Gastric and Duodenal Polyps in Smad4 (Dpc4) Knockout Mice

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Abstract

The SMAD4 (DPC4) gene was initially isolated as a candidate tumor suppressor from the convergent site of homozygous deletions on 18q in a panel of pancreatic carcinoma cell lines. It encodes a common cytoplasmic signaling molecule shared by the transforming growth factor-β, activin, and bone morphogenic pathways. We recently inactivated its mouse homologue Smad4 and demonstrated its role in the malignant progression of benign adenomas to invasive adenocarcinomas by analyzing mice with Apc and Smad4 compound mutations. Although simple Smad4 homozygotes were embryonically lethal, the heterozygotes were fertile and appeared normal up to the age of 1 year. Upon further investigation, however, they have developed inflammatory polyps in the glandular stomach and duodenum. By PCR genotyping and immunohistochemical staining, the wild-type Smad4 allele has been lost in the polypl epithelial cells, i.e., loss of heterozygosity. On the other hand, we have not found any mutations in such genes as K-Ras, H-Ras, N-Ras, p53, or PTEN. Histologically, the polyps are similar to human juvenile polyps showing moderate stromal cell proliferation and infiltrations by eosinophils and plasma cells. In addition, foci of adenocarcinoma with signet ring cells are also found. These results are consistent with a recent report that germ-line SMAD4 mutations are found in a subset of familial juvenile polyposis.

Introduction

Mutations in the SMAD4 (DPC4) gene located on chromosome 18q21 are associated with not only pancreatic cancer (1) but also colorectal carcinoma (2). SMAD4 belongs to the SMAD gene family involved in the signal transduction pathways that are activated through the TGF-β family receptors (3–5). Although TGF-β suppresses normal cell growth, tumors acquire resistance to TGF-β by several mechanisms relatively late in tumorigenesis, usually correlating with invasive changes (6). For example, decreased expression or mutation of TGF-β has been observed in invasive and metastatic carcinomas (7). In addition, LOH and mutations in the SMAD4 gene were reported in invasive and metastatic carcinomas (8). We demonstrated recently that homozygous inactivation of the Smad4 gene causes the polyp adenomas in the Apc mutant mice to progress into invasive and malignant adenocarcinomas (9). The strategy was based on the findings that mouse Smad4 and Apc are mapped on the same chromosome (chromosome 18), 30 cM apart, and that Apc LOH involves loss of the entire chromosome.

Familial juvenile polyposis is an autosomal dominant disease in which individuals are predisposed to inflammatory polyps and gastrointestinal cancer (10). Recently, a subset of juvenile polyposis patients was reported to carry germ-line mutations in the SMAD4 gene (11, 12). Many of the patients had a 4-bp deletion in exon 9 of the SMAD4 gene, which caused a truncation in the MH2 domain near the COOH terminus, resulting in defective signal transduction. Interestingly, another subset of juvenile polyposis patients was reported to carry mutations in the PTEN gene located on chromosome 10q23 and encoding a phosphatidylinositol 3,4,5-phosphate phosphatase (13, 14). The heterozygous Pten knockout mice, however, show pathologic characteristics similar to Cowden disease and its related syndromes, which are caused by mutations in the PTEN gene (15).

Regarding our Smad4 knockout mice (9), the heterozygotes were fertile and appeared normal up to the age of about 1 year, although homozygous mutants were embryonically lethal. Here we report that, upon further observation, the heterozygous mice older than 1 year have developed multiple gastric and duodenal polyps that show much similarity to those in human juvenile polyposis.

Materials and Methods

Generation of Smad4 Knockout Mice. The heterozygous knockout mice we reported recently (9) were originally in the mixed background between 129/Sv and C57BL/6N. They were backcrossed further to the C57BL/6N for six generations.

Polyp Scoring. The number and size of the polyps were scored by a single examiner (K. T.) as described (9, 16, 17).

PCR Detection of Smad4 LOH. Epithelial cells were collected as described earlier (9, 16). Primers were used DPC4F1, PGKR, and DEIR1, as described previously (9).

