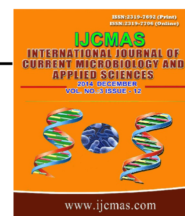

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Original Research Article

High frequency of precore and core promoter mutations in HBV infected population of Punjab in North India

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HBV, HBsAg, HBeAg, precore/core promoter mutations, HCC, BCP, BQW

A B S T R A C T

In HBV infected population of Punjab State (North India), various precore and core promoter mutations were observed. We aimed to determine the various precore and core promoter mutations in Punjab state (North India) by amplifying 32 HBV infected patients and then comparing their sequences with the wild type sequences by using BioEdit sequence comparison software. Various precore and core promoter mutations like 1753-1757, $1762^{T}1764^{A}$ double mutation, $1888^{A/T}$, $1896^{A/C/T}$ and few novel mutations like $1690^{A/T/G}$, $1695^{A/T/G}$ and $1697^{G/C}1698^{A/C/T}1727^{G}1740^{C}$ were observed in our study. These mutations were found more in HBeAg negative patients in our study. We conclude that there is high prevalence of various precore and core promoter mutations in HBV infected population of Punjab state (North India).

Introduction

Hepatitis B virus (HBV) infection is a global health problem, and more than 350 million people in the world are chronic carriers of the HBV [1]. Chronic HBV infection is associated with a wide range of clinical manifestations, from an asymptomatic carrier status with a normal liver histology to severe and chronic liver disease, including cirrhosis and hepatocellular carcinoma (HCC) [2-4].

HBV is a member of *hepadnaviridae* family [5]; a family of enveloped DNA virus with partially double stranded, circular genomes that replicates by reverse transcription [6]. The genome has four overlapping open reading frames (ORFs), encoding the surface, precore/core, polymerase, and X genes [7]. Mutations in the HBV surface (*S*), precore (*PC*) and basal core promoter (*BCP*) genes are observed frequently in HBV infected patients, and studies show that these mutations are associated with the clinical outcomes of HBV disease [8-10]. Patients with core promoter (CP) mutants compared with those with wild-type have also been shown to have a higher risk of development of HCC [11-13]. Most infected patients that are HBeAg(-) harbor HBV variants with mutations in the precore or core promoter region [14].

The most frequent and clinically important mutations in the PC and BCP regions are

G1896A and A1762T/G1764A, respectively; which are often detected in HBeAg-negative chronic HBV infected patients [9]. The predominant precore mutation is a G-to-A change at A1896, which creates a premature stop codon and abolishes the synthesis of HBeAg [15-17]. The most common core promoter mutations involve a two-nucleotide substitution at T1762 and A1764 [14, 18]. These dual mutations (A1762T/G1764A) have been reported in up to 50–80% of patients with HBeAg-negative chronic hepatitis B in Europe and Asia [19], and have been implicated in HCC development [11, 20, and 21].

Enough evidence has emerged to demonstrate that BCP and preC mutants are predisposed to severe and progressive liver diseases after HBV infection, causing an increased risk for hepatocellular carcinoma (HCC) [11, 21-24]. For instance, mutations T1762/A1764 and A1899 have been reported to be independent risk factors for HCC [20], and T1653 and/or V1753 mutations are believed to promote the process of liver degradation [25]. However, the association of these mutations with severe symptoms is manifested in certain populations but not in others [26, 27].

An attempt has been made in this paper to study different precore and core promoter mutations in HBV infected population of Punjab state (North India) as there is lack of information from this area.

Materials and Methods

Samples Collection: The sera were obtained from 32 HBsAg positive patients from different districts of Punjab state (North India) - Rajendra medical college, Patiala, Government hospital, Mansa and Dayanand medical college, Ludhiana from 2006 to 2009. These patients had not taken any antiviral agents such as lamivudine or interferon. All patients were negative for HIV and HCV infection. The ethical committee of Kurukshetra University, Kurukshetra, Haryana had approved the study.

Serological Tests: In all the serum samples, ELISA for HBeAg detection was performed using monoclonal antibody coated ELISA plates procured from Equipar SRL, Italy and the procedure was followed as provided by the supplier. ALT/AST levels (aminotransferases) were performed in all samples as per the protocols supplied with the kits by Span Diagnostics Ltd., Surat, India. HBsAg was detected in serum by using HBsAg strips (IND diagnostic, Canada).

