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Assessment of pathogenicity criteria for constitutional missense mutations of the hereditary nonpolyposis colorectal cancer genes *MLH1* and *MSH2*

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To determine the role played by *MLH1* and *MSH2* missense variants in cancer susceptibility, we have investigated the following genetic and biological characteristics associated with six *MLH1* and four *MSH2* missense changes identified in Italian hereditary nonpolyposis colorectal cancer (HNPCC) families: co-segregation with disease phenotype and/or *bona fide* pathogenetic mutations; presence of the variant in healthy control subjects; evolutionary conservation of the involved aminoacid and type of aminoacid change; and presence/absence of microsatellite instability (MSI) in tumour DNA. Overall, nine variants did not fulfil ≥ 2 pathogenicity criteria. MSI was investigated in tumour samples from carriers of nine different missense mutations. Only 3/9 variants were associated with MSI in tumour DNA. In addition, four variants were not present in affected pedigree members, and five variants were observed in the control population. Based upon these results, we conclude that most *MLH1* and *MSH2* missense changes are unlikely to act as major causative factors in colorectal cancer susceptibility and development.

Keywords: mismatch repair; HNPCC; microsatellite instability; mutation; cancer susceptibility

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome is frequently caused by mutations

of genes involved in the DNA mismatch repair (MMR) apparatus.^{1,2} So far, HNPCC-associated constitutional mutations have been identified in the MMR genes *MLH1*, *MSH2*, and to a lesser extent *PMS1*, *PMS2*, and *MSH6*.³ Approximately 90% of *MSH2* and 70% of *MLH1* mutations lead to the production of significantly shorter polypeptides, whose biological activity is deemed to be abolished or substantially reduced.⁴ The remaining fraction of *MLH1* and *MSH2* molecular changes identified in HNPCC is essentially comprised

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of single aminoacid substitutions, whose functional significance is doubtful.

Analysis of genetic and biological data may provide useful hints about the actual pathogenic role of missense substitutions. Several criteria have recently been pointed out which may be helpful to this purpose.⁵ These include:

- 1) analysis of the results of segregation studies;
- 2) investigation of the entire coding sequence(s) in order to ascertain/exclude the presence of additional mutations;
- 3) population studies aimed at defining whether the mutation can be identified in control chromosomes;
- 4) relative importance of the aminoacid involved, as judged by the degree of evolutionary conservation among distant species;
- 5) type of aminoacid change (conservative versus non-conservative).

In addition, for MMR genes, the presence/absence of microsatellite instability (MSI)⁶ in tumour DNA represents an additional pathogenicity criterion and in fact a very important one since it can be considered as a sort of *in vivo* biological assay aimed at determining the functional status of the MMR machinery.

Most published reports of MMR mutational studies in HNPCC describing missense changes provide no or only partial information on these aspects. During the course of molecular investigation of *MLH1* and *MSH2* constitutional mutations, we have identified 10 different missense substitutions which do not represent well recognised polymorphisms. Therefore, in order to gain information on the clinical relevance of these variants, we undertook an analysis of the above-mentioned characteristics.

Materials and Methods

Patients and Controls

The clinical and familial features of the study population, which included a total of 128 unrelated probands, have been previously described.^{7,8} Briefly, the probands tested were subdivided into four different groups, based on clinical data and family history:

- 1) HNPCC patients, as defined according to the Amsterdam diagnostic criteria;⁹
- 2) suspected HNPCC patients, affected with colorectal cancer (CRC) not fulfilling all of the Amsterdam

criteria, but maintaining a strong clinical suspicion of HNPCC;⁸

- 3) early onset (< 40 years), sporadic CRC patients; and
- 4) probands unaffected by CRC who presented with 1–5 adenomatous polyps and a family history of CRC.

The control population analysed to investigate the frequency of the missense substitutions was represented by healthy blood donors. For each variant identified, the control sample was obtained from individuals residing in the same area as the affected proband(s), with the only exceptions of *MLH1* K618T and *MSH2* N596S, for which regionally matched controls were not available.

Molecular Analysis

For all probands the entire *MLH1* and *MSH2* coding sequences were investigated by single-strand conformation polymorphism (SSCP) analysis on genomic DNA, as previously described.⁷ Screening of additional family members and of control individuals was implemented by either SSCP (*MLH1* variants R265H, V326A, S406N, and V716M; and *MSH2* variant A834T) or restriction-generated PCR¹⁰ (*MLH1* variants R385P and K618T, and *MSH2* variants K246Q, G322D and N596S). All positive samples in the relative and control populations were further investigated by automated direct sequencing to confirm presence of the expected nucleotide changes.

