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RESEARCH ARTICLE

Exploring the genetic variants with HCC prognostic potential in PS and S genes of HBV among treatment Naïve chronically infected patients in Saudi Arabia.

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Abstract

Hepatitis B virus (HBV) infection is a major health concerns worldwide. In 2014, 240 million persons being chronically infected by HBV worldwide (WHO). HBV accounts for 75% of HCC worldwide. Ten HBV genotypes (A-J) with 34 sub-genotypes are reported. HBV diagnosis is primarily done by detecting HBV surface antigen which is coded by the pre surface (PS) and surface (S) genes. Mutations in PS and S genes may result in immune escape of HBV as most B and T cell epitopes are coded by PS and S genes. Besides mutations in surface proteins may also cause their accumulation inside cell and subsequently developing HCC. The aim of this cross sectional study was to analyze the mutation of prognostic importance for HCC development occurring in PS and S genes of HBV. The methodology included staging of the disease using serologic, biochemical profiles of patients. Sequencing the PS and S gene of HBV DNA extracted from serum, questionnaire analysis and collection of medical history of patient genotyping and variant analysis of sequences were performed. Genotype D was most prevalent in the study group, other genotypes detected were A, B C and E. Majority of the patients in this study were in the immune active phase of CHB, only one case was in the immune tolerant phase 15 patients were immune inactive patients and 7 were HBV associated HCC patients. The comparison of variants in the HCC group with other CLD phase revealed one novel C242A transversion variant in genotype D which could be a promising prognostic marker for HCC.

Introduction

Hepatitis B virus (HBV) is a global health problem with about 240 million people persistently infected worldwide (WHO, 2015). Chronic HBV infection is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC). HBV has been divided into ten genotypes, A-J, according to its divergence with 8% or greater in the full genome sequence or 4% divergence or greater in the surface gene sequence. These HBV genotypes have different

geographic, clinical and virologic characteristics. Health complications associated with HBV infection vary greatly and depend on several factors such as age at infection, person's immune status, size of the inoculum at infection, etc. (Asabe et al., 2009; Sharma et al., 2005). About 90-95% adults infected with HBV resolve infection. On the other hand 90% of infants infected with HBV become chronic carriers. The grave consequences associated with CHB are the chronic liver function deficit, cirrhosis, and hepatocellular carcinoma (HCC). Nearly 75% of all HCC cases are associated to HBV.

CHB is characterized by the persistence of high levels of HBV DNA and/or HBsAg and HBeAg for more than six months in blood (Keshvari et al., 2015). CHB causes inflammation of the liver which varies in severity but is always milder than in acute HBV infection. The disease course of CHB can be divided into three phases: high replicative phase or immuno-tolerant phase, low replicative phase or immune clearance phase and non-replicative phase or inactive carrier state (McMahon, 2008). In the high replicative phase the titer of HBsAg, DNA (viral load ≥ 20000 IU/mL) and HBeAg are high with inevident liver disease and normal ALT (Lok and Lai, 1988). In the low replicative immune active phase seroconversion of HBeAg to HBeAb occurs and also DNA levels fall (McMahon, 2008) below 20000 IU/ m. There is an accompanied rise in ALT levels, probably due to immune mediated injury to hepatocytes after a rise in viral load (Liaw et al., 1983). In this phase patients are at high risk of developing cirrhosis (Croagh and Lubel, 2014). Once cirrhosis occurs it persists which cannot be reversed. Nearly one fifth of the persistent HBV cases progress to cirrhosis, increasing the risk of developing HCC by 100 folds.(Ganem D, 2001). Some patients may show moderate levels of liver inflammation and HBV replication despite being negative for HBeAg. These patients may be infected by a mutant HBV that is unable to synthesize HBeAg and are categorized as HBeAg negative CHB. In the non-replicative phase there is little or no evidence of HBV DNA replication with DNA replication markers (HBeAg and HBV DNA in sera) either very low or absent. ALT levels range from normal to as high as 200 IU/L and albumin and globulin ratio is markedly deranged(WHO, 2015). HBV associated hepatocarcinogenesis is a complex multistep multi factorial process. HBV may result in HCC by both direct and indirect means. In the direct role of HBV in hepatocarcinogenesis HBV DNA integrates with Host DNA there by activating several proto oncogenes and inhibiting the tumor suppressor genes by a cis effect. Additionally it can have a trans-activation effect via HBx protein. Besides HBV also accumulates mutations during the chronic course of the disease that may lead to pathogenesis of HCC and in fact play a prognostic role for HCC. The envelope gene of HBV codes for three forms of hepatitis B surface antigen (HBsAg), small (S), middle (M) and large (L), sharing 226 amino acids. Several studies have provided evidence that naturally occurring mutations in the preS region present in M and L but not S are correlated with more progressive liver disease. The preS deletion mutations, in particular, may affect the ratio between the S and L envelope proteins, resulting in endoplasmic reticulum stress leading to more aggressive liver disease and ultimately HCC. Alternatively the indirect route of HBV mediated HCC development occurs in the backdrop of fibrosis and cirrhosis that is the result of immune mediated prolonged injury to liver cells. Deletion mutations in the preS2 start codon mutations are the most commonly reported preS mutations, and are particularly correlated to a more severe HBV-related liver disease leading to liver cirrhosis and development of HCC in chronic HBV patients. Therefore we aimed at determining variants in the PS and S gene of HBV that may play a role in early prognosis of HCC.

Materials and Methods

Study Design and Patient recruitment

This cross sectional study was conducted in HBV infected persons who did not receive any anti-viral drug treatment and had no coinfection by HCV, HDV and HIV. All the subjects were above the age of 20 years. Patients were approached through participating clinicians from hospitals in Jeddah and Gizan region of Saudi Arabia. A total of 151 persons participated in the study and signed the consent form after being informed about the research. The final study group contained 79 patients that satisfied the subject selection criteria. The study design was approved by the King Fahad Medical research Center (KFMRC) ethics committee.

