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


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.

Keywords: juvenile polyposis; SMAD

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. 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, this contention remains controversial and the only proven cause of JPS is a germline mutation in the SMAD4 (DPC4) gene (chromosome 18q21.1). SMAD4 was originally identified by studying homozygous deletions in pancreatic cancer, but this tumour suppressor gene is also mutated in some colorectal carcinomas. SMAD4 protein acts as a trimmer, 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. 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. The TGFβ type I receptor phosphorylates SMAD2 which then complexes with SMAD4. 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. SMAD1 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. Unlike the other known members of the family, SMADs 6 and 7 play a role in inhibiting TGFβ signalling.

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; germline variants in the TGFβ type II receptor may predispose to colon cancer; the down-

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

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\(^\text{3,15}\) after screening exons and intron-exon boundaries using confirmation 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.

### Table 1 Primers and PCR annealing temperature for SMAD amplifications

<table>
<thead>
<tr>
<th>SMAD</th>
<th>Exon/region</th>
<th>Sense primer (5’–3’)</th>
<th>Antisense primer (5’–3’)</th>
<th>Size (bp)</th>
<th>Ta (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>part 1</td>
<td>CTCCTCCCACCACTCAC</td>
<td>TTGAGTCTTTTGTGCA</td>
<td>835</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>part 2</td>
<td>CCACTTCTGAGGCTTCTCA</td>
<td>CGAGGAGGTGCTTTTG</td>
<td>430</td>
<td>48</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>ATTATTAAGGGTATGTCAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>ATTGGGGAACATGCTGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>TCCCTCTTCTTGTGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>AGGGGAACTTGCTGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>GTGCTGAGGCTTCTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correspondence to: Dr R Joulston, Molecular and Population Genetics, Imperial Cancer Research Fund, London WC2A 1PX, UK.

Accepted for publication 17 February 1999
Fisher’s exact test, p>0.5.

Table 2 Genotype frequencies for exon 2 codon 89 polymorphism

<table>
<thead>
<tr>
<th>Cohort</th>
<th>G/G (frequency)</th>
<th>A/G (frequency)</th>
<th>A/A (frequency)</th>
<th>G allele (frequency)</th>
<th>A allele (frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPS</td>
<td>26/31 (0.83)</td>
<td>4/31 (0.13)</td>
<td>1/31 (0.03)</td>
<td>56 (0.90)</td>
<td>6 (0.10)</td>
</tr>
<tr>
<td>Controls</td>
<td>18/25 (0.72)</td>
<td>7/25 (0.28)</td>
<td>0/25 (0)</td>
<td>43 (0.86)</td>
<td>7 (0.14)</td>
</tr>
</tbody>
</table>

Results and discussion

No pathogenic mutations in $SMAD_1$, $SMAD_2$, $SMAD_3$, or $SMAD_5$ were found in any of the patients with JPS screened. Two variants were, however, detected in $SMAD_3$. 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 $SMAD_3$, but also in the human $SMAD_1$, $SMAD_2$, $SMAD_3$, and $SMAD_5$ 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 $SMAD_1$, $SMAD_2$, $SMAD_3$, and $SMAD_5$ as causes of JPS. The other members of the SMAD family—$SMAD_6$, $SMAD_7$, and quite possibly other, undiscovered homologues—have not been excluded, but $SMAD_6$ and $SMAD_7$ seem to be a priori weaker candidates than the other known SMAD genes (see above). The question remains as to why $SMAD_4$ alone is, so far, implicated in JPS. The $SMAD_4$ 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 $SMAD_2$ mutations in cancers, there may exist a degree of redundancy between the other SMAD members, especially $SMAD_2$ and $SMAD_3$, and $SMAD_6$ 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.

We thank the ICRF, CRC, and Coeliac Society for support. Mike Sternberg provided very useful advice on protein structure and function.