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Hepatitis B virus genotype in Iranian patients with hepatocellular carcinoma

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KEYWORDS
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Summary
Objective: Chronic hepatitis B virus (HBV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC). HBV appears to be the most common cause of HCC in Iran. To date, no study has been carried out on the HBV genotype in Iranian HCC patients. This study was undertaken to determine the HBV genotype in Iranian patients with HCC.
Methods: Paraffin-embedded tissue samples from 40 patients (31 males and nine females) with HBV-associated HCC were collected from different pathology centers during 2000—2007. Genotyping of HBV was performed by nested PCR-mediated amplification of the target sequence. PCR products were sequenced, and the genotype of each HBV sequence was determined by comparison with sequences of known genotypes in the GenBank. A phylogenetic tree was constructed.
Results: Phylogenetic analysis revealed that all of the HBV isolates were clustered in genotype D.
Conclusions: Our results concur with other reports from Iran, all showing that genotype D is the only detectable genotype in the different clinical forms of HBV infection in this country.
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Introduction

Hepatitis B virus (HBV) infection is a serious global health problem, with two billion people infected worldwide and 350

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million suffering from chronic HBV infection. Of these, 75% are Asians.[1] Hepatitis B infection is the tenth leading cause of death worldwide, and results in 500 000 to 1.2 million deaths per year caused by chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[2]

HCC represents approximately 6% of all new cancer cases diagnosed worldwide, with more than half of these occurring in China alone. Relatively high incidence rates are also found in Southeast Asia and in sub-Saharan Africa. One of the least curable malignancies, HCC is the third most frequent cause of cancer deaths among men worldwide.[3]

In 1979, Farzadegan et al. reported the prevalence of hepatitis B surface antigen (HBsAg) in Iran to be between 2.5% and 7.2%.[4] A more recent nationwide study showed that the prevalence of HBsAg ranges from 1.7% to over 5% in the different provinces.[5] Although the true prevalence of HCC in Iran is unknown, it is not an uncommon malignancy; 80% of HCC cases in Iran are positive for at least one of the markers of HBV, and this virus appears to be the most common cause of HCC in Iran.[5,6]

HBV genotypes represent naturally occurring strains of HBV that have evolved over the years and reflect the geographical distribution of HBV throughout the world.[7] To date, eight different HBV genotypes have been identified and shown to cluster in different areas of the world.[7,8] Genotype A is pandemic, but most prevalent in northwest Europe, North America, and Central Africa; in Europe, this genotype is more frequent in chronic infection than genotype D.[9] In Japan, HBV genotype B causes a less severe liver disease compared to HBV genotype C, and is not detected in patients with HCC who are less than 30 years old, unlike in other parts of Asia.[10] Genotype C is found in East Asia, Korea, China, Japan, Polynesia, and Vietnam.[11] This genotype causes a more severe liver disease than genotype B in these regions.[12] Genotype D is also more or less pandemic, but is predominant in the Mediterranean area and the Middle East, expanding into India. Genotype E is typical for Africa and genotype F is found in Native American Polynesians.[13] Genotype G, which has a slightly longer genome than other HBV genotypes, has been found in the USA and France. Genotype H has been found in Nicaragua, Mexico, and California. This genotype is the most closely related to genotype F.[14–16]

Some studies from Iran have reported that genotype D is the only detectable genotype in the different clinical forms of HBV infection, including HBsAg carriers, chronic hepatitis B patients, and cirrhotic patients.[17–21] To our knowledge, no study has been carried out to date on the HBV genotype in Iranian HCC patients. Accordingly, the objective of this study was to identify the HBV genotype in Iranian patients with HCC.

Patients and methods

Study population

Formalin-fixed and paraffin-embedded tissue samples from 40 patients (31 males and nine females) with a documented tissue diagnosis of HCC (surgically resected material or biopsy) were provided for analysis. The samples were collected from different pathology centers throughout Iran during 2000—2007. The ethics committee of the Pasteur Institute of Iran approved the study. Hospital records were used to verify age, sex, and virological items such as HBsAg, HIV type 1 (HIV-1) antibody, and hepatitis C virus (HCV) antibody. Patients with positive HBsAg were enrolled in the study. Patients, who were co-infected with HIV-1 or HCV, were excluded.

