RESEARCH ARTICLE

Molecular characterization of hepatitis B virus basal core promoter and precore region of isolates from chronic hepatitis B patients

Israr Ahmad[1], Kafeel Ahmad[2]

Abstract

Objective: To analyse mutations in precore and core promoter regions of hepatitis B virus genome in chronic hepatitis B patients.

Methods: The cross-sectional prospective study was conducted at the Centre of Biotechnology and Microbiology, University of Peshawar and Pakistan Health Research Council (PHRC), Research Centre, Khyber Medical College Peshawar from June 2014 to June 2015, and comprised samples from treatment-naïve chronic hepatitis B patients aged >15 years from three cities of Pakistan. The samples included patients who were both positive and negative for hepatitis B envelope antigen. Viral load, hepatitis B envelope antigen / anti-hepatitis B envelope status, hepatitis B virus enzyme-linked immunosorbent assay and alanine aminotransferase levels were determined. Direct sequencing of basal core promoter and precore regions of hepatitis B virus genome was carried out following a nested polymerase chain reaction approach. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis software version 6.0. Data was analysed using SPSS 16.

Results: Of the 50 patients, 33(66%) were males. The overall mean age was 28.5±11.4 years. Of all the subjects, 25(50%) each were positive and negative for hepatitis B envelope antigen. Precore stop codon mutation G1896A was detected in 19 (38%) isolates; 17(34%) among negative patients and 2(4%) in positive patients. Classic A1762T/G1764A double mutation was noted in 15(30%) isolates. Mutation at position 1764 was observed in 12(48%) samples. A rare G1764T mutation was also detected in 6(12%) isolates. The CG1802-1803 mutation was detected in 47(94%) isolates, while all the 50(100%) isolates had T1858A. The GCAC Kozak sequence was present in 43(86%) isolates; CAA1817-1819 in 49(98%); and G1888 in 49(98%). Overall, 9(18%) isolates had wild-type sequences at all important loci, including positions 1762, 1764 and 1896. The pattern of sequences at genotype specific positions and phylogenetic tree speculates that majority of study isolates belonged to genotype D.

Conclusions: Basal core promoter and precore regions variants along with the preponderances of genotype D-specific mutations suggested a higher risk of hepatocellular carcinoma and poor clinical outcome in such patients.

Keywords: Hepatitis B virus, Basal core promoter, Precore region, Mutation. (JPMA 71: 1575; 2021)

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Introduction

Hepatitis B virus (HBV) infection is a global health problem that has affected millions of people with a significant proportion in chronic stage as HBV carriers.[1] Chronic HBV infection occurs in 20% of the infected patients and leads to severe consequences, like cirrhosis and hepato-cellular carcinoma.[2] HBV infection is more common in the Asia-Pacific region with high morbidity and mortality rates.[3] Pakistan lies in the intermediate prevalence zone where the disease is highly endemic.[4] HBV is a 42nm virus composed of HBV core antigen (HBcAg) surrounded by a lipoprotein layer called HBV envelope antigen (HBeAg) that contains the HBV surface antigen (HBsAg).[5] HBV genome consists of relaxed double-stranded deoxyribonucleic acid (DNA) with a size of 3.2kb.[6,7] The genome has four overlapping open reading frames (ORF) that code for pre-surface/surface (Pre-S/S) antigens, Precore/core (PC/C) antigens, polymerase and X protein.[6,7] HBV genome is susceptible to mutations as the viral DNA polymerase / reverse transcriptase has no proofreading ability.[8] HBV genotypes have implications for HBeAg seroconversion, disease progression, occurrence of core promoter mutations and clinical outcome. Patients with genotype C and D infections have a lower rate of spontaneous HBeAg seroconversion, higher frequency of core promoter and pre-S mutations than those with genotypes A and B infections.[9] Patients with C and D HBV genotypes also are at higher lifetime risk of the development of cirrhosis and hepatocellular carcinoma than those with genotypes A and B.[9] The most prevailing and clinically important mutations are G1896A PC stop codon mutation and dual A1762T/G1764A mutation.[10] Chronic HBeAg-negative hepatitis is prevalent in Mediterranean countries and a large number of infected individuals harbour basal core promoter (BCP) and PC mutations.[11] Treatment with interferon and other drugs in patients infected with mutant viruses is hard due to a higher rate of relapse after treatment termination. A few studies in Pakistan have been conducted on the detection of genotypes and mutations using Line Probe Assay

[1]Pakistan Health Research Council (PHRC) Research Centre, Khyber Medical College, Peshawar; [2]Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar, Pakistan.

