Electronic letters

J Med Genet 2000;**37** (http://jmedgenet.com/cgi/content/full/37/7/e7)

Mutational germline analysis of *hMSH2* and *hMLH1* genes in early onset colorectal cancer patients

EDITOR—Hereditary non-polyposis colorectal cancer (HNPCC) is a heterogeneous autosomal dominant disease with incomplete penetrance. The frequency is estimated at 1:200/1:1000. HNPCC results from constitutional mutation in one of the five human mismatch repair genes (MMR) that have so far been implicated: *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, and *hMSH6*.[1 2] *hMSH2* and *hMLH1* account for the majority of mutations found in HNPCC families (25-70%). The function of MMR genes is to maintain genetic stability and tumour DNA from HNPCC patients shows an accumulation of replication errors exhibiting an instability phenotype at microsatellite loci (MSI).[3 4]

Standard criteria have been established to define HNPCC clinically. These are known as the "Amsterdam criteria" and require that at least two generations be affected by colorectal cancer (CRC), that three or more relatives with histologically verified CRC be present, one of whom is a first degree relative of the other two, and at least one case of CRC be diagnosed before the age of 50.[5] The syndrome is characterised by synchronous and metachronous CRC tumours and by association of certain types of extracolonic tumours, especially endometrial neoplasia.[6]

In 45-86% of cases,[7] genetic testing characterises the mutation in the index patient for each HNPCC family identified and predicts a possible predisposition to colon and/or related syndrome cancer in at risk subjects of the same kindred. In clinical practice, however, besides HNPCC families, there are families with multiple CRC patients that do not fulfil the Amsterdam criteria, or with a single case of early onset CRC, or with multiple primary tumours. These features can suggest an HNPCC syndrome. In these instances, the percentage of germline mutations of MMR genes is low and the lifetime incidence of colorectal cancer is lower than in Amsterdam families.[8 9] However, because of the diagnostic and prognostic value of the correct ascertainment of HNPCC families, efforts should be made in order to identify all cases. Since the search for mutations in the *hMSH2* and *hMLH1* genes is time consuming, expensive, and available only at selected centres, it is important to establish alternative criteria to the Amsterdam criteria in order to select patients in whom MMR gene analysis should be undertaken. Since early onset CRC may suggest a genetic predisposition, we decided to analyse patients with onset of colorectal cancer before the age of 50, with the purpose of identifying clinical and molecular criteria that are simpler than the Amsterdam criteria, but may predict a positive outcome of genetic testing in patients suspected of HNPCC with no definitive demonstration or family history of the disease. We thus carried out molecular analysis for the detection of germline mutations of *hMSH2* and *hMLH1* genes, as well as MSI analysis in matched tumour and normal tissue, in 54 consecutive patients with occurrence of CRC between 20 and 50 years of age. Patients were recruited from various surgical and clinical units. Family history was not considered as an entry criterion, although the family history allowed us to separate these patients into three groups: 24 patients with no family history of CRC who were classified as sporadic; 14 patients with a family history not fulfilling the Amsterdam criteria, mostly reporting only one first degree relative with CRC or more relatives with cancer in other sites, who were classified as having a positive family history; and 16 patients whose family history fulfilled the Amsterdam criteria. Information was collected with regard to the type and site of cancer in the affected subjects (tables 1, 2, and 3). All patients gave their informed consent for the

Table 1 Microsatellite instability (MSI) status and hMSH2 and hMLH1 germline mutations in sporadic early onset colorectal cancer patients

Patient	*Age*	*Colon cancer site*	*Synchronous metachronous cancer*	*MSI*	*PTT*	*SSCP*	*MMR gene alteration/ effect on coding sequence*
SI521	48	R	+	−	−	−	−
CA98	49	R	−	−	−	−	−
SI520	43	Sigmoid	−	−	−	−	−
SI530	41	Rectum	+	−	−	−	−
CA185	40	R	−	+	−	−	−
							*h*MLH1 L260R*
SI532	47	Caecum	Lung	+	−	+	Missense
SI619	44	R	−	−	−	−	−
SI525	40	Rectum	−	−	−	−	−
							*h*MSH2 G321D
SI524	44	R	−	+	−	+	Missense
BA779	46	R	−	+	−	−	−
TO516	47	R	−	−	−	−	−
SI602	45	R	−	−	−	−	−
CA133	48	Sigmoid	−	−	−	−	−
SI510	47	Sigmoid	−	+	−	−	−
SI601	35	Rectum	−	−	−	−	−
							*h*MLH1 L260R*
GE602	37	R	−	+	−	+	Missense
CA474	35	R	−	−	−	−	−
GE604	32	Rectum	−	−	−	−	−
SI518	36	Transverse	−	NT	−	−	−
CA136	35	R	−	+	−	−	−
GE501	26	R	Ileum	−	−	−	−
SI513	26	Rectum	−	−	−	−	−
CA22	27	R	−	+	−	−	−
CA117	33	Transverse	−	+	−	−	−

