Letters to the Editor

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A missense mutation in both *hMSH2* and *APC* in an Ashkenazi Jewish HNPCC kindred: implications for clinical screening

EDITOR—Colorectal carcinoma (CRC), among the most common neoplasms in humans, has a moderately large hereditary component. The two most frequent hereditary CRC syndromes are hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), both dominantly inherited disorders. HNPCC is caused by inherited defects in the DNA mismatch repair (MMR) genes *hMLH1*, *hMSH2*, *PMS1*, and *PMS2*; FAP is caused by mutations in the *APC* gene.[1] Between 1 and 4% of all CRC fully satisfy the international criteria for HNPCC.[2–4] In addition to CRC, these families also have an excess incidence of adenocarcinoma of the endometrium and to a lesser degree, cancer of the stomach, ovary, and other sites.[5] Alterations in *hMSH2* or *hMLH1* account for ~90% of the germline mutations detected in HNPCC.[1] Mutations in *hMSH6* have been described in CRC kindreds that resemble HNPCC but do not always fulfil the above mentioned criteria.[6 7]

Familial adenomatous polyposis (FAP) accounts for less than 1% of CRC; it is characterised by the development of a large number (>100) of colorectal polyps, which, if untreated, will inevitably lead to CRC.[8] The gene underlying FAP, *APC*, was identified in 1991; germline mutations truncating the *APC* gene product are usually found in FAP.[9 10] An interesting missense variant in *APC*, I1307K, was discovered in a 39 year old man with eight colorectal polyps[11] with a family history of colorectal polyps and CRC but not FAP. The T to A polymorphism at nt 3920 in *APC* was postulated to predispose to cancer, not as a direct effect of the protein, but by rendering its region of *APC* hypermutable. The mutation does not seem to be common in populations other than Ashkenazi Jews, where it is found at a frequency of 6.1%[11] (recently we have found the allele in a French Canadian kindred[12]). The relative risk of CRC and other neoplasms associated with I1307K *APC* has been investigated.[13–17] Woodage *et al*[15] concluded that the cumulative risk of CRC among first degree relatives of CRC survivors among carriers compared to non-carriers was 1.9, although this was not statistically significant. By examining a cohort of unselected Ashkenazi Jewish patients with colorectal carcinoma or adenomatous polyps, Gryfe *et al*[16] found statistically significant (p=0.01) relative risks of colorectal neoplasia of 1.72 (95% confidence interval 1.13-2.61) and 1.48 (95% confidence interval 1.08-2.04) compared to controls from New York and Washington, DC, respectively. Recent investigations have indicated that I1307K *APC* is a candidate low penetrance breast cancer susceptibility allele, particularly in heterozygotes for germline mutations in *BRCA1* or *BRCA2*.[15 17] It is hoped that the identification of mutations in CRC susceptibility genes will result in decreased morbidity and mortality by the application of appropriate screening and intervention strategies. To this end, in an Ashkenazi Jewish kindred with HNPCC, we used single strand conformation analysis (SSCA) to search for mutations in MMR genes and an allele specific oligonucleotide hybridisation (ASOH) assay

