Effects of hepatitis B virus infection on human sperm chromosomes

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem worldwide[1-3], especially in Far-East Asia such as China. During HBV infection, HBV can be found in saliva, vaginal secretions, semen and other tissues beyond the liver and blood[4-6]. It is well established that HBV DNA could integrate not only into the host hepatocytes but also into sperm cells[7-16]. Extensive studies have confirmed that HBV DNA integrated into the hepatocytes will increase chromosome instability and cause genetic recombination and hepatocarcinogenesis[17-21]. In this study, in order to clarify the inheritable influence of HBV on sperm chromosomes, we used interspecific \textit{in vitro} fertilization with zona-free hamster eggs to prepare sperm chromosomes and compared the aberration frequencies of sperm chromosomes between patients with hepatitis B and the controls. In addition, FISH technique with HBV DNA as a probe was used to detect HBV sequences in sperm chromosomes and to analyze features of sperm chromosomes integrated HBV DNA. The genetic significance of the results from this investigation was discussed.

MATERIALS AND METHODS

Subjects

Nine men with HBV infection, including 1 subject with acute hepatitis, 6 with chronic hepatitis (2 with chronic active hepatitis, 4 with chronic persistent hepatitis), 2 chronic HBsAg carriers with no clinical symptoms, and 5 healthy men as controls were studied. The age range was from 22 to 38 years old (mean 27), without the history of exposure of radiation and use of mutagenic agent. The status of the markers of HBV infection of the subjects tested was listed in Table 1.

Preparation of sperm chromosomes

The technique of interspecific \textit{in vitro} fertilization of zona-free golden hamster was used to prepare the sperm chromosomes. Those procedures included the treatment of semen samples from the tested subjects, superovulation of golden hamsters and egg processing, insemination and postinsemination culture and preparation of chromosome slides[20,21].

Analysis of aberrations of sperm chromosome

Sperm chromosome slides were stained with 10-fold diluted...
In order to reduce the nonspecific binding, the slides were treated with RNase (Sigma) at 100 mg/L for 60 min at 37 °C, then at 50 mg/L pepsin (Sigma) in 0.01N HCl for 10 min at 37 °C, at last at 1 % polyformaldehyde in PBS for 10 min at room temperature. Chromosomes were denatured at 75 °C for 4 min in 70 % formamide in 2×SSC. In situ hybridization with denatured DNA probe mentioned above was performed with a modification of the procedure described by our previous paper[23]. Briefly, 10 µl hybridization buffer (50 % deionized formamide, 10 % dextran sulfate, and 2×SSC) containing 40 ng/µl biotin-labeled HBV DNA probe, 500 ng/µl sheared salmon sperm DNA was placed on each slide. A coverslip (18×18 mm) was then applied and sealed with rubber cement, followed by overnight incubation in a humidified chamber at 37 °C.

Detection of hybridization signals Post-hybridization washing was carried out, referring to Korenberg’s methods for mapping small DNA probes[24], once in 40 % formamide, 2×SSC for 10 min at 40 °C, then twice, each for 5 min, in 2×SSC at room temperature. Hybridization signals were detected with FITC-conjugated avidin and amplified with goat biotinylated anti-avidin antibody followed by another layer of FITC-avidin (avidin and anti-avidin, Vector Laboratories). To increase the intensity of the hybridization signal, a second round of amplification was applied by sandwich method as above. In order to reduce the nonspecific binding, the slides were preincubated in 4×SSC with 15 % nonfat dry milk for 10 min at 37 °C prior to each step of immunological reactions. The chromosomes were counterstained with propidium iodide (PI, Sigma) and 4, 6-diamidino-2-phenylindole (DAPI, Sigma), 2 µg/ml each in PBS/glycerol (1:9, v/v) containing 0.2 % (1,4)-diazobicyclo-(2, 2, 8) octane (DABCO, Sigma) as an anti-fade agent. Photograghy was taken under a fluorescence microscope (BH-2, Olympus) with the G excitation filter, EY-455 help excitation filter and Y-475 barrier filter, using Fuji ASA 400 color film.

