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Original Article

Lack of Mutation in the Hot Spot Region of the Human P53 Gene in a Number of Iranian Hepatocellular Carcinoma Patients

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ABSTRACT

Background and Objectives: **Mutation directed inactivation of the tumor suppressor gene p53 have been found in countries with high frequency for hepatocellular carcinomas (HCCs). Our goal in the present study was screening of the p53 gene in tumor tissues from HCC affected individuals in southwest Iran for putative mutations in exons 7 and 8 that are known as hot spot regions.**

Materials & Methods**: A total of 25 archival formalin fixed paraffin embedded samples prepared from 1997 to 2006 were collected from hospitals in southwest and northwest of Iran. We examined the codon 249 within the exon 7 using RFLP as well the full-length sequencing of exons 7 and 8.**

Results: **The samples were diagnosed as HCC and classified in four groups, well differentiated (39%), moderately differentiated (54%), poorly differentiated (4.5%) and undifferentiated (2.5%). The hepatitis B virus (HBV) was detected in 16% (n=7) and 11% (n=5) of patient's sera that were affected with liver cirrhosis. No patient was infected with hepatitis C virus. However, in one tumor sample a homozygote mutation was detected at codon 302.**

Conclusion: **In contrast to the numerous reports, particularly from Asian countries, the selected HCC patients in this study show a very low mutation rate in the hot spot regions of the p53 gene. However, further mutation studies have to be conducted for the whole length of the p53 gene for more evaluation.**

Key words: p53 Gene, Hepatocellular carcinoma, Aflatoxin, Liver Cirrhosis

Introduction

The HCC is the leading cause of cancer-related death in the East Asia and counts for the fifth common cancer worldwide (1, 2). The major risk factors for the HCC includes chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, to which 80% of the HCC cases are attributed worldwide (3). Other known risk factors, such as aflatoxin (AFB1), dietary exposure or heavy alcohol consumption, metabolic disorders such as hemochromatosis and .1 antitrypsin deficiency are consider to be involved with the HCC outcome, but they also synergize with each other and

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with chronic viral hepatitis. Although the HCC can develop without preexisting cirrhosis, it occurs in over 80% on the grounds of this condition. Regardless of its etiology, cirrhosis is an independent risk factor of HCC development (4, 5). While activation of the known proto-oncogenes does not seem to play an important role (6), frequent allelic loss on specific chromosomal arms indicates that dysfunction of numerous tumor suppressor may be involved in the development of HCC (7). Among these, the p53 gene, located on the chromosome 17pl3, plays a major role in the regulation of the cell division, and its mutations have been found in many human cancers including the HCC with up to 50% (8, 9).

The prevalence of p53 mutations in HCCs varies significantly between different geographic areas. The p53 mutations have been found in 41% of HCCs from Egypt, 56% from Mozambique, 67% from Senegal and as few as none in HCCs from Australia (10). In certain areas with high dietary exposure to aflatoxin B1 (AFB1), HCC affected patients show high frequency of specific G>T transversion at codon 249 in the p53 gene (11). However, the p53 mutations in HCC patients are detected throughout exons 5-8, especially exons 7 and 8, which are counting to the highly conserved and the most studied exons of the p53 gene (12). Herein, for the first time we analyzed the spectrum of p53 mutations in the tumor tissues of the individuals suffering HCC in Iran, a region with a remarkably high-population prevalence of chronic HBV infection (13).

Materials and Methods

Sample collection

Twenty five cases of hepatocellular carcinoma liver specimens were retrieved from the hospitals' archives from 1997 to 2006 in southwest and northwest of Iran. All of the HCC specimens were fixed in 10% buffered formalin and embedded in paraffin wax, which were stained with haematoxylin and eosin. HCC specimens were graded from I to IV according to Edmondson and Steiner>s classification. As maintained by patient's medical history, the HCC affected individuals were 23 to 80 yr old (median, 44 yr) that had been tested by ELISA for HBV and HCV infection; seven patients (16%) were infected with hepatitis B and 5 (11%) possessed liver cirrhosis but none of patients were infected with hepatitis C. Adjacent non-tumorous liver parenchyma was examined for the presence of cirrhosis.

DNA extraction

One important point for successful DNA extraction from paraffin-embedded tissues is the optimized deparaffinization. DNA extraction from non-tamponated formalin fixed tissue may lead to unsatisfied results, such as DNA degradation, as observed in 19 of 44 archived samples. Genomic DNA was extracted from formalin fixed paraffin embedded samples using QiAamp kit (Qiagen Company, USA) according to the manufacture s protocol. Twenty five samples with good quality were chosen for subsequent steps. The other 20 samples were removed from the study, because of low quality and low concentration. The average of DNA concentration from 25 selected samples was, as has been measured by spectrophotometer, 120 ng/ μl.