Correspondence: Kafeel Ahmad. e-mail: kafeelpbg@gmail.com

capable of only detecting the already known mutations.[12,13] Another local study[14] on the genotypes and BCP/PC mutations using restriction fragment length polymorphism reported genotype D as the prevalent genotype, but it is difficult to deduce that these mutations were not treatment-induced.

The current study was planned to analyse mutations in BCP and PC regions of HBV genome in chronic HBV patients.

Patients and Methods

The cross-sectional prospective study was conducted at the Centre of Biotechnology and Microbiology, University of Peshawar and PHRC Research Centre, Khyber Medical College Peshawar from June 2014 to June 2015. After approval from the institutional ethics review board, the sample size was estimated on the basis of 3% average prevalence of HBeAg positivity, confidence interval (CI) 95% and absolute precision 5% using a standard formula.[15] The patients belonged to different ethnic groups of Pakistan. Those included were HBsAg-positive patients of either gender aged >15 years who were diagnosed at least six months previously and were untreated. The sample included patients who were both positive and negative for HBeAg. Patients having indication of previous treatment with antiviral or nucleoside analogues were excluded.

Sample preparation for nested polymerase chain reaction (PCR) was conducted at the PHRC Research Centre at Khyber Medical College, Peshawar, while nested PCR was carried out at the Centre of Biotechnology and Microbiology, University of Peshawar. Sequencing of PCR products was conducted at the Centre of Excellence in Molecular Biology, Lahore. Serum alanine aminotransferase (ALT) level, taken as a biochemical marker for liver injury,[3] was determined using Innoline kit (Merck) as per the manufacturer's instructions, with ALT level 0-40 IU/L being the standard reference range. Samples were screened for HBeAg and anti-HBeAg status qualitatively using ETI-EBK and ETI-AB-EBK enzyme immunoassay (EIA) kits (DiaSorin SpA, Italy), respectively, according to the manufacturer's instructions.

Viral DNA was extracted from 200µl serum sample using innuPREP Virus ribonucleic acid (RNA) / DNA kit (Analytik Jena AG, Germany) according to manufacturer's instructions, and was stored at -40°C till further use. The 5µl extracted DNA was mixed with 20µl of PCR master mix to make a final reaction volume of 25µl. The reaction tubes were centrifuged at full speed for 5 second. PCR-grade water was added to non-template control (NTC) tube and to each quantification standard tube. Duplex PCR was performed using Robogene HBV quantification kit (Analytik Jena AG, Germany) and Cepheid SmartCycler instrument according to manufacturer's instructions.

Figure-1: (Left to right) Ladder deoxyribonucleic acid (DNA) and purified hepatitis B virus DNA (basal core promoter [BCP] / precore [PC] region).

The PC and BCP regions (1742-1900 bp) were analysed for mutation. Primers were synthesised through GeneLink, USA. The sequences of both inner and outer primers were the same as described in literature except for a single base (G-C) modification at nucleotide position 10 in reverse inner primer.[16]

A 50µl reaction volume for first-round PCR consisted of 5µl template DNA, 1µl (10µM) of each external primer and 25µl of PCR mix (Qiagen, Cat. no 203443) and 19µl ribonuclease (RNase)-free water. Amplification conditions comprised an initial heat activation of Taq DNA polymerase at 95°C for 15min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min for 35 cycles. Final extension was carried out for 10 min at 72°C.

First-round PCR product (1µl) was re-amplified using forward and reverse inner primers in a 50µl reaction mixture. The conditions of the amplification were maintained as in the first round except for the annealing temperature which was 50°C. The amplified product of 307 bp was checked through gel electrophoresis using 2% agarose gel. A 100 bp Ladder DNA (Thermo scientific) was used for size comparison (Figure 1).