Sample collection processing and retrieval of patient medical records.

Participants donated 10 ml of blood which were collected in serum separation (SST) and EDTA tubes (BD Vacutainer®) respectively. Samples were processed by centrifuging at 2000 rpm for 10 minutes and then transferring the serum and plasma into Cryo-vials within 2 hours of collection to prevent DNase, RNase and protease activity. The processed sample aliquots were then stored at -80 ^{o}C until use. Record of patient's personal

profile and contacts were maintained alongside sample collection. Patient's medical history was retrieved from hospital's medical records database. Treatment history was obtained from the hard medical record files of patients. Permission for accessing the patient files was obtained from the hospital record section. All information collected were treated as confidential.

DNA isolation and quantification

DNA was extracted from serum samples using QIAamp Blood mini kit from QIAGEN (QIAGEN, Germany) using manufacturer's protocol. DNA integrity and quantification, was done by spectral measurements of optical density at wavelengths 260nm and 280nm in NanoDrop-2000C, spectrophotometer according to the manufacturer protocol (Thermo Scientific, US). Samples with absorbance ratio at 260/280nm of ≈1.8 are generally accepted as "pure" for DNA. Extracted DNA was stored at -20^{o}C until use.

PCR amplification of PS and S gene visualization and gel extraction

Nested PCR was performed to amplify PS and S regions of the HBV genome using GoTaq® Green Master Mix (Promega, Germany) using the designed gene specific primers. The conditions for the PCR are given in Table 1.The expected size of second PCR products of PS and S HBV genes was ≈564nt and ≈439nt base pair lengths respectively.

The amplified PCR product and DNA size marker (DNA ladder of 100bp or 50bp length) were mixed with 6X loading dye (bromo-phenol blue) and loaded on 2% agarose (Sigma-Aldrich) gel in 1X TBE buffer (Thermo Scientific™) containing 0.5µg Ethidium Bromide/ml(Bio-Rad, Poland) for fluorescence tagging of DNA. The gel electrophoresis was carried out at a constant potential difference of 100 volts, for 40 minutes. The electrphoretogram was visualized under UV in the gel documentation system from Syngene (Syngene, UK).

Positive PCR product with expected sizes of PS (≈564 base pair length) or S (≈439 base pair length) regions were gel purified using Qiagen gel extraction kit (Qiagen, Germany) according to manufacturer protocol. The purified DNA was used as a template for cycle sequencing.

Table 1. PCR conditions used for amplifying PS and S regions of HBV

PS Primers		**5' TO 3'**			NT.Δ		**S Primers**		**5' TO 3'**			NT.Δ	
Forward-outer-PS1s		GGGTCACCTTATTCTTGGGA			2814-2833		Forward-outer-S1s		AGAACATCGCATCAGGACTC			159-178	
Reverse-outer-PS2a		CCCCGCCTGTAACACGAGCA			208-189		Reverse-outer-S2a		CATAGGTATCTTGCGAAGC			642-623	
Forward-inner-PS3s		TTGGGAACAAGATCTACAGC			2828-2847		Forward-inner-S3s		AGGACCCCTGCTCGTGTTAC			181-200	
Reverse-inner-PS4a		GTCCTGATGCGATGTTCTCC			176-157		Reverse-inner-S4a		AGATGATGGGATGGGAATAC			619-600	
Temp	95	95	55	72	72	4	**Temp**	95	95	45	72	72	4
Time	2min	1	30 sec	1min	5 min	∞	**Time**	2min	1 min	1 min	1min	5 min	∞
Cycle	1	40			1	1	**Cycle**	1	45			1	1
NOTE: the nucleotide positions correspond to genomic positions in HBV genotype D reference sequence AF121240													

DNA sequencing

HBV PS and S genes were sequenced in both forward and reverse directions, by automated Sanger dideoxy chain termination method using BigDye® Terminator v3.1 Cycle Sequencing Kit from ABI(Applied Biosystems®, USA).The sequencing conditions were as follows: an initial denaturation step at 96°C for 1 min, followed by 25 cycles of the following steps: denaturation at 96°C for ten seconds, annealing at 50°C for five seconds followed by the extension for one minute at 60°C. At the end of 25 cycles, final extension step at 60^{o}C for four minutes and then the reaction was stopped by cooling at 4^{o}C. The cycle sequencing products was purified using ethanol and 125mM (pH8) EDTA, to remove salts and reagents that could interfere in the reading of sequences in the genetic analyser.The purified cycle sequencing product were mixed with HiDi and the double strands of DNA denatured at

96^{o}C for three minutes and then immediately transferred on ice. The sequences were then read on an ABI 3500 genetic analyser (Life Technologies, Foster City, CA, USA).

Chromatogram processing

The sequence chromatogram were viewed and edited in Geneious software (release version 8.1) (Kearse et al., 2012). The reverse sequences were reverse complemented and aligned to the forward sequences and corrected after visual inspection of peak qualities. The primer regions were trimmed from the sequences before making a consensus sequences. The sequences were submitted to NCBI and can be found at NCBI, BioProject ID PRJNA294996.

Genotyping and reference sequence retrieval

Pre S and S consensus sequences were searched for most similar best aligned sequences using the NCBI web based BLAST alignment tool. The sequence best matched to the query sequence and giving the best alignment score with maximum hits was used to determine the genotype of the query sequence. All the aligned sequences were downloaded as genebank file. Besides the the HBV genotype and sub genotype reference sequences were also downloaded from NCBI taxonomic repository db reference -db_xref="taxon:10407

Variant detection

The sequences were aligned with their reference genotype sequence. The numbering of sequences was done according to the reference sequence corresponding to respective genotypes. The sequences were aligned with the highly annotated genotype-A reference sequence from Genbank (accession # AM282986) (Panjaworayan et al., 2007) Other annotations were obtained from research studies conducted on regulatory domains in primary transcripts and topology of Surface protein of HBV. The "Annotate and predict" tool for variant detection in Geneious software (Kearse et al., 2012) was used to determine the variants with respect to reference sequence (Nielsen et al., 2012).