RESULTS
Analysis of chromosomal quality
Under the same conditions of the experiment, sperm of the subjects with HBV infection as well as healthy controls appeared to be able to penetrate into zona-free hamster oocytes and to develop to the first-cleavage metaphase. However, the quality of metaphase spreads of sperm chromosomes had significant difference between the above two groups (P<0.005, χ²-test). The quality of a number of sperm chromosome plates of the subjects with HBV infection was low, showing generally hard to be stained, stickiness of chromosome, clumping, tortuosity and so on, making the analyzable metaphase spreads greatly decreased. Whereas, few of these phenomena occurred in the controls although a small proportion of sperm chromosome plates of the controls could not be analyzed because of poor separation of chromosomes (Table 2).

Incidence of chromosome aberrations
Among the analyzable metaphase spreads, chromosome aberrations observed included numerical anomaly (aneuploidy), gap, ring chromosome, triradial, dicentric chromosome, pulverization, acentric fragment and deletion, orderly. Of the 233 analyzable sperm metaphase spreads in the hepatitis group, 33 (14.8 %) complements contained chromosome aberrations, being significantly higher than 5 (4.3 %) chromosome aberrations in the control group (P<0.005, χ²-test).

Signals of in situ hybridization
Using specific whole length HBV DNA as probe to perform in situ hybridization on sperm chromosome slides, the sperm metaphases of one patient with chronic persistent hepatitis (subject 6) presented positive signals, all other tested subjects’ spermatozoa (50 per subject), as well as all hamster oocytes (more than 200) did not have FISH signal (Table 1). Of 42 chromosome complements of subject 6 with chronic persistent hepatitis, 9 complements showed clearly twin yellow spots on some chromosomes. In 9 sperm metaphase spreads with positive signals, one had 5 signals on different chromosomes, the rest ranged from 2 to 4 spots involving unrelated

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Table 1 Markers of HBV infection of the tested subjects

| Subjects  | Age | Serum | Seminal fluid |
|-----------|-----|-------|---------------|
|           |     | HBeAg | Anti-HBs | Anti-HBe | HBV-DNA | HBeAg | HBV-DNA |
| Hepatitis |     |       |          |          |         |       |         |
| 1         | 38  | +     | -        | -        | +        | +     | -        |
| 2         | 32  | +     | +        | -        | -        | -     | +        |
| 3         | 25  | +     | -        | -        | +        | -     | -        |
| 4         | 22  | +     | +        | -        | +        | +     | -        |
| 5         | 26  | +     | +        | -        | +        | +     | +        |
| 6         | 23  | +     | +        | -        | +        | +     | +        |
| 7         | 27  | +     | -        | +        | +        | +     | -        |
| 8         | 22  | +     | -        | -        | -        | -     | -        |
| 9         | 25  | +     | -        | -        | +        | +     | +        |
| Controls  |     |       |          |          |         |       |         |
| 1         | 32  | -     | -        | -        | -        | -     | -        |
| 2         | 29  | -     | -        | -        | -        | -     | -        |
| 3         | 23  | -     | -        | -        | -        | -     | -        |
| 4         | 25  | -     | -        | -        | -        | -     | -        |
| 5         | 26  | -     | -        | -        | -        | -     | -        |

*1: acute hepatitis; 2, 3: chronic active hepatitis; 4, 5, 6, 7: chronic persistent hepatitis; 8, 9: chronic HBeAg carriers.
chromosomes. The intensity of signal presented distinct difference among the spots. Positive signals detected were not identical in the chromosomes and in distribution (Figure 1).

**Figure 1** Detection of HBV DNA sequences in sperm chromosomes by FISH with biotinylated whole length HBV DNA probe: (A) a well separated chromosome plate from one patient (subject 6) with chronic persistent hepatitis, with three fluorescent signals on different chromosomes; (B) a poorly separated chromosome plate from the same patient with five fluorescent signals.