PCR product was purified using Gel and PCR Cleanup System Promega (Cat# A9282) as per the manufacturer's instructions. Purified PCR product was sequenced using both forward and reverse inner PCR primers. Mutations were detected by comparing the sequencing results with available reference sequences for different genotypes including genotype A: X70185, genotype B: D00331, genotype C: X01587, genotype D: X72702, genotype E: X75657, genotype F:X75663, and genotype G: AF160501,

using National Center for Biotechnology Information. (NCBI) online tools[17] and BioEdit and Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software. The BCP/PC sequences of all isolates were compared with reference sequences in NCBI GenBank database[17] with accession numbers KC875329, KF471655, JN564405, EU787439, AB674421 using the basic local alignment search tool (BLAST).[17] Reverse and forward sequencing ensured accuracy in detecting mutations. Mutations present in both reverse and forward sequences were considered. Phylogenetic analysis was carried out using MEGA 6.0. Neighbour-joining method employing 1000 times bootstrap. Data were analysed using SPSS 16.

Results

Of the 50 patients, 33(66%) were males. The overall mean age was 28.5±11.4 years. Of all the subjects, 25(50%) each were positive and negative for HBeAg. In terms of ethnic distribution, 16(32%) patients were Pathan, 6(12%) Punjabi, 13(26%) Saraiki, 5(10%) Sindhi, 3(6%) Urdu-speaking, 1(2%) Balochi and 6(12%) belonged to other ethnic groups. Mean ALT level was 49.28±29.84 IU/L and mean viral load was 5.41±6.58 copies/ml (Table 1).

Table-1: Epidemiological, biochemical and molecular characteristics of the patients (n=50).

	Mean±SD	n (%)
Age (years)	28.5±11.4	
Gender		
Male		33 (66)
Female		17 (34)
Ratio	1.9	-
Ethnic groups		
Pathans		16 (32)
Punjabi		06 (12)
Saraiki		13 (26)
Sindhi		05 (10)
Urdu Speaking		03 (6)
Balochi		01 (2)
Others†		06 (12)
HBeAg positive		25 (50)
HBeAg negative		25 (50)
ALT (IU/L)	49.3±29.8	
HBV DNA log10 (copies/ml)	5.99±1.9	
Predominant mutations at BCP and PC region		
1753 T→C/G		12 (24)
1762 A→T		15 (30)
1764 G→A/T		24 (48)
1762 A→T/1764G→A/T		15 (30)
1766 C→G/A/T		17 (34)
1773 T→C		11 (16)
1896 G→A		19 (38)
1899G→A		05 (10)

† Afghani Pathans, Balti, Hindkowan and Bengali; HBeAG: Hepatitis B envelope antigen; BCP: Basal core promoter; PC: Precore; SD: Standard deviation.

Mutations in the BCP/PC regions 1742-1900bp were observed in 41(82%) isolates. The 1764 G→A/T BCP mutation was the most common one detected in 24(48%) isolates. The PC stop codon G→A substitution at 1896 was detected in 19(38%) isolates. The classic A1762T /G1764A/T double mutation was observed in 15(30%) samples. A rare G1764T mutation was detected in 6(12%) isolates; 3(6%) each in HBeAg-negative and HBeAg-positive samples (Table 2). PC stop codon G→A variation at position 1899 was observed in 5(10%) isolates. G1896A and G1899A double mutation at PC region was observed in 5(10%) isolates. Mutations at nucleotide position 1753, 1762, 1764 and 1766 were more prevalent in HBeAg-negative group, but the difference was not significant ($p>0.05$). T→C mutation at position 1773 had similar frequency ($p>0.05$) between the groups. The PC stop codon G→A mutation rate at position 1896 was significantly ($p<0.05$) more

Table-2: Basal core promoter (BCP) and precore (PC) mutation profile of patients negative and positive for hepatitis B envelope antigen (HBeAg)(n=50).