HBV Variants common between HCC and CHB patients

Since all the HCC patients were infected with HBV genotype D, we analysed genotype D sequences from CHB patients for variants which also occurred in HCC. Short oligonucleotide sequences containing 10 bases upstream and downstream of all variants were extracted and used in BLAST across 75460 HBV sequences in the non-redundant, nucleotide database at NCBI. Variants that were not reported in the database were considered unique to study group across all HBV genotypes

Diagnostic Serological and Biochemical tests

Serological markers for infection of HBV, HCV and HIV were analysed using commercially available ELISA kits. The kits for surface antigen (HBs-Ag, envelope antigen (HBeAg) and total core antibody (HBc-Ab), Anti-HCV, Anti-HIV1/2) were obtained from DiaSorin (S.p.A.ViaCrescentino 13040 Saluggia (VC) – Italy). Kit for AFP analysis was ordered from Omega Diagnostics Ltd. (UK). All serological markers were analyzed using manufacturer's instructions. The instruments used were automated ELISA plate washer, and ELISA plate reader from BioTek (BioTek Instruments, Inc. USA). Results for liver function and viral load were obtained from patient records following the day of blood collection.

Diagnosis of HBV associated chronic Liver Disease and staging

CHB is characterized by HBsAg positivity and/or HBV DNA presence for > 6 months. In the early stages HBeAg may be present that reflects the replication of HBV cccDNA in hepatocytes. CHB is differentiated from acute infection by the presence of HBcIgM antibody.
The replicative immune tolerant phase is characterized by HBeAg positive status with a very high viral load of ≥ 20,000 IU/mL with normal liver function, typically ALT levels <19IU/mL in females and <30 IU/mL in males.
The characteristic feature of immune active phase is the elevated liver enzymes particularly the ALT levels≥19IU/mL in females and ≥30 IU/mL in males. Persons with viral load of ≥ 2000 IU/mL are at high risk of

developing cirrhosis. The patients may be HBeAg positive or HBeAg negative. Seroconversion may occur in this phase. Patients in this are usually referred to endoscopy unit for the assessment of liver cirrhosis and liver fibrosis. Persons in the inactive carrier state have persistently normal ALT levels <19IU/mL in females and <30 IU/mL in males. The viral loads are very low or undetected. Patients in this phase are HBeAg negative with or without anti HBeAb being present. Some persons may also clear HBsAg after the non-replicative immune inactive phase and referred to as resolved CHB cases.

Diagnosis of Hepatocellular Carcinoma

HCC was suspected with very high levels of AFP, PTT and/ INR. HCC is confirmed by the presence of liver lesions with or without liver cirrhosis using ultrasonography, MRI or CT scan.

Descriptive and inferential analysis of clinical data

The clinical data of patients in each phase of CLD (duration of disease, AFP, viral load and LFT and CBC) were first analyzed using descriptive statistics, determining the measures of central tendencies and the descriptive at first second and third quartiles. There after comparison of various variables were done between genders to determine the variability among the clinical profiles, which was done by comparing means or medians ±SEM or SD of two or more groups with more than two variables. The comparisons were done by performing two way ANOVA. Overall and genderwise two tailed correlation among variables were also performed by using spearman's correlation statistics. Similarly comparative analysis between the clinical profile of patients with unique clinical trends of presence or absence of a particular variable were also assessed with the correlation analysis among variables in each group. Finally between groups comparisons were done for determining the differences in the clinical profiles in different phases of CLD. The analysis was done using the GraphPad statistical analysis software version 6.

Results

Analysis of Demographic data

In this study total of 79 patients (72 CHB and 7 HBV associated HCC cases) were included from different nationalities (Figure1). The subjects were mainly Saudi nationals. The number of males and females were 44 and 35 respectively. The average ages of males and females were 45.36 years and 45.16 years respectively. The ages of subjects in both genders were proportionally represented with the modal age group being 40-60 years (Figure 2).

