Gastrointestinal Hamartomatous Polyposis in Lkb1 Heterozygous Knockout Mice

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Abstract

Peutz-Jeghers syndrome (PJS) is a hereditary disorder characterized by gastrointestinal hamartomatous polyposis associated with mucocutaneous pigmentation. Germ-line mutations of the gene encoding LKB1 (STK11), a serine/threonine kinase, are identified in most PJS patients. To investigate the role of LKB1 in the PJS phenotypes, we introduced a germ-line mutation in the mouse Lkb1 gene by homologous recombination in mouse embryonic stem cells. In most Lkb1 (+/−) mice >20 weeks of age, hamartomatous polyps developed in the glundular stomach, often in the pyloric region. Small intestinal hamartomas also developed in approximately one-third of the Lkb1 (+/−) mice >50 weeks of age. A genomic PCR and sequence analysis showed that all hamartomas retained both the wild-type and targeted Lkb1 alleles, indicating that allelic loss of the wild-type Lkb1 was not the cause of polypl formation. Moreover, the LKB1 protein level was not reduced in hamartomatous polyps compared with that in the Lkb1 (+/+) normal gastric mucosa. In addition, the remaining allele showed neither missense mutations in the coding sequence nor produced truncated Lkb1 in the hamartoma. Taken together, these data suggest that the wild-type Lkb1 is expressed in the hamartoma at the haploid amount. Accordingly, the gastrointestinal hamartomas appear to develop because of the Lkb1 haploinsufficiency. Although additional genetic events may be critical in hamartoma and adenocarcinoma development, these data strongly suggest that the initiation of polyposis is not the result of loss of heterozygosity in Lkb1.

Introduction

PJS is an autosomal dominant disease characterized by hamartomatous polyps of the gastrointestinal tract as well as mucocutaneous pigmentation of the lips, buccal mucosa, and digits (1, 2). Patients with PJS show an increased risk of developing cancer at relatively young ages (3, 4). Recently, LKB1 (also termed STK11) on chromosome 19p13.3, which encodes a serine/threonine kinase of unknown function, was identified as a gene the mutations of which are responsible for PJS (5, 6). To date, various types of germ-line LKB1 mutations have been identified in PJS families (reviewed in Ref. 7). In addition, LOH on chromosome 19p near LKB1 or somatic mutations in the LKB1 gene have been reported in hamartomas and adenocarcinomas developed in a subset of PJS patients, suggesting that LKB1 is a potential tumor suppressor gene (8–13). Recent cell culture studies have also suggested that LKB1 functions as a tumor suppressor. Forced expression of LKB1 in tumor cell lines results in suppression of cell growth by inducing cell cycle arrests at the G1 phase (14). Phosphorylation of LKB1 by cyclic AMP-dependent kinase or by p90 ribosomal S6 kinase is essential for the suppression of cell growth (15, 16). LKB1 is also required for brahma-related gene 1-induced growth arrest (17). Moreover, LKB1 plays a key role in p53-dependent apoptosis (18). Taken together, mutations in LKB1 may contribute to tumorigenesis through suppression of either growth arrest or apoptosis in epithelial cells. Recently, an Lkb1 gene knockout mouse line has been reported (19). The homozygotes are embryonically lethal because of multiple developmental defects including aberrant vessel formation in the yolk sac and placenta. Expression of vascular endothelial growth factor is deregulated in the Lkb1 homozygous tissues. It is possible that lack of LKB1 supports tumor cell growth through angiogenesis by induction of vascular endothelial growth factor. However, these studies did not investigate whether heterozygous Lkb1 (+/−) mice develop hamartomatous tumors. Here, we describe construction of Lkb1 gene knockout mice and demonstrate that Lkb1 (+/−) mice develop gastrointestinal hamartomas without inactivation of the remaining wild-type Lkb1 allele.

Materials and Methods

Targeting Vector Construction. A partial cDNA fragment of Lkb1 was amplified by RT-PCR using a primer set of E1F (5′-CTA CGG CAA GGT GAA GGA G-3′) and E6R (5′-TAC CCC GTG ATG TTG T-3′). The amplified fragment was used as a probe for screening a 129/SvJ mouse genomic DNA library (Stratagene, La Jolla, CA). A BamHI fragment including exons 1 to 7 was used for vector construction (Fig. 1A).