PCR-Restriction Fragment Length Polymorphism:

Primers for the amplification of the exons 7 and 8 of the P53 gene were selected as previously reported (14). The sequences of used primers were: 5-“CTTGCCACAGGTCTCCCCAA-3“(forward), 5-“ AGGGGTCAGCG CAAGCAGA-3“ (reveres) for the exon 7 and 5“-TTGGGAGTAGATGGAGCCT-3“ (forward), and 5“-AGTGTTAGACTGGAAACTTT-3“(reverse) for the exon 8. Each PCR reaction was performed in 25 μl reaction mixture containing 5 μl of template DNA, 0.5 μl each primer, 0.5 μl dNTP, 0.75 μl $MgCl_2$ (50mM) 0.5 unit Taq polymerase (Fermentase, Denmark), 2.5 μl 10X PCR buffer, betaine with end concentration of 1M (Sigma, Germany) and 5.35 μl ddH2O. The PCR profile for exon 7 was 94 for 3 min, and 35 cycles of 94 for 30 s, 61.5 for 30 s, 72 for 30sec, and final extension at 72 for 5 min, and for exon 8, 35 cycles of 94 for 30 s, 55 for 45 s, 72 for 1 s, and final extension at 72 C for 5 min (Bio-Rad iCycler, Europe, England).

One μl of PCR product was diluted with ratio 1:8 for exon 7 and 1:4 for exon 8 that has been picked up as template with the reverse internal primers of: 5-GTGCAGGGTGGCAAGTCGC-3 and 5-TTCCTTACTGCCTCTTGCTT-3 for exon7 and exon 8 respectively. The amplification condition was similar as has been mentioned above and resulted to products of 176bp length for exon 7 and 231bp for exon 8.

The 176bp of purified DNA fragment of exon 7 of P53 gene was submitted to restriction enzyme HaeIII (Fermentase, Denmark). Digestion was carried out using 0.2 μl HaeIII (10unit s/ μl), 2 μl 10X buffer B, 7

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+PCR Product 10.8 +ddH2O (20 +total volume). This reaction was continued at 37 water incubation for 16 hours. Enzyme HaeIII cleaves 176bp purified DNA product at GG/CC sequence at codon 249-250, generating 115bp and 61bp fragments.

Direct sequencing:

PCR products were direct sequenced with ABI-PRISM-3700 DNA analyzer (Applied Biosystems). The reactions were performed according to the manufacture's instruction with 4 big dyes and the same forward primers as described above. Finally, the obtained sequences were aligned to the p53 gene sequence by NCBI-Blast program (www.ncbi.org).

Results

Twenty five samples were subjected for mutation analysis. Fragments from RFLP analysis indicated no point mutation in codon 249^{ser} (Fig. 1). In the next following step, all the 25 samples were subjected for direct sequencing of exons 7 and 8 of the p53 gene. In just one sample, a homozygote mutation was detected in a patient's tumor tissue with poorly differentiated HCC. This unreported new mutation at codon 302 leads to conversion of prolin (CCC) to leucine (CTC) with unknown consequence (Fig. 2). All the other patients were negative for mutation in the mentioned exons of the p53 gene (Fig. 3).

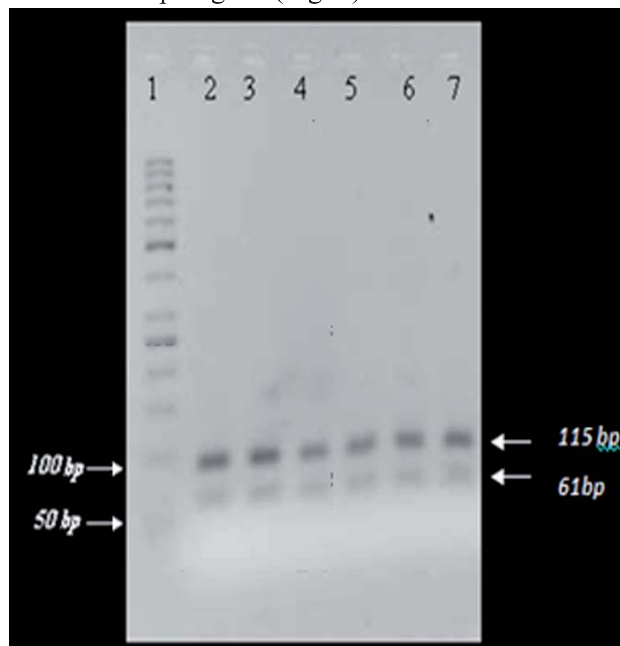

Fig. 1: Two percent agarose gel shows PCR products generated from DNA of some HCC patients (2 to 7) after digestion. The 50 bp DNA size marker (Fermentase, Denmark) was loaded on the gel to the length estimation of fragments. As we show here, all the samples were cut with the enzyme HaeIII indicating wild sequence in the cutting site (codon 249).

