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Smad4 Immunohistochemistry Reflects Genetic Status In Juvenile Polyposis Syndrome

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Abstract

Purpose—Juvenile polyposis (JPS) can be caused by a germline defect of the *SMAD4* gene. Somatic inactivation of *SMAD4* occurs in pancreatic and colorectal cancer and is reflected by loss of SMAD4 immunohistochemistry. Here, SMAD4 immunohistochemistry as a marker of *SMAD4* gene status and the role of SMAD4 in the adenoma-carcinoma sequence in neoplastic progression in JPS are studied.

Experimental Design—20 polyps with a *SMAD4* germline defect and 38 control polyps were studied by SMAD4 immunohistochemistry. Inactivation of the *SMAD4* wild-type allele was studied in dysplastic epithelium and in areas with aberrant SMAD4 expression. *APC*, ß-catenin, p53 and *K-ras* were studied to evaluate the adenoma-carcinoma sequence.

Results—9/20 polyps with a SMAD4 germline defect showed loss of epithelial SMAD4 expression. LOH of *SMAD4* was found in 5 polyps and a somatic stop codon mutation was found in 2 polyps without LOH. Remarkably, somatic inactivation of epithelial SMAD4 did not always coincide with dysplasia and aberrant p53 staining was found in 4 of 6 dysplastic polyps with normal SMAD4 staining. One *K-ras* mutation was found in 9 juvenile polyps with dysplasia. No evidence for Wnt activation was found.

Conclusions—SMAD4 immunohistochemistry mirrors genetic status and provides a specific adjunct in the molecular diagnosis of JPS. However, epithelial SMAD4 inactivation is not required for polyp formation and not obligatory for neoplastic progression in JPS. Instead, different routes to neoplasia in JPS caused by germline *SMAD4* mutation appear operative, including somatic loss of SMAD4 and p53 inactivation without somatic loss of SMAD4.

Keywords

Juvenile Polyposis; colorectal cancer; SMAD4; BMPR1A; LOH

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Introduction

Juvenile polyposis syndrome (JPS) is an autosomal dominant disorder characterized by the presence of distinct juvenile polyps in the gastrointestinal tract and an increased colorectal cancer risk.(1-3) On histology, juvenile polyps have a prominent stromal compartment containing distorted and cystically dilated crypts often lined by reactive epithelium.(4) A germline mutation in the *SMAD4* or *BMPR1A* gene is found in 50% of patients.(5,6) Both genes are involved in the Transforming Growth Factor–Beta/Bone Morphogenic Protein (TGF-ß/BMP) signaling pathway, regulating cell proliferation and differentiation. SMAD4 is a cytoplasmic co-mediator which forms heteromeric complexes with various receptor dependant SMADs. These complexes are translocated to the nucleus and regulate DNA transcription.(7,8) Somatic inactivation of the *SMAD4* tumor suppressor gene occurs in up to 55% of pancreatic cancers, and in other malignancies including colorectal cancer. This occurs either through somatic intragenic mutation with loss of the second allele (loss of heterozygosity, LOH) or deletion of both alleles (homozygous deletion).(9-11)

In JPS the mechanism leading to polyp formation and the role of SMAD4 and BMPR1A is poorly understood. One hypothesis is that juvenile polyps develop through a ‘landscaper defect’ in which the defective cell population lies in the stromal compartment. Neoplasia of the epithelial cells may take place as a result of an abnormal microenvironment.(12,13) Others suggest that inactivation of the second allele in the epithelial cell compartment is likely to initiate polyp formation.(14-16) Different mechanisms of polyp formation may exist for individuals with either a *SMAD4* or *BMPR1A* germline mutation.(12)

In pancreatic cancer, somatic inactivation of *SMAD4* is accurately mirrored by loss of immunohistochemical staining.(17) Similarly, SMAD4 immunohistochemistry may prove a valuable tool in the molecular diagnosis of JPS. Also, this analysis could clarify the role of this gene in juvenile polyp development and disease progression. This understanding has been hampered by lack of studies systematically demonstrating a correlation between SMAD4 immunohistochemistry and *SMAD4* gene status in JPS. Therefore, we investigated SMAD4 protein expression by immunohistochemistry and correlated this result with *SMAD4* gene status in juvenile polyps carrying a *SMAD4* germline defect. In addition, we addressed the role and timing of somatic loss of the wild type *SMAD4* allele and the conventional adenoma-carcinoma sequence in neoplastic progression in JPS.

