Electronic letter

Screening families with endometrial and colorectal cancers for germline mutations

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EDITOR—Endometrial cancer is the most commonly diagnosed cancer of the female reproductive tract in the United States and other western countries.[1] Although several genes may be altered in these cancers,[2] the molecular events in the development of endometrial carcinoma remain poorly defined. Changes in simple sequence repeats in tumour DNA relative to normal DNA, referred to as microsatellite instability (MSI), are a feature of many endometrial carcinomas.[3–7] MSI occurs as a result of failing DNA mismatch repair[8] and is known to accompany defects in the *MLH1*, *MSH2*, *MSH6*, *MSH3*, *PMS2*, and possibly *PMS1* genes. Apart from *MSH3*, all these genes are associated with inherited cancer susceptibility in the context of hereditary non-polyposis colorectal cancer (HNPCC).[3 9–16] Endometrial carcinomas are the most common extracolonic cancers in HNPCC[17] and usually occur at an early age.[18] Women who carry HNPCC mutations have a 22-43% lifetime risk of developing endometrial cancer as compared with 3% for the general population.[19–21] According to a recent report, germline mutations of the DNA mismatch repair gene *MSH6* might be specifically associated with susceptibility to endometrial cancer.[22]

PTEN is a newly isolated tumour suppressor gene located on chromosome 10q23, a region frequently deleted in multiple types of human cancer.[23–25] Inactivation of *PTEN* is the underlying cause of familial Cowden disease.[26] Inactivating mutations in the *PTEN* gene are frequently found in multiple tumour types including brain, breast, prostate, endometrial, and skin carcinomas.[23–25] Knockout mice for *PTEN* die as early embryos, while animals heterozygous for a mutant *PTEN* allele develop a broad spectrum of tumours.[27–29] These observations have established that *PTEN* has multiple target organs including the endometrium.

Recent reports have shown that β-catenin is a multifunctional protein involved in two apparently independent processes. In one it acts as a cell adhesion regulator when coupled with E-cadherin.[30] In the other it acts as an oncogene in the wingless/Wnt signal transduction pathway.[31] The Wnt pathway is highly conserved, and phosphorylation of specific Ser/Thr residues of β-catenin by serine-threonine glycogen synthase kinase (GSK)-3β is a key step in the ubiquitin mediated degradation of β-catenin. Mutation of the APC protein causes an increase in the β-catenin protein, resulting in activation of the Wnt signalling pathway.[32] Hence, structural alterations of the GSK-3β phosphorylation sites of β-catenin would also cause activation of the Wnt pathway. In fact, mutations of *β-catenin* involving specific Ser/Thr sites in exon 3 were observed in a variety of tumours especially in endometrial and colorectal cancers without *APC* mutations.[33–35] Mutation rates of 11-45% have been reported for exon 3 of *β-catenin* in endometrial cancer irrespective of MSI status.[36–38]

The purpose of this study was to explore the genetic basis of familial endometrial cancer in 10 families available to us. If susceptibility to endometrial cancer were associated with defective MMR, or inactivation of a tumour suppressor gene or activation of an oncogene, a heritable mutation in one of these genes would be expected to be present. To test this hypothesis, a comprehensive mutation analysis of the MMR genes *MLH1*, *MSH2*, *PMS1*, *PMS2*, *MSH3*, and *MSH6*, the tumour suppressor gene *PTEN*, and exon 3 of *β-catenin* was undertaken in these families.

Materials and methods

PATIENTS

A total of 10 women with endometrial cancer were studied. Three were from families in which endometrial cancer alone seemed to segregate as an autosomal dominant trait, four were from HNPCC families meeting the modified Amsterdam criteria,[39] and the remaining three with endometrial and colorectal cancer did not fit either classification. The patients were recruited through the Cancer Family Clinic at Karolinska Hospital and the Umeå University Hospital between 1990 and 1996.

SCREENING FOR GERMLINE MUTATIONS

DGGE/CDGE

DNA was extracted from whole blood according to routine procedures. *MLH1* and *MSH2* were screened for mutations by denaturing gradient gel electrophoresis (DGGE).[40 41] *MSH2* exon 5 was studied by constant denaturing gel electrophoresis (CDGE) as described previously.[42] Alterations identified by DGGE/CDGE were verified by sequencing of genomic DNA.