DNA extraction

Sections 5—10 μm wide were prepared from each specimen, avoiding any cross-contamination between samples (using separate disposable items such as gloves, blades and tubes; most importantly the first section of each specimen plus gloves and blade were discarded and a new blade and gloves were used for main sectioning). Sections were subsequently deparaffinized with xylene and digested using digestion buffer containing proteinase K, followed by extensive extraction with phenol/chloroform.[22] The extracted DNA was stored at 4 °C until tested. DNA quality was evaluated by PCR using primers PCO3/PCO4 that amplify a 110-bp product from the human β-globin gene.[23] β-Globin-positive samples were subjected to nested PCR.

Nested PCR

The most conserved regions of S gene sequences were amplified by nested PCR, using primers to amplify the sequence between nt 203 and nt 787, yielding an amplicon of 585 bp. The outer primers were PrsS2 (Forward, nt 2820—2837, 5′-GGGACACCATATTCTTGG) and S1R (reverse, nt 842—821, 5′-TTAGGGTTTAAATGTATACCCA). The inner primers were YS1 (forward, nt 203—221, 5′-GCGGGGTTTTTCTTGTTGA) and YS2 (reverse, nt 787—767, 5′-GGGACTCAAGATGTTGTACAG).[24] Ten microliters of extracted DNA were added to an amplification mixture containing 5 μl of 10× PCR buffer, 1.5 μl of $MgCl_2$ (50 mM), 1 μl of dNTP mix (100 mM each), 1 μl (2.5 U) of Taq polymerase (CinnaGen, Tehran, Iran), and 2 μl of each of the outer primers (10 pmol) in a total volume of 50 μl. The PCR profile was an initial 3 min denaturation at 94 °C, followed by 35 cycles of amplification including denaturation for 45 s at 94 °C, annealing for 60 s at 53 °C, and extension for 90 s at 72 °C. Strand synthesis was completed at 72 °C for 6 min. One microliter of the first-round PCR products was then subjected to second-round PCR under the same conditions but with the inner primers.

DNA sequencing, genotyping, and phylogenetic analysis

The second-round PCR products were purified using a PCR product purification kit (Qiagen, Hilden, Germany), and then sequenced directly at the DNA Sequencing Lab (Kawsar Biotech Co., Tehran, Iran).

A total of 44 reference sequences of HBV genotypes A to H isolates were obtained from GenBank and were used for comparison with the sequences of the isolates in this study. The accession numbers and countries of these sequences are as follows: genotype A, **AB014370** from Japan, **AB222707** from Uzbekistan, **AF297621** from South Africa, **EU410082** from Philippines, **AB194951** from Cameroon; genotype B, **AB073838** from Japan, **AF121243** from Sweden, **M54923**

from Indonesia, AY167098 from Taiwan, X97850 from the UK; genotype C, AB014393 from Japan, AB222714 from Uzbekistan, X75665 from Sweden, X75656 from Polynesia, AB105172 from Hawaii, EU439009 from China; genotype D, AF043593 from Germany, Z35716 from Poland, X80924 from the UK, X65257 from Italy, AB033558 from Japan, AB222709 from Uzbekistan, AF280817 from China, AF121240 from Sweden, X02496 from Switzerland, AY661793 from Turkey, DQ412319 from the Netherlands, AB263407 from Mongolia, AF121239 from Vietnam,

