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Paper

Germline MSH6 mutations are more prevalent in endometrial cancer patient cohorts than Hereditary Non Polyposis Colorectal Cancer cohorts.

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ABSTRACT

Objective: To determine and compare the prevalence of MSH6 (a mismatch repair gene) mutations in a cohort of families with Hereditary Non-Polyposis Colorectal Cancer (HNPCC), and in an unselected cohort of endometrial cancer patients (EC).

Design: Two patient cohorts participated in the study. A cohort of HNPCC families who were known to the Regional Medical Genetics department, and an unselected cohort of patients with a history of EC. All participants received genetic counselling on the implications of molecular testing, and blood was taken for DNA extraction with consent. All samples underwent sequencing and Multiple Ligation probe analysis (MLPA) for mutations in MSH6.

Populations: DNA from one hundred and forty-three probands from HNPCC families and 125 patients with EC were included in the study.

Methods: Molecular analysis of DNA in all participants from both cohorts for mutations in MSH6.

Outcome measures: Prevalence of pathogenic mutations in MSH6.

Results: A truncating mutation in MSH6 was identified in 3.8% (95% CI 1.0-9.5%) of patients in the endometrial cancer cohort, and 2.6% (95% CI 0.5-7.4%) of patients in the HNPCC cohort. A missense mutation was identified in 2.9% and 4.4% of the same cohorts respectively. No genomic rearrangements in MSH6 were identified.

Conclusion: MSH6 mutations are more common in EC patients than HNPCC families. Genomic rearrangements do not contribute to a significant proportion of mutations in MSH6, but missense variants are relatively common and their pathogenicity can be uncertain. HNPCC families may be ascertained through an individual presenting with EC, and recognition of these families is important so that appropriate cancer surveillance can be put in place.

Key Words: Endometrial, Cancer, MSH6, HNPCC.

INTRODUCTION

HNPCC is an autosomal dominant highly penetrant cancer-susceptibility syndrome caused by germline mutations in one of the DNA mismatch repair (MMR) genes, namely MLH1, MSH2 and MSH6[1]. Affected individuals have a predisposition to developing early onset colorectal cancer (CRC) and other HNPCC associated cancers, particularly endometrial cancer (EC)[2].

Diagnosis of HNPCC is dependent on familial clustering of CRC's, and other HNPCC related cancers, early onset cancers, and synchronous and metachronous cancers. Associated with a life time cancer risk of up to 80% [3,4], early diagnosis enables at risk family members to be enrolled in a cancer surveillance programme, thus reducing mortality and morbidity [5-7].

The Amsterdam criteria, developed in 1991 by the International Collaborative Group on Hereditary Non-polyposis Colorectal Cancer (ICG-HNPCC)[8], and subsequently revised in 1999[9], are not diagnostic, but can be used to standardise HNPCC families for comparative multi-centre studies (see Boxes 1 and 2).

MLH1 and MSH2 mutations account for the majority of known mutations in HNPCC families, and can represent between 25%[10] and 49% of Amsterdam criteria positive families[11]. Higher mutation detection rates of 86% have been published, but this may be as a result of founder mutations[12]. MSH6 mutations were first reported in HNPCC kindreds in 1997[13,14], and are less prevalent in HNPCC cohorts with MSH6 mutations estimated to represent approximately 10%

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Box 1:

Amsterdam criteria I

There should be at least three relatives with histologically verified CRC; all of the following criteria should be present:
• One should be a first degree relative of the other two; • At least two successive generations should be affected; • At least one CRC should be diagnosed before age 50; • FAP should be excluded in the CRC case; • Tumours should be verified by pathological examination

Box 2:

Amsterdam criteria II

At least three relatives with an HNPCC associated cancer *
• One affected person is a first degree relative of the other two • At least two successive generations are affected • At least one person was diagnosed before the age of 50 years • Familial adenomatous polyposis has been excluded • Tumours have been verified by pathological examination

*large bowel, endometrium, small bowel, ureter, or renal pelvis, though not including stomach ovary, brain, bladder or skin

of all MMR mutations in HNPCC families[15,16]. Between 2-5% of HNPCC families including Amsterdam I, Amsterdam II, or 'HNPCC like' will have a germline mutation in MSH6[15,17,18]. Mutations have been described in PMS2 and PMS1 in HNPCC kindreds but have not been found to contribute to a significant proportion of families[19,20].

