Compound Disruption of Smad2 Accelerates Malignant Progression of Intestinal Tumors in Apc Knockout Mice

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ABSTRACT

Smad2 is a receptor-regulated Smad that is activated specifically by transforming growth factor β and activin signaling. We disrupted the mouse Smad2 gene by gene targeting. Homozygous Smad2 mutant mice died around E8.5 with impaired visceral endoderm function and deficiency of mesoderm formation. Heterozygotes were fertile and had no apparent abnormality up to at least 1 1/2 year of age. To examine the role of Smad2 inactivation in the process of carcinogenesis, we prepared compound heterozygous mice, which carry both Apc and Smad2 mutations on the same chromosome in the cis-configuration. Compound inactivation of Smad2 in heterozygous Apc mutant mice did not change the total number of intestinal tumors but increased sudden death from intestinal obstruction caused by extremely large tumors. Furthermore, histological examination revealed that Apc/Smad2 cis-compound heterozygotes developed multiple invasive cancers that had never been observed in Apc single heterozygotes. These results indicate that loss of Smad2 does not initiate tumorigenesis by itself but accelerates malignant progression of tumors to invasive cancer in the late stages of carcinogenesis.

INTRODUCTION

TGF-β1 superfamily is a group of multifunctional cytokines that affect cell growth, differentiation, apoptosis, and morphogenesis (1–3). This family consists of >40 family members, including TGF-βs, activins, and BMPs. TGF-β superfamily ligands induce heteromorphic complex formation of cognate type II and type I serine/threonine kinase receptors. Type II receptor kinases then phosphorylate serine and threonine residues in the GS domain of type I receptors, which results in the activation of type I receptor kinases (4). Activated type I receptors signal into cytoplasm through phosphorylation of Smad proteins. Thus far, eight mammalian Smad proteins have been identified. Smad1, Smad2, Smad3, Smad5, and Smad8 are R-Smads, which are directly phosphorylated by type I receptors. Smad2 and Smad3 are activated by the TGF-β type I receptor and the activin type IB receptor, whereas Smad1, Smad5, and Smad8 are activated by BMP type I receptors and activin receptor-like kinase 1. Smad4 is a Co-Smad that binds to activated R-Smads and is a component of transcriptional regulator in the nucleus (5–7). Smad4 interacts with all activated R-Smads and even with β-catenin in activation of the Xenopus twin gene (8). Smad6 and Smad7 are I-Smads, which are induced by TGF-βs, BMPs, IFN-γ, and nuclear factor κB signals (9–11). I-Smads competitively block the activation of R-Smads by type I receptors (12–14).

TGF-β inhibits the growth of many types of cells, including epithelial cells, and loss of TGF-β sensitivity has been implicated in tumorigenesis. Most cancer cell lines have impaired responsiveness to the growth inhibitory activity of TGF-β (15). Of the components involved in TGF-β signal transduction, abnormalities in the TGF-β type II receptor, TGF-β type I receptor, and Smad4 have been extensively characterized. Some tumors, e.g., hereditary nonpolyposis colorectal cancer, develop TGF-β resistance after inactivation of the TGF-β type II receptor (16, 17). Mutations in the TGF-β type I receptor were detected in 30% of ovarian cancer (18). Inactivation of Smad4 (originally termed DPC4) is implicated in carcinogenesis, including that of pancreatic cancer and colorectal cancer (19, 20).

Smad2 is located on human chromosome 18q21.1, where Smad4 is also located. Mutations of Smad2 have also been found associated with colorectal cancers, lung cancers, and hepatocellular carcinoma, although the frequency of Smad2 mutation is much lower than that of Smad4 mutation (21–24). Despite extensive research to detect Smad mutations in cancer specimens, only Smad4 and Smad2 have been found to be mutated (25–27). Because Smad4 is the Co-Smad that is involved in multiple signaling pathways, Smad2 is the only R-Smad that is inactivated in human cancers. However, it remains to be determined whether Smad2 functions as a tumor suppressor gene in the tumor cells carrying Smad2 mutations.

