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RESEARCH ARTICLE

Polymorphism attribution of cSNPs in cancer-related genes located in loss regions with a high frequency of HCC between HBV and health groups

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Abstract Cancer-related genes harbored in the loss regions containing a high frequency of hepatocellular carcinoma (HCC) were selected. Related information was gathered and the coding single nucleotide polymorphism (cSNP) sequences were obtained from the single nucleotide polymorphism (SNP) database. The appropriate primers and oligonucleotide probes were then designed in accordance with the SNP sites, and subsequently, the gene chips for detecting SNPs were constructed. Genomic DNA was extracted from blood samples of healthy controls and from patients with HBV infection. The sequences, including the SNPs, were amplified via polymerase chain reaction (PCR) and labeled using digoxigenin deoxyuridine tri-phosphate (Dig-dUTP). The labeled products were then hybridized with the SNP chips. Results confirmed that the differences in allele frequencies of three SNPs EGFL3 (rs947345), Caspase9 (rs2308950), and E2F2 (rs3218171) were distinct between HBV-infected patients and controls, suggesting that these SNPs ocuring in high frequency in HBV-infected individuals may be associated with susceptibility to HCC.

Keywords tumor-associated genes, Hepatocellular B virus (HBV), single nucleotide polymorphism (SNP), susceptibility

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. In recent years, the worldwide incidence of HCC has risen. Most investigations have focused on the nosogenesis of HCC (Zhang et al., 1994; Br'echot et al., 2000), with most results showing HBV infection as the main reason for HCC occurrence.

Studies on genome polymorphism are an important part in the post-genome era. Single nucleotide polymorphism (SNP) is the most common source of genome polymorphism. Sequencing of the human genome has given us the benefit of finding a lot of SNPs. SNPs located in the gene's coding region are named cSNPs. The majority of cSNPs are single-base substitutions that may or may not lead to amino acid substitutions. Some of the non-synonymous cSNPs, however, can alter a functionally important amino acid residue, and further alter the corresponding protein function.

Chromosomal loss is prevalent in cancer cells. The variations in the coding region of most genes located in the high-frequency loss regions of chromosomes influence gene function. Investigation of cSNPs harbored in loss regions with a high frequency of cancer cell chromosomes can help reveal the genetic mechanism behind tumorigenesis and cancer susceptibility. In our study, the cancer-related genes located in loss regions with a high frequency of HCC were selected. The non-synonymous cSNP sequences were obtained from the SNP database, after which the appropriate primers and oligonucleotide probes were designed in accordance with the SNP sites. Finally, the cSNP chips, including the oligonucleotide probes were constructed. The differences in cSNPs between the healthy group and HBV-infected group were detected by this chip.

2 Materials and methods

2.1 Materials and main reagents

2.1.1 Materials

Sixty eight blood specimens from healthy people were supplied by Tianjin Nankai Hospital. Forty two blood specimens

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from chronically HBV-infected people were supplied by the liver disease clinic of Tianjin Third Central Hospital, Tianjin, China.

2.1.2 Enzymes and reagents

The following enzymes and reagents were used: Taq DNA polymerase and dNTP (TaKaRa Biotechnology Dalian Co., Ltd), nylon membranes (Pall Corporation, American), digoxin and anti-digoxin (Roche Company, Switzerland), DAB condensed liquid (Huamei Biotechnology Company, China).

2.2 Methods

2.2.1 Extraction of DNA and construction of DNA pool

Extraction of DNA was done according to the method using protein K, hydroxybenzene, and chloroform on concrete blood specimens (Niu and Shen, 2000). Construction of the DNA pool was done by taking 100 ng/μL of uniform individual genomic DNA, then intermixing. The healthy group DNA pool was composed of healthy individual genomic DNA. The HBV group DNA pool was composed of HBV- infected genomic DNA.

2.2.2 Preparation of cSNP chips

Forty eight SNPs situated in 25 genes harbored in the high-frequency loss regions 1 p and 8 p and related to hepatocellular carcinoma were included in the chips. At the same time, eight different concentrations of the polymerase chain reaction (PCR)-amplified house-keeping gene G_3PDH were used to detect the hybridization efficiency in the chip. Primers and oligonucleotide probes were designed using primer premier 5.0 based on information about SNP sequences from the SNP database. Each SNP site corresponded to two probes, differing only in the middle base. Probes with the same concentration were arranged on the nylon membrane using the NKG-Microarray III machine. Each chip was irradiated for 3 min with a CL-1000M ultraviolet instrument to strengthen the stability of the combination between the probes and the membrane. The chips were then stored in a refrigerator at 4°C.

