Germline mutations in the TGF-β and Wnt signalling pathways are a rare cause of the “multiple” adenoma phenotype

L Lipton, O M Sieber, H J W Thomas, S V Hodgson, I P M Tomlinson, K Woodford-Richens

The “multiple” colorectal adenoma phenotype is characterised by approximately 5-100 adenomatous polyps of the large bowel, resulting in an increased risk of colorectal cancer. The condition can be inherited as a Mendelian trait, either autosomal dominant or recessive, but can also occur in the form of isolated cases. Some patients with the “multiple” adenoma phenotype are classified as having attenuated polyposis (AAPC). However, most “multiple” adenoma patients have no underlying APC mutation and do not have the extracolonic manifestations sometimes associated with AAPC.

Recently, recessive mutations of the base excision repair (BER) gene involved in the repair of 8-oxo-7,8-dihydro2′(BER) gene previously been excluded as causing multiple adenomas. MutM (MTH1) on chromosome 1p34, was identified in 30% of patients presenting with 15 or more adenomas. MutM is a highly conserved base excision repair (BER) gene involved in the repair of 8-oxo-7,8-dihydro2′deoxyguanosine (8-oxo-G) lesions induced by oxidative damage. Two further BER genes, human homologue of MutT (OGG1) and human homologue of MutT (MTH) have previously been excluded as causing multiple adenomas.

It seems plausible that mutations in APC related genes could cause multiple adenomas by promoting Wnt signalling. APC regulates Wnt signalling by controlling the levels of β-catenin reaching the cell nucleus. Once in the nucleus, β-catenin complexes with DNA binding proteins of the T cell factor (TCF) family and serves as a coactivator of transcription. In the absence of Wnt signalling, levels of β-catenin are minimised by degradation in the proteasome, after phosphorylation by a complex comprising APC, axin, conductin, and glycogen synthase kinase 3-β (GSK3-β). Wnt signalling activates a cascade, which inhibits GSK3-β, allowing β-catenin to escape degradation. Downstream targets of Wnt signalling include c-myc, matrilysin, CD44, urokinase type plasminogen activator receptor, and cyclin D1, involved in cellular proliferation, invasion, and metastasis.

The human APC2 gene (chromosome 19p13.3) encodes a 2302 amino acid protein displaying high homology to APC at the N-terminus, including a series of 20 amino acid repeats, presumably mediating interaction with β-catenin. In SW480 colon cancer cells lacking functional APC protein, transfection with the APC2 gene inhibited β-catenin TCF signalling. Like APC, APC2 regulates the formation of active β-catenin TCF complexes. The region near APC2 shows allelic loss in a variety of human cancers, suggesting that it may function as a tumour suppressor as does its homologue APC.

Table 1 Clinical features of multiple adenoma patients

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>No of patients</th>
<th>Mean</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex M:F</td>
<td>24:23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of diagnosis</td>
<td></td>
<td>48.5</td>
<td>50</td>
<td>18-72</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td>39/47(83%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenomas</td>
<td>47/47(100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoma count</td>
<td></td>
<td>21.8</td>
<td>12</td>
<td>3-100</td>
</tr>
<tr>
<td>Hyperplastic polyps</td>
<td>12/47(25%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>9/47(19%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Characteristics of a further 48 patients screened for BMPR1A were not significantly different.
superfamily of signalling molecules have been shown to cause JPS, with presumably by disrupting the downstream transcription of the same set of target genes. To date, all carriers of SMAD4 and BMPRIA mutations with colorectal adenomas have also had a personal or family history of juvenile polyps, but the possibility remains that some subjects presenting with multiple adenomas harbour germline SMAD4 or BMPRIA mutations.

In all cancer predisposition syndromes, understanding the genetic aetiology underlying the disorder is valuable for screening, particularly in familial cases where “at risk” subjects can be ascertained. We have screened the APC2, GSK3-β, conductin, SMAD4, and BMPRIA genes for germline mutations in 47 “multiple” adenoma patients to assess the possible contribution of these genes to the development of multiple colorectal adenomas.

MATERIALS AND METHODS

Ninety-five patients were recruited through the Cancer Research UK Family Cancer Clinic at St Mark’s Hospital (London, UK) and the Guy’s Hospital Clinical Genetics Unit (London, UK). Inclusion criteria were the presence of between five and 100 synchronous or metachronous colorectal polyps, with or without colorectal cancer, and histological confirmation of adenomatous features in the polyps. Additional phenotypic details (gender, age of onset, histology, polyp number) were available for all patients and are summarised in Table 1. Germline mutations had been excluded in APC (AAPC associated regions) and Mvh using single-strand conformational polymorphism (SSCP) analysis.

