

Original Article

Correlation of Hepatitis B Virus Precore/CorePromoter Mutations with Disease Status in Punjab State in North India - 3

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ABSTRACT

Aim: To find various precore/core promoter mutations and their relatedness with the disease status in HBV infected population of Punjab state in North India.

Materials and Methods: In this study, 53 HBsAg positive patients of different disease states from different districts of Punjab state (India) were taken. Serum samples were collected. DNA was isolated from serum samples. Nested PCR for amplification of precore/ core promoter region was done. PCR products were analysed on agarose gel. Amplified PCR products containing preC/C region were sequenced and analysed for various precore/core promoter mutations by using specific bioinformatics softwares.

Results: Various core promoter mutations (A1762T/G1764A, C1766T/T1768A, G1809A/T/C, C1810T, and A1811C/G); various precore mutations (T1858C, G1862A/T, G1888A/T, and G1896A/T/C) were observed. Approximately, all precore/core promoter mutations were observed more in isolates with advanced liver disease than in isolates with non-advanced liver disease and were found mainly in HBeAg negative patients. There is more prevalence of various precore/core promoter mutations (A1762T/G1764A, C1773T, G1809A/T/C, G1862A/T, and G1896A/T/C) in Hepatocellular carcinoma patients.

Conclusion: We conclude that various precore/core promoter mutations were observed and these mutations were observed more in isolates with advanced liver disease in Punjab in North India. These precore/core promoter mutations were detected more in HBeAg negative patients. Various precore/core promoter mutations were marked in HCC patients in Punjab (North India).

Clinical Significance: These precore/core promoter mutations observed during different stages of the disease acts as biomarkers for the detection of HBV related liver diseases and it helps the clinician/physician for correct treatment of various HBV related liver diseases.

Keywords: HBV; HCC; Precore (PC) mutations; Core Promoter (CP) mutations; PCR

INTRODUCTION

Globally about 2 billion peoples are detected with Hepatitis B Virus (HBV) and out of which 350 million are chronically infected [1]. HBV infection is also linked with various disease states i.e. asymptomatic carriers, chronic hepatitis, liver cirrhosis, and Hepatocellular Carcinoma (HCC) [2]. Finally, approximately 15% –40% of HBV carriers have possibility to develop into cirrhosis, liver failure, or HCC [3].

This virus lacks proofreading activity because of replication by reverse transcriptase enzyme; therefore, new virions possess diverse genetic variability [4]. HBV mutations were observed in all the open reading frames (preS/S, polymerase, precore/core, and X). HBV genome mutations influence the replication fitness of the virus, influence the disease outcome and response to treatment [5]. Certain HBV mutants, including precore/core promoter mutations and pre-S/S deletion mutations, have been found to be related with advanced liver disease and susceptible of HCC development [6,7]. During persistent viral infection, numerous HBV genome mutations have been revealed and linkage between mutations (precore gene and basal core promoter region) and HCC occurrence have been reported [8].

Various studies reveal that the most common mutations in the precore region are G to A substitution 1896 (G1896A), an A to T mutation at nucleotide 1762 (A1762T), a G to A mutation at nucleotide 1764 (G1764A), and the A1762T/G1764A double mutation in BCP region [9-11]. These mutations could prevent the production of HBeAg and contribute to the inefficient immune response that leads to liver cancer [12,13].

Little data exists from different states/regions of India regarding HBV core promoter and precore mutations and their correlation with the disease status. This study was performed to find various precore/ core promoter mutations in HBV infected patients in Punjab state (Northern India), and correlation of these mutations with disease progression.

MATERIAL AND METHODS

Patients

This study comprised of 53 HBsAg positive patients from the

state of Punjab (North India). The serum samples were collected from various districts of Punjab state (Ludhiana, Patiala, and Mansa) and PGIMER, Chandigarh from year 2006 to 2009. Serum samples were taken from the patients and saved at minus 70°C before analysis. This study was permitted by Institutional Ethics Committee, Kurukshetra University, Kurukshetra, Haryana, India.

Serological and biochemical parameters

All serum samples were analyzed for various HBV serological markers (HBsAg and HBeAg) using commercially available kits (Equipar SRL, Italy and IND diagnostic, Canada respectively). Liver function tests (ALT and AST levels) were performed in all serum samples as per the protocols supplied with the kits of Span Diagnostics Ltd., Surat, India.

Different stages of HBV infection: The following different groups of patients were taken for this study:

- Group I (chronic asymptomatic or inactive group)
- Group I included 16 patients and they were chronic asymptomatic (inactive carriers) patients. These patients were analyzed with the presence of HBsAg and absence of HBeAg markers along with normal ALT/AST and were tested twice 6 months apart [14-16].
- Group II (chronic symptomatic group)

This group had 2 subgroups, Group IIa and Group IIb.

- Group IIa
- This group included 15 patients and was called chronic symptomatic (HBeAg–ve) group. These patients were defined by presence of HBsAg marker and absence of HBeAg along with high level of ALT/AST (exceeds 2 times the upper limit of normal).
- Group IIb
- This group included 12 patients and was called chronic symptomatic (HBeAg+ve) group. The patients in this group were analyzed by the presence of HBsAg and HBeAg marker along with normal ALT/AST level [14-16].

• Group III (acute cases group)

This group included 5 patients of acute cases which were defined by HBsAg and IgM-anti-HBc markers with at least 5-10 times increase in aminotransferases (ALT/AST) above the upper limit of normal [16].

• Group IV (HCC cases group)

This group contained 5 HCC patients diagnosed on the basis of recommendations of European Association for the study of the liver [16].

In the present study, Group II & IV together comprised patients with progressive liver disease.

DNA extraction

DNA was isolated from the serum samples by using QIAmp DNA extraction kit (Qiagen, Hilden, Germany). Then isolated DNA was eluted in 100 μ l best quality water (BQW) and was stored at -20°C until further use.

Amplification of precore/core gene

Nested PCR was done for amplification of precore/core promoter region. First round was operated by following conditions (denaturation, 94°C for 30 secs, annealing, 59°C for 50 secs and extension, 72°C for 50 secs) for 40 cycles followed by a concluding extension at 72°C for 10 mins. Initial denaturation step was done at 94°C for 5 mins. The second round PCR was operated by following conditions (denaturation, 94°C for 30 secs, annealing, 58°C for 50 secs and extension, 72°C for 50 secs) for 40 cycles followed by a concluding conditions (denaturation, 94°C for 30 secs, annealing, 58°C for 50 secs and extension, 72°C for 50 secs) for 40 cycles followed by a concluding extension at 72°C for 10 mins. Initial denaturation step was performed at 94°C for 3 mins. The procedure followed was as per the method of Baptista, et al. [17]. The results of second round PCR products were observed on 2% agarose gel as shown in figure 1. Table 1 shows the sequences of primers used in nested PCR.

Direct nucleotide sequencing and CP& PC mutations analysis

Nested PCR products (261 base pairs) were sequenced commercially using an automated sequencer. Nucleotide sequence of all the samples were compared with the available wild type sequences using bioinformatics tool (BioEdit sequence comparison software, v7.2, U.S.A).

Statistical evaluation

For comparison of two independent proportions, the chi square $(\chi 2)$ or Fischer's exact test with Yates correction was used. The statistical differences were calculated using SPSS-20 software. The statistically significant value of p was that which have values lesser than 0.05.

