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Screening *SMAD1*, *SMAD2*, *SMAD3*, and *SMAD5* for germline mutations in juvenile polyposis syndrome

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Abstract

***Background and aims*—Juvenile polyps occur in several Mendelian disorders, whether in association with gastrointestinal cancer alone (juvenile polyposis syndrome, JPS) or as part of known syndromes (Cowden, Gorlin, and Bannayan-Zonana) in association with developmental abnormalities, dysmorphic features, or extraintestinal tumours. Recently, some JPS families were shown to harbour germline mutations in the *SMAD4* (*DPC4*) gene, providing further evidence for the importance of the TGFβ signalling pathway in colorectal cancer. There remains, however, considerable, unexplained genetic heterogeneity in JPS. Other members of the SMAD family are excellent candidates for JPS, especially *SMAD2* (which, like *SMAD4*, is mutated somatically in colorectal cancers), *SMAD3* (which causes colorectal cancer when "knocked out" in mice), *SMAD5*, and *SMAD1*.**

***Methods*—*SMAD1*, *SMAD2*, *SMAD3*, and *SMAD5* were screened for germline mutations in 30 patients with JPS and without *SMAD4* mutations.**

***Results*—No mutations were found in any of these genes. A G–A C89Y polymorphism with possible effects on protein function was found in *SMAD3*, but the frequencies of the G and A alleles did not differ between patients with JPS and controls.**

***Conclusions*—It remains to be determined whether or not this polymorphism is involved in a minor predisposition to colorectal or other carcinomas. *SMAD4* may be the only member of the SMAD family which causes JPS when mutant in the germline. The other genes underlying JPS remain to be identified.**

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Juvenile polyps are hamartomatous lesions of the large bowel, stomach, and small bowel which have a characteristic smooth histological appearance, predominant stroma, cystic spaces, and lack of a smooth muscle core. Multiple juvenile polyps usually occur in a number of Mendelian disorders. Sometimes these polyps occur with specific associated features, for example, in Cowden, Gorlin, and Bannayan-Zonana syndromes.[1] Other patients have juvenile polyps which occur predominantly in the large bowel and stomach and which are associated with progression to carcinoma; these patients are usually said to have juvenile polyposis syndrome (JPS, MIM174900).

Although it has been reported that some patients with JPS have germline *PTEN* mutations,[2] this contention remains controversial and the only proven cause of JPS is a germline mutation in the *SMAD4* (*DPC4*) gene (chromosome 18q21.1).[3] *SMAD4* was originally identified by studying homozygous deletions in pancreatic cancer, but this tumour suppressor gene is also mutated in some colorectal carcinomas.[4] SMAD4 protein acts as a trimer, with the interface between the molecules towards the C terminus. Six other members of the SMAD family have been identified in humans; each of these is a homologue of the others and plays a different role in signal transduction from transforming growth factor β (TGFβ) and other related molecules. Somatic mutations in SMAD2 have been found in colorectal and other cancers.[4 5] Like SMAD4, SMAD2 has activating C terminal domains and autoinhibitory domains at the N terminal: thus, classically, inactivating mutations occur at the C terminal of both proteins, whereas gain of function missense mutations may occur at the N terminal.[6] The TGFβ type I receptor phosphorylates SMAD2 which then complexes with SMAD4.[7] The complex proceeds to the nucleus and initiates transcription. SMADs 2 and 3 are structurally similar and SMAD3 may act in a similar fashion to SMAD2.[8] SMADs 1 and 5 are activated by molecules such as bone morphogenetic protein, and then proceed to the nucleus where they probably interact with SMAD4 and initiate transcription.[9] Unlike the other known members of the family, SMADs 6 and 7 play a role in inhibiting TGFβ signalling.[10 11]

There is accumulating evidence that the TGFβ pathway is of central importance in colorectal carcinogenesis: a large proportion of sporadic colon cancers with microsatellite instability have TGFβ type II receptor mutations[12]; germline variants in the TGFβ type II receptor may predispose to colon cancer[13]; the down-

Abbreviations used in this paper: CSGE, conformation specific gel electrophoresis; JPS, juvenile polyposis syndrome; TGF, transforming growth factor.

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stream targets of TGFβ (SMADs 2 and 4) have been implicated in colorectal tumorigenesis; germline *SMAD4* mutations predispose to juvenile polyps and colon cancer in humans; and germline *Smad3* mutations cause colon cancer in mice.[14] *SMAD4* mutations probably do not account for the majority of JPS cases, however,[3 15] and the other members of the SMAD family are therefore excellent candidate genes for JPS. Based on their known roles and mutations in cancers, it is likely that SMADs 2 and 3 are the best candidates, followed by SMADs 5 and 1, with SMADs 6 and 7 weaker candidates. The genomic structure is known for SMADs 2, 3, and 5. We have previously tested a set of 21 patients with JPS for germline *SMAD4* variants and found just one mutation. Here, we present the results of testing 30 unrelated patients with JPS without *SMAD4* mutations for germline mutations at *SMAD1*, *SMAD2*, *SMAD3*, and *SMAD5*.

