Allelic Loss at SMAD4 in Polyps from Juvenile Polyposis Patients and Use of Fluorescence in Situ Hybridization to Demonstrate Clonal Origin of the Epithelium

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ABSTRACT

Juvenile polyposis syndrome (JPS; Online Mendelian Inheritance in Man2 174900) is a rare Mendelian disorder in which individuals have typical hamartomatous polyps within the gastrointestinal tract. The stromal element of the polyps has classically been thought to be the proliferative component, although epithelial malignancies (largely gastrointestinal cancers) occur more frequently than expected in JPS patients. Germ-line mutations in SMAD4 (DPC4) account for about a third of JPS cases. It has been postulated that the apparent paradox of a stromal lesion predisposing to epithelial malignancy can be resolved by the “landscaper” effect: an abnormal stromal environment affects the development of adjacent epithelial cells, and the resulting regeneration of damaged epithelium leads to an increased risk of cancer. We have found allelic loss at the SMAD4 locus on 18q in polyps from JPS individuals with a germ-line SMAD4 mutation, showing that SMAD4 is acting as a tumor suppressor gene in JPS polyps, as it does in sporadic cancers of the gastrointestinal tract. Interphase fluorescence in situ hybridization showed deletion of one copy of SMAD4 in the epithelial component of JPS polyps, but not in the inflammatory infiltrate. Fluorescence in situ hybridization also suggested that a single copy of SMAD4 was present in stromal fibroblasts of JPS polyps. Thus, biallelic inactivation of SMAD4 occurs in both the epithelium and some of the stromal cells in these lesions, suggesting a common clonal origin. Epithelial malignancies almost certainly develop in juvenile polyposis through direct malignant progression of the epithelial component of the hamartomas. SMAD4/DPC4 probably acts as a “gatekeeper” tumor suppressor in juvenile polyps, and there is no need to invoke a “landscaper hypothesis.”

INTRODUCTION

The hamartomatous polyposis disorders comprise five main conditions including JPS.3 PJS (OMIM 175200), CS (OMIM 158350), Bannayan-Zonana syndrome (OMIM 153480), and GS (OMIM 109400). All of these syndromes show Mendelian dominant inheritance. PJS polyps typically show an arborizing structure with a smooth muscle core. The hamartomas in the other syndromes are all of a JPS type, classically characterized by a rounded polyp composed of cystically dilated glands with a hypercellular stroma (1). Each syndrome is associated with organ-specific malignancies: PJS predisposes to cancers of various sites, including the breast, cervix, and gastrointestinal tract; CS is associated primarily with breast and thyroid cancers; basal cell carcinomas are typical of GS; and JPS patients tend to develop cancers of the colorectum and upper gastrointestinal tract.

The epithelium of PJS polyps has a monoclonal origin, as demonstrated by the finding of allelic loss at the LKB1/STK11 locus on chromosome 19p13.3 (2–4). PJS-associated cancers also show loss of the wild-type allele at this site, strongly suggesting progression from hamartoma to carcinoma. Hamartomas, adenomas, and carcinomas from patients with CS show LOH at the PTEN/MMAC1 locus on chromosome 10q23.3 (5), again suggesting that this gene is acting as a tumor suppressor, with loss of the wild-type gene initiating hamartoma growth and leading to subsequent progression to carcinomas in some cases. Basal cell carcinomas from patients with GS have shown allelic loss in the PITCH region of chromosome 9q31, indicating that the PITCH gene is homozygously inactivated in these hereditary tumors (6); however, because juvenile polyps occur as an infrequent component of this disease, allelic loss has not been investigated in the hamartomas themselves.

Recent work has identified germ-line mutations in the SMAD4 (DPC4) gene on 18q21.1 as a cause of JPS in a subset of families (7, 8). The SMAD4 gene encodes a protein that is a cytoplasmic mediator in the transforming growth factor β signaling pathway. Many of the reported mutations produce SMAD4 proteins that are truncated at the COOH terminus and thus lack normal function. SMAD4 is known to act as a tumor suppressor gene in cancers of the pancreas (9) and the colon (10, 11). The high incidence of colorectal cancer in a large JPS kindred with linkage to 18q21.1 and the subsequent observation of mutations in the SMAD4 gene in this kindred led to the reasoning that SMAD4 might also be acting as a tumor suppressor gene in JPS polyps (7, 8). However, it has recently been postulated that in cancer predisposition syndromes such as JPS, susceptibility genes work through less direct mechanisms (12). It was proposed that the neoplastic progression is driven by initial genetic changes within the stromal cells of the hamartoma (classically the clonal component of these lesions) and that an increased risk of cancer is the result of an altered terrain for epithelial cell growth (the “landscaper” effect). In contrast, classic tumor suppressor genes such as APC were said to function as “gatekeepers,” preventing runaway growth, and the DNA repair proteins such as MLH1 and MSH2 were thought to be acting as “caretakers” of the genome (13).