**Table 2** Analytical results of sperm chromosomes of the tested subjects

| Sub-jects | Number of metaphase spreads observed | Number (%) of analyzable metaphase spreads | Number (%) of normal sperm karyotypes | With aneu-ploidy | With structural aberrations | Rates (%) FISH Results |
|-----------|-------------------------------------|--------------------------------------------|--------------------------------------|----------------|---------------------------|----------------------|
| Hepatitis |                                     |                                            |                                      |                |                           |                      |
| 1         | 73                                  | 23(31.5)                                   | 20(87.0)                             | 1              | 0                         | 13.0                 |
| 2         | 69                                  | 20(29.0)                                   | 15(75.0)                             | 1              | 0                         | 25.0                 |
| 3         | 72                                  | 24(33.3)                                   | 22(91.7)                             | 0              | 1                         | 8.3                  |
| 4         | 68                                  | 29(42.6)                                   | 25(86.2)                             | 0              | 1                         | 13.8                 |
| 5         | 86                                  | 30(34.9)                                   | 28(93.3)                             | 1               | 1                         | 6.7                  |
| 6         | 113                                 | 43(38.1)                                   | 30(69.8)                             | 5               | 2                         | 30.2                 |
| 7         | 82                                  | 19(23.2)                                   | 19(100)                              | 0              | 0                         | 0.0                  |
| 8         | 61                                  | 23(37.7)                                   | 20(87.0)                             | 0              | 0                         | 13.0                 |
| 9         | 48                                  | 12(25.0)                                   | 11(91.7)                             | 1              | 0                         | 8.3                  |
| Total     | 672                                 | 223(33.2)                                  | 190(85.2)                            | 9               | 3                         | 148.0                |
| Controls  |                                     |                                            |                                      |                |                           |                      |
| 1         | 57                                  | 31(54.3)                                   | 29(93.5)                             | 0              | 0                         | 6.5                  |
| 2         | 61                                  | 21(34.4)                                   | 21(100)                              | 0              | 0                         | 0.0                  |
| 3         | 50                                  | 19(38.0)                                   | 19(100)                              | 0              | 0                         | 0.0                  |
| 4         | 36                                  | 17(47.2)                                   | 16(94.1)                             | 1              | 0                         | 5.9                  |
| 5         | 42                                  | 28(66.7)                                   | 26(92.9)                             | 0              | 1                         | 7.1                  |
| Total     | 246                                 | 116(47.2)                                  | 111(95.7)                            | 1              | 1                         | 4.3                  |

g=Gap; r=Ring; tr=Triradial; dic=Dicentric chromosome; pul=Pulverization; ace=Acentric fragment; del=Deletion

*For containing both numerically and structurally chromosomal aberrations simultaneously*

*For containing gap and acentric fragment chromosome aberrations simultaneously*

**DISCUSSION**

Our studies showed that the frequency of sperm chromosomal aberrations was greatly elevated after the infection of hepatitis B virus, and FISH could directly visualize the integration of HBV DNA sequences into sperm chromosomes. These results suggested that HBV infection could produce inheritable biological effects by carrying genetic materials damaged by virus or carrying altered genetic constituent due to the insertions of virus DNA in germ cells. As we know, this is the first report about the influence of biological factors on human sperm chromosomes.

In our study, we unexpectedly found that the sperm chromosomes frequently presented stickiness, faint stain and extreme tortuosity in the tested subjects with HBV infection and analyzable metaphases were low. Nevertheless, these changes were not found in the healthy controls under the strictly identical conditions. A previous report demonstrated that this manifestation had occurred in the chromosomes of somatic cells of HB patients with positive HBsAg. The reasons of causing chromosomal stickiness in the HBV infection patients were unclear. The possibilities we considered might include the following events: First, antigen components of HBV such as core protein might interfere with the assembly of chromosome from chromatin due to its interaction with histones, it was confirmed that the core protein of HBV had participated in the organization of nucleosomes with histones in the hepatitis B virus minichromosome,[26,27] so the condensation degree of chromosome was decreased and staining of chromosome became more difficult. Second, local despiralizations in the chromosomes occurred due to virus or its components. Third, premature chromosome condensation (PCC) might be induced by HBV, because virus-induced PCC is a common phenomenon.[28] Chromosome stickiness itself may be a manifestation of chromosomal pulverizations.

