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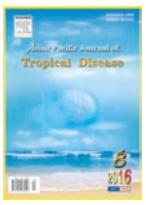

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Evaluation of the frequency of precore/core mutation in patients with chronic hepatitis B, Kerman, Southeast of Iran

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ABSTRACT

Objective: To evaluate the frequency of precore/core mutation in patients with chronic hepatitis B using PCR-restricted fragment length polymorphisms method.
Methods: Sera were obtained from 69 patients with chronic hepatitis B including 30 women (43.5%) and 39 men (56.5%). All patients were tested for the serum alanine aminotransferase, aspartate aminotransferase, the presence of hepatitis Be antigen and hepatitis B surface antigen by electrochemiluminescence and hepatitis B virus DNA load. Precore/core mutation was examined for the presence of a characteristic point mutation at nucleotides A1896G in precore and A1762T and G1764A in core region using nested PCR and restricted fragment length polymorphisms methods.
Results: From total 69 cases with chronic hepatitis B infection, 12 (17.3%) patients had precore mutation and 10 (14.4%) patients had core mutation. From 69 patients, 53 (76.8%) were negative for hepatitis Be antigen and 61 (88.4%) were positive for hepatitis B surface antigen by electrochemiluminescence method. All samples were positive for hepatitis B virus DNA by RT-PCR.
Conclusions: This study suggests that extra molecular methods should be apply for diagnosis and monitoring of mutation in chronic hepatitis B patients synchronic or serological method.

1. Introduction

Hepatitis B infection is one of inflammatory disease of the liver in the world and 350 million people suffer from chronic hepatitis B virus (HBV) infection. HBV is epidemic in Asia and Africa and also is endemic in China. However, a variable pattern has been observed in different regions of the Middle-East[1]. The prevalence of hepatitis B surface antigen (HBsAg) in Iran is between 2.5% and 7.2%; therefore, Iran is an intermediate HBsAg positive area[2]. HBV is classified in Hepadnaviridae family with linear double-stranded DNA genome. According to the variation in sequences of complete genomes, 10 HBV genotypes, A-J have been identified which show a varied geographical distribution[3]. The carrier variability rate for hepatitis B infection is estimated to be 0.1% to 20%. In Iran, HBV genotype D has the highest frequency[4]. HBV disease has been classified into acute, chronic or asymptomatic phase. As the disease progresses to chronic form, chance of the disease developing to cirrhosis and primary hepatocellular carcinoma is increasing. Transmission of disease occurs as vertical (mother to fetus) and horizontal (such as sexual contact, needle stick and intravenous drug use) ways[5,6]. There are four genes encoded by open reading frames of the HBV, called C, X, P and S that produce hepatitis B core antigen (HBcAg) and hepatitis Be antigen (HBeAg), X protein, DNA polymerase and HBsAg, respectively[7]. One of the major genes in HBV is C, which contains two regions that precore encodes 29 amino acid and core encodes 181 amino acid and these proteins produce with two initial codons, which make HBeAg as a virus replication marker[8,9]. Occurrence of mutations in core region at A1762T and G1764A (nt) can alter HBeAg production on transcription level. An another mutation may happen in nucleotide position 1896 (A1896G) [guanine substitutes with adenine] on the precore region, which creates a stop codon, therefore HBeAg can't produce or its concentration will be reduced[10]. These mutations can be found among patients with chronic hepatitis B (CHB). In these patients, the HBV DNA viral load is low, about 10^4–10^5 copies/mL, but some liver enzymes are in high level such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST)[11]. In CHB patients, HBeAg, hepatitis Be antibody and HBV DNA load should be evaluated in sera[12]. Primary analytical tool was ELISA,