Histochemical Analysis. Samples were fixed, sectioned, and stained with H&E or Alcian blue (pH 2.5) as described previously (9). Staining with the periodic acid-Schiff reagent was performed according to Movry (18).

Immunohistochemistry. Essentially the same procedures were used as described previously (9, 17). Samples were stained with the following antibodies: goat polyclonal antibodies against SMAD4 COOH- and NH2-terminal peptides, respectively (identical to corresponding mouse sequences; C-20 and N-16, respectively; Santa Cruz Biotechnology); a rabbit polyclonal antibody against human TGF-β1 (identical to the corresponding mouse sequence; Santa Cruz Biotechnology); a rabbit polyclonal antibody against human TGF-β receptor type II (cross-reacting with the mouse protein; Santa Cruz); a rabbit polyclonal antibody against human adenomatous polyposis coli (cross-reacting with the mouse protein; Santa Cruz Biotechnology); and a rabbit polyclonal antibody against the COOH-terminal residues (amino acids 768–781) of human β-catenin but identical to the corresponding mouse sequence (Sigma Chemical Co.).

Sequencing Analyses. The genomic DNA segments for the mutational hot spots at codons 12, 13, and 61 of the K-Ras (Kras2), H-Ras (Hras1), and N-Ras (Nras) were amplified by PCR and sequenced as described previously (19). The coding sequence for the p53 gene was determined as described (20). The genomic sequence of Pten for the central two-thirds of the coding region was amplified and sequenced as described (21).

Results

We earlier found that the mouse Smad4 (Dpc4) gene maps on chromosome 18, 30 cM distal from Apc. We then inactivated the gene in ES cells by homologous recombination and established a germ line-transmitted mutant strain (9). Although the homozygotes were embryonically lethal, the heterozygotes were fertile and appeared

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3 The abbreviations used are: TGF, transforming growth factor; LOH, loss of heterozygosity; GSK, glycogen synthase kinase.
normal up to the age of ~1 year. Upon examination of 1.5-year-old Smad4 heterozygotes, however, we recently found multiple gastric polyps in all six mice, whereas no such polyps were found in the wild-type littermates. Accordingly, we examined more mice of 25, 30, 50, 65, 75, 85, and 100 weeks of age, respectively (Table 1). At the age of 100 weeks (close to 2 years), gastric polyps were found in all eight mice examined, whereas 3 of 15 mice had such polyps at the age of 50 weeks (~1 year). The polyp multiplicity ranged from one to three per stomach. When 15 heterozygotes at 25 or 30 weeks were examined extensively, however, no polyps were found in any mice.

As shown in Fig. 1A, the gastric polyps were found in the glandular part of the stomach, often in the pyloric region. They showed a pedunculate or sometimes sessile morphology with their sizes ranging from 0.5 to 15 mm in diameter. Their histopathology was characterized by elongated and dilated tubular structures with a moderate stromal cell expansion (Fig. 1, B and C). Although the epithelium that lined polyp glands were mostly hyperplastic showing little cellular atypia (Fig. 1D), foci with obvious dysplastic signs were also observed occasionally with nuclear pseudostratification (Fig. 1E) or abundant mucinous adenocarcinoma cells, including signet ring cells.
At the same time, some infiltrations of eosinophils and plasma cells were observed (data not shown). Interestingly, similar polyps were found in the duodenum as well in mice. Such polyps were not found in the wild-type littermates. The polyp multiplicity in the heterozygotes increased with age up to 15, in mice between 65 and 85 weeks of age. Under a dissection microscope, the duodenal polyps had a sessile morphology, and they were sometimes buried in the epithelial surface. These duodenal polyps included some small “nascent” ones, the histological pictures of which showed mostly hyperplastic epithelium with minimal cellular atypia. However, some foci were found showing abundant mitotic figures and nuclear pseudostratification, with crowding of the crypts.

