Clinical and molecular aspects of MUTYH- and APC-associated polyposis

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Inherited predisposition to colorectal adenomas caused by multiple rare alleles of *MUTYH* but not *OGG1, NUDT1, NTH1* or *NEIL* 1, 2 or 3

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ABSTRACT

Background:

MUTYH-associated polyposis (MAP) is a recessive trait characterised by multiple colorectal adenomas and a high risk of colorectal cancer. MUTYH functions in the DNA base excision repair pathway and has a key role in the repair of oxidative DNA damage.

Objectives:

To assess the contribution of inherited variants in genes involved in base excision repair and oxidative DNA damage including *MUTYH*, *OGG1, NEIL1, NEIL2, NEIL3, NUDT1* and *NTH1* to the multiple colorectal adenoma phenotype.

Methods:

Inherited variants of *MUTYH, OGG1, NEIL1, NEIL2, NEIL3, NUDT1 and NTH1* were sought in 167 unrelated patients with multiple colorectal adenomas whose family histories were consistent with recessive inheritance. These variants were also characterised in ,300 population controls.

Results:

Thirty-three patients (20%) and no controls were *MUTYH* homozygotes or compound heterozygotes (ie, carried two mutations) and therefore had MAP. Eight different pathogenic *MUTYH* mutations were identified, of which four were novel. MAP cases had significantly more adenomas than non-MAP cases (p=0.0009; exact test for trends in proportions) and presented earlier (p=0.013; analysis of variance). Twenty-four protein altering variants were identified upon screening of *OGG1, NEIL1, NEIL2, NEIL3, NUDT1* and *NTH1*. However, all combinations of two (or more) variants that were identified at an individual locus in patients were also seen in controls, and no variants were significantly overrepresented (or under-represented) in cases.

Conclusion:

Multiple rare alleles of *MUTYH* are associated with autosomal recessive MAP, while *OGG1, NEIL1, NEIL2, NEIL3, NUDT1* and *NTH1* do not contribute significantly to autosomal recessive polyposis.

Established genes account for only a small proportion of inherited predisposition to colorectal adenomas (CRAs) and cancer (colorectal cancer (CRC)),[1] and polygenic inheritance involving low penetrance alleles is likely to account for much of the residual familial risk. The identification of these common alleles that confer individually small increases in risk is underway through genome-wide association studies, but clinical application via genetic testing will not be practicable in the short term.[2–4] In contrast, the identification and characterisation of individually rare alleles of high penetrance is important in relation to predictive genetic testing and targeted surveillance in families at high risk of CRC. High penetrance CRC predisposition alleles have been described at a number of loci, including *MLH1, MSH2, MSH6, PMS2, APC* and *MUTYH*.[5] Mutations at these loci promote colorectal tumourigenesis through a variety of mechanisms. *MLH1, MSH2, MSH6 and PMS2* encode proteins that mediate DNA mismatch repair. Inherited mutations in these genes cause hereditary non-polyposis colorectal cancer (HNPCC). Affected individuals develop only small numbers of tumours, but these progress rapidly due, at least in part, to an increased somatic mutation rate in null cells. APC plays roles in regulation of β-catenin, cell adhesion and chromosome segregation. Inherited truncating mutations of *APC* cause the dominantly transmitted disorder familial adenomatous polyposis (FAP) that is characterised by development of hundreds to thousands of premalignant adenomas, a very small proportion of which progress to cancer. Attenuated FAP (AFAP) is characterised by smaller numbers of adenomas (typically <100) and is associated with mutations in specific 59 and 39 regions of *APC*. *MUTYH*-associated polyposis (MAP) is phenotypically similar to AFAP but is transmitted as a recessive trait.[5 6]

MUTYH is a component of the highly conserved base excision repair (BER) system that plays a major role in protecting against oxidative DNA damage and its mutagenic consequences. It removes adenines misincorporated opposite 8oxo-7,8-dihydro-29-deoxyguanosine (8-oxoG)[.7] Its association with colorectal tumourigenesis may, in part, reflect high levels of reactive oxygen species in the colorectum that are generated by commensal bacteria and dietary carcinogens.[8–10] A number of other enzymes work with MUTYH to protect against oxidative DNA damage. Given the established link between biallelic *MUTYH* mutations and the phenotype of multiple CRAs, they also represent candidate CRA and colorectal carcinoma predisposition genes.[11] These genes include OGG1 and NTH1 whose products remove the damaged base from 8-oxoG:C[12] and 8-oxoG:G base-pairs,[13] respectively, and the recently discovered gene family *NEIL1, 2* and 3 whose products have substrate specificities that overlap with OGG1 and NTH1.[14] In addition, NUDT1 functions as an 8-oxoGTPase that hydrolyses oxidised guanine triphosphates in the nucleotide pool, thereby preventing their incorporation into the

nascent strand during DNA replication.[15] In this study we sought inherited mutations at the *MUTYH*, *OGG1, NEIL1, NEIL2, NEIL3, NUDT1 and NTH1* loci in 167 patients who had a phenotype of multiple CRAs resembling FAP or AFAP but in whom no truncating mutations in *APC* had been identified, and in population-based controls.

