ONLINE MUTATION REPORT

No *MSH6* germline mutations in breast cancer families with colorectal and/or endometrial cancer

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Background: The genetic background in breast cancer families with colorectal and/or endometrial cancer is mostly unknown. The functional connection between *MSH6* and the known breast cancer predisposition gene product BRCA1 suggests that the *MSH6* gene may also play a role in breast cancer predisposition.

Methods: We analysed 38 breast cancer families with colorectal and/or endometrial cancer for germline mutations in *MSH6*.

Results: No disease associated mutations were detected among the breast cancer families. However, mutation analysis revealed a Glu995STOP mutation in an atypical HNPCC family. The same mutation was found in a patient with both breast and colorectal carcinoma in our previous study, and haplotype analysis confirmed a common ancestral origin. The Glu995STOP mutation was further examined in an extensive series of 245 colorectal and 142 breast carcinoma patients with a family history of breast, colorectal, and/or endometrial carcinoma, and in 268 healthy population controls, but none was found to carry the mutation.

Conclusions: Our results suggest that *MSH6* may not be the underlying gene in breast cancer families with a history of colorectal and/or endometrial cancer. The Glu995STOP founder mutation is not a familial breast cancer predisposition allele and makes only a limited contribution to colorectal cancer burden in Finland.

Inherited mutations in DNA mismatch repair (MMR) genes cause susceptibility to hereditary non-polyposis colorectal cancer (HNPCC), an autosomal dominant cancer predisposition syndrome characterised by early onset colorectal cancer, and frequently showing extracolonic tumours such as cancers of the endometrium, stomach, ovaries, small bowel, ureter, and renal pelvis.¹ The vast majority of the observed germline mutations in HNPCC families are in the *MLH1* (~50%) and *MSH2* (~40%) genes (www.insight-group.org; International Society for Gastrointestinal Hereditary Tumours). The first germline mutation in *MSH6* was found in a patient with three colorectal tumours and a weak family history of gastrointestinal tumours, indicating atypical HNPCC.² Further reports on families with germline *MSH6* mutations have also shown different clinical features compared with traditional HNPCC phenotype, such as later age of cancer diagnosis, lower penetrance, and predominance of endometrial carcinomas.³⁻⁶ In a recent study by Hendriks *et al*, the cumulative risks at 70 years for *MLH1*, *MSH2*, and *MSH6* mutation carriers were similar for all the HNPCC related tumours combined.⁷ For women, colorectal cancer risk was significantly lower and endometrial cancer risk significantly higher in *MSH6* carriers compared with *MLH1*

and *MSH2* carriers.⁷ In contrast to *MLH1* and *MSH2* mutation carriers, whose colorectal and endometrial tumours very often exhibit high microsatellite instability (MSI-H),⁸⁻¹⁰ tumours from *MSH6* carriers more frequently show a lower degree of MSI (MSI-L) or are microsatellite stable (MSS).^{5 11 12} In breast cancer, MSI has been observed in some sporadic¹³⁻¹⁶ and familial¹⁷ breast tumours and in a few breast tumours of known HNPCC patients.^{18 19}

