Letters to the Editor

Pseudoxanthoma elasticum: evidence for the existence of a pseudogene highly homologous to the *ABCC6* gene

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EDITOR—Pseudoxanthoma elasticum (PXE, MIM 264800) is an inherited disorder of connective tissue in which the elastic fibres of the skin, eyes, and cardiovascular system slowly become calcified, causing a spectrum of disease involving these three organ systems, with highly variable phenotypic expression.[1][2] Mutations in the *ABCC6* gene (previously known as *MRP6*), encoding a 1503 amino acid membrane transporter, have recently been identified by our group and others[3–7] as the genetic defect responsible for PXE. We subsequently designed a strategy for a complete mutational analysis of the *ABCC6* gene, in order to provide accurate molecular and prenatal diagnosis of PXE. During this mutational screening, we have found evidence for the existence of at least one pseudogene highly homologous to the 5' end of *ABCC6*. Sequence variants in this *ABCC6*-like pseudogene could be mistaken for mutations in the *ABCC6* gene and consequently lead to erroneous genotyping results in pedigrees affected with pseudoxanthoma elasticum.

Material and methods

Seven unrelated patients presenting with PXE were evaluated for mutational analysis of the *ABCC6* gene. For each proband, diagnosis of PXE was consistent with previously reported consensus criteria,[8] which include a positive von Kossa stain of a skin biopsy, indicating calcification of elastic fibres, in combination with specific cutaneous and ocular manifestations (angioid streaks).

Whole blood samples were obtained after participants had provided written consent using a form that was approved by the Institutional Review Board of our academic institution. High molecular weight DNA was isolated from peripheral blood leucocytes, using a standard salting out procedure. Primers for amplification of the *ABCC6* gene were designed from the published sequence of human chromosome 16 bacterial artificial chromosome (BAC) clone A-962B4 (GenBank accession number U91318). PCR amplifications of *ABCC6* exon 2 and exon 9 were done in 20 µl volumes with 100 nmol/l of each of the respective PCR primers (table 1), 100 ng of genomic DNA, 100 µmol/l of each dNTP, 1.0 U Ampli*taq* Gold DNA polymerase (PE Biosystems), 10 mmol/l pH 8.3 Tris-HCl, 50 mmol/l KCl, and 1.5 mmol/l MgCl$_2$. The thermal cycling profile used was 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for one minute, followed by one cycle at 72°C for 10 minutes and a soak at 6°C.

RNA was isolated from lymphoblastoid cell lines or skin fibroblasts using Rneasy™ (Qiagen) and was reverse transcribed using random hexamers and Superscript-RT (Gibco BRL). Following reverse transcription, RT-PCR amplifications encompassing exon 2 in the published *ABCC6* cDNA sequence[9] (GenBank accession number AF076622) were done in 20 µl volumes with 4 µl of RT reaction. An aliquot of the amplified product was analysed by ethidium bromide visualisation on a 1.5% agarose gel. A 1/50 dilution was submitted to a nested PCR, using internal specific primers (table 1) and 2.0 U Ampli*taq* Gold DNA polymerase (PE Biosystems), under the following conditions: 95°C for 10 minutes, followed by 20 cycles at 95°C for 30 seconds, annealing temperature gradient ranging from 48°C to 70°C for 30 seconds, and 72°C for one minute 30 seconds, followed by one cycle at 72°C for 10 minutes, and a soak at 10°C, on a Robocycler gradient 96 (Stratagene).

All PCR and RT-PCR fragments were purified using QIAquick Spin PCR Purification Kit (Qiagen) according to the manufacturer's protocol, and 4 µl of the purified PCR products were sequenced using the Big Dye Terminator Ampli*Taq* FS Cycle Sequencing Kit on an automated ABI 310 DNA sequencer (PE Biosystems). DNA sequences were handled with Navigator 2.0 software.

