Alleloype Analysis in Mouse Hepatocellular Carcinomas; Frequent Homozygous Deletion of Mouse Homolog of p16/CDKN2 Gene on Chromosome 4 in Culture

Kazuyoshi Miyasaka, Tetsuya Fukui, and Tomoyuki Kitagawa

Department of Hygienic Chemistry, Hoshi University, 2-4-41, Ebara, Shinagawaku, Tokyo 142, Japan and Department of Pathology, Cancer Institute, 1-37-1, Kamiikebukuro, Toshima-ku, Tokyo 170, Japan.

Received November 7, 1995; accepted December 26, 1995

Because alleloype analysis of many tumors has been important in the identification of new tumor suppressor genes, here we have analyzed hepatocellular carcinomas (HCCs) derived from F1 hybrid mice between C3H and MSM in detail. The analysis showed no allelic loss in primary HCCs, while the loss was detected in tumor cell lines established from HCCs. Recently, a candidate tumor suppressor gene termed p16/CDKN2, which was located near the interferon gene cluster on human chromosome 9p21, was identified by virtue of its frequent homozygous deletion in cell lines derived from many different tumor types. Since frequent allelic imbalances in the D4MT9 locus and loss of heterozygosity in the alpha-interferon gene which was located near the mouse homolg of p16/CDKN2 (mouse p16) gene were detected in tumor cell lines, we investigated homozygous deletion of the mouse p16 gene by the comparative multiplex PCR method. The analysis revealed frequent homozygous deletion of the gene in thirteen of the tumor cell lines (13/25, 52%), but not in primary HCCs (0/25, 0%). These data indicate that gene deletions including the mouse p16 gene on chromosome 4 in tumor cell lines occur during the culture and that allelic imbalances are uncommon in mouse primary HCCs. Our results suggest that mouse p16 plays an important role in mouse hepatocarcinogenesis in vivo in progression or immortalization in vitro.

Key words: hepatocarcinogenesis; mouse homolg; p16/CDKN2; tumor suppressor gene; diethylnitrosamine

Genes which affect cancer growth can be classified into two categories: oncogenes which have positive effects, and tumor suppressor genes which have inhibitory effects on cancer development. Activated oncogenes and/or inactivated tumor suppressor genes are the characteristic genetic alterations and include chromosome duplications, amplifications, deletions and rearrangements. Analyses of these genetic alterations have identified many oncogenes and several tumor suppressor genes have been cloned. The most common oncogenic mutations in human tumors are the ras genes which are found in 10 to 15% of solid tumors, and the most frequently mutated tumor suppressor gene, p53 gene, is mutated in roughly 50% of tumors. More than 100 oncogenes have been characterized, while less than a dozen tumor suppressor genes have been identified to date. The number of the latter is expected to increase to more than 50. A hallmark of tumor suppressor genes including the p53 gene is that they are deleted at high frequency in certain tumor types. The deletions often involve loss of a single allele, so-called loss of heterozygosity (LOH). For LOH, the remaining allele is presumed to be nonfunctional, either because of a preexisting inherited mutation or a secondary somatic mutation.

In human hepatocellular carcinoma (HCC), HCV, HBV and certain oncogenes have been reported as characteristic genetic alterations. The characteristic alterations of tumor suppressor genes, however, are not yet well identified although inactivated p53 gene has been detected only in progressed-liver tumors. It is the authors’ view that part of the growth mechanisms in mouse HCC may conform to the mechanisms of human hepatocarcinogenesis, especially at the early stage of cancer development, so that experimental systems using mouse are essential to elucidate the inactivation mechanism of tumor suppressor gene in HCC. We therefore used C3H/MSM F1 hybrid mice and established an animal model to research the inactivation of tumor suppressor genes in mouse hepatocarcinogenesis. The inbred mouse strain C3H, which presents a high spontaneous incidence of hepatocellular carcinoma, was used as a well-characterized experimental model of hepatocellular carcinoma, and the mouse strain MSM which was inbred at the National Institute of Genetics (Mishima, Japan) is a Japanese wild mouse. To discover the novel HCC-related tumor suppressor genes, we performed the alleloype analysis utilizing microsatellite markers. The microsatellites are simple sequence repeats, often run of (CA)n (n = 10—20) present in very large numbers (>100000 copies) and scattered widely throughout the mouse and other mammalian genomes. Since these repeats often vary in length between inbred strains of mice, the microsatellite markers are frequently used for alleloype analysis of mouse and human cancers. There are now over 4000 polymorphic microsatellites characterized and mapped in the mouse. Since the two mouse strains in the study presented here are evolutionarily 1 million years apart, the degree of polymorphism between C3H and MSM is considerably high. Thus, a total of 100 microsatellite markers were used for alleloype analysis in mouse HCCs and tumor cell lines established from these HCCs.