The genetic background in breast cancer families with colorectal and/or endometrial cancer is mostly unknown. Studies on breast cancer risk in HNPCC have also given conflicting results.^{1 18-22} For example, a statistically significant over-representation of breast cancer was observed in *MLH1* mutation positive families and in families with no detectable *MLH1* or *MSH2* mutations,²³ but the finding was not confirmed in another study.²⁴ The aforementioned studies have mainly focused on classic HNPCC families, and thus the possible effect of *MSH6* may have been overlooked. The *MSH6* protein, along with *MLH1* and *MSH2*, is part of the so called BRCA1 associated genome surveillance complex (BASC), which is suggested to serve as a sensor for DNA damage.²⁵ This large protein complex is comprised of BRCA1 associated DNA repair proteins, all of which possess the ability to bind abnormal DNA structures. Protein-protein interaction between BRCA1, the protein product of a known breast cancer predisposing gene, and the proteins associated with it may indicate that they function in the same DNA damage response pathway.²⁵ Reduced *MSH6* mRNA expression has also been observed in breast tumour derived cell lines.²⁶ In our previous study, we found a germline *MSH6* mutation in a patient with both breast and colorectal carcinoma,²⁷ and a similar patient has been reported by Plaschke *et al*.²⁸ In addition, Hendriks *et al* have reported on an *MSH6* mutation positive breast cancer patient whose tumour showed an MSI-H phenotype and no *MSH6* expression.⁷ In this study, our aim was to evaluate the role of *MSH6* in breast cancer predisposition by analysing 38 breast cancer families with family history of colorectal and/or endometrial cancer for germline *MSH6* mutations. In addition, a previously reported putatively breast cancer associated Finnish *MSH6* mutation was evaluated in an extensive set of breast and colorectal cancer cases.

PATIENTS AND METHODS

Breast cancer families

In the study, 38 families originally ascertained from a group of breast cancer patients at the Departments of Oncology or Clinical Genetics, Helsinki University Central Hospital, Finland, fulfilled the following clinical criteria for this study: (a) at least one breast cancer case, and (b) either endometrial or colorectal carcinoma patient, and (c) additional endometrial or colorectal carcinoma patient or family history of at least one of the following tumours: stomach, small intestine, bile duct, pancreatic, ovarian, kidney, or ureter carcinoma,

adenocarcinoma of the cervix, or malignant brain tumour. Families were classified according to the presence of breast, colorectal, and endometrial cancer cases in the family (table 1). One family fulfilled the Amsterdam criteria, and 10 families the Revised Bethesda Guidelines for HNPCC.^{29 30} No *BRCA1* or *BRCA2* mutations were detected in any of the families, and the presence of the three *MLH1* founder mutations that account for the large majority of all MMR mutations in Finland were excluded. For each family, the index patient and, when available, relatives with endometrial or colorectal carcinoma were analysed for germline *MSH6* mutations, with the total of 42 samples in the analysis.

Breast and colorectal carcinoma patients with family history of cancer

The observed *MSH6* founder mutation was analysed in 142 breast carcinoma patients who themselves or whose first degree relative had colorectal (n = 70) or endometrial (n = 81) carcinoma, and 245 colorectal carcinoma patients who themselves or whose first degree relative had additional colorectal carcinoma (n = 143), breast (n = 86), or endometrial (n = 23) carcinoma. The 142 breast cancer patients belong to the cohorts of 889 unselected³¹ (also unpublished data) and 710 familial breast cancer cases^{32–34} (also unpublished data). None of the 142 families fulfilled the Amsterdam criteria, while 15 families fulfilled the Revised Bethesda Guidelines for HNPCC.^{29 30} Colorectal cancer patient samples were selected from a population based series of 1042 consecutive colorectal cancer cases previously described.^{35 36}

MSI was determined using a polyA marker BAT26, a robust marker for high level MSI detection; 24 of the 245 samples were MSI positive, and 14/245 were shown to harbour a germline mutation in either *MLH1* or *MSH2*^{35 36} (also unpublished data). Three of the 245 families fulfilled the Amsterdam criteria and 83 families fulfilled the Revised Bethesda Guidelines for HNPCC.^{29 30} To evaluate the frequency of the observed *MSH6* mutation in population controls, blood samples from 268 healthy anonymous blood donors were studied.

All specimens were collected and analysed with informed consent and under protocols approved by ethics committees of the Departments of Obstetrics and Gynecology, and Oncology, Helsinki University Central Hospital, Finland, and the Ministry of Social Affairs and Health in Finland.