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Table 1 *Primers for amplification of the ABCC6 gene and cDNA*

Primer	Sequence	Product size (bp)
Genomic		
ABCC6 exon 2 forward	5'-TCT GCG TCC TGG AGT TGT TA-3'	800
ABCC6 exon 2 forward	5'-ATG GGA GTG TAT GCG TAT GT-3'	
ABCC6 exon 9 forward	5'-GGA CAG TGG GGG AAA TAA CG-3'	676
ABCC6 exon 9 reverse	5'-TAG CTG GGC GTG GTG ACA CG-3'	
cDNA (nested PCR)		
ABCC6 exon 2 forward	5'-GAG CCT GAA CCT GCC GCC AC-3'	334
ABCC6 exon 2 reverse	5'-GAA TCA GGA ACA CTG CGA AG-3'	
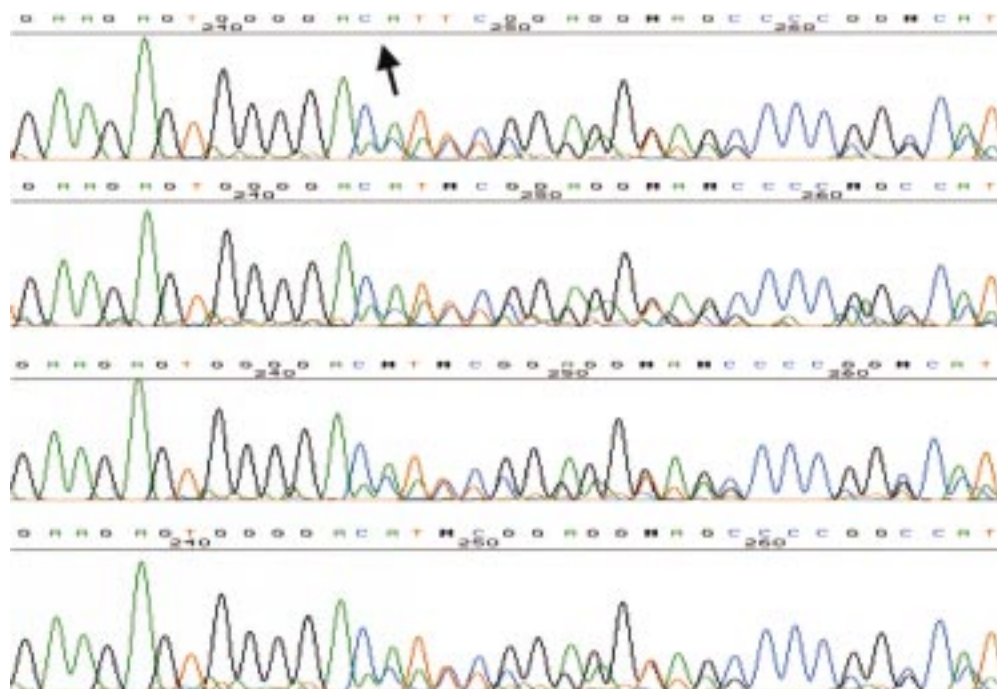
Figure 1 *Detection of a frameshift mutation (c196insT) in what was initially thought to be exon 2 of the ABCC6 gene. Genomic PCR products were sequenced using an antisense primer. Chromatograms show the insertion of an adenine (A) on the antisense strand (arrow), corresponding to a thymine (T) insertion on the sense strand. This single nucleotide insertion is responsible for a frameshift with consequent premature appearance of a stop codon. Heterozygosity for this frameshift mutation is shown here in two PXE patients (lanes 1 and 2) and two controls (lanes 3 and 4), but was also found in all 58 tested controls without exception. Sequencing of the other strand yielded the same result (not shown).*

Results

During our mutational screening of the *ABCC6* gene, we disclosed sequence changes which, although predicted to be truncating mutations, were unexpectedly detected not only in PXE patients but also in all tested controls. We first identified a single nucleotide insertion (c196insT) in the heterozygous state in a sporadic 14 year old female PXE patient. This mutation causes a shift in the reading frame, predicting a premature stop at codon 100 of the ABCC6 protein. Since we found this single nucleotide insertion in six other PXE patients, we initially interpreted this sequence change as a mutational hotspot. However, sequencing of the sporadic case's parents' DNA showed that, although unrelated to each other and phenotypically normal, they were both heterozygous for this frameshift mutation. These results were puzzling; if autosomal dominant inheritance with a de novo mutation[2] had occurred, neither of the unaffected parents should be a carrier, and, conversely, if autosomal recessive transmission had occurred, the proband should be a compound heterozygote on the basis of our results, and, consequently, only one of the parents would be expected to be a carrier of the c196insT mutation. These odd results prompted us to investigate 58 controls, all of whom showed a heterozygous profile for the frameshift mutation in what was thought to be exon 2 of the *ABCC6* gene (fig 1).