MATERIALS AND METHODS

Patients and samples

We studied 167 unrelated patients with multiple CRAs who were identified through regional polyposis registers in the UK and New Zealand, and through the Wales Polyp Study. We also studied ,300 British Caucasian controls (European Collection of Animal Cell Cultures (ECACC), Salisbury, UK). The patients had been screened for truncating mutations in the *APC* gene with negative results and none had a recognised family history of colorectal polyposis in an affected parent (that would have suggested autosomal dominant transmission). Patients were categorised according to the number of CRAs recorded at colonoscopy or colectomy. Group A consisted of 59 patients with 3–10 CRAs. They presented at a mean age of 60 years (range 37–75 years). Group B consisted of 69 patients with 11–100 CRAs. They presented at a mean age of 48 years (range 23–77 years). Group C consisted of 39 patients with >100 CRAs. They presented at a mean age of 44 years (range 13–65 years). Patients with smaller numbers of adenomas were significantly older than those with larger numbers of adenomas ($p = 0.005$; linear regression), consistent with the presence of inherited determinants of disease severity. DNA was prepared from venous blood samples using standard methods. We have previously reported the analysis of *MUTYH* in 111 of the patients.[16] The study was approved by the MREC for Wales (ref. 02/9/22 and 06/MRE09/19).

Identification and characterisation of MUTYH mutations

Numbering of *MUTYH* mutations was based on the α1 transcript (GenBank accession number NM_012222) in which the first 33 nucleotides of exon 3 are coding. The common *MUTYH* mutations Y176C and G393D were assayed as described.[17] In order to identify other rare mutations, cases and controls found to harbour a single common mutation were sequenced for the *MUTYH* open reading frame (ORF) and intronic splice sites as described,[17] as were all patients of non-Northern European ethnic origin. Cases harbouring two rare *MUTYH* mutations may have been missed by this approach. Reverse transcription–PCR (RT–PCR) was performed to study the effect of the sequence variant c.681 G$\rightarrow$A on splicing since this variant involved the last base of exon 8. Blood samples for total RNA preparation were collected and extracted

using the PAXgene Blood RNA kit (Qiagen, Crawley, UK). RNA was reverse transcribed using the SuperScript III First Strand Synthesis System (Invitrogen, Paisley, UK) and cDNA was amplified using primers MYHx6F_Q227Q (5'GTGGCCTACACTGCAGGACC) and MYHx10R_Q227Q (5'AGGGCACTGGCTGCACAG) at an annealing temperature of 58uC. The RT–PCR products were cloned then sequenced using the same primers.

PCR amplification

One hundred and thirty-four cases without two *MUTYH* mutations were screened for mutations in *OGG1, NEIL1, NEIL2, NEIL3, NUDT1* and *NTH*. We amplified exons 1–8 of *OGG1* and 2–5 of *NUDT1* as described.17 The ORFs of *NEIL1, NEIL2, NEIL3* and *NTH1* were amplified as 9, 6, 11 and 5 fragments, respectively. Exons 2–4 of *NEIL2* (spanning the variants R103Q and R257L) were amplified as a 6.5 kb long-distance PCR fragment. Primer sequences are available at http:// www.cardiff.ac.uk/medic/contactsandpeople/c/cheadle-jeremypeter-prof.html.

Mutation analysis

Denaturing high-performance liquid chromatography (dHPLC) was carried out using the 3500HT WAVE system (Transgenomic, Glasgow, UK). To allow the detection of homozygous variants, 5 μl of wild-type PCR product was added to 20 ml of sample product, denatured at 95°C then cooled to 50°C at a rate of 0.5°C per 30 s. dHPLC was carried out at the melting temperatures predicted by Navigator (Version 1.54) software (Transgenomic) with a 12% acetonitrile gradient over 2.5 min (conditions are available at http://www.cardiff.ac. uk/medic/contactsandeople/c/cheadle-jeremy-peter-prof.html). Aberrant elution profiles were identified using Navigator.[18] Direct sequencing was used to characterise variants. Variants were confirmed by amplification refractory mutation system (ARMS) assays or restriction endonuclease digests which were also used to genotype controls. Primer sequences and conditions are available at http://www.cardiff.ac.uk/medic/contactsandpeople/c/cheadle-jeremy-peter-prof.html.