The aim of this study was to investigate whether homozygous inactivation of SMAD4 was present in the polyps of individuals with pure JPS who had a known constitutional SMAD4 mutation. The results have implications not only for the role of SMAD4 as a tumor suppressor in JPS, but also for a wider understanding of how neoplasms develop in a variety of stromal-mediated syndromes.
suppressor gene but also for the clonal origins of JPS polyps and the "landscaper" hypothesis.

MATERIALS AND METHODS

Patients were selected who had pure JPS without any evidence of clinical manifestations associated with other hamartoma syndromes, as determined from detailed pathology reports and family history questionnaires. Constitutional DNA was extracted from peripheral blood using standard methods. JPS polyps retrieved from archives had been reported to be of typical JPS type, with the exception of one polyp that possessed mixed hamartoma/adenoma features. In total, there were 17 polyps from four patients (three of whom were related) carrying a constitutional SMAD4 mutation (14). Polyp material was available from one other patient with a germ-line SMAD4 mutation, but due to lack of normal material, this patient was excluded from the microsatellite studies. Also studied were 21 polyps and 8 carcinomas from 14 individuals who: (a) have had SMAD4 excluded as the cause of their JPS by linkage analysis; (b) have no detectable germ-line SMAD4 mutation, or (c) have not been tested for germ-line SMAD4 mutations. No patient in this study had a PTEN or PITCH mutation.

For the assessment of allelic loss, slides from paraffin-embedded tissue were crudely microdissected using a fine blade or needle to remove polyp tissue from its stalk and any surrounding normal tissue. The stromal and epithelial components were not separated. DNA was extracted using standard methods [for larger polyps, we used the QiaGen (Hilden, Germany) tissue extraction kit, and for smaller polyps, we used proteinase K digestion and phenol/chloroform purification]. PCR was performed in duplicate on polyp/normal samples for seven microsatellite markers (D18S877, D18S851, D18S474, D18S878, ATAD7D07, ATAD82B02, and GATA177C03) that lie at approximately 10 cm intervals along chromosome 18q (Fig. 1) and one marker on chromosome 18p (D18S542). PCR conditions were as follows: 25-μl total volume with approximately 12 ng of DNA as a template with 1× standard PCR buffer, 1.5 mM Mg2+, 0.25 mM deoxynucleotide triphosphates, 0.25 unit of Taq polymerase, and 0.5 mM of each oligonucleotide primer, with the forward primer fluorescence labeled with HEX, FAM, or TET. Cycling conditions consisted of an initial denaturation at 94°C for 5 min; 30 cycles each at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and then a final extension step at 72°C for 10 min. A portion of each PCR product (0.2 μl) was combined with 0.2 μl of Tamra350 size standard (Perkin Elmer Applied Biosystems, Warrington, United Kingdom) and 3 μl of formamide loading buffer. After denaturation at 94°C for 5 min, products were electrophoresed on a 4.25% sequencing gel on an ABI377 semiautomated sequencer for 2 h. Results were analyzed using Genotyper software, with areas under the peaks (including sequencing gel on an ABI377 semiautomated sequencer for 2 h. Results were analyzed using Genotyper software, with areas under the peaks (including

RESULTS

Allelic Loss Detected around SMAD4 in Polyps from Individuals with Germ-line SMAD4 Mutations. A total of 46 polyps and cancers from 18 individuals with JPS were screened for loss of chromosome 18 microsatellites. Four of the individuals (patients 5, 6, and 7, who are from the same family, and patient 2) had had mutations in SMAD4 identified as the cause of their JPS (14). Eight markers were used along chromosome 18 (Fig. 1): (a) D18S542; (b) D18S877; (c) D18S851; (d) D18S474; (e) D18S878; (f) ATAD7D07; (g) ATAD82B02; and (h) GATA177C03. The SMAD4 locus reportedly lies between D18S851 and D18S878 and is thought to be closest to D18S474 (approximately 0.1 Mb distal). Fig. 2 shows the results of the allele loss studies.