Targeted disruption of Smad2 has been reported (28–31). Mice carrying homozygous inactivation of Smad2 died around E7.5–E10.5 from impaired anterior-posterior axis formation and gastrulation defects. Some heterozygous mutants of Smad2 exhibited anomalous development such as cyclopia and mandibular defects (29, 31) but most heterozygotes were viable, fertile, and appeared normal up to at least 1 year of age. No evidence of any roles of Smad2 mutation in carcinogenesis has thus far been observed in these mice.

Mutations in the APC gene are responsible for familial adenomatous polyposis (32, 33). Inactivation of both APC alleles also frequently occurs in sporadic colorectal adenomas (34, 35). Mice carrying APC mutations in their germ line have been established as experimental models of familial adenomatous polyposis (36–38). These mice also develop numerous intestinal adenomas after inactivation of the second APC allele, although tumors are located more in small intestine rather than in colon and rectum. Because both Apc and Smad4/Dpc4 are located on mouse Chr 18, compound heterozygotes carrying both Apc and Smad4 mutations could be obtained by meiotic recombination onto the same chromosome in the cis-configuration. In the tumors of these mice, both Apc and Smad4 were homozygously inactivated by loss of the entire Chr 18, which included the wild-type alleles of both Apc and Smad4. In such mice, intestinal adenomas developed into more malignant tumors than those in the simple APC heterozygote and frequently exhibited invasive growth (39).

Because Smad2 is also located on mouse Chr 18 adjacent to Smad4, we used the same approach to obtain compound heterozygotes of Apc and Smad2 to assess the effects of Smad2 mutations in later stages of carcinogenesis after adenoma formation caused by inactivation of both Apc alleles. We found that loss of Smad2 did not initiate...
tumorigenesis by itself but accelerated progression of adenomas to invasive cancer.

MATERIALS AND METHODS

Isolation of the Murine Smad2 Gene and Construction of Targeting Vectors. The murine Smad2 gene was cloned using mouse Smad2 cDNA (40) as a probe to screen a mouse 129SV genomic library (Stratagene, La Jolla, CA). The targeting vectors were constructed using a 14-kb genomic fragment. A 3-kb fragment in the genomic clone containing exons 3 and 4 was replaced by PGK-neo cassette (Smad2-N vector) or by PGK-neo cassette and nuclear localization signal-conjugated β-galactosidase gene lead by splice acceptor and enhancer sequences (SA-eh-N LacZ; Ref. 41; Smad2-L vector). The neomycin-resistant gene was in reverse orientation relative to the Smad2 gene (Fig. 1A).

Generation of Smad2 Mutant Mice. The vectors were introduced into J1 ES cells (42) by electroporation using a Bio-Rad Gene Pulser (0.4 kV, 250 mF) after linearization by NotI. Cells were selected in 150 μM G418 (Invitrogen Japan, Tokyo, Japan), and G418-resistant colonies were pooled and analyzed by genomic Southern blotting using an EcoRI/KpnI fragment as a probe template (Fig. 1A, probe 2). Chimeric mice were produced by microinjection of Smad2 heterozygous ES cells into E3.5 C57BL6/J blastocysts and transferred to A129/Sv pseudopregnant foster mothers. Chimeric males were mated with C57BL6/J females, and germ-line transmission of the mutated allele was verified by PCR using tail DNA from agouti coat color F1 offspring. Heterozygous males were backcrossed into C57BL6/J inbred mice.

Genotyping of Mice, Embryos, and Intestinal Tumors. Genomic DNA from tips of mouse tails or whole embryos was analyzed by PCR using the following primers: Primer A, 5'-GCTGGCCGGACCTTCACAGTCATCA-3'; Primer B, 5'-AAGCTCCCAGAAGTGGACGTGTCCC-3'; Primer C, 5'-GCTAAAGCGCATGCTCCAGACTGCCTTG-3'; and Primer D, 5'-CCAGCAATGGGAGGCAGACGAAATC-3'. Primers A and D or B and D were used for amplification of wild-type Smad2 allele (800-bp or 400-bp products, respectively) and primers C and D for amplification of targeted Smad2 allele (200-bp products). For genotyping analysis of the intestinal tumors, genomic DNA was extracted using DEXPAT (Takara, Tokyo, Japan) from adenoma tissues and adjacent intestinal tissues following the manufacturer’s recommendations and analyzed by PCR using the following primers: primers B and D for amplification of wild-type Smad2 allele and C and D for targeted Smad2 allele; for amplification of wild-type and 580D alleles of Apc, primers 3, 4, and 5 were used as described previously (43).