Recently a candidate tumor suppressor gene termed p16/CDKN2 which was encoded near the interferon gene cluster on human chromosome 9p21—22 was identified by virtue of its frequent homozygous deletion in cell lines derived from many different tumor types in human. Mouse homolg of the p16/CDKN2 gene (mouse p16) is also located near the interferon gene cluster on mouse chromosome 4. Since we reported that the loss of tumor suppressor gene(s) on the C5—C7 portion of mouse chro-

* To whom correspondence should be addressed.

© 1996 Pharmaceutical Society of Japan
mosome 4 plays an important role in mouse hepatocarcinogenesis in progression in vivo and/or in immortalization in vitro. We also studied the homozygous deletion of this gene in mouse HCCs. Here, we communicate our results, which demonstrate a clear difference between the primary tumors and cell lines established from these tumors.

MATERIALS AND METHODS

Animals Male and female MSM mice were provided by the National Institute of Genetics. MSM mice were mated to C3H mice purchased from Charles River Co. (Atsugi, Japan) in our laboratory. Propagated C3H/MSM (n = 16) and MSM/C3H (n = 2) F1 hybrid mice were used (listed in Table 1).

Induction of Liver Tumors Liver tumors were induced by diethylnitrosamine as follows: group A) F1 hybrid mice received a single intraperitoneal i.p. injection of diethylnitrosamine, 20 μg per g of body weight, at week 6 after birth. 3 days subsequent to a single subcutaneous injection of carbon tetrachloride (0.2 μg per g of body weight in 10 μl sesame oil); group B) F1 hybrid mice received diethylnitrosamine (50 ppm) in drinking water from week 6 after birth, the diethylnitrosamine-prescription was repeated for 2 weeks and then there was an interval of one week; group C) F1 hybrid mice received a single i.p. injection of diethylnitrosamine, 20 μg per g of body weight, at week 2 after birth. The mice were then sacrificed at 50 to 70 weeks after birth as described in Fig. 1, and liver tumors larger than 10 mm in diameter were dissected out. Portions of these tumors were fixed in 10% neutral buffered formalin and paraffin-embedded sections were stained with hematoxylin and eosin for histopathological diagnosis to confirm that the tumors were HCCs. An adjacent portion of each tumor was frozen on dry ice and stored at −80°C until the time of analysis.

Primary Culture The remaining portions of tumors were minced into small fragments (1 mm³) and digested with dispase (1000 U/ml) at 37°C for 2 h. The cells were harvested by centrifugation at 1000 rpm for 5 min, and filtered through nylon mesh of 100 μm. Primary culture was done in Waymouth's medium supplemented with 10% fetal calf serum. The medium was first changed at 24 h and then twice a week thereafter. To establish a cell line of homogenous populations, cells of individual colonies were removed separately with a cloning cylinder; this procedure was repeated one to eight times. About two months later, a tumor cell line of homogenous populations was obtained.

Isolation of DNA High molecular weight DNA was isolated from each tumor tissue and tumor cell line by the SDS–proteinase K method as described previously. DNA samples were dissolved in Tris-EDTA buffer and used.

