

An Investigation of Point Mutations at 7th Exon of Gene P53 in Hepatocellular Carcinoma Patients in Kermanshah Province and the Study of Mutation in Liver Specimens of Mice Exposed to Aflatoxin B₁

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Abstract: Hepatocellular Carcinoma (HCC) is the fifth most common malignancy in the world and the third most common cause of cancer related death worldwide. Incidence of HCC is different in various parts of the world depending on gender, location, dietary aflatoxin exposure and chronic hepatitis B and C infections. The point mutation in p53 gene, 7 exon, 249 codon (AGG→AGT, which leads to the substitution of the remaining arginine to serine) has the highest frequency in patients affected with HCC. Among factors associated with HCC, AFB₁ is considered to be one of the most important causative agents in the formation of HCC in regions with HBV infection and AFB₁ exposed dietary. The aim of this study, is to investigate the status and carcinogenic role of p53 gene, 7 exon and point mutation in HCC affected patients in Kermanshah and the AFB₁ treated mice. Twenty five Formalin-Fixed Paraffin-Embedded (FFPT) tissues related to HCC patients were collected from pathology centers, which were diagnosed using histological methods. Moreover, 16 liver specimens of mice exposed to AF were gathered. Extracted DNA from these cases was amplified by PCR with specific primers. Furthermore to analyze the point mutation of p53 exon 7 in human HCC samples and mice liver specimens, PCR-RFLP and PCR-SSCP were applied. According to the RFLP results, for human samples that were cleaved by restriction enzyme, HaeIII (which cuts GG CC within codon 249 of exon 7) no point mutations were found and all samples were cleaved by this enzyme. The PCR-SSCP results showed two mutations in other codons of exon 7. Also, SSCP for mice PCR products showed that there are clear differences between control specimen and AFB₁ treated samples. This indicated that in addition to the previously mentioned nucleotide mutation, mutation has also occurred in other nucleotides of exon 7. The results of this study indicate that there are not enough evidences to prove the correlation between AFB₁ and HCC index in Kermanshah. However, finding mutation in other codons may suggest the contribution of other risk factors rather than exposure to aflatoxin B₁ to the incidence of HCC. By performing statistical studies, no significant correlation was observed between p53 mutation and histological grade, tumor stage, sex and age. However, there is a direct relationship between these mutations and cirrhosis. PCR results of extracted DNA from the liver of mice exposed to aflatoxin B₁ showed that the used dosage of aflatoxin B₁ in this study is carcinogenesis.

Key words: Hepatocellular carcinoma, aflatoxin, Kermanshah

INTRODUCTION

Hepatocellular Carcinoma (HCC, also called malignant hepatoma) is a primary malignancy that arises from hepatocytes (the major cell type in the liver). HCC is the second leading cause of cancer death in many regions of Asia (Motola-Kuba *et al.*, 2006; Colombo, 2005; Villa and Lo, 2007; Bu *et al.*, 2007). Its incidence peaks between the age 50-70 years and it is more common in men. The development of HCC is a multiple process and several factors are involved. Its risk factors include cirrhosis,

chronic hepatitis, carcinogens and error of metabolism (Motola-Kuba *et al.*, 2006; Llovet and Beaugrand, 2003; Montesano *et al.*, 1997). HCC most commonly appears in a patient with chronic viral hepatitis (hepatitis B or C, 20%) or with cirrhosis (about 80%) (Villa and Lo, 2007; Thomas and Andrew, 2005).

The p53 tumor suppressor gene is the most frequently mutated gene in several human cancers (at least 50% of all human cancers) (Lu *et al.*, 2001; Diller *et al.*, 1990; Kern *et al.*, 1992). The p53 proteins are highly conserved, which also reflects their significance

for the function of the p53 protein and mutations in the conserved regions, which span exon 5-8, have been identified in numerous human cancers (Tanaka *et al.*, 1993). Recent researches have stated that p53 exon 7 (codon 249) is a hotspot for point mutation in HCC and most of the point mutations are G→T transversion at the third base of codon 249 (AGG→AGT) and other are G→C transversion (AGG→AGC) (Lu *et al.*, 2001; Tam *et al.*, 1999; Huang *et al.*, 2003; Liu *et al.*, 2002; Kirk *et al.*, 2000).