2.2.3 PCR amplification and labeling

Polymerase chain reaction was carried out in a Perkin-Elmer thermocycler with a volume of 25 μL and ddH_2O at 18.5 μL, 2.5 μL 10 × PCR buffer, 1 μL (10 μmol/L) each primer, 0.5 μL (10 mmol/L) dNTP (with the proportion of dTTP to Dig-dUTP being 10), 0.1 μL (100 ng/μL) DNA, and 0.5 μL (2 U/μL) Taq DNA polymerase. The touch down-PCR cycling conditions were as follows: denaturing at 94°C for 5 min; five cycles of 94°C 30 s, 64°C 30 s, 72°C 30 s; five cycles of 94°C 30 s, 61°C 30 s, 72°C 30 s; five cycles of 94°C 30 s, 58°C 30 s; 72°C 30 s; 30 cycles of 94°C 30 s, 55°C 30 s, 72°C 30 s; and extending at 72°C for 10 min. The range of amplification products created by PCR is from 80 to 390 bp.

2.2.4 Hybridization, membrane washing and colouring

300 μL hybridization solution was added to a hybridization bag with a cSNP chip in it. Prehybridization was done at 45°C for 30 min. PCR products were incubated in 100°C water for 5 min then in ice water for 5 min. The probe was added to the hybridization bag and incubated at 45°C for 12 h. The membrane was then placed in a container and washed with 2 mL/cm^2 of 2 × SSC, 0.1% SDS, for 2 min at room temperature. The procedure was repeated. Washing was again done with 0.2 × SSC, 0.1% SDS, for 2 min at 42°C. This was repeated as well. The membrane was then washed with TNT solution for 5 min at room temperature. The membranes were then placed in a fresh bag and blocked with 10 × blocking solution, 180 μL TN. These were incubated at 37°C for 30 min. Liquid was removed and the following were added: fresh 10 × blocking solution, 180 μL TN, and 0.3 μL digoxigenin antibody. Incubation was done at 37°C for 30 min. Washing was done with TNT solution for 3 min, and repeated. Incubation of the membrane in DAB solution followed until the color changed to an ideal degree. Finally, the reaction was finished with 1 × PBS. Hybridized chips were then input into the computer by using a scanner, and analyzed with the software chip 3.0 designed by Nankai chromosome Lab china. The A1/A1 (wild-type, WT), A2/A2 (homozygous mutation, MT), and A1/A2 (heterozygous, HT) genotypes were easily distinguished as follows: 3-fold and above, wild-type (A1/A1 genotype); 0.33-fold and below, mutant (A2/A2 genotype); lower than 1.5-fold but higher than 0.67-fold, heterozygous (A1/A2 genotype) (Huber et al., 2002). All the results from hybridization and analyses were repeated three times.

2.2.5 Statistical analysis

The data were denoted as the average $x \pm SD$ (standard deviation). The data between the different groups were compared by t-test analysis.

3 Results

3.1 Detection of hybridization efficiency

The hybridization efficiency was detected by the appearance of the house-keeping gene G_3PDH, and was denoted by a linear equation (Figs. 1 and 2). The hybridization signal data from G_3PDH at different concentrations are linearly distinct at multiple dilutions. The correlative coefficients of the healthy group and the HBV group were 0.9873 and 0.9914, respectively, showing that the hybridization efficiencies of the two chips were high.

