Hepatocellular Carcinoma Caused by Loss of Heterozygosity in Lkb1 Gene Knockout Mice

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Abstract

Germline mutations of the LKB1 gene are associated with Peutz-Jeghers syndrome, which is characterized by mucocutaneous pigmentation and gastrointestinal hamartoma with an increased risk of cancer development. To investigate the role of LKB1 in vivo, we have recently constructed Lkb1 gene knockout mice. Because of Lkb1 gene haplosufficiency, the heterozygous Lkb1 mice develop gastrointestinal polyps of which the histological characteristics resemble those of the Peutz-Jeghers syndrome hamartoma. Here we demonstrate that the Lkb1 (+/-) mice develop hepatocellular carcinomas (HCCs). In Lkb1 (+/-) mice >50 weeks of age, >70% of the male mice developed HCCs, whereas only 20% of the females had HCCs, showing a sex difference in the susceptibility. Histological examinations revealed various types of HCCs, such as “trabecular,” “clear cell,” “pseudoglandular,” and “sarcomatous” types, which were strikingly similar to those found in human HCCs. Western blotting and PCR analyses showed loss of Lkb1 heterozygosity in all of the HCC tissues examined, indicating a tumor suppressor role of LKB1 in the mouse liver. These results suggest that lack of LKB1 is a novel mechanism for HCC development. Thus, the Lkb1 (+/-) knockout mutant should be an important and useful model for human HCC.

Introduction

PJS is an autosomal disorder characterized by mucocutaneous pigmentation and gastrointestinal hamartoma, as well as an increased risk of cancer development (1, 2). LKB1 (also termed as STK11) on chromosome 19p13.3, encoding a serine/threonine kinase, is a gene of which the germ-line mutations are found in PJS (3, 4). Although LKB1 functions have not been thoroughly studied, several pieces of evidence suggest its roles in the cell cycle and apoptosis. Forced expression of LKB1 in tumor cell lines results in cell cycle arrests (5), and phosphorylation of LKB1 is essential for the cell growth suppression (6, 7). LKB1 is also required for the brahma-related gene 1-induced growth arrest (8). In addition, LKB1 plays a key role in the p53-dependent apoptosis (9). On the other hand, a knockout mouse study revealed that LKB1 regulates expression of the vascular endothelial growth factor (10). These data, taken together, suggest that LKB1 mutations may contribute to tumorigenesis through induction of angiogenesis as well as suppression of growth arrest and apoptosis. As we have reported recently, Lkb1 (+/-) mice develop gastrointestinal polyps because of haplosufficiency of Lkb1, with histological characteristics resembling those of human PJS hamartomas (11). We have also found that Lkb1 (+/-) mice develop neoplastic lesions in the liver. To date, several transgenic mouse lines have been established as HCC models. As summarized in Table 1, overexpression of various genes or disruption of the fatty acyl-CoA oxidase gene causes HCC development (12–16). The long latency >1 year of the tumorigenic process in most transgenic lines suggests that additional genetic alterations are required for HCC development in these mice. Consistent with this hypothesis, hepatic dysplasia is found in earlier stages, associated with atypical mitotic figures, increased aneuploidy, chromosomal breakage, and translocations (17, 18). However, precise molecular mechanisms for HCC development have not yet been defined precisely, although these transgenes should act as triggers of a long sequence of events in hepatocarcinogenesis. Here, we characterize HCC developed in Lkb1 (+/-) mice and demonstrate Lkb1 LOH in all of the HCC tissues examined, suggesting a novel molecular mechanism for HCC development.

Materials and Methods

Lkb1 Knockout Mice. Construction of Lkb1 knockout mice was described previously (11). At 3 weeks of age, mice were weaned, numbered, and tails were clipped for genomic DNA preparation. Genotypes of the mice were determined by PCR using primers PGKR (5'-CTA AAG CGC ATG CTC CAG ACT-3') and LKB1EX (5'-GGC GTC CCT AGA CAC ATT TCC-3'). Histological Analyses and Immunohistochemistry. Liver tissues were fixed in PBS-buffered 4% paraformaldehyde, dehydrated, and embedded in paraffin wax before sectioning at 4-μm thickness. For histopathological examinations, sections were stained with H&E. For immunohistochemistry, serial sections were deparaffinized, treated with 0.3% H2O2 in methanol to inactivate endogenous peroxidase, and incubated with anti-PCNA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 200-fold dilution for 60 min. Immunodetection of PCNA was performed using Vector Stain Elite kit (Vector Research, Burlingame, CA).

