

The importance of functional testing in the genetic assessment of Muir-Torre syndrome, a clinical subphenotype of HNPCC

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Received August 25, 2005; Accepted October 3, 2005

Abstract. A majority of families with hereditary nonpolyposis colorectal cancer (HNPCC) are attributable to germline mutations in three DNA mismatch repair (MMR) genes, *MLH1*, *MSH2* and *MSH6*. However, the clinical phenotype appears to reflect a complex interplay between the predisposing mutation and putative constitutional and somatic modifiers. Certain MMR gene mutations predispose to combined occurrence of cutaneous sebaceous gland neoplasms and visceral malignancies, which is known as Muir-Torre syndrome (MTS) and regarded as a phenotypic variant of HNPCC. The sebaceous tumors associated with MTS appear in many patients before visceral malignancies providing important predictability of HNPCC-related integral cancers in mutation carriers. Since most sebaceous skin tumors are, however, sporadic, the contribution of non-truncating mutations found in skin cancer patients is difficult to interpret and genetic assessment of MTS requires a functional test. Here, we studied the repair efficiency of the two *MSH2* missense mutations, L187P and C697F, found in HNPCC families including a few mutation carriers with sebaceous skin tumors. Both mutations were completely deficient in an MMR assay, which together with tumor findings suggested their predisposing role in both internal and skin malignancies in the families.

Introduction

The same germline mutations in DNA mismatch repair (MMR) genes that have a high penetrance in colon malignancies may in some circumstances predispose to cutaneous sebaceous gland tumors and keratoacanthomas. This combined occurrence of at least one visceral neoplasm and at least one sebaceous gland skin tumor in a patient is known as Muir-Torre syndrome (MTS) (1,2). Because of its common genetic predisposition, MTS is currently considered as a subphenotype of hereditary nonpolyposis colorectal cancer (HNPCC) (Lynch syndrome II) (3-7). A recent study on MTS among patients with sebaceous tumors and keratoacanthomas demonstrated that, while the vast majority of sebaceous tumors develop as sporadic forms, the sebaceous tumors which were associated with MTS were multiple and recurrent. Moreover, they showed an early onset and appeared in 60% of patients before visceral malignancies (8), explaining why sebaceous neoplasms are frequently diagnosed without concomitant visceral tumors irrespective of linkage to HNPCC. On the other hand, a probable explanation for the low MTS frequency in known HNPCC kindreds is that skin manifestations are so rare or too minor to be recognized. Consequently, the recognition and diagnosis of MTS is difficult and requires both clinical and molecular assessment.

A defect in the MMR mechanism results in multiple somatic mutations affecting mainly small repeated sequences such as microsatellites and leads to high microsatellite instability (MSI-H). MSI-H phenotype is a hallmark of HNPCC tumors, including MTS-associated skin tumors (7,9). Actually, sebaceous neoplasms have been shown to be the tumors with the highest frequency of MSI-H (10). Unfortunately, a reliable assessment of MTS cannot be solely based on MSI-H phenotype in skin cancer. Most sebaceous tumors seem to be sporadic (8) and an alternative explanation for MSI-H in a tumor may well be a somatic inactivation of the MMR system, e.g. by *MLH1* promoter hypermethylation that is a frequent

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Key words: Muir-Torre syndrome, hereditary nonpolyposis colorectal cancer, MMR, *MSH2*, functional analysis

occurrence in sporadic colorectal cancer and some extracolonic tumors (11,12). Since a diagnosis of MTS in a patient affected by sebaceous neoplasm is of major importance for the patient and the whole family by enabling predictive genetic testing and cancer surveillance of the family members at cancer risk, all patients with MSI-H sebaceous tumors should be considered as possible cases for MTS and HNPCC. If the patient is a mutation carrier and the predicted coding change of the mutation is a substitution of only one amino-acid residue in the respective polypeptide, a functional assay is necessary to verify that the mutation is indeed the underlying cause for MMR deficiency and MSI in the skin tumor.

Materials and methods

MSH2 mutations. The mutations, *MSH2*-L187P (c.560T>C) and *MSH2*-C697F (c.2090G>T), analyzed in the functional assay are both amino-acid substitutions resulting in non-conservative amino-acid changes in conserved residues. According to predictions based on crystal structures of prokaryotic MutS proteins (*MSH2*, MutS homologue 2), the amino-acid, L187, is located in a connector domain suggested to be responsible for the intramolecular interactions in *MSH2*-*MSH6* (MutS α) heterodimer and the amino-acid, C697, is located in the highly conserved ATPase domain (13,14).