Statistical analysis

Variants were assessed for departure from Hardy–Weinberg equilibrium using the $\chi 2$ test or, when the number of genotypes was <5, by using the Monte-Carlo permutation test with 10 000 permutations (HWSIM software http://krunch.med. yale.edu/hwsim/). Differences between the proportion of patients and controls with each variant were analysed using the $\chi 2$ or Fisher's exact tests. Corrections for multiple testing were performed by a permutation test, with random reshuffling of case–control status (when individual genotypes of cases and controls were available). When individual genotypes

for controls were unavailable, correction for multiple testing was performed using spectral decomposition of matrix of pairwise r^2 measures of linkage disequilibrium.[19] The exact test for trend in proportions was used to analyse differences in the proportions of MAP and non-MAP cases in the three phenotype categories (A, B and C). Univariate ANOVA (analysis of variance) was used to compare the mean ages at presentation in MAP and non-MAP cases. The relationship between age at presentation and polyp count was assessed using linear regression analysis.

RESULTS

Mutations at the MUTYH locus

Homozygous or compound heterozygous *MUTYH* mutations confirming MAP were identified in 33/167 patients and 0/354 controls. MAP cases had significantly more adenomas than non-MAP cases (p = 0.0009; exact test for trends in proportions) and presented earlier at 47 (11) (mean (SD)) years vs 54 (15) years (p = 0.013; ANOVA). Twenty-five of these patients have been reported previously.[16] Four novel and likely pathogenic mutations were identified in previously unreported patients, each in combination with one of the two most common northern European mutations, either Y176C or G393D (table 1). The novel variant T474M was also identified as a third mutation in one of these patients, but was considered to be of uncertain clinical significance since it was observed in combination with a known pathogenic mutation (G393D) and the novel truncating mutation (1092delC). The novel mutation c.681 G→A (Q227) was found in combination with Y176C in two unrelated patients. Sequencing of cloned RT–PCR products from one of these patients confirmed that these variants were on different *MUTYH* alleles and that c.681 G→A was associated with skipping of exon 8 (and rarely of exons 8 and 9). In addition, two of 167 cases were identified as apparent *MUTYH* heterozygotes (both for G393D), as were 8/354 controls (not significant).

MAP phenotypes

Thirty-two MAP patients presented symptomatically with rectal bleeding and/or altered bowel habit, and one patient was identified through a screening programme. The mean age at presentation was 47 years (median 48 years, range 13–67 years). Ten patients were specified as having >100 adenomas, 15 had between 10 and 100 adenomas, 1 patient had 5 confirmed adenomas and in 7 cases the adenomas were reported at colectomy simply as being ''multiple'', ''too many to count'', ''numerous'' or ''throughout the colon''. Nineteen of the 33 cases (58%) had CRC (all but one at presentation), diagnosed at a mean age of 50 years. Six patients had more than one primary CRC.

One of the nine previously unreported patients had three gastric adenomas and one of nine female patients was diagnosed with breast cancer aged 71 years. Phenotype data for the nine previously unreported MAP patients are shown in Supplementary table 1.

Variants at the *OGG1, NEIL1, NEIL2, NEIL3, NUDT1* and *NTH1* loci

Sixty-five variants were identified through screening of the *OGG1, NEIL1, NEIL2, NEIL3, NUDT1* and *NTH1* loci in 134 patient samples. Forty-one were silent or intronic changes. The other 24 variants included 22 missense changes, a nonsense change and a splice site alteration (Supplementary table 2). All patient samples containing rare missense variants (minor allele frequencies <15%) or truncating variants were sequenced for the ORF of the corresponding gene to search for second mutations that might support recessive inheritance. We also designed assays for each variant and screened for them in a panel of ~300 British control blood DNA samples (ECACC, Salisbury, UK).

A number of patient samples were found to contain two (or more) rare variants at a single locus, but all pairs of variants seen in patients were also observed in similar proportions of control samples. These combinations were R103Q with R257L in *NEIL2* (shown by cloning and sequencing to be on the same allele), Q172H with R38C in *NEIL3*, and P117R with each of R38C, Q172H, R315Q and R520G in *NEIL3*.

