

# Mononucleotide microsatellite instability and germline *MSH6* mutation analysis in early onset colorectal cancer

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## Abstract

**Germline mutations in the *MSH2* and *MLH1* mismatch repair genes account for most cases of hereditary non-polyposis colon cancer syndrome (HNPCC). In addition, germline *MSH2* and *MLH1* mutations have been detected in patients with non-HNPCC early onset colorectal cancer. Germline *MSH6* mutations appear to be rare in classical HNPCC families, but their frequency in young colorectal cancer cases has not been studied previously. In a population based study of early onset colorectal cancer (<50 years) investigated for tumour microsatellite instability (MSI), we identified a subgroup of tumours with MSI for mono- but not dinucleotide repeat markers (m-MSI+ group). In contrast to tumours with classical MSI for dinucleotide markers (d-MSI+), the m-MSI+ group cancers were mainly left sided (6/7). As *MSH6* mutations in yeast and human cell lines are associated with weak (and preferential mononucleotide) MSI, the complete *MSH6* gene coding region was sequenced in blood DNA from the five m-MSI+ cases available for analysis. A germline non-sense mutation was identified in an isolated case of early onset colorectal cancer (age 43 years). These results support previous findings that germline *MSH6* mutations may not be associated with classical MSI and suggest a role for germline *MSH6* mutations in isolated early onset colorectal cancer.**

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Colorectal cancer (CRC) is one of the commonest cancers in the western world<sup>1</sup> and genetic factors have been clearly implicated in its pathogenesis. Up to 5% of all cases have a recognisable dominantly inherited colorectal cancer susceptibility disorder such as familial polyposis coli (FAP) or hereditary non-polyposis colorectal cancer syndrome (HNPCC). In recent years there has been considerable progress towards defining the genetic basis for colorectal cancer susceptibility. FAP is caused by germline mutations in the APC tumour suppressor gene mutations, which characteristically produce a distinctive clinical

phenotype including florid colonic polyposis. In contrast, HNPCC does not present a distinctive clinical phenotype and the clinical diagnosis of HNPCC depends on a combination of familial clustering of colorectal cancer, susceptibility to early onset and multiple colorectal cancers, and the presence of associated tumours such as endometrial, ovarian, and urothelial cancers.<sup>2</sup> Formalised clinical criteria for the diagnosis of HNPCC ("the Amsterdam criteria") have been adopted widely and define a small group of patients with a strong family history of colorectal cancer.<sup>3</sup> A major advance in colorectal cancer genetics was the mapping and subsequent identification of the molecular basis for HNPCC. Most classical HNPCC kindreds have germline mutations in the *MSH2* or *MLH1* mismatch repair genes.<sup>2-4-8</sup> Cancers from these patients characteristically show a high rate of accumulation of DNA replication errors (RERs) in simple repeat sequences resulting in what has been called microsatellite instability (MSI, MIS, etc). In *MSH2* and *MLH1* deficient tumours, such microsatellite instability has been observed in mono-, di-, tri-, and tetranucleotide repeats.<sup>9-12</sup>

*MSH2* and *MLH1* are human homologues of the *E coli* MutS and MutL components of the *E coli* MutHLS mismatch repair system.<sup>13</sup> In this system, the MutS protein recognises mispaired bases in DNA and recruits other essential protein factors, such as MutL, that function in the mismatch repair reaction. The eukaryotic MMR system is more complex than the bacterial system.<sup>14-15</sup> Eukaryotic MMR involves two different heterodimeric complexes of MutS related proteins. One is a complex of *MSH2* and *MSH6* (sometimes called *GTBP* in humans) that functions in repair of base-base mispairs and small insertion/deletion mispairs, and the other is a complex of *MSH2* and *MSH3* which is involved in repair of insertion/deletion mispairs.<sup>16-18</sup> Similarly, eukaryotic MMR involves two different heterodimeric complexes of MutL related proteins. One is a complex of *MLH1* and *PMS1* (*PMS2* in humans) that functions in the bulk of mismatch repair and the other is a complex of *MLH1* and *MLH3* (*PMS1* in humans) that appears responsible for only a small component of mismatch repair.<sup>19-21</sup> The observation of partially redundant MutS homologue complexes and partially redundant MutL homologue complexes is consistent with the observation that the majority of HNPCC causing mutations are found in *MSH2* and *MLH1*.

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Mutations in these two genes encoding the common subunits of the MutS and MutL homologue complexes, respectively, would be expected to cause the greatest defects in mismatch repair.