Whole-Mount in Situ Hybridization. Dissected embryos were fixed and processed for in situ hybridization as described previously (44). Antisense RNA probes were prepared by in vitro transcription as described previously (45), using the following cDNAs as templates: Brachyury(T) (46); HNF4 (47); and Hex (48).

Fig. 1. Targeted disruption of Smad2 by homologous recombination. A, schematic drawing of the two targeting vectors, the wild-type allele and the targeted alleles. (a) Smad2-L vector and (b) Smad2-N vector. PGK-neo, PGK-promoter connected with neomycin-resistant gene; SA-eh-N LacZ, nuclear localization signal-conjugated β-galactosidase gene lead by splice acceptor and enhancer sequences; DTA, diphtheria toxin A fragment; Not, NotI; Rl, EcoRI; Hind, HindIII; Kpn, KpnI; XbaI, XbaI; Sma, SmaI; Pat, PstI; Acc, AccI. B, Southern blot analysis of genomic DNA from ES clones digested with XbaI: L281, L301, and N9, ES clones with homologous recombination; Ra, random integrants. C, PCR analysis on genomic DNA of littermates from crosses between Smad2 heterozygous (+/-) F1 mice: 1 and 2, wild-type offspring; 3, 4, 5, and 6, heterozygous offspring; wt, PCR products from wild-type alleles; mut, PCR products from targeted alleles.
Generation of Apc and Smad2 cis-Compound Heterozygous Mice. The cis-compound heterozygotes of Apc and Smad2 were constructed as described for construction of the cis-compound heterozygotes of Apc and Smad4 (39).

Pathological Analysis. Gastrointestinal tissues of mice were cut into three pieces, opened, put on a board, and fixed in phosphate-buffered 10% formalin. After fixation and measurement of numbers and sizes of polyps, the tissues were rolled and embedded in paraffin. Paraffin sections were stained with H&E. Other organs were also examined both macroscopically and microscopically.

RESULTS

Generation of Smad2-deficient Mice. We screened an A129/Sv mouse genomic DNA library using a SalI/SacI fragment of mouse Smad2 cDNA as a probe (probe 1) and obtained five independent genomic clones, including exon 2, and six clones covering both exon 3 and exon 4. We then generated two Smad2 targeting vectors using a clone in the latter group in which a part of the Smad2 gene, including exon 3 and exon 4, was replaced by PGK-neo cassette or SA-eh-N.
Phenotypes of Smad2 Mutant Mice. Because the phenotype of homozygous Smad2-deficient embryos had already been reported, we carried out whole-mount in situ hybridization for Brachyury(T), HNF4, and Hex to examine whether our mice had abnormalities similar to those described in previous reports. Brachyury(T) is an early mesoderm marker (46), and HNF4 (47) and Hex (48) are early visceral endoderm markers. Hex is the earliest known visceral endoderm marker exhibiting asymmetric expression in pregastrulation embryos (48). Homozygous Smad2 mutant embryos exhibited no expression of Brachyury(T), HNF4, and Hex (Fig. 2, C, F, I, respectively), suggesting that the Smad2-deficient mice established here had abnormalities in the visceral endoderm similar to those reported by Nomura and Li (29) and could not organize anteroposterior axis and mesoderm formation. We found three anomalous heterozygous Smad2 mutants with cyclopia and mandibular defects as previously reported (29, 31), but most heterozygotes were fertile and exhibited no obvious abnormality up to at least 1 1/2 year of age.

Generation of Apc/Smad2 cis-Compound Heterozygous Mutant Mice. We next mated Smad2 heterozygotes with Apc<sup>530D</sup> mice (43) to obtain compound heterozygotes of Apc and Smad2. Fig. 3 shows the strategy used to generate APC<sup>(+)</sup>/Smad2<sup>(+)</sup> cis-compound heterozygotes, which was originally reported for generation of Apc and Smad4 cis-compound mice (39). We obtained 47 offspring from intercrosses between Apc/Smad2 trans-compound heterozygotes and C57BL/6J mice. Fig. 4A shows a representative result of genotyping analysis of the offspring. Among 47 offspring, there were 13 cis-compound heterozygotes. Eight littermates with Apc heterozygotes mutation were used as controls. The rate of recombination between Apc and Smad2 loci was 13 of 47 equals 0.28, which agreed well with the theoretical rate estimated from the distance (26 cM) between Apc and Smad2 in mouse Chr 18. The cis-compound heterozygotes developed numerous intestinal tumors as observed in the Apc single heterozygotes. As expected from the finding that Apc LOH involves loss of the entire chromosome (39), wild-type alleles of both Apc and Smad2 were lost in the intestinal tumors that developed in the cis-compound heterozygotes (Fig. 4B).

