Original Article

Investigation of Hepatitis B Virus X Gene Mutations in Patients with and without Cirrhosis/Hepatocellular Carcinoma

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Background: Chronic HBV (CH) infection and its consequences including cirrhosis (C) and Hepatocellular Carcinoma (HCC) still represent a major Global health. The relationship between HCC and various mutations of HBx gene has been reported. In the present study, we aimed to determine the sequence variation of HBx gene in patients with Chronic HBV infection or C/HCC.

Materials and Methods : In this cross-sectional study, 15 patients with HBV chronic infection and 13 with C/HCC were included. After viral DNA extraction using commercial kit HBX gene was amplified using an in-house nested-PCR. Then, bi-directional sequencing was performed on the PCR product. The data resulting from sequencing were aligned with reference HBV sequence to identify the mutations.

Results : The mean age of CH and C/HCC groups was 38.23±12.46 and 50.67±14.22 years old, respectively. We found 43 and 20 Amino acid substitutions inside the region of 88–154 from HBx protein in CH and C/HCC groups, respectively. In addition, K130M+V131I mutation was found in 13.34% (2/15) and 30.7% (4/13) of patients in the CH and C/HCC groups, respectively (P=0.36). Furthermore, 10 deletion mutations were observed in both groups with no significant difference (P=0.8).

Conclusion : The results of the present study indicated the relatively high frequency of Amino acid substitutions and deletion, especially in part of region 88-154 from HBx Protein in patients with CH and C/HCC. The findings should be considered in a larger population.

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Key words **: Hepatitis B virus, HBx, Mutations, Cirrhosis, HCC.**

Despite availability of an effective Vaccine against Hepatitis B Virus (HBV), about 220 million chronic HBV (CH) infected patients are at risk of the sequel disease including Cirrhosis (C) and Hepatocellular carcinoma (HCC)[1]. Up to 10 genotypes of HBV have been identified with their own geographic territory; among them, the dominant genotype is D in Iran[2,3].

Hepatocarcinogenesis of HBV is mediated by three factors including Chronic inflammation, Integration of viral DNA in the host genome and Oncoproteins encoded by HBV[4]. Among seven polypeptides encoded by HBV, protein X plays a critical role in Carcinogenesis[5]. The 16-kD HBx protein is composed of 154 Amino acids and manipulates the transcription of cellular and viral genes, signal transduction pathways and Protein degradation; also, it controls the cell cycle and apoptosis[6]. Protein X is encoded by a gene with 462 nucleotides spanning the overlapping pre-core/core promoter, enhancer II, DR1, and DR2. Therefore, mutation inside the X region not only affects its own functionality, but also might influence other related sequences[7]. In addition, several critical cis-elements such as microRNA-binding region, EnhII and the core promoter exist in HBx protein. It has been suggested that mutations which occur naturally in various parts of X gene could be associated with different Hepatitis disease statuses[8]. The reported mutations of X gene could change the function of wild Protein on activation of NF-KB pathway, apoptosis process, P53 interaction and induction of potent responses of T cells[9]. Moreover, these variations affect viral propagation through affecting

Editor's Comment :

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- The results of the present study indicated the relatively high frequency of amino acid substitutions and deletion, especially in amino acids part of 88-154 from HBx protein in patients with CH and C/HCC.

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regulatory sequences of the genome which directs the disease toward Cirrhosis and HCC[10,11]. Moreover, deletions and insertions in C-terminal region of HBx can be related to clinical output and disease severity in patients suffering from Chronic Hepatitis. Therefore, these mutations which might be related to C/HCC can be used as biomarkers to predict disease progression[12]. The present study was conducted to investigate the sequence variations of HBx gene in chronic HBV infection and C/HCC patients.

Materials and Methods

Patient's Selection :

In this study, 47 subjects including those with Chronic Hepatitis (CH) as well as C/HCC were enrolled consecutively from Gastroentrohepatology Unit at Mottahari Clinic and Liver Transplant Research Centers at referral Abu-Ali Sina Hospital, affiliated to Shiraz University of Medical Sciences, from 2013 to 2016. All the enrolled patients had Chronic HBV infection and were divided into CH and C/HCC groups by a Liver Specialist according to Biochemical, Virological and clinical records based on EASL guidelines[13]. Demographic and Clinical data of patients were collected using Medical Records. The CH group consisted of chronic patients who were positive for HBsAg and positive/negative for HBeAg. The C/HCC patients were enrolled based on Ultrasound Scanning, histology grading, Abnormal Liver Function Tests, and α-fetoprotein levels. All the patients were negative for HCV, HDV and HIV. Based on the sequencing of preS1, S2 and S region of these samples, all patients were infected with HBV genotype D[14,15]. Written consent was obtained from each patient before sampling and the study was approved by the Ethics Committee of Shiraz University of Medical Sciences. Five milliliter of venous blood without anti-coagulation was taken from each participant. The sera were separated by centrifuge and stored at -20°C until used.