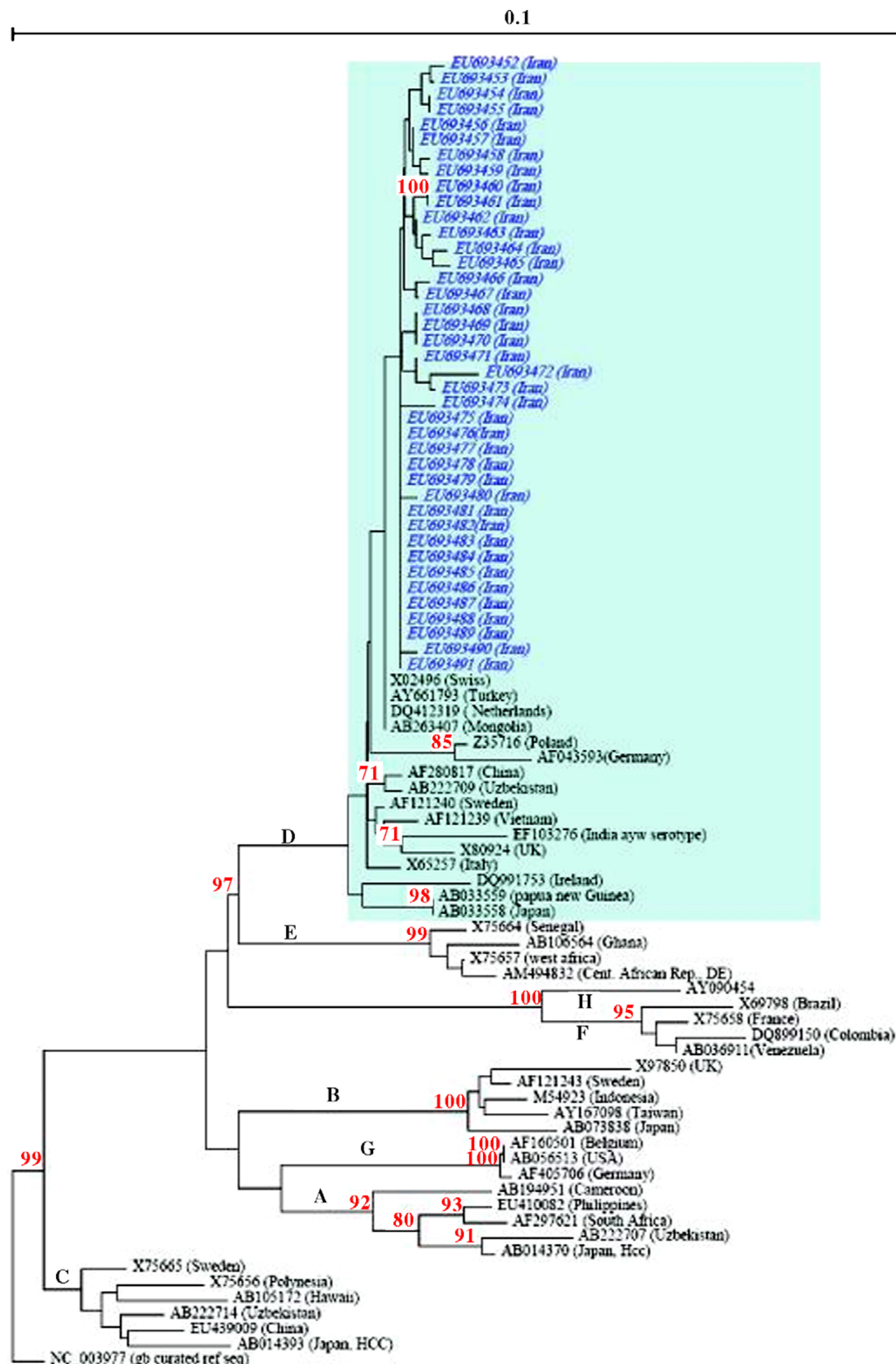

Figure 1 Phylogenetic tree constructed using a Kimura two-parameter matrix and the neighbor-joining method. The numbers represent the percentages of bootstrap replicates (of 1000 totals) for the node. Iranian sequences determined in this study are indicated by '(Iran)' suffix. The letters A—H designate HBV genotypes.

EF103276 from India, DQ991753 from Ireland, AB033559 from Papua New Guinea; genotype E, X75664 from Senegal, AB106564 from Ghana, X75657 from West Africa, AM494832 from Central African Republic; genotype F, AB036911 from Venezuela, X69798 from Brazil, X75658 from France, DQ899150 from Columbia; genotype G, AF405706 from Germany, AF160501 from Belgium, AB056513 from the USA; genotype H, AY090454 from Sweden.

