LETTER TO JMG

Description and functional analysis of a novel in frame mutation linked to hereditary non-polyposis colorectal cancer

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Key points

- Hereditary non-polyposis colorectal cancer (HNPCC) is characterised by a dominantly inherited predisposition to early onset cancer and is linked with malfunction of postreplicative DNA mismatch repair (MMR). A significant proportion of HNPCC associated mutations in the MMR genes give rise to single amino acid substitutions or in frame deletions, which are difficult to interpret.
- It can be attempted to distinguish pathogenic non-truncating mutations from polymorphisms by segregation studies. Also, the biochemical characterisation of a substitution as well as the evolutionary conservation status of a mutated sequence may give some suggestion of its pathogenicity. However, reliable assessment of pathogenicity requires a functional test.
- We describe here a novel *MLH1* mutation, del GAA at codon 71, which was found in two typical HNPCC families. Since the mutation had not been reported before and the predicted coding change was a deletion of only one evolutionarily non-conserved amino acid, the pathogenicity of the mutation was assessed in a functional test. Both the interaction of the *MLH1* variant (del71) with the normal *PMS2* protein and the functionality of the heterodimer of *MLH1* and *PMS2* (*MutLα*) were studied.
- While the heterodimerisation was not abolished, the *MutLα*-del71 variant was completely deficient in an in vitro MMR assay. The result of the functional assay thus confirms the pathogenicity of *MLH1* del71, which is compatible with the typical HNPCC phenotype linked to the mutation.

Hereditary non-polyposis colorectal cancer (HNPCC) is one of the most common cancer syndromes. The cancer susceptibility is dominantly inherited and associated with germline mutations in mismatch repair (MMR) genes. An inherited defect in one allele of a MMR gene and an acquired defect in the other allele leads to hypermutability associated with marked microsatellite instability (MSI) that accelerates tumour progression.¹ To date, over 400 different mutations, affecting mostly the MMR genes *MLH1*, *MSH2*, and *MSH6*, and occurring in over 500 HNPCC kindreds, have been registered in the international database of mutations and polymorphisms found in HNPCC kindreds (<http://www.nfdht.nl>). In addition to colon cancer, MMR gene mutations are known to cause predisposition to a variety of extra-colonic cancers, notably endometrial cancer.² The clinical phenotype including the age at onset and tumour spectrum

vary considerably between families and even in patients from the same family. Part of the phenotypic variation between carriers of similar mutations may be explained by different life styles or the existence of modifying genes.^{3–5} Furthermore, the clinical characteristics in HNPCC families seem to depend on the particular gene involved and the site and type of a mutation in a complex manner.^{6–8}

While a majority of HNPCC associated mutations cause truncations and thus loss of function of the respective polypeptides, the biochemical defects associated with non-truncating mutations that account for one quarter of all mutations are much more difficult to interpret. These alterations appear to be associated with a wide variety of clinical phenotypes, ranging from the lack of any adverse effects to highly increased cancer risk. Occasionally, pathogenic mutations can be distinguished from polymorphisms by segregation studies. Moreover, the conservation status of the mutated sequence as well as the associated biochemical consequences can help the interpretation. Often, however, the lack of clinical samples, insufficient family size, or uncertainty of the biochemical significance of the alteration can hinder conclusions and the nature of the inherited non-truncating mutation should be functionally characterised. A functional assay together with clinical data from a HNPCC kindred enables more accurate predictive testing and counselling of at risk members from families segregating such mutations.

The present study was undertaken to evaluate the pathogenicity of a novel MMR gene mutation *MLH1* del71. The mutation was found in two separate HNPCC families and was linked to clinical features typical of the HNPCC syndrome, such as early age at onset, frequent colon cancers (CRC), and high microsatellite instability (MSI) in tumours.^{1,9} However, since it was an in frame mutation, involving a deletion of only one non-conserved amino acid, its pathogenicity was difficult to interpret. On theoretical grounds, we therefore conducted a functional analysis by an in vitro MMR assay, whose further advantage is that it is not dependent on biological material from mutation carriers and is therefore a convenient choice for international collaborative studies.

MATERIALS AND METHODS

HNPCC kindreds and a novel *MLH1* mutation

A novel mutation, *MLH1* del71, was found in two unrelated HNPCC families (A and B). The pedigrees and the clinical characteristics of the patients are shown in fig 1. In kindred A, three sibs and their uncle had CRC and one distant relative

Abbreviations: HNPCC, hereditary non-polyposis colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; CRC, colorectal cancer; EC, endometrial cancer; WT, wild type

Figure 2 Combined immunoprecipitation and western blot analyses showing interactions of MLH1 WT and MLH1 del71 with wild type PMS2 (MutLα-WT and MutLα-del71). Western blots of total protein extracts (top panel) and immunoprecipitates (IP) obtained with anti-MLH1 (αMLH1) (middle panel) or anti-PMS2 (αPMS2) antibodies (lower panel). Following transfer to the membrane, the proteins were visualised with a mixture of the MLH1 and PMS2 antibodies (see Materials and methods). The figure shows that coexpression of the MLH1 del71 variant with wild type PMS2 yields a stable heterodimer.

detected with western blot analysis with both anti-MLH1 (PharMingen, 13271A) and anti-PMS2 (Calbiochem, NA30-100UG) antibodies.