MSI was investigated on matched paraffin-embedded or frozen tumour and constitutional DNA samples as previously described.^{8,11}

Results

A total of 10 missense changes were observed: eight have been reported by us and/or other groups and two are new (Table 1). Seven variants were identified only once in the patient population, two were found in two independent probands, and one in four probands, for a total of 15 unrelated carriers (Table 1). No frameshift, nonsense, or other mutations were observed, with the only exception of patient A-PD1.

Segregation analysis could be performed for four different mutations in five families (Table 1). The number of relatives affected with CRC who were tested for segregation ranged from two (families A-PD1, A-AV1, R-MD11, and R-RM17) to four (family R-MD4). The only variant – *MLH1* R265H – which was found to co-segregate with the disease phenotype also co-segregated with an *MLH1* truncating mutation. In families A-AV1 and R-RM17 the relatives who were negative for the missense substitutions had developed CRC at 38 and 69 years, respectively. In family R-MD11, the *MLH1* K618T variant was absent in a proband's sister who had developed two synchronous carcinomas of the proximal colon at 53 years. Finally,

Table 1 Genetic, biological and clinical characteristics associated with MMR gene missense variants^a

<i>Mutation</i>	<i>DNA change</i>	<i>Family ID</i>	<i>Phenotype^b</i>	<i>MSI^c</i>	<i>Co-segregation with cancer^d</i>	<i>Allele frequency in control population</i>	<i>Evolutionary conservation^e</i>	<i>Substitution type^f</i>	<i>References</i>
<i>MLH1</i> R265H	CGT→CAT	A-PD1	Amsterdam	+	YES ^g	0/220	YES	cons.	7
<i>MLH1</i> V326A	GTG→GCG	A-AV18	Suspected	–	nt	1/48	NO (I)	cons.	8, 16, 17
<i>MLH1</i> V326A	GTG→GCG	A-AV38	Sporadic	–	na	1/48	NO (I)	cons.	8, 16, 17
<i>MLH1</i> R385P	CGT→CCT	R-RM6	Polyps	–	nt	0/200	NO (A)	not cons.	–
<i>MLH1</i> S406N	AGT→AAT	A-AV30	Sporadic	nt	na	1/92	NO (Q)	not cons.	18
<i>MLH1</i> K618T	AAG→ACG	R-MD11	Suspected	–	NO	1/210	NO (N)	not cons.	19,20
<i>MLH1</i> V716M	GTG→ATG	A-AV23	Sporadic	–	na	0/122	NO (S)	not cons.	8, 21
<i>MLH1</i> V716M	GTG→ATG	A-AV44	Sporadic	–	na	0/122	NO (S)	not cons.	8, 21
<i>MSH2</i> K246Q	AAA→CAA	R-RMG1	Sporadic	+	na	0/200	NO (G)	not cons.	–
<i>MSH2</i> G322D	GGC→GAC	A-AV1	Amsterdam	–	NO	2/244	YES	not cons.	22
<i>MSH2</i> G322D	GGC→GAC	R-RM17	Polyps	nt	NO	2/244	YES	not cons.	22
<i>MSH2</i> G322D	GGC→GAC	R-RM28	Sporadic	nt	na	2/244	YES	not cons.	22
<i>MSH2</i> G322D	GGC→GAC	R-RM33	Sporadic	nt	na	2/244	YES	not cons.	22
<i>MSH2</i> N596S	AAT→AGT	R-MD4	Amsterdam	+	NO	0/226	NO (S)	not cons.	7
<i>MSH2</i> A834T	GCT→ACT	A-AV31	Sporadic	–	na	1/124	NO (V)	not cons.	23

^aFor each missense mutation, parameters not complying with the pathogenicity criteria are indicated in bold characters.

^bAmsterdam: pedigree complying with the Amsterdam criteria; suspected: familial CRC not complying with all of the Amsterdam criteria; sporadic: early-onset (< 40 years) sporadic CRC; polyps: proband with ≥ 1 polyps and family history of CRC.

^c+: MSI-positive; -: MSI-negative; nt: not tested.

^dnt: not tested; na: not applicable.

^eConservation between human and *S. cerevisiae* sequences, based on published *MSH2* and *MLH1* sequences;^{24,25} for non-conserved residues, the yeast aminoacid is indicated within brackets; yeast aminoacids that are identical to those encoded by missense variants are underlined and in bold characters.

^fCons. = conservative.

^gThis variant co-segregated with an *MLH1* truncating mutation in the family.

the *MSH2* N596S mutation identified in the proband from family R-MD4 was present in his affected father and in two affected paternal first cousins, but it was absent in another paternal first cousin who had developed CRC and endometrial carcinoma at 46 and 48 years, respectively.