Material and Methods

Patients and tissue

Archival material from patients with one or more juvenile polyps was collected from The Johns Hopkins Polyposis Registry and clinic (Baltimore, MD, USA) and two academic hospitals in the Netherlands (Academic Medical Center, Amsterdam, and University Medical Center, Utrecht). The study was carried out according to the guidelines of the ethical committee of these institutions and with their approval. Clinical and family history data were examined and polyps were carefully reviewed by an experienced GI pathologist (GJAO) to confirm the diagnosis of JPS or sporadic juvenile polyps. All JPS patients previously underwent genetic analysis through direct sequencing and MLPA analysis.(5) Forty-one patients were included in this study, including 8 patients with a *SMAD4* germline defect, 6 with a *BMPR1A* germline defect and 27 with sporadic juvenile polyps. Polyp tissue was formalin-fixed and paraffinized according to standard procedures.

Immunohistochemistry

Immunohistochemistry was performed using a monoclonal antibody against SMAD4 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, Cat.no. sc-7966, 1:400), p53 (Neomarkers

DO7+BP53-12, Cat.no. MS-738-P, 1:2000) and ß-catenin (BD Transduction Laboratories clone 14, Cat.no. 610154, 1:5000). Briefly, 4 μm sections were deparaffinized, blocked for endogenous peroxidase activity by immersion in 0.3% H2O2 in methanol for 20 min. Antigen retrieval was performed in Tris/EDTA buffer (10 mM/1 mM; pH 9.0) for 10 min at 120°C. Nonspecific binding sites were blocked in PBS with 10% normal goat serum for SMAD4, and in 5% normal goat serum for p53 and ß-catenin, for 10 minutes. This was followed by antibody incubation of 1hour for SMAD4 and p53 at room temperature, and an overnight incubation for ß-catenin at 4°C. Antibody binding was visualized using the Powervision+poly-HRP detection system (ImmunoVision Technologies, Co, Daly City, CA, USA) and PowerDAB (Immunologic, Duiven, The Netherlands, Cat. no. BS03-25) for SMAD4, Powervision+poly-HRP detection system and 3,3-diamino-benzidine (DAB, Sigma D5637) were used for p53 and ß-catenin. Sections were counterstained with haematoxylin.

Scoring of immunohistochemistry

On examination, slides were scored as having either normal, reduced or loss of expression of SMAD4. Normal nuclear staining in the epithelial cells lining normal crypts, or inflammatory cells in the mesenchymal stroma on the same section served as an internal control, i.e. normal expression refers to the same expression as seen in these control cells. Loss of expression was defined as absence of nuclear staining. Reduced expression was graded when a weaker expression, but not a complete absence of nuclear staining, was noted compared to the control cells (Figure 1). p53 immunohistochemistry was scored as either normal constitutive immunoreactivity or as a staining pattern suggesting mutation of the p53 gene, which can be reflected by either very intense immunostaining suggesting a stabilizing p53 mutation or total absence of p53 immunoreactivity consistent with a stopcodon mutation in the p53 gene.(18) ß-catenin immunohistochemistry was scored as either normal membranous or nuclear staining, indicating activation of Wnt signaling.(19) Also, all sections were reviewed for dysplasia (GJAO and FJWK) using standard H&E stained reference slides: Dysplasia was graded according to the standard criteria.(20)

Laser microdissection and DNA isolation

Epithelium of interest was isolated by laser capture microdissection (LCM) using the PALM® Laser Microbeam Microdissection System (Microlaser Technologies, Bernried, Germany) on 8 μm sections counterstained with haematoxylin. DNA was obtained using TK buffer (400 μg/ml of proteinase K and 0.5% Tween 20, 50 mmol/l Tris (pH 9), 1 mmol/l NaCl, 2 mmol/L EDTA). After overnight incubation in 50 μl TK buffer at 56°C, tubes were incubated at 95°C for 10 minutes to inactivate the proteinase K.(21)

LOH analysis

Loss of heterozygosity was assessed using fluorescently labeled primers for the following microsatellites: D18S46, D18S474, D18S858 and D18S64.(16,22,23) Epithelium with aberrant SMAD4 expression was separated from normal SMAD4 stained epithelium using LCM. After PCR amplification the products were separated using the ABI Prism® 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). One μl of the PCR product was mixed with 23 μl formamide and 0.5 μl GeneScanTM ROX-500 (Invitrogen, Carlsbad, CA, USA) as a size marker.