Previous reports have indicated that HBV infection can induce genetic alterations in somatic cells including hepatocytes and blood cells etc, showing increase of the rate of chromosomal aberrations or SCEs.[29-35] It was reported that influenza in spermatocytes of mice could greatly induce the
increase of chromosomal anomalies[38]. Our studies revealed that the incidence of chromosomal aberrations in the HBV infection group was significantly higher than that in the controls. Although in the present study the individual sample sizes and analyzable chromosome numbers were not large enough and it was difficult to compare the differences of rates and types of aberrations among the different clinical and serological states of HBV infection, there were some features worthy to be considered: First, the incidence of chromosome aberrations did not appear to be closely correlated with the seminal fluid status of HBV infection; Second, the chromosome pulverizations were not observed in the controls but were present in 4 sperm cells in the HBV infection group; Third, subject 6 with proven HBV integrations in the sperm chromosomes had prominently higher level of chromosomal aberration than the other HBV infection subjects; Fourth, the chromosome breakages were more frequent in the HBV infection group than that in the controls.

FISH on sperm chromosomes by specific HBV DNA as a probe had showed positive signals in an HBV patient. The results directly indicated that HBV could penetrate blood-testis barrier and enter male germ line and integrate into their genome. So the possibility of vertical transmission of HBV via the germ cells was further confirmed[38,39]. Our preliminary observations showed that HBV integrations into sperm chromosomes had the feature of multi-site integrations and that the predilection site of HBV insertion into chromosomes seemed to be unlikely present. These results were similar with other previous reports about HBV-related hepatocellular carcinoma either in HCC-derived cell lines or in liver tumor samples[37,6764]. During spermatogenesis, the stem cells sequentially undergo proliferation by mitosis and differentiation into spermatocytes and formation into spermatozoa by meiosis. The occurred important events of meiosis were the pairing and recombination of chromosomes during the prophase of meiosis I[37]. Thus it was possible that relocation of viral sequences and multi-site integrations could occur via genetic recombination when HBV was inserted into male reproductive cells, but the genetic effect of initial HBV insertion into various spermatogenic stages must have been greatly various. If the insertion occurred in stem cells, their all daughter cells would carry the integrated form of viral DNA and the effects would be long-term. In our studies, we were surprised by the fact that FISH showed strong signals in a patient using single small DNA probe. The reasonable explanations for this result included that the copies of HBV strand-invasion may be multiple, HBV sequence generated duplications in situ after the invasion into host genome, during the processes of making spermatozoa, unequal cross-over or DNA rearrangements occurred repeatedly due to HBV insertion into DNA of stem cells. According to the sperm chromosome aberration analysis (Table 2), such a subject with HBV integrations had relatively higher incidence of sperm chromosome aberration than that in other subjects without HBV integration. This correlation implied that viral DNA integrations into germ line significantly increased the instability of gametic genome. Thus, HBV integration into sperm cells would create extensively inheritable effects including offspring from such sperm fusion with ovum presenting HBV infection status in newborn infant and producing congenital or hereditary disease (for example, embryonal tumor) by altering genetic constituent and/or inducing mutations[38-40].

We conclude that HBV infection can bring about mutagenic effects on sperm chromosomes which show that the incidence of sperm chromosome aberrations is significantly elevated after the infection of HBV. Integrations of viral DNA into sperm chromosomes with feature of multisites and nonspecific can further increase the instability of sperm chromosomes. Our study suggests that HBV infection can bring about mutagenic effects not only by altering genetic constituent and/or inducing mutations but also possibly by vertical transmission of HBV via the germ line to the next generation.