Initial studies of *MSH6* in HNPCC kindreds focused on kindreds in which tumours showed strong dinucleotide repeat instability and did not identify any mutations.<sup>8, 22</sup> Studies in yeast showed that mutations in *MSH6* did not cause dinucleotide repeat instability but rather caused weak mononucleotide repeat instability and a marked increase in the rate of accumulation of base substitution mutations.<sup>14, 18, 23</sup> Similarly, *MSH6* mutant human tumour cell lines showed much weaker microsatellite instability than tumour cell lines in which *MSH2* or *MLH1* were defective.<sup>14, 24-30</sup> Furthermore, studies with *MSH6* mutant mice showed that they had markedly increased cancer susceptibility and that the tumours in these mice did not show repeat instability, which is similar to the results seen in yeast.<sup>31</sup> These results suggested that mutations in *MSH6* would probably cause cancer susceptibility but the tumours that arose might be distinct from those seen in kindreds inheriting *MSH2* and *MLH1* mutations, at least with regard to their microsatellite instability phenotype. Indeed, two germline *MSH6* mutations have been reported in non-HNPCC familial colorectal cancer kindreds.<sup>32, 33</sup> To investigate further the role of germline *MSH6* mutations in colorectal cancer susceptibility we have performed mutation analysis of *MSH6* in colorectal cancer patients with tumour mononucleotide microsatellite instability (m-MSI).

## Patients and methods

### PATIENTS

Eighty-three patients with early onset colorectal cancer (age <50 years) were analysed for tumour RERs (microsatellite instability (MSI)) using a combination of mono- and dinucleotide microsatellite markers. Patients were ascertained randomly through a population based survey of colorectal cancer in eastern England and the results of MSI for dinucleotide markers on the first 33 patients aged 45 years or less have been reported previously.<sup>34</sup>

### MICROSATELLITE INSTABILITY ANALYSIS OF COLORECTAL CANCERS

For each case (n=83), paraffin embedded tumour and normal tissue sections were obtained and analysed for microsatellite instability with CA dinucleotide microsatellite markers as described previously.<sup>35</sup> In addition, MSI was assessed with two mononucleotide repeat markers chosen from BAT25, BAT26, and BAT40. Tumours were considered as showing conventional dinucleotide repeat instability (d-MSI) if two or more of five dinucleotide repeats tested showed instability and were considered as showing mononucleotide repeat instability also if one or more of two mononucleotide repeats showed instability. Tumours showing mononucleotide but not dinucleotide microsatellite instability were designated m-MSI+. We have used these two defi-

nitions of microsatellite instability to test the hypothesis that there might exist a class of tumours showing only mononucleotide repeat instability. Because all of the tumours designated d-MSI+, but only some of the tumours designated m-MSI+, would fall under the recently published definition of MSI-H,<sup>35</sup> we have generally not used the MSI-H designation in this study to avoid confusion.

### DETECTION OF MUTATION IN *MSH6*

Mutations in *hMSH6* were detected by amplifying individual exons and flanking intron sequences by PCR using the primers devised from the sequence of the *hMSH6* genomic locus (Genbank accession number U73732-U73737). Primer details are available on request. PCR set up was performed with a Tecan Genesis 100 Robotic Workstation and PCR was performed on Perkin Elmer 9600 and 9700 PCR instruments. The PCR products were sequenced on an ABI 377 sequencer (Perkin Elmer/Applied Biosystems) with standard M13 forward and reverse primers and Big Dye terminator chemistry using reagents obtained from Perkin Elmer/Applied Biosystems. In some cases it was necessary to resequence PCR products using primers that prime on internal regions of a PCR product. Analysis of the sequence chromatograms was carried out using Sequencher (Gene Codes Corp, Ann Arbor, MI) as well as by visually examining printed chromatograms to detect sequence changes and heterozygous nucleotides.

## Results

### YOUNG ONSET COLORECTAL CANCER: FREQUENCY AND CHARACTERISTICS OF TUMOUR MICROSATELLITE INSTABILITY

Eighty-three patients with colorectal cancer diagnosed before the age of 50 years were ascertained from a population based study and analysed for tumour MSI with dinucleotide microsatellite markers. An Amsterdam criteria family history of HNPCC was present in two patients. Seven of 83 (8.4%) patients had d-MSI+ tumours (defined as instability at two or more of five dinucleotide markers). This group with classical MSI comprised the two cases with HNPCC (Amsterdam criteria), one patient with a non-HNPCC family history, and four isolated cases. *MLH1* and *MSH2* mutation analysis was performed in the seven cases with d-MSI+ tumours and germline *MLH1* mutations were identified in the two HNPCC cases only.