Because of recent reports that a subset of familial juvenile polyposis kindreds carries germ-line mutations in the **SMAD4** gene, we carefully compared the pathological characteristics of the gastric and duodenal polyps with those of human juvenile polyposis. As in human juvenile polyposis, numerous eosinophils as well as plasma cells were observed, although the extent of the stromal expansion was much less in the mouse polyps, showing less granulation and less infiltration of lymphocytes. These inflammatory pictures are characteristic of the human juvenile polyposis, although the subset of juvenile polyposis that carry **SMAD4** germ-line mutations may be limited to a relatively small fraction. In addition, some villi in the duodenum showed an aberrant appearance under a dissection microscope.

To investigate whether the polyps were initiated because of LOH of the **Smad4** gene, we isolated the epithelial layer from polyps of 1.5-year-old heterozygotes and determined the **Smad4** genotype by PCR. The results in Fig. 3 showed unambiguous LOH in all four polyp epithelial samples. To confirm the LOH histologically, we further stained polyp sections with antibodies against **Smad4** NH2- and COOH-terminal peptides, respectively. As shown in Fig. 3B, the polyp epithelium was not stained by these antibodies, whereas the adjoining normal epithelium was. The same result was obtained with the nascent duodenal polyps as well (Fig. 3C). Accordingly, the **Smad4** LOH is one of the earliest events in polyp formation and likely to be the triggering event for both stomach and duodenal polyp initiation.

It has been established that loss of **Apc** heterozygosity plays a key role in polyp initiation in **Apc**-null knockout mice. **APC** protein

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**Table 1** Gastrointestinal polyps in **Smad4** heterozygotes

<table>
<thead>
<tr>
<th>Age, weeks</th>
<th>No. of polyp-bearing mice/No. of mice examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 (0.5 yr)</td>
<td>Gastric0/11 Duodenal0/11</td>
</tr>
<tr>
<td>30</td>
<td>0/4 0/4</td>
</tr>
<tr>
<td>50 (1 yr)</td>
<td>3/15 1/15</td>
</tr>
<tr>
<td>65</td>
<td>3/3 3/3</td>
</tr>
<tr>
<td>75 (1.5 yr)</td>
<td>6/6 1/6</td>
</tr>
<tr>
<td>85</td>
<td>1/1 1/1</td>
</tr>
<tr>
<td>100 (2 yr)</td>
<td>8/8 8/8</td>
</tr>
</tbody>
</table>

*All gastric polyps were found in the glandular stomach, mostly in the pyloric region.

*Including nascent polyps in the early stage of formation.

*It is likely that we overlooked duodenal polyps in other animals.
binds to β-catenin and stimulates phosphorylation of β-catenin by GSK3β, one of the effectors in the Wnt signaling pathway (24). If β-catenin phosphorylation by GSK3β is blocked by an Apc mutation, unphosphorylated and therefore stable β-catenin accumulates, moves into the nucleus, and activates a new set of genes. To rule out the possibility that the Wnt pathway was activated by either Apc or β-catenin gene mutations in the Smad4 mutant polyps, the subcellular localization of β-catenin was determined by immunohistochemical staining. As shown in Fig. 3D, we could not detect any nuclear localization of β-catenin in the polyps of the Smad4 heterozygotes. On the other hand, a prominent nuclear staining was observed in the polyp epithelium of Apc<sup>716</sup> knockout mice (Fig. 3E). We also determined the sequence of the β-catenin gene for exon 3 that contained the serine and threonine residues to be phosphorylated by GSK3β and responsible for its rapid degradation and the subsequent suppression of the Wnt signaling pathway (24). But we could not find any such β-catenin mutations in the polyp epithelial cells (data not shown).

