Mutation screening of MSH2 and MLH1 mRNA in hereditary non-polyposis colon cancer syndrome

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Abstract

Germline mutations in four human mismatch repair genes (MSH2, MLH1, PMS1, and PMS2) have been reported to cause hereditary non-polyposis colon cancer syndrome (HNPCC). The identification of germline mutations in HNPCC kindreds allows precise diagnosis and accurate predictive testing. To investigate further the genetic epidemiology of HNPCC and the nature and frequency of germline mutations in this disorder, we studied 17 English HNPCC kindreds for germline mutations in MSH2 and MLH1. A previous genetic linkage study had suggested that most English HNPCC families will have mutations in one of these genes. Mutation analysis was performed in a three step process. (1) mRNA extracted from lymphoblastoid cell lines was analysed for gross rearrangements, (2) the in vitro transcription-translation (IVTT) assay was then performed to detect protein truncating mutations, and (3) partial cDNA sequencing of MSH2 or MLH1 was undertaken in families (n=6) linked to MSH2 or MLH1 but without a detectable mutation. Seven different germline mutations were identified in eight of 17 (47%) kindreds (five in MSH2 and three in MLH1). In three cases there was a deletion of a single exon in MSH2 mRNA, three mutations resulted in a truncated protein product, and two missense mutations were identified by direct sequencing. Six mutations were novel. No precise correlation between genotype and phenotype was observed, although a MSH2 missense (Thr905Arg) mutation was associated with a susceptibility to multiple colorectal polyps. Age related risks for colorectal and uterine cancer were similar for MSH2 and MLH1 mutations.

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Key words: hereditary non-polyposis colorectal cancer; MSH2; MLH1.

Hereditary non-polyposis colon cancer syndrome (HNPCC) may account for up to 5% of all colorectal cancers, and is characterised by a dominantly inherited predisposition to early onset (mean 46 years) and multicentric colorectal cancer.¹⁻³ In some families there is also an increased risk of extracolonic cancers, prin-

cipally uterine, ovarian, and urothelial, and HNPCC can be subclassified into Lynch 1 and Lynch 2 in which extracolonic cancers are absent or present respectively. In addition, the Muir-Torre syndrome, which is characterised by the association of sebaceous cyst tumours and internal malignancy, is a further HNPCC variant.⁴ Germline mutations in four human mismatch repair genes, MSH2, MLH1, PMS1, and PMS2, may cause HNPCC,⁵⁻⁹ but there is relatively little information on the genetic epidemiology of HNPCC in different populations. In a previous genetic linkage study of 14 English HNPCC families we found evidence that mutations in MSH2 and MLH1 were likely to account for the majority of families studied.¹⁰ Although presymptomatic molecular genetic diagnosis improves the management of familial colorectal cancer, most HNPCC families are not suitable for linkage based predictive testing. We therefore investigated the frequency, nature, and phenotypic manifestation of MSH2 and MLH1 mutations in 17 English HNPCC families.

Materials and methods

PATIENTS

Lymphoblastoid cell lines were obtained from 17 probands from HNPCC families ascertained from East Anglia and north west England which met the standard criteria of the ICG for the diagnosis of HNPCC (at least three relatives with colorectal cancer in two or more generations, with at least one first degree relationship and with at least one patient diagnosed age <50 years).¹¹ Non-colonic cancers known to be associated with the Lynch 2 phenotype (uterus, ovary, and ureter) occurred in eight families and a diagnosis of Muir-Torre syndrome was made in one kindred (C004). Tumour material was available from an affected member of nine kindreds and these tumours were investigated for microsatellite instability (RER phenotype).

MICROSATELLITE INSTABILITY DNA ANALYSIS

Evidence of microsatellite instability was sought in tumours from affected members of nine families using five microsatellite polymorphisms: D3S1007, D3S1076, D5S346, D17S588, and CA5 on chromosome 2p, as described previously.¹⁰ The finding of microsatellite instability at two or more loci was considered significant.¹⁰

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Table 1 Details of phenotype, number of people with colorectal cancer, linkage to MSH2 and MLH1 (from reference 10), and mutations identified (nucleotide and codons numbered as in references 5 and 6)