Sixty-one of 76 cancers without d-MSI were analysed for mononucleotide repeat instability at two mononucleotide markers chosen from BAT25, BAT26, and BAT40. Microsatellite instability was detected in at least one of two mononucleotide markers (m-MSI+) in seven of 61 cases (11%). Four of these m-MSI+ cases were sporadic cases, while the remaining three came from patients whose families contained two first degree relatives with colorectal cancer but did not satisfy the Amsterdam criteria for HNPCC.

The clinicopathological features of d-MSI+, m-MSI+ only, and MSI- tumours were com-

Table 1 *hMSH6* polymorphisms

Polymorphism	Location	Allele frequency
A to G (Glu to Gly)	116 (exon 1, codon 39)	A=15% G=85%
C to A (silent)	186 (exon 1, codon 62)	C=75% A=25%
A to G (silent)	276 (exon 2, codon 92)	ND
C to T (silent)	540 (exon 3, codon 180)	ND
C to T (intron)	IVS3 (intron 3, -56)	C=92.5% T=7.5%
C to T (silent)	642 (exon 4, codon 214)	C=95% T=5%
A to T (intron)	IVS5 (intron 5, 14)	ND
A to G (intron)	IVS6 (intron 6, 145)	ND
Poly A expansion (intron)	IVS6 (intron 6, -4)	ND
Duplication of ATCT (intron)	IVS7 (intron 7, 31 to 34)	ATCT=60% ATCTATCT=40%
Deletion of a duplicated TTTTGTGTTTAATTCC (intron)	IVS7 (intron 7, -70 to -55)	ND
Insertion of A (intron)	IVS8 (intron 8, 52)	ND
C to G (intron)	IVS8 (intron 8, 54)	ND
Poly A expansion (intron)	IVS9 (intron 9-10)	ND

The location of each indicated change is given as either the cDNA coordinate of the nucleotide change with the affected codon in brackets or the intron number with the intron coordinates of the indicated change in brackets. The term "poly A expansion" refers to a run of As in two different introns where sequencing suggests that different length variants exist or are highly prone to stuttering so that it is not possible to sequence across them.

pared. There were no differences in mean (SD) tumour size (4.8 cm (1.6), 5.5 cm (2.3), and 4.2 cm (1.6) respectively), histological differentiation, or lymph node involvement. However, d-MSI tumours were significantly more frequently right sided in location than those without d-MSI+ (5/7 *v* 15/60). Tumours with m-MSI+ phenotype differed from d-MSI+ tumours by being mainly left sided (6/7 *v* 2/7). We postulated that m-MSI+ tumours might have *MSH6* mutations.

To enable accurate mutation analysis in patients with m-MSI+ tumours, we developed a set of primer pairs for PCR amplification and sequencing of the 10 exons and flanking intron sequences of *hMSH6*. Sequencing of the complete *MSH6* gene coding region was performed in the five m-MSI+ cases for whom peripheral blood DNA was available (two familial and three sporadic). A number of putative polymorphisms were characterised (table 1). These included a number of silent changes, one that caused a Glu to Gly substitution at codon 39 (exon 1), and a number of substitutions, insertions, and deletions in introns. In cases where these changes were suspected to affect either the function or expression of *MSH6*, their allele frequency was determined by analysing DNA from 20 normal controls. In all cases, the detected sequence change was sufficiently common to be considered a polymorphism. However, in one m-MSI+ case, we detected a heterozygous germline nonsense mutation (C642G, Tyr214Ter) (fig 1). Translation of the mutant sequence would be predicted to result in the synthesis of a truncated protein containing only the 213 N-terminal amino acids of the 1260 amino acid long *MSH6* protein. Functional studies in *Saccharomyces cerevisiae* have shown that this truncated protein would be inactive in mismatch repair (unpublished data). The proband presented at the age of 43 years with a left sided, moderately differentiated, 5 cm diameter adenocarcinoma of the large bowel. He was an only child with no family history of cancer. Attempts to determine if there was loss of the wild type *MSH6* allele in