Allelotype Analysis of Microsatellites Microsatellites tested were selected according to previously published information. The primers used were purchased from Research Genetics, Huntsville, and some were kindly provided by Dr. R. Kominami (Niigata University, Niigata, Japan). Standard polymerase chain reactions (PCRs) were performed in a 10 μl volume composed of 100 ng genomic DNA, 0.75 units of Taq polymerase (Promega, Madison), reaction buffer and MgCl₂ concentration specific to each kind of Taq, 170 μM of each nucleotide, and 0.8 μM of each primer. Amplifications were performed with a Perkin Elmer Gene Amp PCR System 9600 (Norwalk, CT) with an initial denaturation step at 94°C for 3 min, followed by 30 to 35 cycles at 94°C for 0.5 min, 58°C for 2 min, and 72°C for 2 min. For some primers, various annealing temperatures between 50 to 65°C were used to increase the yield or specificity. The PCR products were electrophoresed on agarose gels (2.5% NuSieve GTG,

| Table 1. F1 Hybrids Treated by Diethylnitrosamine and Sample Tested |
|---|---|---|---|
| No. | F1 hybrid | Group | Tumor No. | Cell line No. |
| Male | | | | |
| 1 | C3H/MSM F1 | A | 1A | 1a |
| 2 | CH/MSM F1 | 2A,2B | 2a,2b |
| 3 | MSM/C3H F1 | 3A | 3a |
| 4 | MSM/C3H F1 | 4A | 4a |
| 5 | CH/MSM F1 | 5A | 5a |
| 6 | CH/MSM F1 | 6A | 6a |
| 7 | CH/MSM F1 | 7A | 7a |
| 8 | CH/MSM F1 | 8A,8B | 8a,8b |
| 9 | CH/MSM F1 | 9A | 9a |
| 10 | CH/MSM F1 | 10A,10B | 10a,10b |
| Female | | | | |
| 11 | CH/MSM F1 | 11A,11B | 11a,11b |
| 12 | CH/MSM F1 | 12A,12B | 12a,12b |
| 13 | CH/MSM F1 | 13A,13B | 13a,13b |
| 14 | CH/MSM F1 | 14A | 14a |
| 15 | CH/MSM F1 | 15A | 15a |
| 16 | CH/MSM F1 | 16A | 16a |
| 17 | CH/MSM F1 | 17A,17B | 17a,17b |
| 18 | C3H/MSM F1 | B | 18A | 18a |

A total of 25 tumors were induced in 18 F1 hybrids by diethylnitrosamine. Two different tumors were removed from each F1 hybrid of Nos, 2, 8, 10, 11, 12, 13, and 17. Cell lines established from parts of these tumors as described in Materials and Methods.

Group A) Carbon tetrachloride s.c. and Diethylnitrosamine i.p.

Group B) Diethylnitrosamine in drinking water

Group C) Diethylnitrosamine i.p.

Fig. 1. Experimental Protocol
approximately 10 μg of each DNA sample was completely digested with restriction enzyme (50 units of Pst I) in the appropriate buffer. Fragments were separated on 1% agarose gels in Tris–acetate–EDTA buffer; the DNA was then transferred to the nylon filter membranes. LOH was examined with a mouse alpha-interferon gene probe which was kindly provided by Dr. O. Hino (Cancer Institute, Tokyo, Japan). The probe was labeled with [32P]dCTP by random primer extension. Prehybridization and hybridization were performed as described by Kubo et al. The membranes were exposed for autoradiography on Kodak XAR films with intensifying screens at –70 °C for 24 h.

RESULTS

Alleloype Analysis

Alleloype analysis was performed in twenty-five HCCs arising in eighteen F1 hybrid mice.
and twenty-five tumor cell lines established from the
tumor in all autosomes. Histological examination con-
formed that all the analyzed tumors were HCCs (data not
shown). In addition, chromosome X was analyzed in
twelve HCCs and twelve tumor cell lines established from
HCCs in female mice. The microsatellite markers which
were polymorphic between C3H and MSM were chosen
for allelotype analysis, and five loci on each chromosome
as described in Fig. 2 were investigated. To select the
microsatellite markers, each marker was distributed at
maximum intervals below 35 centiMorgan on each chro-
mosome. Whether or not there was an allelic imbalance
or not was determined; for example, in lane 5a of Fig. 3
we judged to be LOH because no MSM-derived allele
was detected in the lane; lanes of “20% normal DNA” and
“80% normal DNA” were judged to be LOH-positive and
LOH-negative, respectively. Some DNAs showed results of
“40 to 60% normal DNA,” and we judged these to be
imbalance in the gene dosage between two alleles rather
than LOH. A representative result of allelotype analysis
is shown in Fig. 4.