Phenotype of Intestinal Tumors in Apc/Smad2 cis-Compound Heterozygous Mice. We compared numbers, sizes, and distributions of intestinal tumors in Apc single heterozygotes and Apc/Smad2 cis-compound heterozygotes (Fig. 5). There were no differences in overall numbers, sizes, or distributions between them (Fig. 5, A–C, respectively; Student’s t test, P < 0.05). However, the compound mice had more polyps > 4 mm in diameter than did the Apc single heterozygotes (Student’s t test, P < 0.05) and frequently developed one or two tumors > 6 mm in diameter (Fig. 5D), whereas only one

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**Fig. 3.** Strategy for construction of Apc<sup>(+/-)</sup>Smad2<sup>(+/-)</sup> cis-compound heterozygotes.

**Fig. 4.** Establishment of Apc<sup>(+/-)</sup>Smad2<sup>(+/-)</sup> cis-compound heterozygous mice. A, genotype of offspring from crosses between Apc<sup>(+/-)</sup>Smad2<sup>(+/-)</sup> cis-compound heterozygotes backcrossed with C57BL/6J. 1, Smad2<sup>(+/-)</sup>Apc<sup>(+)</sup>; 2 and 3, Smad2<sup>(+/-)</sup>Apc<sup>(+/-)</sup>; 4 and 5, Smad2<sup>(+/-)</sup>Apc<sup>(+/-)</sup>; and 6, Smad2<sup>(+)</sup>Apc<sup>(+/-)</sup>. B, genotype of intestinal tumors in the Apc heterozygous and Apc/Smad2 cis-compound heterozygous mice. Control tail DNA (control tail) were extracted from the tail tips of Apc<sup>(+/-)</sup>Smad2<sup>(+/-)</sup> cis-compound heterozygotes. T1 and T2, DNA extracted from tumor tissues; normal, DNA extracted from normal intestinal tissues; wt, PCR products from wild-type alleles; mut, PCR products from targeted alleles.

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LacZ and PGK-neo (Fig. 1A). The targeting vectors were introduced into mouse ES cells by electroporation, and 15 homologous recombinants were obtained. Absence of random integration was confirmed by Southern blotting using a neo fragment as a probe (data not shown). Two clones with L-vector integrated (L281 and L301) and one with N-vector (N9; Fig. 1B) were injected into blastocysts. Three mutants with cyclopia and mandibular defects as previously reported (28, 30), but most heterozygotes were fertile and exhibited no obvious abnormality up to at least 1 1/2 year of age.
Apc single heterozygote developed such a large tumor. The number of large tumors was small, probably because such large intestinal tumors cause lethal intestinal obstruction (Fig. 6, A and B). Five Apc/Smad2 compound mice died suddenly during the experiments by 30 weeks from obstruction ileus caused by the tumors. Sudden death did not occur in the Apc single heterozygotes during the observation period.

Histopathological analysis of intestinal tumors in the compound heterozygotes further confirmed the roles of Smad2 deficiency in acceleration of malignant progression of intestinal tumors. Apc single heterozygotes developed tubular adenomas with uniform tubular structure (Fig. 7 A). In contrast, the tumors that developed in the Apc/Smad2 compound heterozygotes frequently exhibited stromal and vascular invasion (Fig. 7, B–D). These invasive phenotypes were found in 10–15% of intestinal polyps in Apc/Smad2 cis-compound heterozygotes. Tubular structures varied in size and shape, and solid colonies of poorly differentiated carcinoma cells were also observed. Nuclei of the tumor cells were more hyperchromatic than those of Apc single heterozygotes, and nuclear atypia was also enhanced. All of these histological findings indicated that Smad2 deficiency increased the malignancy of tumors resulting from Apc deficiency.

