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Abstract

Polymorphisms of the p53 gene, which participates in DNA repair, can affect the functioning of the p53 protein. The Arg and Pro variants in p53 codon 72 were shown to have different regulation properties of p53-dependent DNA repair target genes that can affect various levels of cytogenetic aberrations in chronic hepatitis B patients. The present study aimed to examine the frequency of chromosomal aberrations and the mitotic index in patients with chronic hepatitis B and their possible association with p53 gene exon 4 codon 72 Arg72Pro (Ex4+119 G>C; rs1042522) polymorphism. Fifty-eight patients with chronic hepatitis B and 30 healthy individuals were genotyped in terms of the p53 gene codon 72 Arg72Pro polymorphism by PCR-RFLP. A 72-h cell culture was performed on the same individuals and evaluated in terms of chromosomal aberrations and mitotic index. A high frequency of chromosomal aberrations and low mitotic index were detected in the patient group compared to the control group. A higher frequency of chromosomal aberrations was detected in both the patient and the control groups with a homozygous proline genotype (13 patients, 3 control subjects) compared to patients and controls with other genotypes [Arg/Pro (38 patients, 20 control subjects) and Arg/Arg (7 patients, 7 control subjects)]. We observed an increased frequency of cytogenetic aberrations in patients with chronic hepatitis B. In addition, a higher frequency of cytogenetic aberrations was observed in p53 variants having the homozygous proline genotype compared to variants having other genotypes both in patients and healthy individuals.

Key words: Hepatitis B; Chromosomal aberrations; Mitotic index; p53 gene codon 72 polymorphism

Introduction

Hepatitis B virus (HBV) is the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) worldwide and ranks 10th among leading causes of death (1 million deaths/year) (1,2). Chronic HBV infection is characterized by a necroinflammatory process involving inflammation and liver regeneration (3,4). Hepatocarcinogenesis is a multistep process influenced by oncogenes and tumor suppressor genes (5). The complex role of HBV in liver carcinogenesis through direct and indirect mechanisms is being debated. Integration of HBV-DNA sequences into the host cell genome can activate cellular genes by a cis-acting mechanism. Chromosomal instability may also result from HBV-DNA integration. HBV-DNA integration into the host cell genome can lead to increased frequencies of chromosomal instability and genetic recombinations (6). Cytogenetic instability of immune system cells in chronic viral infections has been associated with inhibition of the DNA excision repair system and disregulation of apoptosis in target cells (7).

The polymorphisms of genes regulating the DNA repair mechanism and apoptosis affect the baseline level of cytogenetic biomarkers such as chromosomal aberrations (8). Specific mutations of the p53 gene have been strongly implicated in a variety of human cancers (9). The p53 gene representing the key regulator of apoptosis and DNA repair mechanism is of critical importance for the regulation of the cell cycle and the maintenance of genomic integrity. Loss of p53 function has been suggested to be a critical step in multistage hepatocarcinogenesis (10). It is likely that gene polymorphisms can influence p53-related DNA
damage repair and thus provoke chromosome aberrations and decrease mitotic index.

One of the well-known polymorphisms of the p53 gene is Arg72Pro in exon 4 (Ex4+119 G>C; rs1042522), which encodes an arginine-proline substitution in the p53 protein. Two polymorphic variants of wild-type p53 protein have been shown to have some different biochemical and biological properties (11). It has been reported that the Arg variant induces apoptosis more effectively than the Pro variant, which may influence cancer risk (12). The Pro variant has been identified as a risk factor for some malignancies including HCC, but the relationship between this polymorphism and the outcome of HBV infection has not been firmly established (13-15).

Considering all of the above facts, the objective of the present study was to investigate whether there is an association between the mitotic index and chromosomal aberrations and the p53 gene codon 72 Arg72Pro polymorphism in patients with chronic hepatitis B compared to a control group.

**Subjects and Methods**

**Subjects**

Fifty-eight hepatitis B patients receiving interferon therapy in the Department of Gastroenterology, Medical School of Dicle University, were enrolled in the study. Patients with positive hepatitis B surface antigen (HbsAg) and negative hepatitis B antibody (AntiHbs) for more than 6 months were enrolled. Patients with persistently elevated levels of alanine aminotransferase and HBV-DNA received peg-IFN alpha-2a (180 µg/week, Pegasys®, Roche, Switzerland) subcutaneously or peg-IFN alpha-2b (1.5 µg·kg⁻¹·week⁻¹, Pegintron®, Schering, Puerto Rico) subcutaneously. Blood samples were obtained before initiating the treatment. The control group consisted of 30 age- and gender-matched healthy individuals. Patients and controls with any accompanying metabolic or hormonal disorders including hepatosteatosis, hyper- or hypothyroidism, or hyperprolactinemia were excluded. Patients and controls were residents of the same area and came from the same human subpopulation.