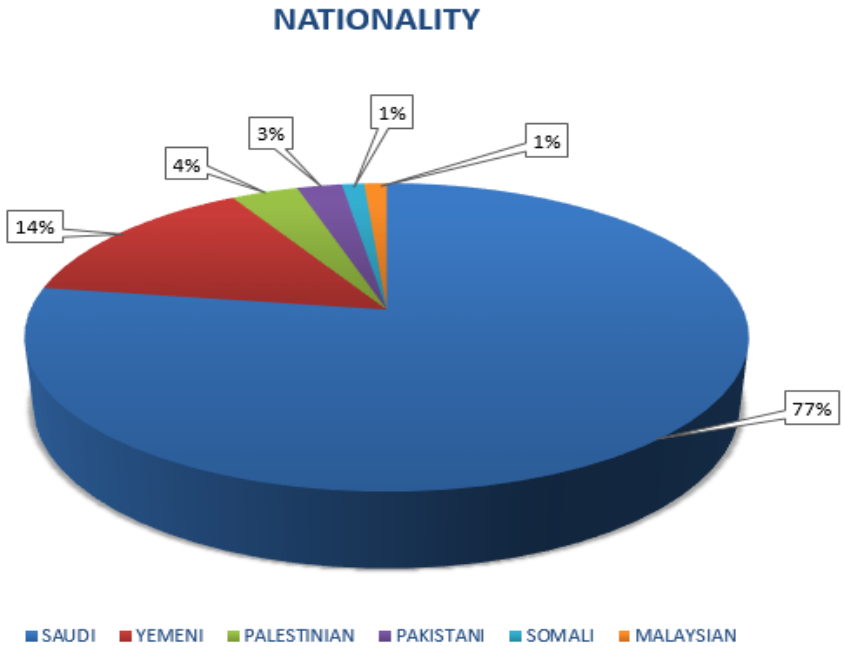

Figure 1. Subject distribution based on their nationalities in the study group

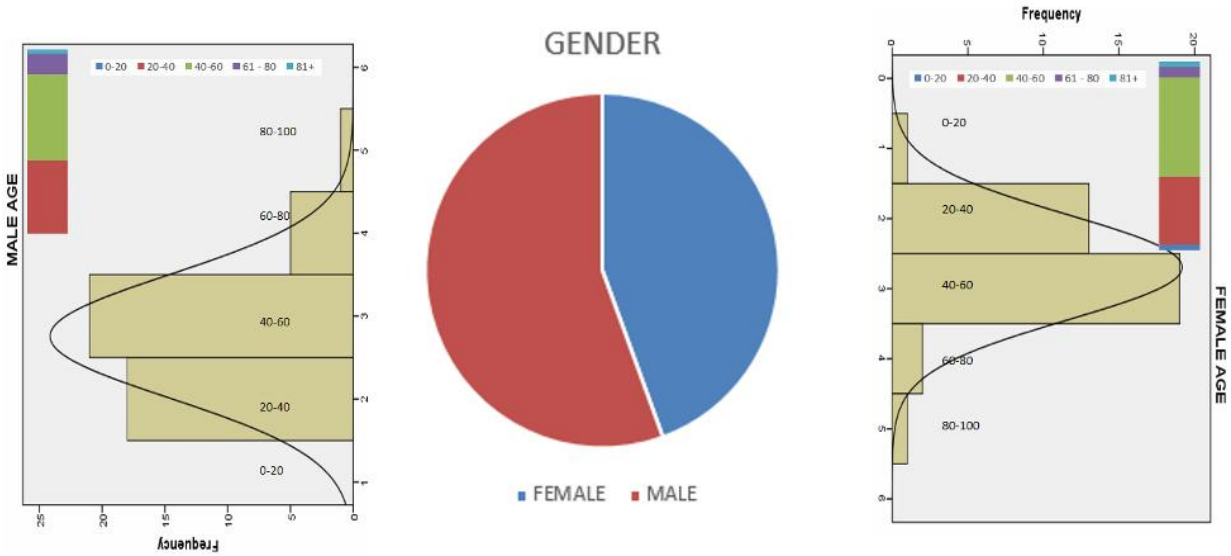

Figure 2. Gender and Age distribution of subjects in the study group

Genotypes

Majority of the subjects were infected with genotype D followed by genotype C, genotype A, genotype E and genotype B (Figure3). Genotype D was detected only in cases from Saudi Arabia, Yemen and Palestine (Figure 4). Genotype B was found only in 2 Pakistan cases, genotype C was detected in low frequency in Saudi, Yemeni and Malaysia cases while genotype E was only detected in 3 Saudi cases. Genotype A was detected in 3 cases from Saudi Arabia, Yemen and Somalia.

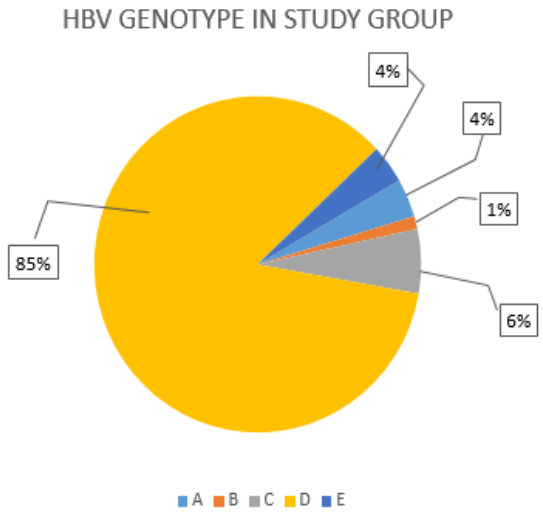

Figure3. HBV genotype composition of study group

Figure 4. Nationality of subjects in which HBV genotype A/B/C/D/E were detected.

Descriptive and inferential analysis of clinical profiles of patients

Serology of the 79 selected patients revealed that the positive samples for HBsAg, HBeAg, HBeAb, HBcAb (total) and HBcIgM were 72, 5, 38, 76 and 1 respectively. The samples negative for HBsAg, HBeAg, HBeAb, HBcAb (total) and HBcIgM were 7, 74, 41, 3 and 78rsspectively (Table 2). The biochemical parameters show that 75 % of the individuals have low AFP, DNA <2000 IU/mL, and all the other liver parameters are normal except for the ALT levels which all males above 50th percentile have ALT >30 IU/ml and the females above 25th (first quartile)percentile have elevated ALT >19 IU/mL (Table 3).

Table2 serology of selected CHB patients

Tests	Positive	Negative
HBsAg	72	7
HBeAg	5	74
HBeAb	38	41
HBcAb total	76	3
HBcIgM	1	78

Table3. Descriptive of Biochemical parameters of selected CHB patients

	Normal Range	Min	25% Percentile	Median	75% Percentile	Max	Mean	SD	SEM	Lower 95% CI of mean	Upper 95% CI of mean	CV
AFP	0-5.4 IU/mL	0	2.2	3	7.175	77200	1350	9963	1286	-1223	3924	737.84%
DNA		19	74	213	1268	2E+08	4E+06	2E+07	4E+06	-3531000	11020000	661.55%
TPROT	64-82 g/L	58	74.25	78	83	101	78.43	7.601	0.9813	76.47	80.4	9.69%
ALB	40.2-47.6 g/L	19	32	36	39	41	35.41	4.825	0.6282	34.15	36.66	13.63%
ALP	50-136 U/L	41	63	77	104.3	778	103.4	102.6	13.25	76.92	129.9	99.23%
AST	15-37 U/L	7	15	21	36.25	277	38.07	51.52	6.651	24.76	51.37	135.33%
ALT	12-78 U/L	14	23	28.5	43.75	137	36.43	22.8	2.943	30.54	42.32	62.57%
male	≥30IU/L	19	27	32	51	111	39.19	19.05	3.421	32.21	46.18	48.60%
female	≥19IU/L	14	20	23	39.5	137	33.48	26.25	4.874	23.5	43.47	78.40%
GGT	5-85 U/L	7	15	27	62	1207	77.16	174.5	23.52	30	124.3	226.08%
TBIL	0-17 μmol/L	2	6	7	11	43	9.212	6.143	0.793	7.625	10.8	66.69%

