005). DNA samples were diluted to 10 ng/µL.

Genetic Analysis

SNP rs3742305 of HMGB1 was selected on the basis of previous findings (Deng et al., 2013), showing evidence of strong association of this SNP with HMGB1. Genotyping of allelic variant (G > C) for rs3742305 was done by PCR-RFLP method. All samples were amplified for target DNA fragment spanning rs3742305 region. The rs3742305 of HMGB1 gene was amplified by using specific pair of primers, sense (5'-3' GTCTCCTTTGCCCAGTGTATCTC) and anti-sense (5'-3' GTACACAGCCTTTGTCTGAGTCTG) designed by using premier primer 5 software.

PCR amplification and Gel Electrophoresis

PCR amplification was carried out with 26 µl of master mix containing 8 µl of Go Taq, 6 µl of water, 1µl of each forward and reverse primer and template with 10µl DNA was added. The reaction conditions were as follows 95 ◦C for 5 minutes, 94 ◦C for 1 minute 57 ◦C for 1 minute and fifteen second and 72◦C for 1 minute and thirty seconds with 40 cycles and **72**◦C for 7 minutes in elongation. Amplified product of 676bp was analyzed by electrophoresis on 2% agarose gel. 2X TBE buffer was prepared from 10X TBE buffer solution and 2 grams of agarose was added to 100 ml of 1X TBE buffer solution. For visualization of DNA, 30 µl of Ethidium Bromide was added to the gel solution. For comparing the fragment size, a 50 bp molecular marker was used (MBI Fermentas, Catalogue no SM1153). The gel was observed in gel DOC system. The PCR amplified product was digested with BCl*I* (recognition site T/GATCA) restriction enzyme

(Qing et al., 2013) at 56 ◦C for 15 hours. The digested product was then analyzed by agarose gel and the required pattern of bands was obtained. 2 % agarose gel solution was used for the separation of DNA fragments. A 50bp ladder was used to compare the band sizes (MBI Fermentas, Catalogue no SM1153).

Statistical Analysis

The mean, frequency and standard deviation was calculated by SPSS.16.0 statistical software (SPSS Inc. Chicago, IL, United States). Genotype, allelic frequency, P- value were calculated by Hardy –Weinberg frequency calculator. $P < 0.01$ considered in Hardy-Weinberg equilibrium was considered significant.

Results

It has been reported that HMGB1 genotypes for 4 SNPs, 2262G/A (rs1045411), 1177G/C (rs3742305), 3814C/G (rs2249825), and rs4540927 (Supic et al., 2015). In our study, we analyzed genotype distribution of GG, GC and CC by entering observed genotype values in the internet calculator of hardy Weinberg equilibrium. The analysis revealed that in Pakistani population we found polymorphism of rs3742305 in HMGB1 gene as represented in Tables 3 and 4. Among 100 HBV patients GG and CC was homozygous genotype and GC was heterozygous genotype. The GG genotype was found in 65(60.84%) of total HBV patients, GC genotypes in 26(34.32%) while CC frequency was lower than GG and GC and detected in 9(4.84%) as shown in Table 4. PCR-amplified product of rs3742305 having size length of 676bp is shown in Fig. 1.

Table 3
Genotype distribution of GG, GC and CC for rs3742305 in HBV Patients and controls

Genotypes for rs3742305			
HBV (N = 100)	**GG**	**GC**	**CC**
	65	26	9
Expected H-W frequency	60.84	34.32	4.84
	60.84%	34.32%	4.84
Controls (N = 50)	**GG**	**GC**	**CC**
	20	23	7
Expected H-W frequency	19.85	23.31	6.85
	39.69%	46.62%	13.69%

Table 4

Allelic distribution of G and C for rs3742305in both patients and controls

Allelic frequency for rs3742305		
HBV(N=100)	**G**	**C**
	156	44
Expected H-W frequency	78%	22%
Controls (N=50)	**G**	**C**
	63	37
Expected H-W frequency	63%	37%