An informed consent was obtained from the patients and control subjects. The Dicle University Medical Faculty Ethics Committee approved the study (#B.30.2.DİC.0.01.00.00/49).

**Lymphocyte cultures and cytogenetic analysis**

For each subject, two whole blood cultures were incubated for 72 h at 37°C in RPMI 1640 medium supplemented with 20% fetal bovine serum, 100 IU/mL penicillin G and 100 µg/mL streptomycin. Lymphocyte growth was stimulated with 1% phytohemagglutinin (16). Colchicine (final concentration 0.06 mg/mL) was added to the cultures 2 h before harvesting time. Subsequently, cells were subjected to a hypotonic solution (0.55% KCl) and fixed with ethanol/glacial acetic acid (3:1). Routine chromosome staining was performed with Giemsa in phosphate buffer. Forty Giemsa-stained metaphases were scored for the percentages of chromosome breaks and chromatid breaks. Chromosome breaks, fragments and deletions were scored as 1; translocation, dicentric chromosomes and rings were scored as 2. Gaps were not included (17,18). Representative metaphase spreads of the above chromosomal aberrations are shown in Figure 1. For the mitotic index values, the percentage of metaphases was assessed in 1000 blast nuclei (19).

**DNA extraction and polymorphism detection**

Genomic DNA was isolated from peripheral leukocytes. The blood samples were submitted to digestion in SDS/proteinase K buffer at 37°C for 6-12 h, followed by two phenol extractions and one chloroform extraction. DNA was then ethanol-precipitated and resuspended in Tris-EDTA buffer (20).

A modified method of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) originally described by Kawajiri et al. (21) was used to identify the p53 gene polymorphism at codon 72 in exon 4 using primers (5’-ATCTACAGTCCCCCTTGCCG-3’) and (5’-GCAACTG ACCGTGCAAGTCA-3’) (15,21). The PCR was performed at 94°C for 4 min to induce initial denatur-
ation, followed by 35 cycles of 40 s at 94°C, 30 s at 56°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. This generated a 296-bp fragment that was then subjected to digestion with Bsh1236I (Fermentas Life Science, Germany) overnight at 37°C. The digested product was visualized on 3% agarose gel, followed by ethidium bromide staining. The presence of the wild-type allele (Arg) was indicated by bands of 169 and 127 bp, whereas no digestion of the mutant allele (Pro) occurred. The sample image of PCR-RFLP products belonging to the aforementioned polymorphism obtained by agarose gel electrophoresis is presented in Figure 2.

Statistical analysis

Data are reported as means ± SD and statistical analyses were performed using the SPSS Statistical package, version 11.5 for Windows (SPSS, Inc., USA). The normality of distribution was evaluated by the Kolmogorov-Smirnov test and the normally distributed data were analyzed by the Student t-test. Non-normally distributed data were analyzed by the chi-square test. All statistical tests were two-sided, and a P value <0.05 was considered to be statistically significant.

Results

Fifty-eight patients with chronic hepatitis B, 38 males and 20 females, and 30 healthy individuals, 18 males and 12 females, were enrolled in the study. The mean ages of the patient and the control groups were 35.03 ± 13.76 and 36.23 ± 13.55, respectively. No statistically significant difference in age or gender distribution was observed between the patient and the control groups (P > 0.05). The demographic characteristics of the study group are listed in Table 1.

The frequency of chromosomal aberrations was significantly higher and the mean mitotic index was significantly lower in the patient group compared to the control group (P < 0.05 and P < 0.05, respectively). No statistically significant difference in age or gender distribution was observed between the patient and the control groups (P > 0.05). The demographic characteristics of the study group are listed in Table 1.

| N       | Patient | Control |
|---------|---------|---------|
| Age, years (means ± SD) | 36.2 ± 16.2 | 34.5 ± 12.5 |

There were no statistical differences between groups (Student t-test).

| N | Patient | Control |
|---|---------|---------|
| Mitotic index | 1.99 ± 0.93* | 3.15 ± 1.29 |
| Chromosomal aberrations | 2.4 ± 0.85* | 0.83 ± 0.21 |

*P < 0.05 compared to control group (Student t-test).

Discussion

There is increasing evidence that HBV infection can cause genetic changes within somatic cells, including he-
Chromosomal aberrations are becoming constantly more apparent. The related to DNA repair, on the frequency of chromosome maintenance (30). Macromolecules and mechanisms responsible for genome repair and checkpoints and by epigenetic effects on abnormalities may be further mediated by accumulated mutations (29). The age-related increase in chromosomal aneuploidy (29). The age-related increase in chromosomal instability and cause genetic recombinations and particularly hepatocellular carcinogenesis (6,24,25).