Samples with two distinctly sized alleles of a particular marker were termed informative. For all informative markers, the allelic imbalance factor was calculated as described by Cawkwell et al.(24) LOH was assumed if the allelic imbalance factor was greater than 1.6 or less than 0.6. Observed losses were confirmed to exclude induced LOH. If retention of

heterozygosity was found, microdissected material was sequenced to establish whether a somatic point mutation of the *SMAD4* gene had occurred.

Mutation analysis

Sequencing of *SMAD4* was performed as described previously.(5) For *APC* and *K-ras* mutation analysis, DNA was isolated from polyps with dysplasia and PCR amplified using Platinum®Taq DNA Polymerase (Invitrogen Corporation, Carsbad, California, USA). Four primers sets covering the mutation cluster region (MCR) in exon 15 of the *APC* gene (25) were used (1For-GAAATAGGATGTAATCAGACG, 1Rev-CGCTCCTGAAGAAAATTCAAC, 2For-ACTGCAGGGTTCTAGTTTATC, 2Rev-GAGCTGGCAATCGAACGACT, 3For-TACTTCTGTCAGTTCACTTGATA, 3Rev-ATTTTTAGGTACTTCTCGCTTG, 4For-AAACACCTCCACCACCTCC, 4Rev-GCATTATTCTTAATTCCACATC). Two primer sets were used for *K-ras* mutation analysis for exon 1 and 2 containing mutational hotspot codons 12, 13 and 61 (Exon1For-CTGGTGGAGTAT TTGATAGT, Exon1Rev-ATG GTCCTGCACCAGTAATA, Exon2For-GTGCACTGTAATAATCCAGAC, Exon2Rev-CCACCTATAATGGTGAATATCT). PCR products were subsequently sequenced using the ABI Prism® 3130 genetic analyzer.

Results

SMAD4 Immunohistochemistry

A total of 58 polyps, including 20 polyps from 8 patients with a *SMAD4* germline defect, 11 polyps from 6 patients with a *BMPR1A* germline defect and 27 sporadic juvenile polyps from 27 patients, were assessed for SMAD4 protein expression using immunohistochemistry (Figure 1). Of 20 polyps with a *SMAD4* germline defect, 9 showed focal reduction or loss of nuclear SMAD4 protein expression in the epithelium (Table 1). In contrast, none of the 11 polyps carrying a *BMPR1A* germline mutation or any of the 27 sporadic juvenile polyps had aberrant SMAD4 expression (data not shown).

LOH and mutation analysis

To assess the implication of aberrant epithelial SMAD4 protein expression, we investigated whether reduction or loss of SMAD4 expression correlated with occurrence of a somatic event in *SMAD4*, i.e. LOH or a somatic point mutation in polyps with a *SMAD4* germline mutation. LOH analysis of the *SMAD4* locus was performed using 4 microsatellite markers. Nine polyps were assessed, all carrying a germline mutation in *SMAD4*, and all had aberrant SMAD4 expression. Results are summarized in Table 2.

Polyp 2.3, 3.1, 8.1, 8.2a and 8.4a with reduction or loss of nuclear SMAD4 expression showed LOH in two or more markers surrounding *SMAD4*, including at least one of two markers closest to the *SMAD4* locus. Retention of heterozygosity was found in polyp 1.1 and 7.3 even though SMAD4 expression was reduced or lost. Subsequent sequence analysis revealed a somatic stop codon mutation in exon 1 (1.1) and exon 2 (7.3) of *SMAD4*, likely resulting in truncation of the protein. In polyp 4.1 and 5.1 with a hemizygous germline deletion of *SMAD4* and immunohistochemical loss of the SMAD4 protein, LOH markers closest to *SMAD4* were non-informative, although more distant markers did show LOH.

