p53 Polymorphisms and Haplotypes as a Possible Predictor of a High-risk Group for Hepatocellular Carcinoma

Shigeaki Sato[1], Takashi Shiraki[1], Yoshiki Inoue[1], Tatsuya Takeshita[2] and Kanehisa Morimoto[2]

[1]Department of Hygiene, Kobe University School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017 and [2]Department of Hygiene and Preventive Medicine, Osaka University School of Medicine, 2-2, Yamadaoka, Suita, Osaka 656-0871, Japan

ABSTRACT

In a case-control study to evaluate the factors involved in the development of hepatocellular carcinoma, polymorphisms of the p53 gene were compared in 68 cases mostly infected with hepatitis C virus (HCV) and 68 controls matched for sex and age: DNA from peripheral blood leukocytes was analyzed by the polymerase chain reaction-single strand conformation polymorphism method and direct sequencing. Polymorphisms analyzed were those in exon 4 (CCC vs. CGC, Pro vs. Arg at codon 72, A1 allele vs. A2 allele), intron 2 (C vs. G at nucleotide 38, A1 vs. A2), intron 3 (C vs. A at nucleotide 65, A1 vs. A2; absence and presence of 16 base pair repeat at nucleotides 24 to 39, A1 vs. A2), intron 6 (A vs. G at nucleotide 62, A1 vs. A2) and intron 7 (C and T vs. T and G at nucleotides 72 and 92, A1 vs. A2). A significantly higher frequency of the allele for CCC (Pro, A1) at codon 72 of exon 4 was found in cases (39%) than in controls (26%) ($p<0.05$). Highly significant linkage of the polymorphisms in exon 4, intron 2, intron 3 and intron 7, and between the intron 3-16 bp duplication and polymorphism in intron 6 also was found. Matched pair analysis showed significantly higher frequencies of certain haplotypes (1-1-1-1-2-2 or 1-1-2-1-2-1 for exon 4, intron 2, intron 3, the intron 3-16 bp duplication, intron 6 and intron 7) in cases than in controls ($p=0.014$, OR=2.27, 95% CI= 1.08-5.12). No preference of specific p53 polymorphisms for specific HCV genotype was detected. These findings suggest that in hepatocarcinogenesis mainly due to HCV infection, genetic factors may be involved and that genetic markers can serve as predictors of a high-risk group for hepatocarcinogenesis.

INTRODUCTION

Cancer prevention consists of three stages; primary prevention, i.e., intervention designed to reduce the risk of getting cancer, secondary prevention, i.e., the early detection and treatment and tertiary prevention, i.e., the prevention of progression of disability through palliative care focusing on quality of life. As for the implementation of effective primary and secondary prevention, detection of high-risk cancer groups is crucially important. High-risk indicators include chronic atrophic gastritis or gastric dysphasia for stomach cancer, family and past histories of colon cancer or polyps and inflammatory bowel diseases for colon cancer and hepatitis C or hepatitis B virus (HCV or HBV) infection or liver cirrhosis for hepatocelluar carcinoma

(HCC). Detection and quantification of environmental carcinogens bound to DNA in human peripheral blood leukocytes or other tissues are also possible indicators of high-risk. DNA adducts of aflatioxin B_1 (1), 4-aminobiphenyl or benz(a)pyrene (2) have been found in human tissues and those of some cancer therapeutic agents such as mitomycin C were also detected (3). DNA methylation is also extensively investigated in cancer patients and smokers (4, 5).

Along with environmental factors the involvement of genetic factors is now well documented (6). Not only for well-established hereditary forms of cancer such as bilateral retinoblastoma, familial adenomatous polyposis or xeroderma pigmentosum but also for some common cancers, relevant genes including HNPCC-related or BRCA genes (7, 8) have been found. Genetic variations of some enzymes involved in the metabolism of chemicals such as cytochromes, N-acetyltransferases, glutathione S-transferases and aldehyde dehydrogenases are also thought to affect the development of some cancers (9).

p53 tumor suppressor gene shows many somatic mutations in many cancers (10, 11). Germ line alterations (genetic polymorphism) of this gene are also closely related to certain types of cancers as in the Li-Fraumeni syndrome patients (12). Genetic polymorphisms of this gene at various sites are also present in cancer patients not categorized as Li-Fraumeni syndrome (13-15). We have analyzed polymorphisms of p53 gene and others involved in the metabolism of chemicals in cases and controls enrolled in a case control study for HCC done in Hyogo Prefecture where the incidence of this cancer is relatively high. The aim of this study was to identify possible genetic predictors of the high-risk group for hepatocellular carcinoma.