Similarly, a heterozygous C to T transition was found at cDNA position 1132 in exon 9 of the *ABCC6* gene. This nucleotide substitution alters the codon (CAG) for glutamine to a stop codon (TAG), predicting termination of translation at position 378 of the ABCC6 protein (Q378X). This nonsense mutation was detected in the heterozygous state in all seven PXE patients and in one healthy volunteer. Mutation Q378X predicted the loss of a *PstI*
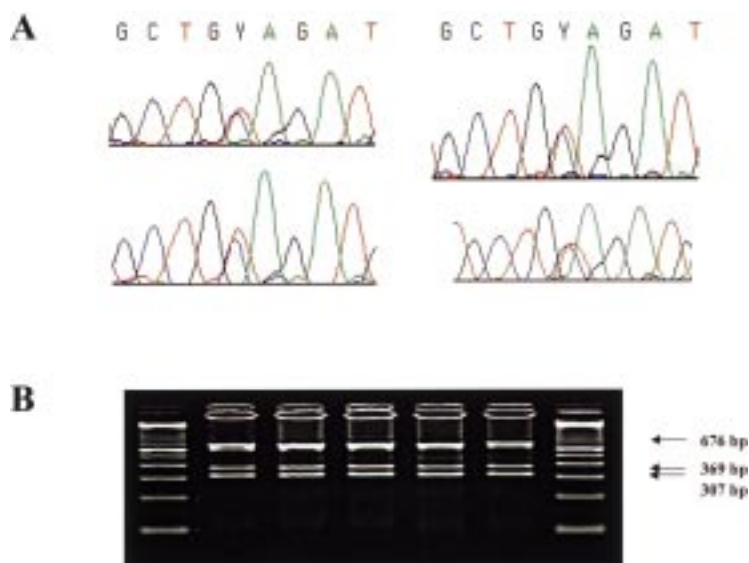
Figure 2 *Detection of a nonsense mutation (Q378X) in what was initially thought to be exon 9 of the ABCC6 gene. (A) Upper panel: identification of a heterozygous nonsense mutation in four patients affected with PXE by direct automated sequencing of exon 9 of the ABCC6 gene. The heterozygous C to T transition alters the codon (CAG) for glutamine to a stop codon (TAG) at position 378 of the ABCC6 protein. The position of the mutation is shown by the letter Y (Y=C and T). (B) Lower panel: mutation c1132C>T (Q378X) predicted the loss of a PstI restriction site. Restriction digests using PstI were performed on PCR amplified exon 9 of the ABCC6 gene. Five healthy volunteers, who although unaffected with PXE display heterozygosity for the Q378X nonsense mutation, are shown. The study of 75 additional white controls yielded the same result. This indicates that rather than being amplified from two genomic copies, the PCR products were being amplified from four genomic copies.*
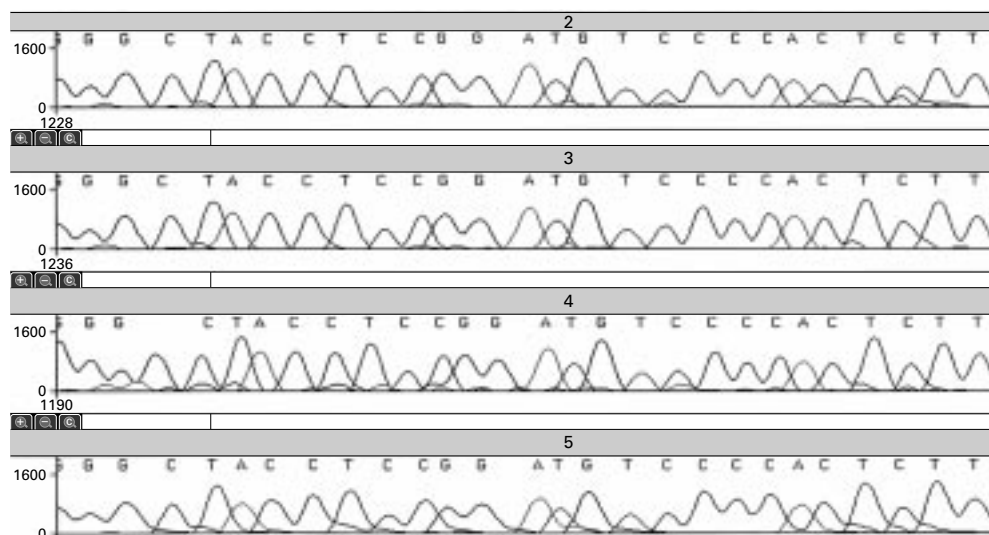
Figure 3 RT-PCR chromatograms of the region corresponding to exon 2 in the ABCC6 cDNA in the four subjects shown in lanes 1-4 in fig 1. ABCC6 mRNA was reverse transcribed and amplified through a nested PCR procedure. Direct automated sequencing of the region corresponding to the mutation detected in exon 2 at the genomic level does not show a thymine insertion in the mRNA and consequently no frameshift is seen. The same nucleotides as in fig 1 are shown, but sequencing was performed with a sense primer. These data indicate that the pseudogene is not expressed.