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but the sensitivity of this method is 1–10 ng and it is not enough for the detection of little amount of antigen or antibody. So the use of ELISA acquires false negative results. Another diagnostic test is electrochemiluminescence (ECL), which is more sensitive than ELISA and can determine lower amount of antigen or antibody, about 0.04 ng/mL, but this method might acquire false negative results too[13]. Therefore, molecular techniques are introduced more sensitive than ELISA and ECL, and in this status, HBV DNA is assessed with PT-PCR method for definite diagnosis[14]. As well as HBV DNA detection, using PCR-restricted fragment length polymorphisms (RFLP) method can determine the evidence of the precore/core mutants in HBV. The presence of mutations in precore/core of HBV genome is important for several points of view: diagnosis, treatment and immunologic evidences[15]. Because of difficult diagnosis and treatment and prolongation of disease duration increasing immunologic responses, the risk of liver cirrhosis and hepatocellular carcinoma will be enhanced. The aim of treatment in CHB patients with HBeAg-positive is converting HBeAg level to negative and acquiring normal ALT level and HBV DNA load become negative or are lower than 2 000 IU/mL[16]. Polyethylene glycol interferon and nucleoside analogs drugs can be used for the treatment of hepatitis B, but the occurrence of mutation can cause resistance to nucleoside analogs, and HBeAg will be negative especially in long term medication[17]. Immunologically, HBcAg and HBeAg are main targets to cytotoxic T lymphocytes but in mutant strains, that HBeAg is low and HBcAg(s) on hepatocytes can be declared by cytotoxic T lymphocytes, so these lesions progress disease to crucial phase[18]. On the other hand, virus can escape from immunity system by reduction in apoptosis mechanism. In this study, the frequency of precore/core mutation was evaluated in patients who have had CHB. Diagnosis of hepatitis B is usually based on serological assays and because these methods are low sensitive in mutation subjects, we used serological methods with molecular methods in patients who have CHB in order to reach true and valid results.

2. Materials and methods

2.1. Patients

In our retrospective study, sera were taken from 69 patients including 30 women (43.5%) and 39 men (56.5%) who had CHB from October 2013 to March 2014 in our Laboratory (Virology Laboratory of the Besat Specialist Clinic, Kerman, Iran). All patients were positive for HBsAg more than 6 months. Clinical examination was done in all samples and based on patients records and serum was classified by gender and age. The serum was separated for biochemical analysis (ALT and AST), serological analysis (HBeAg). The molecular methods for HBV DNA load, nested PCR and RFLP for the detection of precore/core mutation were carried on.

2.2. Serological analysis

ECL method was performed for HBsAg and HBeAg by HBsAgII, Elecsys HBeAg and Cobas analyzers (Roche, Germany).

2.3. Molecular test

2.3.1. HBV DNA load

HBV DNA was extracted from 100 μL of plasma with RIBO-Preb kit (ILS, Russia). For quantification of HBV DNA, real time HBV kit (ILS HBV-Monitor-FRT PCR kit, Russia) was used. Quantitative determination of the amplified products was done with the Rotor-Gene 6000.

2.3.2. Amplification of the precore/core region by PCR

Nested PCR was done for all samples in two steps: in the first step, samples were examined for point mutation at nucleotide 1896 (precore) and in the second step, samples were examined for double mutation A1762T/G1764A (core). Therefore, 5 μL of the extracted DNA was mixed with 15 μL of a PCR reaction mixture (Roche, Germany), containing 10 nmol/L of the primer 1A and 1B and subjected to 35 cycles at 94 ℃ for 1 min, 55 ℃ for 1.2 min, 72 ℃ for 2 min. Also first PCR for core region was done using P1 and P2 primer in the same PCR condition for precore region. Then, nested PCR was done and 5 μL product of the first round of each PCR was mixed with 15 μL of a PCR reaction mixture (Roche, Germany), containing 10 nmol/L of the each primer (2A, 2B, 2C and 2D respectively) for precore region and P3 and P4 primers for core region (Table 1). PCR was done in 30 cycles at 94 ℃ for 1 min, 55 ℃ for 1 min and final extension at 72 ℃ for 1.5 min. The PCR product was analyzed by agarose gel (2%) electrophoresis to see the HBV DNA band under trans-illuminator (BioRad, USA).

