RESEARCH

Novel Susceptibility Locus for Mouse Hepatomas: Evidence for a Conserved Tumor Suppressor Gene

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We have identified previously a putative tumor suppressor gene (TSG) locus at human chromosome (hchr) 7q31 showing that it is altered in a variety of human epithelial tumors. To determine whether this TSG is conserved in mice, we studied loss of heterozygosity (LOH) in chemically induced mouse liver adenomas. The LOH analysis was performed by polymerase chain reaction amplification of 17 (CA)_n microsatellite repeats on mouse chromosome (mchr) 6 A2–C3. Ninety-six of 106 cases (90.6%) had LOH at D6Mit50, and 89.5% had LOH at D6Mit79. These two loci are 0.2 cM apart on mchr 6A2. Another high-LOH site was found in the C3 band. The high incidence of LOH in the 7q-homologous segment of mchr 6 indicates that the human TSG is conserved and is involved in the development of hepatomas.

Although tumor suppressor genes (TSGs) have been studied extensively in human tumors, they have also been shown to be of importance in animal cancer models. All known TSGs have been mapped to mouse chromosomes (mchrs) that are homologous with the human chromosomes (hchrs) that bear the corresponding human loci that code for proteins highly conserved across species (Lehman et al. 1991; Weinberg 1991; Conti et al. 1992; Bowden et al. 1994). For instance, we demonstrated by microcell fusion transfer that introduction of a single hchr 7 can delay the onset of tumors by twofold to threefold and in some cases even repress completely the tumorigenicity of a murine squamous cell carcinoma cell line (Zenklusen et al. 1994a).

We also reported that loss of heterozygosity (LOH) at 7q31.1–q31.2 is a very common event in a variety of human epithelial tumors (Zenklusen et al. 1994b,c, 1995a,b). These studies supported cytogenetic reports indicating that deletions of hchr 7 are common in many different human tumor types, including ovarian, head and neck, gastric, and hemopoietic tumors (Kere et al.

1989; Atkin and Baker 1991, 1993; Osella et al. 1992; Abrahamson et al. 1993).

The mouse hepatic carcinogenesis model has been used by a number of groups as an experimental model for neoplasia (Strom and Faust 1990; Ward et al. 1990; Anderson et al. 1992; Frith et al. 1994; Stanley 1995) and as a bioassay for chemical carcinogens. It is a well-characterized model in which a number of oncogenes (including *c-myc* and *H-ras*) have been shown to be altered frequently (Strom and Faust 1990; Anderson et al. 1992; Stanley 1995), whereas some TSGs commonly affected in human neoplasia (e.g., *p53* and *Rb*) are not altered (Stanley 1995). One possible explanation for this phenomenon is that there are unidentified murine TSGs whose inactivation negates the necessity for *Rb* or *p53* loss (Stanley 1995). This lack of involvement of known TSGs in the mouse liver carcinogenesis model facilitates the study of new TSGs that may be inactivated during chemical carcinogenesis. In the study reported here, we used an infant mouse protocol (Vesselinovich et al. 1975, 1984) because of its simplicity, reproducibility, and uniformity of induced liver tumors. This model has been used previously in carcinogenesis experiments with C57BL/

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To determine whether the broad-range putative TSG on hchr 7 is conserved among species and is involved in mouse hepatocarcinogenesis, we used an extensive set of highly polymorphic markers in the mchr 6 A2–C3 region to assess LOH in tumors induced by B[a]p in the infant mouse carcinogenesis model.

As shown in Figure 2, partial loss of the A2 segment of mchr 6 was a common event in our chemically induced murine hepatomas. LOH occurred in at least one locus in all but one of the tumors assayed. LOH occurred most frequently at D6Mit50 (90.7%) and D6Mit179 (89.5%), which are 0.2 cM apart in the region homologous to hchr 7q distal to the *met* proto-oncogene. In all the cases with LOH assayed, the C3H/HeN allele was retained at this locus. The percentage of LOH in A1-B1, the region of mchr 6 that is homologous to hchr 7q was found to have a Gaussian distribution [by the Kolmogorov-Smirnov test

Differences in B[a]p dose had no significant effect in LOH at any of the loci assayed. However, the B[a]p vehicle and time after treatment (Fig. 3) had significant effects. Although no significant difference was found in the A2 band loci (D6Mit50 and D6Mit179), there were some statistically significant differences in the incidence of allelic loss at the C3 site (D6Mit29). There was a threefold increase in LOH when B[a]p was administered in dimethylsulfoxide (DMSO) (82% of the cases) instead of corn oil (26.5%). Also, there were significant differences ($P < 0.007$) in LOH

Figure 2 Schematic of mchr 6 bands A–C with approximate positions of microsatellite repeat markers used in this study (Copeland et al. 1993; Dietrich et al. 1994), with Kosambi's centiMorgan distances from the centromere indicated by the number at right. The parallel lines indicate homology with hchr regions. The histogram shows percentage LOH for each of the microsatellite markers used.

incidence at this locus at different times after treatment, the percentage of allelic loss at 39 weeks (46.0%) after treatment was lower than the percentage after 52 weeks (60.0%).

