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MUTATION IN BRIEF

Eight Novel *MSH6* Germline Mutations in Patients With Familial and Nonfamilial Colorectal Cancer Selected By Loss of Protein Expression in Tumor Tissue

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Germline mutations in mismatch repair (MMR) genes, predominantly in *MLH1* and *MSH2*, are responsible for hereditary nonpolyposis colorectal cancer (HNPCC), a cancer-susceptibility syndrome with high penetrance. In addition, *MSH6* mutations have been reported to account for about 10% of all germline mismatch repair (MMR) gene mutations in HNPCC patients, and have been associated with a later age of onset of the disease compared to *MLH1* and *MSH2* mutations. Here, we report eight novel germline mutations in *MSH6*. The patients were selected by having developed tumors with loss of MSH6 protein expression. All tumors showed high-level microsatellite instability (MSI-H). Seven mutations resulted in premature stop codons, comprised of two nonsense mutations (c.426G>A [p.W142X], c.2105C>A [p.S702X]), two insertions (c.2611_2614dupATTA [p.I872fsX10], c.3324dupT [p.I1109fsX3]) and three deletions (c.1190_1191delAT [p.Y397fsX3], c.1632_1635delAAAA [p.E544fsX26], c.3513_3514delTA [p.1171fsX5]). In addition, an amino acid substitution of an arginine residue (c.2314C>T [p.R772W]) conserved throughout a wide variety of mutS homologs has been found in a patient not fulfilling the Bethesda criteria for HNPCC. Our results emphasize the suitability of IHC as a pre-selection tool for *MSH6* mutation analysis and the high frequency of germline mutation detection in patients with MSH6-deficient tumors. In addition, our findings point towards a broad variability regarding penetrance associated with *MSH6* germline mutations. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC, MIM# 114500) is a highly penetrant, autosomal dominant cancer-susceptibility syndrome. Affected individuals are at increased risk for developing colorectal, endometrial, extracolonic gastrointestinal, ovarian and ureteral carcinoma and brain tumors (Lynch and de la Chapelle, 1999). A hallmark of most of these malignancies is the contraction/expansion of simple sequence motifs (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993), termed microsatellite instability (MSI). Disease-causing germline mutations in mismatch repair genes (*MSH2* [MIM# 120435; GenBank accession no. AH003235; GDB: 203983], *MLH1* [MIM# 120436; GenBank accession no. AH003234; GDB: 249617], *PMS2* [MIM# 600259; GenBank accession no. U13696; GDB: 386406] and *MSH6* [MIM# 600678; GenBank accession no. U54777; GDB: 632803]) have been described to date (reviewed in Peltomaki, 2001). The majority of germline mutations occurred in *MSH2* and *MLH1* (Liu et al., 1996; reviewed in Peltomaki and Vasen, 1997). At present, approximately 400 different mutations considered to predispose to HNPCC are listed in the HNPCC mutation database (http://www.nfdht.nl). Thirty-one of these mutations were assigned to *MSH6*, although 17 were clear-cut pathogenic in that they presented frameshift and nonsense mutations and mutations affecting the almost invariable intronic GT-AG splice site sequences. The average age at onset of the disease has been shown to be slightly higher in *MSH6* mutation carriers than in *MSH2* and *MLH1* mutation carriers, which might reflect a lower penetrance of *MSH6* mutations (Wagner et al., 2001; Berends et al., 2002). A preference for instability at mononucleotide repeats in MSH6-deficient tumors has been reported by some authors (Verma et al., 1999; Wijnen et al., 1999; Wagner et al., 2001; Plaschke et al., 2002) and is in accordance with recognition of base-base mispairs and insertion-/deletion loops (IDLs) of one base by the MutSα protein complex (MSH2+MSH6) (Acharya et al., 1996); however, this has not been confirmed by others (Wu et al., 1999; Berends et al., 2002). Here, we report eight novel germline mutations found in patients selected for loss of MSH6 expression in their tumor cells.