Table 1
Sequence of primers for nested-PCR precore/core mutation.

Name	Sequence	Polarity
1A	5’-AAGGTCTTACATAAGAGGAC-3’	Forward
1B	5‘-GTACAGTAGAAGAATAAAGC-3’	Reverse
2A	5‘-ATGCAACTTTTTCACCTCTGCC-3’	Forward
2B	5’-ATACGGGTCAATGTCCAGGGCC-3’	Reverse
2C	5’-CCATGCAACTTTTTCACCTCTG-3’	Forward
2D	5’-ATACGGGTCAATGTCCAAGGCC-3’	Reverse
P1	5’GCATGGAGACCACCGTGAAC3’	Forward
P2	5’GGAAAGAAGTCAGAAGGCAA3’	Reverse
P3	5’CATAAGAGGACTCTTGGACT3’	Forward
P4	5’GGCAAAAAAGAGAGTAACTC3’	Reverse

2.3.3. RFLP

After nested PCR amplification for precore/core region, RFLP method was done on positive samples. For precore region, 5 μL of product sample with 190 bp size was mixed with 1 μL *Apa I* (3 000 IU) and 1 μL of *Stul* (1 000 IU) separately (Thermo Scientific, Lithuania) and incubated for 16 h at 37 ℃ and inactivated in 65 ℃ for 5 min. Then, products were analyzed by electrophoresis on a 3% agarose LE gel (Thermo Scientific, Lithuania). DNA fragments were visualized by Red Safe staining. If precore mutation was positive, the band sizes at 105 bp and 85 bp were determined for each sample. For core region, 5 μL of DNA product from nested-PCR with 307 bp size was mixed with 1 μL of *Sau 3A* (300 IU) (Thermo Scientific, Lithuania), and incubated for 16 h at 37 ℃ and inactivated in 65 ℃ for 5 min. Then products were analyzed by electrophoresis on a 3% agarose LE gel (Thermo Scientific, Lithuania). DNA fragments were visualized by Red Safe staining. Expected sizes for bands to be generated from the mutated HBV DNA were 197 bp and 110 bp and there was no digestion occurred in the wild type HBV DNA.

3. Results

3.1. Baseline characteristics

The baseline characteristics of infected patients with CHB was compared in Table 2. The study consisted of 69 patients including

30 (45.4%) females and 39 (56.5%) males. The youngest was 21 and oldest was 63 years old. The average age (mean ± SD) was (42.64 ± 10.40) years. Serological markers of HBV contained HBsAg that was measured for 61 (88.4%) positive and 8 (11.6%) negative. From the patients, 16 (23.2%) were positive and 53 (76.8%) were negative for HBeAg. With PT-PCR, the presence of HBV DNA was confirmed and 63.7% of samples were positive for HBV DNA (Table 2).

Table 2

Basic characteristic in patients with HBeAg-positive and HBeAg-negative.

Variables	HBeAg-negative	HBeAg-positive
Gender (male: female)	33:25	6:5
Age (mean ± SD)	43.4 ± 10.7	39.9 ± 9.2
Normal ALT (*n*, %)	18 (31.0%)	0 (0.0%)
Elevated ALT (*n*, %)	40 (68.9%)	11 (100.0%)
Normal AST (*n*, %)	15 (25.8%)	2 (18.1%)
Elevated AST (*n*, %)	43 (74.1%)	9 (81.8%)
HBV DNA load negative (*n*, %)	25 (43.1%)	0 (0.0%)
HBV DNA positive (mean ± SD)	33 (56.8%)	11 (100.0%)
HBsAg-negative (*n*, %)	8 (13.7%)	0 (0.0%)
HBsAg-positive (*n*, %)	50 (86.2%)	11 (100.0%)