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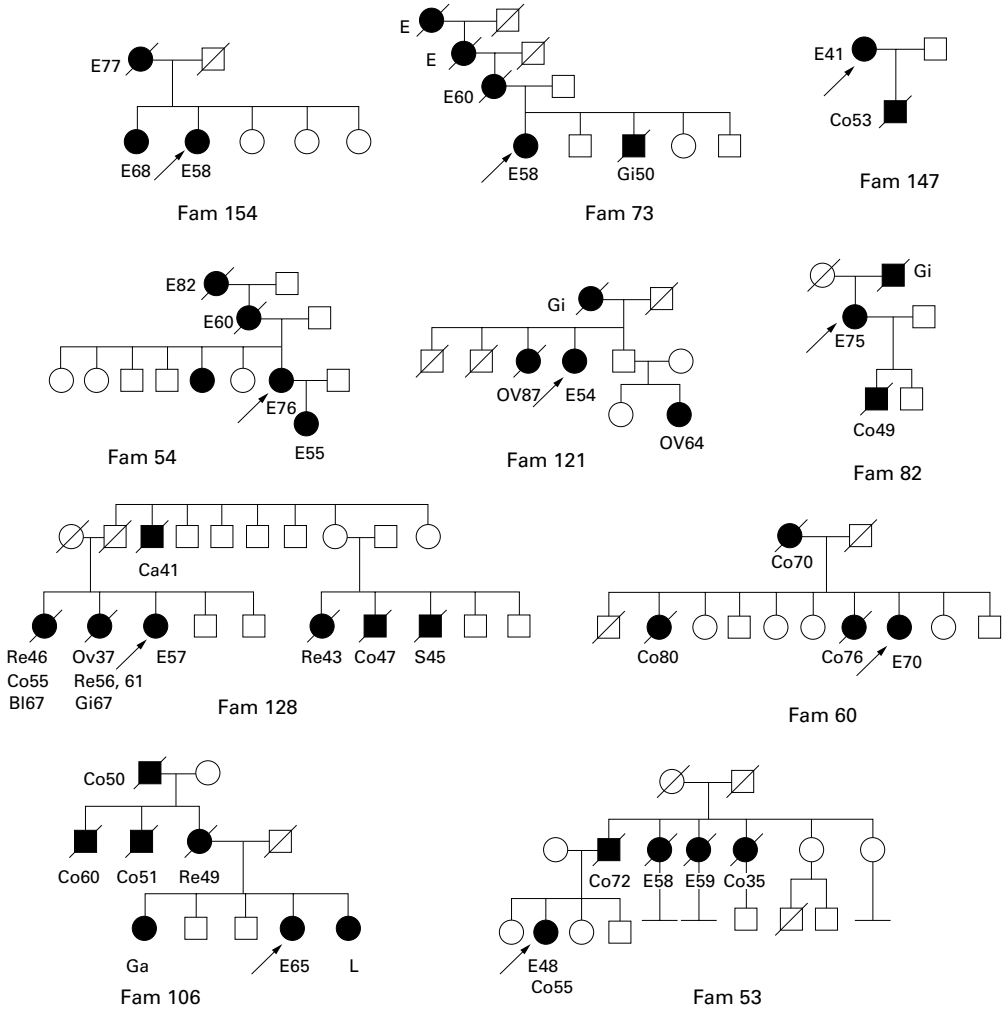

Figure 1 Pedigrees of families included in present study. Filled symbols indicate endometrial cancer (E), colon cancer (Co), rectal cancer (Re), ovarian cancer (Ov), bladder cancer (Bl), glioma (Gi), gastric cancer (Ga), lung cancer (L), and sarcoma (S). The number below each symbol indicates the age at diagnosis. Arrows indicate probands.

RT-PCR/PTT

RNA was extracted from EBV cell lines using the Ultraspec-II RNA PCR kit (Perkin Elmer, Foster City, CA). The random hexamer priming method was used with the GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA) to synthesise cDNA. The protein truncation test (PTT) was carried out according to the manufacturer's instructions (Promega). The cDNA was amplified in two overlapping fragments for *MLH1*, *MSH2*, *MSH3*, *MSH6*, and *PMS2*[12 43 44] and in three fragments for *PMS1*.[12] The *PTEN* cDNA was amplified in two fragments using primers published previously.[45] Alterations identified by RT-PCR/PTT were verified by sequencing of cDNA and genomic DNA.