NOTE: Min-minimum, Max-maximum, SD-standard deviation from mean, SEM-standard error of mean, CI-confidence interval, CV-coefficient of variation

Immuno tolerant phase

Only one patient was diagnosed to be in immune tolerant phase of CHB. The patient was a 20 year old male, Pakistani national infected with HBV genotype C and a patient with Down's syndrome. He presented very high viral load, with slightly increased AFP levels and slight fibrosis of liver. He however had normal liver panel tests and normal CBC.

Immune active phase

Majority of the subjects (56/79) were diagnosed to be in the immune-active phase of CHB. The frequency of HBV genotype in this group was A=2, B=1, C=3, D=49 and E=2. There were 28 males (M) (infected with genotype A=1, C=2, D=24 & E=1) and 28 females (F) (infected with genotype A=1, B=1, C=1, D=24 & E=1). The number of patients in different age bands 20-40, 40-60, 60-80 and 80 above were 24(F/M=12/12), 26(F/M=13/13), 6(F/M=2/4) and 1(F/M=1/0) respectively. The median age in this phase was 44 years (Table 4). The duration of infection in this group ranged from 4 to 30 years. The median duration was 7.5 years (Table 4). Above the median age majority of persons had the disease duration of about 15 years. 75 % of patients had viral loads <2000 IU/mL but had higher ALT levels than the cutoff values. All individuals below 25th percentile were females and had ALT levels >19IU/mL but $\leq$ 22 IU/mL. There were six patients with fibrosis and three with cirrhosis.

Table 4. Descriptive analysis of age and LFT of immune active patients

	N	Min	25% Percen	Median	75% Percen	Max	Mean	SD	SEM	Lower 95% CI of mean	Upper 95% CI of mean
AGE (years)	57	21	31.5	44	52	85	44.32	14.18	1.878	40.55	48.08
DD (years)	56	4	5	7.5	13	30	9.518	5.67	0.7576	8	11.04
AFP (IU/mL)	56	0.8	2.525	3.65	7.05	369.9	31.78	85.91	11.48	8.775	54.79
DNA (IU/mL)	33	19	75	207	1386	2E+06	69572	364346	63425	-59620	198763
TPROT (g/L)	42	58	74.75	78	83.25	101	78.64	7.318	1.129	76.36	80.92
ALB (g/L)	41	19	33	37	40	41	36.22	4.447	0.6945	34.82	37.62
ALP (U/L)	42	42	63	78	99.75	273	92.26	54.57	8.421	75.26	109.3
AST (U/L)	42	11	15	20.5	31	277	40.88	58.77	9.068	22.57	59.19
ALT (U/L)	42	19	23	30.5	44.5	137	37.55	21.27	3.282	30.92	44.18
GGT (U/L)	39	7	13	27	62	369	56.54	83.04	13.3	29.62	83.46
TBIL (µmol/L)	42	2	5	7.5	12.25	43	9.421	6.859	1.058	7.284	11.56

Note: DD-Disease duration, N-number of observations, SD-standard deviation, SEM-standard error of mean, CI-confidence interval

Inactive carrier state

There were 11 patients, four females and seven males that were diagnosed as inactive CHB carriers. The ages of patients varied from 32 to 60 years. All patients were HBeAg negative and except case CHB15 all had undergone seroconversion. All patients had normal PTT and INR the patients presented with an overall normal LFT. There were five individuals having fatty liver, two with liver cirrhosis and three with liver fibrosis. Differential analysis of LFT between males and females revealed a significantly higher levels of AFP ($p<0.001$) in females than males (Table 5). Four patients between the ages 31 to 36 years had resolved CHB. All had normal AFP, LFT and CBC. Owing to the small sample size the difference between the immune active and inactive CHB phase and immune tolerant phase could not be drawn however there was a significantly higher level of AFP associated with immune active phase compared with AFP levels in immune inactive phase.

Table5.Comparison of the biochemical parameters, age and duration of disease between female and males in inactive phase

Test details	Mean (F)	Mean (M)	Mean Diff.	SE of diff.	N(F)	N (M)	t	DF	95% CI of diff.	P Value
AGE (years)	45.75	46.29	-0.5357	35.2	4	7	0.015	98	-70.40 to 69.32	0.9879
DD (years)	14	13.43	0.5714	35.2	4	7	0.016	98	-69.29 to 70.43	0.9871
AFP (IU/mL)	108.6	8.571	100	35.2	4	7	2.841	98	30.17 to 169.9	0.0055**
DNA (IU/mL)	477.8	314	163.8	35.2	4	7	4.652	98	93.89 to 233.6	< 0.0001**
TPROT (g/L)	79.5	80	-0.5	35.2	4	7	0.014	98	-70.36 to 69.36	0.9887
ALB (g/L)	31.5	38.29	-6.786	35.2	4	7	0.193	98	-76.65 to 63.07	0.8475
ALP (U/L)	60	73.43	-13.43	35.2	4	7	0.382	98	-83.29 to 56.43	0.7037
AST (U/L)	22.25	17.14	5.107	35.2	4	7	0.145	98	-64.75 to 74.97	0.8849
ALT (U/L)	16.25	25.29	-9.036	35.2	4	7	0.257	98	-78.90 to 60.82	0.798
GGT (U/L)	11.75	29	-17.25	36.25	4	6	0.476	98	-89.20 to 54.70	0.6353
TBIL (µmol/L)	7.5	7.143	0.3571	35.2	4	7	0.01	98	-69.50 to 70.22	0.9919

NOTE: DD-disease duration, SE-standard error, N-number of observations, F-female, M-male DF-degree of freedom, CI-confidence interval, **significance at P≤ 0.05

Table 6. Comparison of the biochemical parameters, age and duration of disease between individuals having fatty liver and those not having fatty liver in immune inactive phase.