DISCUSSION

We assayed for LOH on mchr 6 in 112 chemically induced hepatomas in C57BL/6N × C3H/HeN mice. High frequencies of LOH were found at D6Mit50 (90.7%), D6Mit179 (89.5%), and D6Mit29 (54.9%). The first two markers map to mchr 6A2, which is homologous to hchr 7q31, and the third maps to mchr 6C3.

The high frequency of LOH at mchr 6A2 in C57BL/6N × C3H/HeN tumors indicates that a TSG relevant to development of hepatic adenomas is located within this region. We reported previously (Zenklusen et al. 1994b,c, 1995a,b) that the hchr 7q31.1 region is frequently lost in several human neoplasias of epithelial origin. As hchr 7q31 is homologous to mchr 6 A2, it is pos-

sible that these observations reflect inactivation of a putative TSG for epithelial tumors that has been conserved among mammalian species. The correlation between sites is supported by the conserved positions of markers on hchr 7 and mchr 6. As shown in Figure 4, the relative position and order of the three commonly used anchors for the long arm of hchr 7 (*COL1A2* at 7q22, *met* at 7q31, and *TCRB* at 7q35) are conserved in mchr 6, allowing us to infer that this segment of mchr 6 closely resembles hchr 7q22–q35. The TSG site we describe in this study is located between *met* and *TCRB*, close to the *met* locus (*met* is 6 cM from the centromere, whereas D6Mit179 and D6Mit50 are at 6.1 cM and 6.3 cM, respectively). In humans, the putative TSG locus at 7q31.1 has also been found to be close to the *met* oncogene between *met* and *TCRB* (Zenklusen et al. 1994b,c, 1995a,b), suggesting that the two putative TSG sites (in humans and mice) are the same and therefore are conserved in these species.

We cannot speculate on relevance of the other region (6C3) that presented a LOH score >50% because of the lack of information on this segment. However, owing to its distance to the F2 band, this region is different from the *Pas-1* locus described in murine lung carcinomas (Gariboldi et al. 1993) and warrants further study to determine if it contains any other putative TSGs.

It is interesting that the two loci described in this paper seemed to be very different. The A2 locus was affected at a very high rate early in carcinogenesis (as the high LOH at 39 weeks suggests) and did not appear to be altered further later (as the unchanged LOH rate at 52 weeks indicated). Therefore, this site seems to be important in the onset of hepatic neoplasia, as it is in other human epithelial tumors (Zenklusen et al. 1994b,c, 1995a,b). Neither the vehicle nor the B[a]p dose affected the incidence of allelic loss at the A2 site. In contrast, the C3 locus appeared to be sensitive to both vehicle and time after treatment (Fig. 3). The higher incidence of LOH later in the carcinogenesis suggests that this site probably harbors a gene important in tumor progression but not essential for tumor initiation. The differences in LOH found when B[a]p was administered in DMSO and in corn oil, suggest that allelic loss at C3 is sensitive to the bioavailability of B[a]p.

It is important to note that although the loci mapped to hchr 7q31.1 homologous region (D6Mit50 and D6Mit179) retained the C3H/HeN allele in all the cases with LOH, the other paren-

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Figure 3 Schematic of mchr 6 bands A–C with approximate positions of microsatellite repeat markers used in this study (Copeland et al. 1993; Dietrich et al. 1994). The parallel lines indicate homology with hchr regions. The histogram shows percentage LOH for each of the microsatellite markers vs. B[a]p vehicle used and time since carcinogen administration (*top*).

tal allele (C57BL/6N) was retained in the cases with LOH at the C3 site (D6Mit29), indicating that the two parental strains differ in susceptibility to the putative TSG inactivation. This evidence further supports the idea that the two susceptibility loci described here are different and that the LOH at the C3 band is independent of the inactivation of the A2 band locus.

It is also interesting that the putative TSG locus that may be related to progression (at C3) retains the allele from the carcinogenesis-resistant strain (C57BL/6N), suggesting that its relative imperviousness to chemical carcinogenesis is conferred at the initiation, more than at the progression, level.

Efforts to clone the hchr 7q31 locus are under way in our laboratory, to identify the putative TSG and determine its function. More data about the C3 band site are needed to establish its relevance to human tumors before it is cloned.