MATERIALS AND METHODS

Subjects

From the patients and hospitals controls enrolled in a case-control study for HCC done in Hyogo Prefecture, 68 cases (51 males and 17 females) and the same number of controls matched for sex and age ($\pm$5 years) who did not have cancer and were not infected with HBV or HCV were selected. Mean age $\pm$S.D. were 61.6$\pm$8.1 years for cases and 60.4$\pm$9.2 years for controls. Peripheral blood was drawn from selected subjects after obtaining their oral informed consent.

This case-control study was done to evaluate the state of hepatitis virus

infection; dietary, drinking and smoking habits; other life styles and the genetic factors that control drinking and the metabolism of chemicals including genetic variants of aldehyde dehydrogenase (ALDH) 2 and glutathione S-transferase (GST) M1. The results of the study will be publish elsewhere.

Analysis of polymorphisms

Genomic DNA was extracted from peripheral blood by the phenol-chloroform method. Polymorphisms in exon 4 (CCC vs. CGC, Pro vs. Arg at codon 72; Bst U1 polymorphism) (16), intron 2 (C vs. G at nucleotide 38) (17), intron 3 (C vs. A at nucleotide 65), intron 3-16 base pair repeat (at nucleotides 24 to 39) (18) and intron 7 (C and T vs. T and G at nucleotides 72 and 92) (19) of p53 gene were analyzed by the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method. And the polymorphism of intron 6 (A vs. G at nucleotide 62; *Msp* I polymorphism) (20) was analyzed by the PCR-restriction fragment length polymorphism method using Msp I. Primers used for amplification of exon 4 were obtained from CLONTECH Laboratories, Inc. (California, USA) and those for introns were designed in the laboratory of the first author and synthesized at Life Technologies, Inc. (Maryland, USA). To confirm the polymorphisms, some PCR products were extracted from the gel and directly sequenced in an ABI PRISM^{TM} 310 sequencer (Perkin-Elmer Corporation, Connecticut, USA). Details of the procedures will be published elsewhere.

Designation of alleles

Allele designation was based on previous publications; the A1 allele is designated for rare types in exon 4 (CCC or Pro) (21), intron 2 (C) (17) and intron 6 (A) (20), and the A1 allele for wild types in the intron 3-16 bp repeat (absence) (18) and intron 7 (C and T) (22). For the polymorphism in intron 3 found in the present study, the A1 allele is designated for wild type (C).

Statistical analysis

Genotype distributions were examined for significance in departure from Hardy-Weinberg equilibrium. Haplotype frequencies were estimated according to the principles outlined by Hill; i.e., the genotype frequency of the double-heterozygote class was computed from other classes (23). The exact test of the matched pair method based on sex and age ($\pm$5 years) was used for the statistical analyses of the case-control studies and odds ratios (OR) and the 95% confidence intervals (95% CI) were

estimated.

RESULTS

Summary results of the case-control study

Preliminary results with all the 108 cases (89 males and 19 females) and 93 controls (63 males and 30 females) enrolled in the study showed that significantly positive correlation with HCC was found with a history of blood transfusion in males and females, family history of HCV hepatitis in males and bachelorhood in males. Apparent but not significant correlation was detected with heavy drinking. No difference was found with ALDH2 genotype distribution between cases and controls. Of 75 cases and 98 controls, the numbers with null type of GSTM1 were 46 (61%) and 45 (46%) respectively, and these were significantly different with the odds ratio of 1.86 and 95% CI of 1.01-3.44 (to be published). In the 68 cases subjected to the p53 polymorphism analysis, 57 (84%) were positive for HCV antibody and only 5 were positive for HBs antigen.

Results on p53 polymorphism

PCR-SSCP showed a novel polymorphism in intron 3. Direct sequencing of the intron revealed that it had A instead of C at nucleotide 65 intron 3 (data not shown).

