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ARTICLE

No association between *MUTYH* and *MSH6* germline mutations in 64 HNPCC patients

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Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant tumour predisposition syndrome caused by germline mutations in mismatch repair (MMR) genes. In contrast to *MLH1* and *MSH2*, germline mutations in *MSH6* are associated with a milder and particularly variable phenotype. Based on the reported interaction of the MMR complex and the base excision repair protein MUTYH, it was hypothesised that *MUTYH* mutations serve as phenotypical modifiers in HNPCC families. Recently, a significantly higher frequency of heterozygosity for *MUTYH* mutations among *MSH6* mutation carriers was reported. We examined 64 *MSH6* mutation carriers (42 truncating mutations, 19 missense mutations and 3 silent mutations) of the German HNPCC Consortium for *MUTYH* mutations by sequencing the whole coding region of the gene. Monoallelic *MUTYH* mutations were identified in 2 of the 64 patients (3.1%), no biallelic *MUTYH* mutation carrier was found. The frequency of *MUTYH* mutations was not significantly higher than that in healthy controls, neither in the whole patient group ($P = 0.30$) nor in different subgroups regarding mutation type. Our results do not support the association between *MSH6* mutations and heterozygosity for *MUTYH* mutations.
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Introduction

The clinical variability observed in autosomal dominant cancer predisposition syndromes may be explained by modifying genes, environmental factors and stochastic effects. In conditions characterised by a variable penetrance, inconsistent manifestation and often sporadic presentation, combination of low-penetrant germline mutations in two or more functionally related genes may aggravate the phenotype and affect the tumour spectrum, exceeding the threshold level to medical attention.

Germline mutations in the MMR genes *MLH1*, *MSH2*, *MSH6* and *PMS2* are known to cause hereditary non-polyposis colorectal cancer (HNPCC/Lynch syndrome), an autosomal dominantly inherited tumour predisposition syndrome associated with colorectal and endometrial cancer and several other extracolonic malignancies.[1] Mutations in the *MSH6* gene account for about 10–15% of all HNPCC germline mutations. The gene products of *MSH6* and *MSH2*

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form the MutSα heterodimeric protein complex, which recognises base–base mismatches and small insertion–deletion loops and initiates the repair by excising the mispaired base.[2] Thus, mutations in *MSH6* decrease the capacity of the MMR system, resulting in augmentation of somatic mutations in tumour suppressor genes and oncogenes.[3] However, the penetrance of MSH6 mutations is lower compared to germline mutations of the MMR genes *MLH1* and *MSH2*. *MSH6* mutation carriers tend to have a later age of onset and a lower incidence of colorectal cancer; the families of these patients often neither fulfil the Amsterdam criteria for HNPCC nor follow the typical autosomal dominant pattern of inheritance.[4] This raises the question whether the *MSH6* mutation alone or in combination with additional inherited susceptibility factors is responsible for the increased tumour risk observed in these patients.

Some aspects of the phenotype of HNPCC have already been associated with variants in other modifying genes, in particular apoptosis-related genes.[5–7] In 2002, Gu *et al*[2] reported an interaction between MSH6 and the base excision repair (BER) protein MUTYH, a DNA glycosylase involved in the repair of oxidative DNA damage. The authors showed that the MSH2/MSH6 complex enhances the binding affinity of MUTYH to the mismatched DNA substrate and the glycosylase activity of MUTYH. Biallelic mutations in *MUTYH* are known to cause MUTYH-associated polyposis, an autosomal recessive precancerous condition of the colorectum.[8–13] The heterozygote frequency in the general population is estimated to be 1–2%.[9,12,14] The risk for colorectal cancer in heterozygote *MUTYH* mutation carriers has been discussed controversially but is apparently low.[15–18]

Due to the functional interaction of the MUTYH protein with the MMR system, it was hypothesised that monoallelic *MUTYH* germline mutations affect the phenotype of HNPCC patients and, in particular, contribute to cancer susceptibility in carriers of an *MSH6* mutation. Recently, Niessen *et al*[19] reported a significantly higher rate of monoallelic *MUTYH* mutations in a group of 20 *MSH6* missense mutation carriers compared to carriers of either *MLH1* or *MSH2* mutations or to healthy controls and concluded that the interaction of mutations in *MSH6* and *MUTYH* may lead to an increased risk for colorectal cancer.