Test details	Mean (fatty liver)	Mean (non fatty liver)	Mean Diff.	SE of diff.	N (fatty liver)	N (non fatty liver)	t	DF	95% CI of diff.	P Value
AGE (years)	44.71	46.09	-1.377	19.88	7	11	0.069	175	-40.60 to 37.85	0.9449
DD (years)	15.14	13.64	1.507	19.88	7	11	0.076	175	-37.72 to 40.73	0.9397
AFP (IU/mL)	63.27	44.95	18.33	19.88	7	11	0.922	175	-20.90 to 57.55	0.3578
DNA (IU/mL)	482.1	373.5	108.6	19.88	7	11	5.464	175	69.37 to 147.8	< 0.0001**
TPROT (g/L)	81	79.82	1.182	19.88	7	11	0.059	175	-38.04 to 40.41	0.9527
ALB (g/L)	34	35.82	-1.818	19.88	7	11	0.091	175	-41.04 to 37.41	0.9272
ALP (U/L)	61.71	68.55	-6.831	19.88	7	11	0.344	175	-46.06 to 32.40	0.7315
AST (U/L)	19.71	19	0.7143	19.88	7	11	0.036	175	-38.51 to 39.94	0.9714
ALT (U/L)	20.57	22	-1.429	19.88	7	11	0.072	175	-40.66 to 37.80	0.9428
GGT (U/L)	14.71	22.1	-7.386	20.26	7	10	0.365	175	-47.37 to 32.60	0.7159
TBIL (µmol/L)	6.857	7.273	-0.4156	19.88	7	11	0.021	175	-39.64 to 38.81	0.9833

NOTE: DD-disease duration, SE-standard error, N-number of observations, DF-degree of freedom, CI-confidence interval, **significance at P≤ 0.05

HCC

A total of seven HCC patients could be recruited in which two were females and five were males. The patients were in the age range of 52 to 70 years, except for the case HCC 10 which was 37 year old male subject. The serology results showed that three HCC cases (HCC 8, 12, 17) were HBsAg negative that could have developed HCC after resolving CHB infection. One patient (HCC12) did not have any serologic indicators of HBV infection and probably had occult HBV infection that could have occurred during blood transfusion. Four of the seven patients had high AFP levels but a very high level of AFP were seen in cases HCC 8 (AFP=77280) and HCC 17(AFP=2256). ALL HCC cases variably had other complication that could pose a risk to HCC. Among the seven

HCC cases four had diabetes of which three had liver cirrhosis and one had fibrosis. Six patients had focal lesions in liver associated with HCC. There was a significantly higher AFP ($p<0.001$) level in HCC than in immune active phase (Table 7). There was no significant difference between the LFT parameters of HCC and immune active phase. Correlation analysis of HCC LFT parameters age and duration of disease revealed a significant positive correlation of ALP, ALT, GGT with TPROT, AST and AFP respectively.

Table 7. Comparison of the biochemical parameters and age of between individuals having HCC and those not having in immune inactive phase.

Test details	**Mean** (HCC)	**Mean** (inact)	**Mean Diff.**	**SE of diff.**	**N** (HCC)	**N** (inact)	**t**	**DF**	**95% CI of diff.**	**P Value**
AGE (years)	61.43	46.09	15.34	1087	7	11	0.0141	143	-2134 to 2165	0.9888
AFP (IU/mL)	11362	44.95	11317	1087	7	11	10.41	143	9167 to 13466	< 0.0001**
TPROT (g/L)	58.71	79.82	-21.1	1087	7	11	0.0194	143	-2170 to 2128	0.9845
ALB (g/L)	23.71	35.82	-12.1	1087	7	11	0.0111	143	-2161 to 2137	0.9911
ALP (U/L)	222	68.55	153.5	1087	7	11	0.1411	143	-1996 to 2303	0.888
AST (U/L)	63.86	19	44.86	1087	7	11	0.0413	143	-2105 to 2194	0.9672
ALT (U/L)	48.86	22	26.86	1087	7	11	0.0247	143	-2123 to 2176	0.9803
GGT (U/L)	280.9	22.1	258.8	1108	7	10	0.2335	143	-1932 to 2450	0.8157
TBIL (µmol/L)	12.14	7.273	4.87	1087	7	11	0.0045	143	-2144 to 2154	0.9964

Note: inact- immune inactive CHB, SE- standard error, N-number of observations, DF-degree of freedom **statistics is significant at $p<0.05$

Table 8. Correlation analysis of the biochemical parameters and age of in individuals having HCC

HCC	DD	PTT	INR	AFP	TPROT	ALB	ALP	AST	ALT	GGT	TBIL
DD (years)		0.8571	0.8571	0.3262	0.7825	0.7825	0.7131	0.2357	0.2667	0.1389	0.4976
PTT (seconds)	-0.1581		1.0000	0.7619	1.1429	0.3810	1.1429	0.8571	1.1429	1.1429	0.0952
INR (ratio)	0.2041	0.2582		0.4286	1.1429	0.5714	0.8571	0.5714	0.5714	0.5714	0.5714
AFP (IU/mL)	0.4325	-0.1595	-0.5149		0.8873	0.5508	0.3778	0.4262	0.5008	0.0381	0.7571
TPROT (g/L)	0.1429	0.0000	0.0000	0.0721		0.1667	0.0067	0.3956	0.1667	0.3536	0.7131
ALB (g/L)	-0.1429	0.4743	0.4082	-0.2703	0.6071		0.3536	0.8397	0.8397	0.9063	0.4976
ALP (U/L)	0.1786	0.0000	-0.2041	0.3964	0.9286	0.4286		0.3024	0.1667	0.1667	0.5948
AST (U/L)	0.5357	0.1581	-0.4082	0.3604	0.3929	-0.1071	0.4643		0.0067	0.0881	0.5560
ALT (U/L)	0.5000	0.0000	-0.4082	0.3063	0.6071	0.1071	0.6071	0.9286		0.0663	0.7825
GGT (U/L)	0.6429	0.0000	-0.4082	0.8108	0.4286	0.0714	0.6071	0.7143	0.7500		0.7825
TBIL (µmol/L)	0.3214	0.7906	0.4082	-0.1442	-0.1786	0.3214	-0.2500	0.2857	0.1429	0.1429	

