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# The genotoxic effects of hepatitis B virus to host DNA

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**Chronic viral hepatitis is the main cause of chronic liver disease, cirrhosis and hepatocellular carcinoma throughout the world. Hepatitis B virus (HBV) has mutagenic effects on somatic cells. HBV may be showing these mutagenic effects through its viral proteins or through integrating into host DNA. The aim of this study was to determine whether HBV has a genotoxic effect on host DNA or not. Peripheral blood lymphocytes of 31 chronic HBV patients and 20 chronic HBV carriers were cultured in order to make cytogenetic evaluation by observing chromosome breakage and cytological evaluation by the micronucleus (MN) test. Their results were compared with 20 healthy controls. For each individual, 100 metaphase chromosome spreads were analysed. Around 190–1091 binucleated cells were observed and MN were scored for each individual. Our results showed significantly higher frequencies of chromosome breaks in chronic HBV patients and in HBV carriers than in the control group. There was no difference in MN scores among HBV patients, HBV carriers and healthy carriers. Based on our data, we conclude that chronic HBV patients and carriers have chromosomal instability and that HBV carriers are as affected as patients because of their same chromosome breakage levels.**

## Introduction

Hepatitis B virus (HBV) infects an estimated 350−400 million people and is the ninth leading cause of death worldwide. Chronic HBV infection can lead to serious clinical complications, including cirrhosis, liver failure requiring transplantation and hepatocellular carcinoma (HCC) (1).

The genome of HBV is ~3.2 kb in size, with four partially overlapping open reading frames encoding surface, core, X proteins and polymerase (2). The complex role of this virus in liver carcinogenesis through direct and indirect mechanisms is still 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. More indirectly, liver cell injury mediated by cellular immune responses may be sufficient to cause liver cancer by promoting cell death and proliferation, and genetic mutations may accumulate in the context of necroinflammatory disease (3). Mutations in critical genes may result from different types of genetic alterations, ranging from subtle sequence changes of a few nucleotides to gross chromosomal abnormalities, including deletions, amplifications and translocations of large DNA fragments (3).

There are several mutation detection protocols for determining the genotoxic effects of physical and chemical agents on DNA (4). Cytogenetic analysis of metaphase chromosome spreads and analysis of binucleated cells in the micronucleus (MN) test can be used for detecting the genotoxic effects of an agent (5).

In this study we used both chromosome breakage counting and MN scoring to analyse the genotoxic effect of HBV on host DNA.

## Materials and methods

### *Subjects*

Thirty-one chronic HBV patients (22 male, 9 female) and 20 chronic HBV carriers (13 male, 7 female) were included in this study. They were interviewed on their life-style habits, such as diet, smoking, alcohol and drug intake, their family history and virus infection process. The Ankara University Ethical Committee approved the study (Approval No. 25-2002/506). Peripheral blood samples (1.5 ml) were collected both from chronic HBV patients and from chronic HBV carriers. Blood samples were also collected from 20 (14 male, 6 female) healthy controls. The ages, smoking habits and ratio of sexes were similar in all study groups. None of the subjects drank alcohol, had special diets or chronic drug use.

### *Culture conditions*

For each subject, two whole blood cultures were incubated for 72 h at 37°C, in RPMI 1640 (SIGMA, R 8758) medium, supplemented with 20% fetal bovine serum (SIGMA, F 9665), 100 IU/ml penicillin G (SIGMA, P 3414) and 100 μg/ml streptomycin (SIGMA, S 0890). Lymphocyte growth was stimulated by 1% phytohemoagglutinin (SIGMA, L 8902) (6).

For cytokinesis-blocked human lymphocyte MN test, cytochalasin-B (Cyt-B) (SERVA, 18015) was added after 44 h at a final concentration of 6 μg/ml. After a total of 72 h culture, cells were harvested by centrifugation, treated twice with 5 ml of a mixture (pH 7.2) of RPMI 1640: deionized water 4:1, supplemented with 2% fetal bovine serum, for 7 min. The cells were again centrifuged and submitted to a mild hypotonic treatment in a mixture of RPMI 1640: deionized water 1:4, supplemented with 2% fetal bovine serum, for 5 min. The centrifuged cells were placed on dry slides and smears were performed. After air drying the slides were fixed with freshly prepared and cold methanol:acetic acid (Riedel-de Haen, 24229: Riedel-de Haen, 27225) (3:1) for 20 min. One day later the slides were stained with 4% Giemsa (MERK, 9204) for 8 min (7).