RESULTS

Overall characteristics of patients

All the serum samples were of male patients and all had mean age 30 ± 10 . These isolates under study had not received any antiviral agents (lamivudine or interferon) and were not having HIV and HCV infection. All patients were HBsAg positive. Out of 53 HBV infected patients, 14 patients were found to be HBeAg positive (26.4%) and 39 patients were found to be HBeAg negative (73.6%). So, more samples were HBeAg negative in our study.

Amplified pre-core/core promoter region

Figure 1 shows the precore/core promoter region as a single band of 261 bp. The precore/core promoter region of 261 bp (1661-1921) was amplified, sequenced and sequence of each sample was compared with the wild type sequence (Accession no.- NC_003977) using BioEdit sequence comparison software tool (U.S.A). The compared sequences are shown in figures (2-8).



Table 1: Details of primers used for amplification of precore/core promoter region of HBV. Sense and antisense primers are denoted by (+) and (-) signs respectively.

Primers	Sequence	Size (bp)	
	Outer primers		
1622F(+)	5'-GAACGCCCATCAGATCCTGC-3'	245	
1966R(-)	545		
	Inner primers		
1661F(+)	5'-GACTCTTGGACTCTCAGC-3'	261	
1921R(-)	201		

Distribution of core promoter mutations in various clinical stages of HBV infection: Various core promoter mutations in different stages of HBV infection were shown (Table 2). A1762T mutation was observed in 30.2% isolates and was observed only in advance liver disease patients (100%). G1764A mutation was observed in 24.5% isolates and was observed only in advance liver disease patients (100%). A1762T/G1764A double mutation was observed in 24.6% isolates and was observed in 100% advanced liver disease patients (100%).

Other core promoter mutations (1756A/C, 1773T, 1802C/1803G, 1809A/T/C, and 1812T) were more significantly associated with advanced liver disease (*p* value less than 0.05).

Distribution of precore mutations in various clinical stages of HBV infection: Various precore mutations in different stages of HBV infection were shown (Table 3). G1896A/T/C mutation was observed in 11.4% samples and was observed in 100% of advanced liver disease patients.

T1858C mutation was observed in 83.1% isolates and was observed more in advanced liver disease patients (59.1%) and less in

non-advanced liver disease patients (40.9%) [*p* value less than 0.05]. So, this mutation was more significantly associated with advance liver disease.

G1862A/T mutation was observed in 84.9% isolates and was observed more in advanced liver disease patients (60%) and less in non-advanced liver disease patients (40%) [p value less than 0.05]. So, this mutation was more significantly associated with advance liver disease.

G1888A/T mutation was observed 83.1% isolates and was observed more in advanced liver disease patients (59.1%) and less in non-advanced liver disease patients (40.9%) [p value less than 0.05]. So, this mutation was more significantly associated with advance liver disease.

Novel core promoter and precore mutations: Various novel core promoter and precore mutations in different stages of HBV infection were shown (Table 4).

Various novel core promoter and precore mutations (1690A/T/G, 1695A/T/G, 1700A/C, 1701A/C, 1703C, 1719G, 1727G, and 1827A/T) were more significantly associated with advanced liver disease (*p* value less than 0.05).

NC_003977 1.13 2.14 3.18 4.112 5.118 6.120 7.121 Clustal Consensus			30 GT CAACGACC - GG A CCAA - GG A CCAA - GG C CAA - GC C. - GG C. - G G G. - G G G. - G G G. - G G G G.	40 GACCTTGAGG AC.C. AGTAAC. A.GCC TA.GCC TA.GCC TA.GCC	50 CATACTTCAA TC	60 A G A C T G T T T G G . G . G .
NC_003977 1.13 2.14 3.18 4.112 5.118 6.120 7.121 Clustal Consensus	70 TTTAAAGACT G G G	80 GGGAGGAGTT 	90 GGGGGAGGAGGAG	100 A T T A G G T T A A . A A A	AGGTCTTTGT T. A. T. A T. A T. A T. A T. A	120 A C T A G G A G G C T
NC_003977 1.L3 2.L4 3.L8 4.L12 5.L18 6.L20 7.L21 Clustal Consensus	130 T G T A G G C A T A . A C C A A T T 	140 AATTGGTCTG .G. TA	150 TT C A C C A G C A C G C G C G C G C G C G C G C G	160 C C A T G C A A C T G	170 1 T T T T C C C C T C C A A A A A A	180 T G C C T A A T C A A T . T C C T C . C
NC_003977 1.L3 2.L4 3.L8 4.L12 5.L18 6.L20 7.L21 Clustal Consensus	190 1	200 A T G T C C T A C T C A A G . T A A 	210 G T T C A A G C C T . C . G A C A C T . T C T T	220 C C A A G C T G T G . T C . A . G . . G . A G . A G . 	230 C C T T G G G T G G C C T T G G G T G G C C T A G G T G G C C T A G G T G G C C T G G G T G G C C T C G G G T G G C C T C C C C C C C C C C C C C C C C C	240 C TTT G G G G C A T C C G . C G T T . A . G
NC_003977 1.L3 2.L4 3.L8 4.L12 5.L18 6.L20 7.L21 Clustal Consensus	250 T G G A C A - T T G T . G G C T . C T . A	260 A C C - C G T A T A G T - C A A A . G . T - A A . A A . A . A A A	270 AAIAAIAA GAAATCCG AGA AAAGGTGA AAAGGTGA AAATTTG AGAATAAA AAAATTTT	A A C A A A T T A G A A C A A A T T A G A G A A G C T G T G A G C T G C T G T G A A A G G T A C T G A G C T A T T G T G	290 A A C T T C T T A G - T T - T G C G A G - T T A C T C A G G A C C G T G G T A G - T T T T C	300 1 1 CGTTTTTCA CGTTTTTGC TGTCTTAA TCTTTTTT

Figure 2: Comparison of nucleotide sequence (L3, L4, L8, L12, L18, L20, and L21) of core promoter/precore region (1661-1921) with wild type (Accession no. – NC_003977) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

NC 003977 8.L25 9.L29 10.L30 11.L32 12.L38 13.L39 14.L41 Clustal Consensus			GTCAACGACC GGAG GGCG GGG GGG GGCT.G GCC	GACCTTGAGG TA TA TA TTG.A TTG.A TTA A.AAG.CCC.	CATACTICAA AGACT GCC. C GCC. C GCC. GC GC	G T T G G G G C G
NC_003977 8.L25 9.L29 10.L30 11.L32 12.L38 13.L39 14.L41 Clustal Consensus	70 T T T A A A G A C T	80 GGGAGGAGTT	90 GGGGGA - GGA 	100 GATTAGGTTA CA	110 AAGGTCTTTG TACTA 	120 G G A G G
NC_003977 8.L25 9.L29 10.L30 11.L32 12.L38 13.L39 14.L41 Clustal Consensus	130 C T G T A G G C A T 	140 A A A T T G G T C T	150 GTTCACCAGC CGT. CGT.	160 A C C A T G C A A C T	170 TTTTTCCCCT CTGCC A	180 T A A T C C C T C .
NC 003977 8.125 9.129 10.130 11.132 12.138 13.139 14.141 Clustal Consensus	190 A T C T C A T G T T T A T A T A T A T A G C T . C	200 C AT G T C C T A C C AT G T C C T A C	210 T G T T C A A G C C T	220 T C C A A G C T G T	230 G C C T T G G G T G G C T T T AA A CCCC TG	240 GGGGC AA.
NC_003977 8.L25 9.L29 10.L30 11.L32 12.L38 13.L39 14.L41 Clustal Consensus	250 A T G G A C A T T G	260 A C C C G T A T A A C C C G T A T A A C C C G T A T A A G A A T C C	270 I I A A A A A A T T T A . A A A A A A T G T G . A A A A A A T T T G . A A A A A A T T T G . A A A A A A T T T G . A A A A A A T T T G . A A A A A A T T T G	GCTATACTGT GAGCTACTGT GAGCTAGTGT GAGCTACTGT GAGCTACTGT GAGCTACTGT GAGCTACTGT GAGCTACTGT CGAGGGTTGG	290 AGTITCTCTC TCTTT GGATTTACTC TCGTT GGAGTTACTC TCGTT GGAGTCTCTC TCTTT GGAGTTACTC TCGTT GGAGTTACTC TCGTT GGAGTTACTC TCGTT G	300 TTTCC TTTCC TTTGT TTTCC TTTGC TTTGC TTTGC