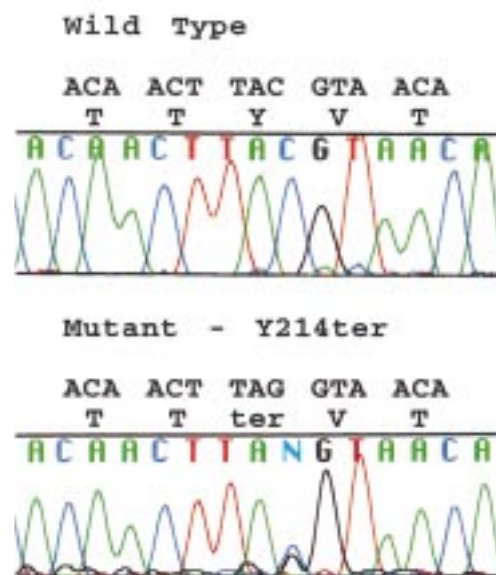


Figure 1 Detection of a germline *MSH6* mutation by sequence analysis. The upper panel shows the sequencing chromatogram from the region of exon 4 containing codon 214 from a wild type control DNA. The DNA and predicted protein sequence are shown above the chromatogram. The lower panel shows the sequencing chromatogram from the same region of a patient DNA sample containing a heterozygous nucleotide at nucleotide 642 of the cDNA sequence. The mutant DNA sequence and predicted mutant protein sequence are indicated above the chromatogram.

tumour tissues were uninformative owing to insufficient quantity and quality of tumour DNA.

## Discussion

The analysis of tumours for MSI using standard microsatellite markers provides a screening strategy for targeting patients with possible germline *MLH1* or *MSH2* mutations. Thus, the overwhelming majority of tumours in HNPCC kindreds with *MSH2* or *MLH1* mutations show MSI. However, the specificity of MSI analysis is reduced by phenocopy tumours in which both alleles of *MSH2* or *MLH1* were inactivated by somatic events. Early age at cancer diagnosis is a characteristic feature of inherited cancer susceptibility and a high frequency of MSI and germline *MSH2/MLH1* mutations has been described in patients with very early onset (<35 years) colorectal cancer.<sup>36</sup> However, the incidence of MSI and germline *MSH2/MLH1* mutations is low in moderately young cases (35–49 years)<sup>34 36 37</sup> suggesting the possible involvement of other genes in these cases. Germline *MSH6* mutations have not been reported in classical HNPCC kindreds, but the presence of a *MSH6* mutation in a Japanese HNPCC family which did not meet Amsterdam criteria led to a suggestion that the phenotype of germline *MSH6* mutations might differ from those of *MSH2* or *MLH1* mutations.<sup>33</sup> Thus there were no cases of early onset (<50 years) colorectal cancer in the Japanese family and extraintestinal (endometrium, ovary, and pancreas) cancers were prominent. A further germline

*MSH6* mutation was also reported from Japan in a 62 year old man with rectal cancer and two colonic adenomas, a weak non-HNPCC family history of gastrointestinal tumours, and MSI in the tumours.<sup>32</sup> These results are similar to those obtained with mutant mice where *MSH6* mutant mice developed cancer at later ages than either *MSH2* or *MLH1* mutant mice.<sup>31 38 39</sup>

Although it seems unlikely that the *MSH6* truncating mutation we identified could not be pathogenic, this possibility should be considered. However, we note that expression of an N-terminal fragment of yeast *MSH6* (that closely corresponds to what would be produced by the human mutant gene we describe) fails to complement the *MSH6* mismatch repair defect in in vitro studies (Das Gupta and Kolodner, unpublished data). Long term follow up of the proband and his children may provide further information on the pathogenicity and penetrance of this mutation. The finding of a germline *MSH6* mutation in an isolated case of young onset colorectal cancer further expands the phenotype of *MSH6* mutations and should prompt further studies of the role of these mutations in early onset colorectal cancer. Recently, Shin *et al.*<sup>40</sup> analysed Korean HNPCC families and 45 sporadic early onset (<40 years) cases for mutations of the mononucleotide repeat in the *MSH6* gene. They reported frameshift alterations in two of 44 suspected HNPCC cases in whom *MSH2* or *MLH1* mutations had not been detected, suggesting that *MSH6* mutations might be found in early onset cases. In this study we targeted these cases with m-MSI for *MSH6* mutation. Although this approach is logical, further research is required to determine if germline *MSH6* mutations can be associated with d-MSI or MSI negative tumours. The results of *MSH6* mice knockout studies would suggest that the latter possibility could occur. A germline *MSH6* mutation was only found in one of the five m-MSI+ tumours analysed. Although this finding clearly shows the potential significance of m-MSI, the four cases without *MSH6* mutations could result from somatic inactivation of *MSH6* or failure to detect a germline mutation (despite sequencing of the complete *MSH6* coding region). Alternatively, germline or somatic mutations in other mismatch repair genes could be implicated in this group; in familial colorectal cancer we found a single m-MSI+ tumour in a patient with a germline *MSH2* mutation (unpublished observation). Our findings and a report of germline *MSH6* mutations in Dutch familial colorectal cancer families<sup>41</sup> indicate a need for further studies of tumour MSI and germline *MSH6* mutation analysis in familial and isolated colorectal cancers.

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