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ARTICLE

Detection of mutations in mismatch repair genes in Portuguese families with hereditary non-polyposis colorectal cancer (HNPCC) by a multi-method approach

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Mutation searching was performed in the *hMSH2* and *hMLH1* genes in 20 Portuguese families representing 124 registered affected individuals. Of the 20, 16 fulfilled the classic 'Amsterdam' criteria for HNPCC, whereas the remaining four families satisfied a modified set of criteria. These criteria required a CRC diagnosed before age 50 years and cancers diagnosed in two other relatives within the HNPCC spectrum. A multi-method approach was performed using the protein truncation test (PTT), single strand conformation polymorphism (SSCP) with two different sets of conditions, heteroduplex analysis (HA) and denaturing gradient gel electrophoresis (DGGE). Putative phenotype–genotype correlations were also explored. Ten different germline mutations were identified. Six of these were found in *hMLH1* in seven families and four in *hMSH2* in four families. SSCP and DGGE had the highest diagnostic yields with the percentage of variants detected above 67% and together HA and PTT had the lowest. No single technique detected all variants. Trends for the absence of extracolonic manifestations were observed in families carrying *hMLH1* germline mutations (four of seven in *hMLH1 vs* one of four in *hMSH2*). Most of the families with rectal cancer were associated with *hMLH1* (six of seven in *hMLH1 vs* two of four in *hMSH2*). A multi-technique approach is necessary to identify a high percentage of germline mutations. Seven novel mutations were found in this Portuguese population. *European Journal of Human Genetics* (2000) **8**, 49–53.

Keywords: mutation detection; hereditary non-polyposis colorectal cancer; single strand conformation polymorphism; heteroduplex analysis; denaturing gradient gel electrophoresis; protein truncation test

Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is responsible for 1–5% of all new colorectal cancer cases and is probably the most frequent hereditary cancer syndrome.[1] Besides familial aggregation, it tends to be associated with young age of onset and a proximal location of colon cancer.[2] Furthermore, affected relatives tend to present a multiplicity of colon tumours and extra-colonic malignancies. In recent years, five DNA mismatch repair genes – *hMSH2*,[3] *hMLH1*,[4,5] *hPMS1*, *hPMS2*[6] and *hMSH6*[7] – were shown to be the genetic determinants of HNPCC. Of these, *hMSH2* and *hMLH1* have been associated with the vast majority of HNPCC families.[8]

Unlike familial adenomatous polyposis (FAP), which is characterised by hundreds of polyps in the colon, there is no reliable clinical marker for HNPCC diagnosis. This led to the International Collaborative Group (ICG-HNPCC) choosing empirical requirements to recruit suspect kindreds with

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HNPCC for gene mapping studies: the Amsterdam criteria.[9] Such families have three cases of colorectal cancer, one of which must be diagnosed earlier than 50 years of age, in more than one generation and one case must be a first degree relative of the other two. Many families with a strong history of dominantly inherited colorectal cancer do not satisfy these stringent criteria but they remain an important group of patients for mutation detection.[10,11] To maximise the yield of mutations several molecular diagnostic methods have been used including protein truncation test (PTT),[12] single strand conformation polymorphism (SSCP)[13,14] heteroduplex analysis (HA)[15] and denaturing gradient gel electrophoresis (DGGE).[16]

The present study was designed to detect germline mutations in a distinct European population and to investigate the ability of different methods to detect these mutations.

Materials and methods

Subjects

Twenty-four affected members from 20 Portuguese HNPCC families were included in this study. Sixteen families fulfilled the classic Amsterdam criteria[9] and four fulfilled a modified version (Patrice Watson, personal communication, 1998). These modifications permit inclusion of families where there are three relatives with confirmed tumour diagnosis with the spectrum of HNPCC (one of them being a first-degree relative of the other two). The spectrum cancers include endometrial, small bowel, ureter and stomach cancer. One of the tumours must be a colorectal cancer diagnosed under the age of 50.

Phenotype characteristics and diagnostic confirmations were obtained through personal interviews and retrieving information from attending physicians, hospital records, death certificates and the Portuguese Oncology Registry. All index cases included in this study gave their written, informed consent to participate and to investigate family data.

