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 (AFAP or AAPC) owing to a germline APC mutation, usually in exons 1-4, exon 9, or the second half of exon 15.1-3 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 gene, human Mut Y homologue (MYH) on chromosome 1p34, were identified in 30% of patients presenting with 15 or more adenomas.4 MYH is a highly conserved base excision repair (BER) gene involved in the repair of 8-oxo-7, 8-dihydro2′-deoxyguanosine (8-oxo-dG) lesions induced by oxidative damage. Two further BER genes, human homologue of MutM (OGG1) and human homologue of MutT (MTH), have previously been excluded as causing multiple adenomas.5

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.6 In the absence of Wnt signalling, levels of β-catenin are minimised by degradation in the proteasome, after phosphorylation by a downstream target of Wnt signalling activates a cascade, which inhibits GSK3-β, allowing β-catenin to escape degradation.7 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.8-10

The human APC2 gene (chromosome 19p13.3) encodes a 2302 amino acid protein displaying high homology to APC. The N-terminus, including a series of 20 amino acid repeats, presumably mediating interaction with β-catenin.11 In SW480 colon cancer cells lacking functional APC protein, transfection with the APC2 gene inhibited β-catenin TCF signalling.12 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.13

Juvenile polyposis syndrome (JPS) is typified by characteristic colorectal polyps with large inflammatory infiltrate, mucin filled cysts, and flattened epithelium.14 They are usually multiple and can occur throughout the intestine, conferring a greatly increased risk of gastrointestinal cancer.15-16 It has been reported that JPS is associated not only with juvenile polyyps, but also with colorectal adenomas.17 Germline mutations of two genes, SMAD4 and BMPR1A, both from the TGF-β pathway, are a rare cause of the “multiple” adenoma phenotype.18

Key points

- “Multiple” (5-100) colorectal adenomas, either showing Mendelian inheritance or occurring as sporadic cases, confer an increased risk of large bowel cancer. In some patients, a diagnosis of attenuated familial adenomatosus polyposis (AFAP or AAPC) can be made owing to the presence of a germline mutation at the 5′ or 3′ end or within exons 9 of the APC gene, a central component of the Wnt signalling pathway. A “multiple” adenoma phenotype can also be caused by recessive mutations in the MYH gene, which is involved in base excision repair.
- In most patients with multiple colorectal adenomas, no underlying APC or MYH mutation can be found and the underlying cause of the disease remains unknown. Wnt signalling components remain good a priori candidates.
- Some patients with juvenile polyposis syndrome, caused by germline SMAD4/DPC4 or ALK3/BMPR1A mutations, also develop adenomas, although all cases described so far have had personal or family histories of juvenile polyps in addition to their adenomas.
- We have screened 47 “multiple” colorectal adenoma patients without APC and MYH mutations for changes in the Wnt pathway components APC2, conductin, and GSK3-β, and in the TGF-β pathway genes SMAD4 and BMPR1A. We found one patient with a putative pathogenic mutation in the BMPR1A gene, but no disease causing alterations in any of the other genes, indicating that these loci rarely account for the “multiple” adenoma phenotype.

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>
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<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</td>
<td>83%</td>
<td></td>
</tr>
<tr>
<td>Adenomas</td>
<td></td>
<td>47/47</td>
<td>100%</td>
<td></td>
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<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></td>
<td>12/47</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td></td>
<td>9/47</td>
<td>19%</td>
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</table>

Characteristics of a further 48 patients screened for BMPR1A were not significantly different.
superfamily of signalling molecules have been shown to cause JPS, presumably by disrupting the downstream transcription of the same set of target genes. To date, all carriers of SMA4 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 SMA4 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, SMA4, 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 MTHFR 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 exon 10), the BMPRIA (Qiagen), and the PCR conditions consisted of 95°C for five minutes, followed by 35 cycles of 95°C for one minute, 55°C for one minute, 72°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 ABI3100 and analysed using Genotype 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 (Promega) and PCRs performed using primer pairs which were 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 x 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 ABI3100 and analysed using Genotype 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 and the upstream primer fluorescein labelled with FAM, 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 MTHFR mutations for alterations in selected components of the TGF-β (BMPRIA, SMA4) and Wnt (APC2, conductin, GSK3-β) signalling pathways, both of which