PCNA Labeling Index. Cell proliferation rate was determined as a PCNA labeling index by scoring the average PCNA-positive cells in four randomly selected microscopic fields (×200) for each PCNA-immunostained section. RT-PCR Analysis. Total RNA was prepared from the liver tissues using ISOGEN solution (Nippon Gene, Toyama, Japan), and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). A pair of primers (EIF; 5'-CTC GTA CGG CAA GGT GAA GGA G-3' and E6R; 5'-TAC AGG CCC GTG GTG ATG TGG T-3') was used to amplify a 585-bp fragment from exon 1 to 6. LOH Analysis. Genomic DNA was extracted using ISOGEN solution (Nippon Gene). Two primer sets were used to detect the targeted Lkb1 allele (PGKR and E6R) and the wild-type Lkb1 allele (PJV; 5'-CAA GGA CAT CAA GCC GGG CAA C-3' and E6R), respectively. For microsatellite analysis, D10Mit42 was chosen, the length of which was 191 bp in the C57Bl/6 and 185 bp in the C57BL/6 background. The primer sequences were obtained from the Jackson Laboratory web site. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Western Immunoblot Analysis. Tissue samples were homogenized and sonicated in lysis buffer [10 mM HEPES (pH 7.4), 50 mM NaCl, 50 mM sodium PPI, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 0.1% Triton X-100, and 500 μM phenylmethylsulfonyl fluoride]. After centrifugation at 2000 × g at 4°C for 10 min, 20 μg of the supernatant protein was separated in a 10% SDS-PAGE. Sheep polyclonal anti-LKB1 antibody (Upstate Biotechnology, Lake Placid, NY) and enhanced chemiluminescence detection technology, Lake Placid, NY) were used to detect the LKB1 protein expression by Western blotting. LKB1 was shown in Fig. 1E, the clear cell type HCC was found in some Lkb1 (+/-) livers, suggesting cytoplasmic glycogen accumulations as often seen in the human HCCs (19). In addition, gland-like structures with large cystic lumen were also observed (Fig. 1F, asterisks), resembling the human pseudoglandular type HCCs (19). Furthermore, the sarcomatous type HCC was also found in a case, with spindle-shape tumor cells invading into the adjacent normal tissue (Fig. 1G, arrowheads). Thus, the histological characteristics of the Lkb1 (+/-) HCCs are strikingly similar to the representative histopathology in human HCCs (19), suggesting that similar genetic or epigenetic changes take place in human and Lkb1 (+/-) mouse HCCs. Importantly, lung metastasis was found in 2 of 19 Lkb1 (+/-) males >50 weeks of age (Fig. 1H).

Cell Replication and Apoptosis in the Hepatic Lesions. To additionally investigate these hepatic lesions, we determined cell proliferation rate by PCNA staining. In the littermate wild-type mouse liver, few PCNA-stained hepatocytes were detected (0.05%), reflecting the quiescent state of most hepatocytes (Fig. 2, A and E). In the nodular foci, on the other hand, many PCNA-positive cells were found (9.3%), indicating continuous proliferation of the dysplastic cells (Fig. 2, C and E). These data, together with the histological findings, suggest that the nodular foci are hepatocellular adenomas. Increased PCNA indices were also found in the HCC sections (5.4%), although not so high as in the nodular foci (Fig. 2, D and E), possibly because of insufficient oxygen or nutrient supply in the large tumors. Importantly, the normal hepatocytes of the Lkb1 (+/-) mice showed a slightly but significantly higher level of PCNA index (0.75%) than those in the wild-type liver, suggesting hepatic hyperplasia caused by the decreased LKB1 level (Fig. 2, B and E).