To further examine the suspected interaction of mutations in both genes, we sequenced the complete coding region of the *MUTYH* gene in 64 *MSH6* mutation carriers recruited by the German HNPCC Consortium and compared the frequency of *MUTYH* mutations among these patients with the frequency of that among healthy controls.

Materials and methods

Patients

The patients included in the study were recruited from six German university hospitals as described.[20] Briefly, in all centres, patient ascertainment, data analysis and documentation were carried out in accordance with the common study protocol. Patients were referred to the study from other hospitals, institutes of human genetics, private practice physicians and private practice human geneticists or came by self-referral. Patients who were enrolled in the study had to fulfil either the Amsterdam criteria II,[21] the original Bethesda guidelines[22] or the revised Bethesda guidelines.[23] Histopathological analysis was controlled at the central reference pathology unit (Department of Pathology, University of Bonn).

We included 64 unrelated index patients from the German HNPCC Consortium who had been examined for germline MMR gene mutations according to the study protocol (see below) and had been found to harbour an *MSH6* germline mutation. Patients with pathogenic mutations in *MLH1* or *MSH2* were not included, but 11 patients were found to have an additional mutation of unknown pathogenic relevance in *MLH1* (nine probands) or *MSH2* (two probands). (For details see Supplementary Table 1). Of the 64 patients, 42 had a pathogenic *MSH6* mutation (frameshift mutation, nonsense mutation or splice-site mutation) and 22 patients had a mutation of unknown pathogenic relevance in *MSH6* (19 missense mutations and 3 silent mutations). Ten patients fulfilled the Amsterdam II criteria, 45 patients met the original Bethesda guidelines and 9 patients the even less stringent revised Bethesda guidelines.[21–23] All patients gave their written informed consent authorising data documentation, examination of tumour tissue for HNPCC characteristics and molecular genetic analysis of genes associated with HNPCC. The study was approved by the ethical committees of all participating clinical centres.

Immunohistochemical staining of MMR proteins

Immunohistochemistry (IHC) for the MMR proteins MLH1, MSH2 and MSH6 was performed according to the study protocol as described previously.[20,24] The level of protein staining in the tumour cells was compared to the protein expression in normal tissue. Tumours were scored to exhibit loss of expression of a repair protein if the nuclei showed no or only very weak immunostaining in comparison to normal tissue.

DNA extraction

Genomic DNA was extracted from peripheral EDTA-anticoagulated blood samples, according to a standard salting-out procedure. With small DNA samples, whole genome amplification was performed using the GenomiPhi DNA amplification kit by GE Healthcare (Chalfont St Giles, Great Britain, UK), according to the recommendations of the manufacturer. The coding sequence of *MSH6* was amplified and sequenced from genomic DNA as described previously.[24,25]

Microsatellite analysis
Analysis for microsatellite instability (MSI) had been performed on matched pairs of tumour DNA and normal DNA using the National Cancer Institute/International Collaborative Group on HNPCC (NCI/ICG-HNPCC) reference marker panel for the evaluation of MSI in colorectal cancer (BAT25, BAT26, D2S123, D5S346 and D17S250). Tumours were scored as highly unstable (MSI-H) if two or more of these five markers exhibited additional alleles, and as stable (MSS) if none of the five markers showed instability. If only one marker showed instability, an additional panel of five markers (BAT40, D10S197, D13S153, MYCL1 and D18S58) was examined. In these cases, the tumour was classified as MSI-H if 3 or more of the 10 markers showed instability, and as low unstable (MSI-L) if only one or two markers showed additional alleles. MSI-H tumours were found in 6 of the Amsterdam-positive patients, 35 of the patients meeting the Bethesda criteria and 6 of the patients fulfilling the revised Bethesda criteria.

