Germline Mutations but Not Somatic Changes at the *MYH* Locus Contribute to the Pathogenesis of Unselected Colorectal Cancers

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***MYH*-associated polyposis is a recently described, autosomal recessive condition comprising multiple colorectal adenomas and cancer. This disease is caused by germline mutations in the base excision repair (BER) gene *MYH*. Genes involved in the BER pathway are thus good candidates for involvement in the pathogenesis of sporadic tumors of the large bowel. We have screened a set of 75 sporadic colorectal cancers for mutations in *MYH*, *MTH1*, and *OGG1*. Allelic loss at *MYH* was also assessed. Selected samples were screened for mutations and allele loss at *APC* and mutations in p53, K-ras, and β -catenin. A panel of 35 colorectal cancer cell lines was screened for *MYH* mRNA and protein expression. One of 75 cancers had bi-allelic germline mutations in *MYH* and on retrospective analysis of medical records this patient was found to have synchronous multiple small adenomas in addition to carcinoma. No somatic *MYH* mutations were found and mRNA and protein were expressed in all of our cell lines. There were no clearly pathogenic mutations in *MTH1* or *OGG1* in any tumor. Bi-allelic germline *MYH* mutations cause ~1 to 3% of unselected colorectal cancers, but appear always to be associated with multiple adenomas. Somatic inactivation of the DNA glycosylases involved in the BER pathway however does not appear to be involved in colorectal tumorigenesis. (*Am J Pathol* 2003, 162:1545–1548)**

cancer, have been shown to play important roles in the pathogenesis of sporadic cancers of the colon and of other sites. The *APC* gene, for example, is mutated in the germline of familial adenomatous polyposis patients and is involved in a majority of sporadic colorectal cancers; 1–3 the mismatch repair loci responsible for hereditary nonpolyposis colon cancer are mutated or silenced in ~10% of cancers of the colorectum; 4 and the transforming growth factor- β pathway member, *SMAD4*, causes juvenile polyposis and is mutated in ~20% of sporadic colorectal cancers. In contrast, the genes responsible for other Mendelian diseases that predispose to colorectal cancer appear to be rarely involved in the pathogenesis of sporadic bowel cancers. Examples include the Peutz-Jeghers hamartoma syndrome gene, *LKB1/STK11* 5 and the second juvenile polyposis gene, *BMPR1A/ALK3*. 6

Al Tassan and colleagues 7 recently found a previously unrecognized autosomal recessive condition comprising multiple colorectal adenomas and cancer. They reported that three affected siblings from a single UK family were compound heterozygotes for nonconservative sequence variants, Y165C and G382D, in the base excision repair (BER) gene *MYH*. 8-Oxo-G is a stable product of oxidative damage and it misrepairs readily with adenine residues resulting in an excess of G:C $\rightarrow$ T:A transversion mutations. Analysis of homologous variants in *Escherichia coli* revealed that the defective proteins had significantly decreased ability to repair 8-oxoG:A defects. Adenomas from the individuals carrying the Y165C and G382D germline sequence variants had a higher than expected incidence of G:C $\rightarrow$ T:A, somatic mutations in the *APC* gene, consistent with defective BER. We have recently confirmed these data in a large series of unrelated patients with multiple colorectal adenomas 8 and found that approximately one-third of patients with between 15 and ~100 adenomas had bi-allelic germline *MYH* mutations. We also screened the related BER genes, *MTH1* and *OGG1*, but found no pathogenic germline changes.

The BER pathway repairs mutations caused by reactive oxygen species that are generated during aerobic metabolism. 9 Oxidative DNA damage has been previ-

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The genes responsible for certain Mendelian diseases associated with colorectal cancer, such as familial adenomatous polyposis and hereditary nonpolyposis colon

Table 1. Patient Characteristics

Age
Range: 30 to 88 years
Median: age 66 years
Sex
45 males
32 females
Tumour site
63 left
14 right
Dukes stage
A—9
B—31
C—36

ously implicated in the etiology of degenerative diseases, aging, and cancer.¹⁰ Levels of 8-oxo-dG have been found to be significantly elevated in carcinomas of the breast,¹¹ lung,^{12,13} and kidney.¹⁴ Although no pathogenic *MYH* mutations have been found to date in sporadic forms of these cancers,¹⁵ mutations in *OGG1* have been found in human lung and kidney tumors.^{16,17} Thus, it appears that genes involved in the BER pathway are good candidates for involvement in the pathogenesis of sporadic tumors of the large bowel.

In this study, we screened 75 unselected, sporadic cancers of the colorectum for mutations in and allelic loss at *MYH*. Forty-eight of these cancers were also screened for mutations in *MTH1* and *OGG1*.