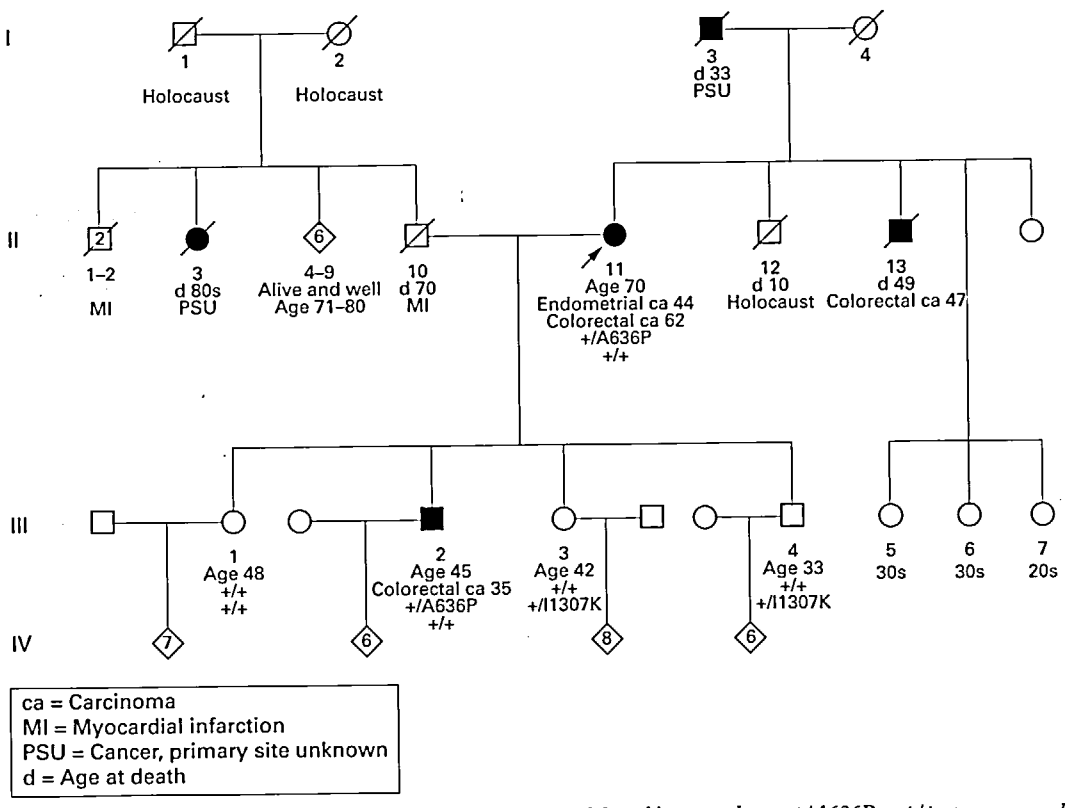

Figure 1 Pedigree of the MON702 kindred. The age at death or the current age of the subjects are shown. +/A636P or +/+=presence or absence of exon 12 mutation in hMSH2; +/I1307K or +/+=presence or absence of I1307K variant in APC.

to identify I1307K *APC*. The proband (fig 1, II.11) was seen by both the medical genetics and colorectal surgery services and a family history was taken, identifying the family as an Amsterdam criteria positive HNPCC kindred (MON702). There are three relatives with CRC, two of the affected subjects are mother and son, and two cases were diagnosed at less than 50 years of age (II.13 and III.2). Informed consent was received for blood donation as approved by the institutional ethics committee. To assess the occurrence of MSI in CRCs from two affected subjects, six high sensitivity MSI markers (dinucleotide repeats D2S123, D18S58, and LNS and mononucleotide repeats BAT25, BAT26, and BAT40)[18 19] were used to evaluate one CRC sample from each patient. The proband's tumour DNA exhibited instability with three of the six microsatellite markers (data not shown) and her son's (III.2, fig 1) tumour DNA showed alterations in allele length compared to matched normal DNA with BAT25, BAT26, and D18S58 (fig 2), defining both neoplasms as MSI$^+$ according to established criteria.[18 20] We then used PCR-SSCA to analyse *hMLH1*[21] and *hMSH2*[22] for heterozygous mutations. A mobility shift was identified in exon 12 of *hMSH2* of genomic DNA amplified from two members of MON702, II.3 and III.2. A germline heterozygous G to C transversion at nucleotide 1905, resulting in a predicted protein sequence change of alanine to proline (A636P), was identified in both subjects showing the mobility shifts. Three unaffected sibs of the affected son (III.1, III.3, and III.4; fig 1) do not carry this mutation. We sequenced all 16 *hMSH2* and 19 *hMLH1* exons in the two affected subjects and did not find another mutation elsewhere in either *hMSH2* or *hMSH1* that might otherwise account for the MSI$^+$ observed.

The A636P mutation appears to be associated with MSI$^+$. To exclude the possibility that it represents a rare polymorphism over-represented in the Ashkenazim, but not associated with HNPCC, we therefore screened 100 Ashkenazi Jews who donated blood anonymously for DNA studies but were not known to have CRC. No carriers of A636P were found by ASOH analysis. Another important question is whether this is a common mutation in Ashkenazi Jewish CRC patients. In total, DNA samples from 196 unselected Jewish CRC patients were successfully amplified by PCR and tested for the presence of the A636P allele. BAT-26 MSI$^+$ was present in the tumour DNA extracted from the neoplasms of 15 (7.6%) of these patients. No carriers of A636P *hMSH2* were identified; these findings further indicate that A636P is not a common sequence variant in this population.