restriction site. To test for the presence or absence of this nucleotide change, we used *Pst*I to digest PCR amplified genomic DNA of 79 additional controls and found all of them to be heterozygotes for the Q378X nonsense mutation (fig 2).

Discussion
Both results are interesting although surprising, since they identify two mutations, one nonsense and the other one inducing a frameshift, expected to cause truncation of the protein and thereby compromise its function. However, these mutations have been shown to be non-pathogenic since they are consistently found in healthy subjects. Among possible explanations for these results, we initially thought of the existence of mutational hotspots, but this hypothesis was ruled out through the discovery of the same mutations in controls. A technical artefact of direct automated sequencing was also considered, but was eliminated through the use of other experimental techniques, including restriction digest experiments. We then checked for possible homologies within the ATP binding cassette (ABC) superfamily.[10] ABC genes are divided into seven distinct subfamilies (*ABC1*, *MDR/TAP*, *MRP*, *ALD*, *OABP*, *GCN20*, and *White*). However, if homologies do exist within the MRP subfamily (subfamily C) to which *ABCC6* belongs, they are not important enough to explain our results, according to our database searches.

Finally, one likely explanation is the existence of a pseudogene with high homology with the 5' end of the *ABCC6* gene, the PCR products being amplified from four rather than two genomic copies. Since we have used intronic primers to amplify *ABCC6* genomic sequences, an *ABCC6*-like pseudogene with introns is expected, as has been, for instance, reported for the gene encoding acid β-glucosidase.[11] Indeed, as is found in Gaucher disease, the existence of a highly homologous *ABCC6* pseudogene hampers the accuracy of molecular diagnosis of PXE, since sequence variants in the pseudogene might be mistaken for pathogenic mutations in the active *ABCC6* gene.

Pseudogenes are thought to arise from tandem gene duplication events caused by chromosome misalignment and unequal crossing over during meiosis. This mechanism would explain the high homology observed in both exonic and intronic sequences of the *ABCC6* gene and pseudogene.

The pseudogene could be located on a different chromosome or could be close to *ABCC6* on chromosome 16. In favour of the later hypothesis is the fact that the short arm of chromosome 16 has been shown to be a site where complex rearrangements have taken place.[12] Further evidence also comes from preliminary FISH experiments which detected double signals at 16p13.1, when fragments of BAC containing the *ABCC6* gene were used as probes.[13]

In order to determine whether the pseudogene is expressed or not, RNA was isolated from skin fibroblasts and lymphoblastoid cell lines and RT-PCR experiments amplifying exon 2 in *ABCC6* cDNA were performed. We found *ABCC6* mRNA to be expressed at low level in cultured skin fibroblasts and lymphoblastoid cell lines from both PXE patients and controls, in agreement with previous data indicating that *ABCC6* is mainly expressed in liver and kidney.[9 14] This prompted us to develop a nested PCR strategy, which proved efficient for the molecular analysis of *ABCC6* mRNA. No frameshift was shown when nested RT-PCR fragments, encompassing the region corresponding to *ABCC6* exon 2, were sequenced (fig 3). This indicates that the pseudogene that

we describe belongs to the unprocessed category.

In conclusion, we have found nonsense and frameshift sequence variations in the *ABCC6* gene, both of which appear to be non-pathogenic, thereby indicating the existence of at least one highly homologous pseudogene, which greatly complicates genotyping in families affected by pseudoxanthoma elasticum. Further studies are needed to map and fully characterise the sequence of the pseudogene(s). However, our results already emphasise the importance of not confusing variants in the pseudogene with pathogenic mutations in the *ABCC6* gene, especially in genetic counselling or prenatal diagnosis.

- Pseudoxanthoma elasticum (PXE) is an inherited systemic disorder of connective tissue with highly variable phenotypic expression.
- Mutations in the *ABCC6* gene were recently identified as the genetic defect responsible for PXE.
- We have characterised two truncating mutations (c196insT and Q378X) in the *ABCC6* gene, always found in the heterozygous state, not only in PXE patients but also in all controls.
- This indicates the existence of a highly homologous pseudogene.
- Sequence variants in the pseudogene should not be confused with mutations in the *ABCC6* gene, especially in genetic counselling or prenatal diagnosis.