Dysplasia and genetic status of SMAD4

With aberrant epithelial SMAD4 protein expression reflecting the occurrence of a somatic event in the SMAD4 tumor suppressor gene, we investigated the association of these phenomena to neoplastic change in juvenile polyps by reviewing all corresponding H&E slides for dysplasia. In 9 of 20 polyps with a *SMAD4* germline defect foci of low-grade

dysplasia were found, two of which contained focal high-grade dysplasia. Four polyps were graded indefinite for dysplasia and 7 negative for dysplasia. (Table 3)

Intriguingly, the presence of dysplasia did not consistently correlate with reduction or loss of nuclear SMAD4 protein expression in juvenile polyps (Table 3 and 4). Polyp 6.2, 6.3 and 7.1 showed dysplasia even though nuclear SMAD4 expression of the epithelium was normal (Figure 2a), whereas, polyp 4.1 and 5.1 showed loss of epithelial SMAD4 expression but had no dysplasia (Figure 1c). Polyp 1.1 and 3.1 had foci of low-grade dysplasia within a larger area of reduced epithelial nuclear SMAD4 expression (Figure 2b) but in polyp 7.3 areas of low-grade dysplasia extended beyond the area showing loss of expression of SMAD4 (Figure 2c).

Remarkably, polyp 8.2 and 8.4 both showed loss of SMAD4 expression in non-neoplastic epithelium (8.2a and 8.4a) but the same sections also contained low-grade dysplasia with normal SMAD4 expression (8.2b and 8.4b) (Figure 2d).

To confirm that SMAD4 immunohistochemistry accurately mirrors the molecular status of *SMAD4*, we aimed to exclude somatic inactivation of SMAD4 in juvenile polyp tissue with dysplasia and a normal SMAD4 staining pattern. Therefore, dysplastic epithelium with normal nuclear SMAD4 expression was microdissected and analyzed for LOH and somatic mutation using non-dysplastic epithelium with normal nuclear SMAD4 expression as a reference. As shown in Table 2, polyp 6.2, 7.1, 8.2b and 8.4b all had retention of heterozygosity of the *SMAD4* locus and no somatic mutations were found.

Role of *APC*, ß-catenin, *K-ras*, and p53 in neoplastic progression in JPS

To investigate whether mutations in the conventional adenoma-carcinoma sequence underlie neoplastic change in juvenile polyps without loss of the wild-type *SMAD4* allele (i.e. with normal SMAD4 protein expression), *APC* (MCR) and *K-ras* mutation analysis, as well as p53 and ß-catenin immunohistochemistry were performed. Results are summarized in Table 3. One somatic *K-ras* mutation was found in codon 12 (GGT → GAT) in polyp 7.3 with low-grade dysplasia and loss of SMAD4 immunostaining. Non-neoplastic areas from the same polyp did not show this *K-ras* mutation. Aberrant p53 staining, suggesting a *p53* mutation, was found in 6 polyps. Five of these polyps contained dysplasia and 1 was graded indefinite for dysplasia. Interestingly four of these polyps (66%) showed normal SMAD4 expression. No mutations were found in the MCR of the *APC* gene and no aberrant ß-catenin expression was found.

Discussion

SMAD4 is one of two known genes responsible for juvenile polyposis syndrome when mutated in the germline. *SMAD4* is a tumor suppressor gene and is frequently inactivated in advanced stages of pancreatic and colorectal cancer. In pancreatic cancer, loss of immunohistochemical labeling in tumor cells reflects genetic status of *SMAD4* with high accuracy.(17)

The role of SMAD4 in JPS polyp formation is poorly understood. Investigators supporting the landscaper theory postulate that juvenile polyps arise primarily due to a stromal defect. The abnormal stroma causes disruption of normal development and regeneration of the overlying epithelium.(12,13) In contrast, other studies provide evidence that LOH of *SMAD4* in the epithelium initiates polyp growth suggesting that SMAD4 acts as a classic tumor suppressor protein in JPS polyps.(14-16) In fact, it is deemed likely that a second hit of the wild type allele initiates growth and neoplastic progression of JPS polyps, which fits the classic tumor suppressor model.(16)

In this study we illuminate the role of SMAD4 in juvenile polyp formation by investigating SMAD4 protein expression and *SMAD4* gene status in juvenile polyps from 8 patients with a germline *SMAD4* mutation. In almost half of all polyps from patients with a *SMAD4* germline defect, focal reduction or loss of nuclear SMAD4 expression in the epithelium was seen. In contrast, no aberrant SMAD4 expression was noted in polyps from patients with a *BMPR1A* mutation, or in any of the sporadic juvenile polyps.