Clinical data of the families. The mutations, *MSH2*-L187P and *MSH2*-C697F, were found in typical and unrelated HNPCC families, in families A and B, and in families C, D, and E, respectively (15-18). The pedigrees and the clinical characteristics of the patients are shown in Fig. 1. Other mutations in *MSH2* or *MLH1* were excluded in the families. All families fulfill the Amsterdam criteria for HNPCC (19,20). Moreover, the families A, C, and possibly E display characteristics of the Muir-Torre syndrome (1,2). In family A, 11 patients had colorectal cancer (CRC) in 5 successive generations and 2 patients had sebaceous gland neoplasm. Two CRC patients and both patients with skin neoplasms were found to be mutation carriers. In kindred C, previously published as family 62 (15-17; www.insight-group.org), 5 patients had CRC in 3 successive generations and one patient had a sebaceous carcinoma. Three patients with CRC and the patient with a sebaceous carcinoma were shown to carry the mutation. In family E, the only studied mutation carrier had endometrial cancer, as did her mother who also was diagnosed with a benign keratoacanthoma in the neck. Families B (family 548) and D (family 934) (18; www.insight-group.org) had several CRCs but no skin malignancies. All human investigations were performed after approval of the Institutional Review Boards of the Universities or local ethics committees.

Microsatellite instability and immunohistochemical analyses. Microsatellite instability and immunohistochemistry for *MSH2* protein expression were studied in CRC tumors of at least one mutation carrier in families A, B, C, and D and in sebaceous tumors in families A and C (Fig. 1). In family E, MSI analysis was performed in an endometrial tumor of a mutation carrier. In families B, C and D, MSI was analyzed

using the Bethesda panel (18,21) while, in family A, only markers Mfd-15 and BAT-26 and, in family E, markers BAT-25, BAT-26 and D5346 were used. Immunohistochemical staining for the *MSH2* protein was performed as described previously (22).

Production of recombinant protein variants and functional assay. Preparation of the mutated *MSH2* cDNAs, expression of the mutated and wild-type proteins by using the Bac-to-Bac Baculovirus expression system (Gibco BRL), and analyses of the protein variants in an *in vitro* MMR assay followed the previously described protocols (23,24). Site-directed mutagenesis was used to introduce the mutations into *MSH2* cDNA. The oligos (5'3', F, forward; R, reverse) used to produce fragments 'a' and 'b' in the first amplifications were as follows (the mutated sites are marked in bold and fragment sizes are in parentheses): *MSH2*-L187P, Fa, CGGATTATT CATACCGTCC; Ra, GAGGAGAGCCTCAGGATTGGAG AACTG (659 bp); Fb, CAGTTCTCCAATCCTGAGGCTCT CCTC; Rb, ATCTTGAACCTCAACACAAGC (1396 bp); and *MSH2*-C697F, Fa, CTTTAGATATGGATCAGGTGG; Ra, CTGCTGACTCAAATGGCACAAAACACC (732 bp); Fb, GGTGTTTTGTGCCATTTGAGTCAGCAG; Rb, TGATT ATGATCCTCTAGTAC (1107 bp). The second amplifications were performed with the primer pairs Fa and Rb. The fragment sizes were 2024 bp for *MSH2*-L187P and 1813 bp for *MSH2*-C697F. The mutated fragments were cloned into the wild-type *MSH2* cDNA with *Bam*HI and *Nde*I, and *Nde*I and *Xho*I, respectively. The mutated cloned fragments were verified by DNA sequencing (ABI PRISM 3100 Genetic Analyser, Applied Biosystems).

For protein production, Sf9 insect cells were co-infected with wild-type or mutated *MSH2* together with wild-type *MSH6* recombinant baculoviruses. Co-infection was used, since both *in vivo* studies in mice (25) and *in vitro* studies in human cells (26,27) have shown that *MSH6* (*Msh6* in a mouse) is unstable without its cognate partner, *MSH2* (*Msh2*).

The functionality of the mutated *MSH2* proteins were studied by complementing *MSH2* deficient (*MSH2*^{-/-}) nuclear extracts (NE) of LoVo cells with the total protein extract of Sf9 cells including overexpressed human *MSH6* and *MSH2* proteins.