1 McKusick VA. Pseudoxanthoma elasticum. In: McKusick VA, ed *Heritable disorders of connective tissue*. 4th ed. New York: Mosby, 1972.

2 Pope FM. Historical evidence for the genetic heterogeneity of pseudoxanthoma elasticum. *Br J Dermatol* 1975;**92**:493-509.

3 Bergen AA, Plomp AS, Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, van Soest S, Baas F, ten Brink JB, de Jong PT. Mutations in ABCC6 cause pseudoxanthoma elasticum. *Nat Genet* 2000;**25**:228-31.

4 Germain DP, Perdu J, Remones V, Jeunemaitre X. Homozygosity for the R1268Q mutation in *MRP6*, the pseudoxanthoma elasticum gene, is not disease-causing. *Biochem Biophys Res Commun* 2000;**274**:297-301.

5 Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards AJ, Terry S, Bercovitch L, de Paepe A, Boyd CD. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. *Nat Genet* 2000;**25**:223-7.

6 Ringpfeil F, Lebwohl MG, Christiano AM, Uitto J. Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. *Proc Natl Acad Sci USA* 2000;**97**:6001-6.

7 Struk B, Cai L, Zach S, Ji W, Chung J, Lumsden A, Stumm M, Huber M, Schaen L, Kim CA, Goldsmith LA, Viljoen D, Figuera LE, Fuchs W, Munier F, Ramesar R, Hohl D, Richards R, Neldner KH, Lindpaintner K. Mutations of the gene encoding the transmembrane transporter ABC-C6 cause pseudoxanthoma elasticum. *J Mol Med* 2000;**78**:282-6.

8 Lebwohl M, Neldner K, Pope FM, de Paepe A, Christiano AM, Boyd CD, Uitto J, McKusick VA. Classification of pseudoxanthoma elasticum: report of a consensus conference. *J Am Acad Dermatol* 1994;**30**:103-7.

9 Kool M, van der Linden M, de Hass M, Baas F, Borst P. Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. *Cancer Res* 1999;**59**:175-82.

10 Borst P, Evers R, Kool M, Wijnholds T. The multidrug resistant protein family. *Biochim Biophys Acta* 1999;**1461**: 347-57.

11 Horowitz M, Wilder S, Horowitz Z, Reiner O, Gelbart T, Beutler E. The human glucocerebrosidase gene and pseudogene: structure and evolution. *Genomics* 1989;**4**:87-96.

12 Loftus BJ, Kim UJ, Sneddon VP, Kalush F, Brandon R, Fuhrmann J, Mason T, Crosby ML, Barnstead M, Cronin L, Deslattes Mays A, Cao Y, Xu RX, Kang HL, Mitchell S, Eichler EE, Harris PC, Venter JC, Adams MD. Genome duplications and other features in 12 Mb of DNA sequence from human chromosome 16p and 16q. *Genomics* 1999;**60**: 295-308.

13 Cai L, Struk B, Adams MD, Ji W, Haaf T, Kang HL, Dho SH, Xu X, Ringpfeil F, Nancarrow J, Zach S, Schaen L, Stumm M, Niu T, Chung J, Lunze K, Verrecchia B, Goldsmith LA, Viljoen D, Figuera LE, Fuchs W, Lebwohl M, Uitto J, Richards R, Hohl D, Ramesar R. A 500-kb region on chromosome 16p13.1 contains the pseudoxanthoma elasticum locus: high-resolution mapping and genomic structure. *J Mol Med* 2000;**78**:36-46.

14 Belinsky MG, Kruh GD. MOAT-E (ARA) is a full length MRP/cMOAT subfamily transporter expressed in kidney and liver. *Br J Cancer* 1999;**80**:1342-9.