Family	Phenotype	No of relatives with colorectal cancer	RER status	Lod scores		Mutation details		
				MSH2	MLH1	Gene	DNA/RNA abnormality	Effect on protein
C001	Lynch 2	3	ND	1.3	-2.23			
C002	Lynch 2	3	ND	1.16	-3.53			
C004	Muir-Torre	5	+	1.52	-5.56			
C003	Lynch 1	7	ND	1.11	-2.98	MSH2	2782C→G Thr905Arg	Missense
C016	Lynch 2	4	+	1.54	-3.46			
C015	Lynch 2	6	ND	1.01	-2.88			
C009	Lynch 2	6	+	-3.39	1.38	MLH1	del 1472A frameshift	Truncated
C006	Lynch 1	3	+	0.01	0.17			
C008	Lynch 1	3	+	-0.37	0.30	MLH1	2163G→A Trp714Stop	Truncated
C022	Lynch 1	3	ND	ND	ND	MSH2	Exon 5 deleted owing to 3' splice site mutation Ggtaaa→Ggtaa	Truncated
C007	Lynch 2	4	+	-0.18	-0.7	MSH2	Deletion exon 3 in RNA, no genomic DNA abnormality	Truncated
C010	Lynch 1	5	+	-0.87	0.27	MLH1	2062G→A Ala681Thr	Missense
C017	Lynch 2	5	+			MSH2	Exon 5 deleted owing to 3' splice site mutation Ggtaaa→Ggtaa	Truncated
C018	Lynch 1	3	ND	ND	ND			
C019	Lynch 1	4	ND	ND	ND			
C020	Lynch 1	6	+	ND	ND			
C021	Lynch 2	3	ND	ND	ND	MSH2	1257C→T Gln397Stop	Truncated

ND = not done.

RNA ANALYSIS

Total RNA was extracted from lymphoblastoid cell lines using TriZol reagent (Life Technologies) according to the manufacturer's instructions. First strand cDNA synthesis was performed using 1 µg RNA and oligo-dT (Promega) with AMV reverse transcriptase (Promega) as directed. Initially MSH2 was amplified in four overlapping fragments as previously described,⁶ and these were analysed by agarose gel electrophoresis for detection of gross rearrangements.

In vitro transcription/translation was performed essentially as described by Liu *et al*¹² using their primer sequences for MSH2, and those of Papadopoulos *et al*⁶ for MLH1. In brief, purified (Wizard purification kit, Promega) RT-PCR DNA was mixed with T7 RNA polymerase and 1 µCi ³⁵S-L-methionine (translation grade, 1000 Ci/mmol at 10 mCi/mol; Amersham Life Sciences) in a rabbit reticulocyte lysate based transcription/translation system (TNT, Promega) and incubated at 30°C for two hours. Protein electro-

phoresis was performed in 4% stacking/12% separating SDS-polyacrylamide gels, the gels fixed, dried under vacuum at 80°C, and then exposed to BIOMAX film (Kodak) for one to seven days.

Any samples with detectable truncated proteins were investigated further by direct cDNA sequencing, both semiautomatic (PRISM Ready reaction dye deoxy terminator cycle sequencing kit, Applied Biosystems) and manual (fmol sequencing system, Promega). Base changes identified in this way were confirmed or denied by either further sequencing or restriction digestion, where possible, of the relevant exon from genomic PCR DNA.

The incidence of the two missense mutations detected (C003; MSH2 Thr905Arg, C010; MLH1 Ala681Thr) in the general population was investigated by restriction digestion. MLH1 Ala681Thr destroys a *Hin*P1I site. Primers MLH1 18F (5' CGT ACC TAT TTT GAG GTA TTG AA 3') and 18R (5' AAG ATT GTA TAG GCC TGT CCT AG 3') were used to amplify a 201 bp product. This was subsequently digested with *Hin*P1I (New England Biolabs), generating 113 bp and 88 bp products in unaffected subjects, but remaining undigested in subjects carrying the alteration.

For detection of MSH2 Thr905Arg, a specific primer was designed such that the presence of the sequence alteration generates a *Hin*fl site. Primers MSH2 16F (5' TTT AAT TAC TAA TGG GAC ATT CAC A 3') and x16T905R rev (5' CAG CTT TTA GCT GTT TTA ACT TGA T 3') generate a 141 bp product. After digestion with *Hin*fl (New England Biolabs), products of 115 bp and 26 bp are seen in subjects carrying the alteration.