R, right; L, left; NT, not tested.
*Mutations not previously reported. Mutations are reported according to Beaudet and Tsui.[15]

Table 2 Microsatellite instability (MSI) status and hMSH2 and hMLH1 germline mutations in early onset colorectal cancer patients with a family history

Patient	*Age*	*Colon cancer site*	*Synchronous metachronous cancer*	*MSI*	*PTT*	*SSCP*	*MMR gene alteration/ effect on coding sequence*
SI509	48	L	Gastric	−	−	−	−
TO502	34	Sigmoid	−	−	−	−	−
SI533	41	Caecum	Ileum	+	−	−	−
SI526	46	Rectum	−	−	−	−	−
SI502	23	Rectum	−	−	−	−	−
SI625	48	R	+	NT	−	−	−
SI511	30	Sigmoid	−	NT	−	−	−
TO622	47	R	−	−	−	−	−
CA68	30	Sigmoid	−	+	−	−	−
SI531	44	Sigmoid	−	+	−	−	−
SI515	49	R	+	−	−	−	−
TO514	46	R	−	−	−	−	−
SI507	32	R	−	NT	−	−	−
TO605	38	R	−	+	−	−	−

R, right; L, left; NT, not tested.

Table 3 Microsatellite instability (MSI) status and hMSH2 and hMLH1 germline mutations in HNPCC patients

Patient	*Age*	*Colon cancer site*	*Synchronous metachronous cancer*	*MSI*	*PTT*	*SSCP*	*MMR gene alteration/ effect on coding sequence*
F93	27	R	−	−	−	−	−
F1	36	R	−	+	−	−	−
SIF1	37	L	+	+	−	+	*h*MLH1 D304V* Missense
F102	29	R	+	+	+	+	*h*MLH1 IVS 8 + A→G Out of frame deletion ex 8
F91	33	R	−	−	−	−	−
F197	43	Sigmoid	−	+	+	+	*h*MLH1 IVS 15 + G→A Out of frame deletion ex 15
G726	20	R	−	+	+	+	*h*MSH2 G845X* Nonsense
F33	35	R	−	+	−	−	−
F100	33	R	−	+	−	−	−
F89	39	R	−	+	−	−	−
F92	45	R	−	+	−	−	−
F90	50	R	−	+	−	+	*h*MLH1 I219V Polymorphism
F719	46	Caecum	−	−	−	+	*h*MSH2 IVS 10 − 10 G→A Polymorphism
F103	47	L	+	−	−	+	*h*MLH1 K618T Polymorphism
SI505	38	L	−	+	+	+	*h*MSH2 IVS 5 + A→T In frame deletion ex 5
CA135	50	R	−	+	+	+	*h*MSH2 399 ins CA* Nonsense

R, right; L, left.
*Mutations not previously reported. Mutations are reported according to Beaudet and Tsui.[15]

collection of personal data and blood and tissue samples. Molecular analysis of at risk relatives of patients with an identified mutation was carried out and their clinical screening outcome was also followed up.

Germline mutations were detected on peripheral blood or lymphoblastoid cell lines by the protein truncation test (PTT), single strand conformation polymorphism (SSCP), and direct DNA sequencing in an ABI PRISM™ 377 DNA SEQUENCER (Perkin Elmer). SSCP was carried out on a non-denaturing 5-20% polyacrylamide gel and run in a Multiphor II System (Pharmacia Biotech, Uppsala, Sweden) at two temperatures, 12°C and 23°C. Genomic DNA sequences corresponding to the 16 *hMSH2* exons and intron-exon junctions were amplified with primer pairs described by Liu *et al*.[10] The 19 *hMLH1* exons and intron-exon junctions were amplified by PCR using primer pairs described by Han *et al*,[11] except for exons 7 and 12 which were amplified using nested PCR with primer pairs described by Kolodner *et al*.[12] The PTT test was performed using primer pairs with a T7 promoter for transcription and a consensus sequence for the initiation of translation in frame with *hMLH1* and *hMSH2*.[13] Primers for *hMLH1* are described by Papadopoulos *et al*.[14] Primers for *hMSH2* are described by Liu *et al*.[10] PCR fragments were transcribed and translated in vitro using TNT Coupled Reticulocytes Lysate System reaction kit (Promega, Madison, WI), incorporating 5 µl ^{35}S methionine.