10 20 30 40 50 60 •••••|•••••|•••••|•••••|•••••|•••••|

Figure 3: Comparison of nucleotide sequence (L25, L29, L30, L32, L38, L39, and L41) of core promoter/precore region (1661-1921) with wild type (Accession no. – NC_003977) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

Association of core promoter mutations with HBeAg status: Various core promoter mutations and their association with HBeAg status were shown (Table 5). A1762T mutation was observed in 30.2% isolates and was observed more in patients with HBeAg negative status than in patients with HBeAg positive status. A1762T mutation was more significantly associated with HBeAg negative status [P value less than 0.05]. G1764A mutation was observed in 24.5% isolates and was observed more in patients with HBeAg negative status than in patients with HBeAg positive status. A1762T mutation was more significantly associated with HBeAg negative status than in 0.05]. 1762T/1764A double mutation was observed in 24.6% isolates and was observed more in patients with HBeAg negative status than in patients with HBeAg positive status. 1762T/1764A double mutation was more significantly associated with HBeAg negative status [*p* value less than 0.05].

Various core promoter mutations (1690, 1695, 1700, 1701, 1703, 1719, 1727, 1773, 1802/1803, 1809, and 1812) were more significantly associated with HBeAg negative status [*p* value less than 0.05].

Association of precore mutations with HBeAg status: Various precore mutations and their association with HBeAg status were observed and shown (Table 6).

G1862A/T mutation was observed in 84.9% isolates and was observed more in patients with HBeAg negative status than in patients with HBeAg positive status. G1862A/T mutation was more significantly associated with HBeAg negative status [p value less than 0.05].