Because LKB1 is required for p53 dependent apoptosis (9), it is possible that disruption of Lkb1 results in suppression of apoptosis. To investigate this possibility, we scored the number of apoptotic bodies in the sections of nodular foci and HCCs. Although few apoptotic bodies were found in the nodular foci (0.18%), the apoptotic rate was rather increased in HCCs (0.9%) than decreased (data not shown). This apoptotic rate in the HCCs was much higher than that of the HCCs in c-myc/TGF-α transgenic mice (0.07%; Ref. 20), suggesting that apoptosis was not suppressed in the Lkb1 (+/-) mouse HCCs.

LOH of the Lkb1 Gene in HCCs. To investigate the molecular mechanism(s) of hepatocarcinogenesis in the Lkb1 (+/-) mice, we analyzed LKB1 protein expression by Western blotting. LKB1 was
expressed in the wild-type liver tissue, and the level in the nontumor part of the \(Lkb1\) (+/−) liver was approximately half of that in the wild-type littermates, reflecting the gene dosage (Fig. 3, A and B). However, in the HCC tissue, expression of LKB1 was virtually abolished, indicating biallelic inactivation of \(Lkb1\). This is in sharp contrast to the situation of the gastric hamartoma caused by haploinsufficiency of \(Lkb1\), where the expression of the wild-type LKB1 remains at the heterozygous level (11). To determine the mechanism of \(Lkb1\) inactivation in HCCs, we performed \(Lkb1\) allele-specific RT-PCR. In the targeted \(Lkb1\) allele, exons 2–4 are replaced with the neomycin resistance gene cassette, causing an alternative splicing of the transcript from exon 1 to 5, which should result in a frame-shift mutation in exon 5 (11). PCR primers for exons 1 and 6 amplified two mRNA species of different lengths from the wild-type and targeted \(Lkb1\) alleles, respectively (Fig. 3C). Whereas the level of the shorter transcript from the targeted \(Lkb1\) allele remained unchanged in either the normal liver or HCCs, the wild-type mRNA level was decreased significantly in the HCC tissues, suggesting inactivation of the wild-type \(Lkb1\) allele. A faint band for the wild-type \(Lkb1\) mRNA in the tumor tissue was likely derived from the nontumor cells of the sinusoid or stroma. Finally, we determined LOH of the \(Lkb1\) gene by genomic PCR analysis (Fig. 3D). The band intensity was reduced significantly for the wild-type \(Lkb1\) allele in all of the HCC samples analyzed, whereas that for the targeted allele remained unaffected, indicating \(Lkb1\) LOH in the HCC tissues. We also verified LOH for a chromosomal region close to \(Lkb1\) by genomic PCR of the \(D10Mit42\) microsatellite marker (data not shown). These data, taken together, strongly suggest that LKB1 plays a role as a tumor suppressor in the liver and that \(Lkb1\) LOH is the cause of the HCCs in our knockout mice.
Table 2 Number of Lkb1 heterozygotes with hepatic lesions

<table>
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<tr>
<th>Age (weeks)</th>
<th>Sex</th>
<th>Mice</th>
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<th>HCC</th>
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<td>M</td>
<td>7</td>
<td>1 (14)</td>
<td>0 (0)</td>
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<td></td>
<td>F</td>
<td>2</td>
<td>1 (50)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>41–50</td>
<td>M</td>
<td>7</td>
<td>7 (100)</td>
<td>2 (29)</td>
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<tr>
<td></td>
<td>F</td>
<td>3</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>51–60</td>
<td>M</td>
<td>7</td>
<td>7 (100)</td>
<td>5 (71)</td>
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<tr>
<td></td>
<td>F</td>
<td>3</td>
<td>1 (33)</td>
<td>1 (33)</td>
</tr>
<tr>
<td>&gt;61</td>
<td>M</td>
<td>12</td>
<td>12 (100)</td>
<td>9 (75)</td>
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<tr>
<td></td>
<td>F</td>
<td>2</td>
<td>2 (100)</td>
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Discussion

We have demonstrated recently that haploinsufficiency of the mouse Lkb1 gene causes gastrointestinal hamartomas (11). In PJS patients, on the other hand, LOH of the LKB1 gene was reported in their gastrointestinal carcinomas, suggesting a tumor suppressor role for LKB1 (21–23). Thus, it is conceivable that loss of the wild-type LKB1 gene is necessary for progression from hamartoma to carcinoma in the PJS gut. In the present study, we have demonstrated biallelic inactivation of the Lkb1 gene in all of the HCC tissues examined, suggesting that lack of LKB1 is also responsible for carcinogenesis in the liver. Although HCC has not been reported in PJS patients, this difference between the human and mouse may be attributable to different susceptibilities to the stimuli that cause LKB1/Lkb1 LOH between the two species. It would also be important to determine whether expression of LKB1 is altered in spontaneous human HCC cases.