Methods

PATIENT SELECTION

Patients with JPS were identified from sources in the UK, Israel, Australia, USA, and Japan. No patient had clinical features suggestive of Cowden, Gorlin, or Bannayan-Zonana syndromes. None had germline *SMAD4* mutations[15] after screening exons and intron-exon boundaries using conformation specific gel electrophoresis (CSGE). Standard diagnostic criteria for JPS were used, in that affected individuals had either five or more juvenile polyps throughout the gastrointestinal tract or any number of juvenile polyps and a family history of JPS. All affected individuals had more than one typical juvenile polyp as confirmed by histology. Fourteen patients had a known family history of JPS. Sixteen patients had no known relative affected with juvenile polyps, although in some cases, relatives had developed colon carcinoma. DNA was extracted from blood samples from appropriate individuals using standard methods.

MUTATION SCREEN

Oligonucleotides were designed to amplify each exon of the *SMAD2*, *SMAD3*, and *SMAD5* genes (including splice sites) specifically from genomic DNA by the polymerase chain reaction (PCR) (table 1). The genomic structure of SMAD1 is not known and cDNA was therefore prepared from a subset of 10 patients with lymphoblastoid lines using the Pharmacia (Uppsala, Sweden) Quick Prep Micro and First Strand Kits; oligonucleotides (table 1) were designed for nested amplification of the *SMAD1* cDNA in four parts. *SMAD3* and *SMAD1* were screened by direct sequencing of purified PCR products in forward and reverse orientations using the ABI Ready Reaction Dye Terminator Cycle Sequencing kit and the 377 Prism sequencer. The search for germline mutations in the *SMAD2* and *SMAD5* genes was performed using CSGE. All samples with possible mutations on sequencing or with CSGE bandshifts were sequenced in forward and reverse orientations after reamplification of the appropriate exon or cDNA region in the PCR. Mutations and polymorphisms were confirmed, if possible, using wild type or mutant specific restriction endonuclease digestion of the PCR product in question.

Table 1 Primers and PCR annealing temperature for SMAD amplifications

SMAD	Exon/region	Sense primer (5'–3')	Antisense primer (5'–3')	Size (bp)	Ta
1	67–901	tgctgactgggttactttttta	aagcaggtgggggcgtatca	835	55°C
	162–543	caaatctcttctgctgtcct	ttccagtggttttagttcat	382	53°C
	497–870	gtcatttactgccgtgtgtg	gaaagggcttcctgggtctg	374	55°C
	799–1714	ctctcccaatagcagttacc	gctcctttgtcagttctcaa	916	54°C
	838–1249	cagcagcacctaccctcact	cctcccctccaacataataa	412	55°C
	1229–1704	attggaaaaggagttcatct	cagttctcaatcgtgtctga	476	52°C
2	1 part 1	tccgctccctccgtcttccatac	cgcgcgcccgcagccctacc	340	67°C
	1 part 2	gacggcggccgggagtgttt	gcgggcgcccaggctttacc	168	63°C
	2	ggtgaaggaagtattctgta	aatgctatgccttattttac	183	50°C
	3	ttttacatcatggtattttg	attttacattaaggaaacat	282	47°C
	4	aatttagcccatttgactgc	gctattccaagaaacagata	473	48°C
	5	ttggatttcttgaacttttt	aacttgaatgcttatgaaca	225	49°C
	6	gctgtgcttgatttgtttta	atgcgtctcaacttctctaa	214	47°C
	7	tttttaaatccttttgtttt	ttatttggctattcattagg	199	46°C
	8	aatctatttttggcttgaat	aatgcctacattatgagtat	342	47°C
	9	ctcatttgtattttgtttca	gttgacatgataggtttatg	198	48°C
	10	atattctaaaacttgtaacc	agaatgcaatgaaacataat	290	47°C
	11	ctgcctgtggacttgaat	tcttgaacttttggatag	150	48°C
3	1	gtcgtccatcctgcctttca	tggtgatgcacttggtgttg	137	55°C
	2–3 part 1	atggccggttgcaggtgtcc	aggcaggccaggcagcatac	205	59°C
	2–3 part 2	cccccggacagttctacctc	tgccgcccacgtgcctacct	183	58°C
	4	gaccaccttccttctgattc	atgaccctgcatgactgacc	120	55°C
	5	tgtctcacctcgcaggttct	tgcacaaggagatactcacc	80	55°C
	6	gtagcccaccctctgtccac	agccacccataccgatgtg	250	58°C
	7	gaggcgtgcggctctactac	tgcctgtgcggctcgtttac	155	58°C
	8	gccctgtttctgtgtttttg	aggcagcacccataactgac	206	55°C
	9	cccacccctttccctatt	aagacacactggaacagc	150	58°C
5	1 part 1	tctccgaagatttgtgtcaa	ctaaagatctgggaatagtg	237	50°C
	1 part 2	aggacagccgagtaaatgtg	tttttccaaattcttctcag	260	50°C
	2	gacttttgatttttgttttt	tgggagctgaaatggacttc	274	51°C
	3	agattttaattattattttt	atgaagtgagtanttctctt	173	45°C
	4	tctgtgtctggtttgttcac	attaaatgtaggaaaatgac	272	47°C
	5	ttttaaggtgttcatctgta	tgttagaggtcacaactcac	284	50°C
	6	aagagggatttgtgatgata	ttaaaacaagtccactaaca	227	49°C

