

ONLINE MUTATION REPORT

Germline *MSH2* and *MLH1* mutational spectrum in HNPCC families from Poland and the Baltic States

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Hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) is an autosomal dominantly inherited syndrome predisposing to the early development of cancers of the colon, rectum, endometrium, small bowel, and urinary tract and accounts for ~5% of all colon cancer cases.¹ There are at least five genes involved in this cancer predisposition and they include *MLH1*,² *MSH2*,³ *MSH6*,⁴ *PMS2*, and *PMS1*.⁵ Currently, more than 300 different mutations have been described in these genes which account for approximately 500 HNPCC kindreds from different parts of the world.⁶ *MLH1* and *MSH2* genes show abnormalities in more than 90% of HNPCC families with identified germline mutations⁷ (<http://www.nfdht.nl>). The majority of reported *MLH1* and *MSH2* mutations are dispersed throughout the 35 exons of these two genes. However, some changes are recurrent and are described as founder mutations in particular populations.⁸⁻¹¹ In order to develop efficient DNA testing, it is important to describe the nature and frequency of mutations that are characteristic of particular ethnic groups. The *MSH2* and *MLH1* mutation spectrum has not been investigated in the eastern European region and therefore there is no knowledge about any recurrent mutations which may significantly aid in the mutation screening procedures for this region. Here, we describe the results of DNA/RNA based mutation sequencing of both the *MSH2* and *MLH1* genes in a series of HNPCC families from Poland (89 cases) and the Baltic States (12 cases).

MATERIAL AND METHODS

Patients

A total of 101 unrelated patients affected by colorectal cancer or an HNPCC associated cancer (endometrium, small bowel, urinary tract) were from 17 families which fulfilled the Amsterdam II criteria¹² and from 84 families matching our modified criteria of suspected HNPCC, one colorectal cancer patient with a first degree relative affected by an HNPCC associated cancer, one of whom was diagnosed under the age of 50 years.¹³ The clinical diagnosis of HNPCC was established or verified at the Hereditary Cancer Centre, Pomeranian Academy of Medicine, Szczecin, Poland. Patients used for this study were ascertained from the following regions: Bydgoszcz (3), Gdańsk (3), Kielce (14), Kraków (3), Legnica (1), Lublin (2), Łódź (1), Olsztyn (13), Poznań (7), Riga (3), Szczecin (33), Tartu (3), Wrocław (5), Vilnius (6), Zielona Góra (4).

DNA isolation

Peripheral blood samples were collected from the patients after obtaining informed consent. DNA was extracted directly from leucocytes by the classical phenol purification method or as described previously.¹⁴

Key points

- The DNA mismatch repair genes *MSH2* and *MLH1* account for a major proportion of hereditary non-polyposis colorectal cancer (HNPCC) families. One approach by which development of an efficient DNA testing procedure can be implemented is to describe the nature and frequency of common mutations in particular ethnic groups.
- We screened 101 HNPCC kindreds from Poland and the Baltic States fulfilling the Amsterdam II diagnostic criteria or suspected HNPCC criteria for mutations in *MSH2* and *MLH1*. Twenty different pathogenic mutations were found, nine in *MSH2* and 11 in *MLH1*. Nine of these had not previously been described. Among families which fulfilled the Amsterdam II criteria, mutations were identified in 59%, and in the remaining kindreds 29% were found to harbour a change in the two genes. Recurrent mutations were found in 50% of examined families with mutations. Two of the most frequent were a substitution of a for t at the splice donor site of intron 5 of *MSH2* and a missense change (A681T) in *MLH1* found in seven and six families, respectively.
- Our results in conjunction with others indicate that a screening system limited to the detection of all reported mutations will allow the identification of the majority of small changes present in coding and flanking intronic regions of *MLH1* and *MSH2* in Polish HNPCC kindreds.

DNA sequencing

All exons and exon-intron junctions of *MLH1* and *MSH2* were amplified using the same protocols as described previously¹⁵ with the same primer sequences as described by Wijnen *et al*^{16, 17} for DGGE but without the M13 and GC clamp sequences at the 5' end. Dye terminator cycle sequencing reactions were performed using the ABI PRISM Dye-terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) according to the manufacturer's recommended protocol. Semi-automated fluorescence analysis was performed using a 373 A DNA Sequencer (ABI, Perkin-Elmer).