REFERENCES

1. Fang JN, Cui LH, Quan ZY, Choi BY, KI M, Park HB. A comparative study on serologic profiles of virus hepatitis B. World J Gastroenterol 2001; 7: 107-110
2. Yan JC, Ma JY, Pan BR, Ma LS. Studies on viral hepatitis B in China. Shi Jiere Haren Xiaohua Zhai 2001; 9: 611-616
3. Rabee C, Pilz T, Klostermann C, Berna M, Schild HH, Sauerbruch T, Caselmann WH. Clinical characteristics and outcome of a cohort of 101 patients with hepatocellular carcinoma. World J Gastroenterol 2001; 7: 206-215
4. Heathcote J, Cameron CH, Dube DS. Hepatitis B antigen in saliva and semen. Lancet 1974; 1: 71-73
5. Darani M, Gerber M, Letter: Hepatitis B antigen in vaginal secretions. Lancet 1974; 2: 1008
6. Dejana A, Lugassy C, Zafrani S, Tiollais P, Brechot C. Detection of hepatitis B virus DNA in pancreas, kidney and skin of two human carriers of the virus. J Gen Virol 1984; 65: 651-655
7. Rogler CE, Sherman M, Su CY, Shafritz DA, Summers J, TAB, Henderson A, Kew M. Deletion in chromosome 11p associated with a hepatitis B integration site in hepatocellular carcinoma. Science 1985; 230: 319-322
8. Hino O. Shows TB, Rogler CE. Hepatitis B virus integration site in hepatocellular carcinoma at chromosome 17;18 translocation. Proc Natl Acad Sci USA 1986; 83: 8338-8342
9. Tokino T, Fukushige S, Nakamura T, Naya S, Murotsu T, Shiga K, Aoki N, Matsubara K. Chromosomal translocation and inverted duplication associated with integrated hepatitis B virus in hepatocellular carcinoma. J Virol 1987; 61: 3948-3954
10. Zhou YZ, Slagle BL, Donehower LA, van Tuinen P, Ledbetter DH, Butel JS. Structural analysis of a hepatitis B virus genome integrated into human spermatozoa. J Virol 1986; 82: 4224-4231
11. Urashima T, Saigo K, Kobayashi S, Imaeaki H, Matsubara H, Koide Y, Asoano T, Kondo Y, Koike K, Isoko K. Identification of hepatitis B virus integration in hepatocellular cancer tissues. J Hepatol 1997; 26: 771-778
12. Pinedo P, Marchio A, Terris B, Mattei MG, Tu ZW, Tiollais P, Dejana A. (3;8) chromosomal translocation associated with hepatitis B virus integration involves the carboxypeptidase N locus. J Virol 1996; 70: 7283-7284
13. Hino O, Tabata S, Hotta Y. Evidence for increased in vitro recombination with insertion of hepatitis B virus DNA. Proc Natl Acad Sci USA 1991; 88: 9248-9252
14. Hino O, Ohtake K, Rogler CE. Features of two hepatitis B virus (HBV) DNA integrations suggest mechanisms of HBV integration. J Virol 1989; 63: 2638-2643
15. Tokino T, Matsubara K. Chromosomal sites for hepatitis B virus integration in human hepatocellular carcinoma. J Virol 1990; 65: 6761-6764
16. Su TS, Wang WL, Yauk YK. Characterization of hepatitis B virus integration in hepatocellular carcinoma. DNA Cell Biol 1998; 17: 415-425
17. Shayan M, Agami R, Shaul Y. HBV integrants of hepatocellular carcinoma cell lines contain an active enhancer. Oncogene 2001; 20: 6811-6819
18. Hadchouel M, Scotto J, Huret JL, Molinie C, Villa E, Degos F, Brechot C. Presence of HBV DNA in spermatozoa: a possible vertical transmission of HBV via the germ line. J Med Virol 1985; 16: 61-66
19. Peng HW, Su TS, Han SH, Ho CK, Ho CH, Ching KN, Chiang BN. Assessment of HBV persistent infection in an adult population in Taiwan. J Med Virol 1988; 24: 405-412
20. Hwang TH, Liu HY, Cai M, Huang J. Chromosomal aberrations induced in human spermatozoa by mitomycin C. Ychian J Gastroenterol 1997; 3: 281-285.