NC_003977	ACTCTTGGAC	TCTCAGCAAT	GTCAACGACC	GACCTTGAGG	C - A T A C T T C A	AAGACTGTTT
<u>15.L42</u>			GG.CAA	A A .	G - C	G .
<u>16.L43</u>			T T A	C.TTCAA	C	. C G .
<u>17.L44</u>			TACAG	A . A G . C A .	C	G .
<u>18.L47</u>			GCC	Α	GCC	G .
<u>19.P1</u>			 A .	A C A .	C	G .
20.P3			 A .	C	C	G .
21.P6			G A	C.A.G.AGAC	GTC	G .
Clustal Consensus				*	* * * * * * *	* * * * * * *
	70	90	00	100	110	120
NC 003977	GTTTÅAAGAĊ	TGGGÅGGAGŤ	TGGGĠGAGGĂ	GATTÁGGTTÁ	AAGGİCTTTĞ	TACTÁGGAGĠ
15.L42						T
16.L43					Τ	T
17.L44						T
18.L47					. T . A	T
19.P1						T
20.P3						T
21.P6				Δ	GT	ΔΤΔ
Clustal Consensus	* * * * * * * * *	* * * * * * * * * *	* * * * * * * * * *	* * * * * * * * *	* **	* *
<u>crustar consensus</u>						
	400		450	400	170	400
	130	140	150	160	1/0	180
NC 003977	CTGTAGGCAT	AAATTGGTCT	GTTCACCAGC	ACCATGCAAC	TTTTTCCCCT	CTGCCTAAT
15.1 42			. CG T .	. T	Α	
16.1.43			CG T	T	Δ	
17 44			CG T	т	Δ	
18 47			CG T	T	Δ	
10.04				T		
INPL			1 1-		Λ	
20 P3			. CG I .	T	· · · · · · A · · ·	
<u>20.P3</u> 21 P6	T 60		. CG T .		A	Τ ΔΔ
<u>20.P3</u> <u>21.P6</u>	T G C	C C A A . T .	. CG T . A . G T . C .	.Т GTССТТ	A C A A A G A	T.AA
<u>20.P3</u> 21.P6 Clustal Consensus	T G C	C C A A . T .	. CG T. . CG T. A. G T. C.	GTCCTT	A C A A A G A * * *	T . A A
<u>20.P3</u> 21.P6 Clustal Consensus	T G C	C C A A . T .		GTCCTT	A C A A A G A	T . A A
<u>13.P1</u> 20.P3 21.P6 Clustal Consensus	T G C	C C A A . T .	210	G. TCCT. T	A C A A A G A	T A A
13.F1 20.P3 21.P6 Clustal Consensus	190 CATCTCATGT	200 	210	G. TCCT. T	ACAAAGA	240 T_G_G_C_T_TG_G
<u>13.F1</u> 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC_003977</u> 15.142	190 CATCTCATGT	200 T - C C A A . T .	210 	G. TCCT. T G. CCTCCAAGCT	ACAAAGA 230 GTGCCTTGGG AA	240 T G G C T T T G G
<u>15.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> <u>15.L42</u> 16.L43	190 	200 1 1 T - C A T G T C C T A C	210 	GTCCTT CCTCCAAGCT	ACAAAGA aCAAAGA 230 GTGCCTTGGG AA	240 T G G C T T - T G G
<u>13.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> <u>15.L42</u> <u>16.L43</u> 17.L44	190 CATCTCATGT	200 T - C C A A . T . T - C A T G T C C T A C A C	210 A C T G T T C A A G 1 1 A C T G T T C A A G T	GTCCTT CCTCCAAGCT	230 GTGCCTTGGG AAAAAAAAAAAAAAAAAAAAAAAAAAAAA	240 T G G C T T T G G
<u>13.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47	190 CATCTCATGT T	200 T - C C A A . T . T - C A T G T C C T A C A C A C	210 A C G T C A A G A C T G T T C A A G C T G T T C A A G C T C T C A A G C T C T C A A G	G. TCCT. T G. CCTCCAAGCT	230 A C A A A G A	240 T G G C T T - T G G
<u>ISJF1</u> 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1	190 G C C A T C T C A T G T T T T	200 	210 	G. TCCT. T CCTCCAAGCT	A C A A A G A	240 T G G C T T - T G G
<u>IS.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> <u>15.L42</u> 16.L43 17.L44 <u>18.L47</u> <u>19.P1</u> 20.P3	190 CATCTCATGT T T T T	200 T - C A T G T C C T A C A C A C A C A C	210 	GTCCTT CCTCCAAGCT	230 1 1 G T G C C T T G G G A	240 T G G C T T - T G G
<u>15.F1</u> 20.P3 21.P6 Clustal Consensus NC_003977 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21 P6	190 CATCTCATGT T T T T T	200 I I T - C A T G T C C T A C A C A C A C A C A C A C	210 	G. TCCT. T CCTCCAAGCT 	230 A A C A A A G A G T G C C T T G G G A	T . A A
<u>IS.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus	190 CATCTCATGT 	200 	210 210 210 A C T G T T C A A G T T T T T T T T T T T T T T T T T T T	G. TCCT. T CCTCCAAGCT 	230 C C A A A G A C C A A A G A C T G C C T T G G G C C T T G G G A C A A A G A C A A A G A	240 T G G C T T T G G C C T . A . G . C .
<u>ISJF1</u> 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 <u>Clustal Consensus</u>	190 CATCTCATGT T T T T T T T	200 I I T - C A T G T C C T A C A C A C A C A C A C A C A C A C A C	210 	220 	230 A C A A A G A A C A A A G A G T G C C T T G G G A A A A A A A A A A A A A	240 T G G C T T - T G G
<u>ISJF1</u> 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC 003977</u> <u>15.L42</u> 16.L43 17.L44 <u>18.L47</u> <u>19.P1</u> 20.P3 21.P6 <u>Clustal Consensus</u>	190 CATCTCATGT 	200 I I T - C A T G T C C T A C A C A C A C A C A C A C A C A C A C	210 	G. TCCT. T 220 CCTCCAAGCT 	230 A C A A A G A G T G C C T T G G G A A 	240 T G G C T T T G G
<u>IS.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> <u>15.L42</u> 16.L43 17.L44 18.L47 <u>19.P1</u> 20.P3 21.P6 Clustal Consensus	190 I I CATCTCATGT T T T T T T Z.CCCC	200 I I T - C A T G T C C T A C A C	210 	G. TCCT. T CCTCCAAGCT 	230 A C A A A G A G T G C C T T G G G 	240 T G G C T T - T G G C C T A G C C
<u>IS.F1</u> 20.P3 21.P6 Clustal Consensus NC_003977 <u>15.L42</u> 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus	190 CATCTCATGT 	200 T - C A T G T C C T A C A	210 	G. TCCT. T CCTCCAAGCT 	230 C A A A G A A C A A A G A G T G C C T T G G G A C A A A G A G T G C C T T G G G 	240 T A A T G G C T T - T G G C C T A . G . C .
<u>ISJF1</u> 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC_003977</u> 15.L42	190 	200 T - C A T G T C C T A C A C	210 	220 T C C T T C C T C C A A G C T A C A G T 280 1 1	230 A C A A A G A	240 T A A
<u>ISJF1</u> 20.P3 21.P6 Clustal Consensus <u>NC 003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus <u>NC 003977</u> 15.L42 16.L43	190 CATCTCATGT T T T CATCTCATGT T <th>200 I I T - C A T G T C C T A C A C</th> <th>210 , I, I A.G, T.C. A.G, T.C. , I, I ACTGTTCAAG , T, I , /th> <th>G. TCCT. T CCTCCAAGCT </th> <th>230 G T G C C T T G G G </th> <th>240 T G G C T T - T G G C C T <</th>	200 I I T - C A T G T C C T A C A C	210 , I, I A.G, T.C. A.G, T.C. , I, I ACTGTTCAAG , T, I ,	G. TCCT. T CCTCCAAGCT 	230 G T G C C T T G G G	240 T G G C T T - T G G C C T <
<u>IS.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> <u>15.L42</u> 16.L43 17.L44 18.L47 <u>19.P1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> <u>15.L42</u> <u>16.L43</u> 17.L44	190 CATCTCATGT 	200 I I T - C A T G T C C T A C A C	210 	G. TCCT. T CCTCCAAGCT 	230 A C A A A G A A C A A A G A G T G C C T T G G G 	T A A 240 T G G C T T G G T G G C T T G G C C C T A G G C T G G G G G G C T T G G G G C T T G G G G G C T T G G G G C T G G G G C T G G G G G C T G G G G G C T G G G G G C T G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G
<u>IS.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47	190 CATCTCATGT	200 I I T - C A T G T C C T A C A A A C A A A A A	210 	220 	230 A C A A A G A A C A A A G A G T G C C T T G G G A C A A A G A G T G C C T T G G G 	Z40 T G G C T T T G G T C G C T T A G C C C T A G C 300 C C C T I T T G G
<u>ISJF1</u> 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 <u>Clustal Consensus</u> <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1	190 CATCTCATGT 	200 I I T - C A T G T C C T A C A A A C A A A	210 	G G A G C T A C T G	A C A A A G A 230 G T G C C T T G G G 1 G T G C C T T G G G A	240 T A A
<u>IS.F1</u> 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus <u>NC_003977</u> 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 20.P3	190 CATCTCATGT T <tr< th=""><th>200 I I T - C A T G T C C T A C A A A</th><th>210 </th><th>G. TCCT. T 220 CCTCCAAGCT CCTCCAAGCT ACA.GT. 280 280 CCTACTG</th><th>230 G T G C C T T G G G </th><th>240 T G G C T T - T G G C C T </th></tr<>	200 I I T - C A T G T C C T A C A A A	210 	G. TCCT. T 220 CCTCCAAGCT CCTCCAAGCT ACA.GT. 280 280 CCTACTG	230 G T G C C T T G G G	240 T G G C T T - T G G C C T
<u>NC_003977</u> <u>15.L42</u> <u>16.L43</u> <u>17.L44</u> <u>18.L47</u> <u>19.P1</u> <u>20.P3</u> <u>21.P6</u> <u>Clustal Consensus</u> <u>NC_003977</u> <u>15.L42</u> <u>16.L43</u> <u>17.L44</u> <u>18.L47</u> <u>19.P1</u> <u>20.P3</u> <u>21.P6</u> <u>21.P6</u> <u>21.P6</u> <u>21.P6</u> <u>21.P6</u> <u>21.P1</u> <u>20.P3</u> <u>21.P4</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P3</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>21.P2</u> <u>2</u>	190 CATCTCATGT CATCTCATGT T T T T T T T T T T T T T T 	200 I I T - C A T G T C C T A C A C C A C A A C C C	210 	G G A G C T A C T G	230 A C A A A G A A C A A A G A G T G C C T T G G G 	Z40 T G G C T T T G G C C T A G C 300 C T C G T T T T G
NC_003977 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus	190 CATCTCATGT TGC 190 CATCTCATGT TTCTCTC TTCCCCC 250 GGCATGGACA A TG.TG	200 T - C A T G T C C T A C A A A C C C T G A . A G	210 	220 T C C T T C C T C C A A G C T A C A G T A C A G T C C T C C A A C T G C A C T C C C C C C A C T G C C C C A C T C C C C C C A C C C C C C C C C C C C C	230 A C A A A G A G T G C C T T G G G A C A A A G A B T G C C T T G G G A A G A A A A G A A G A A A G A A A G A <t< th=""><th>240 T . A A</th></t<>	240 T . A A
13.F1 20.P3 21.P6 Clustal Consensus NC_003977 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus NC_003977 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus 0.P1 20.P3 21.P6 Clustal Consensus	190 CATCTCATGT CATCTCATGT T T T T T T T T 	200 I I T - C A T G T C C T A C A A A C C C C C C C C C A A A A	210 	G G A G C T A C T G A A A A A A C T A C T G	230 G T G C C T T G G G A C A A A G A B T G C C T T G G G A C A A A G A B T G C C T T G G G A A G A A G A A A G A A G A A A A G A A G A A B T G G A G T T A C T B T G G A G T T A C T	240 T G G C T T - T G G C C T . A . G . C . </th
NC 003977 15.L42 16.L43 17.L44 18.L47 19.P1 20.P3 21.P6 Clustal Consensus	190 CATCTCATGT T <tr< th=""><th>200 I I T - C A T G T C C T A C A A A C /th><th>210 </th><th>G. TCCT. T 220 CCTCCAAGCT CCTCCAAGCT ACA.GT. 280 280 CCTACTG A</th><th>230 A C A A A G A A C A A A G A G T G C C T T G G G </th><th>240 T G G C T T - T G G C C T . A. G. C. </th></tr<>	200 I I T - C A T G T C C T A C A A A C 	210 	G. TCCT. T 220 CCTCCAAGCT CCTCCAAGCT ACA.GT. 280 280 CCTACTG A	230 A C A A A G A A C A A A G A G T G C C T T G G G 	240 T G G C T T - T G G C C T . A. G. C.
<u>NC_003977</u> <u>15.L42</u> <u>16.L43</u> <u>17.L44</u> <u>18.L47</u> <u>19.P1</u> <u>20.P3</u> <u>21.P6</u> <u>Clustal Consensus</u> <u>NC_003977</u> <u>15.L42</u> <u>16.L43</u> <u>17.L44</u> <u>18.L47</u> <u>19.P1</u> <u>20.P3</u> <u>21.P6</u> <u>Clustal Consensus</u> <u>21.P6</u> <u>Clustal Consensus</u>	190 CATCTCATGT	200 I I T - C A T G T C C T A C A C C C A C C C A A A C C C	210 	G. TCCT. T 220 CCTCCAAGCT CCTCCAAGCT 	230 I I G T G C C T T G G G AA 	T A A 240 T G G C T T T G G T G G C T T T G G C C T A G G C C C T A G G C C C T A G G C C C T G C T G C C G C T G C T G G G G