DNA Extraction: DNA was extracted from the serum samples by using QIAmp extraction kit from Qiagen ((Hilden, Germany). Finally the extracted DNA was suspended in 100 μl best quality water (BQW) and stored in -20°C in deep freezer.

Amplification of PreCore/Core Region: Polymerase chain reaction (PCR) for the amplification of precore/core promoter region was done using nested PCR. First round PCR was performed utilizing primer 1622F (5′-GAACGCCCATCA GATCC TGC-3′) and 1966R (5′-GTCAGAAGGC AAAAACGAGAG-3′) primers. The PCR was performed for 40 cycles involving denaturation at 94°C for 30 sec, annealing at 57°C for 50 sec and extension at 72°C for 50 sec. The initial denaturation was at 94°C for 5 minutes and final extension was performed at 72°C for 10 min. Second round PCR was performed using 1661F (5′-GACTCTTGGACT CTCAGC-3′) and 1921R (5′-TTTATACG GGTCAATGTC-3′) primers as per the method of Baptista *et al.,* [13]. A detail of primers was given in table 1. The PCR was performed for 40 cycles

involving denaturation at 94°C for 30 sec, annealing at 42°C for 30 sec, extension at 72°C for 50 sec. Initial denaturation was at 94°C for 3 min, and final extension was at 72°C for 10 minutes. The results were analyzed on 2% agarose gel as shown in Figure 1.

Sequencing of Samples and analysis: The 261 bp amplified products of precore/core region were sequenced. Nucleotide sequence of each sample was compared with the wild type sequence (Accession no.- GRS08538) using BioEdit sequence comparison software tool (U.S.A).

Result and Discussion

ALT level and HBeAg status: - The assays of ALT and HBeAg showed that 59% of the samples had higher ALT level and 75% of the samples were negative for HBeAg.

Amplified Pre Core/Core promoter Region: - Nested PCR was performed using 1622F and 1966R primers in the first round and 1661F and 1921R primers in the second round as per the method of Baptista *et al.,* [13]. Figure 1 show that precore/core promoter region as a single band of 261 bp.

Comparison of sequenced precore/core promoter region with wild type sequence: - 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.- GRS08538) using BioEdit sequence comparison software tool (U.S.A). The compared sequences are shown in figure 2, 3, 4 and 5.

Common precore/core promoter mutations observed

On comparison with wild type, following precore/core promoter mutations were observed in 32 samples:-

Core promoter mutations

1753-1757: - These are the one of the commonly observed core promoter mutations. 1753^{A} mutation was observed in 3% of the studied samples. Wild type nt. was T at position 1753. $1756^{A/C}$ was observed in 9% of the studied samples. Wild type nt. was G at position 1756. 1757^{G} mutation was observed in 96% of the studied samples. Wild type nt. was A at position 1757.

1762^{T} 1764^{A} : - This double mutation is one of the commonest core promoter mutation. 1762^{T} mutation was found in 31% of the studied samples. Wild type nt. was A at position 1762. 1764^{A} mutation was found in 28% of the studied samples. Wild type nt. was G at position 1764. 1762^{T} 1764^{A} double mutation was observed in 28% of the studied samples.

1766^{T} 1768^{A} : - This double mutation was found in 3% of the studied samples. Wild type nt. was C at position 1766 and T at position 1768.

1773^{T}:- This is one of the core promoter mutations. 1773^{T} mutation was observed in 100% of the studied samples. Wild type nt. was C at position 1773.

$1809^{A/T}1812^{T}$:- These are the missense mutations found in our studied samples as observed by Baptista *et al.,* [13] who found that $1809^{T}1812^{T}$ mutation is missense mutation and is found in 80% of African blacks and represent wild-type sequence in Southern African isolates. This missense mutation was observed in 93% of the studied samples. Mutations at 1809 were observed in 97% of the studied samples. Wild type nt. was G at position 1809. Mutation at 1812 was observed in 94% of

the studied samples. Wild type nt. was C at position 1812.