Results

Details of the clinical features and previous genetic linkage results¹⁰ for the 17 families are given in table 1.

ANALYSIS OF MSH2 AND MLH1 mRNA

Large deletions of MSH2 mRNA were detected in three kindreds (C017, C022, C007). Sequencing of the mutant mRNA and genomic

Figure 1 SDS-PAGE of in vitro transcription-translation products from MLH1, showing truncated proteins in fragment 2 (codons 325-756) in tracks 4 (C008; Trp714Stop) and 5 (C009; 1472delA).

DNA showed an identical deletion of exon 5 secondary to a 3' splice site mutation in two kindreds (table 1). In C007 a deletion of exon 3 was detected in the MSH2 mRNA, but no abnormality was identified in genomic DNA.

PROTEIN TRUNCATION ANALYSIS

MSH2 and MLH1 cDNA was analysed for the presence of protein truncating mutations in the 14 kindreds without visible mRNA abnormalities. In vitro transcription-translation (IVTT) assay showed abnormalities in MLH1 in two kindreds: a single base frameshift deletion within exon 13 in C009 and a nonsense mutation in exon 19 in C008 (fig 1), and an alteration in MSH2 in one kindred, a nonsense mutation in exon 7 in C021 (table 1).

DETECTION OF MSH2 AND MLH1 MUTATIONS BY DIRECT SEQUENCING

To investigate if any of the 11 kindreds without an apparent mutation on mRNA and IVTT assay might have missense mutations, we partially sequenced MSH2 or MLH1 cDNA in six kindreds where linkage had previously been suggested (MSH2: C001, C002, C003, C004, C016, and MLH1: C010). Abnormalities were defined in two kindreds. In C003 there was a Thr→Arg substitution at codon 905 in exon 16 of MSH2 (fig 2). The sequence change tracked with disease status and haplotype and was identified in all five affected subjects, but was not detected in 56 normal controls (data not shown). In C010 an Ala→Thr substitution was identified at codon 681 in exon 18 of MLH1 (table 1). The sequence change also tracked with the disease and haplotype and was present in all five affected relatives but not in 53 normal controls (data not shown). No mutation was identified in four kindreds (C001, C002, C004, C016) showing linkage to chromosome 2p16 markers despite sequencing >60% of the MSH2 coding sequence in each case.

GENOTYPE-PHENOTYPE CORRELATIONS

There were no significant differences between the incidence of Lynch 1 and Lynch 2 phenotypes according to whether MSH2 or MLH1 was mutated. Thus, of five families with MSH2 mutations, two had a Lynch 1 phenotype and three a Lynch 2 phenotype. Of three kindreds with MLH1 mutations, two had Lynch 1 and one a Lynch 2 phenotype. However, C003 kindred was notable because the phenotype overlapped with an attenuated polyposis phenotype and two of six relatives with the Thr905Arg substitution in MSH2 had ~100 adenomatous polyps. Age related risks for HNPCC related cancers were calculated for 50 subjects with MSH2 and MLH1 mutations. The age related risks for cancer for gene carriers were 15%, 69%, and 89% at 40, 50, and 60 years respectively. The predicted cancer risks at the age of 50 years were similar in subjects with MSH2 mutations (n=27) and MLH1 mutations (n=23) for (1) all cancers (66% and 71% respectively), (2) colorectal cancer (62% v 67%), and (3) Lynch 2 extracolonic cancers (uterus, ovary, and ureter) (26% and 29%).

REPLICATION ERRORS IN COLORECTAL CANCERS
Replication errors were detected at two or more of five loci analysed in colorectal cancers from all of the nine families available for analysis (table 1).