For detecting chromosomal breakage 2 h before harvesting, colchicine (0.2 μg/ml; SIGMA, C-97) was added. Chromosome spreads were obtained following standard techniques (0.075 M KCl hypotonic shock, methanol:acetic acid 3:1 fixation) (8).

### *Cytogenetics*

For each sample, 190–1091 binucleated cells were observed to assess the frequencies of MN. The cells scored for MN had to be clearly seen as binucleate. The number of MN in each binucleate cell were scored (9). MN were accepted only when (i) they were separated from the main nuclei, but included within the corresponding cytoplasm, (ii) they had a chromatin structure similar to that of the main nuclei, (iii) they were coplanar to the main nuclei (10) and (iv) they were no greater than one-third the volume of the main nuclei (11). A hundred Giemsa-stained metaphases were scored for the percentages of

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chromosome breakage. Chromatid breakage, chromosome breakage, fragments and deletions were accepted as 1; translocation, dicentric and rings were accepted as 2 breakage. Gaps were not included (7,12).

For the mitotic index (MI) values, the percentage of metaphases was assessed on 1000 blast nuclei (8). We also calculated the cytokinesis-blocked proliferation index (CBPI) according to the following formula: (considering NI to NIV cells from 1 to 4 nuclei) CBPI = [NI + 2NII + 3(NIII + NIV)]/total (5).

*Statistical analysis*

Differences among HBV patients, HBV carriers and the control group in terms of age, MI and CBPI were evaluated by one-way analysis of variance (ANOVA). The chi-square test was used to test differences among groups for sex and smoking habits. Test of normality was assessed by the Kolmogorov–Smirnov test with Lilliefors significance correction. The degree of association between the virus infection period and its relation with the patient's and carrier's breakage and MN percentage were assessed by Spearman's correlation coefficient. Comparison of patient's and carrier's breakage and MN percentage with respect to family history were analysed using Mann–Whitney *U*-test. Differences among the three groups for MN and breakage percentages were evaluated by Kruskal–Wallis variance analysis. When the *P*-value from the Kruskal–Wallis test statistics is statistically significant, multiple comparison test was used to know the groups that differed from each other (13).

## Results

There were 22 male (71%) and 9 female (29%) subjects, their ages ranging from 18 to 60 years (mean 38 years) in the chronic HBV patient group. In the HBV carrier group, there were 13 male (65%), and 7 female (35%) subjects, their ages ranging from 24 to 50 years (mean 37.5 years). A total of 20 subjects with a mean age of 34 years (from 25 to 42 years), 70% of whom are male, were included in the control group (Table I).

There were no significant differences among the three groups with respect to age, sex and smoking habit ($P = 0.32$, $P = 0.90$ and $P = 0.72$, respectively).

We calculated MI and CBPI for each individual (Table I). Mean MI and CBPI were not statistically different among the three groups ($P = 0.966$, $P = 0.674$, respectively) (Table II).

We counted chromosome breakage frequency (in %) for each individual (except two patients for whom metaphase spreads could not be obtained) (Table I) and then calculated their median value for each group (Figure 1). Median chromosome breakage frequency was 2 (min–max: 0–8) for patients, 2 (min–max: 0–7) for carriers and 1 (min–max: 0–3) for control group. Result of comparisons between the groups for chromosome breakage percentages were as follows: control versus carrier, $P = 0.007$; control versus patient, $P = 0.031$; carrier versus patient, $P = 0.413$. We found that the frequency of chromosome breaks in metaphase chromosome spreads of chronic HBV patients and carriers was significantly higher than the control group. Surprisingly, there was no significant difference between patients and carriers.

Multi-aberrant cells are defined as cells containing >10 chromosomal aberrations (7). We saw cells with a maximum of three breakages in different patients; thus, we did not see multi-aberrant cells in any of our subjects.

The induction of MN in HBV patients, carriers and controls was evaluated by scoring 190–1091 binucleated cells (in ‰) for each individual (Figure 2). Median MN frequency was 4 (min–max: 0–16) for patients; 3.0 (min–max: 0–11) for carriers and 3.8 (min–max: 0–10) for control group. There was no significant difference among the three groups in terms of MN frequency ($P = 0.99$).