Mutation	HBeAg Negative (n=25) n (%)	HBeAg Positive (n=25) n (%)	*p*-value
1753 T→C	08(32)	03(12)	0.05
1753 T→G	01(04)	00(0)	
1762 A→T	10(40)	05(20)	0.05
1764 G→A	12(48)	06(24)	0.05
1764 G→T	03(12)	03(12)	
1766 C→A	02(08)	01(04)	0.05
1766 C→G	06(24)	05(20)	
1766 C→T	02(08)	02(08)	
1773 T→C		05(20)	0.05
1814 A	25(50)	25(50)	0.05
1858 T	25(50)	25(0)	0.05
1862 G	25(50)	25(50)	0.05
1888 G→T	-	01(04)	0.05
1896 G→A	17(68)	02(08)	0.00
1899 G→A	04(16)	01(04)	0.05

Table-3: Genotype-specific variants in Basal core promoter (BCP) and precore (PC) gene (n=50).

Position	Nucleotide sequence	Occurrence	n (%)
1802-1803	CG	Genotype A, D, E	47(94)
	CT	Rare variant	01(02)
	AG	Rare variant	01(02)
	CA	Rare variant	01(02)
1809-1812	GCAC	non-A genotype	43(86)
	CCAC	Rare variant	02(04)
	GAAC	Rare variant	03(06)
	TCAC	Rare variant	01(02)
	AAAA	Rare variant	01(02)
1817-1819	TAA	Genotype G	01(02)
	CAA	Wild-type	49(98)
1858	T	Genotype B, D, E	50(100)
1888	A	subtype A1	01(02)
	G	Wild-type	49(98)

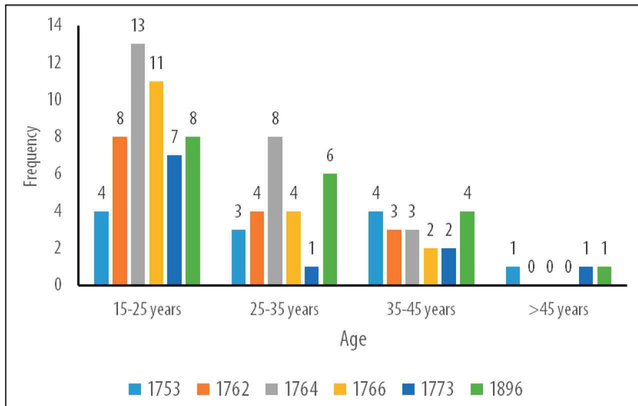

Figure-2: Age-wise distribution of Basal core promoter (BCP) and precore (PC) mutations.

frequent in HBeAg-negative patients compared to HBeAg-positive patients (Table 2).

Higher rates of mutations were observed at position 1753, 1762, 1764, 1766, 1773 and 1896 in those aged <45 years (Figure 2). Mutations at position 1753, 1762, 1764, 1766 and 1773 were more prevalent in samples with viral load >4.0 log10 copies/ml), but the difference was not significant (*p*>0.05). However, PC stop codon mutation G→A at position 1896 was associated with increased HBV DNA levels (*p*<0.05). Similarly, mutation frequency at the same Position was significantly higher at elevated >42 IU/L ALT levels (*p*<0.05). Rest of the mutations did not show any significant association with ALT levels (*p*>0.05).

The BCP/PC sequences of all isolates were compared and a phylogenetic tree was constructed (Figure 3).

Of all the genotypes 9(18%) were wild type (Figure 4). Further, 46(92%) isolates had a CG sequence at position1802-1803 which is a characteristic of A, D and E genotypes. CT nucleotide sequence at location 1802-1803 was detected in 2(4%); 1(92%) unusual AG sequence and 1(2%) rare CA sequence at this position. GCAC wild type Kozak sequence at position 1809-1812, which affects HBeAg expression and is commonly found in genotype D, was detected in 43(86%) samples. Rare variant sequences GAAC, CCAC and TCAC at the same position were detected in 3(6%), 2(4%) and 1(2%) sample respectively. Wild-type CAA sequence at position 1817-1819 was present in 49(98%) samples, while its variant sequence TAA specific to genotype G was found in 1(2%) sample. The wild type 1858T nucleotide in PC region associated with genotype B, D and E was noticed in all 50(100%) samples. A rare 1888T variant nucleotide was