Table 1 Family history of breast, colorectal, and endometrial cancer among the families studied	
Breast, colorectal, and endometrial cancer patients in the families	No. of families
≥3 breast cancer patients	28
with at least one colorectal cancer patient in a family	16
with at least one endometrial cancer patient in a family	6
with at least one colorectal and one endometrial cancer patient in a family	6
2 breast cancer patients	7
with at least one colorectal cancer patient in a family	4
with at least one endometrial cancer patient in a family	2
with at least one colorectal and one endometrial cancer patient in a family	1
1 breast cancer patient	3
with at least one colorectal and one endometrial cancer patient in a family	2
with several primary tumours (at least breast and colorectal/endometrial)	1
Total	38

Mutation analysis

All 10 exons and exon–intron boundaries were screened for germline *MSH6* mutations by conformation sensitive gel electrophoresis (CSGE), with modifications previously described.²⁷ Briefly, genomic DNA was amplified by PCR, with one of the primers being end labelled with γ -³³P-dATP (PerkinElmer Life Sciences, Boston, MA, USA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). PCR products were then denatured for 10 minutes at 95°C, and heteroduplexes were allowed to form by letting the samples slowly cool to room temperature. Samples were run on mildly denaturing CSGE gels (10% acrylamide, 10% ethylene glycol, 15% formamide) either at 35 W for 3–4 hours or at 3 W overnight. All samples with an abnormal band shift were reamplified and sequenced by direct sequencing using BigDye Terminator Cycle Sequencing kit (version 3.1) and ABI 310 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Haplotype analysis

Haplotype analysis was performed in order to evaluate the possible common ancestry of the observed *MSH6* mutation. The polymorphic microsatellite markers used were: D2S119, D2S2298, D2S2174, D2S2240, and D2S2378. The analysis was done by end labelling one of the primers with γ -³³P-dATP (PerkinElmer Life Sciences) and separating the denatured PCR products in a mildly denaturing 10% polyacrylamide gel.

RESULTS AND DISCUSSION

Identification of mismatches that have occurred during replication or recombination of homologous but non-identical DNA sequences is accomplished by two heterodimeric complexes, MSH α and MSH β . The former, comprising of MSH2 and MSH6, is primarily responsible for the recognition of single nucleotide mismatches and small insertion/deletion loops,^{37–39} whereas the latter, with MSH2 and MSH3, preferentially recognises insertion/deletion loops.^{39 40} The partial functional redundancy of the two complexes may explain both the rarity of germline *MSH6* mutations in HNPCC and the atypical HNPCC phenotype common to families with such mutations. Interestingly, in contrast to *MSH6*, where several disease causing germline mutations have been identified in HNPCC and atypical HNPCC, such mutations are thus far lacking for *MSH3*.

The role of germline mutations in MMR genes in breast cancer predisposition has remained controversial, though major contribution appears unlikely. Results from most epidemiological studies suggest that breast tumours are not associated with HNPCC syndrome,^{1 22 24} although Scott *et al*²³ reported a 15 fold excess in lifetime breast cancer risk among *MLH1* mutation carriers. As the aforementioned studies have mostly concentrated on classic HNPCC families with either *MLH1*, *MSH2*, or unknown mutation status, it may be that the effect of *MSH6* has been overlooked as germline mutations in *MSH6* predispose mainly to atypical HNPCC. We²⁷ and others²⁸ have also reported on *MSH6* mutation carriers with both breast and colorectal carcinoma. Colorectal cancer is a common feature in breast cancer families, and 16% of Finnish breast cancer families include also colorectal cancer patients.⁴¹ The underlying predisposition gene in such families is unknown. The aim of this study was to formally analyse the role of *MSH6* in breast cancer families who also have endometrial and/or colorectal cancer patients.