Mutation analysis of the *MSH6* gene
In general, mutation analysis of the *MSH6* gene was performed according to the study protocol if the patient's tumour tissue showed an isolated loss of MSH6 in the immunohistochemical staining or – when no tumour tissue was available – if the patient's family met the Amsterdam criteria and no pathogenic germline mutation in *MLH1* or *MSH2* was found. Individual patients were examined for other reasons at the discretion of the respective centres. Mutations were either categorized as pathogenic (frameshift mutations, nonsense mutations, splice site mutations and one deletion of two exons), unclassified variants (missense mutations and silent mutations, that have not been functionally tested so far and are not described in the literature as polymorphisms) or polymorphisms (if described in the databases and literature; known polymorphisms are not shown in the Supplementary Table 1). The mutations in our 64 patients are distributed over the whole *MSH6* gene. An apparent clustering of mutations in exon 4 can be explained by the size of this exon containing more than one-third of the *MSH6* cDNA.

Screening for germline mutations in the *MUTYH* gene
Screening for *MUTYH* mutations was performed by amplifying and sequencing the whole coding region and the flanking exon–intron boundaries of the *MUTYH* gene as described previously.[10] We applied the description of the coding sequence used by Al-Tassan *et al*[8] (GenBank: U63329.1) for mutation description and not the actual reference sequence of the coding *MUTYH* sequence (GenBank: NM_012222.1). All mutations were confirmed by a second independent PCR.

***MUTYH* mutation frequency in Caucasian controls**
Data on the *MUTYH* mutation frequency in the general population were taken from three published studies in which large groups of normal Caucasian controls had been screened for mutations in the whole coding region of the *MUTYH* gene.[9,12,14] One of the three control groups encompasses 116 German probands (healthy blood donors).[14] In all 577 control individuals, 9 monoallelic *MUTYH* mutations were identified (some of them of unknown functional relevance), indicating a frequency of around 1.6% in the general population. In the German control group, monoallelic *MUTYH* mutations were found in two patients (1.7%).

Statistical analysis
The statistical comparison (frequency of monoallelic *MUTYH* mutations in the different subgroups of our patients) was performed using Fisher's exact test for categorical variables. A *P*-value of <0.05 was considered to be statistically significant.

Results
We sequenced the whole coding region of the *MUTYH* gene in 64 carriers of an *MSH6* germline mutation and identified two monoallelic *MUTYH* mutations (3.1%). No patient harboured a biallelic *MUTYH* mutation. The frequencies of the previously reported *MUTYH* polymorphisms were consistent with published data.[9,11,12] The inclusion criteria, results of the tumour tissue examination and mutation analysis are summarised in Table 1.

The patient with the pathogenic *MSH6* mutation, c.3324_3325insT;p.Ile1109TyrfsX3, harboured the truncating *MUTYH* mutation c.247C>T;p.Arg83X that had been described earlier.[9] The patient had been diagnosed with ovarian cancer at the age of 54 and with colorectal cancer at the age of 55 years and, therefore, met the Bethesda criteria. Her tumour tissue showed MSI-H and a nuclear loss of the MSH6 protein. No other family members could be investigated.

The second patient with the silent *MSH6* mutation, c.1770C>T;p.Pro590, of unknown functional relevance carried the *MUTYH* missense mutation c.502C>T; p.Arg168Cys that leads to an amino-acid exchange in an evolutionarily conserved residue, suggesting pathogenicity.[11] She had been diagnosed with an MSS rectal cancer at the age of 51 years, and her family history met the Amsterdam criteria. Her tumour tissue was positive for all tested MMR proteins in immunohistochemical staining. In addition, the patient harboured the *MLH1* missense mutation c.2146G>A;p.Val716Met, which is suspected of being a rare polymorphism.[26–28] Unfortunately, no other family members were available for further investigation.

Thus, one of the two *MUTYH* mutations was found in the 42 patients harbouring a pathogenic (truncating) *MSH6*

Table 1 Mutation status and results of MSA and IHC in 64 *MSH6* mutation carriers according to clinical HNPCC criteria