Table I shows the genotype distributions of polymorphisms and the A1 allele frequencies in exon 4 and introns 2, 3, 6 and 7. All the data showed a good fit to the Hardy-Weinberg equilibrium, with no significant deviation from equilibrium at the 5% level. When the distribution of each genotype was compared between cases and controls there was no significant difference with any polymorphism. However, when the numbers of alleles of the respective polymorphisms were compared the rare A1 allele was significantly higher in exon 4 of the cases. A similar result was obtained for intron 2, but it was not significant at the 5% level by Fisher's exact test. It was, however, significant at p=0.039 by the chi-square test. No significant differences in the distributions of polymorphism were detected in introns 3 to 7. It should be noted that the distributions of polymorphisms in exon 4 and intron 2 were very close. The A2 allele in the intron 3-16 bp duplication and A1 allele in intron 6 are very rare in the Japanese when compared with the Caucasian, as previously reported (24). Three polymorphisms in exon 4 and introns 2 and 3 showed strong linkage disequilibrium and the same was true for exon 4 and introns 2 and 7. Rare alleles in the intron 3-16 bp

duplication and intron 6 (A2 for the intron 3-16 bp duplication and A1 for intron 6) numbered only three each, but there was strong linkage disequilibrium with each other (data not shown).

Estimated extended haplotype frequencies for the six polymorphisms are shown in Table II. About 95% of the linkages are classified as three haplotypes; 2-2-1-1-2-1, 1-1-1-1-2-2 and 1-1-2-1-2-1. It should be noted that almost all the sites with rare alleles in exon 4 and intron 2 also showed a rare allele in intron 3 or 7, but never at both sites.

Table III shows matched pair analysis with respect to the presence of the 1-1-1-1-2-2 or 1-1-2-1-2-1 allele. The chromosomes individually have rare alleles in exon 4 and introns 2 and 7 or in exon 4 and introns 2 and 3, whereas they have common rare alleles in exon 4 and intron 2. Matching was done by sex and age (±5 years), and the number of pairs counted for presence/absence of the above two types of chromosomes. The number of pairs was 25 for cases (+)/controls (-) and 11 for cases (-)/controls (+), showing a significant difference (p=0.014, OR=2.27, 95% CI=1.08-5.12). The rare alleles (A1) in exon 4 and intron 2 may therefore be associated with high risk of hepatocarcinogenesis.

p53 polymorphisms and HCV genotype

Of the 57 HCV positives among 68 cases, HCV genotypes of 52 were available. The relationship of the p53 polymorphism in exon 4 to the HCV genotype is shown in Table IV. For those with the 2-1 and 2-2 haplotypes, the HCV genotype distributions were almost the same, 1b predominated followed by 2a. This is similar to the distributions reported for Japanese HCV carriers (25). The numbers of 1-1 cases for each HCV genotype were too few to make a statistical correlation. A very similar tendency was found for the p53 polymorphisms in introns 2, 3 and 7, indicating absence of preference of p53 polymorphisms for a specific HCV genotype.

DISCUSSION

HCC in Japan today is caused mostly by HCV infection (26), HBV infection as the direct cause having significantly decreased (27). The drinking of alcohol is involved in producing liver damage and carcinogenesis caused by HCV infection (28). Compared with these extrinsic factors, the intrinsic or genetic factors relevant to hepatocarcinogenesis have yet to be determined. Because evaluation of the genetic

markers that may be involved in hepatocarcinogenesis was the purpose of our case-control study the genetic polymorphism of some enzymes and p53 gene was selected. Polymorphisms of p53 gene in relation to breast, lung, colorectal and cervical cancers have already been investigated (13-15). p53 polymorphism in hepatocellular carcinoma patients, however has not. In this study we found a significant increase in the number of rare alleles in exon 4 (A1) at codon 72 (CCC) and intron 2 (A1) in DNA obtained from peripheral blood leukocytes of HCC patients as compared with the DNA of the control subjects. Although the difference was not very marked between the two groups it may account for the genetic susceptibility of some individuals to hepatocarcinogenesis. Most of the cases in this study were infected with HCV and some with HBV. No marked difference in the distribution of polymorphisms in exon 4 or intron 2 was found for those positive and negative for HCV antibody, but statistical analysis was not possible due to the small number of seronegatives. The control subjects were selected from individuals not infected with these hepatitis viruses, so it was not possible to investigate whether the rare types of polymorphisms in exon 4 (A1) and intron 2 (A1) of the p53 gene work preferentially at certain stages of the infection by or proliferation of hepatitis viruses. We can only speculate that the polymorphisms of the p53 gene may be involved in certain steps in hepatocarcinogenesis triggered by virus infection or other agents. It is reasonable to conclude that the polymorphism in exon 4, rather than that in intron 2, is responsible for this mechanism. Because the polymorphisms in exon 4 and intron 2 were closely linked, the significant correlation of the latter to hepatocarcinogenesis may simply be a phenomenon that derives from this close linkage, although a germ line mutation outside the coding region of p53 may possibly be suggested to affect this gene expression (29).