Lkb1 Knockout Mice. Mouse ES cells RW4 (Genome Systems, St. Louis, MO) were used. Homologous recombinants were screened by PCR using primers PGKR (5′-CTG CAT CCN ATC ATG TGC TGG-3′) and LKB1EX (5′-GGG GAA ACG CCC GTG ATG GAG G-3′) and confirmed by genomic Southern analysis. Genotyping of the mice was performed by PCR using the primers E6R and PGKR. A BglII-BamHI fragment probe was used for genotyping by Southern hybridization (Fig. 1A).

Histological Analyses. The methods have been described previously (20). The primary antibodies anti-αSMa monoclonal antibody (Progen Biotechnik, Heidelberg, Germany) and anti-β-catenin polyclonal antibody (Sigma Chemical Co. St. Louis, MO) were used at 50- and 500-fold dilutions, respectively.

RT-PCR Analysis. Total RNA was prepared from the glandular stomach using ISOBEX solution (Nippon Gene, Toyama, Japan), and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Two primer sets were used to amplify a 585-bp fragment from exons 1 to 6 (E1F and E6R) and an 809-bp fragment from exons 1 to 8 (E1F and E8R; 5′-TGT CTG GGC GTG TTT GAA TAG G-3′), respectively.

LOH Analysis. Genomic DNA was extracted as described (20). Two primer sets were used to detect the targeted allele (PGKR and 5′-GTC ATC AAC AGG ATG CTC A-3′) and the wild-type allele (5′-TAC TCC CTC CAG CTC ATT GAC G-3′ and 5′-GAG GTC GGA CAT GAT TGC G-3′), respectively.

In Situ Hybridization. Sense and antisense RNA probes were prepared from a BglII-PstI (1025-bp) fragment of the mouse Lkb1 cDNA. Deparaffinized sections were treated with 0.2 M hydrochloric acid, followed by 5 μg/ml of protease K. Subsequently, they were fixed in 4% paraformaldehyde and treated with triethanolamine buffer with stepwise additions of acetic acid

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3 The abbreviations used are: PJS, Peutz-Jeghers syndrome; JPS, juvenile polyposis syndrome; LOH, loss of heterozygosity; ApC, adenomatous polyposis coli (gene); αSMa, α-smooth muscle actin; ES, embryonic stem; RT-PCR, reverse transcription-PCR; TGF, transforming growth factor.
anhydride. After prehybridization, the specimens were hybridized with digoxigenin-labeled RNA probes overnight. DIG nucleic acid detection kit (Roche) was used for signal detection.

**Western Immunoblot Analysis.** Tissue samples were homogenized and sonicated in lysis buffer [10 mm HEPES (pH 7.4), 50 mm NaCl, 50 mm sodium Pp, 50 mm NaF, 5 mm EDTA, 5 mm EGTA, 100 μm Na2VO₄, 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 were separated in a 10% SDS-polyacrylamide gel. Sheep polyclonal anti-LKB1 antibody (Upstate Biotechnology, Lake Placid, NY) and ECL detection system (Amerin a 10% SDS-polyacrylamide gel. Sheep polyclonal anti-LKB1 antibody (Invitrogen) at 37°C for 1 h with shaking. Epithelial cells were collected by centrifugation at 1000 rpm for 5 min, suspended in culture medium as described (21), and seeded on collagen-coated culture dishes to avoid contamination of interstitial cells.

**Sequencing Analyses.** cDNA fragments that covered the Lkb1 full coding region were amplified with three sets of primers as follows: 5'-CGA AGG GGA CGA CAA AGA-3' and 5'-GAA CAA TGC CCT GGC TGT GTA G-3' for the 5' part; 5'-CTG CGG CAT CGG CAT GTG A-3' and 5'-CTG CGG TCC TAG GTG A-3' for the middle part; and 5'-TAT TAC GCG CCG AAG TGC TCC T-3' and 5'-TAT TAC GCG CCG AAG TGC TCC T-3' for the 3' part. Amplified cDNA fragments were sequenced directly using the same primers.

**Results**

**Generation of Lkb1 Gene Knockout Mice.** The Lkb1 gene was inactivated in ES cells by homologous recombination in which exons 2, 3, and 4 were deleted and replaced with a neomycin resistance gene cassette (Fig. 1A). The chimeric mice derived from a recombinant ES clone transmitted the targeted Lkb1 allele to the offspring. The progeny genotypes were examined by genomic Southern analysis (Fig. 1B). No homozygous mutant pups were obtained by intercrossing the heterozygotes, indicating that Lkb1 (+/-) mice were embryonically lethal. This is consistent with a previous report analyzing another Lkb1 knockout mutant (19).