Aflatoxins are toxic fungal metabolites produced by *Aspergillus* species. Aflatoxin B₁ (AFB₁) is a member of a family of difuranocoumarins produced by *Aspergillus flavus* and related fungi and is the most potent natural hepatotoxin and carcinogenic of the aflatoxins and is an established risk factor for hepatocarcinogenesis in human (Tam *et al.*, 1999; Liu *et al.*, 2002). The liver can also be the target of AFB₁ where ingestion of crops and animal feed is common (Tam *et al.*, 1999; Lee *et al.*, 1998; Bedard *et al.*, 2005). AFB₁ is biotransformed by cytochromes P450, lipoxygenase and prostaglandin H synthase to highly reactive AFB₁-8, 9-*exo*-epoxide, which changes to AFB₁-N⁷-Gua and AFB₁-FAPY, a toxic products that includes a G to T mutation of the p53 at codon 249 up-regulating growth factor II that leads to a reduction of apoptosis and HCC formation (Smela *et al.*, 2001).

The aim of the present study, was to use a rapid and simple method for detecting these mutations in clinical isolates and in treated mouse liver with AFB₁. The Polymerase Chain Reaction (PCR) using degenerate oligonucleotide primers have made it possible to amplify gene more quickly and easily. Single-Strand Confirmation Polymorphism (SSCP) is a developed method for detecting mutations that relies upon the ability of one or more nucleotide changes to alter the electrophoretic mobility of single-strand DNA molecules under nondenaturation conditions (Xie *et al.*, 2002; Hayashi, 1991). Because of technical simplicity and relatively high sensitivity, SSCP has become one of the most popular strategies for detecting genetic variations and mutations.

MATERIALS AND METHODS

Tumor samples: Tissue specimen used in the study was paraffin embedded and collected from Kermanshah hospitals. Thirty samples of liver cancer were collected

and were stained with HE and examined under microscope. Finally, 25 samples were identified containing HCC. Furthermore, mouse liver spacemen were collected from Razi Institute (Karaj-Iran). From 16 samples, which were collected, 6 samples were used as control and 10 samples were isolated from AFB₁ treated mouse.

DNA extraction: In this study, human DNA was extracted by phenol-chloroform method. DNA was extracted from 1-4 sections (10 µm) of paraffin-embedded tissue blocks with xylene, ethanol and phenol-chloroform method and dissolved in 50 µL of distilled water (Greer *et al.*, 1994). Also, mouse DNA was extracted from frizzed liver samples. For DNA extraction, frizzed liver sample was homogenized with NET buffer (1:8 w v⁻¹) and then was treated with 50 mg mL⁻¹ RNase and 0.2% SDS. Furthermore, phenol-chloroform method was used and the provided DNA was dissolved in 50 µL of distilled water. The concentration of DNA was determined using spectrophotometer.

PCR: The exon 7 of human p53 gene was amplified using the following primers (Table 1). For PCR, a volume of 10 µL of the DNA template solution was added to 41.5 µL reaction mixture containing 25 µL dd H₂O, 5 µL 10X PCR buffer, 3 µL 50 mM MgCl₂, 2 µL 10 mM dNTP, 2 µL each primer, 1.5 µL betain and 1 µL 5 U µL⁻¹ taq polymerase enzyme. Amplification was carried out in UK thermocycler (Model FTGRAD₂D) with temperature program consisting of initial denaturation (5 min at 94°C), 30 amplification cycles (1 min at 94°C, 1 min at 60°C and 1 min at 72°C) and the final extension (10 min at 72°C).

Also, PCR reaction was carried out for exon7 of mouse p53 gene. For this purpose, a volume of 6 µL of DNA template solution was added to 44 µL reaction mixture containing 33 µL ddH₂O, 5 µL 10X PCR buffer, 2 µL MgCl₂, 1 µL dNTP, 1 µL each primer and 1 µL Taq polymerase. Then amplification was carried out with temperature program consisting of initial denaturation (1 min at 94°C), 30 amplification cycles (30 sec at 94°C, 1 min at 69°C, 1 min at 71°C) and the final extension (5 min at 70°C). The amplification products were visualized by staining with ethidium bromide, after electrophoresis on 1.7% agarose gel (Sambrook, 2001).

RFLP: PCR products, which were derived from exon7 of p53 gene were digested by HaeIII (BsuRI) (with 4 bp

Table 1: Primers for human and mouse exon7 of p53 genes analysis

Gene	Primer name	Primer sequence	Ref.
Human p53 exon7	GW-XI-1C	CTTGCCACAGGTCTCCCCAA3"5	(Liu <i>et al.</i> , 2002)
	GW-XI-1D	5'TGTGCAGGGTGGCAAGTGCC3'	(Liu <i>et al.</i> , 2002)
Mouse p53 exon7	MP7-56F	5'CAGGTACCTGTAGTGAGGTAGGG3'	(Tam <i>et al.</i> , 1999)
	MP7-56R	5'TGGAACAGAAACAGGCAGAAAGCTG3'	(Tam <i>et al.</i> , 1999)

recognition sites) restriction enzyme. The restriction enzyme digestion reaction system was as follows: 1 μ L HaeIII, 2 μ L 10 \times PCR buffer, 5 μ L DNA sample, 12 μ L ddH₂O (20 μ L total volume). This reaction system was incubated at 37°C for 4 h and checked with 4% agarose and 10% polyacrylamid gel electrophoresis.