Significant correlations of the polymorphism at codon 72 (A1 allele, Pro type) in exon 4 have been reported in lung (14) and breast (15) cancers. In uterine cervical cancer associated with human papilloma virus (HPV), the A2 allele or Arg type of polymorphism at this position is reported to predominate over that of the A1 or Pro type and this is explained by the higher susceptibility of the Arg-type p53 protein to degradation by the E6 from HPV (30), but it is important to note that separate observations have also been made (31, 32). In our study reported here, there was no significant correlation for polymorphisms in intron 3, intron 3-16 bp duplication, intron 6 or in intron 7 alone.

The association of the polymorphism in exon 4 or intron 2 with hepatocellular carcinoma was statistically significant but was not of the all or none type. More genetic markers associated with hepatocellular carcinoma may be needed to identify the high-risk group. Even though the association of individual markers with this cancer may not be marked, the association of many combined markers, including the genetic polymorphisms in exon 4 and intron 2, should provide a good indicator of high risk.

ACKNOWLEDGEMENTS

We are indebted to the hospitals that participated in this case control study and provided data on, as well as the blood samples of, the study subjects. This work was supported in part by research grants from the Shinryokukai, the alumni association of Kobe University School of Medicine and from Hyogo Prefecture.

REFERENCES

1. Harrison J. C. and Garner R. C. (1991) Immunological and HPLC detection of aflatoxin adducts in human tissues after an acute poisoning incident in S. E. Asia. Carcinogenesis, **12**, 741-743.
2. Alexandrov K., Rojas M., Geneste O., Castegnaro M., Camus A. M., Petruzzelli S., Giuntini C. and Bartsch H. (1992) An improved fluorometric assay for dosimetry of benzo(a)pyrene diolepoxide-DNA adducts in smokers' lung: Comparisons with total bulky adducts and aryl hydrocarbon hydroxylase activity. Cancer Res., **52**, 6248-6253.
3. Kato, S., Yamashita, K., Kim, T., Tajiri, T., Onda, M. and Sato, S. (1988) Modification of DNA by mitomycin C in cancer patients detected by ^{32}P-postlabeling analysis. Mutation Res., **202**, 85-91.
4. Mustonen, R. and Hemminki, K. (1992) 7-Methylguanine levels in DNA of smokers' and non-smokers' total white blood cells, granulocytes and lymphocytes. Carcinogenesis, **13**, 1951-1955.
5. Kato, S., Bowman, E. D., Harrington, A. M., Blomeke, B. and Shields, P. G. (1995) Human lung carcinogen-DNA adduct levels mediated by genetic polymorphisms *in vivo*. J. Natl. Cancer Inst., **87**, 902-907.
6. Perera, F. P. (1996) Molecular epidemiology: insights into cancer susceptibility,

risk assessment, and prevention. J. Natl. Cancer Inst., **88**, 496-509.