Direct sequencing of MSH6 and β-catenin

The individual exons and flanking intron sequences of *MSH6* were amplified using primers and cycling conditions described previously.[46] Exon 3 of β-catenin was studied as described previously.[47] Fifty ng of genomic DNA were amplified in each reaction under the following conditions: one cycle of 96°C for two minutes, 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for one minute, and one cycle of 72°C for 10 minutes. The PCR product was purified using Microcolumn (Amicon). Fifty ng of the PCR product was sequenced using internal primers,[47] Thermo

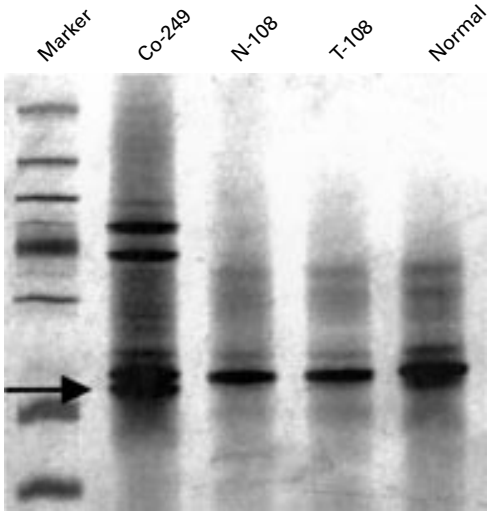

Figure 2 A MSH2 germline mutation detected by DGGE. Lane 1, marker; lane 2, index patient of family 82; lanes 3-4, normal and tumour DNA from the son of the index patient; lane 5, normal control.

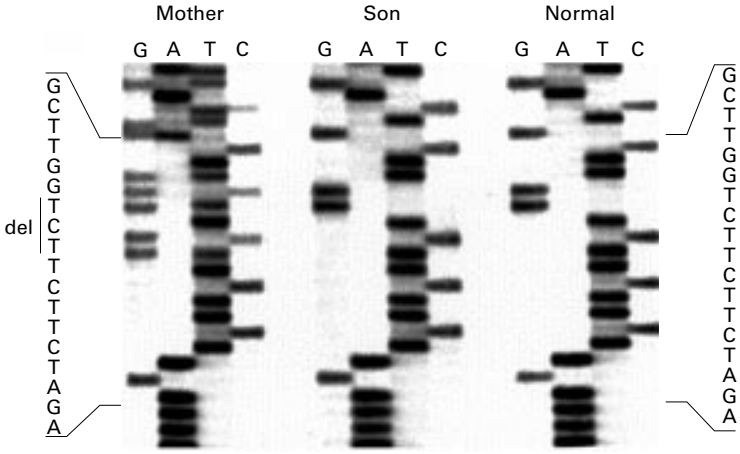

Figure 3 Genomic DNA sequencing for family 82. The index patient was heterozygous for a TCT deletion in MSH2 (left) while the son of the index patient (middle) and a normal control (right) showed the wild type sequence.

Sequenase (Amersham Corp), and ^{33}P-labelled ddNTPs (Amersham) according to the manufacturer's instructions. Single base substitutions in exons 2 and 3 of *MSH6* found by direct sequencing were further investigated in newly collected samples and in normal controls by specific variant detection techniques. Primer sequences and PCR conditions are available from the authors upon request.

MSI analysis

A previous study showed that mononucleotide markers are much more sensitive in identifying MSI as compared to dinucleotide markers[48]; in fact, BAT-26 alone has been suggested to be sufficient for diagnosis of microsatellite instability.[49] Tumour analysis was therefore performed with two mononucleotide repeat markers, BAT-25 and BAT-26, and the tumours were considered MSI-H if both markers showed sequence alterations.[50] If only one of BAT-25 or BAT-26 was positive, a panel of 10 dinucleotide markers (D3S1283, D3S647, D3S1514, D2S119, D2S147, LNSCA1 (*APC*), *TP53*, D3S1029, D3S1611, D3S1298) was studied as previously described[51] and tumours with mutations in more than 40% of informative markers were considered MSI-H.[52]

Results and discussion

Ten women with endometrial cancer were screened for germline mutations in eight different genes. *MLH1* and *MSH2* were first evaluated by DGGE on genomic DNA, followed by RT-PCR and PTT on cDNA. The cDNA sequences of *PTEN*, *PMS1*, *PMS2*, *MSH3*, and *MSH6* were investigated by RT-PCR and PTT; additionally, *MSH6* exons and flanking introns were sequenced from genomic DNA. *β-catenin* exon 3 was studied by direct sequencing of genomic DNA. The mutation screening strategy chosen should allow the detection of most pathogenic alterations in the MMR and tumour suppressor genes studied, with the exception of possible mutations in the promoter or 3' untranslated region.

DGGE and sequence analysis showed a germline mutation in *MSH2* (in frame deletion of bases 279 to 281 of exon 2) in a woman with endometrial cancer from family 82 (figs 1, 2, and 3, table 1). The index patient was 75 years old and had a son affected with colon cancer at 49 years of age. The endometrial cancer of the index patient was MSI negative, while the colon cancer of her son was MSI positive. Segregation analysis showed that the son, despite having an MSI positive colon cancer at a young age, did not have the *MSH2* mutation. Furthermore, the alteration was not observed in 142 HNPCC families or families with a history of colorectal cancer.[53] We conclude that this mutation was not associated with colon cancer and it remains unclear whether it was important in the development of endometrial cancer in this family.