Allelic frequency of G was shown to be 156(78%) and for C, 44(22%) as shown in Table 5. In control individuals GG and CC was homozygous genotype and GC was heterozygous genotype. The GG genotype was found in 20(39.69%), GC in 23(46.62%) while CC frequency was lower than GG and GC and detected in 7(13.69%). Allelic frequency of G is 63% and C is 37%.Chi square and P-value calculated using Chi square and P-value calculator is 0.01 and degree of freedom is 2. Three types of rs3742305 genotypes in HBV infected patients, wild type homozygous GG, minor homozygous CC and minor heterozygous GC. Genotypic distribution of GG 65(60.84%), GC 26 (34.32%) and CC 9(4.84%). Allelic frequency of G is 156(78%) and C is 44(22%) and in control individuals 20(39.69%), 23(46.62%) and 7(13.69%) for GG, GC and CC respectively. Hardy-Weinberg equilibrium by chi square test showed that P-value is 0.01 (Table 5), which confirmed that there is significant association between polymorphism of HMGB1 and HBV patients and studied group was in hardy-Weinberg equilibrium. Figure 2 shows the PCR RFLP assay, where the GC genotype shows band length of 676bp, 496bp and 180bp and GG shows band length of 676bp only.

Table 5
Allelic distribution of G and C for rs3742305in both patients and controls

Allelic frequency for rs3742305		
HBV(N = 100)	**G**	**C**
	156	44
Expected H-W frequency	78%	22%
Controls (N = 50)	**G**	**C**
	63	37
Expected H-W frequency	63%	37%

Discussion

HMGB1 is highly conserved non-histone nuclear DNA binding protein that contributes to architecture of chromatin DNA (Reeves, 2010). It also acts as inflammatory cytokine in response to immune mediated diseases (Kaneko et al., 2017). In this study it is evident that most of the individuals of GG genotype having chronic hepatitis B infection (CHB) were male. It means CHB infection has association with GG genotype and CC genotype is less prevalent in population. The most of polymorphism occurs in CHB patients while in SHB infected patients, very little polymorphism occur although SHB infected patients have acute-on-chronic liver failure with reduction in liver size. The fractions calculated reveal that in HMGB1 gene 1176 G/G genotype is more susceptible to CHB with more progressive status towards HBV infection and allele G is clearly related with infection production. The more persistence of 1176 G/G genotypes in HBV patients may leads to liver carcinoma results into liver failure and CHB progress towards SHB. The homozygous 1176 C/C is less progressive then 1176 G/G while 1176 G/C have very little involvement in disease progression (Deng et al., 2013).

The uniqueness of this study is homozygous genotype which is involved in progression of disease. The homozygous GG genotype is associated with disease progression in HBV patients. In immune responses liver plays an important role. Parenchyma and non-parenchyma cells activate toll like receptors and Receptor for advanced glycation end-products. These receptors identify the HMGB1 in pathogenesis of liver disease (Chen et al., 2013; Sopipong et al., 2011) Extracellular HMGB1 have critical role in activation of hepatic injury depending upon receptors and post translational modification (Deng et al., 2013). It implicates in several immune-mediated diseases. About six polymorphism and four mutations were firstly identified and HMGB1 genotypes effect on the risks of SIRS and sepsis. Many researches reveal that polymorphism in HMGB1 leads to early and late death in individuals. Association between genetic variations and disease severity are also identified like polymorphism in HMGB1 leads to systemic inflammatory response syndrome (SIRS) (Kornblit et al., 2008). The tag SNPs in the entire gene involves in dysfunction syndrome (Zeng et al., 2011). The 1176G/G genotype of HMGB1 gene associates with

HBV infection leads towards disease elevation and current study confirmed this association and progress status similar to work of study.

Conclusion

In conclusion, our result shows that HMGB1 1176 G/G genotype has progression towards HBV infection and have high risks towards liver cirrhosis and hepatocellular carcinoma as compare to others as 1176 C/C and 1176 G/C and it also represent that there is association of HMGB1 which is a proinflammatory mediator between 1176 G/C polymorphism of HMGB1 and hepatitis B virus infection. It shows the role of HMGB1 in pathophysiology of diseases related to HBV and in progression of HBV infection towards severity in the form of hepatocellular carcinoma. The future prospects of this study are that it provides a clue for researchers in finding the pathogenicity of HBV infection and finding of other restriction sites involve in causing of polymorphism association with HBV disease pathogenesis. It will help in risk assessment and prediction of clinical outcomes and provides a clue for diagnostics of acute and chronic hepatitis B. It will offer the counseling for precautions in life style changes to high-risk individual.