Precore mutations

$1862^{A/T}$: - This is one of the precore mutations. This mutation was present in 97% of the studied samples. Wild type nt. was G at position 1862.

$1888^{A/T}$: - This is also one of the precore mutations. $1888^{A/T}$ mutation was found in 94% of the studied samples. Wild type nt. was G at position 1888.

$1896^{A/C/T}$: - This is one of the commonest precore mutations. This mutation was found in 12% of the studied samples. Wild type nt. was G at position 1896.

Other less common precore/core promoter mutations

1719^{G}: - This is one of the core promoter mutations. 1719^{G} mutation was observed in 97% of the studied samples. Wild type nt. was T at position 1719.

$1850^{A/C}$: - This is one of the precore mutations. $1850^{A/C}$ mutation was observed in 94% of the studied samples. Wild type nt. was T at position 1850.

1858^{C}:- This is one of the precore mutations. 1858^{C} mutation was present in 91% of the studied samples. Wild type nt. was T at position 1858.

$1915^{A/G}$: - This is one of the precore mutations. $1915^{A/G}$ mutation was present in all the studied samples (100%). Wild type nt. was T at position 1915.

Novel precore/core promoter mutations

$1690^{A/T/G}$: - $1690^{A/T/G}$ mutation was observed for the first time in Indian HBV infected hepatitis samples in our study. $1690^{A/T/G}$ mutation was found in 65% of studied samples. Wild type nt. was C at position 1690.

$1695^{A/T/G}$: - This mutation was observed for the first time in Indian HBV infected hepatitis samples in our study. $1695^{A/T/G}$ mutation was found in 43% of studied samples. Wild type nt. was C at position 1695.

$1697^{G/C}1698^{A/C/T}1727^{G}1740^{C}$: - This mutation was one of the novel core promoter mutation found in HBV infected samples. $1697^{G/C}$ mutation was observed in 31% of the studied samples. Wild type nt. was T at position 1697. $1698^{A/C/T}$ mutation was observed in 34% of the studied samples. Wild type nt. was G at position 1698. 1727^{G} mutation was observed in 3% of the studied samples. Wild type nt. was A at position 1727. 1740^{C} mutation was observed in 9% of the studied samples. Wild type nt. was T at position 1740.

Deletion: - Deletion mutations were found mainly at nt. position 1695 (15%), 1703 (46%) and 1727 (25%).

Insertions: - Insertion mutations were found in 34% of the studied samples. Insertion of C was found at position 1700-1701 in P27, insertion of A was found at position 1719-1720 in P40, insertion of A was found at position 1727-1728 in P74, insertion of A was found at position 1748-1749 in P31, insertion of G was found at position 1748-1749 in P33, P40 and P45, insertion of C was found at position 1756-1757 in P19, insertion of CG was found at position 1757-1758 in P77, insertion of C was found at position 1911-1912 in P33, insertion of A was found at position 1911-1912 in P35, insertion of C was found at position 1914-1915 in P81 and insertion of C was found at position 1915-1916 in P22.

Precore and core promoter mutations

We observed various precore/core promoter mutations described above in the results in the HBV infected patients.

We observed **1757^{G}** mutation in 96% of the studied samples. As per the observation of Sendi *et al.,* 2009 [28], high level (>10^{5} copies/mL) of serum HBV DNA was directly associated with the presence of core promoter double 1762^{T} 1764^{A} mutations together with 1757^{G}.

We observed **1762^{T} 1764^{A} double mutation** in 28% of the studied samples. In our study this double mutation was found more in HBeAg negative patients so these results are in accordance with the results of Chauhan *et al.,* Singh *et al.,* and Kumar *et al.,* [29-31] which showed that 1762^{T} and 1764^{A} mutations were higher in HBeAg negative patients with high ALT. 1762^{T} and 1764^{A} mutations suppress HBeAg synthesis and may contribute to hepatocarcinogenesis [13, 32]. The BCP mutants may enhance HBV virulence by increasing host immune response to HBV-infected hepatocytes, increasing viral replication or altering the coding region for the X [33-36]. Previously reported results [37], suggested that patients with the BCP T1762/A1764 mutant exhibited significantly higher serum HBV DNA levels compared to those with the BCP A1762□G1764 wild-type strain, regardless of the PC 1896 status.