Results

Studied tumors showed MSI and loss of *MSH2* protein. Both MSI and immunohistochemical (IHC) analyses suggested MMR deficiency in the tumors (Fig. 1). As was previously published, in families B, C and D, all of the studied tumors including one CRC in each family and the sebaceous carcinoma in family C showed MSI-H (18). In family A, one CRC showed instability with both studied markers and the sebaceous adenoma showed instability with marker Mfd-15 while marker BAT-26 was stable. In family E, the studied endometrial cancer showed typical MSI with all studied markers, BAT-25, BAT-26 and D5346. Furthermore, *MSH2* protein was lost in all studied tumor samples including a sebaceous neoplasm in families A and C.

Both *MSH2* protein variants were deficient in the functional assay. In order to determine whether the two missense

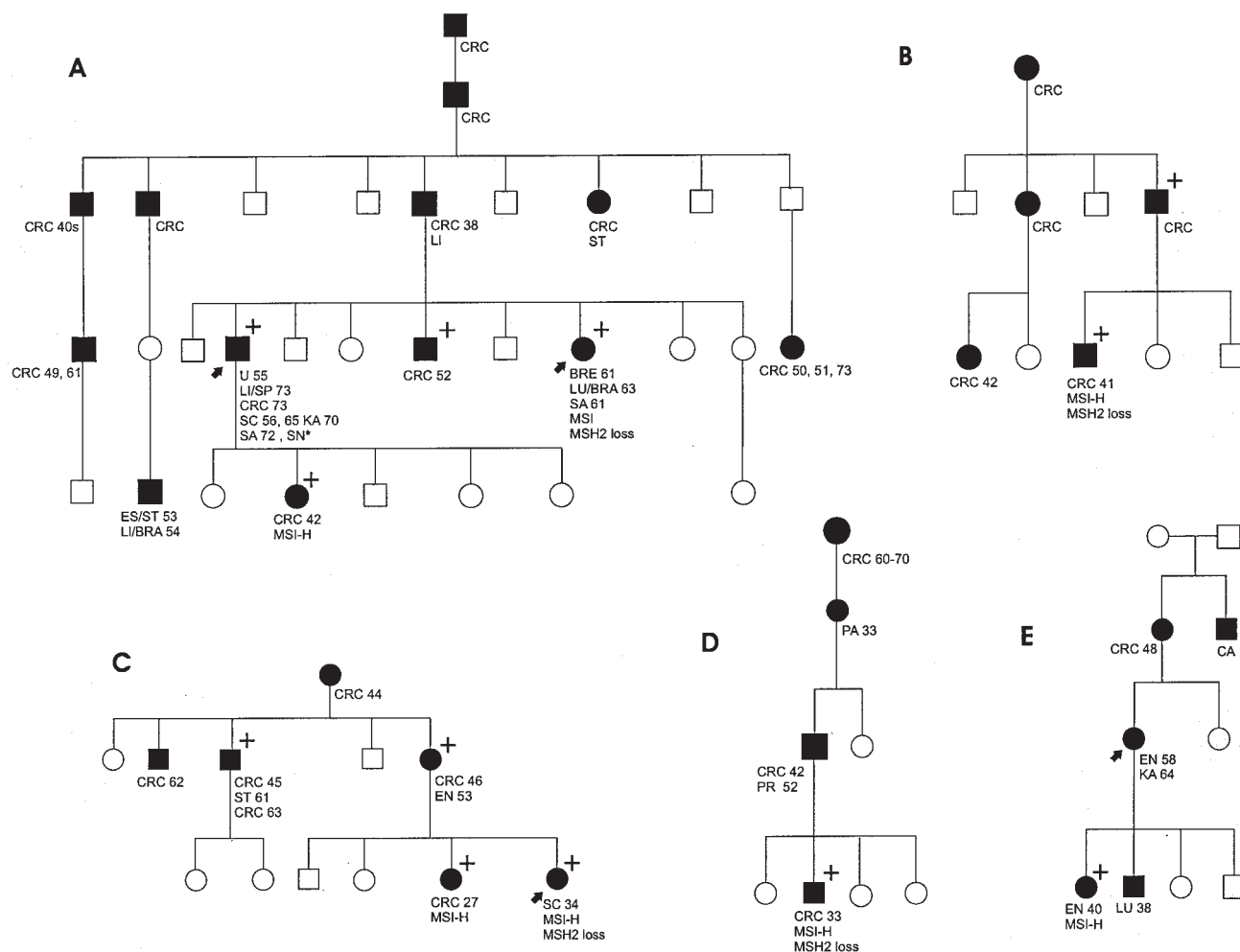


Figure 1. Pedigrees of families A, B, C, D and E. Plus (+), patients verified as carrying the mutation *MSH2*-L187P in families A and B and *MSH2*-C697F in families C, D and E. Arrow, patients diagnosed with sebaceous gland neoplasms and/or keratoacanthomas. Tumor types, ages at onset, and results from MSI and immunohistochemical studies are marked when available. Abbreviations: CRC, colorectal cancer; EN, endometrial cancer; ST, stomach cancer; U, ureter cancer; LI, liver cancer; ES, esophagus cancer; BRE, breast cancer; BRA, brain cancer; PA, pancreatic cancer; SP, spleen cancer; PR, prostate cancer; LU, lung cancer; CA, cancer (location unknown), SA, sebaceous adenoma; SC, sebaceous carcinoma; KA, keratoacanthoma; SN, other skin neoplasm. *In addition to the tumors listed, the patient exhibited 5 basal cell carcinomas, 3 seborrheic keratosis, 3 sebaceous hyperplasias, 2 sebaceous epitheliomas, 1 squamous cell carcinoma, and 1 malignant melanoma.