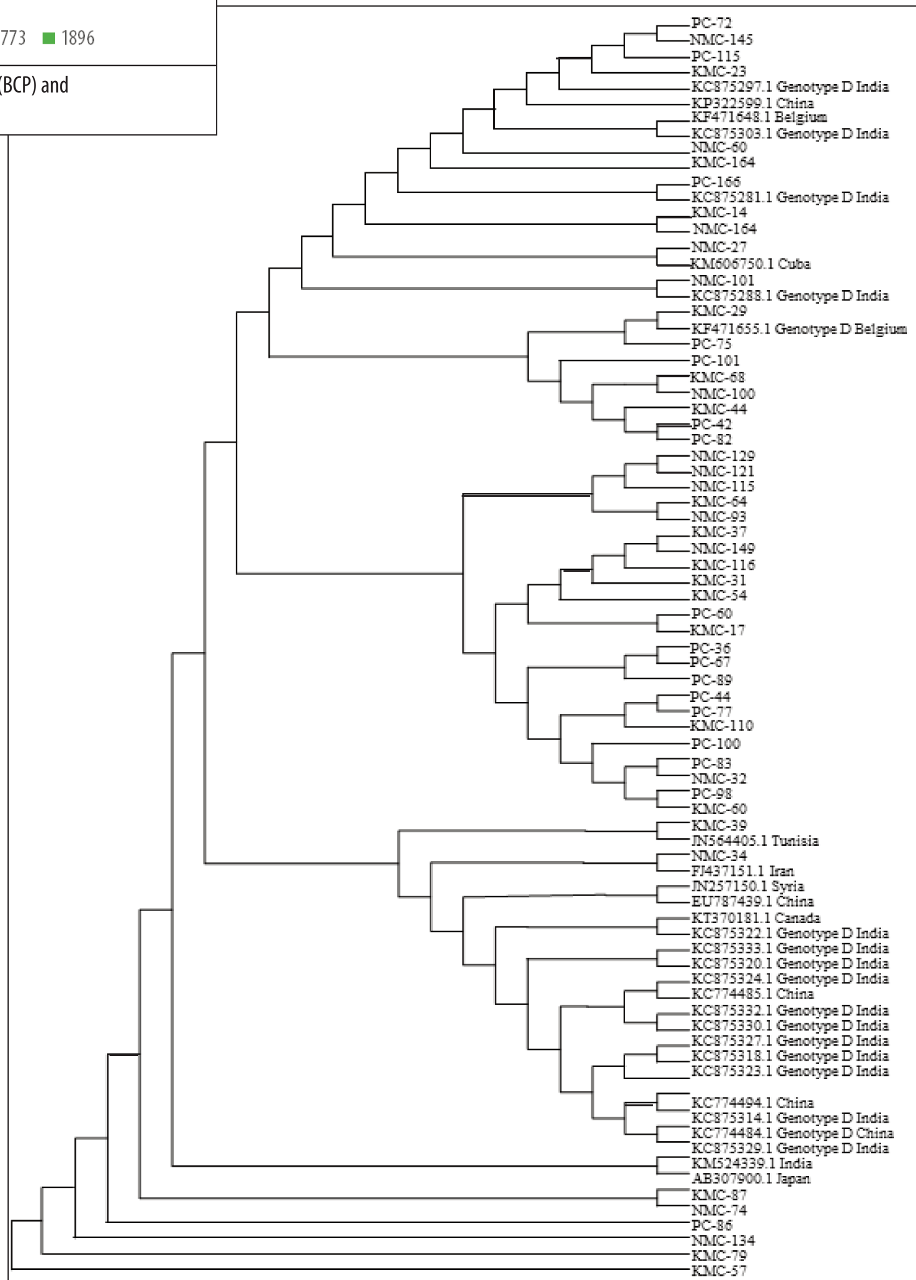

Figure-3: Phylogenetic presentation of hepatitis B virus basal core promoter (BCP) and precore (PC) sequences.

Figure--4: Demographic, biochemical and molecular characteristics of the patients.