7. Toft, N. J. and Arends, M. J. (1998) DNA mismatch repair and colorectal cancer. J. Pathol., **185**, 123-129.
8. Cornelisse, C. J., Cornelis, R. S. and Devilee, P. (1996) Genes responsible for familial breast cancer. Pathol. Res. Pract., **192**, 684-693.
9. Nyberg, F., Hou, S. M., Hemminki, K., Lambert, B. and Pershagen, G. (1998) Glutathione S-transferase mul and N-acetyltransferase 2 genetic polymorphisms and exposure to tobacco smoke in nonsmoking and smoking lung cancer patients and population controls. Cancer Epidemiol. Biomarkers Prev., **7**, 875-883.
10. Prokocimer, M. and Rotter, V. (1994) Structure and function of p53 in normal cells and their aberrations in cancer cells: projection on the hematologic cell lineages. Blood, **84**, 2391-2411.
11. Strauss, B. S. (1997) Silent and multiple mutations in p53 and the question of the hypermutability of tumors. Carcinogenesis, **18**, 1445-1452.
12. Akashi, M. and Koeffler, H. P. (1998) Li-Fraumeni syndrome and the role of the p53 tumor suppressor gene in cancer susceptibility. Clin. Obstet. Gynecol., **41**, 172-199.
13. Sjalander, A., Birgander, R., Athlin, L., Stenling, R., Rutegard, J., Beckman, L. and Beckman, G. (1995) p53 germ line haplotypes associated with increased risk for colorectal cancer. Carcinogenesis, **16**, 1461-1464.
14. Birgander, R., Sjalander, A., Rannug, A., Alexandrie, A.-K., Sundberg, M. I., Seidegard, J., Tornling, G., Beckman, G. and Beckman, L. (1995) p53 polymorphisms and haplotypes in lung cancer. Carcinogenesis, **16**, 2233-2236.
15. Sjalander, A., Birgander, R., Hallmans, G., Cajander, S., Lenner, P., Athlin, L., Beckman, G. and Beckman, L. (1996) p53 polymorphisms and haplotypes in breast cancer. Carcinogenesis, **17**, 1313-1316.
16. Matlashewski, G. J., Tuck, S., Pim, D., Lamp, P., Schneider, J. and Crawford, L. V. (1987) Primary structure polymorphism at amino acid residue 72 of human p53. Mol. Cell. Biol., **7**, 961-963.
17. Pleasants, L. M. and Hansen, M. F. (1994) Identification of a polymorphism in intron 2 of the p53 gene. Hum. Genet., **93**, 607-603.
18. Lazar, V., Hazard, F., Bertin, F., Janin, N., Bellet, D. and Bressac, B. (1993) Simple sequence repeat polymorphism within the p53 gene. Oncogene, **8**, 1703-1705.

19. Yamada, Y., Yoshida, T., Hayashi, K., Sekiya, T., Yokota, J., Hirohashi, S., Nakatani, K., Nakano, H., Sugimura, T. and Terada, M. (1991) p53 gene mutations in gastric cancer metastases and in gastric cancer cell lines derived from metastases. Cancer Res. **51**, 5800-5805.
20. de la Calle-Martin, O., Romero, M., Fabregat, V., Ercilla, G., Vives, J. and Yague, J. (1990) MspI polymorphism of the human p53 gene. Nucleic Acids Res., **18**, 4963.
21. Harris, N., Brill, E., Shohat, O., Prokocimer, M., Wolf, D., Arai, N. and Rotter, V. (1986) Molecular basis for heterogeneity of the human p53 protein, Mol. Cell. Biol., **6**, 4650-4656.
22. Prosser, J. and Condie, A. (1991) Biallelic ApaI polymorphism of the human p53 gene (TP53). Nucleic Acids Res., **19**, 4799.
23. Hill, W. G. (1974) Estimation of linkage disequilibrium in randomly mating populations. Heredity, **33**, 229-239.
24. Hiyama, K., Takeda, M., Shirotani, Y., Ishioka, S., Awaya, Y., Inai, K., Inyaku, K., Hozawa, S., Inamizu, T. and Yamakido, M. (1994) Duplication polymorphism within the third intron of the p53 gene is a rare event in Japanese population. Jpn. J. Human Genet., **39**, 193-195.
25. Yuki, N., Hayashi, M., Mita, E., Hagiwara, H., Oshita, M., Ohkawa, K., Katayama, K., Kasahara, A., Fusamoto, H. and Kamade, T. (1995) Clinical characteristics and antibody profiles of chronic hepatitis C patients; relation to hepatitis C virus genotypes. J. Med. Virol., **45**, 162-167.
26. Esumi, M. and Shikata, T. (1994) Hepatitis C virus and liver diseases. Pathol. Int., **44**, 85-95.
27. Shiratori, Y., Shiina, s., Zhang, P. Y., Ohno, E., Okudaira, T., Payawal, D. A., Ono-Nita, S. K., Imamura, M., Kato, N. and Omata, M. (1997) Does dual infection by hepatitis B and C viruses play an important role in the pathogenesis of hepatocellular carcinoma in Japan? Cancer, **80**, 2060-2067.
28. Brechot, C., Nalpas, B. and Feitelson, M. A. (1996) Interactions between alcohol and hepatitis viruses in the liver. Clin. Lab. Med., **16**, 273-287.
29. Avigad, S., Barel, D., Blau, O., Malka, A., Zoldan, M., Mor, C., Fogel, M., Cohen, I. J., Stark, B., Goshen, Y., Stein, J. and Zaizov, R. (1997) A novel germ line p53 mutation in intron 6 in diverse childhood malignancies. Oncogene, **14**, 1541-1545.

30. Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I. M., Matlashewski, G. and Banks, L. (1998) Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. Nature, **393**, 229-234.

31. Helland, A., Langerod, A., Johnsen, H., Olsen, A. O., Skovlund, E, Borresen-Dale, A.-L. et al. (1998) p53 polymorphism and risk of cervical cancer. Nature, **396**, 530-532.

32. Klaes, R., Ridder, R., Schaefer, U., Benner, A. and Doeberitz, M. V. (1999) No evidence of p53 allele-specific predisposition in human papillomavirus-associated cervical cancer. J. Mol. Med., **77**, 299-302.

Table I. p53 genotypes and A1 allele frequencies in cases and controls. [a]

Polymorphism		p53 genotype			Allele frequency		χ^2HW[c]
		1-1	2-1	2-2	A1	p[b]	
Exon 4	Cases	12	29	27	0.390	0.038	0.727
	Controls	7	22	39	0.265		1.940
Intron 2	Cases	12	29	27	0.390	0.053	0.727
	Controls	8	21	39	0.272		3.301
Intron 3	Cases	55	11	2	0.890	0.135	2.101
	Controls	61	6	1	0.941		2.806
Intron 3-	Cases	67	1	0	0.993	0.624	0.004
16 bp dupl	Controls	66	2	0	0.985		0.015
Intron 6	Cases	0	1	67	0.007	0.624	0.004
	Controls	0	2	66	0.015		0.015
Intron 7	Cases	36	27	5	0.728	0.322	0.000
	Controls	42	23	3	0.787		0.004

[a] Number of cases and controls was 68 each.

[b] p values are differences between the cases and controls: Fisher's exact test.

[c] Chi-square values for deviation from Hardy-Weinberg proportions.

Table II. Estimated extended haplotype frequencies in six p53 polymorphisms in cases and controls.

Exon 4	Intron 2	Intron 3	Intron 3-16 bp dupl	Intron 6	Intron 7	Cases	Controls
2	2	1	1	2	1	0.610	0.713
1	1	1	1	2	2	0.272	0.184
1	1	2	1	2	1	0.103	0.051
2	2	1	1	2	2	0.000	0.015
1	1	1	1	2	1	0.007	0.007
1	1	2	1	2	2	0.000	0.007
1	1	2	2	1	1	0.007	0.000
1	1	1	2	1	2	0.000	0.007
1	1	1	2	1	1	0.000	0.007
2	1	1	1	2	1	0.000	0.007

Table III. Matched pair analysis with respect to the presence of 1-1-1-1-2-2 or 1-1-2-1-2-1 alleles in the order of exon 4, introns 2 and 3, intron 3-16 bp duplication and introns 6 and 7.

		Controls	
		+	-
Cases	+	15	25
	-	11	17

p =0.014, OR=2.27, 95% CI=1.08-5.12

Exact test of matched pair analysis using the F distribution gave p<0.025, which is significant.

"+" indicates the number of subjects with at least the 1-1-1-1-2-2 or 1-1-2-1-2-1 allele and "-" the number of subjects bearing neither allele.

Table IV. Number of cases showing individual HCV genotypes for each type of polymorphism in exon 4 of p53 gene.

p53 genotype	HCV genotype				
	1b	2a	2b	3	Total
1-1	2	2	1	1	6
2-1	18	4	1	1	24
2-2	16	5	0	1	22