Neither breakage nor MN percentage was normally distributed ($P < 0.05$ for all groups).

When stratification of the data according to smoking status was made, sample size within each stratum was too small

**Table I.** MI, breakage percentage, CBPI, BNC and MN values with demographic data for all subjects

| Group | Age | Sex | SH | MI | B (%) | CBPI | BNC | MN (‰) |
|---|---|---|---|---|---|---|---|---|
| P1 | 39 | M | 1 | 4.2 | 2 | 1.65 | 970 | 8.24 |
| P2 | 54 | M | 0 | 1.2 | 3 | 1.47 | 504 | 3.96 |
| P3 | 58 | M | 1 | 3.0 | 2 | 1.47 | 750 | 8 |
| P4 | 36 | M | 0 | 4.9 | 1 | 1.60 | 585 | 5.12 |
| P5 | 48 | M | 1 | 2.6 | 4 | 1.66 | 943 | 2.12 |
| P6 | 32 | M | 0 | 6.4 | 0 | 1.54 | 1039 | 2.88 |
| P7 | 38 | M | 0 | 5.5 | 2 | 1.77 | 335 | 5.97 |
| P8 | 27 | M | 2 | 1.0 | 1 | 1.66 | 1091 | 0.91 |
| P9 | 42 | M | 2 | 3.5 | 2 | 1.62 | 1000 | 4 |
| P10 | 27 | M | 2 | 7.1 | 0 | 1.62 | 1012 | 0.98 |
| P11 | 22 | M | 0 | 4.2 | 2 | 1.83 | 1000 | 8 |
| P12 | 18 | F | 0 | 5.8 | 3 | 1.77 | 1000 | 0 |
| P13 | 47 | F | 0 | 3.7 | 2 | 1.70 | 720 | 4.16 |
| P14 | 38 | F | 0 | 4.2 | 8 | 1.57 | 1010 | 5.94 |
| P15 | 37 | M | 0 | 4.0 | 0 | 1.37 | 236 | 4.23 |
| P16 | 22 | M | 0 | * | * | 1.37 | 1000 | 7 |
| P17 | 60 | F | 0 | 1.8 | 0 | 1.36 | 960 | 7.29 |
| P18 | 50 | M | 0 | 2.8 | 4 | 1.40 | 1000 | 16 |
| P19 | 29 | M | 0 | 3.7 | 2 | 1.69 | 1000 | 5 |
| P20 | 42 | F | 0 | 3.2 | 4 | 1.34 | 741 | 2.69 |
| P21 | 22 | M | 0 | 1.8 | 1 | 1.19 | 866 | 6.92 |
| P22 | 50 | M | 2 | * | * | 1.34 | 1000 | 9 |
| P23 | 36 | M | 1 | 3.9 | 2 | 1.60 | 209 | 0 |
| P24 | 24 | M | 0 | 4.0 | 1 | 1.80 | 829 | 0 |
| P25 | 32 | F | 0 | 4.0 | 1 | 1.79 | 1000 | 0 |
| P26 | 49 | F | 1 | 2.7 | 2 | 1.76 | 1000 | 3 |
| P27 | 48 | M | 0 | 2.8 | 5 | 1.70 | 1000 | 1 |
| P28 | 21 | M | 0 | 2.8 | 0 | 1.86 | 1000 | 3 |
| P29 | 46 | F | 0 | 1.2 | 2 | 1.63 | 1000 | 4 |
| P30 | 38 | F | 0 | 6.2 | 0 | 1.68 | 1000 | 6 |
| P31 | 48 | M | 0 | 6.2 | 1 | 1.67 | 550 | 1.81 |
| C1 | 48 | F | 0 | 2.1 | 1 | 1.49 | 1000 | 3 |