Altogether, eight germline alterations in the coding and seven in the non-coding region were observed in the 38 families (table 2). Six of the coding variants were silent substitutions, five of which have been reported as polymorphisms (www.insight-group.org; International Society for Gastrointestinal Hereditary Tumours). In addition, both

Table 2 Germline *MSH6* alterations in breast cancer families

Location	Nucleotide change	Effect on protein	Families with the variant	Variant previously described (reference no.)
Exon 1	116G→A	Gly39Glu	4	4, 42, 43
Exon 1	186C→A	Arg62Arg	15	4, 42, 43
Exon 2	276A→G	Pro92Pro	9	4, 42, 43
Exon 3	540T→C	Asp180Asp	15	4, 5, 43
Intron 3	628-56C→T		9*	4, 43
Exon 4	642C→T	Tyr214Tyr	8*	4, 5, 43
Exon 4	1186C→G	Leu396Val	2	4, 5
Exon 4	1875C→T	Ser625Ser	1	
Exon 5	3306T→A	Thr1102Thr	1	4
Intron 5	3438+14A→T		20	4, 43
Intron 6	3557-18delT/insT		†	
Intron 7	3646+29delCTAT		35*	
Intron 7	3647-54 del17		9*	4, 43
Intron 9	4002-27delT		‡	
3'UTR	4083+49G→C		1	

*Also homozygotes for the rare allele; †microsatellite with 12-14xT (12/13, n=25; 13/14, n=13); ‡microsatellite with 17-18xT, all samples heterozygotes (17/18).

missense changes (Gly39Glu and Leu396Val) have been previously classified as polymorphisms, and functional analysis of the latter has shown that a plasmid with the Leu396Val gene is able to complement the mismatch repair defect of an *msh3 msh6* double mutant strain.⁴ Intronic variants observed in this study were common and located outside the conserved splice sites, suggesting that they may not have functional significance. It is possible that some mutations may have been missed in this study, as the CSGE method is known to be less than 100% sensitive and we did not look for large genomic rearrangements. In addition, the sample size in the mutation screening is quite small. Despite these limitations, the absence of disease associated mutations suggests that *MSH6* germline mutations are not, or only rarely, the cause of breast cancer in families with family history of colorectal and/or endometrial cancer.

In the course of this study, a complete mutation analysis of an additional family in which the colorectal tumour showed no *MSH6* expression in immunohistochemical analysis revealed the same Glu995STOP mutation that was seen in our previous study in a patient with both breast and colorectal carcinoma.²⁷ Glu995STOP is a protein truncating

mutation deleting 365 amino acids from the C terminal end of the protein, including domains that are conserved in many MutS homologues and participate in, for example, DNA binding, ATPase activity, and protein dimerisation^{44, 45} (www.ensembl.org/homo_sapiens/). The mutation positive families that are not known to be related originate from the same geographical region of Southern Finland, and haplotype analysis confirmed the common ancestral origin of the mutation (fig 1). Both families show atypical HNPCC, with predominance of endometrial carcinoma. Both families do, however, fulfil the revised Bethesda Guidelines for HNPCC³⁰; family 5130 with the presence of a colorectal cancer patient diagnosed before 50 years of age, and family 2342 with the presence of both colorectal and endometrial carcinoma in the same patient.

Recurrent founder mutations provide another approach to evaluate the presence of susceptibility gene mutations in more extensive sets of patients and families, and have been useful in cancer susceptibility gene studies in Finland.^{31, 35, 36} To further analyse the importance of this particular Glu995STOP mutation among Finnish breast and colorectal carcinoma patients with family history of cancer, we studied its frequency in 245 colorectal cancer patients who themselves or whose first degree relative had been diagnosed with additional colorectal carcinoma or breast or endometrial carcinoma, and in 142 breast cancer patients who themselves or whose first degree relative had colorectal or endometrial carcinoma. Of these 387 patients, 72 (18.6%; 26 colorectal and 46 breast cancer patients) were from the same geographical region as the mutation carriers. None was found to carry the mutation. The mutation was also absent in 268 anonymous Finnish blood donors. This suggests that the Glu995STOP is a new Finnish *MSH6* founder mutation that may have some relevance in a geographically restricted area, but its contribution to colorectal cancer burden is limited. The results also indicate that the Glu995STOP is not a familial breast cancer predisposition allele.