10 20 30 40 50 60

Figure 4: Comparison of nucleotide sequence (L42, L43, L44, L47, P1, P3, and P6) of core promoter/precore region (1661-1921) with wild type (Accession no. – NC_003977) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

G1888A/T mutation was observed in 83.1% and was observed more in patients with HBeAg negative status than in patients with HBeAg positive status. G1888A/T mutation was more significantly associated with HBeAg negative status [p value less than 0.05].

G1896A/T/C mutation was observed in 11.4% isolates and was observed more in patients with HBeAg negative status than in patients with HBeAg positive status. G1896A/T/C mutation was more significantly associated with HBeAg negative status [p value less than 0.05].

Precore /core promoter mutations and HCC risk: Various core promoter and precore mutations were found to be associated with Hepatocellular Carcinoma (HCC) risk and shown (Table 7).

DISCUSSION

In this work, we performed a molecular study of preC/C gene to analyze the prevalence of various precore and core promoter mutations in various disease stages of Hepatitis B virus and correlation of various mutations with the disease status in the state of Punjab (North India).

Our study observed A1762T/G1764A double mutation in 24.6% patients and was observed in 100% of the patients with advanced liver disease. This double mutation was found more in HBeAg negative patients and was observed in 40% of the HCC patients. Our results confirmed with the findings of Wang W, et al. [18] who observed that mutations (1762/1764 and 1896/1899) were the causative factor for

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NC_003977 22.P10 23.P13 24.P14 25.P19 26.P22 27.P23 28.P24 Clustal Consensus	ACTCTTGGAC	TCTCAGCAAT	GTCAACGACC GCAACGACC GCAA GCAA GCAA GCAA	GACCTTGAGG CGACA. ACA. AGA.ACA. AGA.ACA. AGACA. AGACA.	CATACTTCAA - C	AGACTGTTTG
NC_003977 22.P10 23.P13 24.P14 25.P19 26.P22 27.P23 28.P24 Clustal Consensus	70 T T T A A A G A C T	80 G G G A G G A G G A G T T	90 G G G G G G A G G A G 	100 A T T A G - G T T A	110 A A G G T C T T T G	120 T A C T A G G A G G . T
NC_003977 22.P10 23.P13 24.P14 25.P19 26.P22 27.P23 28.P24 Clustal Consensus	130 C T G T A G G C A T	140 A A A T T G G T C T	150 GTTCACCAGC .CGT. .CGT.	160 A C C A T G C A A C T	170 T T T T T C C C C T 	180 C T G C C T A A T C
NC_003977 22.P10 23.P13 24.P14 25.P19 26.P22 27.P23 28.P24 Clustal Consensus	190 A T C T C A T G T T A A A A A A A A A A	200 C A T G T C C T A C C A T G T C C T A C C C C C	210 T G T T C A A G C C T	220 T C C A A G C T G T	230 G C C T T G G G T G A A A A 	240 G C T T T G G G G C
NC_003977 22.P10 23.P13 24.P14 25.P19 26.P22 27.P23 28.P24 Clustal Consensus	250 A T G G A C A T T G A 	260 A C C C G - T A T A	A A . A A A . A A A . A A A . A A A . A A A			

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Figure 5: Comparison of nucleotide sequence (P10, P13, P14, P19, P22, P23, and P24) of core promoter/precore region (1661-1921) with wild type (Accession no. – NC_003977) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

HBeAg negativity, liver function injury and also acted as biomarkers for HCC progression. Our results also matched with the Brazilian study of Chachá SGF, et al. [19], who observed high prevalence of precore and basal core promoter mutations. They also observed that A1762T/G1764A mutant was independently related with progressive forms of liver fibrosis, hepatic cirrhosis, and HCC.

We observed G1896A/T/C mutation in 11.4% patients and were observed only in advanced liver disease patients. G1896A/T/C mutation was observed more in HBeAg negative patient and was observed in 100% HCC patients. Our study confirmed the findings of Yang HI, et al. [20] who observed that G1896A mutation blocks HBeAg formation and this mutation was found to be related with hepatocarcinogenesis. C1766T/T1768A mutation was observed in 1.9% patients and these patients belonged to advance liver disease. C1766T/T1768A mutation was observed only in patients with HBeAg negative status and was observed in 20% HCC patients. Our study matched with the findings of Nishizawa T, et al. [21] who observed C1766T/T1768A mutations in the BCP region of genotype A increase viral replication and down regulate HBeAg expression. As per above study we observed C1766T/T1768A mutation only in HBeAg negative patients.

G1809A/T/C mutation was reported in 86.8% patients and was observed more in advanced liver disease patients and less in nonadvanced liver disease patients. G1809A/T/C mutation was observed more in patients with HBeAg negative status than in patients with HBeAg positive status and was observed in 60% HCC patients.

