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• LIVER CANCER •

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

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Abstract

AIM: To investigate the frequency of genomic instability in murine hepatocellular carcinoma (HCC) cell lines Hca/A2-P (P) and Hca/163-F(F) with low and high metastatic capacity, and to explore its association with the occurrence and metastasis of hepatocellular carcinomas.

METHODS: Forty microsatellite markers were randomly selected to examine P and F cells for genomic instability using PCR-simple sequence length polymorphism (PCR-SSLP) analysis.

RESULTS: Allelic genes on the chromosomes of P cell line with thirty informative microsatellite loci were paralleled to those of inbred strain C_3H mouse, while those of F cell line with 28 loci were paralleled to those of inbred strain C_3H 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.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent human cancers worldwide and has ranked second in China since 1990s[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[11-15]. 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[16,17]. 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 altertions 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/16-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℃ with 5% CO_2. Inbred strain C_3H 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 C_3H mice using the standard phenol/chloroform extraction and ethanol precipitation methods[18]. 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℃ 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[19]. 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 $MgCl_2$, 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℃ for 12 minutes, DNA amplification was performed for 15 cycles of 94℃ for 30 seconds, 63℃ for 60 seconds (decreased 0.5℃ of each cycle), and 72℃ for 90 seconds, and then for 25 cycles of 94℃ for 30 seconds, 56℃ for 60 seconds, and 72℃ for 90 seconds, with a final extension at 72℃ 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

Table 1 Characteristics of microsatellite loci and summary of microsatellite alterations in mouse HCC cell lines

Locus name	Position (cM)[a]	Allele size (bp)[a]	Annealing (℃)	Hum homology region[a]	Hca/A2-P	Hca/16A3-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	loss
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

[a]Data from GenBank.

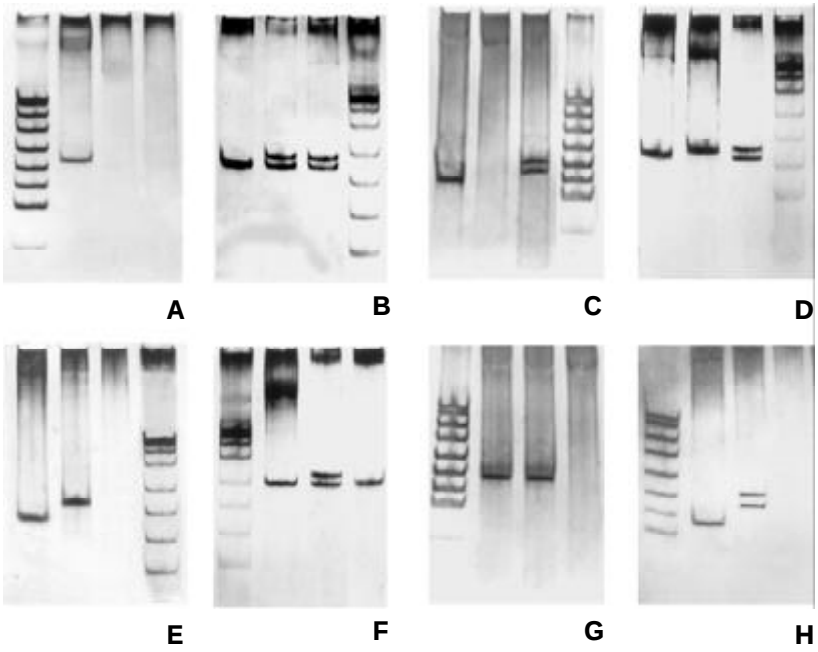

Figure 1 Genomic instability in murine hepatocellular carcinomas with low and high metastatic capacity, PCR-SSLP. A: D3Mit18, B: D3Nds2, C: D3Mit17, D: D7Mit17, E: D11Mit5, F: D11Mit11, G: D16Mit5, H: D16Mit4.

microsatellite loci were same as that of C_3H 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 was 37.5% in P cell line. 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 was 42.5% in F 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. In contrast to inbred strain C_3H mice, the allelotype sizes of 25 microsatellite loci were same as those of C_3H mice, 8 loci with loss of alleles, extra bands showing at 4 loci and band shifting at 3 loci in P cell line, while 23 loci were same as that of C_3H mice, allele loss at 11 loci, extra bands showing at 4 loci and band shifting at 1 locus in F cell line.

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 C_3H 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.

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 humen and mice tumors[26]. 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[27,28]. 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 prohibitin[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

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 D16Mit4 locus in P cells, allelic loss was found in F cells due to continuous genomic instability during the development and progression of tumors. Genomic instability at some chromosomes of HCC in mice were studied and some loci related to hepatocarcinogenesis and progression were obtained which might be of great significance in searching new tumor associated genes.

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.

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