In comparison to MLH1 and MSH2, the phenotype of MSH6 is characterised by a later age of onset of CRC, incomplete penetrance, and a higher risk and later age of onset of EC in female MSH6 carriers[15,21]. MSH6 mutation carriers may be missed amongst analysis of HNPCC families if the Amsterdam criteria are used as selection criteria[22] It is likely that MSH6 mutations may occur at a higher prevalence in a cohort of EC patients in comparison to HNPCC cohorts that have been selected by the Amsterdam criteria which are characteristic of HNPCC families with a mutation in MLH1 or MSH2. A few studies have looked at the prevalence of MSH6 mutations in EC patients with estimates between 1.7% of patients with EC less than 50 years[23], and 4.7% identified in EC patients un-selected for age or family history[24].

In this study we sought to determine the prevalence of MSH6 mutations in our Northern Ireland HNPCC cohort with less restrictive inclusion criteria than the Amsterdam II criteria, in an attempt to include as many MSH6 phenotype families as possible given the probable later onset of colon cancers; and also determine the prevalence of mutations in an unselected cohort of EC patients.

METHODS

Subject Recruitment

The study was granted ethical approval by the Office for Research Ethics Committees Northern Ireland (ORECNI). Two patient cohorts were recruited; HNPCC and endometrial cancer patient cohorts. Sample sizes were calculated from previous studies with estimated prevalence figures of MSH6 of 9%[16] and 8%[25] respectively in each cohort, giving a target size of 197 cases for an estimate of prevalence with 95% confidence intervals no wider than +/- 4% for the HNPCC cohort and a target size of 177 cases for an estimate of prevalence with 95% confidence intervals no wider than +/- 4% for the endometrial cohort.

The HNPCC cohort was known to the regional genetics department, and had received genetic counselling in the past, with blood taken for diagnostic testing of MLH1 and MSH2 or DNA storage. All families who met the Amsterdam I and II criteria were included. In addition, inclusion criteria was extended to families with a clustering of CRC, or other HNPCC related cancer, with at least three affected family members (age not restricted), or at least two family members if the age of onset was below 50 years with pedigrees suggestive of autosomal dominant inheritance, or an individual with CRC diagnosed less than 35 years – similar to the Bethesda criteria. Probands were contacted with information regarding the study, and a consent form with a stamped addressed envelope (SAE) to return if they wished to participate. One hundred and forty-three participants in total were recruited to this cohort 35 meeting the Amsterdam I, 6 Amsterdam II, and 102 classified as 'HNPCC like'.

Patients with a history of EC (back to 01/01/99) were identified by means of a pathology coding database covering all patients from the Eastern Health Board in Northern Ireland and recruited consecutively.

All potential participants were contacted with a participant information sheet, with a detachable reply slip, to be returned in the enclosed SAE, for those keen to participate. For patients with returned reply slips, a clinic appointment was offered to discuss the study with genetic counselling, and obtain medical details, family history, informed consent and a blood sample for DNA extraction. One hundred and eighty-eight potential participants were contacted, and one hundred and twenty-five participants were recruited to this cohort. Age ranged from twenty-six to eighty-four, with a mean age of 58.9 years. Mean body mass index (BMI) in this cohort was 30.65, ranging from 18.64-53.15.

MSH6 Sequencing

All DNA was or had been extracted by the Nucleic Acid Extraction Centre (NAEC), Belfast City Hospital, and stored at -80˚C. Working dilutions in X1 TE were made at 5ng/µl.

All ten exons and at least 20 base pair (bp) of flanking intronic sequence was subject to direct sequencing analysis. PCR products for nine of ten exons of MSH6 were obtained using Applied Biosystems VariantSEQr™ Requencing System (product number: RSS000012234_02). All reactions were carried out using standard reaction mix and conditions as determined by ABI. Exon 1 primers were as follows:

TABLE I:

Number of mutations identified in each cohort

	HNPCC (115)	95% CI	Endometrial (105)	95% CI	Total 220
Truncating	3 (2.6%)	0.5% - 7.4%	4 (3.8%)	1.0% - 9.5%	7 (3.2%)
Missense	5 (4.4%)		3 (2.9%)		8 (3.6%)
Total	8 (7.0%)	3.1% - 13.2%	7 (6.7%)	2.7% - 13.3%	15 (6.8%)

1F PCR; TCCGTCCGACAGAACGGTTG, 1R PCR; ATGCTCCAGACTCGACCCG, using a standard 25µl reaction mix with 3.4µl of 25mm $MgCl_2$ and 0.4µl of 5U/µl Expand DNA polymerase (ABI) at an annealing temperature of 60°C.