We observed **1766^{T} 1768^{A}** mutations in 3% of the studied samples. Recently, a longitudinal study demonstrated that T1762/A1764 mutation could be detected 4-7 years prior to HCC in most cases, while T1766/A1768 mutation occurred only at or near the stage of HCC, suggesting T1766/A1768 may be a more valuable predictive marker for HCC [38]. Because several reports demonstrated that T1766/A1768 mutation enhanced viral replication [39, 40], high viral load caused by this mutation might contribute to hepatocarcinogenesis.

Some *in vitro* study showed that the T1762/A1764 double mutation had no significant effect on replication capacity in comparison to the wild type [40]. However, all CP mutants bearing additional mutations in this region, i.e., mutations at the position of V1753/T1762/A1764, T1762/A1764 /A1768 or V1753/T1762/A1764/A1768 exhibited to markedly enhance viral replication than the wild type [40, 41], supporting a carcinogenetic potential of this mutation pattern. Thus, it is reasonable to think these mutations in BCP may play a synergistic role on enhancing HBV carcinogenesis.

1773^{T} mutation was observed in 100% of the studied samples. We found 1773^{T} mutation more in HBeAg negative samples as per the report of Chen *et al.,* 2005 [42] who reported that the HBeAg-negative patients had a significantly higher frequency of core promoter nucleotides C1753 and T1773 and precore nucleotides T1846, A1896, and A1899 than HBeAg-positive patients.

$1809^{A/T}1812^{T}$ mutation was observed in 93% of the studied samples as per observed by Singh J *et al.* and Kumar R *et al.,* [30, 31]. These were the missense mutations found in our studied samples as observed by Baptista *et al.,* [13] who found that $1809^{T}1812^{T}$ mutation is missense mutation and is found in 80% of African blacks and represent wild-type sequence in Southern African isolates.

$1862^{A/T}$ mutation was present in 97% of the studied samples. We observed $1862^{A/T}$ mutation more in HBeAg negative samples as per the study of Hou *et al.,* 2002 [43]

observed that the G to T substitution at position 1862 leads to an amino acid change in codon 17 of the precore protein of the virus, which is part of a signal peptidase recognition motif and In vitro translation experiments showed that this variant has greatly reduced capacity to produce hepatitis B e antigen (HBeAg) from its precore protein precursor. Kramvis *et al.,* 1998 [44] observed that the 1862 mutation and other missense mutations (1899) and deletions detected in the precore gene, may disrupt HBV DNA replication and/or signal peptide cleavage leading to HBeAg-negativity.

$1888^{A/T}$ mutation was found in 94% of the studied samples as observed by Kumar *et al.,* [31]. 1888^{A} mutation has a stabilizing effect on encapsidation signal as observed by Kramvis *et al.,* [44] and it also possibly affects reverse transcription, and hence this mutation affects the translation of the core protein as observed by Rogozin *et al.,* [45].

$1896^{A/C/T}$ mutation was found in 12% of the studied samples and was found more in HBeAg negative samples. The G to A substitution at nucleotide 1896 prevents the production of HBeAg by introducing a premature stop codon into the open reading frame (ORF) of the precore region. The frequency of the G1896A mutation was found to be higher in the HBeAg negative group as observed by Mangia *et al.,* [46]; Grandjacques *et al.,* [47] as observed by our study.

1719^{G} mutation was observed in 97% of the studied samples. It is one of core promoter mutations. Yin *et al.,* 2011 [48] which showed that mutation at nt.1719 along with mutations at nt.1674, nt.1762, nt.1764, nt.1846, nt.1896, and nt.1913 in genotype C were significantly associated with CHB, cirrhosis, and HCC (Severe liver disease).

$1850^{A/C}$ is one of the precore mutations and was observed in 94% of the studied samples. This mutation was observed more in HBeAg negative patients. Chen *et al.,* 2006 [49] observed that T1773, C1802, G1803, T1846, A1850, and C1858 mutations might have significant correlation with HBeAg nonseroconversion.

