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 (1, 2). Lkb1 mice develop gastrointestinal polyps of which the histological characteristics resemble those of the Peutz-Jeghers syndrome hamartomas. 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 tumour 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 (+/-) mutant should be an important and useful model for human HCC.

Introduction

PJS (Peutz-Jeghers syndrome) 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 arrest (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 haploinsufficiency 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. In agreement 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 that 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 PKGR (5’-CTA AAG CGC ATG ATG ATG) 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 (E1F; 5’-CTC GTA CGG CAA GGT GAA GGA G-3’ and E6R; 5’-TAC AGG CCC GTG GTG ATG TTG 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 (PKGR and E6R) and the wild-type Lkb1 allele (PJW; 5’-CCG CAA GGT GAA GGA G-3’ and E6R; 5’-TAC AGG CCC GTG GTG ATG TTG T-3’) was used to amplify a 585-bp fragment from exon 1 to 6.

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3 The abbreviations used are: PJS, Peutz-Jeghers syndrome; LOH, loss of heterozygosity; RT-PCR, reverse transcription-PCR; TGF, transforming growth factor; HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen.

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, 100 μM Na3VO4, 0.1% Triton X-100, and 500 μM phenylmethylsulfonyl fluoride]. After centrifugation at 2000 x 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, Inc.) was used as a primary antibody for LKB1 detection.

Results

**Heaptic Lesions in Lkb1 (+/−) Mice.** We have demonstrated recently that Lkb1 (+/−) mice develop gastrointestinal polyps in which submucosal muscle layers form an arborized network, resembling the PIIS hamartomas (11). Because PIIS patients show an increased risk of developing both benign and malignant tumors in extraintestinal organs, we carefully examined various tissues for any neoplastic lesions. Although the Lkb1 (+/−) mice had no tumors during the first 30 weeks, they developed small nodular foci in the liver afterward (Fig. 1A). These nodules of usually 1–2 mm in diameter, were found in 1 of 7 male mice (14%) between 31 and 40 weeks of age (Table 2). After 41 weeks, all 26 of the male mice necropsied (100%) had nodular foci in the liver (Table 2). Such hepatic lesions were more abundant and severe in the male mice than in the females, indicating a sex difference in the susceptibility. In addition to these small nodules, the Lkb1 (+/−) mice developed large HCC tumors with marked vascularization (Fig. 1B). The youngest male mouse that had HCC was 48-week-old, >10 weeks older than that with the nodular foci (Table 2). Therefore, the incidence of HCC increased gradually with age, and 9 of 12 males (75%) >61 weeks of age developed HCCs. These results suggest that some of the nodular foci progressed into HCCs (see below).

**Histological Characteristics of HCC in Lkb1 (+/−) Mice.** Histological sections of the Lkb1 (+/−) liver stained with H&E revealed that the nodular foci consisted predominantly of large dysplastic hepatocytes with normal nuclear:cytoplasm ratio (Fig. 1C). The “clear-cell” type hepatocytes were also observed in ~20% of the nodules (data not shown). On the other hand, HCCs developed in the Lkb1 (+/−) mice showed a variety of histological types as shown in Fig. 1, D–G. The most abundant histopathology was the trabecular type, consisting of well-differentiated carcinoma cells (Fig. 1D). This type is observed in most human HCCs, from which other HCC types are derived (19). Several other types of HCCs were also observed. As 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 suppression of Lkb1 results in suppression of apoptosis. To investigate this possibility, we scored the number of apoptotic cells 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

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Table 1: Representative transgenic models with spontaneous HCC