The transcript from the targeted Lkb1 allele was examined by RT-PCR analysis using primers to amplify cDNA fragments for exons 1–6 and for exons 1–8, respectively (Fig. 1A). The wild-type Lkb1 cDNA fragment of the expected size was amplified with either primer set (Fig. 1C, arrows). Smaller fragments were also amplified with both primer sets only when Lkb1 (+/-) cDNA was used as the template (Fig. 1C, arrowheads). The length of such shorter cDNA fragments precisely matched the predicted size of the exons 2, 3, and 4-deleted Lkb1 cDNA. These results suggest that some alternative splicing events took place in the transcript from the targeted Lkb1 allele. However, such alternatively spliced mRNA, in which exon 1 is ligated to exon 5, should result in a frame-shift mutation after codon 97, eliminating most of the kinase domain (residues 50–337).

**Gastrointestinal Polyps in Lkb1 (+/-) Mice.** Lkb1 (+/-) mice developed normally and showed no overt phenotypes up to 20 weeks of age. However, gastric polyps developed in 93% of Lkb1 (+/-) mice >20 weeks of age (Fig. 2). After 40 weeks of age, the incidence of the gastric polyps reached 100%. Polyps developed in the glandular stomach, often in the pyloric region (Fig. 3A). Polyps were also found in the small intestine of the aged Lkb1 (+/-) mice (Figs. 2 and 3B). The incidence of the small intestinal polyps in the heterozygotes >50 weeks was 31% (Fig. 2).

Some Lkb1 (+/-) mice became moribund after 50 weeks of age, which was attributed to pyloric constriction and obstruction of the stomach. Others survived beyond 70 weeks of age (data not shown).

**Histological Characteristics of Gastrointestinal Polyps.** Although smaller gastric polyps were of sessile morphology, larger ones were pedunculated (Fig. 3C). They consisted of glandular and cystic epithelial layers. A bromodeoxyuridine incorporation assay showed that tumor cells proliferated continuously in the polyps (Fig. 3D). However, they did not show any dysplastic morphology, but the normal epithelial layers of different types were mixed in the polyp tissue (Fig. 3E, inset). These histological characteristics indicate that the Lkb1 (+/-) polyps are hamartomas like those in human PJS. To...
further compare the pathological characteristics of the \textit{Lkb1} (+/−) with PJS polyps, we immunohistochemically studied the mouse gastric polyp sections using an anti-αSM 

The PJS hamartomas show a particular histology where benign glands are surrounded by fronds of lamina propria containing the muscularis mucosae. In the \textit{Lkb1} (+/−) mice, as expected, an arborizing network of smooth muscle bundles was stained with anti-αSM antibody, extending into branching fronds in the polyp tissue (Fig. 3F). Thus, the \textit{Lkb1} (+/−) polyps exhibit strikingly similar characteristics to those of PJS. The small intestinal polyps were also composed of the normal epithelial cell layers (Fig. 3G). Few adenomatous changes were observed in any hamartomas examined, which is consistent with the PJS hamartomas that show low rates of neoplastic changes (22).

These histological characteristics of the \textit{Lkb1} (+/−) hamartomas are very different from those of the \textit{Apc} knockout (\textit{Apc}Δ/−) mouse intestinal polyps, where proliferating dysplastic adenoma cells are found without smooth muscle fibers of an arborizing pattern (20). To rule out the possibility that the Wnt signaling pathway is activated in the \textit{Lkb1} (+/−) hamartomas, we determined the subcellular localization of β-catenin by immunostaining. When the Wnt pathway is activated, β-catenin is stabilized and translocated to the nucleus, as we have demonstrated previously in the \textit{Apc}Δ/− polyps (23). In the \textit{Lkb1} (+/−) polyps, however, β-catenin was localized to the basolateral membrane and remained outside the nucleus in the polyp epithelial cells (Fig. 3, H and I). These results suggest that the Wnt pathway is not activated in the hamartomatous polyps, which is consistent with a previous report that neither β-catenin mutation nor APC LOH is detected in PJS hamartomas (9, 12).