A higher frequency of sister chromatid exchange and a lower mitotic index were observed in the peripheral lymphocytes of patients with chronic HBV infection or cirrhosis. It has been suggested that this is the result of direct genotoxic effects of HBV and genotoxicity may contribute to the development of hepatocellular carcinoma (26).

We observed a high frequency of chromosomal aberrations and low mitotic index in the peripheral lymphocytes of patients with chronic hepatitis B. This result supports the view that HBV has genotoxic and apoptotic effects on the host genome.

Although the rate of chromosomal aberrations was very low in the control group, advanced age was proposed as the major causative factor in the development of chromosomal aberrations in these healthy individuals. Aging is related to an increased frequency of genomic instability and changes in chromosome morphology (27,28). Examples of age-related genomic instability include karyotype alterations, 2q31 deletions or losses of entire chromosomes resulting in aneuploidy (29). The age-related increase in chromosomal abnormalities may be further mediated by accumulated mutations in genes coding proteins required for DNA replication and repair and checkpoints and by epigenetic effects on macromolecules and mechanisms responsible for genome maintenance (30).

The influence of gene polymorphisms, particularly those related to DNA repair, on the frequency of chromosome aberrations is becoming constantly more apparent. The protective or predisposing role of certain DNA repair genes regarding chromosome aberrations has been observed (31,32). The influence of polymorphisms on the p53 gene, the key regulator of the DNA repair processes, and on the frequency of chromosome aberrations is an interesting aspect. The most important polymorphism of the p53 gene in the general population, characterized by an Arg to Pro substitution at codon 72 in the transactivation domain of exon 4, has been investigated as a risk modifier in several cancer models including HCC and HBV-related HCC (15,33). The variant Pro allele has been shown to be less efficient in suppressing cell transformation and inducing apoptosis and to be less sensitive to degradation by viral oncopgenic proteins (11). However, other recent reports have pointed out that the Arg variant is a less efficient DNA repair molecule and therefore Arg/Arg carriers may have an increased frequency of chromosomal aberrations and a high level of genomic instability (34,35).

In the present study, the genotype distribution of p53 gene codon 72 polymorphism did not differ significantly between patients and control subjects. We also observed no significant association between mitotic index and p53 gene codon 72 polymorphism. However, both in patients and healthy controls with a homozygous proline genotype, we observed higher rates of chromosomal aberrations when compared to patients and control individuals with other genotypes (Arg/Pro and Arg/Arg), which are supposed to be associated with the risk of developing HCC.

The present study has some limitations. The first was the limited number of patients who participated in the study. We performed this study in the southeastern region of Turkey where some technical and economic issues prevented us from increasing the sample size. Second, the duration of chronic HBV infection may influence the development of chromosomal aberrations. There was a wide variation in the duration of chronic HBV infection in our study. Also, homogeneity of the duration of chronic HBV infection may be of significance in determining the relationship of HBV infection with chromosomal instability. Leveri et al. (36) mentioned that variation in susceptibility to chromosomal aberrations and mitotic index is partially attributed to the duration of HBV infection as well as to the demographic characteristics of the patients such as age, gender, ethnicity, exposure to aflatoxin, cigarette smoking, and alcohol consumption. A larger number of individuals and a longer duration of follow-up are required to confirm and extend the present results.

We demonstrate that the homozygous proline variant (Pro/Pro) of p53 codon 72 has a higher susceptibility to genomic instability than homozygous arginine (Arg/Arg) and heterozygous arginine/proline (Arg/Pro) variants. However, the higher frequency of chromosomal aberrations in healthy controls with the homozygous proline genotype indicates that the Pro variant affects the occurrence of genomic instability independently from the hepatitis B virus. Our results

Table 3. Chromosomal aberrations observed in patients and control individuals with the homozygous proline genotype (Pro/Pro) and other genotypes (Arg/Pro and Arg/Arg).

|       | N  | Chromosomal aberrations |
|-------|----|--------------------------|
| Patient |    |                          |
| Pro/Pro | 13 | 7.31 ± 2.57*             |
| Arg/Pro | 38 | 3.16 ± 0.80              |
| Arg/Arg | 7  | 4.29 ± 4.28              |
| Control |    |                          |
| Pro/Pro |  3 | 3.33 ± 2.88*             |
| Arg/Pro | 20 | 0.25 ± 0.24              |
| Arg/Arg |  7 | 1.43 ± 0.92              |

Data are reported as means ± SD. *P < 0.05, Pro/Pro compared to Arg/Pro and Arg/Arg (Student t-test).
support the view that the Pro variant is less effective than the Arg variant in inducing apoptosis and inhibiting cellular transformation. These results agree with studies that assert that the homozygous proline genotype is directly related to the development of HCC in healthy populations. Also, our results disagree with reports suggesting that the Arg form might be less potent in reducing genomic instability, and perhaps cancer predisposition (34-36).

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