3.2. The incidence rate of precore/core mutations

Nested-PCR test was performed with specific primers. The presence of precore/core mutations was determined by digestion of the PCR product with *Apa I*/*Stul* and *Sau 3A* enzymes, which cleaved the A1896G and A1762T/G1746A mutants, respectively. When amplified DNA fragments digested with *Apa I*, the wild type derived product was cleaved to a 85 bp fragment. On the other hand, precore mutant was digested to a 85 bp fragment while the wild type derived product was resistant to *Stul* enzyme. These enzymes produced two fragments of 85 bp and 20 bp from a 105 bp PCR product. In electrophoresis separated two bands (105 bp and 85 bp), the third band (20 bp) migrated to the end of the gel. Therefore, the samples were classified into four groups (precore mutant, wild type, mixed and undetectable) and in this study, their rate was 12 (17.4%), 5 (7.2%), 35 (50.7%), 1 (1.4%), respectively. Another enzyme that used for core region was *Sau 3A*. Expected sizes from the mutated HBV DNA were 197 bp and 110 bp, and there was no digestion concurred in the wild type HBV DNA. From 69 samples, 49 nested-PCR were positive which were analyzed for core mutation. Incidence rate of the mutant, wild type, mixed and undetectable cases was 10 (14.5%), 18 (26.1%), 12 (17.4%), 2 (2.9%) respectively. Therefore, there was not significant correlation between gender and precore/core mutation rate. The incidence rate of the highest precore mutation was 9 (75%) in 30–50 years old and in core mutations was 5 (50%) in same range (Table 3). There was not significant relation between age and precore (P = 0.16), and core (P = 0.93) mutations (Table 4). From 12 mutations in precore region, 11 (91.7%) cases were seen in HBsAg-positive patients (P = 0.7) and in core region, all of mutants (100%) were seen in HBsAg-positive patients (P = 0.4). In precore region, 11 (91.7%) cases were seen in HBeAg-negative patients (P = 0.3) and in core region, 6 (60%) of mutants were seen in HBsAg-negative patients (P = 0.4). Serum ALT and AST was measured and correlated with precore/core mutation rates. ALT and AST in precore mutants had no significant difference between 4 groups (wild, mutant, mixed and undetectable) (P = 0.21, P = 0.22) respectively. But mean of ALT in core mutants had significant difference between 4 groups (P = 0.006, P = 0.011). The mean ± SD of ALT in core mutants was 212.1 ± 130.5 and in mixed group was 143.5 ± 44.3. This showed that ALT level in mutant group was higher than mixed group. About AST, there was no difference between 4 groups (Figure 1).

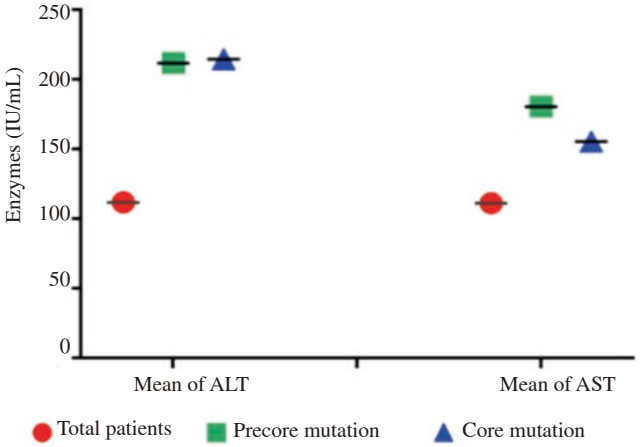

Figure 1. Comparison of mean of ALT and AST enzymes in patients with precore and core mutants.

Table 3

Distribution of sex and age group in patients with precore and core mutants.

Type of mutation		G1896A	A1762T/G1764A
Sex (male, female)		58.3%, 41.7%	70%, 30%
Age group	< 30 years	0%	10%
	30–50 years	75%	50%
	> 50 years	25%	40%

Table 4

Basic characteristic in patients with HBV DNA positive and negative.

