ELECTRONIC LETTER

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

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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 2] 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.[3] *MYH* 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 MutM (*OGG1*) and human homologue of MutT (*MTH*) have previously been excluded as causing multiple adenomas.[4]

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.[5] 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).[6 7] Wnt signalling activates a cascade, which inhibits GSK3-β, allowing β-catenin to escape degradation.[8] 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.[9 10]

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.[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 polyps, but also with colorectal adenomas.[17] Germline mutations of two genes, *SMAD4* and *BMPR1A*, both from the TGF-β

Table 1 Clinical features of multiple adenoma patients

Patient characteristics	No of patients	Mean	Median	Range
Sex M:F	24:23			
Age of diagnosis		48.5	50	18–72
Family history	39/47 (83%)			
Adenomas	47/47 (100%)			
Adenoma count		21.8	12	3–100
Hyperplasic polyps	12/47 (25%)			
Colorectal cancer	9/47 (19%)			

Characteristics of a further 48 patients screened for *BMPR1A* were not significantly different.

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 adenomatous polyposis (AFAP or AAPC) can be made owing to the presence of a germline mutation at the 5′ or 3′ end or within exon 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.

superfamily of signalling molecules have been shown to cause JPS,[18–20] presumably by disrupting the downstream transcription of the same set of target genes. To date, all carriers of *SMAD4* and *BMPR1A* 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 *BMPR1A* 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 *BMPR1A* 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 *MYH* 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 $MgCl_2$ (Promega), 1 mmol/l $MgCl_2$, 200 μmol/l dNTPs, 200 nmol/l of each primers, 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, 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 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. Primer sequences are 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 ^{35}S 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 *BMPR1A* and *SMAD4* genes was performed using previously published primers.[21 22] 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.

Fragments showing aberrant migration for each of the genes were reamplified, purified using Qiaquick columns (Qiagen), and then sequenced in both forward and reverse orientations using the ABI Big Dye Terminator kit (PE Applied Biosystems) in parallel with control samples.

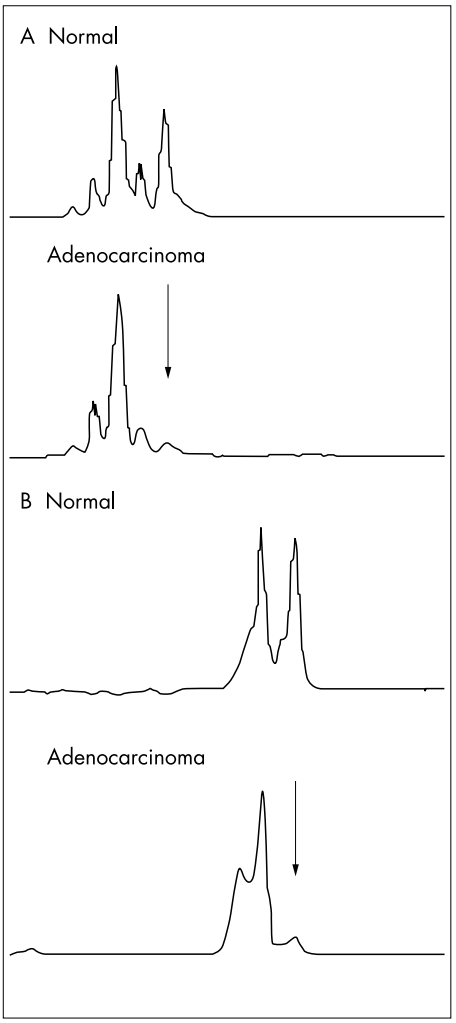

Figure 1 Loss of heterozygosity analysis at the *BMPR1A* locus in an adenocarcinoma from a patient with a germline *BMPR1A* mutation. Shown are the results from microsatellites (A) ALK3CA and (B) ALK3GGAA lying 43 Mb and 73 Mb proximal to the *BMPR1A* gene, respectively. The allele showing LOH is arrowed.

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[22] with the forward primer fluorescently 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 *MYH* mutations for alterations in selected components of the TGF-β (*BMPR1A, SMAD4*) and Wnt (*APC2*, conductin, *GSK3*-β) signalling pathways, both of which

Table 2 Mutations of the *BMPR1A*, *SMAD4*, *APC2*, conductin, and *GSK3β* genes (based on Genbank accessions gi[|]4757853; gi[|]i865656; gi[|]4514536; gi[|]20558133; gi[|]21361339, respectively) in multiple adenoma patients

Patients	Gene/exon	Mutation	Predicted effect of mutation
A1, F2, A7*, E6	*BMPR1A* exon 3	nt 4 C>A	T2P
H1, H3, F4	*BMPR1A* intron 6	IVS6 –26 T>A	
H2	*BMPR1A* exon 7	nt 435 G>A	Silent P145P
A8*	*BMPR1A* exon 9	nt 777 G>A	Silent A259A
D1	*BMPR1A* exon 10	c.360 del 3 bp (nt1079-1081)	Loss of histidine residue
H2	*BMPR1A* intron 11	IVS11 –11 T>C homozygote	
E3	*BMPR1A* intron 11	IVS11 –11 T>C heterozygote	
D1	*APC2* exon 4	nt. 419 T>C	F130S
E6	*APC2* exon 6	nt 687 G>A	Silent
A1	*APC2* exon 8A	nt 967, G>C	G323R
D3, H6	*APC2* exon 10	nt. 1317 C>T	Silent
A1, A4, A5, B4, H1, H2	Conductin exon 6	nt.635 C>T	Silent
D2, D6, E3	Conductin exon 5	IVS6+19 G>T	

*indicates patients screened in the second cohort of 48 multiple adenoma patients.

have been implicated in colorectal tumourigenesis. Screening of the TGF-β superfamily members, *BMPR1A* and *SMAD4*, identified a single putative pathogenic change in *BMPR1A* (patient DI), 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 *BMPR1A* locus was detected in an adenocarcinoma from this patient (fig 1). Besides this alteration, two intronic polymorphisms (IVS6 –26t/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),[20] were detected in *BMPR1A*. No pathogenic sequence changes or polymorphisms were detected in the *SMAD4* gene. To clarify the involvement of *BMPR1A* 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* in colorectal tumourigenesis in "multiple" adenoma patients. No sequence variants were found in *GSK3*-β in any of our 47 patients, consistent with previous studies failing to detect pathogenic somatic mutations in colorectal adenomas/carcinomas.[24]

Somatic mutations of conductin have been found in about a quarter of microsatellite unstable cancers.[25] 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 germline 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.[26]

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 *BMPR1A* 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.[27] 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* (AAPC associated mutations), *MYH*, and perhaps *BMPR1A* 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.

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