![Figure 3](image)

**Fig. 3.** Control PCR Using Mixed DNA Templates Isolated from
Normal Kidney and Sample 5a

Sample 5a shows that loss of whole MSM-derived allele in chromosome 4 was
gradually added to normal DNA isolated from kidney of an F1 hybrid mouse.
Microsatellites near the D4Mit19 locus were PCR-amplified, run on an agarose
gel and photographed as described in “Materials and Methods.”

![Figure 4](image)

**Fig. 4.** Representative Result Showing LOH on Chromosomes

---

**Table 2.** a) Results of Allelotype Analysis in Tumor Cell Lines (Except for Chromosomes 2, 4, 7 and 13)

<table>
<thead>
<tr>
<th>Chromosome 1</th>
<th>Chromosome 8</th>
<th>Chromosome 12</th>
<th>Chromosome 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1Mit14</td>
<td>M*</td>
<td>D8Mit1</td>
<td>M*</td>
</tr>
<tr>
<td>D1Mit18</td>
<td>M*</td>
<td>D8Mit4</td>
<td>M*</td>
</tr>
<tr>
<td>D1Mit8</td>
<td>M*</td>
<td>D8Mit6</td>
<td>M*</td>
</tr>
<tr>
<td>D1Mit15</td>
<td>M*</td>
<td>D8Mit40</td>
<td>—</td>
</tr>
<tr>
<td>D1Mit17</td>
<td>—</td>
<td>C</td>
<td>D8Mit14</td>
</tr>
<tr>
<td>Chromosome 3</td>
<td>Chromosome 9</td>
<td>Chromosome 14</td>
<td>Chromosome 18</td>
</tr>
<tr>
<td>D3Mit54</td>
<td>—</td>
<td>D9Mit2</td>
<td>—</td>
</tr>
<tr>
<td>D3Mit21</td>
<td>M*</td>
<td>D9Mit22</td>
<td>—</td>
</tr>
<tr>
<td>D3Mit7</td>
<td>M*</td>
<td>D9NdS2</td>
<td>C*</td>
</tr>
<tr>
<td>D3Mit12</td>
<td>M*</td>
<td>D9Mit20</td>
<td>—</td>
</tr>
<tr>
<td>D3Mit18</td>
<td>—</td>
<td>D9Mit18</td>
<td>—</td>
</tr>
<tr>
<td>Chromosome 5</td>
<td>Chromosome 10</td>
<td>Chromosome 15</td>
<td>Chromosome 19</td>
</tr>
<tr>
<td>D5Mit1</td>
<td>—</td>
<td>D10Mit2</td>
<td>—</td>
</tr>
<tr>
<td>D5Mit5</td>
<td>—</td>
<td>D10Mit15</td>
<td>—</td>
</tr>
<tr>
<td>D5Mit9</td>
<td>M*</td>
<td>D10Mit7</td>
<td>C*</td>
</tr>
<tr>
<td>D5Mit27</td>
<td>M*</td>
<td>D10Mit10</td>
<td>C*</td>
</tr>
<tr>
<td>D5Mit34</td>
<td>—</td>
<td>D10Mit25</td>
<td>C*</td>
</tr>
<tr>
<td>Chromosome 6</td>
<td>Chromosome 11</td>
<td>Chromosome 16</td>
<td>Chromosome X</td>
</tr>
<tr>
<td>D6Mit1</td>
<td>—</td>
<td>D11Mit1</td>
<td>M</td>
</tr>
<tr>
<td>D6Mit4</td>
<td>—</td>
<td>D11NdS9</td>
<td>M</td>
</tr>
<tr>
<td>D6Mit230</td>
<td>—</td>
<td>D11Mit15</td>
<td>M</td>
</tr>
<tr>
<td>D6Mit11</td>
<td>C</td>
<td>D11Mit10</td>
<td>M</td>
</tr>
<tr>
<td>D6Mit14</td>
<td>—</td>
<td>D11Mit102</td>
<td>M</td>
</tr>
</tbody>
</table>