PCR-SSCP: PCR products were directly subjected to silver staining SSCP analysis according to the method of (Peng *et al.*, 1995). Five micro litter of PCR products were denatured in 30 μ L of 98% formamid, 10 mmol mL⁻¹ NaOH, 20 mmol L⁻¹ EDTA, 0.05% (w v⁻¹) bromophenol blue and 0.05% (w v⁻¹) xylene cyanol, at 98°C for 5 min. The samples were immediately located on an 8% polyacrylamide gel and run at 1.25 V cm⁻¹ in 1 \times TBE in 4°C for approximately 3 h. After electrophoresis, the gels were fixed and stained with silver.

RESULTS

PCR products: Total DNA was extracted from paraffin embedded tissue blocks and frizzed samples. The PCR reaction was performed by mentioned primers for human and mouse p 53 genes, respectively. After PCR products were resolved on a 1.7% agarose gel, single band of 196 and 256 bp was observed for human and mouse p53 genes, respectively and no band was observed in the control having no template DNA (Fig. 1-2).

RFLP: The 196 bp DNA fragment, which was derived from exon7 of p53 gene, was submitted to restriction

enzyme HaeIII digestion. Enzyme HaeIII was cleaved a GG/CC sequence at codons 249-250, generate 99, 66 bp and 2 small fragments (12 and 30 bp) from 196 bp PCR product. It is necessary to mention that 12 and 30 bp fragments are small fragments and can not be detected in 4% agarose gel. Also, the 12 bp fragment (due to its exceptionally small length) can not be detected by polyacrylamid gel (Fig. 3).

PCR-SSCP: In order to study other probable mutations, SSCP technique in the polyacrylamid gel was used. The results showed that 2 samples (No. 1 and 5) have different banding patterns in comparison to those of a healthy person (Fig. 4). At the next step of study, the mice, which

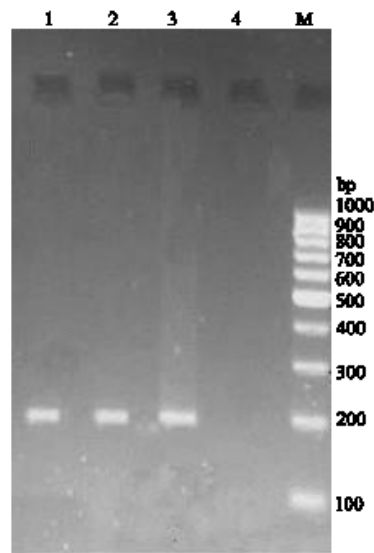


Fig. 1: The PCR products of p53 exon7 for human HCC samples. Lane 1-3: PCR products (196 bp). Lane 4: negative control. M referred to 1000 bp marker

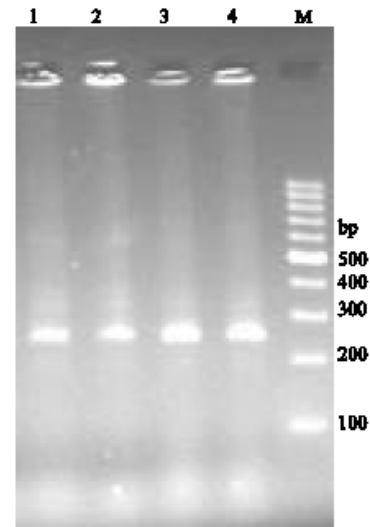


Fig. 2: The PCR products of p53 exon7 for AFB₁ treated mice. Lane 1-4: PCR products (256 bp). M referred to 1000 bp marker

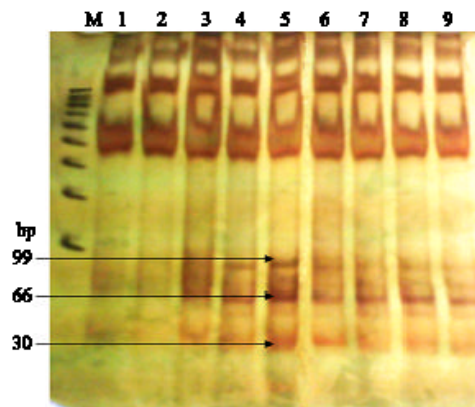


Fig. 3: PCR-RFLP of p53 exon7. M referred to 100 bp marker. Lane 2 is PCR product samples. Lane 1 and 3-9 are digested PCR products.