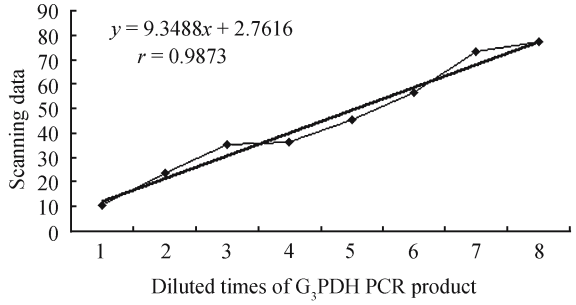

Fig. 1 The detection of hybridization efficiency in control

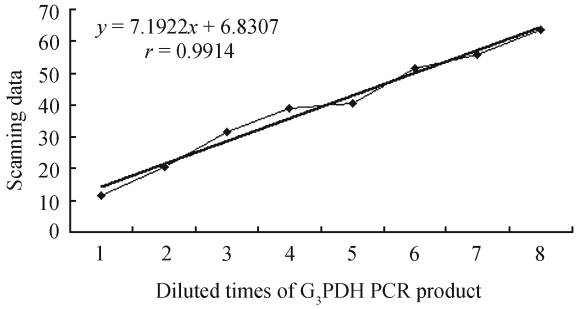

Fig. 2 The detection of hybridization efficiency in HBV infectors

3.2 Results of SNP chip hybridization

The hybridization results from the healthy group and the HBV group are shown in Figs. 3 and 4, respectively. The different sites are labeled by arrowheads. Results show that there are differences in the SNPs of tumor-related genes of the healthy group and the HBV group. The detected polymorphic sites in the HBV group were EGFL3 (rs947345), Caspase9 (rs2308950), and E2F2 (rs3218171). The scanning data of these three SNPs were all lower than 0.33. The exact data are 0.0247 ± 0.0087, 0.0220 ± 0.002, and 0.2522 ± 0.0075, respectively.

3.3 The gene frequency of different sites

Allele frequencies (Xu et al., 1999) of SNPs in the healthy and HBV group are shown in Table 1. Results from statistical analysis showed that gene frequencies of three sites were different between the healthy and the HBV group. The difference in frequencies of rs947345 and rs2308950 in the two groups is significantly distinct. The difference in frequency of rs3218171 between the two groups is also significant.

Table 1 Allelic frequencies of SNPs in the healthy and HBV groups (%)

Gene name	Sequence number of SNP	Polymorphic site	Gene frequency (healthy control)	Gene frequency (HBV infected)
EGFL3	rs947345	A	44.64	2.99**
		G	55.36	97.01**
Caspase9	rs2308950	G	97.91	4.10**
		A	2.09	95.90**
E2F2	rs3218171	G	26.19	22.13*
		T	73.81	77.87*

Note: ** means $p < 0.05$ (the significant difference between two groups); * means $0.1 > p > 0.05$ (the distinct difference between two groups).

4 Discussion

We took many measures to ensure the reliability of the chips. The following were ensured: the repeat units from left to right of the chip; setting the dots for hybridization control; designing the oligonucleotide probes (including the perfect match and mismatch probes) in accordance with the conservative area of the household gene G_3PDH to detect the preciseness of the chips; and, prevention of non-specific band occurence by using touch-down PCR cycling conditions and heightening the dependability of the hybridization. Further, a blank site was set to detect the uniformity of chips' backgrounds.

The nylon membranes used as the texture of the chips increased the quality of writing, and hybridization was

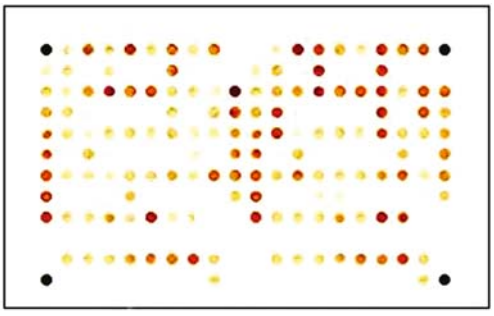

Fig. 3 The hybridization results of healthy controls

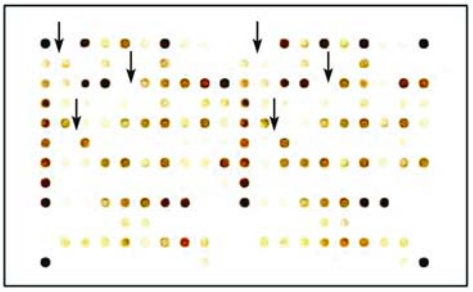

Fig. 4 The hybridization results of the HBV-infected group

Note: Probes of Figs. 3 and 4 are repeated twice on the chip. These are repeat units from left to right. The coordinate sites (controlling sites of the probes) on the chip are denoted by four black dots. The first line corresponds to PCR products with different concentrations. Two probes corresponding to the polymorphism sequence of each SNP begin from the second line to the tenth, while the probe located above is the natural one, and on the underside is the polymorphic one. The first, second and lowermost two dots in the tenth line are blank dots.

mature. Different kinds of fluorescent disturbing factors also had no effect on hybridization and in the showing of colour of DAB. In addition, the 3' end of the oligonucleotide probes were linked tails with the same kind of base, thus strengthening the link between probes and the membrane, as well as the repetition of hybridization. Construction of the DNA pools assisted in detection of the polymorphic sites with high frequency in the HBV group.

