Genomic instability of murine hepatocellular carcinomas with low and high metastatic capacities

Shu-Hui Zhang, Wen-Ming Cong, Jing-Quan Shi, Hong Wei

Shu-Hui Zhang, Wen-Ming Cong, Department of Pathology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China
Jing-Quan Shi, Department of Pathology, Southwestern Hospital, Third Military Medical University, Chongqing 400038, China
Hong Wei, Laboratory Animal Center, Third Military Medical University, Chongqing 400038, China

Supported by the National Natural Science Foundation of China, No. 39900173 and the Major State Key Basic Research Development Program of China, No. G20000161061

Correspondence to: Dr. Shu-Hui Zhang, Department of Pathology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200433, China. zhangshuhui100@sohu.com

RESULTS: Allelic genes on the chromosomes of P cell line with thirty informative microsatellite loci were paralleled to those of inbred strain C3H mouse, while those of F cell line with 28 loci were paralleled to those of inbred strain C57BL/6 mice. The frequency of microsatellite alterations was 37.5% and 42.5% in P cell line and F cell line, respectively. There were different alterations of allelic band 9 at loci between P and F cells, among which, the frequency of microsatellite alterations was most commonly seen on chromosomes 3, 7, 11 and 16.

CONCLUSION: Genomic instability in mouse chromosomes 3, 7, 11 and 16 may play a more important role in the development and progression of HCC in mice. It is suggested that these two sub-clones derived from a same hepatic tumor in homozygous mouse present different genetic features.

Zhang SH, Cong WM, Shi JQ, Wei H. Genomic instability of murine hepatocellular carcinomas with low and high metastatic capacities. World J Gastroenterol 2004; 10(4): 521-524 http://www.wjg.net/v1007-9327/10/521.asp

INTRODUCTION
Hepatocellular carcinoma (HCC) is one of the most frequent human cancers worldwide and has ranked second in China since 1990[1-3]. The development and progression of HCC are considered as a complex process involving genetic alterations, such as chromosomal deletions, chromosomal translocation, point mutations, and gene amplification. These changes can lead to activation of oncogenes or inactivation of tumor suppressor genes at various stages of HCC[4,5]. Genetic instability or genomic instability in human cancers can be divided into two types: microsatellite instability (MSI) which is usually equated with DNA polymerase errors, and chromosomal instability or loss of heterozygosity (LOH) which can result from errors in chromosome partitioning. Both LOH and MSI are considered as phenotypes of genomic instability[6,7]. LOH is frequently observed on chromosomes 1p, 4q, 5q, 8p, 8q, 9p, 10q, 11p, 13q, 14q, 16q, 17p and 22q in HCC, suggesting that tumor suppressor genes may take part in hepatocarcinogenesis[8-10]. MSI and mutations of defective mismatch repair genes can occur in hepatocytes in some chronic hepatitis, cirrhosis and HCC[11-13]. Inbred strain mice provide the guarantee to study on comparison, reliability and accuracy of molecular genetics in neoplasms, because of their characteristics such as high genetic stability, phenotypic uniformity and homozygous alleles. Moreover, it is very valuable to understand the various molecular changes of development and progression in carcinogenesis[14,15]. We examined genomic instability with microsatellite markers at 40 loci on four chromosomes in HCC with low and high metastatic capacity in mice and analyzed the association of microsatellite alterations and metastatic abilities, in order to provide experimental data for finding new tumor suppressor genes and metastasis associated genes.

MATERIALS AND METHODS
Hepatocellular carcinoma cell lines Hca/A2-P (P) and Hca/163-F(F) with low and high metastatic capacity were used in this study. Two cell lines were routinely cultured in 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C with 5% CO2. Inbred strain C57BL/6 mice were provided by Sino-British SIPPR/BK Laboratory Animal Center (Shanghai, China).

DNA extraction
Genomic DNA was extracted from cancer cells and normal liver tissues of inbred C57BL/6J mice using the standard phenol/chloroform extraction and ethanol precipitation methods[16]. Briefly, normal tissues and cancer cells were incubated with 2 ml lysis/digestion buffer (1% sodium dodecyl sulfate, 1 mM EDTA, 50 mM Tris at pH 8.5, and 100 µg proteinase K/ml) at 52 °C for 16 h. The digested lysate was subjected to two further extractions with an equal volume of chloroform:phenol:isoamyl alcohol (24:25:1). After centrifugation, DNA was precipitated from the aqueous phase by two volumes of cold absolute ethanol and collected with a glass rod[17]. The DNA was further purified with RNase digestion, two steps of phenol/chloroform extractions, and precipitated and collected as described above. The concentration of DNA was determined with both spectrophotometric and fluorometric methods.