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NC_003977 29.P25 30.P26 31.P27 32.P29 33.P30 34.P31 35.P33 Clustal Consensus	ACTCTTGGAC	T CT C A G C A A T	GTCAACGACC .GACC.A.A. G.AC.G GC.AT.A AC.AC.A AC.CC.A.A GAC.TT	GACCTTGAGG C.TGCA.GC- ATGCAGC- AC.T.GC.C. .TT.CCC- ACTAAA.CC- ACTTGCAGC- TTGCAGC-	CATACTTCAA	A G A C T G T T T G
NC_003977 29.P25 30.P26 31.P27 32.P29 33.P30 34.P31 35.P33 Clustal Consensus	70 T T T A A A G A C T	80 GGGAGGAGTT	90 GGGGGA - GGA	100 GATTAGGTTA	110 A A G G T C T T T G T . A T . A T . A T . A T . A T . A T . A	120 T A C T A G G A G G . T
NC_003977 29.P25 30.P26 31.P27 32.P29 33.P30 34.P31 35.P33 Clustal Consensus	130 C T G T A G G C A T A C T G	140 A A A T T G G T C T C . T T . G .	150 GTTCACCAGC CGT. CGT. TCGG.G.T. CGT. CGT. CGT. CGT. CGT.	160 A C C A T G C A A C T	170 T T T T T T C C C C T A A A A A A A A A A A A A A A A A 	180 C T G C C T A A T C
NC_003977 29.P25 30.P26 31.P27 32.P29 33.P30 34.P31 35.P33 Clustal Consensus	190 A T C T C A T G T T 	200 C A T G T C C T A C G T	210 T G T T C A A G C C T	220 T C C A A G C T G T T C C	230 G C C T T G G G T G G C C T T G G G T G G T A G T A A A A A A	240 G C T T T G G G G G C A T G T T C A A C
NC_003977 29.P25 30.P26 31.P27 32.P29 33.P30 34.P31 35.P33 Clustal Consensus	250 A T G G A C A T T G G G G A	260 A C C C G T A T A A T T C C C A . A	270 A A A T T G G A G A A A A T T G G A G A A A A A A A A A A A A	280 II C T A C T G T G G A	290 l G T T A C T C T C G	300 I I T T T T T G C C T T

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Figure 6: Comparison of nucleotide sequence (P25, P26, P27, P29, P30, P31, and P33) of core promoter/precore region (1661-1921) with wild type (Accession no. – NC_003977) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

Page -008

C1810T mutation was reported in 3.8% patients and these patients belonged to advanced liver disease. C1810T mutation was observed only in patients with HBeAg negative serology and was observed in 20% HCC patients. A1811C/G mutation was reported in 3.8% patients who belonged to advanced liver disease and was not found in HCC patients. C1812T mutation was found in 81.2% patients and was observed more in advanced liver disease patients than in non-advanced liver disease patients. C1812T mutation was observed more in patients with HBeAg negative status than in patients with HBeAg negative status than in patients. Our study matched with the study of Makondo E, et al. [22] who observed that mutations (1762T1764A, Kozak sequence mutation 1809-1812, 1814-1816, G1896A with C1858T and G1862T) were causative factor for high HBeAg-negativity.

G1862A/T mutation was observed in 84.9% patients and was observed more in advanced liver disease patients and less in nonadvanced liver disease patients. G1862A/T mutation was observed more in patients with HBeAg negative serology than in patients with HBeAg positive serology and was observed in 60% HCC patients. Our study matched with the findings of Kramvis A, et al. [23] who observed that 1862 mutation along with other missense mutations and deletions in the precore region, may disturb HBV DNA replication and/or signal peptide breakage leading to HBeAg-negativity. Viral replication disruption may stimulate assimilation of replicative intermediates (unencapsidated) and lead to hepatocarcinogenesis. So as per above study we observed 1862 mutation more in HBeAg negative patients and in 60% of HCC patients.

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36.P35

37.P40

38.P43

<u>39.P45</u> 40.P60

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Figure 7: Comparis	son of nucl	eotide s	seque	nce (I	P35, I	P40, F	P43, P	45, F	960, I	P64,	and F	P72)	of co	ore p	orom	notei	r/prec	ore i	regio	on (1661	-1921) wit	h wi	ld ty	pe (/	Acce	essi
no. – NC 003977)	usina BioE	Edit Sof	tware.	Dot	(.) sh	ows t	he sim	nilarit	v and	1 (-) \$	seaue	ence	sho	ws c	lelet	ion.				,					,	· ·		

A.G

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ССА

G1888A/T mutation was observed in 83.1% patients and was observed more in advanced liver disease patients and less in nonadvanced liver disease patients. G1888A/T mutation was observed more in patients with HBeAg negative status than in patients with HBeAg positive status and was observed in 40% HCC patients. Our study matched with the findings of Kimbi G, et al. [24] but we did not conduct genotypic study. As per Kimbi G, et al. [24] who observed that G1888A mutation was unique to subgenotype A1 and occurs hardly in other genotypes and in HBV patients outside Africa. This mutation could influence the translation of the core protein. It also stabilizes the encapsidation signal and affecting virus reverse transcription process. The newly introduced start codon at position 1888 may have a significant part in the regulation of the translational ability of a following start codon.

G1915A/C mutation was observed in 15.1% patients and was observed equally in advanced liver disease patients (50%) and in non-advanced liver disease patients (50%). G1915A/C mutation was observed more in patients with HBeAg negative status than in patients with HBeAg positive status and was found in 40% HCC patients. Our study matched with the study of Kumar R, et al. [25] who observed novel precore mutation 1915A/G in HBV infected population of Punjab state (North India).

We observed various core promoter/precore mutations (1753A, A1762T, G1764A, C1766T, T1768A, and G1896A) in this study which were found more in advanced liver disease patients, associated with high HBeAg negativity and found more in HCC patients. These observations matched with the study of Trinks J, et al. [26] in which

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GA.

GACCTTGAGG CATACTTCAA AGACTG

 CGA....CA
 GC....

 T..A...CAC
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 CGA.A
 GA

 GC....
 GC....

GC

CA

C A

Page -009

NC_003977 43.P74 44.P75 45.P76 46.P77 47.P78 48.P79 49.P81 Clustal Consensus		20 T C T C A G C A A T	30 GTCAACGACC CGA. GA. GA. GA. GA.	40 GACCTTGAGG T.GC T.GC T.GC T.GC T.GC T.GC T.GC	50 CATACTTCAA - C	60 A G A C T G T T T G G G G G
NC 003977 43.P74 44.P75 45.P76 46.P77 47.P78 48.P79 49.P81 Clustal Consensus	70 T T T A A A - G A C 	80 T G G G A G G A G T	90 T G G G G G A G G A	100 G A T T A G G - T C . C G .	110 T A A A G G T C T T 	120 T G T A C T A G G A T T T T T T T T
NC_003977 43.P74 44.P75 45.P76 46.P77 47.P78 48.P79 49.P81 Clustal Consensus	130 G G C T G T A G G C	140 A T A A A T T G G T	150 CTGTTCACCA CG CG CG CG CG CG CG	160 G C A C C A T G C A T . T T . T T . T .	170 A C T T T T T T C C C A. A. A. A. A. A. A. A. A. A.	180 CTCTGCCTAA
NC_003977 43.P74 44.P75 45.P76 46.P77 47.P78 48.P79 49.P81 Clustal Consensus	190 II T C A T C T C A T G T T T T T T	200 T T C A T G T C C T A C A C A C A C A C A C A C A C	210 A C T G T T C A A G T T T T T T T	220 C C T C C A A G C T 	230 G T G C C T T G G G A A A A A A A	240 T G G C T T T G G G
NC_003977 43.P74 44.P75 45.P76 46.P77 47.P78 48.P79 49.P81 Clustal Consensus	250 G C A T G G A C A T	260 T G A C C C - G T A	270 T A A A	280 G A G A G C T G T G T G G A G C T A C - - G G A G C T A C - T G G A G C T A C - T G G A G C T A C -	290 TGGTTACTA TGTAGAGTT TGTGGAGTTA TGTGGAGTTA TGTGGAGTTA	300

Figure 8: Comparison of nucleotide sequence (P74, P75, P76, P77, P78, P79, and P81) of core promoter/precore region (1661-1921) with wild type (Accession no. – NC_003977) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

various core promoter/precore mutations like T1753C, A1762T, G1764A, C1766T, T1768A G1896A, G2092T and T2107C were related with acute liver failure and lead to chronic hepatitis.