HNPCC inclusion criteria	*MSH6 mutation*		*MSI-H tumours*	*Loss of MSH6 in IHC*	*Unclassified variant in MLH1 or MSH2*	*MUTYH mutation*
Amsterdam	Pathogenic mutation	6	5	5	2	0
	Unclassified missense mutation	2	0	1	1	0
	Unclassified silent mutation	2	1	0	1	1
Bethesda	Pathogenic mutation	29	23	22	4	1
	Unclassified missense mutation	15	12	6	2	0
	Unclassified silent mutation	1	0	0	0	0
Revised Bethesda	Pathogenic mutation	7	6	7	0	0
	Unclassified missense mutation	2	0	0	1	0
	Unclassified silent mutation	0	0	0	0	0

mutation (2.4%) and the other in one of the three patients with a silent *MSH6* mutation. No *MUTYH* mutation occurred in the 19 patients with an *MSH6* missense mutation. (For clinical and molecular genetic details, see Supplementary Table 1). Regarding the clinical inclusion criteria, one *MUTYH* mutation was found among the 10 patients whose family history met the Amsterdam criteria (10.0%), and one in the group of 45 patients fulfilling the Bethesda criteria (2.2%). No *MUTYH* mutation was found in the nine patients meeting the revised Bethesda criteria.

The heterozygote frequency in the general population was estimated to be around 1.6%, based on the published results of a complete *MUTYH* screening among 577 normal Caucasian controls.[9,12,14] Hence, the frequency of heterozygous *MUTYH* mutations among our *MSH6* mutation carriers did not differ significantly from the frequency observed in healthy individuals, neither in the whole cohort of 64 patients ($P=0.30$) nor in different subgroups (pathogenic *MSH6* mutations, $P=0.51$; *MSH6* missense mutations, $P=0.75$). In contrast to Niessen *et al*,[19] we found no increased frequency of *MUTYH* mutations among our *MSH6* missense mutation carriers, although the difference between both studies was not significant ($P=0.106$). Based on our sample sizes, the statistical power of our study to detect the same difference in the frequencies of *MUTYH* mutations between *MSH6* missense mutation carriers and normal controls as seen by Niessen *et al*[19] (ie 20 *vs* 1.5%) was 79%. If all *MSH6* mutation carriers were considered (64 patients), the power was >99%.

Discussion

It is a reasonable hypothesis to assume that the combination of low-penetrant germline mutations in two or more functionally related genes explains the phenotype variability in hereditary tumour predisposition syndromes characterised by an incomplete penetrance (and sporadic appearance) like that in *MSH6* related HNPCC families.

Interactions between MSH6 and the BER protein, MUTYH, were first described by Gu *et al*.[2] These authors could demonstrate that the MSH2/MSH6 heterodimer stimulates the DNA binding and glycosylase activities of MUTYH to misincorporated adenines opposite 8-OxoG. Interestingly, they found a physical interaction between MUTYH and MSH6 that was substantially decreased in MT1 cell lines with compound heterozygous *MSH6* missense mutations (Asp1213Val and Val1260Ile) in the C-terminal region of MSH6. These cells are deficient for base–base mismatch repair.

Since the interaction of the MMR system and the BER system had been reported, two studies were undertaken to investigate a possible modifying effect of *MUTYH* mutations on the course of disease in HNPCC patients. Ashton *et al*[29] could not find a higher frequency of *MUTYH* mutations among *MLH1* and *MSH2* mutation carriers (the type of mutation was not reported) and mutation-negative HNPCC patients, compared to each other and to normal controls: they screened 442 HNPCC patients for the presence of the two common *MUTYH* mutations, Y165C and G382D, and identified monoallelic *MUTYH* mutations in two of the 209 *MLH1* or *MSH2* mutation carriers (all fulfilled the Amsterdam or the Bethesda criteria). In the group of 233 mutation-negative HNPCC patients, 3 harboured a monoallelic and 2 biallelic *MUTYH* mutations.

Niessen *et al*[19] examined 76 carriers of MMR germline mutations (25 with mutations in *MLH1*, 26 in *MSH2* and 25 in *MSH6*) for *MUTYH* variants. Of 20 patients carrying a *MSH6* missense mutation, 4 (20%) were found to have a monoallelic *MUTYH* mutation. The proportion was significantly increased compared to normal controls (1.5%; $P=0.001$) and to a group of 134 CRC patients without an MMR mutation (0.7%; $P=0.002$). The frequency of *MUTYH* mutations among the *MLH1* and *MSH2* missense mutation carriers and among the carriers of truncating mutations in the MMR genes was not significantly different from the frequency in the healthy controls. In summary, the combined results of the two studies indicate that an interaction of monoallelic *MUTYH* and

MMR germline mutations might be relevant only in *MSH6* missense mutation carriers, but not in the more penetrant *MLH1*, *MSH2* and truncating *MSH6* mutations.