Variables	HBV DNA load negative	HBV DNA load positive
Age (mean ± SD)	45.9 ± 9.2	40.74 ± 10.5
Gender (male: female)	15:10	24:20
Normal ALT (*n*, %)	10 (40%)	8 (18.1%)
Elevated ALT (*n*, %)	15 (60%)	36 (81.8%)
Normal AST (*n*, %)	7 (28%)	10 (22.7%)
Elevated AST (*n*, %)	18 (72%)	34 (77.2%)
HBeAg-negative (*n*, %)	25 (100%)	28 (63.6%)
HBeAg-positive (*n*, %)	0 (0%)	16 (36.3%)
HBSAg-negative (*n*, %)	6 (24%)	2 (4.5%)
HBSAg-positive (*n*, %)	19 (76%)	42 (95.4%)

We classified HBV DNA level into 4 groups that levels of HBV DNA in serum were related to the core mutation rate (P = 0.002), but in precore mutation, levels of HBV DNA had no significant correlation with HBV DNA level (P = 0.17) (Table 5). In contrast, CHB patients infected with the precore/core virus had a significant lower HBV DNA level than those infected with precore/core mutant viruses and those with precore virus had lower HBV DNA levels (P = 0.009). In this study, we determined that 91.7% of precore mutants were positive for HBsAg and negative for HBeAg. All core mutants were positive for HBsAg and 60% were negative for HBeAg. However, there was no significant correlation between these serological markers and mutations. Precore and core mutants were more frequently found in HBeAg-negative patients than in HBeAg-positive patients.

Table 5
Basic characteristic in patients with precore /core mutation.

Variables	With G1896A mutation	Without G1896A mutation	With A1762T/ G1746A mutation	Without A1762T/ G1746A mutation	Undetectable
Age (mean ± SD)	44.50 ± 9.32	40.30 ± 10.04	42.60 ± 13.50	42.06 ± 10.22	49.00 ± 7.21
Gender (male: female)	7:5	22:30	8:2	17:23	2:1
Normal ALT (*n*, %)	5 (41.6%)	8 (20.0%)	1 (10.0%)	6 (20.0%)	1 (33.3%)
Elevated ALT (*n*, %)	7 (58.3%)	32 (80.0%)	9 (90.0%)	24 (80.0%)	2 (66.6%)
Normal AST (*n*, %)	5 (41.6%)	8 (20.0%)	1 (10.0%)	8 (26.6%)	1 (33.3%)
Elevated AST (*n*, %)	7 (58.3%)	32 (80.0%)	9 (90.0%)	22 (73.3%)	2 (66.6%)
HBV DNA negative	6 (50.0%)	10 (25.0%)	1 (90.0%)	5 (16.6%)	1 (33.3%)
HBV DNA positive	6 (50.0%)	30 (75.0%)	9 (10.0%)	25 (83.3%)	2 (66.6%)
HBeAg-negative (*n*, %)	11 (91.6%)	29 (72.5%)	6 (60.0%)	23 (76.6%)	2 (66.6%)
HBeAg-positive (*n*, %)	1 (8.3%)	11 (27.5%)	4 (40.0%)	7 (23.3%)	1 (33.3%)
HBsAg-negative (*n*, %)	1 (8.3%)	4 (10.0%)	0 (0.0%)	3 (10.0%)	0 (0.0%)
HBsAg-positive (*n*, %)	11 (91.6%)	36 (90.0%)	10 (100.0%)	27 (90.0%)	3 (100.0%)