MATERIALS AND METHODS

We selected 26 patients from cohorts that were recruited through Bethesda guidelines for HNPCC (Rodriguez-Bigas et al., 1997). These cohorts are currently under analysis for MMR gene mutations and such mutations have been found and partially reported for *MLH1*, *MSH2* and *MSH6* (for example: Plaschke et al., Int J Cancer, 2002). In order to facilitate the identification of additional *MSH6* mutations, we selected patients who fulfilled all of the following criteria: 1) the patients had developed tumors that were either MSI-L (n=11) or MSI-H (n=15); 2) expressed MLH1 and MSH2 in their tumor cells, whereby MLH1 should have had no relative reduction in staining intensity when compared to non-tumorous cells; 3) no previous analyses for *MSH6* mutations in the germline and for MSH6 expression in the tumor; and 4) no identified *MLH1* or *MSH2* mutation, if analyzed. In addition, a series of 57 unselected colorectal cancer patients was analyzed for MSI and MMR protein expression. All analyzed patients were Caucasians. Written informed consent was obtained from the patients investigated.

Immunohistochemical staining was performed on 5-μm-thick formalin-fixed, paraffin-embedded tumor sections using mouse monoclonal antibodies for MSH6 (clone 44, Transduction Laboratories, Lexington, UK, 250 μg/ml, 1:50), MSH2 (clone FE11, Oncogene™ Research Products, Cambridge, Mass., 100 μg/ml, 1:100) and MLH1 (clone G168-15, PharMingen Int., San Diego, Calif., 1 mg/ml, 1:200) as described previously (Plaschke et al., 2002). Loss of expression in the tumor cells was considered solely when there was normal nuclear staining in adjacent non-neoplastic cells, which served as internal controls.

Analysis for microsatellite instability was performed on paired samples of lymphocyte DNA and paraffin-embedded or fresh-frozen tumor tissue. We applied the five markers of the reference panel (BAT25, BAT26, D5S346, D17S250, D18S58) according to the international guidelines for the evaluation of MSI in colorectal cancer (Boland et al., 1998) for all tumors. Additional mono- (BAT40, GTMSIn9, TGFβRIIpA) and dinucleotide (D2S123, D3S1300, D3S1619, D10S197) repeats were applied for some of the tumors as previously described (Dietmaier et al., 1997; Plaschke et al., 2000). Tumors were classified as highly unstable (MSI-H) if at least 30% of the markers showed instabilities.

Mutation analysis was performed on genomic DNA isolated from peripheral blood lymphocytes according to standard protocols. After PCR-amplification (Taq-polymerase, Applied Biosystems, Weiterstadt, Germany) all exons of *MSH6* including flanking intronic regions were directly sequenced using the Thermo Sequenase Fluorescent Cycle Sequencing kit and Automated Laser Fluorescence (A.L.F.express) sequencing devices (both

Amersham Biosciences, Freiburg, Germany) as described by Plaschke et al. (2000). Identified mutations were confirmed on at least two independent PCR products. Mutation nomenclature is according to Antonarakis et al. (1998) and den Dunnen and Antonarakis (2000).

Since the missense mutation identified in patient DD1 removed a restriction site of the endonuclease *AciI,* we digested PCR products of 95 healthy Caucasian controls with this enzyme to screen for the presence of this mutation in the normal population.

RESULTS AND DISCUSSION

Performing immunohistochemistry as a pre-screening method for mutation analysis of mismatch repair genes in patients with colorectal cancer, we identified eight tumors showing loss of MSH6 expression and retained expression of MSH2 and MLH1. Seven (47%) of the 15 MSI-H tumors of the selected Bethesda positive patients showed loss of MSH6 expression. One of these patients (DD2) fulfilled the strict Amsterdam criteria for HNPCC (Vasen et al., 1999). The patient and family histories of tumor disease are given in Table 1. Possible explanations for the remaining 8 (53%) MSI-H tumors are mutations within MLH1, MSH2 or MSH6 that affect protein function but not stability, or the involvement of other genes. None of the 11 MSI-L tumors showed loss of MSH6 expression. The eighth patient (DD1) was diagnosed with colon cancer at the age of 75 and was the only one who developed an MSH6-negative tumor among the series of 57 unselected colorectal cancers patients (Fig. 1). All tumors showed instability for at least 40% of markers tested and were classified MSI-H, whereby the tumors of 4 patients (DD1, DD2, HD1 and RG2) showed a prevalence of instability at mononucleotide markers (Table 2).