To identify a potential interaction between mutations of the two genes, we screened 64 *MSH6* mutation carriers of the German HNPCC Consortium for *MUTYH* mutations and found two monoallelic mutations among all patients. In contrast to the findings of Niessen *et al*,[19] no *MUTYH* mutation was identified in the 19 carriers of an *MSH6* missense mutation. Compared to the general population, the frequency of *MUTYH* mutations was not increased in our sample, neither in the whole group of 64 patients nor in different subgroups regarding mutation type.

One explanation for the divergent findings might be a different composition of the two patient groups. In fact, both samples seem to vary in certain aspects, but are similar in others. The clinical inclusion criteria for HNPCC diagnostics mentioned by Niessen *et al*[19] are comparable to those of the German HNPCC consortium (Amsterdam or Bethesda), suggesting no relevant ascertainment bias between the groups, at least in the majority of patients. Since neither the 20 *MSH6* missense mutations nor the IHC and MSI results are described in detail by Niessen *et al*,[19] the degree of correlation between the two groups cannot be determined exactly. The diverse proportion of *MSH6*-truncating and missense mutations in both studies (5/20 *vs* 42/19) may indicate a difference in the spectrum of *MSH6* missense variants. However, two of the three different *MSH6* missense mutations published by Niessen *et al*[19] (all had a *MUTYH* mutation) were also identified in our patients (c.1186C>G;p.Leu396Val; c.431G>T;p. Ser144Ile), indicating a substantial overlap.

Notwithstanding, in the majority of our *MSH6* missense mutation carriers (13/19), mutation analysis in *MSH6* was performed only if the patient's tumour tissue was MSI-H or showed a loss of MSH6 in IHC whereas all tumours of the four *MSH6/MUTYH* mutation carriers examined by Niessen *et al*[19] showed normal IHC staining for MSH6 and only one tumour tissue was MSI-H. According to these results of Niessen *et al*,[19] a combined *MSH6/MUTYH* mutation might be relevant only in case of low-penetrant *MSH6* missense mutations, which neither alter IHC nor microsatellite stability. As a consequence, the standard diagnostic criteria used for *MSH6* mutation analysis (MSI-H, IHC) would be inappropriate to detect the patients identified by Niessen *et al*.[19]

It cannot be ruled out that the difference in the frequency of *MUTYH* mutations between the German and the Dutch patients occurred by chance due to the limited number of *MSH6* missense mutation carriers. It will be difficult to recruit much larger cohorts of *MSH6* missense mutation carriers in the near future; however, further studies are needed to clarify this issue.

In conclusion, according to our results, the frequency of *MUTYH* mutations that was identified in a large cohort of *MSH6* mutation carriers who fulfilled the clinical criteria for HNPCC is not increased compared to normal controls. The over-representation of heterozygous *MUTYH* mutations in *MSH6* missense mutation carriers reported by Niessen *et al*[19] remains to be explained.

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Appendix

The German HNPCC-Consortium consists of the following centres (in alphabetical order): clinical centres in Bochum (in addition to authors: F Brasch, JT Epplen, S Hahn, C Pox, S Stemmler, A Tannapfel and J Willert), Bonn (in addition to authors: S Uhlhaas, M Sengteller, W Friedl, N Friedrichs and R Buettner), Düsseldorf (in addition to authors: B Betz, T Goecke, G Möslein and C Poremba), Dresden (in addition to authors: DE Aust, F Balck, A Bier, R Höhl, FR Kreuz, SR Pistorius and J Plaschke), Heidelberg (in addition to authors: F Cremer, M Keller, P Kienle, HP Knaebel, M von Knebel-Doeberitz, U Mazitschek and M Tariverdian), München/Regensburg (in addition to authors: A Laner, B Schönfeld, E Holinski-Feder, H Vogelsang, R Langer, S Dechant and P Rümmele) and centre for documentation and biometry in Leipzig (in addition to authors: M Loeffler, M Herold, U Enders and J Schaefer).

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