To examine the possibility of additional mutations in other genes that may be responsible for the polyp formation in the Smad4 heterozygous mice, we determined the sequences of the mutational hot spots at codons 12, 13, and 61 of Kirsten-Ras (Kras2), Harvey-Ras (Hras1), and N-Ras (Nras) genes but did not find any mutations (data not shown). We also determined the sequence of the central two-thirds (all exons other than 1, 4, or 9) of the p53 gene (Trp53) coding region that contained all mutational hot spots, without any mutations found (data not shown). Because another subset of familial juvenile polyposis was reported to carry germ-line mutations in the PTEN gene (25, 26), a gene encoding a phosphatidylinositol 3,4,5-phosphate phosphatase (27–29), whose germ-line mutations are responsible also for Cowden disease (13, 30), we further investigated its mouse homologue Pten in the polyps of the Smad4 heterozygotes. However, we found neither LOH nor mutations in the coding sequence in any polyp DNA samples (data not shown).

Discussion

The similarities between the polyps in Smad4 heterozygous mice and those in human juvenile polyposis are more important than the differences. Although juvenile polyposis primarily affects the colon, at times it involves the entire gastrointestinal tract or presents as isolated gastric juvenile polyposis. Polyps may be present in early infancy or, more commonly, are discovered in childhood or early adulthood (31). Histologically, juvenile polyps are characterized by abundant stroma infiltrated by a variable number of lymphocytes, plasma cells, neutrophils, and eosinophils and by dilated, often cystic, mucinous glands (31). Familial juvenile polyposis predisposes to gastrointestinal cancer and possibly to pancreatic cancer (32–34). In fact, the gastric and duodenal polyps in the Smad4 heterozygous mice resembled human juvenile polyps because of their moderate stromal cell expansion and prominent infiltrations by eosinophils and plasma cells. In addition, we have found some foci showing clear signs of adenocarcinoma with signet ring cells. The differences of the mouse disease from the human juvenile polyposis are the onset of the disease late in the mouse life (mostly >1 year) and relatively moderate pathological pictures, which can be explained by the less
advanced state of the disease than those found clinically in most human juvenile polyposis.

Although juvenile polyps are often classified as hamartomatous polyps compared with adenomatous polyps (10), most pathologists have interpreted juvenile polyps as inflammatory lesions with reparative changes (22). Hamartomas are nonneoplastic nodules composed of an abnormal overgrowth of indigenous cell types with evidence of an underlying developmental etiology (35). For example, we demonstrated recently that hamartomatous polyps develop in the cecum and the proximal colon of mice heterozygous for the Cdx2 gene. The tumors started to develop as outpouching pouches at 11.5 days of gestation and formed partial duplications of the gut, which were contained later as hamartomatous polyps (36). The polyps in the Smad4 heterozygous mice, however, were not such hamartomas but developed late in their lives and more appropriately described as inflammatory polyps.

Recently, it was reported that another subset of juvenile polyposis patients carried germ-line mutations in the PTEN gene (25), although three earlier reports found no evidence of germ-line PTEN mutations in 21 juvenile polyposis syndrome families and 16 sporadic cases (37). On the other hand, germ-line mutations in the PTEN gene have been found in two related autosomal dominant hamartomatous polyposis syndromes, Cowden disease and Bannayan-Ruvalcaba-Riley syndrome (13, 14). Although Cowden disease and its related syndromes predispose to malignant cancer of the breast and the thyroid, the polyposis in the gastrointestinal tract is of benign nature. In contrast, the juvenile polyps of the gastrointestinal tract are reported to predispose to malignant changes in more than one-half of cases (32, 33). Accordingly, questions arise whether mutations in the same gene can cause two separate disease entities, or the diagnosis for these diseases may not always be clear and unambiguous. In this regard, it is worth noting that heterozygous mice for the PTEN gene knockout mutation show hyperplastic-neoplastic changes in the prostate, skin, and colon, which are more characteristic of Cowden disease and related syndromes than those of juvenile polyposis (37).

In conclusion, we have demonstrated that Smad4 heterozygous mice develop gastric and duodenal polyps because of LOH of the Smad4 gene late in their lives. This animal model should provide a useful means to investigate and develop treatment for a subset of the human familial juvenile polyposis that carries germ-line SMD4 mutations.

References


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