<table>
<thead>
<tr>
<th>Transgenic</th>
<th>Gene Symbol</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Alb/MT*</td>
<td>c-myc (mouse)/TGF-α (human)</td>
<td>Santoni-Rugiu, E. et al., 1996b</td>
</tr>
<tr>
<td>MT</td>
<td>PML-RARA (human)</td>
<td>David, G. et al., 1997b</td>
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<tr>
<td>Alb</td>
<td>SV40 T antigen</td>
<td>Sandgren, E.P. et al., 1989b</td>
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<tr>
<td>Alb</td>
<td>H-ras (G12V, human)</td>
<td>Sandgren, E.P. et al., 1989b</td>
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<tr>
<td>TRE</td>
<td>MET (human)</td>
<td>Wang, R. et al., 2001</td>
</tr>
<tr>
<td>Alb</td>
<td>c-myc (mouse)</td>
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</tr>
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<td>MT</td>
<td>TGF-α (human)</td>
<td>Kim, C-M. et al., 1991b</td>
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<tr>
<td>Native</td>
<td>Hepatitis B virus X protein</td>
<td>Damsford, I.A. et al., 1998b</td>
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<td>Alb</td>
<td>Hepatitis B surface antigen</td>
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<td>Geller, S.A. et al., 1994a</td>
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<tr>
<td>Native</td>
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<td>Alb</td>
<td>uPA (mouse)</td>
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<tr>
<td>HBV</td>
<td>Hepatitis C virus core protein</td>
<td>Deane, N.G. et al., 2001</td>
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<tr>
<td>LFABP</td>
<td>cyclin D1 (mouse)</td>
<td>Sakata, H. et al., 1996c</td>
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<tr>
<td>MT</td>
<td>HGF (mouse)</td>
<td>Fan, C-Y. et al., 1998c</td>
</tr>
</tbody>
</table>

* Abb, mouse albumin; MT, mouse metallothionein; TRE, tetracyclin-responsive element; HBV, hepatitis B virus; LFABP, rat liver fatty acid-binding protein; PML, promyelocytic leukemia; RAR, retinoic acid receptor α; uPA, urokinase-type plasminogen activator; HGF, hepatocyte growth factor; AOX, fatty acyl-CoA oxidase.

* These papers have been summarized in 12.

* These papers have been summarized in 13.

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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.

Fig. 1. Morphological features of the Lkb1 (+/−) mouse hepatic lesions. A and B, gross appearance of multiple small nodules near surface (A; arrowheads) and HCCs (B; arrowheads). C–H, representative histological sections (H & E). Nodular foci (NdFc) consisting of dysplastic hepatocytes (C; arrows), HCCs with trabecular type (D), clear cell type (E), pseudoglandular type with cystic lumens (F; +), sarcomatous types with intraliver invasion (G; arrowheads), and metastatic foci (Mx) in the lung (Lg; H). T and N in D show the tumor (i.e., HCCs) and normal tissues with their border indicated by arrowheads. Bars, A and B, 10 mm; C, 100 μm; and D–H, 200 μm.
Table 2 Number of Lkb1 heterozygotes with hepatic lesions

<table>
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<tr>
<th>Age (weeks)</th>
<th>Sex</th>
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<th>Nodular Foci</th>
<th>HCC</th>
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<td>31–40</td>
<td>M</td>
<td>7</td>
<td>1 (14)</td>
<td>0 (0)</td>
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<tr>
<td></td>
<td>F</td>
<td>2</td>
<td>1 (50)</td>
<td>0 (0)</td>
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<tr>
<td>41–50</td>
<td>M</td>
<td>7</td>
<td>7 (100)</td>
<td>2 (29)</td>
</tr>
<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>
</tr>
<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>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2</td>
<td>2 (100)</td>
<td>0 (0)</td>
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</table>

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 α1-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, homozygous knockout line and dominant-negative transgenic line, also show an 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.
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Fig. 3. Inactivation of the wild-type Lkb1 allele in HCC. A and B. Western blot analysis of non-tumor (N) and tumor (T) liver tissues. Gel photograph (A) and mean band intensities shown in bar graph with ± SD (B). The mouse genotypes are shown on top, and the position of LKB1 band on the right. C. allele-specific RT-PCR analysis of wild-type mouse liver (W), and non-tumor (N) and tumor (T) tissues of Lkb1/+/− liver. Band positions for wild-type Lkb1 gene (WT) and targeted Lkb1 gene (KO) are shown.

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References

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