| C2 | 25 | F | 1 | 5.2 | 2 | 1.81 | 1000 | 6 |
| C3 | 46 | M | 0 | 5.9 | 1 | 1.74 | 1040 | 10 |
| C4 | 39 | F | 0 | 3.1 | 3 | 1.29 | 1000 | 14 |
| C5 | 48 | M | 2 | 4.2 | 3 | 1.34 | 1000 | 6 |
| C6 | 38 | M | 0 | 3.0 | 3 | 1.17 | 423 | 2.36 |
| C7 | 33 | F | 0 | 0.9 | 2 | 1.34 | 1000 | 2 |
| C8 | 32 | M | 2 | 3.0 | 3 | 1.33 | 854 | 9.36 |
| C9 | 27 | F | 0 | 4.3 | 0 | 1.64 | 676 | 2.95 |
| C10 | 47 | M | 0 | 6.0 | 0 | 1.86 | 707 | 0 |
| C11 | 24 | F | 0 | 1.5 | 3 | 1.48 | 837 | 10.75 |
| C12 | 47 | M | 0 | 4.5 | 1 | 1.60 | 1000 | 2 |
| C13 | 50 | M | 0 | 5.0 | 7 | 1.67 | 1000 | 5 |
| C14 | 25 | M | 1 | 4.6 | 1 | 1.80 | 1000 | 0 |
| C15 | 31 | M | 2 | 2.8 | 6 | 1.83 | 727 | 9.62 |
| C16 | 42 | M | 1 | 6.0 | 1 | 1.74 | 1002 | 3.99 |
| C17 | 36 | M | 1 | 1.0 | 6 | 1.54 | 878 | 1.13 |
| C18 | 38 | M | 1 | 2.9 | 5 | 1.78 | 975 | 3.07 |
| C19 | 42 | F | 0 | 3.2 | 1 | 1.70 | 356 | 8.42 |
| C20 | 31 | M | 0 | 4.6 | 1 | 1.86 | 1000 | 3 |
| Ct1 | 25 | F | 0 | 3.1 | 1 | 1.58 | 566 | 3.53 |
| Ct2 | 29 | M | 0 | 2.7 | 2 | 1.36 | 1000 | 10 |
| Ct3 | 32 | M | 0 | 6.4 | 0 | 1.26 | 274 | 7.29 |
| Ct4 | 26 | F | 1 | 3.7 | 0 | 1.42 | 332 | 6.02 |
| Ct5 | 27 | M | 0 | 2.5 | 1 | 1.96 | 1000 | 4 |
| Ct6 | 41 | F | 1 | 3.6 | 3 | 1.60 | 915 | 5.46 |
| Ct7 | 30 | M | 2 | 4.9 | 0 | 1.70 | 548 | 3.64 |
| Ct8 | 30 | F | 0 | 4.8 | 1 | 1.58 | 475 | 4.21 |
| Ct9 | 28 | F | 0 | 4.4 | 0 | 1.79 | 1000 | 3 |
| Ct10 | 41 | M | 2 | 3.0 | 2 | 1.63 | 579 | 1.72 |
| Ct11 | 32 | M | 0 | 4.4 | 0 | 1.69 | 710 | 2.81 |
| Ct12 | 28 | M | 0 | 3.2 | 0 | 1.74 | 440 | 4.54 |
| Ct13 | 28 | F | 0 | 4.8 | 0 | 1.56 | 1000 | 1 |
| Ct14 | 40 | M | 2 | 3.3 | 2 | 1.73 | 1000 | 0 |
| Ct15 | 42 | M | 2 | 4.6 | 0 | 1.93 | 1000 | 1 |
| Ct16 | 40 | M | 0 | 4.0 | 0 | 1.81 | 1000 | 3 |
| Ct17 | 41 | M | 0 | 4.0 | 1 | 1.63 | 1000 | 7 |
| Ct18 | 40 | M | 0 | 3.0 | 1 | 1.66 | 1000 | 2 |
| Ct19 | 39 | M | 0 | 2.6 | 2 | 1.65 | 1000 | 4 |
| Ct20 | 41 | M | 2 | 3.2 | 3 | 1.54 | 790 | 10.12 |