Allelic loss of microsatellites near the SMAD4 locus was detected in multiple polyps from two of the individuals studied who had known constitutional mutations (patients 2 and 5; Fig. 3), whereas microsatellites proximal and distal to the SMAD4 locus did not show loss. In all cases, it was the wild-type allele that was lost, as ascertained by genotyping family members. Patient 2 was informative for six of the eight markers, and in all six juvenile polyps studied, loss was apparent for one or more microsatellites. The marker that showed the most frequent loss in juvenile polyps from this patient was D18S474, the marker closest to SMAD4. In one polyp (polyp 2.4) from patient 2, only this marker was lost, whereas the other polyps showed loss over a greater distance. Polyp 2.1 from this individual shows loss of all informative markers along the long arm of chromosome 18q, indicating that a major event led to the loss of the second functional copy of SMAD4; this is interesting to observe in a benign lesion which for many years was thought to be nonneoplastic and without malignant potential. It is apparent that different regions of the chromosome have been lost in different polyps, indicating that different mechanisms may be involved in inactivation of the second copy of SMAD4.

Patient 5 also possesses a constitutional SMAD4 mutation and showed LOH of one or more markers in seven of nine juvenile polyps studied. The two markers that encompass SMAD4 (D18S851 and D18S878) showed loss in five of seven polyps. In all cases, the

Fig. 1. Positions of each of the microsatellite markers on chromosome 18 used for the LOH analysis. The position of SMAD4 is shown in bold.
putative wild-type allele was lost. The marker closest to \textit{SMAD4}, D18S474, was uninformative in this individual. The other two polyps of these seven (polyps 5.6 and 5.8) showed loss of just one marker each, neither of which was close to \textit{SMAD4}. It is possible that polyps 5.6 and 5.8 and the polyps that did not show any loss of any marker (polyps 5.3 and 5.9) had their second copy of \textit{SMAD4} inactivated by an alternative mechanism such as point mutation. There is also the possibility that with only crude microdissection, there is contaminating normal tissue confounding the detection of loss in a fashion that is inconsistent between different markers. Patients 6 and 7, who are from the same family as patient 5, showed no loss in either of two JPS polyps, but these individuals were poorly informative at many markers.

Nine patients (patients 1, 3, 4, 13, 14, 15, 16, 17, and 18) had no
detectable germ-line SMAD4 mutation. Of 16 JPS hamartomas from these individuals, 10 showed no allelic loss at any marker, and the remainder showed loss of just one marker. Loss was observed close to SMAD4 in only two polyps (polyps 1.1 and 13.2). One patient had a mixed juvenile/adenomatous polyp (polyp 3.5), and this polyp showed loss around SMAD4. Of eight cancers (from patients 3, 4, 17, and 18), six (polyps 3.2, 3.3, 3.4, 4.2, 17.1, and 18.1) lost alleles at markers around SMAD4. Two cancers (polyps 3.6 and 4.1) showed no loss around SMAD4. Patients 3, 17, and 18 are from the same family. Gastrointestinal cancers and the mixed polyp from this family appear to show loss of a large portion of 18q encompassing SMAD4, although some markers are uninformative. This family has had germ-line mutations in SMAD4 excluded by both linkage analysis and mutation screening. The observed allelic loss in these cancers almost certainly reflects the loss of 18q that is found in approximately 60% of sporadic colorectal cancers (10, 11), although no studies of small bowel cancers have been performed.

Single polyps from patients 8 and 11 lost only D18S851, although markers nearer to SMAD4 failed in the PCR; therefore, it is possible that they may also be lost. A single marker (ATA7D07) was lost in the sole polyp from patient 9. No loss was observed in single polyps from patients 10 and 12. None of these four patients has been screened for mutations in SMAD4, and the loss of 18q observed in some of these polyps may reflect a “second hit.”