Following the report[11] that I1307K *APC* is common in Ashkenazi Jewish CRC families, we investigated whether this mutation was present in the MON702 family. CRCs from subjects carrying this variant are usually MSI negative.[11 23] It was, therefore, not surprising that I1307 *APC* was not present in the affected subjects in MON702, whose CRCs were MSI$^+$. However, unaffected subjects III.3 and III.4 were found to carry I1307K *APC*; these positive ASOH results were confirmed by sequencing. We also observed I1307K *APC* in eight out of 153 samples from Ashkenazi Jewish controls (5.2%), a similar figure to those reported previously.[11 15]

Most of the *hMSH2* mutations described so far in HNPCC result in a truncated protein and allow an immediate inference of pathogenicity. In the absence of large kindreds with numerous affected subjects, it is difficult to evaluate the pathogenicity of non-truncating mutations. Ideally, previously conducted functional studies can shed light on the aetiological significance of missense mutations in small kindreds such as MON702; inactivating missense mutations at the ATP binding site of hMSH2 have been

Figure 2 Microsatellite analysis of CRC removed from III.2 in the MON702 kindred. For each locus, the left lane shows the results from the neoplastic tissue; the right lane was loaded with amplification products from adjacent normal tissue. This sample was scored as MSI for BAT25, BAT26, and D18S58; thus, the neoplasm was considered MSI positive.

described.[24] On the other hand, six missense mutations found in HNPCC patients were found not to affect protein-protein interactions of hMSH2 and hMSH6 or hMSH3.[25] Unfortunately, however, amino acid 636 of the hMSH2 protein has not been directly examined in any of the published studies on the function of *hMSH2*.[24-27] The *APC* gene has been examined in *E coli* using a colorimetric assay based on an in frame fusion with the β-galactosidase gene.[28] Andreutti-Zaugg *et al*[29] designed a fusion protein assay in *S cerevisiae* in which *hMSH2* is inserted upstream of the *ADE2* gene, in the same reading frame. When this plasmid is expressed in *S cerevisiae*, high levels of active Ade2 protein are formed, resulting in white colonies in a colorimetric assay. Truncating mutations in the *hMSH2* gene lead to truncated fusion protein, hence red colonies, reflecting lack of Ade2 activity. Surprisingly, some missense changes, including the HNPCC mutation G674D at the ATP binding site, resulted in pink colonies, reflecting partial activity of the ADE2 reporter gene. Since protein expression was similar, the reduced enzymatic activity of the fusion protein was inferred to be the result of incorrect protein folding, leading to a denatured protein.[29] We introduced A636P *hMSH2* into plasmid pCA57 by site directed mutagenesis (QuikChange kit, Stratagene), creating an A636P *hMSH2::ADE2* fusion protein. A mutant protein of the correct relative molecular mass was observed to be expressed at comparable levels to its wild type counterpart by immunoblotting analysis of cellular extracts. Next, the phenotype of the A636P fusion protein was determined in the colorimetric in vivo assay.[29] Colonies from cells containing the pCA57-*hMSH2* A636P mutant plasmid were pink. This indicated a partial loss of function of the

fusion protein, and suggested that the mutation does affect hMSH2 protein folding in *S cerevisiae* and by implication may affect structure and function in human cells. This finding further validates the *hMSH2::ADE2* assay as capable of detecting certain non-truncating mutations in *hMSH2*.

In MON702, an Amsterdam criteria positive HNPCC kindred, we found that the CRCs from two affected subjects (II.3 and III.2) showed MSI$^+$. A missense G to C (nucleotide 1905) transversion mutation in *hMSH2* was identified at codon 636 in both these subjects, leading to a predicted protein change of alanine to proline in the hMSH2 protein. This mutation has not been previously published, but was recently posted on the HNPCC ICG database (http://www.nfdht.nl, Bressac-de Paillerets, unpublished data). However, these investigators were uncertain about the pathogenicity of the A636P mutation. We consider the A636P missense mutation to be pathogenic for the following reasons: (1) the mutation segregates with the disease, and three colonoscopy negative relatives do not carry the mutation; (2) the mutation was not found in 100 Ashkenazi Jewish controls, who were not known to have CRC; (3) no other truncating or missense mutation was identified on complete screening of *hMLH1* or *hMSH2* by SSCA and direct sequencing in affected subjects; (4) the mutation substitutes an amino acid belonging to a different polarity group and can be inferred to affect protein folding as shown in the *hMSH2::ADE2* fusion assay.