Microsatellite markers and polymerase chain reaction
The characteristics of microsatellite loci used in this study are
shown in Table 1. The polymerase chain reaction mixture contained more than 20 ng of genomic DNA, 200 µmol/L of each dNTP, 1.5 mM MgCl₂, 0.5 units of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA), 0.5 µmol/L of each primer, and 10×AmpliTaq Gold PCR buffer in a final volume of 10 µL. After denaturation at 94°C for 12 minutes, DNA amplification was performed for 15 cycles of 94°C for 30 seconds, 63°C for 60 seconds (decreased 0.5°C of each cycle), and 72°C for 90 seconds, and then for 25 cycles of 94°C for 30 seconds, 56°C for 60 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 10 minutes. PCRs were run in a Biometra thermocycler (Biometra, Germany). The PCR products were electrophoresized on 8% denatured polyacrylamide gel under a constant voltage of 30 V/cm for simple sequence length polymorphism (PCR-SSLP) analysis. The gel was stained with silver staining after electrophoresis.

Identification of genomic instability

We scored only the bands in cancer cells by preceding or succeeding compared with those in normal samples. Loss or gain of band(s) and clearly detectable changes in intensity were scored[20]. Scoring was done by two independent observers. A change of band intensities was defined as an increase or decrease of the signal intensity by ≥50% in tumor DNA compared to normal DNA by gray scanning function in an image analysis software.

RESULTS

Microsatellite alterations in HCC cell lines with low and high metastatic capacity in mice

Genomic instability was examined using 40 microsatellite markers spanning 4 chromosomes in HCC with low and high metastatic capacity in mice. The results showed that thirty

| Locus name | Position (cM) | Allele size (bp) | Annealing (°C) | Hum homology region | Hca/ A2-P | Hca/ 16A 3-F |
|------------|--------------|------------------|----------------|---------------------|----------|-------------|
| D3Mit21    | 14.2         | 218              | 58             | 3q24-q28            | additional band | additional band |
| D3Nds2     | 23.1         | 115              | 60             | 4q26-q28            | additional band | additional band |
| D3Mit22    | 25.1         | 240              | 55             | 4q25-q28            | 240       | 240         |
| D3Mit13    | 42.6         | 220              | 58             | 1q21                | 230       | 230         |
| D3Mit15    | 45.9         | 145              | 55             | 1p36-q12,1q23-31    | 145       | 145         |
| D3Mit16    | 45.9         | 186              | 53             | 1p36-q12,1q23-31    | 186       | 186         |
| D3Mit17    | 50.3         | 180              | 54             | 1p13-p22            | loss      | additional band |
| D3Mit18    | 54.6         | 214              | 57             | 4q28-q31            | loss      | loss         |
| D3Mit19    | 66.7         | 176              | 60             | 4q25-q28            | 176       | 176         |
| D3Mit147   | 79.4         | 134              | 60             | 1p31                | 134       | 134         |
| D7Mit20    | 5.5          | 107              | 62             | 19q13.2             | loss      | loss         |
| D7Mit18    | 25.1         | 120              | 62             | 11p15-p14           | shifted   | shifted      |
| D7Mit16    | 29.5         | 248              | 64             | 15q11-q13           | 248       | 248         |
| D7Mit17    | 37.2         | 162              | 56             | 15q24-q26           | shifted   | additional band |
| D7Mit19    | 31.7         | 135              | 60             | 15q14               | 135       | 135         |
| D7Mit10    | 62.3         | 150              | 62             | 10q24-q26           | 150       | 150         |
| D7Mit12    | 62.3         | 197              | 56             | 10q24-q26           | 197       | 197         |
| D7Mit13    | 62.3         | 195              | 60             | 10q24-q26           | 195       | 195         |
| D7Mit14    | 64.5         | 147              | 58             | 10q24.3-ter         | 147       | 147         |
| D7Mit15    | 66.7         | 138              | 62             | 10q26               | loss      | loss         |
| D11Mit1    | 2.2          | 153              | 58             | 7p13-p11            | 153       | 153         |
| D11Mit2    | 4.4          | 140              | 58             | 7p                   | 140       | 140         |
| D11Mit4    | 36.1         | 242              | 58             | 17p13-p11           | loss      | loss         |
| D11Mit5    | 36.1         | 188              | 55             | 17p13-p11           | shifted   | loss         |
| D11Mit7    | 40.4         | 144              | 64             | 17p13               | loss      | loss         |
| D11Mit8    | 42.6         | 155              | 59             | 17p13               | loss      | loss         |
| D11Mit10   | 64.5         | 100              | 55             | 17q11-qter          | 100       | 100         |
| D11Mit13   | 65.6         | 162              | 63             | 17q24-q25           | 162       | 162         |
| D11Mit11   | 72.1         | 238              | 64             | 17q11-qter          | additional band | 238 |
| D11Mit12   | 75.4         | 147              | 59             | 17q25               | 147       | 147         |
| D16Mit9    | 4.0          | 132              | 58             | 16q13               | 132       | 132         |
| D16Mit1    | 17.5         | 106              | 61             | 22q11               | 106       | 106         |
| D16Mit2    | 17.5         | 189              | 57             | 22q11               | 189       | loss        |
| D16Mit3    | 23           | 100              | 59             | 3q11-q13            | 100       | loss        |
| D16Mit4    | 25.1         | 123              | 58             | 3q13                | shifted   | additional band |
| D16Mit5    | 32.8         | 160              | 59             | 3q21-qter           | 160       | loss        |
| D16Mit6    | 45.9         | 190              | 59             | 3p11                | 190       | 190         |
| D16Mit7    | 45.9         | 162              | 60             | 3p11                | loss      | loss        |
| D16Mit225  | 58           | 198              | 58             | 21q22               | 198       | 198         |
| D16Mit71   | 69.2         | 154              | 58             | 21q22               | loss      | loss        |