mutations in *MSH2* might contribute to the MMR deficiency seen in sebaceous tumors in the few mutation carriers in families A and C, we tested the recombinant MutS α variants (*MSH2*/*MSH6*) in the *in vitro* MMR assay. Since the heteroduplex DNA molecules including G-T mismatch are not all repairable, they are added in excess in the assay, and the repair percentage of the proficient wild-type controls are used as a reference level. As shown in Fig. 2, nuclear extracts (NE) of MMR-proficient TK6 cells repaired 27% (SD $\pm$ 6%) of the added heteroduplex DNA, whereas NEs of LoVo (*MSH2*^{-/-}) cells that are deficient in MMR showed 6% (SD $\pm$ 3%) repair. The wild-type MutS α (MutS α -WT, *MSH2*-WT/*MSH6*-WT), purified and total protein extract, complemented the deficient LoVo NEs repairing 40% (SD $\pm$ 9%) and 45% (SD $\pm$ 10%) of heteroduplex DNA, respectively (Fig. 2, lanes 4 and 5), while complementation with MutS α -L187P and MutS α -C697F exhibited repair efficiency of 6% (SD $\pm$ 2%) and 8% (SD $\pm$ 3%), respectively, approximately in the same levels as the negative control. With regard to the repair percentages, the *MSH2* proteins were both deficient in MMR.

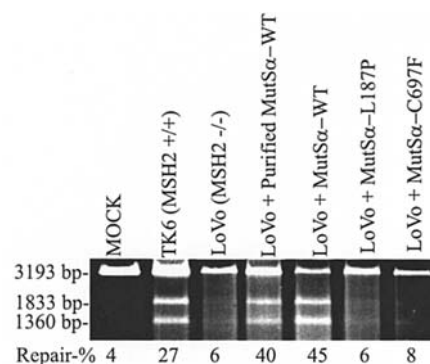


Figure 2. Complementation of MMR deficient nuclear extracts with MutS α variants (above). Mock, heteroduplex DNA with no protein; TK6, MMR-proficient NE used as a positive control; and Lovo, MMR-deficient NE used as a negative control. Marker left, migration of the unreplicated linearized plasmid DNA (3193 bp) and of the two fragments (1833 and 1360 bp) produced following correction of the GT mispair, which makes the DNA susceptible to cleavage with the restriction endonuclease *Bgl*III. Numbers below panel, fraction (%) of repaired DNA. The repair efficiencies were quantified by comparing the intensities of the uncorrected and corrected DNA fragments using Image-Pro 4.0 (Media Cybernetics). The values are an average of three independent experiments.

Discussion

The recognition and diagnosis of MTS is difficult and requires both clinical and molecular assessment. The present study was undertaken to reveal the importance of functional testing in the genetic assessment of Muir-Torre syndrome.