P, patient; C, carrier; Ct, control; M, male; F, female; SH, smoking habit; 0, never; 1, 1–10 cigarettes/day; 2, 11–20 cigarettes/day; MI, mitotic index; B, breakage; CBPI, cytokinesis-blocked proliferating index; BNC, binuclear cell; MN, micronucleus; *, no metaphase spreads.



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**Table II.** Descriptive statistics for MN, chromosome breakage frequencies, MI and CBPI of patients, carriers and control group

| | MN | | Breakage | | MI | | CBPI | |
|---|---|---|---|---|---|---|---|---|
| | $\overline{X} \pm SD$ | Median (min–max) | $\overline{X} \pm SD$ | Median (min–max) | $\overline{X} \pm SD$ | Median (min–max) | $\overline{X} \pm SD$ | Median (min–max) |
| Control | 4.22 ± 2.77 | 3.82 (0–10.12) | 0.95 ± 1.05 | 1 (0–3) | 3.81 ± 0.99 | 3.65 (2.5–6.4) | 1.64 ± 0.17 | 1.64 (1.26–1.96) |
| Carrier | 4.58 ± 3.40 | 3 (0–11) | 2.5 ± 2.06 | 2 (0–7) | 3.69 ± 1.59 | 3.70 (0.9–6.0) | 1.60 ± 0.22 | 1.66 (1.17–1.86) |
| Patient | 4.43 ± 3.47 | 4 (0–16) | 1.96 ± 1.78 | 2 (0–8) | 3.74 ± 1.62 | 3.70 (1.0–7.1) | 1.60 ± 0.17 | 1.63 (1.19–1.86) |
| *P* | 0.987* | | 0.018* | | 0.966** | | 0.674** | |

MI, mitotic index; CBPI, cytokinesis-blocked proliferating index; MN, micronucleus; *, Kruskal–Wallis ANOVA; **, one-way ANOVA.

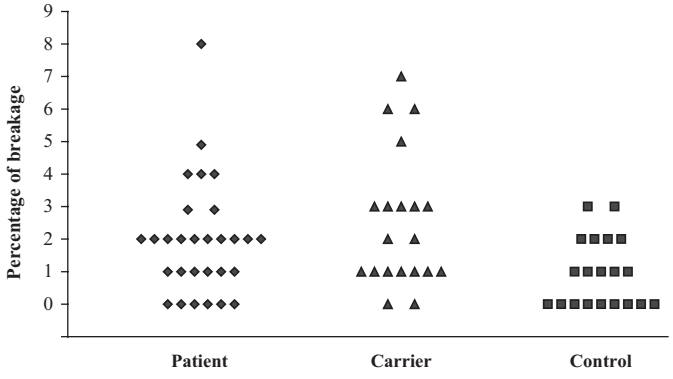


**Fig. 1.** Distribution of chromosome breakage percentage in HBV patients, carriers and controls. Closed diamonds, different from control ($P = 0.007$), but not different from carrier ($P = 0.413$) (multiple comparison test); closed triangles, different from control, $P = 0.031$ (multiple comparison test).

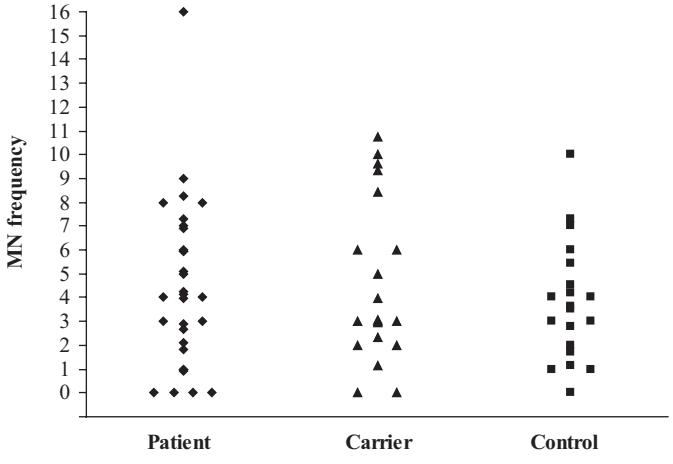


**Fig. 2.** Distribution of MN frequency (‰) in HBV patients, carriers and controls. No significant difference among the three groups ($P = 0.859$) (Kruskal–Wallis ANOVA).

for statistical analysis to be performed satisfactorily. For this reason, MN and breakage percentage were evaluated among the three groups in terms of smoking habit as smokers and non-smokers. There were no significant differences among the three groups in terms of breakage and MN percentage for smokers and non-smokers ($P = 0.154$, $P = 0.786$ for smokers; $P = 0.062$, $P = 0.876$ for non-smokers, respectively).

The data about age, sex, smoking habit, MI, breakage percentage, CBPI, BNC and MN of each subject are shown in Table I.

Descriptive statistics results for MN, chromosome breakage frequencies, MI and CBPI of patients, carriers and control group are shown in Table II.

Comparisons of breakage and MN percentage of the patients and carriers with respect to family history were not significant ($P = 0.67$, $P = 0.41$ for patients and $P = 0.87$, $P = 0.20$ for carriers, respectively).