Hereditary and somatic DNA mismatch repair gene mutations in sporadic endometrial carcinoma

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EDITOR—Endometrial cancer (EC) is the second most common malignancy in the hereditary non-polyposis colorectal cancer (HNPCC) syndrome.[1] In a recent large study, cumulative cancer incidences by the age of 70 in HNPCC mutation carriers were: colorectal 82%, endometrial 60%, gastric 13%, and ovarian 12%.[2] Interestingly, in female mutation carriers the incidence of endometrial cancer (60%) exceeded that of colorectal cancer (CRC) (54%), as had been suggested earlier.[2 3]

Predisposition to HNPCC is the result of germline mutations in the mismatch repair genes.[4] Detectable mutations in the two major genes, *MLH1* and *MSH2*, account for some 3% of all colorectal cancers.[5] One might therefore assume that a similar proportion of all endometrial cancer patients would have such mutations; however, in a number of studies addressing this question, extremely few germline mutations have been found. Summarising the studies by Katabuchi *et al*,[6] Kobayashi *et al*,[7] Lim *et al*,[8] Gurin *et al*,[9] and Kowalski *et al*,[10] only one germline mutation (in *MLH1*) was found in a total of 352 EC patients (0.3%). In these studies, mutations were sought in all patients whose tumours were microsatellite instability (MSI) positive.

Recent reports have suggested that *MSH6* might account for many endometrial cancers and that families with these mutations show atypical features of HNPCC with endometrial and ovarian cancers outnumbering colorectal cancers.[11 12] Additionally, MSI, a hallmark of HNPCC, was low in most tumours associated with *MSH6* mutations or was preferentially shown by mononucleotide repeats rather than dinucleotide repeats.[12–14] Previous studies have reported that 9-25% of sporadic endometrial cancers display microsatellite instability.[7 9 15–18] In the majority of cases, this instability arises through hypermethylation of the *MLH1* promoter region.[9 19–21] This epigenetic change results in reduced (or no) expression of the *MLH1* transcript.[22]

This study was undertaken to revisit the issue of microsatellite instability and mismatch repair gene mutations in sporadic endometrial cancer. By initially studying tumour tissue, both germline and somatic mutations were evaluated in the *MSH2*, *MLH1*, and *MSH6* genes in a retrospective series of microsatellite stable and microsatellite unstable endometrial cancers.

Material and methods
All patients diagnosed with endometrial adenocarcinoma between October 1996 and February 1998 at the Ohio State University Hospital were considered retrospectively. Among these 85 patients, archival tissue was available from 74.

After appropriate investigational review board (IRB) approval, these 74 charts were reviewed and the tissue blocks recovered. Histological sections were made, stained with haematoxylin and eosin, and the histological diagnosis critically re-evaluated. Sections 50 μm thick were cut from regions of the tumour containing as high a proportion of tumour cells as possible (typically >50%). To obtain nonmalignant tissue, sections were obtained either from tissue emanating from other organs, primarily lymph nodes, that were histologically cancer free, or alternatively sections were made from parts of the endometrial tissue that had no cancer cells. All materials were unlinked from their identifiers before being subjected to DNA extraction and genetic analyses.

Tissue sections were deparaffinised with two xylene washes. Rehydration was accomplished through 20 minute incubations in decreasing concentrations of alcohol (100%, 80%, 50%) at room temperature followed by an overnight incubation in double distilled water at 4°C. DNA was extracted by lysis of the tissue for 18 hours at 55°C with 1 mg/ml proteinase K in 400 μl of buffer (10 mmol/l Tris, 400 mmol/l NaCl, 2 mmol/l EDTA, and 0.7% sodium dodecyl sulphate, pH 8.2). Degraded proteins were precipitated with 2.5 volumes of saturated NaCl after centrifugation. DNA was precipitated with 2.5 volumes of 100% ethanol at –20°C, washed in 70% cold ethanol, then dissolved in 50 μl of TE buffer (10 mmol/l Tris and 1 mmol/l EDTA, pH 8.0).

Microsatellite sequences were amplified using the Bethesda panel.[13] Owing to limited availability of normal tissue, tumour DNA was used for MSI determination without its corresponding normal DNA pair. Amplifications were done in 15 μl PCR reaction volumes using 1 μl of each 8 μmol/l primer (the 5' primer is fluorescently labelled), 10 ng of genomic DNA, and 8 μl of Qiagen's HotStar*Taq* Master Mix. The thermal cycling profile was one cycle at 95°C for 12 minutes, followed by 45 cycles at 95°C for 10 seconds, 55°C for 15 seconds, and 72°C for 30 seconds, followed by one cycle at 72°C for 30 minutes, followed by a soak at 4°C. Respective PCR reactions for each marker were pooled together and loaded on to the PE3700 automated sequencer. Allele sizing and calling was done using Genotyper software (Applied Biosystems). For polymorphic markers D2S123, D5S346, and D17S250 samples were scored as MSI positive if more than two alleles