Two single base substitutions in *MSH6* were identified (families 154 and 106 in table 1). Both alterations have been published as non-pathogenic polymorphisms.[54 55] Interestingly, the index patients of these two families harboured both variants. To evaluate the pathogenicity of these variants, we analysed 30 patients with a family history of endometrial or

Table 1 Characteristics of 10 endometrial cancer and endometrial/colorectal cancer families and mutation screening results

			Mutation screening							
Fam No	*Tumour type*	*MSI status*	*MLH1 DGGE/PTT*	*MSH2 DGGE/PTT*	*MSH3 PTT*	*MSH6 PTT/sequencing*	*PMS1 PTT*	*PMS2 PTT*	*PTEN PTT*	*β–catenin sequencing of exon 3*
154	3 EC*	–†	–/–	–/–	–	–/pm‡	–	–	–	–
73	4 EC+Gi	ND §	–/–	–/–	–	–/–	–	–	–	–
54	4 EC	ND	–/–	–/–	–	–/–	–	–	–	–
121	2 EC+1 CRC¶	–	–/–	–/–	–	–/–	–	–	–	–
53	3 EC+3 CRC	–	–/–	–/–	–	–/–	–	–	–	–
147	1 EC+1 CRC	–	–/–	–/–	–	–/–	–	–	–	–
128	1 EC+4 CRC	–	–/–	–/–	–	–/–	–	–	–	–
60	1 EC+3 CRC	–	–/–	–/–	–	–/–	–	–	–	–
106	1 EC+4 CRC	ND	–/–	pm ** /–	–	–/pm ††	–	–	–	–
82	1 EC+1 CRC	–	–/–	mut‡‡/–	–	–/–	–	–	–	–

*Endometrial cancer.
†MSI analysis and mutation screening was negative.
‡Polymorphism was CCA→CCG (Pro→Pro) at 276 of exon 2, and GAT→GAC (ASP→ASP) at 540 in *MSH6*.
§MSI analysis was not done.
¶Colorectal cancer.
**Polymorphism was C→G (Ala→Ala) at 984 of exon 6 in *MSH2*.
††Polymorphism was CCA→CCG (Pro→Pro) at 276 of exon 2, and GAT→GAC (ASP→ASP) at 540 in *MSH6*.
‡‡Mutation was del TCT at 279–281 of exon 3 in *MSH2*.

colorectal cancer and 192 anonymous normal controls. Variant CCA→CCG (Pro→Pro) at codon 92 was found in 24% of the patients and 23.5% of normal controls, while variant GAT→GAC (Asp→Asp) was identified in 41% of the patients and 44% of normal controls. We also investigated the coexistence of these variants and found no statistically significant difference between the numbers of subjects with compound variants among our cases (7/30, 23%) and controls (23/192, 12%). The comparable frequencies observed in the patients and controls suggested that these variants were likely to be harmless polymorphisms. Finally, *PTEN* and *β-catenin* showed no germline mutations in this subset of endometrial cancer or endometrial and colorectal cancer families.

All available endometrial tumours (one tumour from seven families each) were MSI negative, including the tumour that harboured a germline mutation in *MSH2* described above. Endometrial tumours from HNPCC families are typically MSI positive.[3 16 22] Our finding of MSI negative tumours together with the lack of germline mutations in MMR genes suggests a non-significant role for these genes in the present relatively limited series of families with endometrial cancer. Even though the actual genes predisposing to endometrial and colorectal cancer in these families remain to be identified, the possibility of an increased cancer risk owing to inherited susceptibility should be considered in the counselling of such families.

- We recruited 10 families affected by either endometrial cancer alone or with both endometrial and colorectal cancer and carried out mutation screening of six MMR genes (*MLH1*, *MSH2*, *MSH3*, *MSH6*, *PMS1*, and *PMS2*). We also investigated the whole coding sequence of *PTEN* and a specific mutable site of *β-catenin*. No germline mutation with a clear pathogenic role was identified in any of the genes studied.
- Seven available endometrial tumours from the present families were MSI negative indicating that efficient mismatch repair was maintained in these tumours.
- The lack of clear cut pathogenic germline mutations of MMR genes suggests a limited role for these genes in familial endometrial cancer and implies that as yet unknown susceptibility genes are likely to be involved in such families.

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