ID	Age (years)	Ethnic group	Gender	ALT (IU/L)	Log10 Copies/ml	HBeAg	1753	1762	1764	1766	1768	1802-1803	1773	1809-1812	1814	1858	1862	1888	1896	1899
KMC-14†	15	Pathan	M	18	7.74	Negative	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
KMC-17	22	Pathan	F	17	3.71	Negative	C	T	A	C	T	CG	T	GCAC	A	T	G	G	A	G
KMC-23	20	Pathan	M	62	4.86	Positive	T	A	T	G	T	CG	T	GCAC	A	T	G	G	G	G
KMC-29	53	Pathan	M	32	8.83	Positive	T	A	G	C	T	CG	T	TCAC	A	T	G	T	G	G
KMC-31	19	Pathan	F	58	2.60	Negative	T	A	G	C	T	CG	C	GCAC	A	T	G	G	A	G
KMC-37	41	Pathan	M	28	4.22	Negative	C	T	A	C	T	CG	C	GAAC	A	T	G	G	A	G
KMC-39	35	Pathan	M	62	4.21	Negative	C	T	A	C	T	CG	T	GCAC	A	T	G	G	G	G
KMC-44	24	Pathan	M	138	7.69	Positive	T	A	T	G	T	CG	T	GCAC	A	T	G	G	G	G
KMC-54	29	Pathan	M	36	5.67	Negative	T	A	A	C	T	CG	T	GCAC	A	T	G	G	A	G
KMC-57†	15	Pathan	M	79	9.70	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
KMC-60	19	Pathan	M	23	3.53	Negative	T	A	G	G	T	CG	T	GCAC	A	T	G	G	A	G
KMC-68	28	Afghani Pathan	M	31	5.40	Negative	T	T	A	C	T	CG	T	GCAC	A	T	G	G	G	G
KMC-79	60	Pathan	F	40	9.70	Positive	T	A	G	C	T	CA	C	CCAC	A	A	G	G	G	G
KMC-87	20	Afghani Pathan	F	56	9.70	Positive	T	A	G	C	T	CG	C	GCAC	A	T	G	G	G	G
KMC-110	24	Hindko	F	25	4.80	Negative	T	A	A	A	T	CG	T	GCAC	A	T	G	G	A	A
KMC-116	15	Pathan	M	96	5.98	Positive	C	T	A	C	T	CG	C	GAAC	A	T	G	G	A	G
KMC-164†	30	Pathan	F	38	5.74	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
NMC-27	18	Punjabi	F	50	9.70	Negative	T	A	G	G	T	CG	T	GCAC	A	T	G	G	G	G
NMC-32	38	Saraiki	F	16	3.59	Negative	T	A	G	G	T	CG	T	GCAC	A	T	G	G	A	A
NMC-34	40	Saraiki	M	23	6.22	Negative	C	T	A	C	T	CG	T	GCAC	A	T	G	G	G	G
NMC-60†	25	Saraiki	M	107	4.67	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
NMC-64	55	Saraiki	M	18	7.44	Negative	G	A	G	C	T	CG	T	GCAC	A	T	G	G	A	G
NMC-74	45	Saraiki	M	34	9.70	Negative	T	A	G	C	T	CG	C	GCAC	A	T	G	G	G	G
NMC-93	45	Urdu	F	37	8.32	Negative	T	A	G	C	T	CG	T	GCAC	A	T	G	G	A	G
NMC-100	25	Saraiki	F	36	6.72	Positive	C	T	A	C	T	CG	T	GCAC	A	T	G	G	G	G
NMC-101	35	Saraiki	M	81	4.76	Positive	T	A	G	G	T	CG	T	GCAC	A	T	G	G	G	G
NMC-115	32	Saraiki	F	32	4.76	Negative	T	T	A	C	T	CG	T	GCAC	A	T	G	G	A	G
NMC-121	20	Saraiki	M	92	6.48	Positive	T	T	A	C	T	CG	T	GCAC	A	T	G	G	G	G
NMC-129	45	Saraiki	M	63	5.99	Positive	C	T	A	C	T	CG	T	GCAC	A	T	G	G	G	G
NMC-134	25	Punjabi	M	15	3.80	Negative	T	T	A	T	T	CT	T	GCAC	A	T	G	G	G	G
NMC-145†	35	Punjabi	M	32	5.60	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
NMC-149	35	Saraiki	F	15	3.75	Negative	C	T	A	C	T	CG	C	GAAC	A	T	G	G	A	G
NMC-164	25	Saraiki	M	36	5.88	Negative	C	T	A	C	T	CG	T	GCAC	A	T	G	G	G	G
PC-36	26	Sindhi	M	77	7.08	Negative	C	A	T	G	T	CG	T	GCAC	A	T	G	G	A	G
PC-42	19	Punjabi	M	81	4.76	Positive	T	A	G	A	T	CG	T	AAAA	A	T	G	G	G	G
PC-44	20	Punjabi	M	39	4.56	Negative	T	T	G	C	T	CG	C	GCAC	A	T	G	G	A	A
PC-60†	26	Punjabi	M	47	6.20	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
PC-67	28	Balti	F	32	4.13	Negative	T	A	A	A	T	CG	T	GCAC	A	T	G	G	A	G
PC-72†	15	Sindhi	M	51	6.07	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
PC-75	15	Sindhi	M	97	6.07	Positive	T	A	G	C	T	CG	T	CCAC	A	T	G	G	G	G
PC-77	15	Bangali	M	24	4.67	Positive	T	A	G	C	T	CG	C	GCAC	A	T	G	G	A	A
PC-82	23	Saraiki	M	34	6.07	Positive	T	A	G	C	T	AG	T	GCAC	A	T	G	G	G	G
PC-83	24	Sindhi	M	47	4.55	Negative	T	A	T	G	T	CG	C	GCAC	A	T	G	G	A	A
PC-86	23	Bangali	F	68	8.30	Positive	T	A	A	T	T	CG	C	GCAC	A	T	G	G	G	G
PC-89	42	Sindhi	M	24	6.45	Negative	C	A	G	T	T	CG	T	GCAC	A	T	G	G	A	G
PC-98	28	Urdu	F	41	5.12	Negative	T	A	T	G	T	CG	T	GCAC	A	T	G	G	A	G
PC-100	22	Sindhi	F	58	8.12	Positive	T	T	A	G	T	CG	T	GCAC	A	T	G	G	G	G
PC-101†	22	Pathan	M	118	6.07	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
PC-115†	44	Pathan	M	96	5.98	Positive	T	A	G	C	T	CG	T	GCAC	A	T	G	G	G	G
PC-165	22	Balochi	M	17	5.85	Positive	T	A	T	G	T	CG	T	GCAC	A	T	G	G	G	G