Aberrant SMAD4 immunostaining in JPS showed clear correlation with somatic inactivation of the *SMAD4* gene. A second hit of the wild-type *SMAD4* allele was found in 7 of 9 polyps with aberrant SMAD4 expression. This included LOH in five polyps and a somatic stop codon mutation resulting in truncation of the SMAD4 protein in two others. It proved difficult to assess LOH status using the microsatellite technique in two polyps (4.1 and 5.1) from two patients with a hemizygous germline deletion of all 11 exons of *SMAD4*. LOH analysis gave unreliable or non-informative results because the full extent of the germline deletion was not known. However, markers located further away from the *SMAD4* gene locus did show LOH in these polyps.

These results clearly demonstrate that aberrant nuclear SMAD4 protein expression in JPS patients is indicative of somatic inactivation through LOH or somatic mutation, as has previously been shown in pancreatic cancer.(17) Furthermore, reduction or loss of epithelial SMAD4 expression in the polyps of individuals with JPS is specific for the presence of a *SMAD4* germline defect, ranging from missense mutations to hemizygous deletions. Therefore, SMAD4 immuunohistochermistry can be used as a first screening method in the molecular diagnosis of JPS. An underlying germline *SMAD4* mutation is likely if reduced (compared to surrounding stroma) or absent SMAD4 expression is found in the epithelial component of a juvenile polyp. However, normal SMAD4 expression is less predictive of germline status.

Moreover, since focal loss of epithelial SMAD4 expression was found only in a subset of juvenile polyps with a *SMAD4* germline mutation, inactivation of the wild type *SMAD4* allele in the epithelium is not required for polyp initiation and formation, but rather occurs as a late event during polyp growth and neoplastic progression. This concurs with previous observations in mouse models of juvenile polyposis reporting that haploinsufficiency is sufficient for polyp initiation.(26,27)

One study by Kim et al. reported that targeted inactivation of *Smad4* in stromal T-cells leads to a JPS-like phenotype and epithelial cancers of the gastrointestinal tract in mice, whereas inactivation in the epithelium does not.(28) Although our results argue that inactivation of *SMAD4* occurs in the epithelium and not in the stroma of juvenile polyps, we cannot eliminate the concept that haploinsufficiency of *SMAD4* in cells of the stromal compartment contributes to juvenile polyp initiation as per the landscaper theory. In fact, our finding that epithelial inactivation of *SMAD4* is not required for polyp initiation suggests that this may indeed be the case.

Surprisingly, we found that the majority of polyps with dysplasia showed normal SMAD4 protein expression (66%), whereas loss of SMAD4 expression was slightly more common in non-neoplastic polyps than in juvenile polyps with dysplasia (44% vs. 33%) (Table 4). With regard to SMAD4 in neoplastic progression, this finding suggests that neoplastic change of the epithelium in juvenile polyps with a *SMAD4* germline defect is not necessarily initiated by inactivation of the wild-type *SMAD4* allele (Figure 2a), conflicting with the proposed gatekeeper function of SMAD4 in JPS.(16) Rather, these results suggest an alternative pathway leading to neoplasia in JPS with somatic inactivation of *SMAD4* as a late event during neoplastic progression, in accordance with its role in the conventional adenoma-

carcinoma sequence in colorectal cancer.(29) Although in our study evidence for (early) Wnt-pathway activation was not found p53 accumulation occurred in 4 of 6 polyps with dysplasia and normal SMAD4 immunostaining. Others did report somatic *APC* mutations in dysplastic juvenile polyps (30), and our study used only *APC* MCR mutation analysis and ß-catenin immunohistochemistry; however also others did not find support for a major role for Wnt pathway activation in early neoplastic development in JPS.(14,27,31)

On the other hand, somatic inactivation of *SMAD4* also occurred in epithelium without morphological features of dysplasia in 44% of juvenile polyps (Table 4, Figure 1c). In some cases this was observed on the very same section containing areas of low-grade dysplasia with normal SMAD4 expression (Figure 2d).