Viral DNA Extraction and HBx Gene Amplification:

HBV DNA was extracted from 200 µL of each Serum samples by viral DNA extraction kit (Cinnagen Inc. Tehran, Iran), according to the manufacturer*'s instructions.* A nested PCR assay was performed using specific outer and inner primers (Table 2). Primers design was carried out with NCBI homepage primer designing software based on genomic sequences of B, C and D HBV genotypes. Due to overlapping of gene X with precore/core promoter, enhancer II, DR1 and DR2, the primers were designed in such a way that they amplified both X gene and these overlapping regions.

In the amplification stage, each PCR mixture at the first round contained 0.5 pmol of each outer primer, 5µL of extracted DNA, 1.5 mM MgCl2, 1U Taq DNA polymerase (CinnagenInc, Tehran, Iran) and 200 mM of each dNTPs (25 µL total volume) and PCR condition: 95°C for 5 min, 28 cycles of 94°C for 35 seconds, 58°C for 45, 72°C for 40 seconds and 72°C for 3 minutes. The second round PCR was performed in a similar amount of PCR mixture and also cycling time parameters with set 2 of primers but the number of cycles was 35 and annealing temperature was 56°C.

Sequencing and Multiple Sequences Alignment :

After purifying the PCR products from the gel electrophoresis by using PCR Product Purification Kit (MN Inc, Germany), bi-directional sequencing was performed using nested internal primers. The results from sequencing were aligned with a group of reference genomic sequences of HBV from data bank by using MEGA7 software to identify the mutations. Every difference between reference sequences and PCR products was considered as mutation.

Statistical Analysis :

Epi Info™ software and SPSS 22 were used for statistical analysis. Chi square test was used for analysis of the mutation data and P value <0.05 was considered significant. Age, ALT, AST were expressed as Mean ± SD.

Results

In this study, 15 out of 23 CH samples and 13 out of 24C/HCC samples had acceptable quality of sequencing data. The mean age of the participants was 38.23±12.46 and 50.67±14.22 years in the CH and C/HCC groups, respectively. Totally, 12 patients (80%) in the CH and 12 (92.3%) in C/HCC groups were male and the rest in each group were female. The level of ALT (P=0.016) and AST (P=0.002) and age (P=0.029) were significantly higher in the C/HCC group than the CH group. Demographic and clinical data for the patients in both groups are shown in Table 1.

In the case of detected mutations, in total, 53 and 25 substitutions were detected in the X Protein sequence of CH and C/HCC groups, respectively. However, no significant difference was determined. Also, 43 and 20 Amino acid substitutions were found in Amino acids sequence 88–154 of HBx Protein in the CH and C/HCC groups, respectively. Moreover, 13.34% (2/15) of the patients in the CH group and 30.7% (4/13) of those in the C/HCC group had K130M+V131I double mutation (p=0.36). Additionally, we also showed K130N (n=2), K130Y (n=1) and K130C (n=1), V131L (n=3) mutations in the CH group and K130Q (n=1) and V131L

Table1 — *Demographic and clinical data of the study groups*

Characteristics	CH group (N=15)	C/HCC group (N=13)	P value
Gender :			
Male	12(80%)	12 (92.3%)	-
Female	3(20%)	1 (7.7%)	
Mean Age±SD	38.23±12.46	50.67±14.22	0.029
ALT*±SD	47.08±43.93	119.2±98.73	0.016
AST*±SD	31.58±21.29	130.9±106	0.002

ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; CH: Chronic Hepatitis; C/HCC: Cirrhosis/Hepatocellar Carcinoma

(n=2) mutations in the C/HCC group. Furthermore, H94Y(3), I127N(n=1)/T(n=2)/F(n=1), F132Y(n=3) mutations in the CH group, H94Y(n=2), I127L/T/S(5) and F132Y(n=1) mutations in the C/HCC group were observed. Interestingly, 10 deletion mutations were determined in each group albeit no statistically significant difference was seen. These mutations included single deletion at position 135 in the CH group and 101 in the C/HCC group. Moreover, there were some partial deletions at the 48-72 and the C-termini of X Protein region; they included Del47-72(1), Del77-end (1), Del130-133(3), Del 129-131(1), Del 130-132(1) in the CH group and Del47-72(2), Del77-end (2), Del76-end (1), Del47-72(1), Del74-152(1), Del74-149 and Del130-133(2) in the C/HCC group. However, based on the deleted residues and blocks, any special difference was extrapolated when comparing the two groups (Table 3).