*Data from GenBank.
microsatellite loci were same as that of C3H mice, they were D3Mit15, D3Mit16, D3Mit19, D3Mit22, D4Mit1, D4Mit2, D7Mit10, D7Mit12, D7Mit13, D7Mit14, D7Mit16, D7Mit19, D7Nds2, D11Mit1, D11Mit2, D11Mit10, D11Mit12, D11Mit13, D15Mit17, D15Nds1, D16Mit1 and D16Mit7. The results of microsatellite alterations in mouse HCC cell lines are shown in Table 1. Examples of genomic instability in hepatocellular carcinomas with low and high metastatic capacity in mice by PCR-SSLP method are shown in Figure 1. The frequency of microsatellite alterations on chromosomes 3, 7, 11 and 16 was 40%, 40%, 50% and 20% in P cell line. The frequency of microsatellite alterations on chromosomes 3, 7, 11 and 16 was 40%, 40%, 40% and 50% in F cell line. Microsatellite alterations at D3Mit18, D3Mit21, D3Nds2, D7Mit15, D7Mit18, D7Mit20, D11Mit4, D11Mit7, D11Mit8 and D16Mit7 in P cell line were same as those in F cell line. Microsatellite alterations at D3Mit17, D7Mit17, D11Mit5, D15Nds2, D16Mit2, D16Mit3, D16Mit4 and D16Mit5 in P cell line were different from those in F cell line. These results suggested that there were different genetic alterations in hepatocellular carcinoma (HCC) cell lines with high and low metastatic capacity derived from a homozygous mouse hepatic tumor.