Consequently, the role of SMAD4 in neoplastic progression of juvenile polyps remains unclear. Although SMAD4 inactivation is seen in a clonal pattern it occurs seemingly independent of microscopically evident neoplastic change. Perhaps the most likely scenario is that two pathways causing neoplasia occur. In juvenile polyps carrying a *SMAD4* germline defect, an increased selective pressure leading to early stage inactivation of this gene may exist. This molecular marker of neoplasia can be visualized by loss of SMAD4 immunohistochemical staining but may on microscopy of the H&E section not yet be recognizable as dysplasia. Alternatively, selective pressure may also be increased on other genes capable of initiating neoplastic change, such as *p53*. This could be a direct result of the *SMAD4* germline defect or from the abnormal microenvironment present in juvenile polyps. In addition, epigenetic silencing of genes may be important in this model. Somatic inactivation of *SMAD4* may then occur at a later stage, possibly leading to acceleration of the neoplastic progression. Alternatively, retention of a wild type *SMAD4* allele may also enable polyps to benefit from the tumor promoting actions by the TGF-ß signalling pathway. (8) Although this study was performed with a limited number of patients, several important conclusions can be drawn. First, we found that SMAD4 immunohistochemistry accurately reflects *SMAD4* status in polyps of the juvenile polyposis syndrome and can be a useful and specific adjunct to the molecular diagnosis of JPS. Second, somatic inactivation of *SMAD4* occurs in the epithelium but is presumably not a prerequisite for neoplastic change. Our results suggest that various pathways can lead to neoplasia in juvenile polyposis caused by germline mutation of *SMAD4*. One pathway initiated by somatic loss of *SMAD4* and another characterized by p53 inactivation with retention of the wild-type *SMAD4* allele.

Statement of Translational Relevance

The current study shows that SMAD4 immunohostochemistry mirrors genetic status and can be used as a first screening method in the molecular diagnosis of JPS. A germline *SMAD4* mutation is likely if absent or reduced SMAD4 expression is found in a juvenile polyp. In addition, this study increases our understanding of juvenile polyposis pathogenesis. It is shown that biallelic *SMAD4* inactivation is not required for polyp formation and not obligatory for neoplastic progression in juvenile polyps. Moreover, different routes to neoplasia in juvenile polyposis caused by germline *SMAD4* mutation appear operative, including somatic loss of *SMAD4* and p53 inactivation without somatic loss of *SMAD4*.

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Abbreviations

JPS	juvenile polyposis syndrome
LOH	loss of heterozygosity
MCR	mutation cluster region

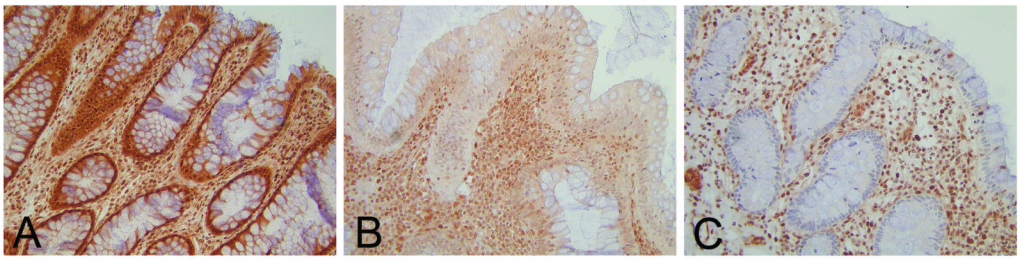

Figure 1.

SMAD4 Immunohistochemical scoring. SMAD4 IHC was scored as normal (A), reduced (B) or loss of SMAD4 expression (C). Nuclear staining in the epithelial cells lining normal crypts or inflammatory cells in the mesenchymal stroma on the same section served as internal control. Note loss of SMAD4 expression in non-neoplastic epithelium in C. Magnification 20X.

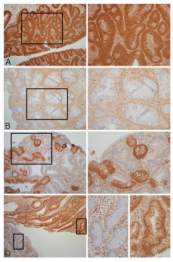

Figure 2.
SMAD4 IHC and dysplasia. A: Dysplasia with normal epithelial SMAD4 expression. B: Dysplasia within area of reduced SMAD4 expression. C: Dysplasia extending beyond area of SMAD4 loss. E: Dysplasia with normal SMAD4 expression and non-neoplastic epithelium with loss of SMAD4 expression adjacent on one section. Magnification left panel 10X (figure 2E 5X) with 20X zoom (right panel).

Table 1

SMAD4 immunohistochemistry in juvenile polyps from patients with a *SMAD4* germline mutation.