The I1307K missense mutation in *APC* has been reported to predispose to CRC. Virtually restricted to the Ashkenazim, it is not associated with classical FAP. We wished to study MON702 for this mutation, because it has been suggested that I1307K *APC* may be more frequent in CRC kindreds carrying MMR gene mutations.[19] On testing the living members of the kindred for this mutation, we found that I1307K *APC* was indeed present in the family, but was carried by those who have neither CRC nor polyps. The proband and her affected son do not carry the mutation. As well, analysis of the sole available archival pathology specimen from the father (fig 1, II.10) suggested that the mutation is coming from his side of the family. There are no confirmed cases of CRC in his 10 sibs. In the MON702 HNPCC kindred, affected A636P *hMSH2* carriers do not carry the I1307K *APC* variant; therefore, our present study does not suggest a biological relationship between mutations in MMR genes and I1307K *APC*. Previously, we reported[12] a French Canadian kindred (MON1061) in which I1307K *APC* was segregating with a novel truncating mutation in *hMLH1* (1784delT). There was no consistent relationship of the presence or absence of I1307K *APC* with CRC, but all tested subjects with early onset CRC were heterozygous for the *hMLH1* mutation.

Clearly, further work is required to resolve this issue, including follow up of these families and ascertainment of subjects carrying both a germline mutation in the *hMSH2* gene and I1307K *APC*. Until that time caution should be exercised in deciding to discontinue cancer surveillance of a person in an Ashkenazi Jewish CRC family when MMR gene mutation analysis is negative, even in a family where a germline MMR mutation has been identified, unless status at *APC* codon 1307 has been determined. Recent studies of Ashkenazi Jewish populations have resulted in odds ratios of under 2 for the risk of CRC associated with the I1307K allele of *APC*.[11 13–16] Ashkenazi Jews carrying the I1307K allele but who have no family history of CRC have a risk of colon cancer that is only marginally increased, and more evidence that knowledge of I1307K status can prevent CRC is required to support general screening efforts. However, in an Ashkenazi Jewish family with multiple cases of CRC, *APC* I1307K determination of all subjects who request mutation analysis may be justified.

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A PCR test for the detection of hypermethylated alleles at the retinoblastoma locus

EDITOR—The development of retinoblastoma, a childhood malignancy of the eye, is initiated by mutations in both alleles of the retinoblastoma gene (*RB1*). Mutations changing the nucleotide sequence at this locus and chromosomal mechanisms resulting in loss of heterozygosity (LOH) are the most common events responsible for gene inactivation.[1] However, in the *RB1* gene, as well as in other tumour suppressor genes including *BRCA1*, *p15*, and *p16*, hypermethylation of 5' regulatory regions may also cause gene inactivation and, consequently, tumour development.[2-6] Methylation of the CpG rich island at the 5' end of the *RB1* gene is observed in some 10% of unilateral sporadic retinoblastomas.[3 4 7] Recently, the methylation pattern of tumours with hypermethylated *RB1* alleles was analysed in detail by the bisulphite genomic sequencing technique.[8] In most of these tumours, most CpG sites were methylated (75-100%). In one of seven tumours only, the density of methylated CpG sites was more variable, ranging from 25% to 62.5%.

Mutation analysis in tumours from patients with sporadic unilateral retinoblastoma is required for accurate risk prediction in relatives.[1] Consequently, we have devised a methylation specific PCR assay (MSP)[5 9] for rapid and reliable detection of methylation at the *RB1* promoter. Bisulphite treatment of denatured DNA converts all unmethylated cytosines to uracil leaving methylated cytosines in CpG dinucleotides unaltered. After bisulphite treatment, a methylated allele differs from the unmethylated allele in nucleotide sequence at all CpG positions. The downstream PCR primer (RBcom) was designed to bind to the bisulphite treated sense strand irrespective of the methylation status (fig 1). Binding of RBcom to a methylated or unmethylated allele results in different complementary strands in the first round of the PCR. Upstream primers are designed to bind to the DNA strand synthesised in the first round of PCR. Primer RBmet binds to the strand derived from the methylated allele, whereas primer RBunmet will only bind to the DNA strand generated from the unmethylated *RB1* allele. The methylated allele results in a 201 bp PCR product, whereas the unmethylated allele results in a 154 bp PCR product. As unmodified genomic DNA is not amplified, incomplete modification of genomic DNA in the bisulphite reaction will not yield specific PCR products (not shown). However, mutations affecting the primer binding sites might result in diminished or absent amplification of the corresponding PCR product and thus false negative results.