-- both alleles retained, C, loss of C3H-derived allele. M, loss of MSM-derived allele. * imbalance in the gene dosage between two alleles and the allele indicated "C" or "M" is smaller than the other allele.
Table 2. b) Results of Allelotype Analysis in Tumor Cell Lines in Chromosome 4

<table>
<thead>
<tr>
<th>Allele</th>
<th>1a</th>
<th>2a</th>
<th>2b</th>
<th>3a</th>
<th>4a</th>
<th>5a</th>
<th>6a</th>
<th>7a</th>
<th>8a</th>
<th>8b</th>
<th>9a</th>
<th>10a</th>
<th>10b</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4MT18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C*</td>
<td>C*</td>
<td>M</td>
<td>M</td>
<td>C</td>
<td>-</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D4MT4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C*</td>
<td>C*</td>
<td>M</td>
<td>M</td>
<td>C</td>
<td>-</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D4MT9</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>M</td>
<td>M</td>
<td>C</td>
<td>M</td>
<td>C</td>
<td>M</td>
<td>-</td>
<td>M*</td>
</tr>
<tr>
<td>D4MT12</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>M</td>
<td>M</td>
<td>C</td>
<td>M</td>
<td>C</td>
<td>M</td>
<td>-</td>
<td>M*</td>
</tr>
<tr>
<td>D4MT51</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>M</td>
<td>M</td>
<td>C</td>
<td>C</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>11a</td>
<td>11b</td>
<td>12a</td>
<td>12b</td>
<td>13a</td>
<td>13b</td>
<td>14a</td>
<td>15a</td>
<td>16a</td>
<td>17a</td>
<td>17b</td>
<td>18a</td>
<td>-</td>
</tr>
</tbody>
</table>

- alleles retained, C, loss of CSE-derived allele, M, loss of MSM-derived allele. *, imbalance in the gene dosage between two alleles and the allele indicated “*C” or “*M” is smaller than the other allele.

Table 3. Summary of Results of Allelotype Analysis in Tumor Cell Lines

<table>
<thead>
<tr>
<th>Cell line No.</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>1</td>
</tr>
<tr>
<td>2a</td>
<td>2</td>
</tr>
<tr>
<td>2b</td>
<td>2</td>
</tr>
<tr>
<td>3a</td>
<td>3</td>
</tr>
<tr>
<td>4a</td>
<td>4</td>
</tr>
<tr>
<td>5a</td>
<td>5</td>
</tr>
<tr>
<td>6a</td>
<td>6</td>
</tr>
<tr>
<td>7a</td>
<td>7</td>
</tr>
<tr>
<td>8a</td>
<td>8</td>
</tr>
<tr>
<td>9a</td>
<td>9</td>
</tr>
<tr>
<td>10a</td>
<td>10</td>
</tr>
<tr>
<td>10b</td>
<td>10</td>
</tr>
<tr>
<td>11a</td>
<td>11</td>
</tr>
<tr>
<td>11b</td>
<td>11</td>
</tr>
<tr>
<td>12a</td>
<td>12</td>
</tr>
<tr>
<td>12b</td>
<td>12</td>
</tr>
<tr>
<td>13a</td>
<td>13</td>
</tr>
<tr>
<td>13b</td>
<td>13</td>
</tr>
<tr>
<td>14a</td>
<td>14</td>
</tr>
<tr>
<td>15a</td>
<td>15</td>
</tr>
<tr>
<td>16a</td>
<td>16</td>
</tr>
<tr>
<td>17a</td>
<td>17</td>
</tr>
<tr>
<td>17b</td>
<td>17</td>
</tr>
<tr>
<td>18a</td>
<td>18</td>
</tr>
<tr>
<td>A (%)</td>
<td>4</td>
</tr>
<tr>
<td>B (%)</td>
<td>8</td>
</tr>
<tr>
<td>C (%)</td>
<td>8</td>
</tr>
</tbody>
</table>

A sample which showed both LOH and imbalance in the gene dosage between two alleles was treated as LOH. —, alleles retained. M, loss of MSM-derived allele. I, imbalance in the gene dosage between two alleles (the sample showed no LOH). ND, not determined. A, percent with LOH. B, percent with imbalance in the gene dosage between two alleles. C, percent with allelic imbalance. a) Because of duplication of part of the samples, this is not the sum of A and B.

found, however.

Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism, RFLP analysis of the mouse alpha-interferon gene was carried out in twenty-five HCCs and two cell lines, and LOHs were observed in the tumor cell lines but not in primary HCCs (data not shown).

Comparative Multiplex PCR Analysis in Mouse p16

A candidate tumor suppressor gene termed p16/CDKN2 was encoded near the interferon gene cluster on human chromosome 9p21—22, and the mouse p16 gene was also located near the mouse interferon gene cluster on the C5—C7 portion of mouse chromosome 4 (Fig. 6). The standard comparative multiplex PCR analysis in the mouse p16 gene was performed (Fig. 7). Because our analysis was PCR-based, the multiplex PCR was used to comparatively amplify the target locus (mouse p16) and a marker locus outside the region of interest. For example, the marker, D6RCK2, was used as an internal control in the experiment. When signals of the D6RCK2 marker in normal DNAs and tumor cell line DNA were of equal intensity, the signal of the mouse p16 gene in tumor cell line DNA was observed to be significantly deleted (Fig. 7a). Therefore, the coamplification of control and locus of interest in DNA tested provided a means of control for PCR amplification and enabled the relative level of target signals.
Fig. 5. Number of Chromosomes Affected with LOH and/or Imbalance in the Gene Dosage between Two Alleles at One or More Markers in Individual Tumor Cell Lines

Fig. 6. Genetic Linkage Map and Comparative Maps for Human Homologous Mouse p16

The name of each locus is indicated to the right of the chromosome, and the distance from the centromere is indicated to the left (centiMorgan).

sequences to be quantified. The sensitivity and quantity in comparative multiplex PCR analysis are shown in Fig. 7b. Since normal DNA was gradually added to the cell line which showed homozygous deletion of mouse p16, the signal intensities of the mouse p16 gene were in proportion to DNA content added although the signals of PCR-amplified products of D6RCK2 marker were almost equal. Comparative analysis of the mouse p16 gene was thus performed by this procedure. Because of the high intensity of the signal of the mouse p16 gene, however, it seemed difficult to detect the homozygous deletion of the gene when the content of contaminated normal DNA was heavy. In the experiment presented here, a case of tumor DNA with a normal DNA contamination rate of about 20% (Fig. 7b) was judged to be the homozygous deletion.

The results of comparative multiplex PCR analysis in tumor cell lines are shown in Fig. 8. Signals of the PCR-amplified product by each control primer were detected in all tumor cell lines, and were almost equal. Frequent homozygous deletions were detected in exon 1 and exon 2 of the mouse p16 gene (13/25; 52%; 10/25, 40%, respectively). Samples 1a, 2a, 4a, 11a, 11b, 12a, 12b, 13b, 17a, and 17b showed homozygous deletion of both exons of the gene, while samples 6a, 13a, and 16a showed homozygous deletion in exon 1 but not in exon 2. Thus, part of the tumor cell lines had lost exon 1 of the mouse p16 gene, but retained exon 2. So, although some tumor cell lines showed LOH but not homozygous deletion, the retained allele might delete an extremely small region such as only a part of exon 1 of the mouse p16 gene on chromosome 4. In primary tumors, however, both exon 1
Fig. 8. Representative Result Showing Homozygous Deletion of Mouse p16 in Tumor Cell Lines by Comparative Multiplex PCR Analysis
a) Analysis in exon 1 of mouse p16 gene. b) Analysis in exon 2 of mouse p16 gene. Number above each lane indicates sample number. Samples which are shown as “H” at the bottom of each lane show homozygous deletion of the mouse p16 gene. Sample numbers 6a, 15a, and 16a show homozygous deletion in exon 1 of mouse p16 gene but not in exon 2 of this gene.

Fig. 9. Homozygous Deletions of Mouse p16 in Tumor Cells at the Early Phase of Culture
Although the sample was taken only 3d after starting primary culture (lane 1a-3d) shows the homozygous deletion of the mouse p16 gene, the hepatocellular carcinoma-derived tumor cells after treatment with dispase (lane 1a-0d) showed no homozygous deletion of the gene.

and exon 2 of the gene were amplified and the homozygous deletions were not detected (0/25, 0%).