Discussion

Germline MSH2 and MLH1 mutations were identified in eight of 17 (47%) HNPCC families investigated. In a previous study, seven of the 17 families had shown probable linkage to MSH2 (n=6) or MLH1 (n=1).¹⁰ Germline mutations were detected here in one of six families with apparent linkage to MSH2 (lod score >1) and in the single family linked to MLH1. In addition, a germline mutation was detected in four families which were uninformative for linkage analysis. Six of the seven distinct mutations we detected were novel. The MSH2 exon 5 splice site mutation identified in two families was also detected by Liu *et al*¹² in three of 29 HNPCC families analysed. However, haplotyping of C017 and C022 has not shown evidence of a common haplotype (unpublished observations). We detected deletions of MSH2 mRNA in three of 17 kindreds (17%) compared to seven of 29 (24%) studied

Figure 2 Semiautomatic sequencing electrophoretogram of codons 904-905 of MSH2 from C003, showing heterozygosity at the second position of codon 905 (ACA→AGA; Thr905Arg).

by Liu *et al.*¹² using similar methods. In both studies, there are examples of patients with mRNA deletions in which a genomic sequence abnormality could not be identified. Many previously reported mutations in MSH2 and MLH1 are predicted to result in a truncated protein product,^{6 8 12-15} but this may reflect the popularity of the *in vitro* transcription-translation assay for mutation detection in HNPCC. Direct sequencing identified apparent missense mutations in two of our families (25% of identified mutations). These sequence variants are likely to be pathogenic as both (1) lead to substitutions of conserved amino acids, (2) track with the disease phenotype (colorectal cancer or multiple polyps or both), and (3) were not detected in normal controls. Although HNPCC is clinically heterogeneous with Lynch 1, Lynch 2, and Muir-Torre subtypes often distinguished, in practice these divisions may not be clear cut,¹⁶ and there are no clear genotype-phenotype correlations. Thus mutations in MSH2 and MLH1 can cause Lynch 1, Lynch 2, and Muir-Torre phenotypes.¹² The age related cancer risks we calculated were based on data for all known gene carriers in a family. As all the families we analysed were selected because they satisfied the Amsterdam criteria, these risks may not be accurate for MSH2 or MLH1 mutations in non-Amsterdam criteria families. Most HNPCC patients have few polyps, but sometimes the distinction between HNPCC and attenuated familial polyposis coli can be equivocal. Mutations in the 5' exons of the APC gene may cause an attenuated FAP phenotype.¹⁷ In our series, family C003 was notable because of the frequency of colorectal polyps in family members, including two who had ~100 polyps in colectomy specimens. However, this family is not linked to the APC gene and the Thr905Arg substitution in MSH2 is likely to be pathogenic. The identification of further families with this mutation will help resolve whether this mutation per se is associated with frequent polyps, or whether the observed phenotype in C003 is a result of modifier genes.¹⁸

We found that although analysis of the MSH2 and MLH1 genes by inspection of transcript size and the protein truncation test can rapidly identify germline mutations, most HNPCC kindreds satisfying the ICG diagnostic criteria did not have an identifiable mutation using these methods (11 of 17). Other groups have also reported a lower than expected mutation detection rate.^{12 19} This may reflect inefficiency of the mutation detection techniques used or that, in contrast to the predictions from genetic linkage studies, MSH2 and MLH1 might not account for most HNPCC families. Our strategy would not detect mutations which resulted in an absent or unstable transcript. Buerstedde *et al.*¹⁹ recently reported the highest mutation detection rate (6/10 Swiss HNPCC families) by sequencing each MSH2 and MLH1 exon and flanking sequence. However, even this labour intensive approach will not identify large germline deletions. Finally, we only analysed one person

from each family and it is possible that some of these may by chance have been a phenocopy. In contrast to Finland, where most HNPCC families are accounted for by two ancestral mutations,^{8 20} there is no evidence for founder mutations in English HNPCC families. Thus, apart from the exon 5 splice mutation, none of our other six mutations were detected in a second family, and analysis of our families by a specific restriction site assay did not detect the MLH1 mutation (codon 519 + 1 T frame-shift) reported by Kolodner *et al.*¹³ in a very large family from north east England (data not shown). Although five families linked to chromosome 2p16 did not have an identifiable MSH2 mutation, we cannot exclude the possibility that these families have a mutation in the mismatch repair enzyme gene (GTBP or p160), which also maps to this region.^{21 22} Although Jass *et al.*²³ have reported that only 12 of 19 HNPCC kindreds satisfying the ICG criteria had tumour microsatellite instability, all HNPCC families in our data set were RER positive. Further studies of RER status and MSH2 and MLH1 mutation analysis in different centres are required to resolve the genetic epidemiology of HNPCC fully.

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