Materials and Methods

Frozen samples of 75 colorectal carcinomas from a sequential series were taken from colectomy specimens from St. Mark's Hospital, London. Histological review showed that all hematoxylin and eosin-stained sections taken contemporaneously from the same tumor consisted of >75% carcinoma. Cases with inflammatory bowel disease or a known Mendelian cancer syndrome were excluded. For each tumor, the following clinicopathological data were collected: patient age, Dukes stage, grade, and anatomical site in colon (Table 1). DNA was extracted from each specimen and paired normal bowel using standard methods.

Fluorescent single-strand conformational polymorphism analysis was performed to screen for variants in the coding regions and exon-intron boundaries of the *MYH*, *MTH1*, and *OGG1* genes. Oligonucleotides and reaction conditions used to amplify each fragment are available from the authors. Samples were run at 18°C and 24°C on the ABI3100 capillary sequencer. All tumors with bandshifts on fluorescent single-strand conformational polymorphism analysis were sequenced in forward and reverse orientations for that exon using a new, unlabeled polymerase chain reaction product, the ABI Big Dye Terminator Ready Reaction Mix (PE Applied Biosystems, Warrington, UK) and the ABI 377 semiautomated sequencer. All sequencing reactions were performed alongside the paired, normal DNA sample.

Loss of heterozygosity (LOH, allelic loss) analysis was performed at the microsatellite marker D1S2677 (located

~2.5 kb from the *MYH* locus) in the cancer samples, using standard protocols, dye-labeled oligonucleotides, and the ABI377 sequencer. Allelic loss was scored if the dosage of one allele in the tumor decreased by 50% or more relative to the other allele, after correcting for the relative allele peak areas using constitutional DNA. LOH was also assessed by direct inspection of sequence electropherograms for samples with mutations or polymorphisms within the *MYH* gene.

In selected samples, we screened for mutations in *APC* (exon 15 regions D-I), β -catenin (exon 3), and K-ras (exon 1) by direct sequencing (forward and reverse). p53 was screened by fluorescent single-strand conformational polymorphism analysis essentially as described above. The oligonucleotides and polymerase chain reaction conditions used are available from the authors. LOH at *APC* was assessed as above using the microsatellites D5S346 and D5S656.

A panel of 35 colorectal cancer cell lines (C10, C106, C32, C70, C75, C80, C84, C99, CACO2, COLO205, COLO320, COLO678, COLO741, GD2D, GP5D, H716, HCA46, HCA7, HCT8, HRA19, HT29, HT55, LOVO, LS123, LS174T, PC/JW, RKO, SKCO1, SW1222, SW1417, SW403, SW480, SW620, SW837, SW948) was screened for loss of MYH protein expression using Western blots and the muty11-second and muty12-second antibodies (Autogen Bioclear). The same cell lines were screened for *MYH* mRNA expression using reverse transcriptase-polymerase chain reaction (Muty-cdna oligonucleotides: forward primer, CAGAGGCTTTGAAGGC-TACC; reverse primer, TGCAGCATGACCTCTGAGAC).

Results and Discussion

In our series of 75 colorectal cancers, we found two pathogenic *MYH* mutations in a single tumor (no. 1351). These mutations were the previously reported G382D change and a novel 3-bp deletion (1395-7 delGGA) (Figure 1), which resulted in removal of a glutamic acid residue within a highly conserved region of the MYH protein. These two mutations were allelic and analysis of normal tissue from this patient showed both mutations also to be present in the germline. The patient presented at age 53 with a right-sided, moderately differentiated, Dukes B cancer. Retrospective analysis of the full medical records of this patient showed no reported family history of colorectal tumors, but did reveal the presence of multiple small adenomas synchronous with the carcinoma. The cancer showed no mutations in *APC*, β -catenin, or p53 and no LOH at D5S346 or D5S656. There was a K-ras codon 12 mutation, GGT→TGT, of a type in keeping with defective oxidative repair. This finding clearly supports the previous evidence that recessive germline *MYH* mutations cause multiple colorectal adenomas and cancer.

In two cancers, we found missense *MYH* variants, which were not known polymorphisms, present as heterozygotes with the wild type. In both cases, these variants were also present in the constitutional DNA. One of the changes (tumor no. 565) was a known variant,

Figure 1. Tumor 1351: 3-bp deletion (1395-7 del GGA).

R295C, reported to be of uncertain functional significance.⁸ The patient presented at age 66 with a left-sided, poorly differentiated Dukes B cancer. There was no known family history of colorectal tumors or report of synchronous or metachronous adenomas. There was no allelic loss at *MYH* in this tumor as assessed using the microsatellite D1S2677 or by inspection of the raw sequence. Screening for *APC* mutations in this cancer revealed a 2-bp deletion (4120-1 del AT) in exon 15G. There were no mutations found in p53 and a K-ras codon 12 mutation, GGT→GTT.