HNPCC and MTS syndromes are characterized by a dominantly inherited predisposition to early onset cancer, HNPCC to internal cancers and MTS by coincidence of at least one sebaceous gland skin tumor and one internal malignancy (1,2). High-grade MSI, which reflects a loss of MMR, is a hallmark of both tumor types (7,9). Indeed, MTS patients have been shown to have a similarly high probability of an underlining MMR defect as patients with a family history fulfilling the diagnostic Amsterdam Criteria I for HNPCC (10,19). Thus, the awareness of MTS provides powerful predictability of colorectal and other HNPCC integral cancers in mutation carriers, increasing the importance of its diagnosis.

In spite of its importance, the recognition of sebaceous neoplasms in HNPCC kindreds is quite exceptional. An explanation might be that many HNPCC-associated mutations have a lower susceptibility to sebaceous skin tumors than to colorectal and other internal cancers with the result that skin manifestations are totally absent or too subtle to be recognized in HNPCC kindreds. Accordingly, in MTS the vast majority of mutations have been identified in *MSH2*, only three mutations in *MLH1* and none in *MSH6*, supporting some kind of genotype correlation for MTS in HNPCC (10,17). Forty-one different germline MMR gene mutations have been published in patients diagnosed with MTS (8,10,17,28) and 38 of those cause truncations, and thus loss-of-function of the respective polypeptides, while about 30% of all mutations in HNPCC are non-truncating (29; www.insight-group.org). Assuming that loss-of-function mutations especially predispose to MTS, we aimed to address the question of whether and how missense mutations, which are infrequently reported from MTS families, cause susceptibility to sebaceous gland neoplasms and the syndrome.

We studied the repair efficiency of the two *MSH2* missense mutations, L187P and C697F, found in classical HNPCC families with and without sebaceous tumors raising the suspicion of MTS (Fig. 1). Family A, carrying *MSH2*-L187P, included 11 CRC patients and two siblings affected by sebaceous gland tumors. The patient with sebaceous carcinomas also had ureter, liver, spleen, and colorectal cancer as well as many other skin neoplasms including sebaceous adenoma and keratoacanthoma. To our knowledge, this was the first time the mutation *MSH2*-L187P was associated with MTS phenotype. Previously, *MSH2*-L187P was associated with the typical HNPCC kindred, with family B, including 5 CRCs but no skin malignancies (18). The three HNPCC kindreds carrying the mutation *MSH2*-C697F show many CRCs and a sebaceous carcinoma in family C (15,16), 3 CRCs at an early age of onset but no skin malignancies in family D (18), and CRC, 2 endometrial cancers, and a keratoacanthoma in family E. Altogether, two sebaceous lesions were analyzed to have MSI and loss of MSH2 protein suggesting MMR deficiency. Previously, *MSH2*-C697F and another suspected MTS mutation, *MSH2*-R524P, have been studied to be pathogenic in a yeast functional assay (30). In accordance with

those, the present study, which is performed in a human homologous system, specifically sorted complete MMR deficiency of the mutated proteins MSH2-L187P and MSH2-C697F suggesting their predisposing role in internal and skin malignancies.

The fact that the same missense mutations gave rise to HNPCC phenotypes with and without MTS implies that, in addition to the predisposing mutation, MTS phenotype requires other contributing factors. In general, it is unknown why certain organs are more cancer-prone than others in HNPCC, and several hypotheses exist, many of which also apply to skin cancer (25). Skin is continuously exposed to exogenous mutagens and the MMR system participates in the elimination of such lesions (31). Differential exposure rates to genotoxic agents may offer a possible explanation why only a fraction of carriers of the same mutation develop skin cancer. Our study, together with the literature data, suggests that the type of mutation (truncating vs. non-truncating) is not critical for MTS phenotype as long as the mutation results in an MMR defect. Our recent study on functional significance and clinical phenotype of non-truncating mutations in *MLH1* clearly distinguished alterations with severe functional defects from those not or slightly impaired in protein function, which was mirrored by different phenotypic characteristics (32). The present functional testing of the two missense mutations in *MSH2* supports the notion that Muir-Torre subphenotype of HNPCC is associated with severe functional defects in the repair capability of the mutated proteins, which is compatible with a phenotype fulfilling the Amsterdam criteria in the families.

Acknowledgements

We thank Professor J. Jiricny for providing us with the *MSH2* and *MSH6* cDNA clones, TK6 cell line and purified MutS α and M. Korhonen for technical help. This work was supported by grants from the Sigrid Juselius Foundation, The Academy of Finland, and the Finnish Cancer Foundation.

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