Although it has been reported that LKB1 plays a role in the cell cycle arrest and p53-dependent apoptosis (5–9), we did not find any significant suppression of apoptosis in the Lkb1 mouse HCCs compared with other transgenic models. However, it is conceivable that Lkb1 LOH suppresses elimination of the hepatocytes with mutations during early stages of hepatocarcinogenesis, allowing transformed cells to colonize. Such a molecular mechanism for HCC development was suggested in other HCC mouse models as well (12). In TGF-α/c-myc transgenic mice, hepatocyte dysplasia with atypical mitotic figures is observed before the HCC development (17). In addition, transplantation of such dysplastic tissues to nude mice gives rise to HCC, suggesting the presence of “initiated” cells in the dysplastic livers (17). In other transgenic mice overexpressing hepatitis B virus antigens, mutant α-1-antitrypsin or urokinase-type plasminogen activator, the transgene product is toxic to but not directly tumorigenic in the hepatocytes (12). During the regeneration from the chronic liver injury caused by the transgene product, transformed hepatocytes appear to be selected and form tumors in these models. Accordingly, it is conceivable that Lkb1 LOH causes similar changes to those in the early transformed hepatocytes in the transgenic models. To characterize such changes, it should be interesting to investigate the gene expression profile in HCCs in the Lkb1 (+/-) mice using gene microarrays.

It has been demonstrated recently that SMAD4, a cytoplasmic messenger of the TGF-β signal, can bind to LKB1 through LIP1 (for LKB1 interacting protein 1), and that inhibition of LKB1 suppresses TGF-β signaling (24). Thus, it is conceivable that TGF-β signaling is down-regulated in HCCs of the Lkb1 (+/-) mice. Because TGF-β acts as a potent inhibitor of cell growth, and an apoptosis inducer for epithelial cells and hepatocytes (25, 26), a decreased responsiveness to TGF-β may contribute to HCC development. In the HCCs of TGF-α/c-myc mice, expression of TGF-β type II receptor is down-regulated (27). Furthermore, two mutant mouse lines of the TGF-β type II receptor gene, heterozygous knockout line and dominant-negative transgenic line, also show increased susceptibility to chemical liver carcinogenesis (28, 29). Therefore, another mechanism for hepatocarcinogenesis in the Lkb1 (+/-) mice may be inhibition of the TGF-β signal, causing suppression of growth inhibition and apoptosis.

Lkb1 (+/-) mice develop small nodular foci of hepatocellular adenoma with continuous cell proliferation, which may be a precursor of HCC. In the present study, we have determined Lkb1 LOH in HCC but not yet in these adenomatous nodules, because of the limited sample.

Fig. 2. Liver cell proliferation rate determined by PCNA staining. A–D, PCNA immunostaining of the sections of wild-type liver (A), nontumor region of Lkb1 (+/-) liver (B), nodular focus (C), and HCC (D). PCNA-stained nuclei are seen in dark brown. The fatty change observed in the normal part (B, C, or D) is not caused by Lkb1 mutation directly, because it is detected also in the age-matched control mice. E, mean PCNA indices shown as percentages of stained cells with ±SD. WT, wild-type mouse liver; NT, nontumor part of Lkb1 (+/-) liver; NdFc, nodular foci; Bars, 100 μm, and *, P < 0.05.
amounts. To precisely understand the molecular mechanisms of hepatocellular adenoma initiation and its progression to HCC, it should be determined whether Lkb1 LOH occurs in these early lesions. Such an analysis is in progress using samples obtained by laser-captured microdissection.

In conclusion, we have demonstrated that biallelic inactivation of Lkb1 gene defective in Peutz-Jeghers syndrome. Nature (Lond.), 391: 184–187, 1998.


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