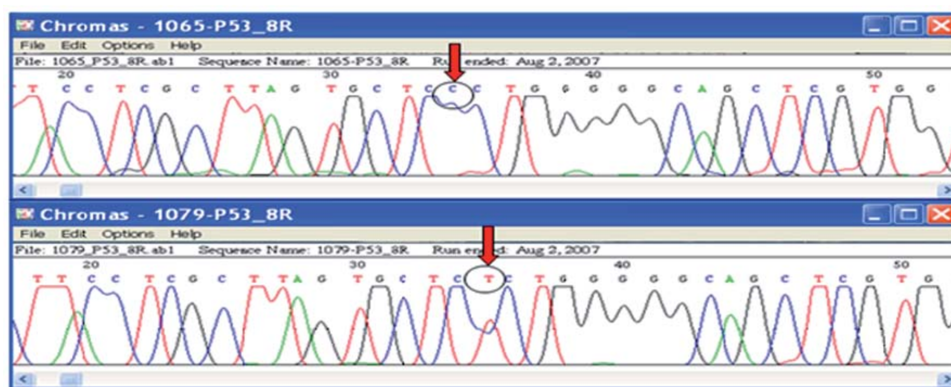

Fig. 2: Partial sequence of the p53 gene from a sample (upper) without mutation and a sample with mutation in codon 302 (lower) indicating homozygote C>T conversion

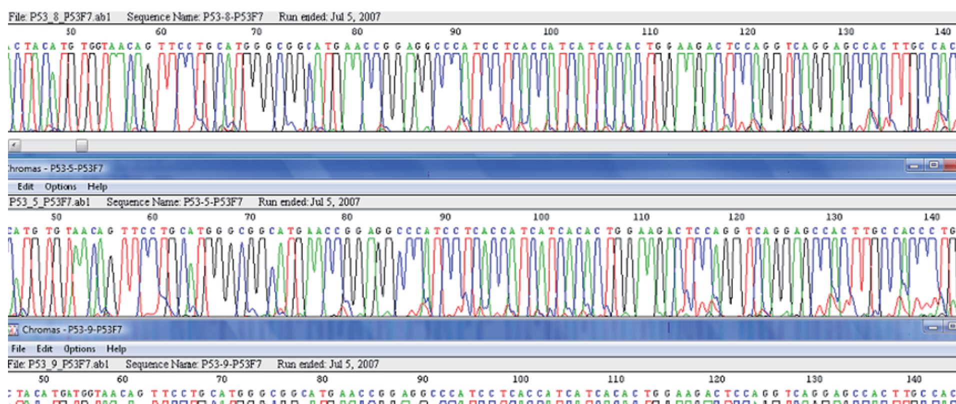

Fig. 3: Direct sequencing profile of 3 samples that were negative for mutation in exon 7 of the p53 gene

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Discussion

Mutations in the p53 have been identified in majority of human cancers (15), but locations of mutations in HCC are not identical in different geographic regions (16). The most striking example of specific mutation in the p53 gene is a G to T transversion in the third base of codon 249. This mutation has been detected in 10%-70% of HCC patients from areas with a high exposure to AFB1 (10) but not in HCC affected patients in regions with negligible exposure to AFB1 (14). However, we could not observe any alterations in exon 7 especially at codon 249 of the p53 gene. Our results are consistent with the low prevalence of mutation in geographic areas, such as developed countries with low-level exposure to the AFB1 (17, 18). Due to the co-existence of the mutation at codon 249 and the AFB1 contamination, other factors than aflatoxin B1 may be responsible in HCC abnormalities in the present study. Alternatively, the individuals that were subjected here, may possess an over expression of the glutathione S transferase M1 (GSTM1) enzyme, which is known to be involve in the aflatoxin metabolism that leads to the neutralization of the AFB1 (17). Moreover, in our study, an unreported missense mutation was observed in exon 8 from a HCC patient with grade IV tumor. The pathogenicity of the detected mutation needs though to be approved by more evaluations. Commonly, the deamination of cytosine to thymine leads to C>T conversion. Increased level of nitric oxide and/or inhibition of a DNA repair enzyme in the hepatocytes may cause such DNA deamination (8, 11). Probably, mutations in the p53 occur in the last genetic event in carcinogenesis of the liver (13). It has been reported that domains IV and V of the p53 are particular conspicuous in poorly differentiated HCC in respect to wide distribution in well and moderately differentiated HCC (13).

Conclusion

Overall finding suggests that the HCC patients show less mutation in the hot spot regions of the p53 gene as has been expected, at least in conducted patients in this report. The locations of mutations within the p53 tumor suppressor gene differ in HCCs from different geographical regions (14). We suggest also searching other areas of the p53 gene for putative mutations to better understanding the undertaken mechanisms involving the etiology of HCC and to define the 'homemade' or private hotspot region within the p53 gene, particularly from Iran.

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