No variants showed departure from Hardy–Weinberg equilibrium amongst the cases, and all except for four unique missense mutations (I321T in *OGG1*, P208S in *NEIL1*, R164T in *NEIL2* and D132V in *NEIL3*) were also found in controls. None was significantly under-or over-represented in cases versus controls after correction for multiple testing (Supplementary table 2). One of 134 cases and 2/359 controls were heterozygotes for the novel nonsense mutation Q90X in *NTH1*. Five of 134 cases were heterozygous for the previously reported splice site variant 434+2T→C in *NEIL1* (dbSNP reference rs5745908) as were 8/360 controls ($p = 0.15$).

DISCUSSION

Patients were selected for study if they had multiple CRAs but no dominant family history of polyposis and no truncating mutation of *APC*. Twenty percent (33/167) were found to carry biallelic *MUTYH* mutations and were classified as MAP patients. All had AFAP-like or classical FAP-like phenotypes, most with between tens and hundreds of macroscopic CRAs. None had >1000 adenomas (as is seen in severe FAP due to mutations of *APC* at codon 1309) and only 1 of the 59 patients with 3–10 adenomas had MAP. These data support the emerging picture of the MAP colorectal phenotype.[20–23]

Table 1 Novel *MUTYH* mutations: evidence for/against pathogenicity

Patient	Exon	AA change	Nucleotide change	Predicted effect	Conservation		Frequency in controls†	Likely to be pathogenic
					AA	Evolution*		
2058	8	G213E	c.638 G→A	Missense	NC	C	0/724	Yes
0748 1232	8	Q227	c.681 G→A	Aberrant splicing	N/A	N/A	0/722	Yes
0376	9	N235S	c.704 A→G	Missense	SC	C	0/722	Yes
4891	12	N/A	c.1092delC	Frameshift	N/A	N/A		Yes
4891	14	T474M	c.1421 C→T	Missense	SC	SC	0/724	Unknown

All novel mutations were found in conjunction with either Y176C or G393D. Amino acid (AA) substitutions were non-conservative (NC) or semi-conservative (SC) changes.
*Alignment of human (NP_036354), mouse (NP_573513), *Arabidopsis* (NP_193010), fission yeast (NP_594145), *Escherichia coli* (NP_417436) and *Bacillus stearothermophilus* (1RRQA) MUTYH homologues was carried out using Clustal W. Residues were determined to be semi-conserved (SC) or conserved (C) through evolution based on the retention of identical amino acids in some or all of the organisms, respectively.
†Variants were typed in British control samples (supplied by European Collection of Animal Cell Cultures).
N/A, not applicable.

In addition, we observed adenomas of the stomach and duodenum in MAP cases, but screening for upper gastrointestinal disease had been inconsistent in our patient series. Systematic studies have not been reported yet. The early mean age for symptomatic presentation with MAP (47 years) and the high prevalence of CRC at presentation (58%) suggest that the roll out of population-based bowel screening programmes for average risk older adults will have little impact on MAP-related CRC. A proactive approach to gene testing and additional surveillance of the siblings of MAP index cases is required. The relatively high incidence of synchronous cancers also makes complete colonoscopy the surveillance procedure of choice, as in AFAP.

Four novel likely pathogenic *MUTYH* mutations were identified, one of which (c.681 G→A) was found in two unrelated MAP cases. At present, many centres screen only for these common mutations (G393D or Y176C) in a diagnostic setting, but the growing list of rare mutations21 23 highlights the need for comprehensive screening of the *MUTYH* gene. We identified no patients with biallelic mutations that were likely to be pathogenic in *OGG1, NEIL1, NEIL2, NEIL3, NUDT1* or *NTH1*, and conclude that these genes do not contribute significantly to polyposis determined by autosomal recessive inheritance. Our study does not exclude a contribution of these genes to polygenic determination of the polyposis phenotype and we did not investigate families showing autosomal dominant inheritance. The truncating mutations Q90X in *NTH1* and 434+2T→Cin *NEIL1* were identified in sporadic multiple CRA cases but were also at similar frequencies in controls. We noted that an alternative translation initiation signal at codon 102 in NTH1 could lead to a partially deleted but functional *NTH1* protein from the Q90X allele.[24]

A smaller previous study[25] did not identify pathogenic mutations in *OGG1* or *NUDT1* in patients with multiple CRAs, and another[26] investigated *NTHL1, NEIL1, NEIL2, MPG, TDG, UNG* and *SMUG1* in CRC families with negative results. There is considerable functional redundancy within repair systems for oxidative DNA damage. Not only do the substrates of the BER glycosylases overlap, but the mismatch repair complex MSH2/MSH6, the CSB protein and BRCA1 and BRCA2 have also been implicated in the repair of 8-oxoG. So far, of the many genes involved in BER, only *MUTYH* has an established role in colorectal tumourigenesis.

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