All PCR products were subject to clean up using ExoSAP-IT®† to remove excess primer dimer, unincorporated dNTPs, and non-specific DNA products. Sequencing reactions were carried out using BigDye® terminator Ready Reaction Mix v1.1 from ABI®, according to manufacturer's instructions.

Additional primers were required to sequence exons 7 and 8 because of a poly T at the 5′ end of exon 7, which resulted in slippage during the sequencing reaction, and polymorphisms situated at the 3′, (c.3646 +35_38delATCT) of exon 7, and 5′, (c.3647 -51_-35 del 17), and 3′ (c.3802-42insT) end of exon 8, which made sequence of the exons unreadable when the polymorphisms were present in the heterozygous state. Additional sequencing primers for exon 7 (7F Seq; TTGTGATTTTTTTTTTTTAAG, 7R Seq; TAGTCTTCAAATGAGAAG) and 8 (8F Seq; GAGTTACTTCCTTATGCA , 8R Seq; GAAGTGCCCTCTCAAAAAACC) were designed. Electrophoresis was carried out by the Queen's University Belfast genomic core facility on an ABI 3730 DNA analyser.

MLPA Analysis

All samples were subject to MLPA analysis using SALSA MLPA KIT POO8 MSH6/PMS2 from MRC-Holland. Reaction mix and conditions are as determined by MRC-Holland. Electrophoresis was carried on an ABI 3100 Avant DNA analyzer using a GeneScan™ - **500 ROX**™ size standard. From the raw data generated, peak heights of each amplification product were exported to Excel worksheets designed by Dr Andrew Wallace, National Genetics Reference Laboratory, Manchester, so that the result of each sample could be 'normalised'.

Statistical Methods

The cohort sizes necessary to obtain estimates with adequate precision were initially calculated using a Normal approximation to the binomial sampling distribution provided by the StatCalc program in the EpiInfo package (http://www.cdc.gov/EpiInfo/). The Stata package (http://www.stata.com) was used to give the exact binomial confidence limits for a proportion (Table I).

RESULTS

MSH6

Good quality sequence was obtained for all 10 exons of MSH6 in 220 participants, 115 from HNPCC cohort, and 105 from endometrial cohort. Results with exact binomial confidence limits for a proportion are shown in table I. Given that the pathogenicity of the missense mutations identified has yet to be determined, arguably the proportion of the truncating mutations is more relevant.

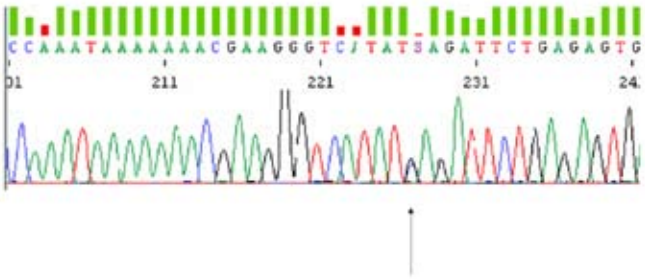

Fig 1. Sequencing analysis showing a truncating mutation c.755 C>G (p.Ser252X) in exon 4 of MSH6

All sequences were viewed with reference to a control sample (with a known mutation in MLH1 or MSH2), and reference sequences (www.ensemble.org, www.ncbi.nlm.nih.gov). All variants are described with reference to den Dunnen *et al*[26].

TABLE II:

Truncating mutations identified

Nucleotide Change	Protein Change	Mutation	Exon	Cohort	Classification
c.642 C>A	(p.Tyr214X)	Truncating	4	HNPCC	HNPCC like
c.755 C>G (figure 1)	(p.Ser252X)	Truncating	4	EC	HNPCC like
c.755 C>G	(p.Ser252X)	Truncating	4	EC	Not HNPCC
c.3103 C>T	(p.Arg1035X)	Truncating	4	EC	HNPCC like
c.3261 delC	(p.Pro1087Pro fs X3)	Truncating	5	HNPCC	HNPCC like
c.3840_3846delGGAGACT	(p.Gln1280_Thr1282>GlnfsX45)	Truncating	9	HNPCC	HNPCC like
c.3938_3941dupTTCA	(p.Gln1314HisfsX14)	Truncating	9	EC	Amsterdam II

Table III:

Missense variants identified

Nucleotide Change	Protein Change	Polyphen prediction	Exon	Cohort	Classification
c.663 A>C	(p.Glu221Asp)	Benign	4	EC	HNPCC like
[c.866 G>A]+[c.867C>A]	[p.Gly289Asp]+[p.Gly289Gly].	Benign	4	HNPCC	HNPCC like
c.1508 C>G	(p.Ser503Cys)	Possibly damaging	4	HNPCC	Amsterdam I
c.1508 C>G	(p.Ser503Cys)	Possibly damaging	4	EC	Not HNPCC
c.1739 C>T	(p.Ser580Leu)	probably damaging	4	HNPCC	HNPCC like
c.3217 C>T	(p.Pro1073Ser)	Benign	5	HNPCC	Amsterdam II
c.3929 G>C	(p.Glu1310Asp)	Benign	9	HNPCC	HNPCC like
c.3963 A>T	(p.Arg1321Ser)	possibly damaging	9	EC	HNPCC like

All truncating and missense variants identified are shown in tables II and III (example figure 1) with their corresponding cohort and family classification. Participants from the endometrial cohort with a variant identified were also classified according to Amsterdam I, II, 'HNPCC like', or, a family history that was not in keeping with HNPCC. All missense variants were subject to analysis by PolyPhen (www.coot.embl.de/PolyPhen/), a tool that predicts the potential impact of an amino acid substitution on the structure and function of a human protein (see table III for predictions). Further population, family and functional studies were not carried out to evaluate the missense variants but further work is planned.

MLPA

All samples underwent MLPA analysis. Out of 268 successful MLPA results, no aberration in copy number was identified.

DISCUSSION

Fifteen variants in all were identified from the two cohorts, seven of which resulted in premature STOP codon (truncating mutations), and were therefore considered pathogenic. A further eight missense mutations were identified, of which the functional significance is not known at this time. A summary of results is shown in table I. At the time of study design, only estimates of total prevalence were available[16,25] and the cohort sizes actually attained in the study were smaller than planned. This is reflected in lower precision (wider confidence limits) in the estimates than had originally been specified.

HNPCC Cohort

A definite pathogenic mutation was identified in 2.6% of the HNPCC cohort. These results are comparable with other studies carried out on Amsterdam, Amsterdam II and 'HNPCC like' families – estimates range between 2% and 5% - who have a germline mutation in MSH6[15,17,18]. Further work on the missense variants is required to determine their pathogenicity, as the yield of MSH6 mutations in the HNPCC cohort could increase up to 7% if these are found to be significant.

Endometrial cohort

A truncating germline mutation was identified in 3.8% of the endometrial cohort. This is higher than that obtained for the HNPCC cohort of whom the majority were 'HNPCC like', thereby broadening the criteria to fit with the described characteristics of a MSH6 phenotype[15,21]. This prevalence figure is also likely to increase following further work (including immunohistochemistry), being carried out on the missense variants as some of these may be pathogenic.

There are few studies looking at MSH6 in EC. Comparison can be difficult to make between studies because of pre-selection of some study groups of EC patients by age restriction or tumour microsatellite instability. Goodfellow[26] estimated the minimum prevalence of inherited MSH6 mutations in EC to be 1.6%, from a sub-population of an EC cohort, selected for molecular analysis, the majority of which showed tumour microsatellite instability (MSI). A comparable figure is seen by Berends[23] one MSH6 mutation identified in a cohort of 58 EC patients diagnosed less than 50 years whose families fulfilled the Amsterdam II criteria. Higher figures of 4.7%[24], and 8.3%[25], have been observed in EC cohorts not restricted by age or the limitations of the Amsterdam II criteria, but where the majority of tumours exhibit MSI, although the significance of the latter study will be limited by its relatively small cohort.

As well as the heterogeneous populations studied, the variability in frequency of MSH6 mutations in both HNPCC and endometrial cohorts can also be accounted for by the sensitivity of techniques used to identify variants, the use of MSI and IHC to target molecular screening of MMR genes, and the interpretation of missense mutations which occur relatively frequently in MSH6[23]. Founder mutations in certain populations can also contribute to higher than average prevalence rates of MMR genes[12]. Further work on functional, population and family studies is required to determine the pathogenicity of the eight missense mutations identified.

Genomic Rearrangements

Genomic rearrangements account for between 17%[28] to 54.8% of pathogenic mutations in MLH1 and MSH2 in HNPCC families[29]. The prevalence of genomic rearrangements in MSH6 is less well studied, but it had been estimated that rearrangements may account for 10-20% of mutations in MSH6[30]. No genomic rearrangements were identified in our HNPCC cohort consistent with findings by Charbonnier[31]and Wagner[18]. Likewise genomic rearrangements were not detected in any of our EC patients in keeping with findings by Ollikaninen[32]. Studies that have identified MSH6 genomic rearrangements have been particularly large HNPCC

TABLE IV:

Classification of families with a MSH6 variant identified

	Truncating	Missense
Amsterdam I	0	1
Amsterdam II	1	1
'HNPCC like'	5	5
Not HNPCC	1	1

cohorts[33], or EC cohorts pre-selected by MSI[24], yielding a genomic rearrangement in MSH6 in less than 1% of the chosen population.