† denotes wild-type sequences

identified in 1(2%) sample and the wild type 1888G nucleotide was detected in the rest of the samples (Table 3).

Discussion

To our knowledge, the current study is the first to report direct sequence analysis of BCP and PC regions from Pakistan on the status of HBV mutations.

Majority of the patients were aged <45 years, suggesting a high HBV prevalence in younger age group in Pakistan which is in line with literature.[12] Overall, a high frequency (38%) of 1896 (G→A) mutation was observed. This mutation changes codon 28 into a stop codon abolishing HBeAg expression. A study reported an overall 27% PC mutation frequency in HBV cases using line probe assay.[12] A similar study reported 18% frequency of PC stop codon mutation.[13] The occurrence of HBeAg-negative status despite the absence of A1896 mutation in some samples, and occurrence of HBeAg-positive status in some samples with coexisting A1896 mutation suggests the existence of mixed viral infection where both wild-type and mutant viruses coexist.[12] The reported prevalence of A1896 stop codon mutation varies from 12% to 85% in different studies.[11-13,18-21] Geographical disparity in the prevalence of the PC mutants is genotype-dependent, which, in turn, is related to the existence or absence of C or T at location 1858. PC mutation is confined to non-A genotypes harbouring thymidine at position 1858 which base pairs with A1896 and stabilises the encapsidation signal epsilon required for viral replication.[20] On the other hand, the presence of cytosine at position 1858 in genotype A precludes the emergence of the A1896 mutants since C forms a wobble pair with A at position 1896 and destabilises epsilon, consequently decreasing replication capability.[8] Owing to a single mutation requirement needed for epsilon stability, the emergence of A1896 PC variant is mostly common in HBV genotype D; the leading genotype in the Mediterranean region, followed by genotypes B and C in Japan and Southeast Asia, and is occasionally detected in genotype A which is the most common type in North America and northern Europe.[21]