Discussion

HCC ranks the sixth among the most prevalent cancers, represents the third cancer-related death across the world and is mainly caused by HBV[16]. Mutation in HBx, especially in the COOH-terminal region, has been suggested to direct the disease progression toward HCC. Thus, investigation of HBx mutations can lead to confirming the predictors of end-stage Liver disease by HBV.

Amino acids from 52-65 and 88-154 in HBx protein have a key role in the transactivation, transcription and replication of the HBV genome[17]. In this study, we detected 43 and 20 Amino acid substitutions in 88-154 region of HBx Protein in the CH and C/HCC groups, respectively. In addition, R56L(1), S65P(1) and Del47-72(3) mutations were found in 52-65 region of HBx in the C/HCC group and L58H(1) and Del47-72(1) Amino acid substitutions were found in the CH group. Mani, *et al* reported H52Y and S64T from patients suffering from Chronic Liver Disease. In addition, they observed T36A mutation in 4 participants with Chronic Liver Disease[18].

Moreover, 13.34% (2/15) of patients

Table 3 — *The frequency of HBx variations in the CH and C/HCC groups*

CH(n=13)	C/HCC(n=15)	*P value*
Substitution mutation		
M1T(1)	-	0.48
C6G (1)	-	0.48
P11H(1),	-	0.48
A12T(1),	-	0.48
D14N(1),	-	0.48
H30Y(1),	-	0.48
S31P(1),	-	0.48
S38A(2), H30Y(1),	S38A(1),	0.29
S41Y(2),	S41Y(1),	0.47
-	R56L(1),	0.55
L58H(1),	-	0.48
-	S65P(1)	0.55
-	R72C(1)	0.55
R78S(1),	-	0.48
-	Q87P(1)	0.55
F88L(1), F88S(1),	F88S(2), F88L(1)	0.59
H94Y(2),	H94Y(2)	0.65
R96W(1),	-	0.48
L98I(1),	-	0.48
G107D(1),	-	0.48
F112V(2)	F112V(1)	0.47
D114Q(1),	-	0.48
E121G(4)	E121G(1)	0.33
E122D(1),	E122D(1),	0.72
I127N(1), I127T(2), I127F(1),	I127L(1), I127T(3), I127S(1)	0.61
R128I(1),	-	0.48
K130N(2), K130Y(1), K130M(2), K130C(1),	K130M(5), K130Q(1)	0.83
V131L(3), V131I(1)	V131I(4), V131L(2)	0.72
F132Y(3),	F132Y(1)	0.29
V133I(1)	-	0.48
G135E(1),	-	0.48
C137Y(1),	-	0.48
	R138T(1)	0.55
H139A(1), H139D(1),	-	0.24
K140M(1),	-	0.48
A144P(2),	A144T(2)	0.65
A146E(1), A146P(1), A146T(1),	-	0.12
P147L(2)	-	0.24
C148W(2)	C148W(1)	0.47
N149H(2)	-	0.24
T152I(1)	-	0.48
-	I283L(1)	0.55
Deletion Mutation		
Del47-72(1), Del77-end (1), Del 101(2), Del 130-133(3), Del 129-131(1), Del 130-132(1), Del 135(1)	Del 47-72(2), Del 77-end (2), Del 76-end (1), Del47-72(1), Del 74-152(1), Del74-149, Del 101(1), Del 130-133(2)	0.8

CH: Chronic Hepatitis; C/HCC: Cirrhosis/Hepatocellar Carcinoma

Table 2 — *The sequences of primers used in the nested PCR*

	Sequence	Position	Product Size
HBx Forward 1	5'-CGATCCATACTGCGGAACT-3'	1262-1946	685 bp
HBx Reverse 1	5'-GTAACTCCACAGWAGCTCCA-3'		
HBx Forward 2	5'-GCTTGYTTTGCTCGCAG-3'	1288-1886	599 bp
HBx Reverse 2	5'-CAAGGCACAGCTTGGAG-3'		

in the CH group and 30.7% (4/13) in the C/HCC group had K130M+V131I double mutation. Additionally, we found K130Y/N/C/L (n=7) mutations in the CH group and K130Q/L(n=3) in the C/HCC group. In the same line, Mani, *et al* showed that 27% of patients in different stages of HBV infection had (K130M+V131I) double mutation[18]. Double mutation (K130M+V131I) affects the cell cycle regulation, DNA repairing mechanism, and HBeAg expression[19]. In this regard, Shi, *et al* for the first time reported HBx10-144 double mutation that may be involved in progression toward HCC[20]. 130 and 131 sites in HBx overlap with A1762T and G1764A sites in the basal core promoter, which are common substitutions in HCC[21]. Liu, *et al* reported that K130M/V131I mutation promoted transcription activity of hypoxia-inducible factor-1 (HIF-1) which is enhanced in human tumors[22]. It is believed that the interaction of HIF-1 and HBx is caused by formation of a stronger secondary structure in HBx as a result of this double mutation[22].