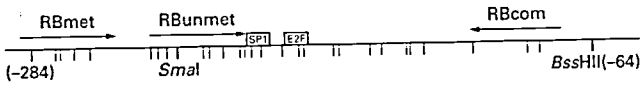

Figure 1 Relevant part of the RB1 promoter. For sequence see T'Ang et al.[10] The positions of the MSP primers RBmet, RBunmet, and RBcom are indicated by arrows. Recognition sites for transcription factors SP1 and E2F as well as for methylation sensitive restriction enzymes BssHII and SmaI are shown. Positions of CpG dinucleotides are given by vertical lines. BssHII restriction site is located 64 bp upstream of the initiation codon. Primer sequences: RBcom: 5'-CCTACCCCRACTCCCRTTACAAAAATAATTTCAAC-3'; RBmet: 5'-GCGTTTTAGTTCGCGTATCGATTAGCGTTTTAG-3'; RBunmet: 5'-TGGTGGGTTTGGGAGTTTTGTGGATGTGATGTT-3'. R=adenine and guanine were added during synthesis of primer RBcom in equal amounts to avoid preferentially binding to methylated or unmethylated template.

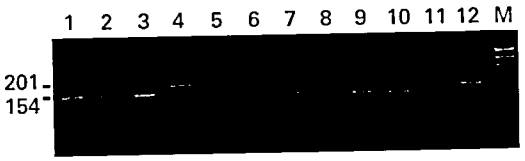

Figure 2 MSP analysis of hypermethylated (lanes 2, 4, 7, 11, and 12) and unmethylated (lanes 1, 3, 5, 8, 9, and 10) retinoblastomas; PCR failure (lane 6); marker(M) = MspI digested pUC19 DNA. The positions of the 201 bp PCR product representing the methylated allele and the 154 bp PCR product representing the unmethylated allele are indicated; 5 µg genomic DNA was bisulphite treated as previously described.[9 11] Amplifications were performed in a reaction volume of 25 µl containing 2 µl of bisulphite treated DNA (Perkin Elmer, PE9600). Conditions: 10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.2 µmol/l of each dNTP, 0.25 µmol/l of primer RBcom, 0.3 µmol/l of primer RBmet, 0.05 µmol/l of primer RBunmet, and 0.5 units AmpliTaq (Perkin Elmer). Cycling conditions: 94°C for five minutes for one cycle; 95°C for 15 seconds, 64°C for 15 seconds, and 72°C for 30 seconds for 35 cycles, followed by 72°C for five minutes. PCR products were separated on 3% agarose gels.

To establish the MSP at the *RB1* promoter, 20 tumour DNA samples, which had previously been characterised in detail,[1] were used. Characterisation included genotyping with intragenic markers, DNA sequencing, and analysis of the methylation status by Southern blot hybridisation of genomic DNA digested with methylation sensitive restriction enzyme *Bss*HII.[3] All tumours showed loss of heterozygosity (LOH) at the intragenic polymorphic loci RBi2 and RB1.20, and an inactivating mutation was identified in the remaining allele. In eight tumours a methylated allele was identified by Southern blot hybridisation. In each of these samples, a 201 bp PCR product was clearly visible when tested in the MSP assay (fig 2, lanes 2, 4, 7, 11, and 12). As expected, DNA from tumour and blood cells not containing a methylated *RB1* allele produced a 154 bp PCR product only (fig 2, lanes 1, 3, 5, 8, 9, and 10). No PCR product was obtained from unmodified genomic DNA under the conditions used here (data not shown). Trace amounts of the 154 bp PCR product were also detectable in tumours with LOH at intragenic loci. This finding might be the result of an unhomogeneous cell population consisting of cells with varying degrees of hypermethylation and non-tumour cells. Therefore, the presence of a 154 bp PCR product in the *RB1*-MSP assay does not necessarily indicate the presence of an unmethylated *RB1* allele in the tumour. A 201 bp PCR product specific for the