Nucleotide sequences were aligned with CLUSTAL W program using BioEdit software (BioEdit Sequence Alignment Editor software, Department of Microbiology, North California State University) and confirmed by visual inspection. Genetic distance was estimated using the Kimura two-parameter matrix.[25] A phylogenetic tree was constructed by the neighbor-joining (NJ) method.[26] Bootstrap resampling and reconstruction were carried out 1000 times to confirm the reliability of the phylogenetic tree.[27] The analyses and calculated nucleotide differences within and between the isolate sequences were carried out by MEGA program, version 4.[28]

The nucleotide sequences of Iranian HBV isolates reported in this article can be found in the GenBank database under accession numbers EU693452 through EU693491.

Results

A total of 40 patients with HBV-associated HCC were enrolled in this study. Thirty-one (77.5%) of them were male and nine (22.5%) were female. The mean age of the patients at diagnosis of HCC was 59.25 years (range 18—87 years).

Phylogenetic analysis results show the Iranian isolates clustered in the genotype D branch with other genotype D HBV reference genes (Figure 1).

Discussion

Hepatitis B genotyping has received immense attention recently and its clinical implications are being investigated extensively throughout the world. This is the first study on HBV genotype in Iranian patients with HCC. This study shows genotype D as the only detectable genotype in Iranian HCC patients.

It has been demonstrated that HBV genotype can affect the clinical outcome of chronic HBV infection.[29] For instance, genotype D of HBV is associated with more severe liver disease than genotype A and may lead to HCC in young patients.[30,31] It has also been reported that genotype D of HBV has a lower response rate to lamivudine in comparison with genotype A of HBV.[32] Therefore, an understanding of HBV genotype distribution seems to be of great importance for the better management of HBV infection by medical personnel and for the improvement of public health.

The relationship between HBV genotype and HCC is inconclusive. In a study by Kao et al.[33] in HCC patients older than 50 years, genotype C was the most prevalent, whereas in patients younger than 50 years, genotype B was the most common. These results are partly in conflict with those of Orito et al.[10] from Japanese patients, where genotype B-infected patients with HCC were older than HCC patients with genotype C. Another study conducted by Sumi and colleagues reported that although genotype C in patients was associated with a slower development of HCC than genotype B, there was no difference in the life-time risk of development of HCC.[34] Chan et al. reported that HBV genotype C, particularly subgenotype Ce, increased the risk of HCC in chronic hepatitis B patients.[35] Genotype D was associated with more severe liver disease than genotype A in India.[31] Baig et al.[36] showed that in Pakistan, HBV genotype D was most prevalent among HCC patients. Genotype D was also the most prevalent genotype in reports from other parts of the Middle East, like Saudi Arabia, Yemen, and Turkey.[37—39]

The first report of HBV genotyping of 26 HBV isolates from Iranian chronically HBV-infected individuals revealed that HBV genotype D is dominant in Iran.[17] Another study by same scholar in 147 HBV-infected patients also revealed that HBV genotype D is prominent in Iran.[20]

In a study by Alavian et al.,[18] HBV genotypes were determined in 109 HBsAg-positive patients including 95 (86.4%) with chronic hepatitis, 11 (10%) with liver cirrhosis, and three (2.7%) inactive carriers by using INNO-LiPA methodology, which is based on the reverse hybridization principle. Genotype D was the only detected type found in all patients. Mojiri et al.[21] also reported genotype D as the only type of HBV found in different clinical forms of HBV infections, including cirrhosis, among the residents of southwest Iran.

Our study shows genotype D as the only detectable genotype in Iranian patients with HBV-associated HCC. Because all of the patients had genotype D, no correlation could be observed between different genotypes and epidemiological or clinical factors. Our results concur with other reports from Iran,[17—21] all showing that genotype D is the only detectable genotype in the different clinical forms of HBV infection in this country. This unique distribution of HBV-D infection may be related to geographical location as well as ethnic background.

In conclusion this is the first study on HBV genotype in Iranian HCC patients, and reveals that HBV genotype D is the only detectable genotype in these patients.

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Conflict of interest: No conflict of interest to declare.

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