In vitro mismatch repair assay

In vitro MMR assay was carried out according to a previous report.⁸ A total of 75 µg of nuclear extract (NE) of HCT116 colorectal cancer cell line, which lacks functional MLH1 and is deficient in MMR, was complemented with the total protein extract including overexpressed MLH1 and PMS2 proteins. The circular DNA heteroduplexes, which contain a G•T mismatch 369 bp downstream from a single strand nick, were used as substrates.¹⁷⁻¹⁹ For the assay, the total protein extracts were estimated to contain equal quantities of recombinant MLH1 (MutLα-WT, 2.3 µg; MutLα-del71, 25.0 µg). NE of the MMR proficient cell line TK6 (75 µg), purified MutLα (0.3 µg), and MutLα-WT were used as positive controls in the assay. MMR deficient HCT116 NE was a negative control. The presence of a G•T mismatch makes the heteroduplexes not susceptible to cleavage by the endonuclease *Bgl*II. The repair reaction converts the G•T heteroduplex to the A•T homoduplex, whereafter *Bgl*II can cleave the molecule and the repair efficiency can be measured by the cleavage efficiency. Since the heteroduplex molecules are not all repairable they are always added in excess in the assay, and the repair percentage of the proficient wild type controls is used as a reference level.

RESULTS

All five tumours available for MSI analysis from family A, as well as the index tumour from family B, showed high instability. Mutation analyses and sequencing showed an in frame mutation, *MLH1* del71, which deletes bases GAA (nucleotides 211-213) in exon 3 corresponding to a glutamic acid in the polypeptide. This mutation had not been reported before in HNPCC families (<http://www.nfdht.nl>).

The co-immunoprecipitation assay was performed in order to study the effect of mutation *MLH1* del71 on heterodimerisation of MLH1 and PMS2 proteins. As shown in fig 2, the mutation might have reduced but did not abolish MutLα assembly. However, the complex MLH1 del71/PMS2 (MutLα-del71) failed to complement MMR deficient HCT116 nuclear extract (fig 3). While proficient extracts TK6, HCT116 + purified MutLα, and HCT116 + MutLα-WT repaired 18%, 17%, and 37 % of G•T heteroduplexes, respectively, HCT116 + MutLα-del71 repaired 0% of them. The result of the MMR assay thus confirmed the pathogenicity of the mutation.

DISCUSSION

The present study was undertaken to test the pathogenicity of a novel mutation *MLH1* del71, which was found in two unrelated HNPCC families. Both families displayed typical clinical

Figure 3 MMR activity of MMR deficient nuclear extract of HCT116 cells complemented with wild type MutLα (purified and total protein extract) and mutated MutLα-del71. TK6 is a MMR proficient and HCT116 a MMR deficient control (see Materials and methods). The fragment lengths on the right indicate the migration of the unrepaired linearised plasmid DNA (3193 bp) and of the two fragments (1833 bp and 1360 bp) produced following correction of the G•T mismatch, which makes the DNA susceptible to cleavage with the restriction endonuclease *Bgl*II. Percentages below the gel represent fractions of repaired DNA and display absolute MMR deficiency (0%) of MutLα-del71. The figure represents an ethidium bromide stained 1% agarose gel.

features, such as early age at onset, predominance of colon carcinomas, and high MSI in tumours. However, the alteration was a 3 bp deletion maintaining the coding sequence in frame and deleting only one amino acid from the protein. Moreover, the deleted codon was not evolutionarily conserved and the mutation has not been reported before in HNPCC families. Thus, interpretation was difficult and reliable assessment of pathogenicity required a functional test. In vitro MMR assay, the method used here, provides an opportunity to find out if a mutation prevents the protein from repairing mismatches. If tumours from the mutation carriers are MSI positive, the method can confirm that MSI results from the mutation in question. If tumours are MSI positive but the mutation found functions in the in vitro MMR assay it suggests that either there is another germline mutation or an epigenetic inactivation of a MMR gene, which causes MSI. If tumours are MSI negative, the MMR mechanism is functioning and not associated with disease. The in vitro MMR assay was recently used to test six *MLH1* mutations⁸ and four *MSH6* mutations.²⁰ The results suggested that the mutations linked to typical HNPCC imparted a MMR deficient phenotype on the altered polypeptide, whereas the mutations associated with atypical or putative HNPCC did not. However, the results did not exclude the possibility that the latter mutations affect biochemical events preceding the MMR function in vivo.

Here, we showed that *MLH1* del71 might have reduced but did not abolish MutLα assembly. However, the protein complex MLH1 del71/PMS2 (MutLα-del71) was totally deficient in MMR reaction. Accordingly, the analysis of the interaction between MLH1 and PMS2 can be indicative, but the result can be difficult to interpret when used as a functional test. This is important since over 60% of *MLH1* mutations are not located in the known MLH1/PMS2 interaction region (<http://www.nfdht.nl>),^{21, 22} and thus presumably do not or only slightly hamper their heterodimerisation. The results of the in vitro MMR assay confirmed the pathogenicity of *MLH1* del71. Codon 71 is located in the region, which has been reported to function in ATP binding and hydrolysis^{23, 24} and, accordingly, can be expected to be sensitive to alterations. Our result showed that a mutation can be pathogenic irrespective of the conservational status of the altered

sequence. Finally, the confirmed pathogenicity of *MLH1* del71 is compatible with the typical HNPCC phenotype linked to the mutation, emphasising the importance of comparing clinical and experimental data.

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