The homozygous deletions of the mouse p16 gene were detected in some cell lines at the early phase in primary culture. As shown in Fig. 9, tumor cells only 3d after the start of primary culture (lane, 1a-3d) showed the homozygous deletion of this gene, but hepatocellular carcinoma-derived liver cells which were treated with dispase (lane, 1a-0d) did not show the deletion. This primary HCC thus retained both alleles of the mouse p16 gene.

DISCUSSION

The present study demonstrated; 1) completely LOH-negative results in all autosomes and chromosome X in primary HCCs; 2) very frequent LOHs on chromosome 4 and infrequent LOHs on other chromosomes in tumor cell lines; and 3) frequent homozygous deletions of the mouse p16 gene in tumor cell lines but not in primary HCCs.

The allelotyping analysis of multiple tumors including HCC has been helpful in the effort to research candidate tumor suppressor genes in human. In animals, however, the application of analytical methods has been rare because of a lack of DNA markers for the analysis. We have recently established an animal hepatocarcinogenesis model which enable us to research loci of the inactivated tumor suppressor genes utilizing the microsatellite markers and diethylaminoinduced HCCs present in C3H/MSM F1 hybrid mice. We earlier demonstrated that these tumors had no point mutation of c-Ha-ras codon 61. This result has indicated a clear difference in HCC development between C3H mouse and C3H/MSM F1 hybrid mouse because c-Ha-ras activation is common in C3H mouse HCC. We therefore expect that other mechanisms, except for the activation of c-Ha-ras codon 61, may be present in cancer development in mouse, and we performed the allelotyping analyses to research the candidate tumor suppressor genes.

The criterion for LOH is that a relative intensity of the alleles in tumor DNA differs from the relative intensity of two alleles in non-tumor DNA by one factor. This criterion, however, is not stringent enough to support LOH in some cases. For example, when a DNA specimen has a twofold or threefold increase in the copy number of one allele, instead of loss of the other allele by duplication or multiplication, it fails to distinguish the loss of the one allele from an increased copy number of the other allele. The criterion is reliable for detecting an imbalance in the gene dosage between two alleles in a tumor. In the study
presented here, therefore, we investigated not only LOH but also such allelic imbalance on each chromosome in more detail.

We performed the allelotype analysis utilizing a total of 100 microsatellite markers for the primary HCCs, and detected no allelic imbalance. At first, it seemed that because of the contamination of normal DNA which was derived from normal cells such as nonparenchymal and/or extramedullary hematopoietic cells, allelic imbalances might not be able to be detected in primary HCCs. To overcome this problem, we cultured the tumor cells to avoid contamination by the nonparenchymal or extramedullary hematopoietic cells, and obtained twenty-five tumor cell lines from twenty-five tumors arising in eighteen mice. We then analyzed the cultured tumor cells, and found highly frequent allelic imbalances on chromosome 4 and infrequent allelic imbalances on the other chromosomes.

It seems that one or more putative tumor suppressor genes may be encoded on chromosome 4. The common region of allelic imbalance on chromosome 4 has a homologous syteny on human chromosome 9p. In human HCCs, LOH has been found at relatively high frequency for 1p, 4q, 5q, 8p, 10q, 11p, 13q, 16p, 16q, and 17p, although LOH on mouse chromosome 4 may be essential to hepatocarcinogenesis. While LOH on human chromosome 9 has not been documented for human HCCs, such change at the interferon gene cluster locus has been observed in human acute lymphatic leukemias, malignant gliomas, malignant melanomas, malignant mesotheliomas, bladder cancers, head and neck cancers, small-cell and non-small-cell lung cancers and ovarian tumors. Recently, Kamb and his colleagues and Nobori and his colleagues also discovered a cell cycle-regulating gene existing at the same site. Since mouse chromosome 4 (and human chromosome 9) do not contain other known tumor suppressor genes such as APC, WT, RB, p53, MEN, NF1 or DCC, the possibility that this gene may well be responsible for mouse hepatocarcinogenesis deserves consideration. This candidate tumor suppressor gene termed p16/CDKN2 encodes an inhibitor of cyclin dependent kinase 4 and regulates the cell cycle. Regulation of cell cycle progression in eukaryotes is controlled by cyclin dependent kinase, whose catalytic activity is modulated by association with different cyclins which function as regulatory subunits. In mammalian cells, several classes of cyclins have been identified which may associate with different cyclin dependent kinase catalytic subunits.