Cycle conditions: four minutes 94°C initial denaturation; 94°C for 30 seconds, 0.5–1 minute at specified temperature (Ta), 72°C for 0.5–1 minute for 35 cycles; and a final extension step of seven minutes at 72°C. For SMAD1, the gene was amplified in two segments initially (67–901, 799–1714, GenBank U59912), followed by two nested PCRs within each of those regions as shown.

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Table 2 Genotype frequencies for exon 2 codon 89 polymorphism

Cohort	G/G (frequency)	A/G (frequency)	A/A (frequency)	G allele (frequency)	A allele (frequency)
JPS	26/31 (0.83)	4/31 (0.13)	1/31 (0.03)	56 (0.90)	6 (0.10)
Controls	18/25 (0.72)	7/25 (0.28)	0/25 (0)	43 (0.86)	7 (0.14)

Fisher's exact test, p>0.5.
JPS, juvenile polyposis syndrome.

Results and discussion

No pathogenic mutations in *SMAD1, SMAD2, SMAD3*, or *SMAD5* were found in any of the patients with JPS screened. Two variants were, however, detected in *SMAD3*. One of these represents a known synonymous G–A polymorphism at position 3 of codon 103, encoding alanine. The second polymorphism in exon two has not been reported previously and is a non-synonymous G–A difference in position 2 of codon 89. This base change results in a cysteine to tyrosine amino acid substitution. The polymorphism was confirmed using *Fnu* 4H1 restriction enzyme digestion of a 490 base pair fragment which was amplified using the forward primer of exon2–3 part one, and the reverse primer of exon2–3 part 2 (table 1). Digestion with *Fnu* 4H1 was performed on the patient cohort and a panel of 25 control individuals (table 2). The observed frequency of the codon 89 alleles was very similar in both of these groups, suggesting that this polymorphism is not a factor important in the development of JPS. However, this polymorphism may not be without functional effect, as the change involves loss of a cysteine residue (within a β pleated sheet) which is conserved evolutionarily not only in the human, rat, mouse, *Drosophila*, and *C elegans* homologues of *SMAD3*, but also in the human *SMAD1, SMAD2, SMAD3*, and *SMAD5* genes. This polymorphism is therefore a candidate locus for conferring a minor genetic predisposition to colorectal or other gastrointestinal carcinomas, especially given the occurrence of colorectal cancer in *Smad3* "knockout" mice.[14]

We have excluded germline mutations in *SMAD1, SMAD2, SMAD3*, and *SMAD5* as causes of JPS. The other members of the SMAD family—*SMAD6, SMAD7*, and quite possibly other, undiscovered homologues—have not been excluded, but *SMAD6* and *SMAD7* seem to be a priori weaker candidates than the other known SMAD genes (see above). The question remains as to why *SMAD4* alone is, so far, implicated in JPS. The SMAD4 protein seems to have the central role in signal transduction through the SMAD pathway, interacting with all the other SMAD molecules and forming an unusual trimeric structure, which is probably disrupted by pathogenic mutations in JPS. Despite the finding of occasional *SMAD2* mutations in cancers, there may exist a degree of redundancy between the other SMAD members, especially SMADs 2 and 3, SMADs 1 and 5, and SMADs 6 and 7 which means that germline mutations in any one of these genes do not lead to juvenile polyposis. There is considerable unexplained genetic heterogeneity in juvenile polyposis, with remaining potential for discovering new colon cancer genes by studying these families.

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