Abbreviations

HMGB1: High Mobility Group Box Protein 1

WHO: World Health Organization

SNP: Single Nucleotide Polymorphisms

Declarations

Ethics approval and consent to participate:

The study has been approved by ethical review board of Islamic International University Islamabad.

Consent to publication:

All authors approved the submission of the manuscript for publication

Availability of data and material:

The data is available and can be used for the academic or research purposes.

Competing interests:

The authors have no conflict of interest.

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Authors Contribution:

ST is principal investigator of the study. ST, ZK, ZZP and US wrote the manuscript and analyzed the data. ZZP and US improved the study. ZK performed experiments. MA, and HA assisted ST in providing technical support.

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Not applicable

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Figures

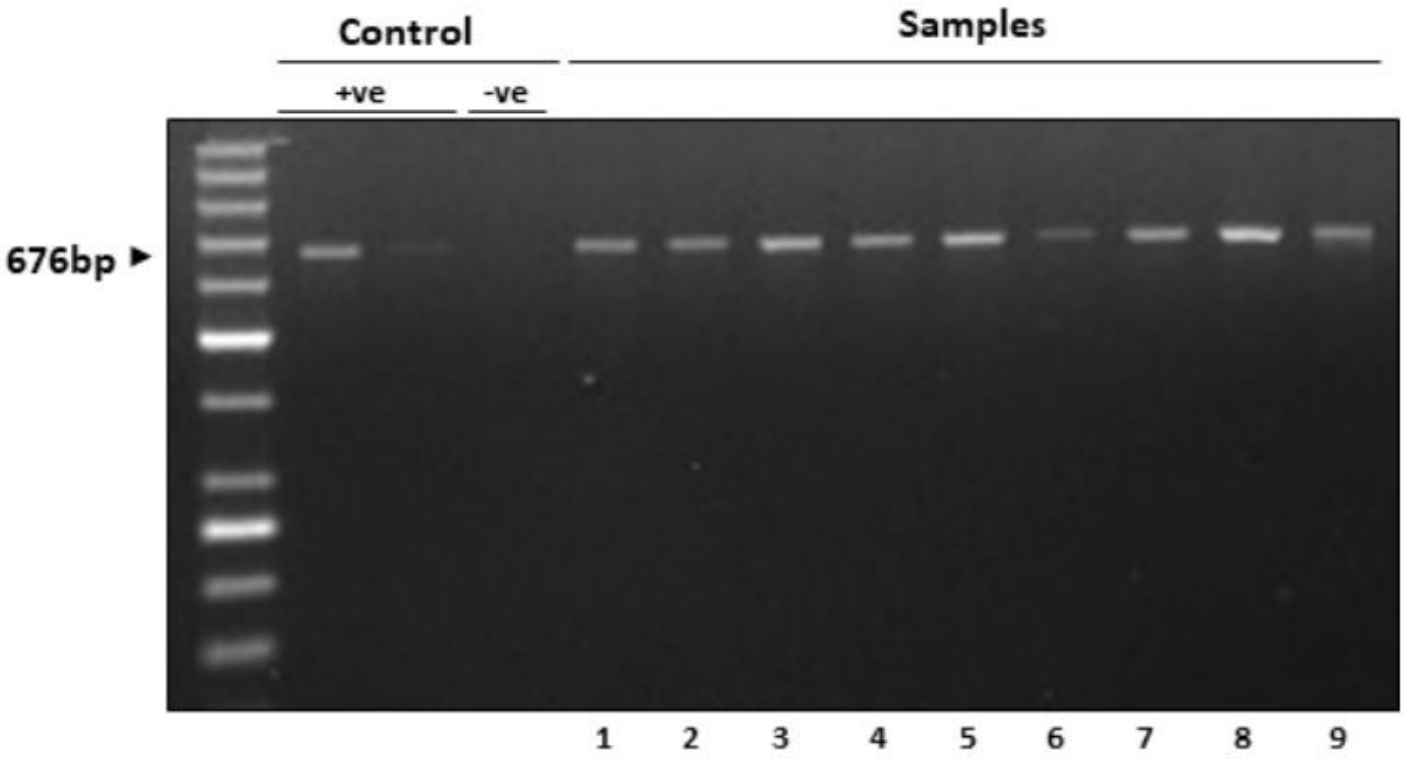

Figure 1

PCR- Amplified product of rs3742305: Lane M: 50bp, DNA ladder, Lane 1-9; Amplified product of rs3742305, NTC; Negative Control, +ve Cont: Positive Control