RNA based sequencing

RNA sequencing template was used for 15 patients in whom mutations had not previously been detected by genomic DNA based sequencing. Total RNA was extracted from fresh lymphocytes using Trizol (Gibco BRL, Life Technologies Inc,

Table 1 Polish and Baltic States families with *MSH2* and *MLH1* germline mutations and rare exonic variants of unknown significance

Case No/family ID	Gene/exon or intron change	Position of nucleotide	Consequence
1/9154	<i>MSH2</i> /2	c.del273TCT	92delL
2/1524	<i>MSH2</i>/3	c.613G>T	E205X
3/1882	<i>MSH2</i> /SD5	c.942+3a>t	In frame del exon 5
4/4814	<i>MSH2</i> /SD5	c.942+3a>t	In frame del exon 5
5/5888	<i>MSH2</i> /SD5	c.942+3a>t	In frame del exon 5
6/6797	<i>MSH2</i> /SD5	c.942+3a>t	In frame del exon 5
7/LT7*	<i>MSH2</i> /SD5	c.942+3a>t	In frame del exon 5
8/15105	<i>MSH2</i> /SD5	c.942+3a>t	In frame del exon 5
9/17237	<i>MSH2</i> /SD5	c.942+3a>t	In frame del exon 5
10/1296	<i>MSH2</i> /7	c.1216C>T	R406X
11/6474	<i>MSH2</i> /7	c.1216C>T	R406X
12/7167	<i>MSH2</i>/SD10	c.1661+5g>c	Out of frame del exon10
13/6609	<i>MSH2</i>/12	c.1771-1772insA	Frameshift
14/6506	<i>MSH2</i> /13	c.2131C>T	R711X
15/6810	<i>MSH2</i>/SD13	c.2210+1g>c	Out of frame del exon13
16/3213	<i>MSH2</i>/14	c.2388delT	Frameshift
17/2796	<i>MLH1</i>/1	c.37delG	Frameshift
18/7640	<i>MLH1</i> /1	c.83C>T	P28L
19/6353	<i>MLH1</i> /1	c.83C>T	P28L
20/8162	<i>MLH1</i> /2	c.184C>T	Q62X
21/2575	<i>MLH1</i> /4	c.350C>T	T117M
22/2683	<i>MLH1</i>/4	c.356-357insAA	Frameshift
23/4370	<i>MLH1</i> /SD8	c.677G>T	Splice
24/4102	<i>MLH1</i>/10	c.883delAGgt	Out of frame del exon10
25/6774	<i>MLH1</i> /SD12	c.1409+1g>c	Splice
26/7576	<i>MLH1</i> /13	c.1489-1490insC	Frameshift
27/7848	<i>MLH1</i>/14	c.1668delCCA	553delT
28/6434	<i>MLH1</i> /15	c.1672G>T	E558X
29/1821	<i>MLH1</i> /18	c.2041G>A	A681T
30/1881	<i>MLH1</i> /18	c.2041G>A	A681T
31/7266	<i>MLH1</i> /18	c.2041G>A	A681T
32./6802	<i>MLH1</i> /18	c.2041G>A	A681T
33/8998	<i>MLH1</i> /18	c.2041G>A	A681T
34/ LT-11*	<i>MLH1</i> /18	c.2041G>A	A681T
35/6609	<i>MSH2</i> /15	c.2558A>C	E853A pathogenic?
36/ A1127†	<i>MLH1</i> /1	c.55A>T	I19F pathogenic?
37/9855	<i>MLH1</i> /10	c.875T>C	L292P pathogenic?
38/10388	<i>MLH1</i> /10	c.875T>C	L292P pathogenic?
39/3412	<i>MLH1</i> /18	c.2059C>T	R687W pathogenic?

*From Lithuania.

†From Estonia.

Bold: mutations not found previously in other populations.