The other heterozygous *MYH* change (tumor no. 338) was a novel mutation, V61E. This change does not involve a conserved amino acid and the human mutant residue is actually the wild type in rat and mouse; it seems unlikely, therefore, that this variant has pathogenic effects. The patient presented at age 86 with a left-sided, moderately differentiated, Dukes C cancer. No personal history of multiple adenomas or any family history of note was reported. Although this patient was not informative at D1S2677, direct inspection of the raw sequence showed clear loss of the germline wild-type *MYH* allele in the cancer (Figure 2). Screening for *APC* mutations in this cancer revealed a G→T transversion at 1317 (E1317X) in exon 15G. There was no LOH at D5S346 or D5S656. No mutations were found in either K-ras or p53. Overall, allelic loss was uncommon close to *MYH*. Sixty-nine tumors were both informative and did not show microsatellite instability at D1S2677, of which 16 (23%) showed LOH.

The previously described *MYH* polymorphisms in exon 2 (V22 mol/L), exon 12 (Q324H),¹⁸ and exon 16 (S501F)⁷ were found with respective allele frequencies of 9%, 29%, and 1% in our patients. These variants were all present in both the constitutional and tumor DNA and

Figure 2. Tumor 338: loss of germline wild-type *MYH* allele (val→glu, V61E, T→A).

their frequencies were similar to those previously reported in controls.⁷

Although no somatic mutations were found in *MYH*, we also addressed the possibility that *MYH* mRNA or protein was lost from sporadic colorectal cancers by other mechanisms, such as transcriptional silencing. Although some subtle quantitative effects were not excluded we found that all 35 cell lines showed expression of *MYH* mRNA and protein.

No clearly pathogenic mutations were found in *MTH1* or *OGG1*. The previously described polymorphisms, V83 mol/L and D119D in *MTH1*¹⁹ and G308E²⁰ and S326C²¹ in *OGG1*, were found with respective allele frequencies of 1%, 15%, 1%, and 24%. It has been suggested that the S326C polymorphism plays an important role in the risk for smoking- and alcohol-related orolaryngeal cancer²² and is associated with an increased risk of lung cancer.²³ All of the polymorphisms found in this panel of tumors occurred at rates not significantly different from those found in the normal population,^{7,19,23} suggesting that, subject to the limitations of our sample size, they do not play a major role in the risk of colorectal cancer (details not shown).

Our data clearly support the existence of the recessive *MYH*-associated polyposis syndrome. It is interesting that our patient with bi-allelic *MYH* mutations had no detected *APC* mutations or LOH. Although we did not screen the entire *APC* gene, our data suggest the entirely plausible possibility that *MYH* mutations do not specifically lead to *APC* hypermutation, but can also lead to increased mutations of other, unknown genes involved in the pathogenesis of colorectal tumors. Based on an estimated frequency of 2 to 3% for pathogenic *MYH* alleles⁸ and a lifetime risk of colorectal cancer of 3 to 4%, we expect that ~1 to 3% of all colorectal cancers would result from *MYH* changes, a frequency that is higher than familial adenomatous polyposis and probably comparable with hereditary nonpolyposis colon cancer. Our finding that 1 of 75 unselected colorectal cancer patients has bi-allelic mutations is consistent with this expectation. Our data do indicate, however, that colorectal cancer in the absence of multiple adenomas is unlikely to result from bi-allelic *MYH* mutations, thereby emphasizing the need for accurate reporting of polyps synchronous with carcinoma.

There is little suggestion from our data that *MYH* mutant/wild-type heterozygotes are at an increased risk of colorectal cancer. Our V61E carrier's cancer had lost the wild-type *MYH* allele and had a somatic mutation at *APC*

typical of those associated with *MYH*-associated polyposis,⁷ but this was most likely to be coincidental. Given our findings, it appears unlikely that *MYH* mutations act as subpolymorphic, low penetrance susceptibility alleles as we proposed for *APC* E1317Q in colorectal tumors²⁴ and has recently been shown for *CHEK2* in breast cancers.²⁵ A large case-control study is however required to confirm that there is no increased risk in *MYH* heterozygotes.

We found no somatic *MYH* mutations (except a moderate frequency of LOH) and no absence of mRNA or protein. On the one hand, these findings are to some extent expected, because it is evident that a colorectal tumor will, by chance, usually acquire two *APC* mutations before it has acquired two *MYH* mutations and two *APC* mutations. *MYH*-associated polyposis and hereditary nonpolyposis colon cancer have a similar prevalence and both involve defective DNA repair of mismatched bases. It is therefore a little surprising given these parallels and that MLH1 function is lost in ~10% sporadic colorectal cancers^{26,27} that *MYH* appears to have no such role. Future analyses are, we suggest, likely to reveal that mismatch repair and oxidative repair genes predispose to bowel tumors through fundamentally different mechanisms.

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