DISCUSSION

Smad2 is a receptor-regulated Smad that participates specifically in TGF-\(\beta\) and activin signaling. Targeted disruption of Smad2 caused embryonal lethality around E8.5 through dysfunction of visceral endoderm in the homozygous embryo. We observed neither expression of Brachyury(T) nor mesoderm induction in our homologous Smad2 mutant mice as observed in Smad2 targeted mice reported by Nomura and Li (29) and Weinstein et al. (30).

Heterozygous mice appeared normal up to at least 1 1/2 year of age. Because Smad2 is located on Chr 18 close to Apc and second hits occur as a result of loss of the entire Chr 18 containing the wild-type allele (39), inactivation of the wild-type allele of Smad2 is also expected to occur at rates similar to that of Apc in the intestinal epithelia. However, Smad2 heterozygotes did not develop any gastrointestinal tumors, suggesting that homozygous deletion of Smad2 does not initiate tumor formation at least in intestinal epithelia of mice. This is in contrast to the recent observation by Takaku et al. (49) that Smad4 heterozygotes frequently develop gastric and duodenal polyps after 1 year.

TGF-\(\beta\) has growth inhibitory activity in various cells, and most carcinoma cell lines were found to be resistant to the growth inhibitory activity of TGF-\(\beta\) (15). Loss of TGF-\(\beta\) signaling was thus expected to contribute to tumorigenicity by disturbance of cell growth arrest. However, our study together with the previous report on Apc/Smad4 cis-compound mice by Takaku et al. (39) show that deficiency of Smad2 or Smad4, which are the only Smads mutated in human cancers, could not initiate excess and autonomous cell growth in intestinal epithelia of mice for up to 1 year. In Apc mutant mice, however, tumor growth and invasion were accelerated by loss of Smad2 or Smad4. Acceleration of tumor cell growth was more pronounced with loss of Smad4 than with that of Smad2. Loss of Smad4 reduced the number of polyps in small intestine, but the size and

Fig. 6. A large intestinal tumors at 7 mm in diameter in the proximal jejunum of an Apc/Smad2 cis-compound mouse caused lethal intestinal obstruction and sudden death at 20 weeks of age.
relative numbers of colorectal polyps were increased (39). Furthermore, tumor invasion was observed more frequently in Apc/Smad4 cis-compound heterozygotes than in Apc/Smad2 heterozygotes (>50% versus 10–15% of polyps). Signet ring cells were found in the intestinal polyps of Apc/Smad4 heterozygotes but not in Apc/Smad2 heterozygotes. In this respect, Smad2 deficiency may be partially compensated for by Smad3, which transduces TGF-β signals together with Smad2. Homozygous Smad3 mutant mice were reported to be viable and to develop multiple colorectal cancers (50), although no Smad3 mutation has been reported in human cancer, and Smad3-targeted mice reported by other authors did not develop colorectal cancer (51, 52). Smad4 is the only Co-Smad found in mice and is involved in both TGF-β/activin and BMP signaling pathways. Moreover, Smad4 has been reported to function in roles other than TGF-β superfamily signaling such as Wnt signaling (8). These differences between Smad2 and Smad4 function may contribute to the differences in severity in phenotypes of tumors between Apc/Smad4- and Apc/Smad2 cis-compound mice. Notably, Smad4 mutations are observed with high frequency in pancreatic cancer in humans (18), but no pancreatic cancer developed in either Apc/Smad2 cis-compound heterozygotes or Apc/Smad4 cis-compound heterozygotes (39).

Resistance to TGF-β signaling is usually acquired in the late stages of carcinogenesis (15, 20). Targeted deletion of the TGF-β1 gene was also reported to cause colorectal cancer (53) and rapid progression of epidermal cells to skin cancer (54). Furthermore, overexpression of TGF-β1 in skin of mice reduced the incidence of benign papillomas but increased the rate of development of invasive cancer (55). Although it is unknown how both overexpression and deletion of TGF-β1 increase the rate of malignant progression of cancer (56), this study has clearly shown that deficient Smad2 signaling increase the rate of initiation of cancer invasion. Additional studies are required to reveal the role of impaired TGF-β signaling in cancer progression.

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