Table 2 *MSH2* and *MLH1* polymorphisms in HNPCC families

Gene/exon or intron	Position of sequence alteration	Consequence	Frequency of heterozygous cases
<i>(A) Frequent polymorphisms</i>			
<i>MSH2</i> /1	c.211+9c/g	No consequence – polymorphism	32/101
<i>MSH2</i> /6	c.965A/G	G322D	19/101
<i>MSH2</i> /9	c.1511-9t/a	No consequence – polymorphism	14/101
<i>MSH2</i> /10	c.1661+12g/a	No consequence – polymorphism	33/101
<i>MLH1</i> /8	c.655A/G	I219V	31/101
<i>MLH1</i> /13	c.1558+14a/g	No consequence – polymorphism	5/101
<i>MLH1</i> /14	c.1668-19a/g	No consequence – polymorphism	43/101
<i>(B) Rare polymorphisms-silent mutations</i>			
Family ID	Gene/exon	Position of nucleotide change	
PK2801	<i>MSH2</i> /7	c.1224T>C	
WB8028	<i>MSH2</i> /10	c.1563T>C	
CE6219	<i>MLH1</i> /18	c.2040C>T	
LM2328	<i>MLH1</i> /19	c.2323T>A	

Gaithersburg, MD). cDNA was synthesised with c therm polymerase (Roche Diagnostics) using 0.5 µg total RNA and specific primers complementary to the 3' end of *MLH1* and to the 3' end of *MSH2*. PCR products were size fractionated by agarose gel electrophoresis. Sequencing of the cDNA was as described previously.¹⁸

RESULTS

Unequivocal mutations were detected in 34 families; 16 mutations were identified in *MSH2* and 18 mutations in *MLH1*. Among families which fulfilled the Amsterdam II criteria mutations were identified in 59% of kindreds. There were three families that harboured *MSH2* changes and seven

families associated with *MLH1* gene mutations. From the patients matching our modified criteria of suspected HNPCC, mutations were detected in 29% of families, 13 in *MSH2* and 11 in *MLH1*.

Recurrent mutations were identified in 17 (50%) families with mutations. Two of the most frequent changes were a substitution of A to T in the splice donor site of intron 5 of the *MSH2* gene, which represents the most frequent *MSH2* mutation reported⁸ and a missense change, a A681T in *MLH1*, found

in seven and six families, respectively. Mutations not described previously in other populations were found in nine families (highlighted in table 1, fig 1). The pathogenic nature of the g to c change at 1661+5 was confirmed at the RNA level by identifying an aberrant transcript which was the result of exon 10 skipping which resulted in the creation of stop codon. RNA based sequencing resulted in the detection of an aberrant *MLH1* transcript which was the result of a loss of exon 16, but no change could be identified in genomic DNA.¹⁸ In addition to