NOTE : DD-disease duration, Numbers in black font represent correlation coefficient '**r**', p value is represented in blue font.
Highlights yellow represent significant correlation at $p<0.05$

Genetic variants common among HCC and various phases of CLD

All the HCC patients were infected by HBV genotype D. Therefore only genotype D sample sequences could be compared with the prevalent variants in HCC samples. The variants were detected with respect to genotype D reference sequence AF 121240. There were total 13 variants observed in all HCC samples in both PS and S regions. Most of the variants were found to be naturally occurring in other genotypes at the corresponding domain and nucleotide positions, and therefore may not have any significant effect on structure or function of the surface protein. Two novel mutations C242A and C479A were observed in the CYL1 and major hydrophilic region. (MHR) (Table 10). Variant C242A in the CYL1 domain frequently occurred in HCC and non-HCC samples (Table 10). The non HCC samples having C242A variants were in immune active phase of CHB. There was no trend

observed in the HCC associated risk factors such as diabetes, fatty liver, fibrosis cirrhosis and smoking. There was no correlation found among the LFT parameters age, duration of disease, AFP and the viral loads (Table 9).
Four deletions variants were also observed in HCC and non HCC samples. All of these deletions occurred in the beginning of the PS2 region and overlapped the B/T epitope determining regions. Two of the samples had 3 base in frame deletions at genomic positions 34 to 36. Two HCC samples showed a 27 base in frame deletion spanning from nucleotide positions 34 to 57. One non –HCC sample showed a six base deletion from nucleotide positions 52 to 57 (Table 10).

Table9. Spearman's correlation analysis between variables in the group having C242Atransversion

	AGE	DD	AFP	DNA	TPROT	ALB	ALP	AST	ALT	GGT	TBIL
AGE (year)		1	0.0333333	0.33333	0.4666667	0.2333333	0.4333333	0.1666667	0.3	0.0666667	0.766667
DD (years)	0.5		1	1	0.3333333	0	1	1	0.3333333	1	1
AFP (IU/mL)	0.9746794	0.5		0.33333	0.4333333	0.1333333	0.35	0.35	0.2333333	0.0833333	0.95
DNA (IU/mL)	0.7378648	-0.5	0.8		0.75	0.5	0.75	0.3333333	0.3333333	0.0833333	1.083333
TPROT (g/L)	-0.289474	-1	-0.410391	-0.4		0.2	0.8333333	0.8333333	1	0.8333333	0.366667
ALB (g/L)	-0.552632	-0.866025	-0.718185	-0.3162	0.7631579		0.1333333	1	0.3	0.3	0.9
ALP (U/L)	0.4616903	-0.5	0.6	0.4	-0.102598	-0.718185		1.05	0.0833333	0.2333333	0.233333
AST (U/L)	0.7181848	-0.5	0.6	0.8	0.1538968	0.0512989	0		0.5166667	0.2333333	0.683333
ALT (U/L)	0.615587	-1	0.7	0.8	0.0512989	-0.564288	0.9	0.4		0.0833333	0.35
GGT (U/L)	0.8720816	-0.5	0.9	1	-0.102598	-0.564288	0.7	0.7	0.9		0.783333
TBIL (µmol/L)	0.2051957	0.5	0.1	0	-0.46169	0.1025978	-0.7	0.3	-0.6	-0.2	

NOTE : DD-disease duration, Numbers in black font represent correlation coefficient '**r',** p value is represented in blue font.
Highlights yellow represent significant correlation at p<0.05

Table10 variants common among HCC and other CLD phases of CHB

Variant Sequence	GENOMIC Δ	δ TYPE	NT. δ	CODON δ	AAδ	RAW *f*	Δ in Codon	Δ in protein domain
120PS, G2PS, HCC14PS	2,965	TV	A -> C	ACC -> CCC	T -> P	3	1	PS1, NTCP-A, S2/SPROM,
HCC8PS	2,965	TV	A -> T	ACC -> TCC	T -> S	1	1	PS1, NTCP-A, S2/SPROM,
G8PS, HCC8PS	2,992	TS	G -> A	GCT -> ACT	A -> T	2	1	PS1, NTCP-A, S2/SPROM,
HCC14PS	3,008	TS	T -> C	CTG -> CCG	L -> P	1	2	PS1, S2/S PROM, BTEPI
HCC8PS	3,103	TS	A -> G	ACC -> GCC	T -> A	1	1	PS1, S2/S PROM, BTEPI
100PS, 101PS, 104PS, 112PS, 114PS, 117PS, 121PS, 124PS, 125PS, 131PS, 132PS, 133PS, 136PS, 25PS, 30PS, 34PS, 39PS, 43PS, 44PS, 45PS, 50PS, 58PS, 68 PS , 69PS, 76PS, 81PS, 82PS, 85PS, 89PS, HCC14PS, HCC15PS, HCC17PS	3,154	TS	A -> G	AAC ->GAC	N -> D	32	1	PS1, CORE-B/P, BTEPI
G8PS, HCC8PS	2	TV	T -> A	CTC -> CAC	L -> H	2	1	PS1, S2/S PROM, BTEPI
109PS, 117PS, 125PS, 127PS, 15PS, 17PS, 22PS, 25PS, HCC14PS	37	TS	A -> G	AGT ->GGT	S -> G	9	3	PS2, BEPI
101PS, G8PS, HCC8PS	34-36	Del	3BASES	27 BASE DELETION in G8PS, HCC8PS		3		PS2, BEPI
G8PS, HCC8PS	37-48	Del	15BASES			2		PS2, BEPI
130PS, 94PS, G3PS, G8PS, HCC8PS	49-51	Del	3BASES			5		PS2, BTEPI
G8PS, HCC8PS	52-57	Del	6BASES			2		PS2, BTEPI
89PS, HCC18 PS	148	TS	G -> A	GCT -> ACT	A -> T	2	3	PS2, BTEPI
116 S , 126 S, 37 S, 40 S, HCC-18 S	225	TS	G -> A	AGA ->AAA	R -> K	5	2	TM1
129 S, 17 S, 30S, 53 S, 85 S, HCC 14 S, HC	242	TV	C -> A	CAG ->AAG	Q -> K	7	1	CYL1-TEPI
HCC-18 S	479	TV	C -> A	CTA -> ATA	L -> I	1	1	AGL
HCC 14 S, HC 17 S	555	TV	A -> T	TAT -> TTT	Y -> F	2	2	AGL-"a-BEPI