21. Kamiguchi Y, Mikamo K. An improved, efficient method for analyzing human sperm chromosomes using zona-free hamster ova. Am J Hum Genet 1986; 38: 724-740
22 Sambrook J, Fritsch EF, Maniatis. Molecular cloning—a laboratory manual. 3rd edition, New york: Clod Spring Habor Laboratory Press 2001: 1.21-1.83
23 Huang JM, Huang TH, Fang XW, Zhuang TG, Liu HX. Detection of aneuploidy in human sperm by FISH. Zhonghua Yixue Yichuanxue Zazhi 1997; 14: 248-249
24 Korenberg JR, Chen XN. Human cDNA mapping using a high-resolution R-banding technique and fluorescence in situ hybridization. Cytogenet Cell Genet 1995; 69: 196-200
25 Vianello MG, Fasce LC, Mura MS, Andreoni G, Scalise G. Cytogenic analysis in HBsAg positive subjects, healthy carriers and patients with acute-phase hepatitis. Bol Ist Sieroter Milan 1979; 58: 266-272
26 Bock CT, Schranz P, Schroder CH, Zentgraf H. Hepatitis B virus genome is organized into nucleosomes in the infected cell. Virus Genes 1994; 8: 215-229
27 Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, Zentgraf H. Structural organization of the hepatitis B virus minichromosome. J Mol Biol 2001; 307: 183-196
28 Aula P. Virus-induced premature chromosome condensation (PCC) in single cells and G-bands of PCC-chromatin. Hereditas 1973; 74: 81-87
29 Chen H, Chiu TS, Chen PJ, Chen DS. Cytogenetic studies on human liver cancer cell lines. Cancer Genet Cytogenet 1993; 65: 163-166
30 Marchio A, Meddeb M, Pinseau P, Danglot G, Tiollais P, Bernheim A, Dejean A. Recurrent chromosomal abnormalities in hepatocellular carcinoma detected by comparative genomic hybridization. Genes Chromosomes Cancer 1997; 18: 59-65
31 Pang E, Wong N, Lai PB, To KF, Lau JW, Johnson PJ. A comprehensive karyotypic analysis on a newly developed hepatocellular carcinoma cell line, HKCl-1, by spectral karyotyping and comparative genomic hybridization. Cancer Genet Cytogenet 2000; 121: 9-16
32 Livezey KW, Negorev D, Simon D. Hepatitis B virus-transfected Hep G2 cells demonstrate genetic alterations and de novo viral integration in cells replicating HBV. Mutat Res 2000; 452: 163-178
33 Livezey KW, Negorev D, Simon D. Increased chromosomal alterations and micronuclei formation in human hepatoma HepG2 cells transfected with the hepatitis B virus HBX gene. Mutat Res 2002; 505: 63-74
34 Wang PL, Liu SF, Shen SL, Dong XY. A study of sister-chromatid exchange in peripheral lymphocytes of hepatitis B patients with HBsAg positive and their children. Mutat Res 1986; 175: 249-254
35 Chatterjee B, Ghosh PK. Constitutive heterochromatin polymorphism and chromosome damage in viral hepatitis. M utat Res 1989; 210: 49-57
36 Sharma G, Polasa H. Cytogenetic effects of influenza virus infection on male germ cells of mice. Hum Genet 1978; 45: 179-187
37 Hecht NB. The making of a spermatozoon: a molecular perspective. Dev Gen 1995; 16: 95-103
38 Naumova AK, Kisselev LL. Biological consequences of interactions between hepatitis B virus and human nonhepatic cellular genomes. Biomed Sci 1990; 1: 233-238
39 Kim H, Lee MJ, Kim MR, Chung IP, Kim YM, Lee JY, Jang JJ. Expression of cyclin D1, cyclin E, cdk4 and loss of heterozygosity of 8p, 13q, 17p in hepatocellular carcinoma: comparison study of childhood and adult hepatocellular carcinoma. Liver 2000; 20: 173-178
40 Tanaka T, Miyamoto H, Hino O, Kuragawa T, Koike M, Iizuka T, Sakamoto H. Primary hepatocellular carcinoma with hepatitis B virus-DNA integration in a 4-year-old boy. Hum Pathol 1986; 17: 202-204

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