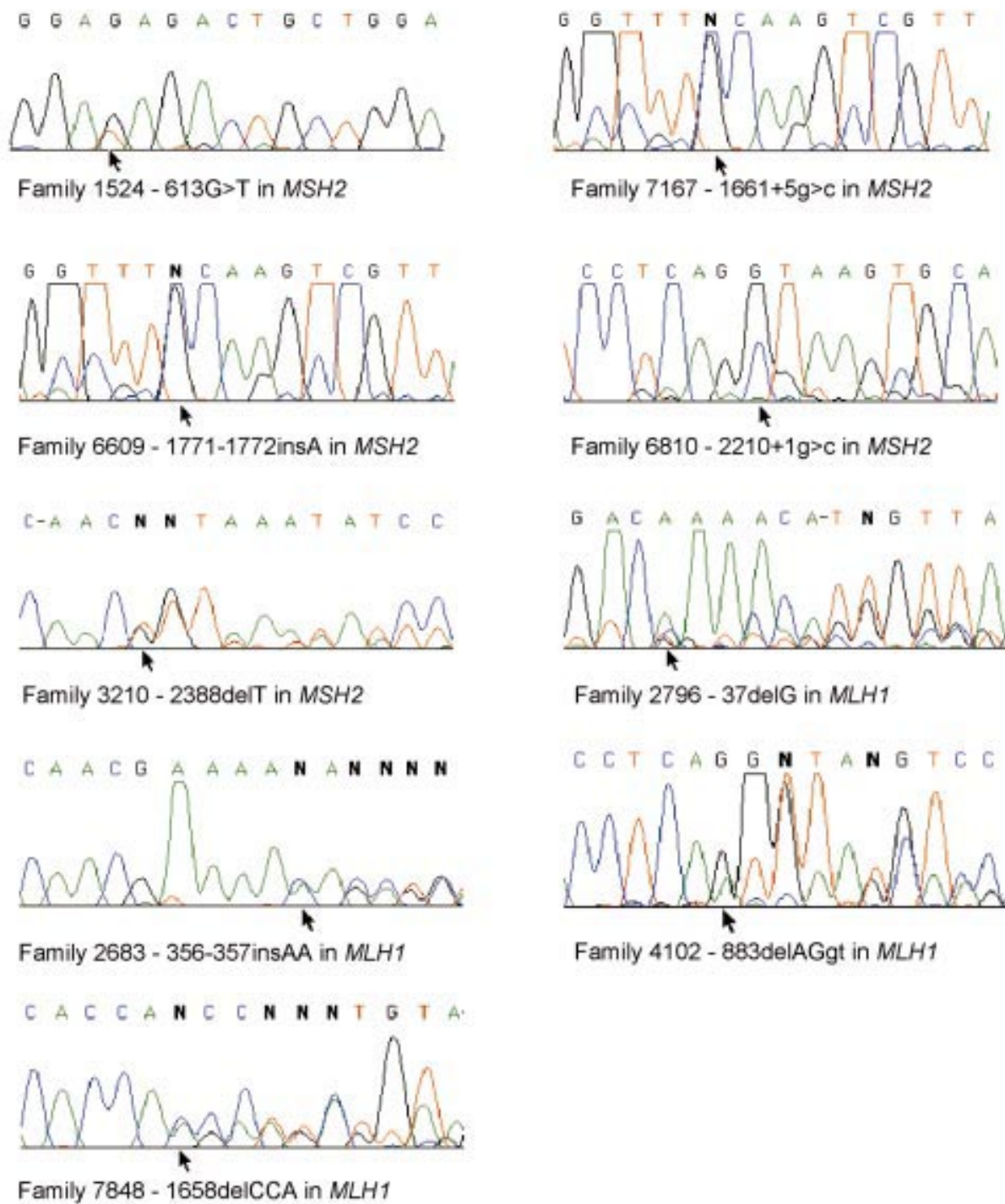


Figure 1 *MLH1* and *MSH2* mutations not described in other populations. Sequence chromatograms of cases 2, 12, 13, 15, 16, 17, 22, 24, and 27.

Table 3 Characteristics of novel missense alterations of unknown significance

Missense alterations (amino acid substitution)	Gene	Change polarity	Location in functional domain	Absence in general population*	Matched AMS or sHNPCC criteria	Cosegregations with disease
E853A	<i>MSH2</i>	Yes	?	Yes	sHNPCC(+)	No
I19F	<i>MLH1</i>	No	Yes - ATPase	Yes	sHNPCC(+)	NE
L292P	<i>MLH1</i>	No	Yes - ATPase	Yes	sHNPCC(+)	NE
R687W	<i>MLH1</i>	Yes	Yes - potentialy PMS2 binding	Yes	sHNPCC(+)	Yes

*Alteration not found in any of 100 normal controls.
NE, not examined.

unequivocal mutations, sequence variants of uncertain pathological significance were detected in five families. One of these alterations (c.875T>C) occurred in two patients (table 1). Frequent and rare *MSH2* and *MLH1* polymorphisms are summarised in table 2.

A summary of novel missense alterations of unknown significance is shown in table 3.