Our study matched with the study of Gil-García AI, et al. [27], who showed that the coexistence of the mutations A1762T/G1774A and G1896A could lead to a high-risk of carcinogenosis. As per above study, we also observed A1762T/G1764A and G1896A in this study.

CONCLUSION

In conclusion, we demonstrated various precore/core promoter mutations (A1762T, G1764A, A1762T/G1764A, C1766T/T1768A, C1773T, T1802C/T1803G, G1809A/T/C, C1810T, A1811C/G, C1812T, T1850A/C, T1858C, G1862A/T, G1888A/T, G1896A/T/C

and G1915A/C) in the studied samples and these mutations were associated more in patients with advanced liver disease. These mutations were observed more in HBeAg negative patients. Various novel precore/core promoter mutations (C1695A/T/G, G1700A/C, G1701A/C, A1703C, T1719G, and C1827A/T) were observed. Various precore/core promoter mutations (C1695A/T/G, G1700A/C, A1703C, T1719G, A1762T/G1764A, C1773T, T1802C/T1803G, G1809A/T/C, C1827A/T, G1862A/T, and G1896A/T/C) were marked in HCC patients in Punjab (North India).

CLINICAL SIGNIFICANCE

The above precore/core promoter mutations observed during different stages of the disease acts as biomarkers for the detection

<u>NC_003977</u> 50. <u>M1</u> 51.M2 52.M3 53.M4 Clustal Consensus	10 A C T C T T G G A C	20 T C T C A G C A A T	30 G T C A A C G A C C G C A A C G A C C G C A A C G A C C G G G A A G G G A A	40 GACCTTGAGG C.ACA. AT.CA. AT.CA. ATGCA AGAT.A	50 CATACTTCAA GC	60 A G A C T G T T T G
NC_003977 50.M1 51.M2 52.M3 53.M4 Clustal Consensus	70 T T T A A G A C T G	80 G G G A G G A G T T	90 GGGGGGAGGAG	100 ATTAGGTTAA A	110 AGGTCTTTGT A	120 A C T A G G A G G C T
<u>NC_003977</u> 50.M1 51.M2 52.M3 53.M4 Clustal Consensus	130 TGTAGGCATA	140 II A A T T G G T C T G 	150 T T C A C C A G C A C G C G T C G T	160 I.CATGCAACT 	170 I I TTTTCCCCCTC A A A	180 T G C C T A A T C A
<u>NC_003977</u> 50. <u>M1</u> 51.M2 52.M3 53.M4 Clustal Consensus	190 II T C T C A T G T T C TA. TA. TA.	200 A T G T C C T A C T C	210 G T T C A A G C C T T	220 C C A A G C T G T G 	230 I.CTTGGGTGG A A	240 C T T T G G G G C A
<u>NC_003977</u> 50.M1 51.M2 52.M3 53.M4 Clustal Consensus	250 T G G A C A T T G A	260 C C C G T A T A A A . A . A A	A A A A A A A A A A A A A A A A G A			

Figure 9: Comparison of nucleotide sequence (M1, M2, M3, and M4) of core promoter/precore region (1661-1921) with wild type (Accession no. - NC_003977) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

Table 2: Variou	able 2: Various core promoter mutations in different stages of HBV infection and their <i>p</i> value.												
Sr. No.	Mutation	Group I	Group Ila	Group IIb	Group III	Group IV	p value						
1.	1753A	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	NA						
2.	1756A/C	0 (0%)	2 (33.4%)	0 (0%)	2 (33.3%)	2 (33.3%)	0.014						
3.	1757A	3 (50%)	1 (16.7%)	0 (0%)	0 (0%)	2 (33.3%)	NA						
4.	1762T	0 (0%)	13 (81.3%)	0 (0%)	0 (0%)	3 (18.7%)	NA						
5.	1764A	0 (0%)	11 (84.6%)	0 (0%)	0 (0%)	2 (15.4%)	NA						
6.	1762T/1764A	0 (0%)	11 (84.6%)	0 (0%)	0 (0%)	2 (15.4%)	NA						
7.	1766T/1768A	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	NA						
8.	1773T	15 (31.3%)	13 (27.1%)	11 (22.9%)	5 (10.4%)	4 (8.3%)	0.000						
9.	1802C/1803G	16 (32%)	13 (26%)	12 (24%)	5 (10%)	4 (8%)	0.000						
10.	1809A/T/C	13 (28.3%)	13 (28.3%)	12 (26.1%)	5 (10.9%)	3 (6.4%)	0.000						
11.	1810T	0 (0%)	1 (50%)	0 (0%)	0 (0%)	1 (50%)	NA						
12.	1811C/G	0 (0%)	1 (50%)	1 (50%)	0 (0%)	0 (0%)	NA						
13.	1812T	13 (30.3%)	12 (27.9%)	11 (25.6%)	5 (11.6%)	2 (4.6%)	0.000						

ISSN: 2766-5070

 Table 3: Various precore mutations in different stages of HBV infection and their p value.

Sr. No.	Mutation	Group I	Group Ila	Group IIb	Group III	Group IV	<i>p</i> value
1.	1850A/C	13 (28.9%)	13 (28.9%)	12 (26.6%)	5 (11.2%)	2 (4.4%)	0.000
2.	1858C	13 (29.5%)	13 (29.5%)	12 (27.3%)	5 (11.4%)	1 (2.3%)	0.000
3.	1862A/T	13 (28.9%)	13 (28.9%)	11 (24.5%)	5 (11.1%)	3 (6.6%)	0.000
4.	1888A/T	13 (29.6%)	12 (27.3%)	12 (27.3%)	5 (11.3%)	2 (4.5%)	0.000
5.	1896A/T/C	0 (0%)	1 (16.6%)	0 (0%)	0 (0%)	5 (83.4%)	NA
6.	1915A/C	4 (50%)	2 (25%)	0 (0%)	0 (0%)	2 (25%)	NA

Table 4: Various	able 4: Various novel core promoter and precore mutations in different stages of HBV infection and their <i>p</i> value.											
Sr. No.	Mutation	Group I	Group Ila	Group Ilb	Group III	Group IV	p value					
1.	1690A/T/G	10 (43.5%)	4 (17.4%)	3 (13.1%)	4 (17.4%)	2 (8.6%)	0.000					
2.	1695A/T/G	6 (23.1%)	7 (26.9%)	6 (23.1%)	3 (11.5%)	4 (15.4%)	0.000					
3.	1700A/C	12 (33.4%)	13 (36.1%)	6 (16.7%)	1 (2.7%)	4 (11.1%)	0.000					
4.	1701A/C	3 (18.8%)	5 (31.2%)	6 (37.4%)	1 (6.3%)	1 (6.3%)	0.000					
5.	1703C	12 (30.8%)	9 (23.1%)	10 (25.6%)	5 (12.8%)	3 (7.7%)	0.000					
6.	1719G	12 (26.7%)	13 (28.9%)	12 (26.7%)	5 (11.1%)	3 (6.6%)	0.000					
7.	1727G	1 (25%)	2 (50%)	0 (0%)	0 (0%)	1 (25%)	0.046					
8.	1827A/T	16 (30.8%)	15 (28.8%)	12 (23.1%)	5 (9.6%)	4 (7.7%)	0.000					