All the associations between virus infection period and its relation with the breakage and MN percentage of the patients and carriers were insignificant ($r = 0.28$, $P = 0.15$; $r = 0.30$, $P = 0.11$ for patients and $r = 0.03$, $P = 0.90$; $r = 0.43$, $P = 0.06$ for carriers, respectively).

## Discussion

HBV is the one of the most important infectious agents affecting human health, with ~25–40% of infected people having serious clinical complications, including cirrhosis and hepatocellular carcinoma (14).

Some studies have shown that HBV has mutagenic effects on somatic cells. HBV genome can integrate into the host genome and these non-specific integrations exist within different regions, leading to chromosomal instability (15). HBV DNA integrations are targeted not only into host hepatocyte genomes but also into other cells known to carry HBV, such as blood cells. Integrations increase chromosomal instability and cause genetic recombinations (16) and particularly, hepatocellular carcinogenesis (17). Recent studies have shown that HBV infection can cause genetic changes within somatic cells, including hepatocytes and blood cells and this can be demonstrated as increased chromosomal breakage (15). These were cell line studies of hepatocyte and cancer cells (18).

In this study, we evaluated HBV patients and carriers individually. Their results were compared with a control group with respect to genomic instability. In order to detect the mutagenic effects of HBV within the host genome at cytologic and cytogenetic level, MN and breakage evaluations were performed. These techniques are used to detect the effect of chromosomal damage triggered by physical and chemical agents (8). In MN test, cytokinesis is inhibited by Cyt-B without inhibiting mitosis. Dividing cells can be detected by observation of binuclear cells (19). Breakage evaluation is performed in metaphase spreads. By using these two tests, not only chromosomal damage within metaphase spreads are observed but also MN detection of cells in interphase is performed, so a rapid observation of large cell populations is possible (8).

MN and breakage frequencies are affected by age, alcohol and smoking habit (20). In this study, age and smoking habit are homogenously distributed among HBV patients, carriers and the control group.

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HBV synthesizes different viral proteins. These proteins are important for their sites of action and for their induction of chromosomal instability. For example, HBV core protein has been shown to make contact with histones, to affect the chromosomal structure and to change chromosome condensation (15). Another study was related with the HBx gene that integrates into host genome and is expressed in most of the hepatocellular carcinomas. HBx disturbs cell cycle control points and cellular proliferation. Although the effects of HBV in transformation are not well understood, it is estimated that it is related to the interaction between HBV and the DNA repair system. Moreover, it is shown that HBx prefers to bind damaged DNA and leads to aberrant DNA repair ability. All these studies have shown that HBx disturbed control mechanisms of the genomic stability. This leads to a susceptibility to a mutation accumulation (18).

In our study, breakage frequency was significantly different between the control group and HBV patients and carrier group. This shows that HBV increases chromosome breakage and causes instability. It is notable that no difference was detected between chronic HBV patients and carrier group. So, HBV causes instability whether it causes manifest disease or maintains carrier status.

It is also interesting that there was no significant difference between patient, carrier and control groups in MN frequencies ($P = 0.99$). We thought that this finding might appear for several reasons. First, acentric fragments are one of the causes of MN formation, but all acentric fragments do not become MN at the first cell division. Some can survive, replicate or convert to MN in a second or subsequent division (21). In our MN assay, we investigated cells just after the first cell division before cytokinesis. Therefore, we probably might have seen and counted our fragments as breakages, but could not see them as MN. They could exist in cells with two or more nuclei. In addition, we saw chromosome and chromatid breakages more than acentric fragments in our study. We would not expect to see all these chromosome and chromatid breakages as MN. Second, sometimes the real MN formation cannot be evaluated efficiently because of cytoplasmic remnants (22). In our study, suspicious MNs were not included, hence the real result could be higher than the recorded MN rates. Nevertheless, it is difficult to understand chromosome breakage occurrence without the formation of MN. A third explanation for this situation may be methodological differences. However, although both assays were performed on peripheral blood lymphocytes under comparable conditions, this does not mean that results from the two assays will necessarily be in agreement (21).

In conclusion, HBV patients and carriers have increased rates of chromosome breakage and instability. Because of similar breakage frequencies between patients and carriers, it is suggested that HBV carriers should be considered as HBV patients from a cytogenetic point of view.

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