Homologous regions between mouse and human chromosomes have been defined[21]. Mouse chromosome 3 is syntenic to certain regions of human chromosomes 1p13-p22, 1p36-q12, 1q23-31, 3q24-q28, 4q25-q31 and 8. Previous studies suggested that 4q25 and 4q26-27 deletions occurred in human HCC[22,23]. Epidermal growth factor locus is located at the former region. Cyclin A and interleukin-2 loci are located at the latter region, which has a HBV integrated position. Mouse chromosome 7 is syntenic to certain regions of human chromosomes 10q26-ter, 11p15, 15q and 19q13. High frequencies of loss of heterozygosity (LOH) were observed on chromosome 10q26 in human hepatocellular carcinoma[24]. Ras and Bax genes are located at 1.9cM distance from D7Mit18 locus. More than 50% of colon adenocarcinomas with human microsatellite mutator phenotype examined were found to have frameshift mutations in a tract of eight deoxyguanosines [(G)8] within BAX, a gene that promotes apoptosis[25]. Allelic loss and mutation of Ha-ras were seen in a variety of human and mouse tumors[26,27]. Mouse chromosome 11 is syntenic to certain regions of human chromosomes 17p13 and 17q21-qter. Previous studies have shown that high frequencies of LOH were found on chromosomes 17p13.1 and 17p13.3 in human HCC[28]. Some studies have demonstrated LOH at the p53 locus on chromosome 11 and mutational inactivation of the remaining p53 allele in a significant percentage of carcinomas[29], which also harbored for BRCA1 tumor suppressor gene and candidate suppressor protein[30,31]. In the present study, we found that the allelic loss at D3Mit18, D7Mit15, D7Mit20, D11Mit4, D11Mit7 and D11Mit8 on 17p13 was present in P and F cells. We found an extra band at D3Mit21 (IL-2 locus), D3Nds2 (EGF locus) and D7Mit18 in P and F cells. These results suggested that chromosomes 3, 7 and 11 might carry candidate tumor suppressor genes, and play important roles in the development of HCC in mice. Additionally, allelic loss occurred at D3Mit17 of P cells, while extra band appeared in F cells. Band shifting was present at D7Mit17 locus in P cells, while extra band appeared in F cells, suggesting that different

DISCUSSION

In the present study, we found that the frequent genomic instability on chromosomes 3, 7, 11 and 16 existed in hepatocellular carcinoma (HCC) cell lines Hca/A2-P(P) and Hca/163-F(F) with low and high metastatic capacity, both of which were derived from an inbred C3H mouse. Microsatellite alterations at D3Mit18, D3Mit21, D3Nds2, D7Mit15, D7Mit18, D7Mit20, D11Mit4, D11Mit7, D11Mit8 and D16Mit7 in P cell line were same as those in F cell line. These results suggested that there were tumor suppressor genes in these points, inactivation of them might play an important role in the development of HCC in mice. Microsatellite alterations at D3Mit17, D7Mit17, D11Mit5, D15Nds2, D16Mit2, D16Mit3, D16Mit4 and D16Mit5 in P cell line were different from those in F cell line. These results suggested that there were different genetic alterations in hepatocellular carcinoma (HCC) cell lines with high and low metastatic capacity derived from a homozygous mouse hepatic tumor.
sub-clones of the same tumor have heterogeneity.

Mouse chromosome 16 is syntenic to certain regions of human chromosomes 3q21-ter, 12q, 16p, 21q and 22q. It was reported that human chromosome 3q carried a tumor suppressor gene for osteosarcoma, because this chromosomal region was frequently deleted in this type of tumors[32]. LOH was frequently observed on 22q in HCC[33]. Mafune et al[34] found that mouse chromosome 16 was deleted in a clone of mouse fibrosarcoma 505 cells, suggesting that chromosome 16 may have a cognate of such candidate tumor suppressor genes and hence deletion of the region might confer malignancy related with metastasis. In the present study, genomic instability at D16Mit2, D16Mit3, D16Mit4 and D16Mit5 was found in mouse HCC with a high metastatic capacity. It was suggested that genes in chromosome 16 were closely related to tumor metastasis, their mutation could result in metastasis of HCC. Band shifting occurred at 505 cells, suggesting that chromosome 16 may have a cognate region of the region might confer malignancy related with metastasis.

In conclusion, genomic instability in mouse chromosomes 3, 7, 11 and 16 may play an important role in the development and progression of HCC in mice.