**FISH Demonstrates that Individuals with SMAD4 Germ-line Mutations Show Clonal Origin of Epithelial Cells and Some Stromal Cells in JPS Polyps.** FISH was performed on paraffin blocks from three JPS polyps from three patients (patients 2, 5, and 19) who have germ-line SMAD4 mutations. Patient 19 was not included in the allelic loss study because of a lack of normal material. PAC clone (224 j 22) was used as a probe for 18q21. To ensure that SMAD4 was the target for deletion studies, exons 2 and 11 of this gene were amplified in 100% of clones tested, and the PAC clone was mapped to 18q21 using normal metaphase spreads. Using this probe, we observed only one signal in epithelial cells of juvenile polyps (90%, 95%, and 82% from patients 2, 19, and 5, respectively; Fig. 4, a–c). Infiltrating stromal lymphocytes showed two signals in over 90% of nuclei from all three polyps (Fig. 4f). Intriguingly, the stromal fibroblasts and pericytect fibroblasts from each polyp also showed just one signal in between 83% and 90% of cells, respectively (Fig. 4, d and e).

To control for counting whole nuclei, an 18-centromere probe was also hybridized to the same sections analyzed using PAC 224 j 22; the 18-centromere probe showed two signals in the great majority of cells of both stromal (87%) and epithelial (85%) origin (Fig. 4g). To control for hybridizing efficiency, PAC 224 j 22 was used as a probe against both normal colon and appendix sections from an unrelated individual. Two signals were observed in the majority of stromal and epithelial cells (94% and 90% of colon and appendix, respectively; Fig. 4h). To ensure that the cells counted were of the origin indicated by their morphology (stromal, epithelial, or inflammatory infiltrate), Giemsa staining of the same section used for FISH was performed, and antibodies

![image](cancerres.aacrjournals.org)
polyps using FISH. FISH found no loss of SMAD4. However, we found loss of SMAD4 (Fig. 5). This was true of juvenile polyps from many different patients, dissimilar to adenomas, with an abundance of entrapped epithelium.

Isolated allelic loss are false positives resulting from variation in experiments being performed in duplicate, some of these examples of changes unrelated to tumorigenesis. It is even possible that despite all possibility to explain these isolated losses is background genetic might be important in the pathogenesis of these JPS polyps. Another possibility to explain these isolated losses is background genetic changes unrelated to tumorigenesis. It is even possible that despite all experiments being performed in duplicate, some of these examples of isolated allelic loss are false positives resulting from variation in allelic amplification efficiency.

Other patients without detectable SMAD4 mutations showed allelic loss in JPS polyps at occasional microsatellites on 18q. It is possible that these changes, regardless of whether or not they target SMAD4, might be important in the pathogenesis of these JPS polyps. Another possibility to explain these isolated losses is background genetic changes unrelated to tumorigenesis. It is even possible that despite all experiments being performed in duplicate, some of these examples of isolated allelic loss are false positives resulting from variation in allelic amplification efficiency.

Contrary to previous studies (1, 12, 17), our results suggest that the juvenile polyp in JPS is not simply a stromal lesion with an excess of connective tissue. Microscopically, the polyps in this study were not dissimilar to adenomas, with an abundance of entrapped epithelium (Fig. 5). This was true of juvenile polyps from many different patients, both with and without SMAD4 mutations. Consistent with these appearances, we found loss of SMAD4 in epithelial cells from JPS polyps using FISH. FISH found no loss of SMAD4 in stromal lymphocytes but did find loss in the less numerous stromal fibroblasts and pericytial myofibroblasts. This result suggests that the epithelium of JPS polyps is clonal and that part of the stroma is also derived from the same clone, contrary to general histological theory. Loss of SMAD4 in both epithelium and stroma can also explain how microsatellite analysis can readily and consistently detect allele loss at multiple markers on 18q in crudely microdissected polyps, despite the presence of the considerable inflammatory infiltrate.

Hamartomas from tuberous sclerosis patients contain several tissue components and have been shown to be clonal (18), neodifferentiation of melanoma cells into stroma has recently been observed (19), and malignant mixed Müllerian tumors of the ovary have a monoclonal origin of epithelial and mesenchymal cells (20). We cannot exclude the possibility that stromal and epithelial cells in JPS polyps arise from different clones, both of which have independently lost SMAD4; presumably, polyp growth would not occur without mutations in both tissue components. We are, moreover, loathe to exclude the final explanation, artifact, for our findings, although there is no obvious source for this, and we made every attempt to avoid methodological problems: (a) tissue sections were 9-μm thick; (b) two signals were observed in the nuclei of inflammatory cells; (c) normal tissue showed two FISH signals; (d) an 18-centromere probe showed two signals in JPS polyps; and (e) all FISH experiments were performed in triplicate.