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.
have been implicated in colorectal tumorigenesis. 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 SMAD5.22 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 -26/a and IVS11 –11T>C), two silent point mutations (nt 435 G>A and nt 777 G>A), and one previously reported missense polymorphism (nt 4 C>A, T2P),23 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, GSK3-β, 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 found in the 5′ region of APC2, which comprises the majority of the known β-catenin binding/regulation 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 as disease causing owing to its absence in other affected family members. Our findings therefore do not support a role for APC2 or SMAD4 in colorectal tumourigenesis in “multiple” adenoma patients.

Somatic mutations of conductin have been found in about a quarter of microsatellite unstable cancers.24 All mutations were frameshifts in mononucleotide repeats and involved the dishevelled and axin (DIX) domain, although biallelic mutations were not found. Two conductin variants were seen in our study population. One polymorphism in intron 6 (IVS6 +19 G>T) was present in 8/47 (17.0%) of patients and one silent variant in exon 6 (nt.635 C>T) was present in 3/47 (6.3%) of patients. No clearly pathogenic changes were found.

Our analysis of selected members of the TGF-β and Wnt pathways provides little evidence implicating the involvement of germ line mutations in these two signalling cascades, other than in APC, in the pathogenesis of multiple colorectal adenomas. This finding is supported by previous studies excluding germline β-catenin variants in “multiple” adenoma patients.25

The histology of the polyps in our group of mutation negative “multiple” adenoma patients did not differ in any evident way from that of sporadic or FAP adenomas and thus gives no clue as to genetic aetiology. The one person who was found to have an apparently pathogenic mutation in BMPRIA had no juvenile polyps reported. He was diagnosed at the age of 58 with eight tubular adenomas of mild to moderate dysplasia distributed throughout the colon and had no significant family history. This may indicate that this gene contributes, albeit rarely, to a subset of patients who present only with multiple adenomas. The absence of mutations in genes involved in the Wnt and TGF-β signalling suggests that other, as yet unidentified, predisposition genes and pathways exist. Possible candidates include the CRAC1 locus on chromosome 15q14-q22.26 In conclusion, the “multiple” adenoma phenotype cannot generally be attributed to germline mutations in genes involved in the Wnt and TGF-β signalling pathways. Patients and families who present in this manner could be offered mutation analysis of the APC (APC associated mutations), MYH, and perhaps BMPRIA genes, although the number of pathogenic mutations identified in the last of these will be relatively small. Further work is needed to unravel the genetic aetiology of multiple adenomas, but given the homogeneity of the phenotype, this is likely to rely on a combination of genetic linkage analysis and examination of candidate genes in alternative pathways leading to adenoma formation.

ACKNOWLEDGEMENTS

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Table 2 Mutations of the BMPRIA, SMAD4, APC2, conductin, and GSK3β genes (based on Genbank accessions gi[|4757853; gi[|865656; gi[|4514356; gi[|20558133; gi[|21361339, respectively) in multiple adenoma patients

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<thead>
<tr>
<th>Patients</th>
<th>Gene/exon</th>
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<th>Predicted effect of mutation</th>
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<tr>
<td>A1, F2, A7*, E6</td>
<td>BMPRIA exon 3</td>
<td>nt 4 C&gt;A</td>
<td>T2P</td>
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<td>BMPRIA exon 7</td>
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<td>nt 777 G&gt;A</td>
<td>Silent A259A</td>
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<td>c.360 del 3 bp (n1079-1081)</td>
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<td>E3</td>
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* indicates patients screened in the second cohort of 48 multiple adenoma patients.
REFERENCES