Molecular methods

DNA was extracted from venous blood using the guanidine/HCl method.[17] Lymphoblastoid cell lines were established for RNA extraction, as described previously.[18] cDNA was synthesised by reverse transcription using Superscript (Gibco BRL, Paisley, UK) and amplified by RT-PCR.

PTT PTT assay was performed as described.[19,20] cDNA sequences of *hMSH2* and *hMLH1* were amplified in overlapping fragments using primers already reported.[21,22] The resulting products were then translated using the TNT7 Quick kit (Promega, Madison, WI, USA) and resolved by 12% SDS-PAGE.

SSCP Primers for PCR DNA amplification for the 16 *hMSH2* and 19 *hMLH1* exons have been described.[23,24] PCR products were tested under two sets of SSCP conditions. The first was 1 × MDE gel (FMC Bioproducts, Rockland, MD, USA) in 0.6 × TBE with 5% glycerol run at about 20°C, and the second was 8% acrylamide gel (Sigma, St Louis, MO, USA) (acrylamide:bisacrylamide, 49:1) in 0.6 × TBE run at 4°C. Following electrophoresis through a 24 cm long gel for 16 hours at 200 V, DNA bands were visualised by silver staining.

Heteroduplex analysis Fragments were resolved simultaneously with the SSCPs in MDE gels.

DGGE DGGE was performed using primers described.[25,26] To determine optical DGGE conditions, DNA melting behaviour was simulated using the MELT 87 program, provided by Dr Lerman.[27]

Sequence analysis In the Lisbon laboratory, DNA sequencing used primers as for DGGE but without the GC-clamp. The amplified fragment was purified using QIAquick PCR purification Kit Protocol (QIAGEN, Hilden, Germany). [γ-^{32}P]dATP end-labelled primer was used for cycle sequencing using the fmol DNA Sequencing System kit (Promega). The products were resolved in a 7% Long Ranger (JT Baker, Deventer, Netherlands) acrylamide gels containing 7 M urea.

In the Newcastle laboratory, sequencing reactions were carried out using T7 Sequenase v 2.0 PCR product sequencing kits (Amersham Life Science, Amersham, UK) and the products were run on 6% polyacrylamide, 7 M urea gels at 60 W.

Results

Clinical data

Data from 124 affected individuals were obtained, 51% were male. Besides colorectal cancer, 4% had stomach cancer, 3.5% brain tumours and 2.6% urinary tract cancer. Of females patient, 25% had endometrial cancer and 3.3% had ovarian cancer. Six families had no extra-colonic manifestations. Rectal cancer was documented in 45% of the families and in 14% of affected individuals. No differences were present regarding tumour diagnosis distribution between Amsterdam families and those with modified criteria. The mean age of diagnosis was the same in the Amsterdam and modified criteria families, 48.9 ± 15.8 and 48.5 ± 11.2 years respectively.

Molecular characterisation

A summary of the germline mutations found in 11 of the 20 families examined appears in Table 1.

Novel mutations In seven families we found seven mutations which have not been reported previously, (ICG-HNPCC database www.nfdht.nl).

A frameshift mutation at *hMSH2* codon 787 leading to premature protein truncation was found in family 22. Three novel nonsense mutations were seen, at *hMLH1* codon 721 in family 3, at *hMSH2* codon 183 in family 4 and at *hMSH2* codon 518 in family 19. In this last kinship, family 19, a second variant *hMSH2* codon 322 glycine to asparagine was

observed which as been reported both as a pathological change[28] and as a polymorphism.[21,28,29]

Three missense variants were observed. In family 1 at *hMLH1* codon 659 there is an arginine to leucine change; in family 14 at *hMLH1* codon 607 there is a leucine to histidine change; and in family 24 a methionine replaces a valine at *hMLH1* codon 213.

Other mutations Three germline mutations identified in these Portuguese patients have been reported previously. Family 42 has a splice site mutation at *hMSH2* intron 5 which causes an in-frame deletion of exon 5.[30] Families 2 and 8 share the same nonsense mutation at *hMLH1* codon 488. In family 28, an AAG→GCG base change at *hMLH1* codon 618 results in a lysine to alanine substitution (ICG-HNPCC database www.nfdht.nl).