Previously, after detecting a germ-line deletion of 10q in a patient who had juvenile polyps and multiple congenital abnormalities, Jacoby et al. (17) used allele loss/FISH analysis to show somatic deletion of chromosome 10q22 in juvenile polyps. The putative locus contained within the 10q22 region was termed JP1 and was postulated to account for a large proportion of JPS cases. Loss of chromosome 10q led to the conclusions that JPS may be allelic to CS that had been mapped to the same region and that PTEN may be the “hamartoma” gene. Most of the patients in the study of Jacoby et al. (17) who showed allelic loss were less than 10 years of age, and, given the multiple abnormalities of the patient with the interstitial deletion, it was possible that many of the patients in the study of Jacoby et al. (17) actually had CS. Not all patients could have had CS, however, because Marsh et al. (21) concluded that the minimal region of allele loss in the patients of Jacoby et al. (17) did not include the CS locus. Soon afterward, PTEN mutations were shown to cause CS, but the timing was such that Jacoby et al. (17) were unable to show germ-line PTEN mutation in any of their patients. Therefore, there is no evidence that the 10q22 changes in their polyps were “second hits,” and their significance for tumorigenesis was unknown.

Jacoby et al. (17) found that their patients’ polyps harbored 10q deletions in inflammatory lymphocytes in the lamina propria, suggesting that the juvenile polyp may be a lymphomatous neoplasm. This is contrary to our findings. There were potentially important methodological differences between our work and that of Jacoby et al. (17). It has been demonstrated that when using FISH, sections of 6 μm contain almost no nuclei that are uncut (22). Therefore, it may not be appropriate to conclude that loss has occurred using the 5-μm sections used by Jacoby et al. (17), especially if cells comprising the tumor vary in size from normal tissues and/or if stromal and epithelial cells are of different sizes. Jacoby et al. (17) used much lower thresholds than those used in this study to assess allelic loss using microsatellites. Whereas any threshold for the analysis of lesions of uncertain clonal origin must, to some extent, be arbitrary, the lower thresholds of Jacoby et al. (17) are likely to increase sensitivity but run the risk of false positives. For the FISH analysis, Jacoby et al. (17) did not hybridize their 10q probe to control sections to check for differences in probe hybridization efficiency, and they used a chromosome 21 control probe that may itself have undergone changes in polyps or be subject to important differences from the 10q probe.

![Fig. 5. H&E-stained section (×10 magnification) of a juvenile polyp from patient 2 showing an abundance of entrapped epithelium and a stromal component consisting mainly of the inflammatory infiltrate.](image-url)
Our data show that, on the reasonable assumption that loss of the wild-type SMAD4 initiates tumorigenesis, the epithelium of JPS polyps is intimately involved in the formation of the hamartoma and its subsequent progression to carcinoma. In our subset of JPS patients with germ-line SMAD4 mutations, the polyps appear to be composed of an abundance of entrapped epithelium that has become cystically dilated, with the stroma largely containing lymphocytes and other inflammatory cells. We have also found good evidence of loss of wild-type SMAD4 in the epithelium, stromal fibroblasts, and pericryptal myofibroblasts of JPS polyps, a counterintuitive result that may require confirmation by other workers. The causes of differentiation of a single cell of origin into stroma and epithelium within the polyp are, of course, unknown, but it certainly appears that the classical categorization of JPS polyps as simply stromal lesions is incorrect. Thus, the “landscaper” hypothesis, which was developed to explain how epithelial cancers could supposedly arise from the stromal component of a JPS polyp, has no experimental basis and does not apply here, even if it may do so elsewhere. It is therefore not surprising to observe an increase in the incidence of gastrointestinal cancer in patients with JPS. It also becomes much easier to explain how occasional JPS patients can develop cancer very early in life (23). SMAD4 therefore appears to act as a tumor suppressor gene of the “gatekeeper” type in the epithelium of both JPS polyps and sporadic cancers.

ACKNOWLEDGMENTS

Invaluable assistance was provided by the Histopathology Unit and by the Equipment Park, Imperial Cancer Research Fund (London, United Kingdom). Thank you to Jeremy Jass for supplying invaluable samples.

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