Promoter Region

Sequencing is highly sensitive for detection of mutations in the coding regions; however the promoter region of MSH6 was not sequenced in this study.

Previous studies have mainly concentrated on coding regions, and exonic / intronic boundaries. Two deletions of the MSH6 promoter region have been described in HNPCC families[22,33], but other studies looking at the promoter region of MSH6 in EC cohorts[27] or in HNPCC patients negative for a mutation in MLH1 or MSH2[17], did not identify any pathogenic mutations. Studies looking specifically at the promoter region of the more prevalent MMR genes, MLH1 and MSH2, identified three possible pathogenic mutations in the promoter area in 141 HNPCC patients and patients with early onset CRC (<45 years)[34]. Given that MSH6 mutations occur at a relatively low rate in both HNPCC and EC patients, we can assume that mutations of the promoter region in either cohort are unlikely to significantly alter the prevalence figures calculated from this study.

Redundancy of MSH6 mutations

Although germline mutations in MSH6 are distributed throughout the length of MSH6 displaying little redundancy, the majority of pathogenic mutations identified are in exon 4[15], with fifty-seven percent of truncating mutations identified in this study (4/7) occurring in exon 4, the largest of MSH6 exons, indicating that analysis of MSH6 in HNPCC families without a known mutation should commence at exon 4.

MSH6 phenotype

None of the truncating mutations identified in this study met the original Amsterdam criteria, the majority having a 'HNPCC like' phenotype, with a later age of cancer onset, and non-penetrance in family members (Table IV). This further supports current evidence that application of the Amsterdam criteria to HNPCC families to select for molecular testing will result in a significant proportion of MSH6 mutations being missed[15,22].

Previously unidentified HNPCC families

Eighteen out of 125 participants (14.4%) from the endometrial cohort had a significant previously unidentified HNPCC phenotype. Five of these participants had a variant identified, three truncating mutations and two missense mutations. These findings are in keeping with findings from other studies where previously unidentified HNPCC families have been ascertained through an individual with EC[23]. Increased awareness of HNPCC and other hereditary cancer syndromes amongst physicians/surgeons directly involved with the care of cancer patients such as gynaecologists, surgeons, oncologists, and general practitioners is essential for their identification.

Endometrial Cancer

HNPCC, traditionally identified as a condition with a genetic predisposition to CRC, has now been recognised as conferring a significant risk of EC to females, particularly those with a mutation in MSH6. In addition to other Mendelian inherited syndromes with a predisposition to EC such as Muir Torré, Cowden and Turcot syndrome, there are families who show a clustering of EC alone that do not have an identifiable molecular basis. Un-identified genes or predisposing low penetrant polymorphisms may contribute. The importance of environmental factors conferring a risk to the development of EC cannot be underestimated. Obesity is associated with increased levels of endogenous oestrogens, and is a significant risk factor for the development of EC. Mean BMI in the endometrial cohort was 30.65 and ranged from 18.64-53.15, with only 28 (22.4%) of participants having a BMI within the normal range (<25). In comparison it is estimated that 44% of UK females over 16 years have a BMI within the normal range. (Figures published by the Department of Health and estimated by the Health Survey for England 2003; www.dh.gov.uk).

The mean BMI for the four participants from the endometrial cohort with a truncating mutation in MSH6 was 26.2, ranging from 22.36-31.05. One of these participants had a BMI in the overweight range, and one had a BMI in the obese range. It is likely that obesity has an additive effect to the underlying risk form a MMR mutation, but larger studies would be required to determine this.

CONCLUSION

From this study we have identified the minimum prevalence of pathogenic mutations in MSH6 to be higher in an unselected cohort of EC patients, than a cohort of HNPCC patients who have been selected by criteria 'widened' from the traditional Amsterdam II criteria, in keeping with the described phenotype of MSH6. Unlike the other more common MMR genes, genomic rearrangements do not contribute to a large proportion of mutations in MSH6.

HNPCC families may not be identified if patients present with HNPCC associated cancers, such as gynaecological cancer, rather than the more commonly recognised phenotype of CRC. Clinicians should be vigilant to this possibility when presented with a history of endometrial cancer in young women. Further work on immunohistochemistry of possible missense variants may increase the true frequency of mutations in MSH6.

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