Polymorphisms were also revealed in *hMLH1* exons 8[21] and 17[31] and introns 13[32] and 14,[21,31] and *hMSH2* intron 1[33] and exon 6[21,29] and intron 13.[34] In this small group of individuals these variants occurred at frequencies comparable with other populations.

Discussion

Nature and distribution of mutations

In the present study, 10 different germline mutations were identified in 11 out of 20 families, seven of these have not been reported previously. From this study the spectrum of the mutations detected in the *hMLH1* and *hMSH2* genes appears to be very heterogeneous among these HNPCC patients, since only one mutation was found in more than one family.

Some mutations have become enriched in certain populations.[25] In contrast, in this small sample set, there is no evidence for a founder HNPCC mutation in these two genes in the Portuguese population. We found equal distribution of mutations in *hMLH1* (55%) and *hMSH2* (45%).

Our results also confirm that nonsense mutations occur preferentially in the *hMSH2* gene, whereas both missense and nonsense mutations occur in *hMLH1*.[8]

Mutation detection strategy

In the present study using a variety of techniques we were able to detect 17 variants, 10 of which are mutations and 7 polymorphisms. The informativeness obtained by the different methods used for mutation searching were 75% for SSCP using MDE gels, 88% for SSCP using 49:1 acrylamide–bisacrylamide, 19% for heteroduplex analysis and 50% for PTT. DGGE, performed in two different laboratories, detected overlapping sets of variants with success rates of 67% and 71%. By using both sets of conditions,[25,26] 94% mutations were detectable by DGGE, as shown in Table 1.

These data demonstrate the importance of a combination of more than one technique to increase the specificity and sensitivity of mutation detection.

SSCP and DGGE are shown to be highly sensitive techniques. Since these techniques show similar levels of informativeness then the decision as to which method to set up depends on factors such as the equipment available and the previous experience of each individual centre.

Regarding PTT and heteroduplex analysis, although their mutation detection rates are low they may have some value. In the case of heteroduplex analysis its simplicity should be considered and the fact that it can be resolved simultaneously with SSCP in MDE gels. Under these conditions, we often achieved a superior sensitivity in resolving mobility differences between homoduplex and heteroduplex strands,

Table 1 *hMLH1* and *hMSH2* germline mutations identified

Family No.	*Mutation*	*Nucleotide change*	*Location*	*Previously reported*	*Informative method*[a]
hMLH1					
1	R659L	G→T at 1976	EX17	no	SM, SA, D
3	Y721X	T→A at 2163	EX19	no	SM, P, D
14	L607H	T→A at 1820	EX16	no	SA, D
2, 8	R488X	C→T at 1459	EX13	yes (ICG[c])	SM, SA, D
24	V213M	G→A at 637	EX8	no	D[d]
28	K618A	AA→GC 1852	EX16	yes (ICG[c])	SM, SA, D
hMSH2					
4	Q183X	C→T at 547	EX3	no	SM, SA, P, D
19	G322D[b]	G→A at 965	EX6	yes (21, 28, 29)	SM, SA, D
19	Q518X	C→T at 1552	EX10	no	SA, P, D
22	2360insTT	ins TT at 2360	EX14	no	SA, H, D
42	IVS5+3A→T	A→T at 942 +3	IVS5	yes (30)	SM, SA

[a]SSCP MDE (SM); SSCP 49:1 (SA); HA on MDE (H); PTT (P); DGGE (D)
[b]we suggest that this is a polymorphism and not a pathological change (see text)
[c]ICG is the ICG-HNPCC database at www.nfdht.nl
[d]V213M was not tested by SSCP or heteroduplex analysis

when compared with conventional non-denaturing polyacrylamide gel electrophoresis. On the other hand, PTT has the advantage of requiring analysis of only two PCR products per gene and is quick and simple to use.[35,36] Of the three truncating mutations not detected by PTT, one (MSH2 IVS5 +3A→T) results in exon skipping. It is possible that this deleted mRNA species is unstable and not represented in the population amplified by RT-PCR. Similarly, a bias in expression may underlie the failure to detect the two truncating mutations (MLH1 R488X and MSH2 2360 insTT).