In spite of mutations in the D and E domain including H94Y(3), I127N/T/F(n=4) and F132Y(n=3) in the CH group and H94Y(n=2), I127L/T/S(n=5), and F132Y(n=1) in the C/HCC group, there was not any correlation between the groups and mutations. D and E functional domains in HBx Protein are associated with nuclear transactivation, signal transduction as mutations in these domains may be modulating its transactivation property[21]. It has been reported that H94Y, I127T, K130M, V131I and F132Y/I/R mutations that are located in the D and E domains might be related to modulation of HBx transactivation property[21]. In addition, I127T+K130M+V131I triple mutation was reported with progression of Liver Disease[21].

This study also demonstrated that 10 small and partial deletions of HBx Protein in both groups separately. There were some deletions in the C-termini of X protein region including Del 47-72(1), Del 77-end (1), Del 130-133(3), Del 129-131(1) and Del 130-132(1) in the CH group and Del 47-72(2), Del 77-end (2), Del 76-end (1), Del 47-72(1), Del 74-152(1), Del 74-149 and Del 130-133(2) in the C/HCC group. Deletion in the COOH-terminal of HBx is a frequent event in Hepatocellular Carcinoma[23]. Some studies have reported X gene containing different deletions in the COOH-terminal region of HCC patients[24,25]. Al-Anazi *et al* reported that there was evidence for an effect of deletion mutation in HBx on cell cycle regulators[24]. They showed that HBx-WT enhanced modulation of p21, p27 and cyclin D1, whereas truncated forms of HBx (61-124) inhibited p53 expression significantly. Similarly, truncated forms including HBx (1-94) and HBx (61-154) suppressed the expression of PARP and Bax efficiently. Fu, *et al* reported that HBx-d382 deletion mutant (128-145aa) enhanced the cell proliferation[26]. The author found out that C-terminal truncations and deletion mutations, in contrast, attenuated the HBx ability to promote transcription activity of HIF-1[22]. In the present study, we did not find any difference between the frequencies of deletion mutations in C-terminal of HBx in C/HCC patients in comparison with CH patients that may be related to sample size. Salarnia, *et al* reported that deletion and insertion mutations in C-terminal of HBx were more frequent in cirrhotic patients compared to chronic HBV patients[27]. Recently, it was reported that C-terminal truncated HBx by downregulating TXNIP initiated hepato-carcinogenesis[28].

Moreover Li, *et al* reported that HCC-related mutations mainly resided in the HBx transactivation domain, immune epitopes, viral promoter and protein/miRNA binding sites[29]. In B cell epitope region including aa36, aa44 and aa50, we did not detect any mutation. Xie et al. reported that T36P/S/A mutation in the B cell epitope was not significantly higher in HCC than non-HCC patients infected with genotype A/C/D. Muroyama, *et al* and Cho, *et al* reported that A44V and G50R were significantly higher in the HCC group than non-HCC (genotype A/D). Also, in aa118 and aa 123 related BH3-like motif, core promoter and EnhII, NRE region we did not detect any mutation; However, in aa127 related BH3-like motif, core promoter and NRE regions we reported 4 and 5 mutations in CH and C/HCC, respectively. Fan, *et al* reported L123S and a silent mutation in aa118 that were significantly higher in the HCC group than non-HCC ones (genotype D1)

Mutations have various biological outcomes, and based on the mutation type, they play different roles in the HBV infection outcome. Moreover, some of these mutations can be used as prognosis and predict the outcome of the disease. Relatively small sample size as well as lack of available data regarding the viral replication parameters could be the limitations of this study.

Conclusion

The present study showed that amino acid substitutions in the HBV-HBx gene, especially in amino acids 88-154, are frequent in patients with CH and C/HCC. These mutations might be related to HBV-associated liver injury and progression of infection. However, a more detailed study on a larger population of HBV-infected patients is recommended to confirm this claim.

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Conflicts of interest : All the authors declared that there is no conflict of interest.

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