Analysis of MSI was carried out on normal and tumoral tissue DNA by amplification of two mononucleotide repeats (BAT 25 and 26) and the dinucleotide repeats D2S123, D5S136, and D3S1611. A sample was considered positive for MSI when two of five PCR products of tumoral DNA showed the presence of novel bands that were not visible in the PCR products of the corresponding normal tissue DNA.

In 38 patients classified as sporadic and with familial clustering of colon cancer, 13 tumours out of 34 tested (38%) showed MSI, and in three patients (8%) germline mutations were detected (tables 1, 2, and 4). The three mutations are missense. Mutation G321D in exon 6 of

Table 4 Frequency of microsatellite instability (MSI) positive tumours and hMSH2 and hMLH1 germline mutations in early onset colorectal cancer patients

No of patients		*MSI positive (%)*	*hMSH2, hMLH1 germline mutations (%)*	*% of mutations in MSI positive tumours*
Non-HNPCC	38	13 (38)*	3 (8)	23
HNPCC	16	12 (75)	6 (37)	50
Total	54	25 (50)*	9 (17)	36

*Four patients not tested.

hMSH2 found in patient S1524 has been previously described as causative of disease.[16] Mutation L260R in exon 9 of *hMLH1* was detected in patients S1532 and GE602. This mutation has not been previously described and we could not find it in more than 100 normal subjects examined. Laboratory contamination was excluded by multiple controls with new samples of DNA obtained separately from both patients. The two patients come from different regions of Italy and deny any relationship between their families. The mother of patient GE602 showed the same mutation and had two villous adenomas on the control colonoscopy done at the age of 62. No other mutation positive subjects were found in seven healthy adult first degree relatives of these patients.

In young patients belonging to HNPCC families, 12 tumour tissues (75%) were MSI positive; of these, nine presented a constitutional MMR gene shift on SSCP and five a truncated PTT product (table 3). All alterations were characterised. Three mutations have not been previously reported as pathogenetic germline mutations, G845X and 399 insCA, resulting in stop codons, found in the *hMSH2* gene in patients G726 and CA135 respectively, and a missense alteration at nt 912 of *hMLH1* with a change Asp→Val (D304V) in patient SIF1. To our knowledge, this change has never been identified in healthy persons. We examined 15 members of this kindred; six of these were positive, four with colon or endometrial cancer and two under 20 years of age with normal colonoscopy. We considered this mutation of clinical significance.

Patients F719 and F103, whose tumours did not show MSI, exhibited DNA germline missense changes. The first is an already known DNA polymorphism and the second has been described as causative of disease,[11] but in our case, given the stability of MS, we considered it a neutral mutation. This raises the question of the pathogenetic significance of missense mutations whose effects are not obvious without further analysis. As shown in tables 1, 2, and 3, right sided colon cancer, including the caecum, was involved in 33 patients (61%), while synchronous and/or metachronous tumours were found in 11 patients (20%).