Because of the high frequency of missense mutations especially in *hMLH1* it is important to use methods which analyse DNA directly rather than the protein product. In this respect PTT, although attractive for a first screen, is not sufficient because it does not detect missense mutations. In addition PTT must be performed using mRNA from fresh pellets of lymphoblastoid cell lines, otherwise a significant number of alternatively spliced fragments will be found which could be misinterpreted as truncated products.[36]

Family selection criteria

Not only is the molecular genetic strategy for mutation detection important for achieving a high yield of mutations but also careful family recruitment. The success of mutation detection was high with both the Amsterdam families, nine mutations in 16 families, and the families conforming with the modified criteria, two mutations in four families. This rate of mutation detection accords with Scottish and Dutch studies.[11,37] Thus by enlarging the set of families studied with this modification ten additional 'at-risk' first degree relatives are identified for predictive testing who otherwise would have been missed.

Genotype–phenotype correlations

Genotype–phenotype correlations in HNPCC are not well defined. In this small set of families we observe a trend for the absence of extracolonic malignancies in families carrying *hMLH1* germline mutations (four of seven in *hMLH1* and one in four in *hMSH2*). Most of the families with rectal cancer were associated with *hMLH1* (six of seven in *hMLH1* compared with two of four in *hMSH2*).

Clinical implications

In contrast to the seven families which can be counselled based on mutations causing definitive changes in the *hMLH1* or *hMSH2* protein, we face the problem of three novel missense mutations in which the pathogenic nature is unclear.

In order to resolve these issues further criteria should be analysed. The evolutionary conservation of the amino acid changes should be considered. Two out of these three mutations (hMLH1 codon 607 leu→his and codon 659 arg→leu) change the amino acid polarity and are therefore likely to alter protein folding. Segregation of these variants through the extended families should be analysed. Only with these criteria satisfied is it possible to distinguish missense mutations that lead to cancer susceptibility from polymorphisms without clinical significance. The missense variant, *hMSH2* G322D is recorded as both a pathological change[11] and a polymorphism.[21,28,29] In family 19 this variant occurs together with a clearly pathological nonsense variant *hMSH2* Q518X, suggesting the polymorphic nature of G322D.

Of the 20 families included in this study we report here that approximately 35% may be attributed to *hMLH1* and 20% to *hMSH2* mutations. In the remaining 45% of apparently hereditary non-polyposis colon cancer families, no mutation has been detected. This could be due to a lack of sensitivity in the methods we have used,[38] mutations in regions of the genes we have overlooked, mutations in other DNA mismatch–repair genes such as *PMS1*, *PMS2*, and *hMSH6*, or other HNPCC genes yet to be identified. In addition it is possible that these families complying with Amsterdam criteria for HNPCC have clustering of colorectal cancer due to epigenetic factors. Analysis of tumours from affected family members for microsatellite instability can help to establish whether or not familial cancers are due to mismatch–repair gene defects.[10] Colorectal cancer diagnosed at the extremely young age of less than 35 years has been shown to be highly indicative of HNPCC.[11]

In conclusion, on the basis of the present data, the best strategy begins with the careful recruitment of the families. There is a high probability of finding a mutation in classic Amsterdam but also in modified criteria families. Mutation analysis should be performed in the *hMSH2* and *hMLH1* genes by complementary strategies in order to increase the informativeness of the mutation search. Of greatest value are denaturing gradient gel electrophoresis (DGGE) using two sets of conditions, or single strand conformation polymorphism (SCCP) using two sets of conditions, followed by direct sequencing of any exons indicative of variation. For the negative cases informativeness is increased by adding other techniques such as heteroduplex analysis (HA) or PTT. To determine the biological consequence especially in the case of missense mutations a functional assay system is required.

Once a pathological mutation is identified, predictive diagnostic testing for other members of the family should be straightforward. This will allow invasive clinical investigations to be focused on those at increased risk and will accelerate recruitment to the international chemoprevention study CAPP2.[39]

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