**Expression of \textit{Lkb1} in Hamartomas.** To determine whether loss of the LKB1 function caused hamartomas in the gastrointestinal epithelium, LOH for the \textit{Lkb1} gene was analyzed in the polyp tissues. A genomic PCR analysis using allele-specific primers showed both the wild-type and targeted \textit{Lkb1} alleles in all hamartomas examined (Fig. 4A). Then the nucleotide sequence was determined for the \textit{Lkb1} mRNA expressed in the hamartoma epithelial cells. As a result of direct sequencing analyses, no \textit{Lkb1} mutation was found in any of the four independent polyps examined (data not shown). Finally, LKB1 protein expression was examined by Western blotting. In \textit{Lkb1} (+/−)
mice, the protein expression was decreased to 47% of that in the wild-type mice, reflecting the **Lkb1** gene dosage (Fig. 4, **B** and **C**). Interestingly, the same level of LKB1 as in the normal stomach was expressed in the gastric hamartoma tissues of the **Lkb1** (**/H11001/**/H11002**) mice. No shorter protein bands were detected that could be derived from the targeted allele encoding the NH2-terminal 97 residues. Taken together, these data exclude the possibility for biallelic **Lkb1** inactivation as the cause of the hamartoma initiation.

To determine the cell types that express **Lkb1** in the hamartomatous polyps, we performed an *in situ* hybridization analysis. The **Lkb1** mRNA was predominantly detected in the hamartoma epithelium, as well as in the normal epithelium of the gastric mucosa (Fig. 4, **D** and **F**). This result is consistent with a previous report that shows LKB1 expression in the polypl epithelium of PJS patients (24). These data, taken together, suggest that another genetic event(s) in addition to the heterozygous inactivation of **Lkb1** may be required for the hamartoma formation.

**Hepatocellular Carcinoma in **Lkb1** (+/*−*) Mice.** In extraintestinal organs, most **Lkb1** (+/*−*) mice developed hepatocellular carcinoma after 30 weeks of age, suggesting that LKB1 plays a tumor suppressor role in the liver. Different from PJS, we could not find any other neoplastic lesions in **Lkb1** (+/*−*) mice (data not shown).

**Discussion**

**Lkb1** is the gene the mutations of which are associated with PJS (5, 6), and various types of germ-line **Lkb1** mutations have been reported in PJS (8). The present study using **Lkb1** knockout mice provides the first direct evidence that a heterozygous **Lkb1** mutation is responsible for gastrointestinal hamartomas that share the histological features with those in human PJS. However, the polyp localization in **Lkb1** (**/H11001/**/H11002**) mice is not necessarily identical with that in PJS, because polyps develop frequently in the small intestine as well as in the stomach and colon. It is conceivable that the additional genetic change that affects hamartoma formation (see below) differs between mice and human. According to some studies, LOH or somatic mutations of **Lkb1** are found in informative cases of hamartomas and adenocarcinomas in PJS patients, suggesting that **Lkb1** is a tumor suppressor gene (8–13). It has been also proposed that a somatic mutation in another gene may be required for the neoplastic changes of the PJS polyps (12, 13). However, it has not been determined whether loss of
the LKB1 function is essential for the hamartoma initiation. Here, we have excluded Lkb1 LOH in the gastric hamartomas of the Lkb1 heterozygous mice. Furthermore, we have demonstrated LKB1 expression at the haploid level in the hamartoma tissues. These data strongly suggest that biallelic inactivation of Lkb1 is not necessarily required for the hamartoma development. It is conceivable that additional genetic alterations in other genes trigger the hamartoma formation. Considering the LKB1 functions, it is possible that LOH in the Lkb1 gene contributes to progression of the hamartomas to malignant tumors. Because LKB1 is involved in the cell cycle arrest and p53-dependent apoptosis (14, 17, 18), reduction in its level may lead to suppression of growth arrest and apoptosis and help accumulate somatic mutations. Such cells might be predisposed to transformation. Gene chip analysis may help identify the gene mutation(s) that trigger the hamartoma formation. In this report, we have shown that the Wnt signaling cascade was not activated in the Lkb1 (+/−) hamartomas. The result is consistent with a previous report that showed β-catenin mutation in addition to LKB1 LOH only in adenomatous lesions of the hamartomatous polyps (9, 12). Thus, activation of the Wnt pathway may be an important step in hamartoma-carcinoma progression.

Previously, we demonstrated that heterozygous Smad4 knockout mice develop gastrointestinal hamartomas that have similar histopathology to those in JPS, such as stromal expansion or association with melanin spots of the oral mucosa, lips and digits: a syndrome of diagnostic significance. Nat. Engl. J. Med., 241: 992–1005, 1994.


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