Cyclin D1 is rate-limiting for progression through G1, and functions as an oncogene in cooperation with the ras gene. Ectopic expression of cyclin D1 can overcome growth arrest mediated by the retinoblastoma protein. Cyclin D1 can bind to the retinoblastoma protein and is able to stimulate phosphorylation of the retinoblastoma protein by cyclin dependent kinase 4, its predominant kinase partner. Retinoblastoma protein phosphorylation by cyclin–cyclin dependent kinase 4 is inhibited by p16/CDKN2 and may reflect a potential of this gene to block cell cycle progression. Thus, the mouse p16 gene acts as a brake at one point of the cell cycle, and may be inactivated during cancer development in a variety of tumors.

We studied the possibility of homozygous deletion of the mouse p16 gene because the human p16 gene was identified by virtue of its frequent homozygous deletion in cell lines derived from many different tumor types. For effective detection of homozygous deletion of the mouse p16 gene, comparative multiplex PCR analysis was performed. In primary tumor, both exon 1 and exon 2 of the mouse p16 gene were PCR-amplified, while analysis of the tumor cultured cells revealed highly frequent homozygous deletion of this gene and gave clear-cut positive results.

However, this procedure of primary culture brings up a problem of whether only the immortalized cells which have a growth advantage in tumor are cultured. We assume that allelic loss and/or homozygous deletion of the mouse p16 gene on chromosome 4 are the events responsible for the growth advantage for part of the tumor cells in the tumor tissue throughout the primary culture. Otherwise, the already immortalized cells which have a growth advantage by inactivation of the mouse p16 gene in the primary tumor would survive throughout the primary culture. Our interpretation is correct, the allelic imbalances detected may not be early events but later events in vivo in the mouse hepatocarcinogenesis. For human it has been pointed out that part of the cases of homozygous deletion of the human p16 gene may be an event in vitro. Therefore, the discrepancy between the frequency of homozygous deletion of the mouse p16 gene seen in tumor cell lines and in primary tumors may be due to culturing procedure and not to the contamination of normal DNA extracted from the nonparenchymal cells or extramedullary hematopoietic cells. However, the homozygous deletion of the mouse p16 gene has been detected in tumor cells only 3d after the primary culture has began. Because these primary hepatocellular carcinomas show no homozygous deletion, this result supports that the detected homozygous deletion of the mouse p16 gene may be an early event during primary culture or that immortalized cells which have lost the gene are present in the primary tumor. Since some tumor cell lines monoclonal on mouse chromosome 4 have retained one allele of the mouse p16 gene, the cells do not show the homozygous deletion of this gene. Although the inactivation mechanism of the mouse p16 gene in such cell lines is not yet understood, the mechanism except for homozygous deletion of the gene may be present; for example, methylation inactivation of the gene or the retained allele may delete an extremely small region such as exon 1 alone. It is noteworthy that homozygous deletions of the mouse p16 gene have been detected in half of these cell lines and that other cell lines retain at least one allele. This fact may be important for future studies of underlying mechanisms of the deletion meaningful for mouse HCC development and human tumor cells. Thus it is conceivable that homozygous deletion of the mouse p16 gene is not an initial or very early event in mouse hepatocarcinogenesis but instead occurs during the progression of carcinogenesis. Hino and his colleagues, however, suggested that another tumor suppressor gene may be located near the mouse p16 gene. Therefore, an unknown tumor suppressor gene located in this deleted region may be also inhibited.
Finally, neither the c-Ha-ras gene nor the p16 gene are common at the early stage including initiation in mouse hepatocarcinogenesis, and the lack of allelic imbalances in our diethylnitrosamine-induced primary HCCs may be interpreted in the following ways: allelic imbalances occurred involving regions other than those studied; or tumor suppressor genes were inhibited by point mutations.

Acknowledgments  The authors are grateful to Dr. H. Kanda and Ms. K. Nomura for their encouragement. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture and the Ministry of Health and Welfare in Japan.

REFERENCES