1858^{C} mutation was present in 91% of the studied samples. Studies revealed that 1858^{C} strains developed core promoter mutations more frequently, and hence associated with more severe liver disease (Chan *et al.,* [50] Hou *et al.,* [51]). 1858^{C} was observed in Asian, African and middle eastern isolates, while North American and European isolates have a T at nt. 1858 (Lok *et al.,* [52] as per our observation of 1858^{C} in Indian (Punjab) isolates.

$1915^{A/G}$ mutation was present in all the studied samples (100%). We observed this mutation in all chronic patients who were untreated. Similar observations were made in untreated patients from many studies (Laskus *et al.,* [53], so this observation also supports our results.

We also observed core promoter mutations like $1690^{A/T/G}$, $1695^{A/T/G}$, $1700^{A/C}$, and 1703^{C} in our study and these mutations were first time observed in North Indian patients (Punjab).

In conclusion, we observed various precore and core promoter mutations (1762^{T} 1764^{A} and $1896^{A/C/T}$) and some novel precore and core promoter mutations like $1690^{A/T/G}$, $1695^{A/T/G}$, $1700^{A/C}$, 1703^{C} in HBV infected population of Punjab state (North India). These mutations were found more in HBeAg negative samples with high ALT level. So we can say that there is more prevalence of various precore and core promoter mutations in the Punjab population (North India).

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′	345
1966R(-)	5′-GTCAGAAGGCAAAAACGAGAG-3′	
Inner primers		
1661F(+)	5′-GACTCTTGGACTCTCAGC-3′	261
1921R(-)	5′-TTTATACGGGTCAATGTC-3′	

Figure.1 Amplification of precore/core promoter region. Lanes 2-7 show amplified products of preC/CP region. Lanes 1 shows marker DNA

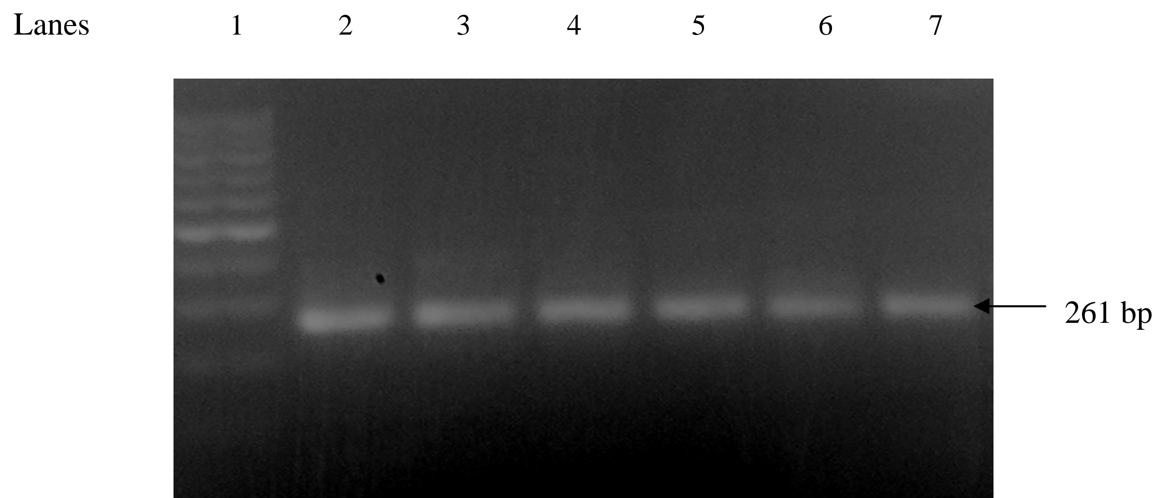

Figure.2 Comparison of nucleotide sequence (L3, L4, L29, L42, L44, P1, P3 and P8) of core promoter/precore region (1661-1921) with wild type (Accession no. - GRS08538) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion

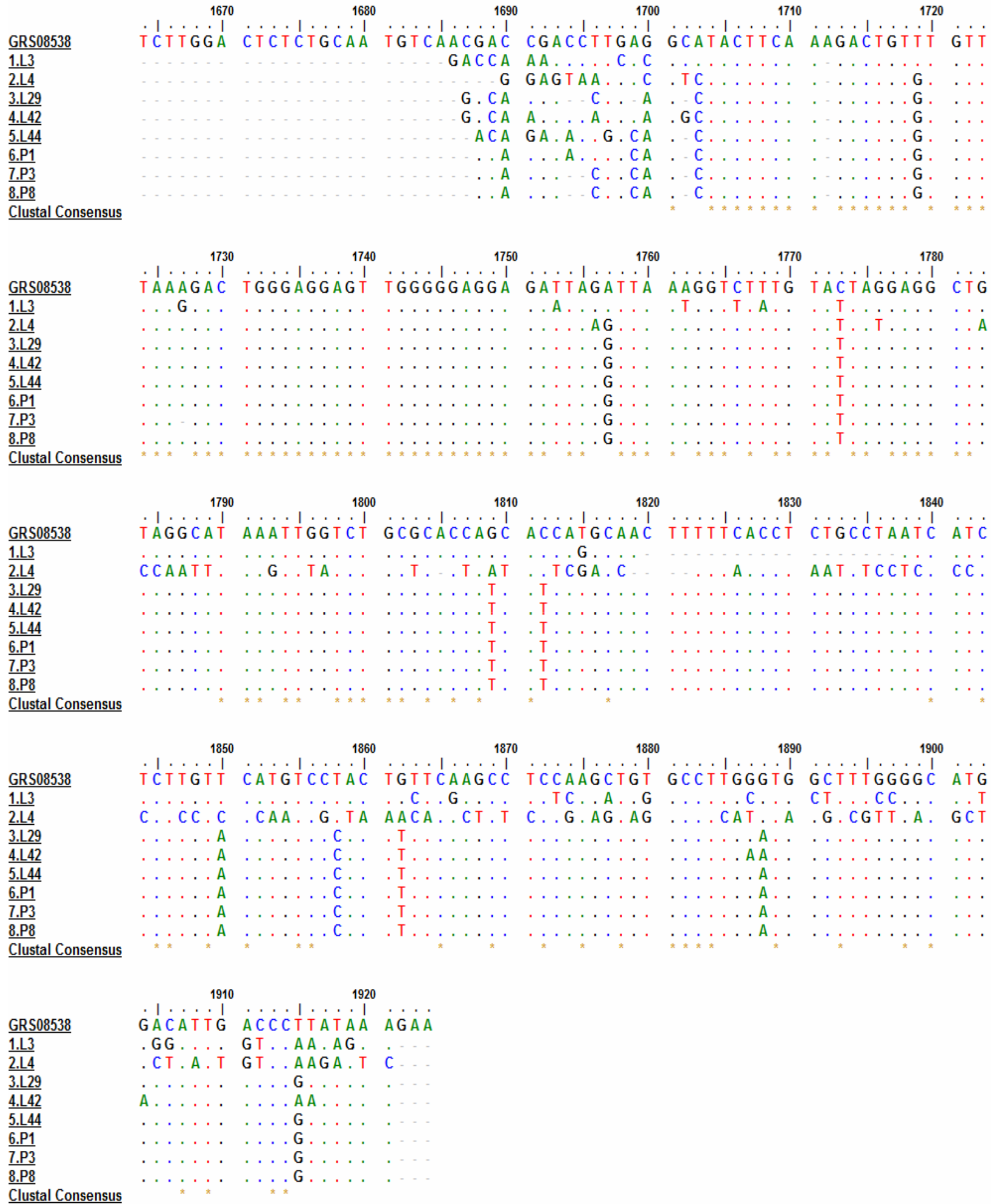

Figure.3 Comparison of nucleotide sequence (P14, P19, P22, P23, P25, P26, P27 and P30) of core promoter/precore region (1661-1921) with wild type (Accession no. - GRS08538) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion

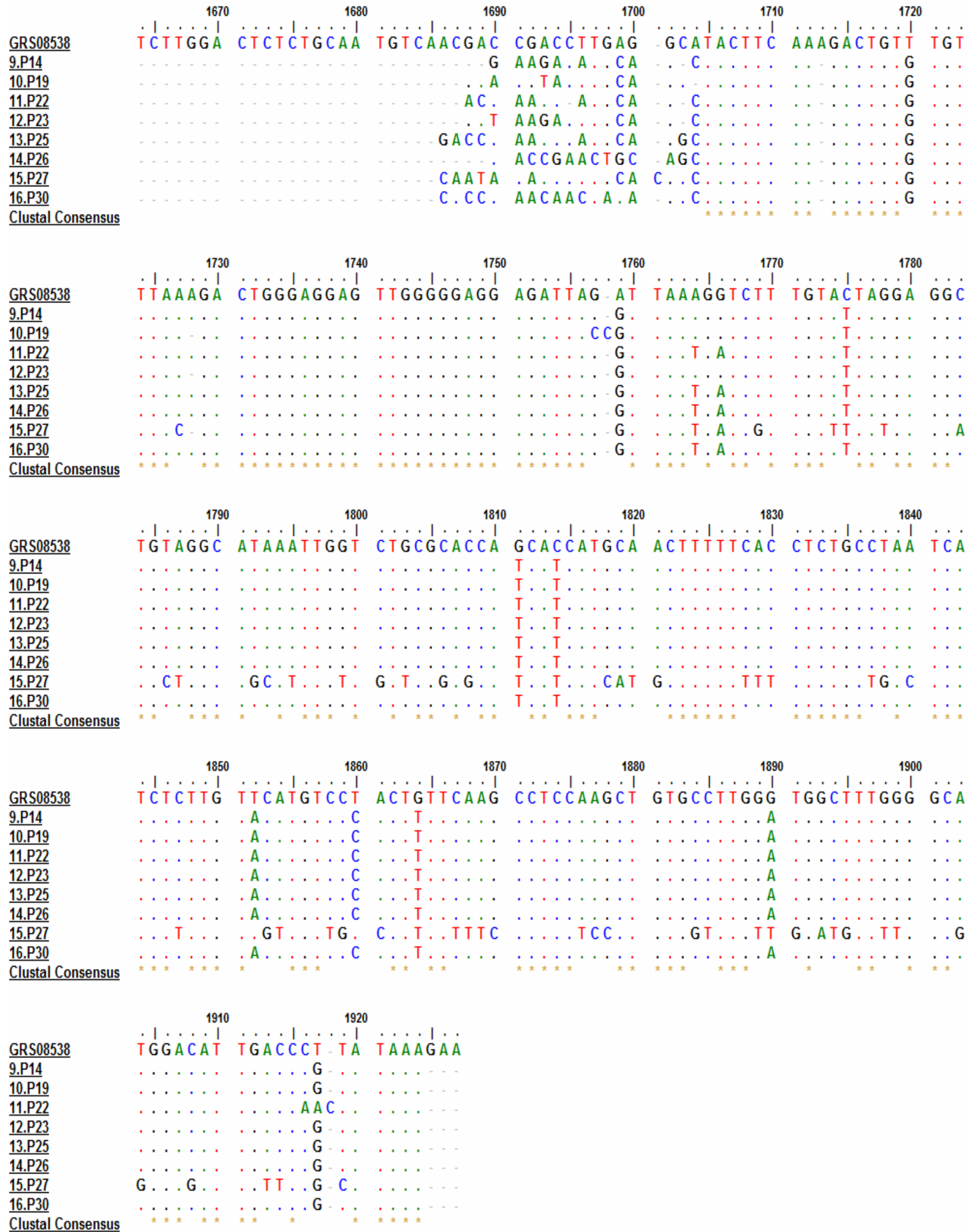

Figure.4 Comparison of nucleotide sequence (P31, P33, P35, P40, P43, P45, P64 and P72) of core promoter/precore region (1661-1921) with wild type (Accession no. - GRS08538) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion

Figure.5 Comparison of nucleotide sequence (P74, P75, P76, P77, P78, P79, P81 and M4) of core promoter/precore region (1661-1921) with wild type (Accession no. - GRS08538) using BioEdit Software. Dot (.) shows the similarity and (-) sequence shows deletion.