Table 1. Characteristics of the Families with Novel *MSH6* Germline Mutations

Patient	Gender	Tumor history of the index patient (age at diagnosis)	Affected relatives tumors (age at diagnosis)	Bethesda Criterion
BN1	M	ascending colon (31)	father: prostate (76) mother: breast (66) maternal aunt: breast (52)	B4
HD1	F	lymphoma (44), ascending colon (55), endometrial (57)	maternal aunt: colorectal (75), gynecological (~60) maternal grandfather: gastric (~60) paternal aunt: gynecological (~60) paternal aunt: gynecological (~75)	B2
HD2	M	cecal colon (40)	maternal grandmother: colorectal (~50), kidney (~75) maternal uncle: brain (58) maternal aunt: breast (unknown)	B4
DD1	F	ascending colon (75)	none	none
DD2	F	rectal (51)	brother: cecal colon (47) father: rectal (54) niece: lymphoma (28)	Amst
RG1	F	ovarian (54), ascending colon (55)	twin sister: endometrial (52) mother: gastric (52), breast (54) maternal aunt: breast (66) gastric (69) maternal aunt: endometrial (73) maternal uncle: brain (~70) father: colon (73) paternal uncle: prostate (73)	B2
RG2	M	sigmoid colon (33)	none	B4
UK1	F	ascending colon (36), ovarian (45) endometrial (45)	mother: ampullary (78); maternal grandmother: liver metastases (45); father: gastric/esophageal (69)	B2, B4

(Amst: Amsterdam; B: Bethesda; ~: exact age of onset of the disease is not known)

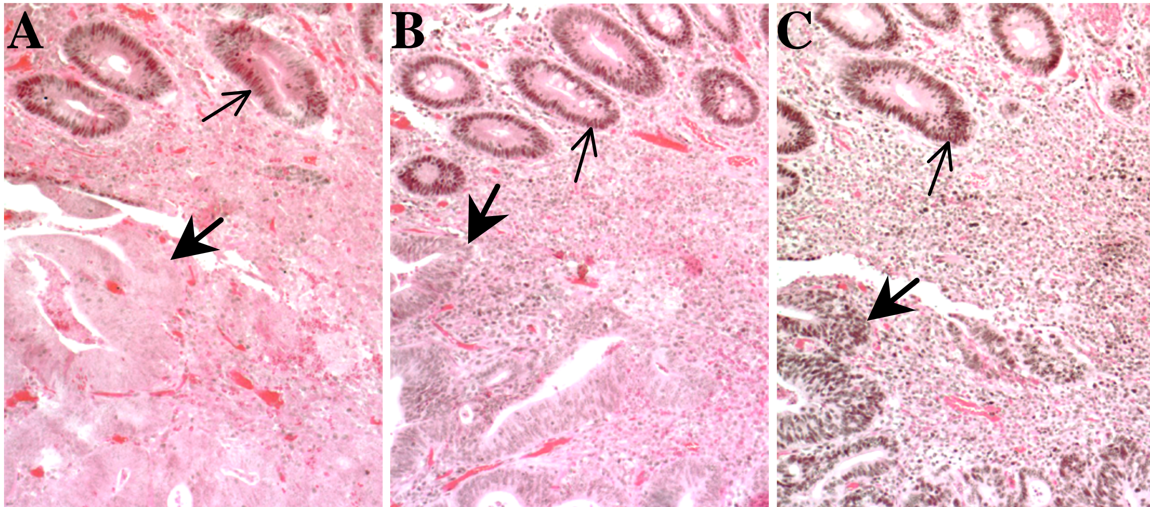

Figure 1. Immunohistochemical staining of colon tumor sections from patient DD1, harboring the MSH6 missense mutation R772W. The tumor showed: **A:** loss of MSH6 expression; **B:** reduced expression of MSH2, as reported for MSH6-negative tumors (Plaschke et al., 2002); and **C:** normal expression of MLH1. Bold arrows (➔) mark tumor cells, normal arrows (→) mark normal epithelial crypts.