4. Discussion

HBV has high rate of mutations especially in chronic phase because viral enzyme with RNA-dependent DNA polymerase function has no proof-reading function[19]. These mutations can alter clinical course of disease and progress disease to critical conditions such as hepatic cellular cancer, cirrhosis and death[20]. Different studies showed that precore/core mutations occur in acute and chronic phase but the prevalence of them is higher in chronic phase. According to some researches, precore region forms the hairpin structure in the stem loop of encapsidation signal, where nucleotide 1896 is located opposite to nucleotide 1858 (cytosine or thymine in various genotypes). In G1896A mutation, these locations paired together and U-G substitute with U-A pairing[21]. In core mutation, A1762T/ G1746A, AGG sequences alter to TGA[22]. In this study, we found that precore and core mutation rate in CHB patients were about 14.4% and 15.9% respectively. While in 2013, Yang *et al*. reported these mutations were 90% and 10% for precore and core[23]. Sharma *et al*. demonstrated that among patients with CHB, 32% had precore mutations and 17.3% had core mutations[24]. In 9 HBeAg-negative samples, we identified that 17.3% had precore mutation and in 7 HBeAg-negative samples, 13.4% had the core mutation. Funk *et al*.[25] showed that precore mutation was 50% in HBeAg-negative patients in Asia and Kumar *et al*. reported that 64.7% were HBeAg-negative in core[26]. Another study reported that the incidence of precore and core mutations in HBeAg-negative patients were higher than HBeAg-positive[24]. Our study showed that the precore/core was more frequently found in HBeAg-negative patients more than HBeAg-positive patients. Examinations showed that HBV DNA level is very low or undetectable in chronic phase of disease[24]. Here, 81.8% of precore and 40% of core mutants had low HBV DNA level (closely 10^5 copies/mL). Our results confirmed that in chronic phase and in presence of precore/core mutations, HBV DNA level become low. Elevated ALT enzyme is also used as marker of viral hepatitis or liver damage[27-29]. Increased ALT was more than 1.5 times upper limit of normal and AST was more than 1.2 times upper limit of normal on 2 monthly determinations observed in CHB phase[30]. This study determined higher level of ALT and AST in precore/ core mutation presentation. According to our collected data, among 69 patient which were tested for HBsAg with ECL, 61 (88.4%) were positive whereas all samples (100%) were positive about HBV DNA. Then, HBeAg were tested with ECL and 16 (23.1%) were positive but all samples of HBV DNA were positive (100%) by using RT-PCR method. About 30 years ago, different immunoassay methods supplied for HBV diagnosis and had been evaluated like molecular methods until now. Serological methods have low sensitivity and in some phase of disease, serological changes are not good enough. For example, HBsAg and HBeAg are good determinants for diagnosis, but sometimes become undetectable because of the variation of their plasma levels in different phases, mutations like precore/core and low sensitivity of diagnostic methods. HBsAg is the first serological marker that appears within 4–10 weeks after infection[27-29,31,32]. The disappearance of HBsAg occurs in clearance phase by immunity system. In precore/core mutations, HBeAg production stops or decreases into undetectable level and because of low sensitivity in ECL (0.1 IU/mL) methods, there is a deficiency for diagnosis. It has been shown that the tests which are negative for HBsAg by ECL, are positive with PCR[33]. Another research was done in Iran, among 63 samples, 100% were positive by PCR but 90.5% were positive with ECL for HBsAg detection. In our study, two diagnostic methods were used: ECL and RT-PCR for definitive HBV diagnosis. We found that the ECL method is more sensitive than ELISA for diagnosis and ECL comparing with ELISA has the degree of imprecision about 5.0% and 14.9% respectively[34]. RT-PCR is sensitive enough for HBV DNA detection which means that RT-PCR is definitive diagnostic method that gains accurate results even in absence serological marker.

Prominent prevalence of precore/core mutation in middle-aged adult group was reported in our study and we reported low prevalence in geriatric and pediatric groups. Our result supported by another studies showed a high prevalence of this mutation among middle-aged patients[15,35,36].

In conclusion, an interme-diate prevalence of precore/core mutation among patients with CHB was reported in Kerman Province. A significant association between precore/core mutations and its role in causing HBeAg-negativity were evaluated. However, our results suggested a significant relation of precore/core and patients with CHB and liver cirrhosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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