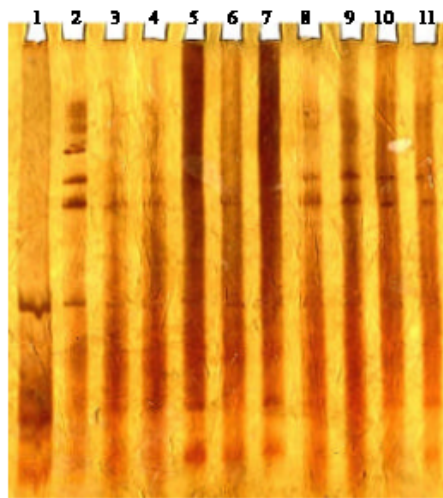


Fig. 4: PCR-SSCP of p53 exon7 for human HCC samples. Lane 1 is dsDNA. Lane 2 and 6 have point mutations of p53 exon7. Lane 3, 4, 5, 7, 8, 9, 10 and 11 do not have point mutations of p53 exon7



Fig. 5: PCR-SSCP of p53 exon7 for AFB1 treated mice. Lane 5 and 8 are dsDNA and control, respectively. Lane 1, 2, 3, 4, 6, 7 and 9 are AFB₁ treated samples

were treated by AFB₁ were studied using PCR-SSCP. According to Fig. 5, results showed that there are clearly differences between control and treated samples and furthermore electrophoretic pattern for all aflatoxin treated samples differ in comparison to the control. Also, the banding pattern can differ in aflatoxin treated samples. In Fig. 5, 2 samples can be observed, which differ from other aflatoxin treated samples. This indicates that in addition to the mentioned nucleotide mutation, mutation also occurs in other nucleotide of exon7.

DISCUSSION

Hepatocellular Carcinoma (HCC) is the fifth most common and the third most deadly cancer worldwide. More than half a million cases are identified and about a similar number die of the disease each year. HCC is closely associated with chronic liver disease and as many as 80% of cases occur in cirrhotic liver (Bu *et al.*, 2007; Thomas and Andrew, 2005).

The tumor suppressor gene p53 is found to be involved in the carcinogenesis of diverse types of cancer. The p53 gene is mutated in at least 50% of human cancers, including most tumor types (Lu *et al.*, 2001). Some domains of p53 protein are highly conserved, which also reflect their significance for its functions. The exons that encode the domains are hotspots for point mutation. If point mutations occur in these sites, the peptides that are translated from these templates will affect the correct folding of the p53 protein. Hence, the cell cycle suppression function of p53 will be affected, which will result in the loss of all proliferation control (Kern *et al.*, 1992; Tanaka *et al.*, 1993; Huang *et al.*, 2003). Investigation has found that p53 exon7 (codon 249) is a hotspot for point mutation in Hepatocellular Carcinoma (HCC) and most of the point mutations are G→T changes at the third base of codon 249 (Argenin→Serine) and others are G→C (Argenin→Serine). Point mutations of codon 249 will lead to the loss at the HaeIII restriction site in the tumors genomic DNA (GGCC→GTCC) (Llovet and Beaugrand, 2003; Tanaka *et al.*, 1993; Lee *et al.*, 1998; Garcia *et al.*, 2000). Therefore, PCR-RFLP should be an expedient and convenient method to detect the point mutation of p53 codon 249 (Lu *et al.*, 2001; Tam *et al.*, 1999; Huang *et al.*, 2003; Liu *et al.*, 2002; Lee *et al.*, 1998).

AFB₁, present in foods contaminated by certain *Aspergillus* sp., is a well-known carcinogen, which induces G:C→T:A transversion mutation (Chan *et al.*, 2003). The high frequency of G→C transversion mutations at codon 249 of p53 gene in high AFB₁ regions is in agreement with the mutation specificity of AFB₁ (Kirk *et al.*, 2000).

In this study, according to the RFLP results, point mutation were not found in p53 exon7 and all samples were cleaved with HaeIII restriction enzyme. Therefore, in order to detect other mutations in this exon, PCR-SSCP at polyacrylamide gel was used. The PCR-SSCP results indicated that, only two samples differ from control samples. This indicated that in two samples another mutation occurred, which was the result of

HCC. The PCR-SSCP for mice PCR products showed that there are clear differences between control and aflatoxin treated samples. In addition, electrophoretic pattern for all aflatoxin treated samples differ in comparison to the control. Also, in two samples banding pattern differed with the control. These indicated in addition to nucleotide mutation, mutations also occur in other nucleotide of exon 7.

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