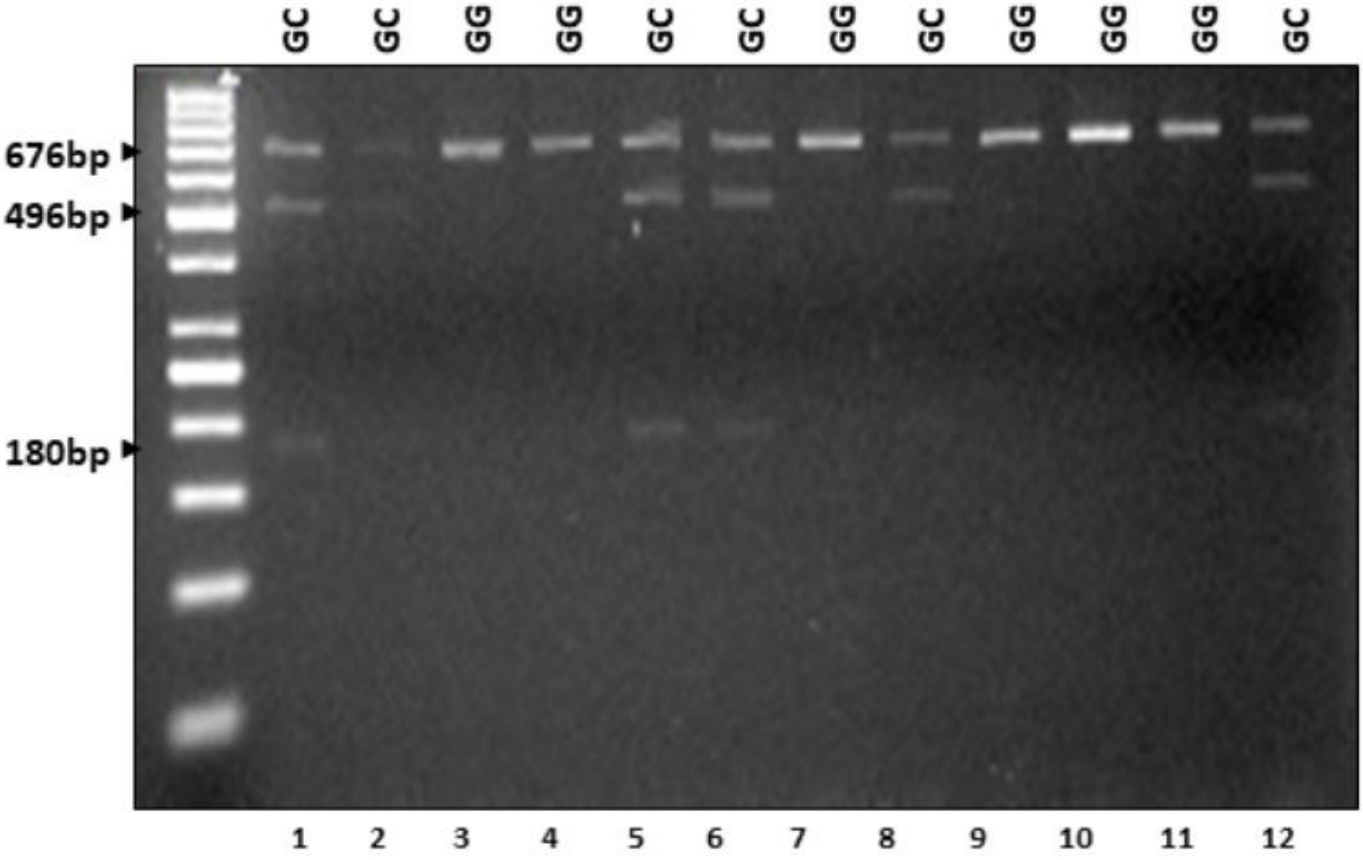

Figure 2

RFLP Analysis of rs3742305: Lane M: 50bp DNA ladder, GC genotype shows 676bp, 496bp and 180bp and GG shows 676bp.