Patient	polyp	Exon	Mutation	Effect	SMAD4 IHC		
					normal	reduced	loss
1	1.1	10	c.1411-1435del25	p.G471FfsX25		■	
	1.2	10	c.1411-1435del25	p.G471FfsX25	■		
2*	2.1	8	c.970 T>C	p.C324R	■		
	2.2	8	c.970 T>C	p.C324R	■		
	2.3	8	c.970 T>C	p.C324R		■	
3*	3.1	8	c.970 T>C	p.C324R		■	
	3.2	8	c.970 T>C	p.C324R	■		
4†	4.1	1-11	hemizygous deletion				■
5†	5.1	1-11	hemizygous deletion				■
	5.2	1-11	hemizygous deletion		■		
6	6.1	8	c.989 A>G	p.E330G	■		
	6.2	8	c.989 A>G	p.E330G	■		
	6.3	8	c.989 A>G	p.E330G	■		
7	7.1	9	c.1193 G>A	p.W398X	■		
	7.2	9	c.1193 G>A	p.W398X	■		
	7.3	9	c.1193 G>A	p.W398X			■
8	8.1	8	971delG	p.C324FfsX12			■
	8.2	8	971delG	p.C324FfsX12			■
	8.3	8	971delG	p.C324FfsX12	■		
	8.4	8	971delG	p.C324FfsX12			■

*Patient 2 and 3 and

†patient 4 and 5 were from the same family

Table 2

SMAD4 LOH and mutation analysis in juvenile polyps from patients with a *SMAD4* germline mutation.

Patient	Polyp	SMAD4 IHC	LOH analysis				Somatic mutation
			D18s46		D18s474	D18s858 / D18s64	
1	1.1	reduced					c.170 T>A p.L57X
2*	2.3	reduced					
3*	3.1	reduced					
4†	4.1	loss					
5†	5.1	loss					
6	6.2	normal					
7	7.1	normal					
	7.3	loss					c.403 C>T p.R135X
8	8.1	loss					
	8.2a	loss					
	8.2b	normal					
	8.4a	loss					
	8.4b	normal					

*Patient 2 and 3 and

†patient 4 and 5 are from the same family

LOH

Non-informative

Retention

Table 3

Dysplasia in juvenile polyps and SMAD4, *APC*, ß-catenin, *K-ras* and p53 status.

Patient	polyp	Dysplasia	SMAD4 IHC	*APC* mutation	*K-ras* mutation	ß-catenin IHC	p53 IHC
1	1.1	LGD	reduced	NM	failed	M	N
	1.2	Neg.	normal	-	-	M	N
2*	2.1	Indef.	normal	-	-	M	N
	2.2	Neg.	normal	-	-	M	N
	2.3	Indef.	reduced	-	-	M	N
3*	3.1	LGD	reduced	NM	NM	M	N
	3.2	Indef.	normal	-	-	M	N
4†	4.1	Neg.	loss	-	-	M	N
5†	5.1	Neg.	loss	-	-	M	N
	5.2	Neg.	normal	-	-	M	N
6	6.1	Neg.	normal	-	-	M	N
	6.2	HGD	normal	NM	NM	M	A
	6.3	HGD	normal	NM	NM	M	A
7	7.1	LGD	normal	NM	NM	M	A
	7.2	LGD	normal	NM	NM	M	N
	7.3	LGD	loss	NM	Codon 12	M	A
8	8.1	Indef.	loss	-	-	M	A
	8.2a	Neg.	loss	-	-	M	N
	8.2b	LGD	normal	NM	NM	M	A
	8.3	Neg.	normal	-	-	M	N
	8.4a	Neg.	loss	-	-	M	N
	8.4b	LGD	normal	NM	NM	M	N

*Patient 2 and 3 and

†patient 4 and 5 were from the same family

LGD: low-grade dysplasia. HGD: high-grade dysplasia. Indef.: indefinite for dysplasia. Neg.: negative for dysplasia. NM: no mutation found. M: membranous ß-catenin staining pattern. N: normal p53 staining pattern. A: aberrant p53 staining pattern (i.e. overexpression of absent staining).

Table 4

Correlation between dysplasia and SMAD4 immunostaining in juvenile polyps.

	Normal SMAD4 IHC	Reduced/lost SMAD4 IHC	Total
No dysplasia	5 (56%)	4 (44%)	9
Indefinite for dysplasia	2 (50%)	2 (50%)	4
Dysplasia (LGD and HGD)	6 (66%)	3 (33%)	9