METHODS

Animals

One hundred twelve (C57BL/6N × C3H/HeN) F₁ male

mice were used in these experiments. They were housed in groups of six or less in plastic cages, with unlimited water and food. The animals were divided into six groups; hepatic adenomas were induced in each animal at 15 days of age by a single intraperitoneal injection of either 125, 250, or 375 µg of B[a]p in DMSO or corn oil. One subgroup of each of the six animal groups was killed 39 weeks later, and their livers collected. The remaining animals were killed 52 weeks after treatment. All liver samples were fixed in Statfix (Statpath, Riderwood, MA) and embedded in paraffin. The number of tumors obtained in each subgroup is shown in Table 1.

DNA Extraction from Tumors

Eight-micrometer-thick tissue sections were cut from the paraffin-embedded blocks and placed on glass slides. Unstained sections were microdissected to separate normal and nonneoplastic tissue. As a reference, consecutive serial sections were stained with hematoxylin and eosin. High-molecular-weight DNA was extracted from tumor sections by standard proteinase K digestion followed by Chelex extraction (Ausubel et al. 1992) and ethanol precipitation of the supernatant.

(CA)_n Microsatellite Repeat Amplification Analysis

Seventeen (CA)_n microsatellite repeats on mchr 6 (Copeland et al. 1993; Dietrich et al. 1994) were amplified with

Figure 4 Schematic of mchr 6 (left) and hchr 7 (right) showing anchor genes (in boldface). The numbers at the right of the markers indicate the genetic distances from the centromere on mchr 6 and are derived from the MAPMAKER program (Kosambi's centiMorgans). The thick vertical bar at the right of hchr 7 indicates the smallest common deleted region found in human epithelial tumors and the probable location of the TSG (Zenklusen et al. 1994b,c, 1995a,b).

a Thermocycler 9600 (Perkin-Elmer Cetus, Norwalk, CT). Each 25- μ l reaction mixture contained 2.5 μ l of 10 $\times$ standard PCR buffer (Perkin-Elmer Cetus), 100 ng of DNA, 1 unit of *Taq* polymerase, 400 pM each primer, and 200 mM each dNTP. Hot-start PCR was performed by using a *Taq*

polymerase-specific antibody that inactivates the enzyme and is released during the first denaturation cycle (*Taq* Start; Clontech, Palo Alto, CA). The DNA was amplified with 30 cycles of 20 sec of denaturation at 94°C, 30 sec of annealing at 55°C and 20 sec of extension at 72°C. The number of cycles used was in the linear part of the amplification process (i.e., before product saturation) permitting us to expect equal optical density of both alleles if no LOH had occurred.

The PCR products were separated in a 3.5% MetaPhor agarose (FMC Bioproducts, Rockland, ME) gel by electrophoresis at 5.5 V/cm² for 3 hr in TBE buffer (89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA at pH 7.5) with 0.5 mg/ml of ethidium bromide in TBE and a standard loading buffer (Ausubel et al. 1992). The gel was photographed with a Fotodyne 3-4400 ultraviolet transilluminator (Fotodyne, Inc., New Berlin, WI) and Polaroid Positive-Negative 4 $\times$ 5 Instant Film (Polaroid Corp., Cambridge, MA).

The nonradioactive method used in this study has several advantages over its radioactive counterpart (Kemp et al. 1993; Ah-See et al. 1993; Nawroz et al. 1994) besides avoiding the use of radioisotopes. The PCR products are separated in a horizontal high-resolution agarose gel that allows the screening of 96 reactions in a single electrophoretic run, dramatically increasing the speed of the overall process. The use of this system did not decrease the sensitivity of allele detection, because the type of agarose used resolves, in horizontal runs, fragments only 2 bp different in length. The amount of template we used for the PCR reactions was also within the range used by other groups performing this type of analysis (Kemp et al. 1993; Ah-see et al. 1993; Nawroz et al. 1994).

Allelic-loss Determination

Because in most cases one of the band disappeared completely, the allelic loss of a marker was determined by visual examination by two reviewers blinded to one another's reading. In five cases where a residual band could be seen, we used densitometric measurements. We conducted a series of titrations using different proportions of homozygous and heterozygous samples (inbred parental strain DNA and F₁ DNA) to assess the influence of stromal-tissue contamination of our amplification reactions. We determined that we could detect as little as 30% contamination of heterozygous template in the homozygous DNA (data not shown). In view of these results, we considered a sample to have LOH if an entire band was absent or if one band's intensity was <30% of the normal band's intensity by densitometry (Zenklusen et al. 1994b).

Statistical Analysis

The normality of the percentage LOH distributions was tested by using Kolmogorov-Smirnov continuous cumulative distribution test (Zar 1991).

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Table 1. Distribution of Tumors Obtained in Treatment Subgroups

B[a]P dose	Vehicle	39 Weeks	52 Weeks	Total
125 μ g	DMSO	3	10	13
	Corn Oil	4	9	13
250 μ g	DMSO	7	14	21
	Corn Oil	9	2	11
375 μ g	DMSO	4	26	30
	Corn Oil	11	13	24
Total		38	74	112

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