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                        1670       1680       1690       1700       1710       1720
                  .|....|    ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ...
GRS08538          TCTTGGA   CTCTCTGCAA  TGTCAACGAC  CGACCTTGAG  GCATACTTCA  AAGACTGTTT  GTT
25.P74            -------   ----------  -------CGA  ....T.GC..  C--.......  .-......G.  ...
26.P75            -------   ----------  ---------G  A...T.GC..  C--.......  .-......G.  ...
27.P76            -------   ----------  -------.GA  ....T-GC..  C--.......  .-......G.  ...
28.P77            -------   ----------  ------..GA  ....T-GC..  C--.......  .-......G.  ...
29.P78            -------   ----------  --------GA  ....T.GC..  C--.......  .-......G.  ...
30.P79            -------   ----------  ----------  -------AGA  C--.......  .-......G.  ...
31.P81            -------   ----------  ----------  --..T.GC..  C.C.......  .-......G.  ...
32.M4             -------   ----------  -------G.CA A...T.G-..  C--.......  .-......G.  ...
Clustal Consensus                                                  *******  *  ******  *   ***

                        1730       1740       1750       1760       1770       1780
                  .|....|    ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ...
GRS08538          TAAA-GA   CTGGGAGGAG  TTGGGGGAGG  AGATTAGA--  TTAAAGGTCT  TTGTACTAGG  AGG
25.P74            ....A..   ..........  ..........  .......G--  ..........  .....T....  ...
26.P75            ....-..   ..........  ..........  .......G--  ..........  .....T....  ...
27.P76            ....-..   ..........  ..........  .......G--  ..........  .....T....  ...
28.P77            ....-..   ..........  ..........  ......CGCG  ..........  .....T....  ...
29.P78            ....-..   ..........  ..........  .......G--  ..........  .....T....  ...
30.P79            ....-..   ..........  ..........  .......G--  ..........  .....T....  ...
31.P81            ....-..   ........G.  ..........  .......G--  ..........  .....T....  ...
32.M4             ....-..   ..........  ..........  .......G--  ..........  .....T....  ...
Clustal Consensus **   **   ******** *  **********  ******      **********  ***** ****  ***

                        1790       1800       1810       1820       1830       1840
                  .|....|    ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ...
GRS08538          CTGTAGG   CATAAATTGG  TCTGCGCACC  AGCACCATGC  AACTTTTTCA  CCTCTGCCTA  ATC
25.P74            .......   ..........  ..........  .T..T.....  ..........  ..........  ...
26.P75            .......   ..........  ..........  .T..T.....  ..........  ..........  ...
27.P76            .......   ..........  ..........  .T..T.....  ..........  ..........  ...
28.P77            .......   ..........  ..........  .T..T.....  ..........  ..........  ...
29.P78            .......   ..........  ..........  .T..T.....  ..........  ..........  ...
30.P79            .......   ..........  ..........  .T..T.....  ..........  ..........  ...
31.P81            .......   ..........  ..........  .T..T.....  ..........  ..........  ...
32.M4             .......   ..........  ..........  .T..T.....  ..........  ..........  ...
Clustal Consensus *******   **********  **********  *  ** *****  **********  **********  ***

                        1850       1860       1870       1880       1890       1900
                  .|....|    ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ...
GRS08538          ATCTCTT   GTTCATGTCC  TACTGTTCAA  GCCTCCAAGC  TGTGCCTTGG  GTGGCTTTGG  GGC
25.P74            .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
26.P75            .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
27.P76            .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
28.P77            .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
29.P78            .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
30.P79            .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
31.P81            .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
32.M4             .......   ..A.......  C...T.....  ..........  ..........  A.........  ...
Clustal Consensus *******   ** *******   *** *****  **********  **********   *********  ***

                        1910       1920
                  .|....|    ....|....|  ....|...
GRS08538          ATGGACA   TTGACCC-TT  ATAAAGAA
25.P74            .......   .......-G.  .....---
26.P75            .......   .......-G.  .....---
27.P76            .......   .......-G.  .....---
28.P77            .......   .......-G.  .....---
29.P78            .......   .......-G.  .....---
30.P79            .......   .......-G.  .....---
31.P81            .......   .......CG.  .....---
32.M4             .......   .......-G.  .....---
Clustal Consensus *******   *******   *  *****
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Competing interests

The authors declare that they have no competing interests.

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