The coding region of *MSH6* was sequenced from genomic DNA of these 8 patients. Germline mutations were identified in all patients (Table 2). The mutations are not reported in the HNPCC database (http://www.nfdht.nl) and, to our knowledge, have not been described in the literature. Seven mutations resulted in premature stop codons comprising two nonsense mutations, two insertions and three deletions. The eighth mutation was a C>T transition at nucleotide 2314, identified in patient DD1. This mutation was not found in 95 healthy controls. Sequencing of the complete coding region of *MSH6* for this subject did not reveal any sequence alterations other than previously described common polymorphisms (Plaschke et al., 2000). The mutation results in a substitution of arginine at codon 772 by tryptophan. The Arg772 residue is conserved for structural integrity between the *mutS* homologs of man, yeast (*Saccharomyces cerevisiae*), worm (*Caenorhabditis elegans*), plant (*Arabidopsis thaliana*) and bacteria (*Escherichia coli, Thermus aquaticus, Streptococcus pneumoniae*) (Culligan and Hays, 2000; Obmolova et al., 2000). The mutation of the respective arginine residue in the *mutS* gene of *E. coli* (R305) has been shown to cause a dominant negative mutator effect when expressed from a multicopy plasmid in a *mutS* wild type background (Wu and Maurinius, 1994). The results obtained in bacteria might not directly compare to humans, because the bacterial mutS protein forms a homodimer, whereas several mutS homologs have been evolved in humans functioning in hetereodimers. Therefore, *MSH6* mutations still leave the MutSβ complex (MSH2+MSH3) unaffected, and this protein complex has also been shown to be involved in MMR (Acharya et al., 1996; Palombo et al., 1996). The same c.2314C>T mutation was found as somatic hit in an endometrial tumor of a patient with an *MSH6* germline mutation (Goodfellow et al., 2003). Additional support for the functional impairment of *MSH6* upon substitution of the conserved R772 residue might be taken from somatic point mutations affecting a different nucleotide but the same amino acid residue (c.2315G>A [p.R772Q]), being identified in a colon and a gastric cancer, both with the MSI-H phenotype (Ohmiya et al., 2001). Although, different substitutions of the same amino acid can be functional and non-functional, respectively.

Due to lack of known family histories of HNPCC-related tumors in patients BN1, DD1 and RG2, it cannot be ruled out that these patients have *de novo* mutations. The R772W missense mutation was identified in a patient with late onset of the disease, which might be interpreted as a reduced penetrance of this missense mutation, compared to protein truncating mutations. On the other hand, the respective tumor showed complete loss of protein expression. Therefore, protein depleting *MSH6* mutations may be associated with a broad variability in penetrance. The majority of *MSH6* germline mutations reported here and in the literature has been identified in patients who do not fulfill the strict Amsterdam criteria for HNPCC (Kolodner et al., 1999; Wijnen et al., 1999; Wu et al., 1999; Berends et al., 2002). Moreover, we identified *MSH6* germline mutations in patients that were even not suspected for HNPCC (Plaschke et al., 2002). An increased frequency of endometrial carcinomas, the second most common

tumor entity in HNPCC, has been shown to be associated with *MSH6* germline mutations, compared to *MLH1* and *MSH2* mutations (Wijnen et al., 1999). A recent study on unselected endometrial carcinomas also identified germline mutations in patients without a family history of cancers suggestive of HNPCC (Goodfellow et al., 2003). The identification of *MSH6* germline mutations in patients not selectable by anamnestic data on disease histories indicates that some patients and their relatives would benefit from *MSH6* screening of all colorectal and endometrial tumors.

Table 2. Novel *MSH6* Germline Mutations

Pat.-ID	Exon	Codon	Nucleotide Change[1] (c.)	Consequence (p.)	MSI[2] Mono	MSI Di
DD2	2	142	426G>A	W142X	3/4	3/7
RG2	4	397	1190_1191delAT	Y397fsX3	2/2	1/3
HD2	4	544	1632_1635delAAAA	E544fsX26	2/2	3/3
UK1	4	702	2105C>A	S702X	2/3	1/3
DD1	4	772	2314C>T	R772W	4/5	3/7
HD1	4	872	2611_2614dupATTA	I872fsX10	2/2	0/3
RG1	5	1109	3324dupT	I1109fsX3	2/2	3/3
BN1	6	1171	3513_3514delTA	D1171fsX5	2/2	0/1

([1] according to GenBank accession no. U54777 version 2; nucleotide numbering starts with the A of the start codon)
([2] MSI: microsatellite instability; Mono: mononucleotide repeat marker; Di: dinucleotide repeat marker)

The germline mutations reported here are scattered throughout the gene, which is in line with the data on *MSH6* mutations deposited in the HNPCC mutation database and reported in the literature (Kolodner et al., 1999; Wijnen et al., 1999; Wu et al., 1999; Berends et al., 2002; Plaschke et al., 2002). Our results emphasize the suitability of IHC for the selection of patients to be included in *MSH6* mutation analysis. To our knowledge, no solely somatic *MSH6* inactivation has been reported in MSH6-negative tumors, not deficient for MLH1 or MSH2. Together with previously reported findings (Plaschke et al., 2002), our data indicate, that high frequencies of germline mutation detection can be achieved in patients with MSH6-deficient tumors, even when including patients that do not fulfill Bethesda guidelines for HNPCC.

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