DISCUSSION

The appropriate management of patients from HNPCC families decreases the risk of cancer and results in a better life expectancy. The identification of HNPCC families remains problematical, as the Amsterdam II criteria which rely on pedigree and clinical data are very restrictive. Indeed the above Amsterdam II criteria cannot be matched in the majority of families with *MSH2* or *MLH1* mutations. At present, the only method to diagnose HNPCC unequivocally in suspected cases of this disorder is to identify constitutional mutations in the genes associated with this disorder. The most accurate method of detecting germline mutations are molecular analyses using either DNA or RNA templates for DNA sequencing analysis. These techniques are complex, time consuming, and expensive. Given the limited resources for routine mutation screening, it is essential to develop DNA testing protocols further. One of the most efficient ways of achieving this is to describe the nature and frequency of population specific mutations in order to target those changes first. From the results presented here, it is apparent that two mutations (a to t substitution in the splice donor site of intron 5 of the *MSH2* gene and missense A681T in *MLH1*) account for about 40% (12/34) of mutations identified in Polish HNPCC families. Two other mutations (1216C>T in *MSH2* and 83C>T in *MLH1*) occurred in two families (one of which has not previously been reported) and eight mutations (del273TCT, 2131C>T in *MSH2* and 184C>T, 350C>T, 667G>T, g>c at 1409+1, ins C at 1490, and 1672G>T in *MLH1*) were observed previously in other populations. The remaining nine mutations (613G>T, g>c at 1661+5, ins A at 1772, g>c at 2210+1, delT at 2388 in *MSH2* and delG at 37, ins AA at 356, delAGgt at 883, delCCA at 1658 in *MLH1*) were found to be potentially specific for Polish families as they have not previously been described. Therefore, the characteristics of mutations identified from Poland and the Baltic States suggest that it is worthwhile to develop mutation testing focusing on these changes as a first approach. The frequency of mutations in families that fulfilled the Amsterdam II criteria was approximately 60% and in families suspected of HNPCC around 30%, which is comparable with the frequency of mutations identified by other authors for different populations.¹⁹ Therefore, it can be predicted that the sensitivity of testing of this population for the reported mutations could be very high. Certainly, the sensitivity of this testing approach will not achieve 100%. False negative results could potentially occur in families with missense mutations. In our series, more than 10% of *MSH2/MLH1* mutations involved such an amino acid substitution (table 3). The missense alteration 2558A→C at codon 853 in exon 15, which resulted in a substitution of glutamic acid

for alanine, seems to be a neutral rare polymorphism as it did not cosegregate with the disease in the family. In spite of the location in important functional domains in the *MLH1* protein, two missense alterations, I19F and L292P, could not be exclusively associated with the disease as they may represent polymorphisms. The missense alteration 2059C→T at codon 687 in exon 18 that resulted in substitution of arginine for tryptophan appears to be a pathogenic alteration. The sensitivity of finding *MSH2/MLH1* mutations can be further improved by the detection of large genomic deletions or rearrangements. Their contribution may be as high as 36% of all *MSH2* mutations according to Dutch HNPCC mutation analysis.²⁰ *MLH1* mutations are mainly single nucleotide changes. The only country with a high proportion of *MLH1* deletion is Finland, where a founder effect associated with a 3.5 kb deletion encompassing exon 16 has been identified.¹⁰ Our studies of RNA transcripts suggest that the frequency of large intragenic deletions without involvement of the 5' and 3' ends of the *MSH2* or *MLH1* genes is low in the population studied here. However, more extensive studies are necessary in order to describe the actual proportion of such changes among HNPCC families in this population. Loss of exon 16 detected at the RNA level may suggest the presence of a large genomic deletion. This is not, however, caused by the 3.5 kb genomic deletion observed frequently in Finland, since in experiments using long PCR with primers for exons 15 and 17 we observed only the product of normal length.¹⁸ Two of the most frequent mutations identified in Poland were also found in Lithuanian families, suggesting a common history. Poland and the Baltic States may have more common mutations than reported here since the number of samples from Estonia, Latvia, and Lithuania were too small to make the appropriate comparisons. In summary, it seems likely that the *MSH2* and *MLH1* changes described here are representative of the majority of HNPCC mutations in families from this region. Therefore, we believe it is justified to develop a DNA testing strategy based on the preferential analysis of changes identified from this population.

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Databases: *MLH1* OMIM: 120436; GDB: 249617; GenbankNW_000249; HGMD: *MLH1* <http://www.nfdht.nl/database/mlh1.htm> (hMLH1 Mutations Database). *MSH2* OMIM: 120435; GDB: 203983; GenBank:NM_000251; HGMD: *MSH2* <http://www.nfdht.nl/database/msh2.htm> (hMSH2 Mutation Database).

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