Molecular testing for detection of germline mutation for HNPCC is extremely valuable but certainly not simple, owing to heterogeneity of the disease, to the incomplete penetrance, and because of the relatively low percentage of mutations found. Even though HNPCC is reported to constitute almost 1-10% of colorectal cancers, many HNPCC related cancers probably go unrecognised. In clinical practice, it is often impossible to select patients whose families fulfil the Amsterdam criteria because of the small family size, the impracticability of compiling a verified family history, and the lack of a characteristic clinical phenotype. Various strategies have therefore been proposed to identify cases of non-Amsterdam HNPCC. Aaltonen *et al*[17] recently tested a series of 509 consecutive tumour tissues for MSI and found that 12% of tumours exhibited MSI; of these, 2% had germline mutation of *hMSH2* or *hMLH1*. Patients with germline mutations had a positive family history and, in several cases, fulfilled the Amsterdam criteria. Evidence was therefore provided that analysis for MSI followed by analysis of MMR genes can identify cases of familial colon cancer. In an editorial on this study, Lynch and Smyrk[18] criticised this approach because, since almost all patients with an identified mutation had a family history, mutational analysis could have been performed directly. They concluded that reliance on family history is the most cost effective way to identify HNPCC. Since the aim of our study was to identify HNPCC patients in apparently sporadic cases of CRC, we chose to select patients for the early occurrence of cancer. This is in accordance with recent studies. Having identified 27% of MSI positive tumours and 8% of MMR germline mutations in families not fulfilling the Amsterdam criteria, Wijnen *et al*[9] proposed a logistic method for estimating the probability of detecting germline mutations. According to this method, the early age of diagnosis and the MSI status are clinical and molecular conditions that can improve the rate of detection of MMR gene mutations.

Farrington *et al*[19] examined a cohort of 50 patients with extremely early onset of CRC, without considering the family history. They found a high sensitivity and sensibility of MSI status and suggested genetic testing for all cancer patients <30 years of age.

Furthermore, Cravo *et al*[21] reviewed the Bethesda guidelines[22] for indications of MSI testing, concluding that the clinical criteria of age <45 years, right side of tumour, and mucinous component are easier to implement than the Bethesda guidelines and sufficiently discriminating to proceed to MSI testing. Our results show that, although MSI was present in 13/34 tumours examined in sporadic and in non-Amsterdam patients, only three germline mutations have been found; this contrasts with HNPCC patients, in whom six pathogenetic germline mutations have been detected on 11 MS unstable tumours. Our data are in accordance with previous studies[9 22] and we confirm that, in the absence of a significant family history, all patients with CRC under the age of 50 should undergo MSI status testing followed, in positive cases, by molecular analysis of the two most frequently involved MMR genes. Right sided location of cancer should also be taken into consideration as an associated risk factor. The low percentage of mutations in young non-HNPCC patients carrying unstable tumours could be because of mutation of other MMR genes, which are not tested because they are rarely involved in HNPCC, or in genes not yet isolated. Very recently, the involvement of the *hMSH6* gene has been hypothesised for early onset colorectal cancer with MSI for mononucleotide repeat markers[23]; the *MED-1* gene was cloned which interacts with *h*MLH1 protein and its mutant form is associated with MSI.[24] It is possible that mutations of these genes might be responsible for some MSI positive HNPCC cases.

The cloning of MMR genes has heralded a new era for the genetic counselling of patients with a family history of non-polyposis colorectal cancer. On the basis of such information, people should be able to make decisions about screening and prevention strategies with a more precise estimation of their risks. However, all the current data on the risk of developing CRC associated with MMR gene mutations have so far come from large, high risk families. This constitutes a rather obvious bias, which can be avoided by assessing the presence of MMR gene mutations in affected subjects belonging to small families, families not fulfilling the Amsterdam criteria, or in single cases of early onset of CRC. In addition, to be really certain about the risks of carriers, we need to identify many different MMR gene mutations in order to evaluate their possible meaning and penetrance. However, we cannot ignore the fact that differences in cancer risk conferred by mutated MMR genes may be the consequence of modifier factors, either genetic or environmental, that also run in families. Thus, gene carriers in high risk families might have a greater risk not just because they are gene carriers but also because they carry a greater burden of other risk modifiers. Searching for MMR gene mutations and characterising the different status (affected or not) of gene carriers could contribute to the identification of such modifiers.

This work was supported by AIRC, Associazione Italiana Ricerca sul Cancro (Progetto Speciale Tumori Ereditari del Colon), MURST, Ministero dell' Università e della Ricerca Scientifica e Tecnologica, Cofinanziamento 1998, CNR Progetto Finalizzato ACRO 94.1142.039.

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J Med Genet 2000 37: e7
doi: 10.1136/jmg.37.7.e7

PostScript

CORRECTION

In the July 2000 issue of the journal (*J Med Genet* 2000;**37**:e7), in the electronic letter by Montera *et al*, two mutations were wrongly described in table 3. For patient F102, the mutation should have read hMLH1 IVS7 −2 a→g, out of frame del exon 8. For patient CA135, it should have read hMSH2 400 ins CA, nonsense.

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