In the current study, all samples harboured thymidine at position 1858 which speculates the preponderance of genotype D in Pakistani patients, as has been previously reported.[12,13] A study reported T1858 nucleotide among all D genotype samples.[20] Cytosine at position 1858 primarily exists in genotype A and is common in sub-genotype C1, but not in C2.[22] A1896 and A1899 simultaneous mutations were detected in 5(10%) of the isolates and 4 of these belonged to HBeAg negative samples. It is worth mentioning that all the samples having G1899A mutation had a G1896A coupled mutation and no A1899 was found alone. A more severe disease progression has been reported in HBV patients harbouring A1896 and A1899 double mutation, but not in patients with only A1896 mutation.[23] Another study reported the presence of A1896 and A1899 double mutation in 26% inactive carriers and 44% chronic hepatitis patients.[2] A significantly higher frequency of this double mutation has been reported in genotype D6 compared to other sub-genotypes of D.[24] Like the existence of genotype-specific variant T1858 in all samples, loci 1814 and 1862 were highly conserved having A1814 and G1862 in all the isolates except for the presence of T1888 instead of G1888 in one sample. Genotypes A, C and D differed significantly in terms of G1862T and T1858C variations where the existence of T1858 was associated with genotype D and T1862 and C1858 variants were highly prevalent in genotype A.[25] G1862T mutation has been suggested to interfere with the cleavage of signal peptide and contributes to HBeAg phenotype.[18] The diminished replication ability of genotype A is attributed to G1862T mutation and the inhibitory effect of G1862T on genome replication is relieved by G1899A mutation which restores core protein expression.[26]

The double 1762/1764 mutation (1762 A→T /1764 G→A/T) in BCP region was detected in 15(30%) patients, which is in accordance with a study in India which reported 32% frequency.[21] In another report, relatively higher incidence of paired 1762/1764 mutation was reported in isolates with genotypes A (64%) and C (66.7%), while the lowest frequency (30.5%) was detected among genotype D patients.[25] A rare G1764T mutation was detected for the first time in 12% of the studied isolates which has been previously reported from Tunisia with 3.4% frequency in genotype D7 and 19.5% frequency in genotype D1.[27] The frequency of double mutation at 1762/1764 was not statistically different between HBeAg-positive and HBeAg-negative isolates, as stated by others.[22] However, some studies have reported the association of T1762/A1764 double mutation more with HBeAg-negative than with HBeAg-positive phenotypes.[2]

Mutations in the BCP region at position 1753, 1762, 1764, 1766, 1773 and genotype-specific variations at position 1802-1803 and the Kozak sequence at 1809-1812 were prominent. Wild-type Kozak sequence GCAC at 1809-1812 was detected in majority of samples as reported by others in majority of non-A genotype isolates.[28] The absence of TCAT sequence at position at 1809-1812 and A nucleotide at position 1888, characteristics of genotype A1, suggests the absence of this genotype in the studied samples. The existence of wild-type sequences in majority of isolates at genotype-specific loci suggests genotypic stability in the

studied population.

Although the pre-S and S regions, important for genotyping, were not sequenced, genotypes of the isolates were speculated on the basis of BCP/PC region sequencing using two approaches. These approaches involved genotyping on the basis of genotype-specific variation in the BCP/PC regions and utilising maximum identity scores of the studied sequences with all the NCBI GenBank database[17] sequences of HBV genotypes with the help of BLAST software. Based on these comparisons, the speculated predominant genotype among the studied isolates was the D genotype, which has been reported previously as well.[12,13] Phylogenetic tree of the study isolates with known sequences from GenBank[17] showed that majority of our samples belonged to genotype D isolates from India, Turkey, Tunisia, China and Iran. Sequence analysis of genotype-specific loci and phylogenetic analysis speculates that majority of our isolates belonged to genotype D. Owing to the occurrence of genotype D specific mutations/variants, such patients have lower chances of spontaneous HBeAg seroconversion, higher frequency of BCP mutations than PC mutations and increased lifetime risk of cirrhosis, HCC and poor treatment outcome.

Conclusion

The prevalence of PC stop codon mutation G1896A was higher in HBeAg-negative samples than in HBeAg-positive samples. The existence of wild-type sequences in majority of isolates at genotype-specific loci suggested genotypic stability in the studied population. BCP region showed more variation than PC region, highlighting the risk of hepatocellular carcinoma in these patients.

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