The frequency of the missense changes in a reference population was investigated for all variants. Five of these were found in control chromosomes (Table 1).

Biological information derived from interspecies sequence comparison and from the chemical–physical properties of wild-type and mutant aminoacids indicated that only two mutated aminoacid residues were identical between human and *S. cerevisiae* gene sequences, and that 8/10 aminoacid changes were non-conservative. The amino acid introduced by the DNA sequence change at position 596 of human *MSH2* corresponded to that found at the analogous position in the yeast gene, suggesting that it is not likely to significantly modify the polypeptide function (Table 1).

Matched analysis of constitutional and tumour DNA available from 11 samples, corresponding to nine mis-

sense variants, showed that only three cases, carrying different mutations, displayed MSI (Table 1).

Discussion

The present study was aimed at ascertaining the clinical significance of missense mutations of the *MLH1* and *MSH2* genes through the analysis of genetic and biological pathogenicity indicators. We found that all of the rare missense variants investigated did not comply with at least one pathogenicity criterion. A major pathogenetic role is probably excluded for two variants (*MLH1* V326A and K618T) based on four criteria, for two variants (*MSH2* G322D and A834T) based on three criteria, and for five variants (*MLH1* R265H, R385P, S406N and V716M; *MSH2* N596S) based on two criteria (including concomitance with a truncating mutation for the *MLH1* R265H variant).

The remaining missense mutation, *MSH2* K246Q, did not fulfil only one criterion, ie involvement of a non-conserved aminoacid residue, which is not sufficient to rule out functional consequences. In addition, a role for MMR dysfunction in tumour development is strongly

suggested by the finding of MSI in neoplastic DNA from the carrier of this variant.

On the other hand, absence of MSI and/or the identification of carriers in the control population strongly suggest that the *MLH1* variants V326A, R385P, S406N, K618T, V716M, and the *MSH2* variants G322D and A834T do not significantly impair MMR function. Clearly, absence of MSI by itself does not provide unequivocal evidence for this conclusion, since CRCs may also develop through pathways unrelated to MMR deficiency, by analogy to the majority of CRCs arising in the general population. However, involvement of alternative pathways would be expected to occur only occasionally in MMR gene mutation carriers, as also indicated by our finding that all 12 tumours tested in carriers of protein-truncating mutations showed MSI.¹² Likewise, it is possible that some of the healthy carriers identified in the control population are at increased risk of cancer. However, in view of the marked genetic and allelic heterogeneity of HNPCC, the likelihood of identifying carriers of specific mutations in relatively small control samples, such as those investigated in this study, would be extremely low. It should also be considered that the frequencies of missense variants in control and patient populations may show inter-ethnic differences, as demonstrated for the *MLH1* V384D change.¹³

Overall, these results indicate that the majority of missense mutations detected in the MMR genes *MLH1* and *MSH2* are unlikely to act as major causative factors in HNPCC-related cancer development. Several other missense variants have been reported in the literature.³ Indications for their involvement in cancer susceptibility have been provided for only a few of them, like *MLH1* G67W. This mutation, which occurs in a highly conserved *MLH1* domain, has been found at the homozygous state in individuals presenting with severe and very early clinical manifestations, as well as with MSI in non-tumoral cells.¹⁴

Partly at variance with our results, data derived from a novel functional *in vitro* assay indicate that the majority of *MLH1* rare missense variants behave like *bona fide* pathogenetic – frameshift or nonsense – mutations.¹⁵ Although most of the missense mutations identified in the present study are different from those investigated *in vitro*, three had also been tested with the *in vitro* assay. The results are concordant for two variants (R265H and V326A) and discordant for one (K618T). By contrast to several lines of evidence against a major pathogenetic role obtained in this study,

the latter variant tested positive *in vitro*, as assessed by the absence of a mutator phenotype in K618T transformants. The reasons for this discrepancy are not clear. Since the biological mechanisms underlying the test are not yet clarified, it is possible that the *in vitro* test may not be able to assign correctly a functional significance to all *MLH1* molecular variants. It is also possible that some variants might induce subtle alterations of the MMR system, which can be revealed by the *in vitro* test, but are not detectable with currently used MSI assays.

In conclusion, systematic application of the approach used in this study should be encouraged in order to gain further knowledge on this issue and to provide useful elements for counselling carriers of rare MMR gene missense variants. Achievement of these aims could be facilitated through the institution of a missense mutation database for MMR genes, integrating results of clinical and *in vitro* studies.

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