For fluorescence SSCP analysis, genomic DNA was isolated from peripheral blood using standard methods. Primer pairs to amplify overlapping fragments were designed for the coding regions including exon-intron boundaries of the APC2 (exon 1 to codon 1388 in exon 14, including the “20 amino acid repeat” region), conductin, and GSK3-β genes and are available from the authors on request. The entire conductin sequence was not available on a public database and was therefore derived by performing a BLAST search of the cDNA against the HTGS sequence database. Each 25 μl PCR reaction contained 1× PCR reaction buffer without MgCl₂, (Promega), 1 mmol/L MgCl₂, 200 μmol/L dNTPs, 200 μmol/L of each primer, 50 ng of genomic DNA, and 1 U of Taq DNA polymerase (Qiagen), and the PCR conditions consisted of 95°C for five minutes, followed by 35 cycles of 95°C for one minute, 55°C or 60°C for one minute, and a final extension step at 72°C for 10 minutes. The resulting PCR products were screened for variants by being run at 18°C and 24°C on the ABI 3100 and analysed using Genotyper 2.5 software (Perkin-Elmer Applied Biosystems). This method is approximately 90% sensitive in mutation detection.

For the APC2 protein truncation test (PTT) for exon 14, cDNA was synthesised using the First Strand Synthesis kit (Promega) and PCRs performed using primer pairs which spanned exon 14, with the forward primer tagged with a T7 RNA polymerase binding site and an in-frame start codon. Primers were available from the authors on request. In vitro coupled transcription translation was performed on the tagged PCR products using the TNT Rabbit Reticulocyte Lysate Kit (Promega), incorporating 35S methionine. The resulting “proteins” were separated according to size on a 12.5% polyacrylamide resolving gel. Once fixed and dried, gels were exposed to film overnight and developed.

Exon by exon polymerase chain reaction amplification of the BMPRIA and SMAD4 genes was performed using previously published primers. Resulting PCR products were screened for germline mutations causing conformational changes using the PHAST mini-gel SSCP analysis system (Pharmacia) according to the manufacturer’s instructions.

Results showing aberrant migration for each of the genes were reamplified, purified using Qiaquick columns

![Figure 1](http://jmg.bmj.com/)

**Figure 1** Loss of heterozygosity analysis at the BMPRIA locus in an adenocarcinoma from a patient with a germline BMPRIA mutation. Shown are the results from microsatellites (A) ALK3CA and (B) ALK3GGAA lying 43 Mb and 73 Mb proximal to the BMPRIA gene, respectively. The allele showing LOH is arrowed.

For loss of heterozygosity (LOH) analysis, 5 × 10 μm unstained tumour sections were dewaxed and dissected into an appropriate amount of digestion buffer (1 × magnesium free buffer, 20 μg/ml proteinase K) using a haematoxylin and eosin stained slide as a guide for the area to be microdissected. The resulting tumour DNA was PCR amplified alongside the blood DNA from the patient using three microsatellite markers D10S573, ALK3CA, and ALK3GGAA with the forward primer fluorescently labelled with TAMRA, TET, or HEX. Products were run on the ABI377 and results were analysed using Genotyper™ software, with areas under the peaks (including stutter bands) compared for all informative (heterozygous) markers. Allelic loss was considered present if the relative ratio of normal:tumour peak areas was less than 0.5, or greater than 2, thereby allowing for contaminating normal tissue within the microdissected tumour.

RESULTS AND DISCUSSION

We have screened a set of 47 “multiple” adenoma patients without germline APC and Mvh mutations for alterations in selected components of the TGF-β (BMPRIA, SMAD4) and Wnt (APC2, conductin, GSK3-β) signalling pathways, both of which
have been implicated in colorectal tumourigenesis. Screening of the TGF-β superfamily members, BMPRIA and SMAD4, identified a single putative pathogenic change in BMPRIA (patient D1), a 3 bp deletion at codon 360 resulting in the loss of a histidine residue. This amino acid is highly conserved and lies within the kinase domain of the protein, which is essential for the activation of the downstream targets SMAD1 and SMAD5.23 In accordance with a pathogenic effect, LOH at the BMPRIA locus was detected in an adenocarcinoma from this patient (fig 1). Besides this alteration, two intronic polymorphisms (IVS6 –261a and IVS11 –11T>C), two silent mutations (nt 435 G>A and nt 777 G>A), and one previously reported missense polymorphism (nt 4 C>A, T2P),24 were detected in BMPRIA. No pathogenic sequence changes or polymorphisms were detected in the SMAD4 gene. To clarify the involvement of BMPRIA mutations in causing a “multiple” adenoma phenotype, a second series of 48 APC and MYH mutation negative patients was screened for SMAD4 changes, but no additional pathogenic variants were identified.

Mutation analysis of three genes involved in Wnt signalling, APC2, GSK3β, and conductin, showed no clearly pathogenic changes in the original set of 47 patients. Two silent (nt 687 G>A and nt 1317 C>T) and two novel missense (nt 419 T>C, F140S and nt 967 G>C, G322R) mutations were identified in BMPRIA. In accordance with the known β-catenin binding/activation domains (table 2), the F140S variant in exon 4 occurred at an amino acid that is not evolutionarily conserved and doubt must therefore be cast on its significance. The G322R missense variant was excluded not evolutionarily conserved and doubt must therefore be cast on its significance. The G322R missense variant was excluded.

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