REFERENCES

1. Wu MC, Shen F. Progress in research of liver surgery in China. World J Gastroenterol 2000; 6: 773-776
2. Tang ZY. Hepatocellular carcinoma--cause, treatment and metastasis. World J Gastroenterol 2001; 7: 445-454
3. Bosch FX, Ribes J, Borras J. Epidemiology of primary liver cancer. Semin Liver Dis 1999; 19: 271-285
4. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002; 31: 339-346
5. Feitelson MA, Sun B, Satiroglu Tufan NL, Liu J, Pan J, Lian Z. Genetic mechanisms of hepatocarcinogenesis. Oncogene 2002; 21: 2593-2604
6. Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. Proc Natl Acad Sci U S A 2003; 100: 776-781
7. Kawai H, Suda T, Aoyagi Y, Isokawa O, Mita Y, Waguri N, Kuroiwa T, Igarashi M, Tsukada K, Mori S, Shimizu T, Suzuki Y, Abe Y, Takahashi T, Nomoto M, Asakura H. Quantitative evaluation of genomic instability as a possible predictor for development of hepatocellular carcinoma: comparison of loss of heterozygosity and replication error. Hepatology 2000; 31: 1246-1250
8. Buendia MA. Genetics of hepatocellular carcinoma. Semin Cancer Biol 2000; 10: 185-200
9. Nagai H, Pineau P, Tiollais P, Buendia MA, Dejean A. Comprehensive allelotyping of human hepatocellular carcinoma. Nat Genet 1997; 14: 2927-2933
10. Li SP, Wang HY, Li QJ, Zhang CQ, Fung QS, Huang P, Yu XJ, Huang LX, Li Yang W, Zeng XG. Genome-wide analyses on loss of heterozygosity in hepatocellular carcinoma in Southern China. J Hepatol 2001; 34: 840-849
11. Dore MP, Realdi G, Mura D, Onida A, Massarelli G, Dettori G, Hiroshi K, Atamura T, Momoi J, Kim YS, Konishi N, Matsuoka K. Genetic aberrations in early stage hepatocellular carcinomas: a study based on PCR.Liver 1993; 13: 259-261
12. Chou YH, Chung KC, Jeng LB, Chen TC, Liaw YF. Frequent allelic loss on chromosomes 4q and 16q associated with human hepatocellular carcinoma in Taiwan. Cancer Let 1998; 123: 1-6
13. Nagai H, Poniglittikmongkol M, Fujimoto J, Yamamoto H, Kim YS, Konishi N, Matsuoka K. Genetic aberrations in early stage hepatocellular carcinomas. Cancer 1998; 82: 454-461
14. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, Peruch M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science 1997; 275: 967-969
15. Lunnickzky Y, Antal S, Unger E, Wunderlich L, Hidvegi E, Safrrany G. Carcinogenic alterations in murine liver, lung, and uterine tumors induced by in utero exposure to ionizing radiation. Mol Carcinog 1998; 21: 100-110
16. Rashid A, Wang JS, Qian GS, Lu BX, Hamilton SR, Groopman JD. Genetic alterations in hepatocellular carcinomas: association between loss of chromosome 4q and p53 gene mutations. Br J Cancer 1999; 80: 59-66
17. Zhao X, Li J, He Y, Lan F, Fu L, Guo J, Zhao R, Ye Y, He M, Chong W, Chen J, Zhang L, Yang N, Xu B, Wu M, Du G, Gu J. A novel growth suppressor gene on chromosome 1p7p13.3 with a high frequency of mutation in human hepatocellular carcinoma. Cancer Res 2001; 61: 7383-7387
18. Fuji H, Bied MA, Zhou W, Weitzenman SA, Baylin SB, Gabrielsson E. Methylation of the HIC-1 candidate tumor suppressor gene in human breast cancer. Oncogene 2000; 16: 2159-2164
19. Jepe ER, Liu XT, Kiehlbauch JL, Mcclung JK, Dell’Orco RT. Prognostic in breast cancer cell lines: loss of antiproliferative activity is linked to 3’ untranslated region mutations. Cell Growth Differ 1996; 7: 871-878
20. Sato T, Sakamoto T, Takita K, Saito H, Okui K, Nakamura Y. The human prohibitin (PHB) gene family and its somatic mutations in human tumors. Genomics 1993; 17: 762-764
21. Kruzelock RP, Murphy EC, Strong LC, Naylor SL, Hansen MF. Localization of a novel tumor suppressor locus on human chromosome 17p13.3 with a high frequency of mutation in human hepatocellular carcinoma. Cancer Res 1997; 57: 105-106
22. Takahashi K, Kudo J, Ishibashi H, Hirata Y, Niho Y. Frequent loss of heterozygosity on chromosome 22 in hepatocellular carcinoma. Hepatology 1993; 17: 794-799
23. Mafune Y, Asakura H, Komarni R. Altered losses and metastatic ability of mouse tumor cell clones derived from a heterozygous mouse. Oncogene 1994; 9: 2191-2196

Edited by Xu JY and Wang XL