Mutations of certain genes in HCC patients with HBV infection have been investigated (Yamamoto et al., 1995). Okamoto's results showed that allelic loss and dot mutation occurred in p53 because of HBV infection. The structure of the p53 protein is exceptional. The mutative p53 and HBsAg form a steady compound which can increase the susceptibility to HCC (Okamoto et al., 1993). In our study, we designed cSNP chips of tumor-related genes harbored in loss regions with high frequencies of hepatocellular carcinoma genes. We then detected the polymorphism of these cSNPs in a HBV group and a healthy group, and analyzed for the first time the relationship between HBV infection and susceptibility to HCC.

A difference in the cSNPs between the two groups was seen. Some cSNPs, such as EGFL3 (rs947345), Caspase9 (rs2308950) and E2F2 (rs3218171) appeared with high frequency in the HBV group. Further, these polymorphic sites lie in the coding region of the genes, which may result in changes to the gene sequence and affect the relevant protein's function. This may then affect the embedding of the virus genome and the host genome. EGFL3 is concerned with the *ras* signal transduction pathway (Nakayama et al., 1998). Caspase9 is a key factor in the mitochondrial pathway of apoptosis (Lundberg and Weinberg, 1999). E2F2 is a kind of transcription factor in mammals. It plays important roles in the cell cycle and in apoptosis (Moroni et al., 2001). We also detected the cSNPs of tumor-related genes in 20 HCC samples infected with HBV in our additional investigation. There are six polymorphic sites in HCC patients. Three of these sites were seen in the HBV group, while Caspase9 (rs2308938, rs2308941) and BNIP3L (rs1055806) only occurred in the HCC patients. The variance in frequency of these six sites increased by degrees in the healthy group, the HBV group, and the HCC patients, thus showing that the high frequency cSNPs in the HBV group and the HCC patients infected with HBV may be related to HCC susceptibility. The variations in gene coding regions play an important role in the carcinogenesis and development of HCC, and may result in changes in gene function and interaction among genes, which can then increase the frequency of the disease. In future studies, we intend to investigate the effecting of variant genes on proteins by several methods including cell transfection.

References

Br´echot C, Gozuacik D, Murakami Y, Paterlini-Brechot P (2000). Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Cancer Biology, 10: 211–231

Huber M, Mundlein A, Dornstauder E, Schneeberger C, Tempfer C B, Mueller M W, Schmidt W M (2002). Accessing single nucleotide polymorphisms in genomic DNA by direct multiplex polymerase chain reaction amplification on oligonucleotide microarrays. Analytical Biochemistry, 303(1): 25–33

Lundberg A S, Weinberg R A (1999). Control of the cell cycle and apoptosis. European Journal of Cancer, 35(4): 531–539

Moroni M C, Hickman E S, Lazzerini D E, Caprara G, Colli E, Cecconi F, Muller H, Helin K (2001). APAF1 is a transcriptional target for E2F and p53. Nature Cell Biology, 3(6): 552–558

Nakayama M, Nakajima D, Nagase T, Nomura N, Seki N, Ohara O (1998). Identification of high-molecular-weight proteins with multiple EGF-like motifs by motif-trap screening. Genomics, 51(1): 27–34

Niu J Y, Shen H B (2000). Application and purification of DNA from clotted blood. Journal of Nanjing Medical University, 20(5): 389–340 (in Chinese)

Okamoto M, Ohtsu H, Miyaki M, Yonekawa H (1993). No allelic loss at the p53 locus in 1,2-dimethylhydrazine-induced mouse colon tumors: PCR-SSCP analysis with sequence-tagged microsatellite site primers. Carcinogenesis, 14(7): 1483–1486

Xu C D, Jiang S H, Chen S N, Yang Y Q, Fan L A, Xu J Y (1999). Study on HLA-DQA1 allelic frequency in children with duodenal ulcer. Chinese Journal of Digestion, 19(3): 172–174 (in Chinese)

Yamamoto K, Kato M, Kurosawa K (1995). Analysis of HBV-DNA precore mutation in Chronic liver disease developed to hepatocellular carcinoma. International Hepatology Communications, 3: 159–164

Zhang F X, Liang S D, Yuan A L (1994). Study on the relationship between HCV, HBV and liver cirrhosis, hepatocellular carcinoma. Cancer, 13(2): 144–146 (in Chinese)