Legends : *across 75460 HBV sequences at NCBI (nr/nt). δ =change, Δ=Position, AA = amino acid. Average length of S sequences 399 (nt), Nucleotide= NT., *f*= Frequency, CDS=Coding sequence, NOTE: Prefix CHB and suffix omitted from sample ID. Coding domains → CYL1= cytoplasmic loop 1, TM1= transmembrane domain 1, ENV=envelope protein, TM2=trans-membrane domain 2, AGL= major antigenic loop, AGL-'a'= 'a' determinant in the antigenic loop.

Discussion

Majority of the individuals (56/79) in this study were found to be in immune active phase of CHB. Due to heterogeneous nature of serologic and biochemical profiles of patients, there is often confusion in the classification of patients as immune active and immune inactive patients (Kim et al., 2011). The classification is important from disease management point of view. An interesting finding was that the individuals with fatty liver had lower viral loads than those without fatty liver. A large scale study conducted in Taiwan has found an inverse relationship of fatty liver with the viral load but a positive correlation with ALT levels (Cheng et al., 2013). This supports our observation.

Patients in the immune inactive low replicative phase had normal LFT and CBC. In woman the AFP level was significantly higher than in males. However studies conducted earlier have not shown any significant association between female gender and AFP levels (Liu et al., 2014). Five out of 11 individuals had fatty liver, two with liver cirrhosis and three with liver fibrosis. Earlier studies have indicated the risk association of fatty liver and the development of fibrosis and cirrhosis (Minakari et al., 2009; Williams et al., 2015). Four patients between the ages of 31 to 36 years had resolved CHB within 7 to 9 years after first occurrence of acute liver disease. Studies have reported spontaneous resolution of HBV by the loss of HBsAg that may occur naturally. Clearance of HBsAg has been estimated to be 0.5 to 2 percent in Western patients and only about 0.1 to 0.8 percent in Asian countries(Liu et al., 2010).

Only seven HBV associated HCC case could be recruited. The number of samples was not enough to make a conclusive finding. Most of the samples were collected from the emergency wards that limited the access to HCC patients. Also many were terminally ill. The relatives or the patient did not agree to participate in the study. Majority of the patients were above the age of 50 years. It was interesting to find young HCC male patient at the young age of 37 years. One study has indicated worse first year survival in male patients under the age of 40 years (Chen et al., 2006). However prognosis improved after first year of HCC development. Another interesting finding was that three HCC patient between the ages 67 to 85 were HBsAg negative. According to one study patients who resolve CHB after 50 years of age were more likely to develop HCC(Yuen et al., 2008). Two of the three HBsAg negative patients had developed HCC after resolving CHB. One HBsAg negative patient showed no serological markers but HBV DNA was amplified from the patient's serum sample. Suggesting that he had occult infection. He was known to have received blood transfusion in Saudi Arabia which could have been the source of infection. Four of the HCC patients had elevated AFP levels with one patient showing exceptionally high AFP level(Murugavel et al., 2008). AFP is widely used as a diagnostic marker for HCC however it yields unsatisfactory results in early diagnosis of HCC (Zhao, 2013). The comparative analysis of LFT of HCC, immune active and immune inactive patients revealed no significant difference. The only significant difference observed was in the AFP which was significantly higher than both active and inactive phases of CHB. Other important feature was the increased PTT in all the HCC patients. Normal levels of PTT and INR were observed in all other phases of CHB.

Most of the variants detected in HCC samples were those which naturally occurred in other genotypes on the corresponding variant position and therefore might be insignificant. However there were two novel mutations which may be significant in pathophysiology of chronic liver disease and HCC. One of the two novel mutation was frequently observed in the CYL1 domain causing a C242A transversion that results in a non-synonymous Q187K change in amino acid. Another novel mutation was observed just before the antigenic 'a' determinant in the AGL or MHR. Deletion mutations in the N terminal end of pre S2 domain were also observed. This position is reported to be a mutational hotspot and is commonly reported in HCC samples (Zhang et al., 2012). Therefore samples with deletion mutations in this region may have a worse prognosis. Studies have reported that the non S2/S promoter deletion do not differ in the amount of surface protein production and secretion. However since these mutation overlap the B and T cell epitope recognition sites they may escape recognition by immune cells.

Conclusion

Owing to small sample size we were not able to find other variants that could be of prognostic importance. However one variant which occurred frequently in the CYL1 domain causing a C242A transversion that results in a non-synonymous Q187K change in amino acid, holds promise as a prognostic marker for HCC. This variant was not reported and its uniqueness was checked by BLAST across 75460 HBV sequences in the non-redundant, nucleotide database on 25th September 2015 on NCBI database. More research need to be done to explore new variants and explore their pathophysiologic effect in disease progression.

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