Sr. No.	Mutations	HBeAg positive	HBeAg negative	<i>p</i> value
1.	1690	1690A = 1/13 (7.7%)	1690A = 12/13 (92.3%)	0.000
		1690T = 0/13 (0%)	1690T = 3/3 (100%)	0.000
		1690G = 2/7 (28.6%)	1690G = 5/7 (71.4%)	0.000
2.	1695	1695A = 1/8 (12.5%)	1695A = 7/8 (87.5%)	0.000
		1695T = 6/17 (35.3%)	1695T = 11/17 (64.7%)	0.000
		1695G = 0/1 (0%)	1695G = 1/1 (100%)	0.000
3.	1700	1700A = 4/24 (16.6%)	1700A = 20/24 (83.4%)	0.000
		1700C = 3/12 (25%)	1700C = 9/12 (75%)	0.000
٨	1701	1701A = 6/8 (75%)	1701A = 2/8 (25%)	0.000
ч.		1701C = 1/8 (12.5%)	1701C = 7/8 (87.5%)	0.000
5.	1703	1703C = 11/39 (28.2%)	1703C = 28/39 (71.8%)	0.000
6.	1719	1719G = 14/45 (31.1%)	1719G = 31/45 (68.9%)	0.000
7.	1727	1727G = 0/4 (0%)	1727G = 4/4 (100%)	0.046
8.	1753	1753A = 0/1 (0%)	1753A = 1/1 (100%)	NA
9.	1756	1756A = 0/6 (0%)	1756A = 6/6 (100%)	NA
10.	1757	1757A = 0/6 (0%)	1757A = 6/6 (100%)	NA
11.	1762	1762T = 1/16 (6.2%)	1762T = 15/16 (93.8%)	0.000
12.	1764	1764A = 1/13 (7.7%)	1764A = 12/13 (92.3%)	0.000
13.	1762/1764	1762T/1764A = 1/13 (7.7%)	1762T/1764A = 12/13 (92.3%)	0.000
14.	1766/1768	1766T/1768A = 0/1 (0%)	1766T/1768A = 1/1 (100%)	NA
15.	1773	1773T = 14/48 (29.2%)	1773T = 34/48 (70.8%)	0.000
16.	1802/1803	1802C/1803G = 14/50 (28%)	1802C/1803G = 36/50 (72%)	0.000
17.	1809	1809A = 0/1 (0%)	1809A = 1/1 (100%)	0.000
		1809T = 14/44 (31.8%)	1809T = 30/44 (68.2%)	0.000
		1809C = 0/1 (0%)	1809C = 1/1 (100%)	0.000
18.	1810	1810T = 0/2 (0%)	1810T = 2/2 (100%)	NA
19.	1811	1811C = 1/1 (100%)	1811C = 0/1 (0%)	NA
		1811G = 0/1 (0%)	1811G = 1/1 (100%)	NA
20.	1812	1812T = 13/43 (30.3%)	1812T = 30/43 (69.7%)	0.000
			× /	

ISSN: 2766-5070

Table 6: Various precore mutations and their association with HBeAg status and their p value.						
Sr. No.	Mutations	HBeAg positive	HBeAg negative	<i>p</i> value		
1.	1827	1827A = 14/51 (27.7%)	1827A = 37/51 (72.5%)	0.000		
		1827T = 0/1 (0%)	1827T = 1/1 (100%)	0.000		
2.	1850	1850A = 14/44 (31.8%)	1850A = 30/44 (68.2%)	0.000		
		1850C = 0/1 (0%)	1850C = 1/1 (100%)	0.000		
3.	1858	1858C = 14/44 (31.8%)	1858C = 30/44 (68.2%)	0.000		
4.	1862	1862A = 0/1 (0%)	1862A = 1/1 (100%)	0.000		
		1862T = 13/44 (29.5%)	1862T = 31/44 (70.5%)	0.000		
5.	1888	1888A = 14/43 (28.2%)	1888A = 29/43 (28.2%)	0.000		
		1888T = 0/1 (0%)	1888T = 1/1 (100%)	0.000		
6.	1896	1896A = 1/2 (50%)	1896A = 1/2 (50%)	0.014		
		1896T = 0/2 (0%)	1896T = 2/2 (100%)	0.000		
		1896C = 0/2 (0%)	1896C = 2/2 (100%)	0.000		
7.	1915	1915A = 0/7 (0%)	1915A = 7/7 (100%)	0.005		
		1915C = 1/1 (100%)	1915C = 0/1 (0%)	0.000		

Table 7: Various core promoter and precore mutations and Hepatocellular Carcinoma (HCC) percentage.

Sr. No.	Nucleotide position	Mutation	HCC percentage
4	1600	1690A	1/5 (20%)
1.	1090	1690G	1/5 (20%)
2	1695	1695A	1/5 (20%)
	1000	1695T	3/5 (60%)
3.	1700	1700C	4/5 (80%)
4.	1701	1701A	1/5 (20%)
5.	1703	1703C	3/5 (60%)
6.	1719	1719G	3/5 (60%)
7.	1753	1753A	1/5 (20%)
0	1750	1756A	1/5 (20%)
0.	1756	1756C	1/5 (20%)
9.	1757	1757A	2/5 (40%)
10.	1762	1762T	3/5 (60%)
11.	1764	1764A	2/5 (40%)
12.	1762/1764	1762T/1764A	2/5 (40%)
13.	1766/1768	1766T/1768A	1/5 (20%)
14.	1773	1773T	4/5 (80%)
15.	1802/1803	1802C/T/1803G	4/5 (80%)
16	1800	1809A	1/5 (20%)
10.	1809	1809T	2/5 (40%)
17.	1810	1810T	1/5 (20%)
18.	1812	1812T	2/5 (40%)
10	1827	1827A	3/5 (60%)
19.	1027	1827T	1/5 (20%)
20	1850	1850A	1/5 (20%)
		1850C	1/5 (20%)
21.	1858	1858C	1/5 (20%)
22	1862	1862A	1/5 (20%)
<i>LL</i> .	1002	1862T	2/5 (40%)
23.	1888	1888A	1/5 (20%)
		1888T	1/5 (20%)
	1000	1896A	2/5 (40%)
24.	1896	18961	2/5 (40%)
		18960	1/5 (20%)
25.	1915	1915A 1915C	1/5 (20%) 1/5 (20%)
		19100	1/3 (2070)

of HBV related liver diseases and it helps the clinician/physician for correct treatment of various HBV related liver diseases.

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INSTITUTIONAL ETHICS COMMITTEE STATEMENT

The study was reviewed and approved by the Institutional Ethics committee, Kurukshetra University, Kurukshetra, Haryana, India

INFORMED CONSENT STATEMENT

All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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