In the other *MSH6* positive family (5130), a *CHEK2* 1100delC mutation has also been identified.²⁷ Both mutations were found in the index patient diagnosed with both breast and colorectal carcinoma at the age of 34 years, and in her mother, who had been diagnosed with benign meningioma. As the *CHEK2* 1100delC is now known to act as a low penetrance breast cancer allele^{46, 47} and *MSH6* is known to predispose to colorectal cancer, it appears likely that the breast carcinoma from the index patient was caused by the *CHEK2* mutation and the colorectal tumour by the *MSH6*

Figure 1 Pedigrees of the families with the germline *MSH6* Glu995STOP mutation: (A) 5130, (B) 2342. Proband is indicated by an arrow. Tumour site(s) and age at diagnosis are indicated: Br, breast; Co, colon; En, endometrium; Ki, kidney; Men, meningioma; Pr, prostate; Ve, ventricle. *Diagnosis could not be verified from medical documents; the age presented is the at death. Haplotypes are shown beneath the individuals, with the shaded haplotype referring to a shared disease haplotype.

mutation. This is further supported by the MSH6 immunohistochemical staining and MSI analyses on the colorectal and breast tumours: while the colorectal tumour shows no MSH6 expression and exhibits MSI-H, the breast tumour is MSS and shows only slightly reduced MSH6 expression as compared with adjacent normal tissue. The index patients' sister was diagnosed with breast cancer at the age of 38 years, but she was not found to carry either of the mutations and her tumour is most likely sporadic. Alternatively, there may also be a currently unknown genetic factor or an undetected *BRCA1/2* mutation underlying the early onset breast cancer cases in this family. In another *MSH6* mutation carrier affected with both colorectal and breast carcinoma, the breast tumour did not exhibit MSI or loss of MSH6 expression, and it was suggested that also that breast tumour is most likely not due to mismatch repair deficiency.²⁸ Despite the *MSH6* positive breast cancer patient whose tumour exhibits MSI-H and loss of MSH6 expression,⁷ in most published cases the mutation status of the breast cancer patient is unknown or the patient with breast cancer has not been a mutation carrier.^{4 5 11 12 48 49} Therefore, many breast cancer cases among *MSH6* families may be sporadic or due to other breast cancer predisposition alleles.

Both breast and colorectal carcinomas are common tumours that often occur together in families. This co-existence may be due to chance clustering of two common cancers, but there may also be genetic factors that predispose to both cancer types.⁵⁰ Mutations in *LKB1* that predispose to Peutz-Jeghers syndrome have been associated with an increased risk for both breast and colorectal cancer.⁵¹ Elevated colorectal cancer risk has also been reported for *BRCA1* and *BRCA2* mutation carriers, although the results have been inconsistent.^{52–55} In addition, the studies on whether the breast cancer risk is increased among HNPCC families have given contradictory results.^{1 18–22 24} Altogether, germline mutations in all of these genes may explain only a small fraction of families with both of these tumour types. The presence of families with a strong family history of both breast and colorectal carcinomas and no mutations in the known predisposition genes have led to the suggestion that there may be a novel gene(s) associated, when mutated, to an increased risk for both cancer types.⁵⁰ Recently, the *CHEK2* 1100delC mutation was found to be associated with families with both breast and colorectal cancer, and was suggested to underlie the hereditary breast and colorectal cancer syndrome HBCC.⁵⁶ However, the observation was not confirmed in a study by Kilpivaara *et al.*⁴¹ Furthermore, no statistically significant increase in the 1100delC mutation frequency has been observed either in familial or in sporadic colorectal cancer,^{41 56} or in patients with multiple adenomas,⁵⁷ suggesting that its effect on colorectal cancer is very low or non-existent.

The absence of germline *MSH6* mutations in our set of breast cancer families and patients studied here do not exclude the possibility that *MSH6* mutations may be associated with an increased breast cancer risk in the context of *MSH6* mutation carrier HNPCC families. However, our results suggest that *MSH6* is not a breast cancer predisposition gene that would manifest itself in breast cancer patients and families with the family history of colorectal and/or endometrial cancer. Other breast/colorectal cancer susceptibility genes are likely to be the cause in such families.

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