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Germline epimutation of *MLH1* in individuals with multiple cancers

Catherine M Suter[1], David I K Martin[2,3] & Robyn L Ward[1,4]

Epigenetic silencing can mimic genetic mutation by abolishing expression of a gene. We hypothesized that an epimutation could occur in any gene as a germline event that predisposes to disease and looked for examples in tumor suppressor genes in individuals with cancer. Here we report two individuals with soma-wide, allele-specific and mosaic hypermethylation of the DNA mismatch repair gene *MLH1*. Both individuals lack evidence of genetic mutation in any mismatch repair gene but have had multiple primary tumors that show mismatch repair deficiency, and both meet clinical criteria for hereditary nonpolyposis colorectal cancer. The epimutation was also present in spermatozoa of one of the individuals, indicating a germline defect and the potential for transmission to offspring. Germline epimutation provides a mechanism for phenocopying of genetic disease. The mosaicism and nonmendelian inheritance that are characteristic of epigenetic states could produce patterns of disease risk that resemble those of polygenic or complex traits.

Epigenetic silencing is a stable but reversible alteration of gene function mediated by histone modification, cytosine methylation, the binding of nuclear proteins to chromatin and interactions among these[1,2]. It does not require, or generally involve, changes in DNA sequence. Errors in the elaborate apparatus of epigenetic silencing possessed by higher eukaryotes can lead to 'epimutation'[3–5], which we define as epigenetic silencing of a gene that is not normally silenced, or epigenetic activation of a gene that is normally silent. Germline epimutations are known in plants, examples being methylation and transcriptional silencing of the gene *Lcyc* in toadflax (*Linaria vulgaris*)[6], paramutation in maize[5,7] and the *clark kent* alleles of SUPERMAN in *Arabidopsis thaliana*[8]. Similar phenomena in mammals could be an unrecognized source of phenotypic effects, which might manifest as disease. The increasingly detailed understanding of the genetics of human disease suggests a strategy to identify epimutations: screen for methylation of known disease-associated genes in affected individuals who do not carry a mutation in the relevant gene. Tumor suppressors are good candidates for this strategy because there is a clear relationship between their inactivation and the development of cancer. Germline mutations in tumor suppressor genes can predispose to cancer[9]. Tumor suppressors are also commonly methylated (and inactivated) in the course of neoplastic progression[10], but the causal relationship between hypermethylation and tumorigenesis has not been established.

We hypothesized that some individuals are predisposed to develop cancer because they carry germline epimutations of tumor suppressor genes. We selected 94 individuals for this study: 18 with hyperplastic polyposis[11], 11 with personal histories of colorectal cancer and 65 with a family history of colorectal cancer but without deleterious germline changes in *MSH2*, *MLH1* or *APC*[12]. We screened a subset of 44 individuals for promoter methylation of *MLH1*, *CDKN2A*, *TMEFF2*, *HIC1*, *RASSF1*, *BRCA1*, *APC* (promoters 1A and 1B), *BLM* and *MGMT*. We subjected bisulfite-modified DNA from peripheral blood to either combined bisulfite-restriction analysis (COBRA)[13] or methylation-specific PCR (MSP)[14]. We identified one individual (TT) with methylation of the *MLH1* (mutL homolog 1) promoter (**Fig. 1**). We

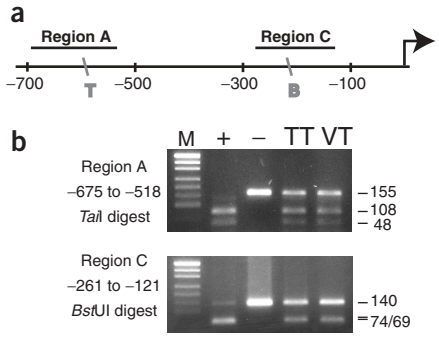

Figure 1 Methylation of *MLH1* in peripheral blood from individuals TT and VT. (**a**) The *MLH1* promoter showing the two regions screened by COBRA, with sites for the restrictions enzymes *Tai*I (T) and *Bst*UI (B). (**b**) COBRA of regions A and C of the *MLH1* promoter. The location of each region relative to the transcription start site is shown on the left. Amplicons of regions A and C generated from methylated template will digest with *Tai*I or *Bst*U1, respectively. PCR products from unmethylated template will not digest. M, pUC19/*Msp*I DNA ladder; +, RKO colon cancer cell line; –, healthy control peripheral blood.

[1]Department of Medical Oncology, St Vincent's Hospital, Sydney, Australia. [2]Victor Chang Cardiac Research Institute, Sydney, Australia. Schools of [3]Biotechnology and Biomolecular Sciences and [4]Medicine, University of New South Wales, Sydney, Australia. Correspondence should be addressed to D.I.K.M (d.martin@victorchang.unsw.edu.au).

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Table 1 Clinical details of individuals TT and VT

Individual (current age, sex)	Cancer	Age at diagnosis	MLH1 IHC	MSI	*MLH1* LOH
TT (64 y, male)	Colorectal, caecum	43	–	+	+[a]
	Colorectal, descending colon	44	NA	NA	NA
	Duodenal, 3 synchronous	51	–	+	+[a,b]
	Ampulla of Vater	59	–	+	+
VT (65 y, female)	Colorectal, caecum	46	–	+	+[a]
	Endometrial	53	–	+	ND
	Melanoma	57	NA	NA	NA
	Breast, infiltrating ductal	63	–	+	+[a]

[a]Loss of the A allele. [b]Two of three cancers showed LOH.
IHC, immunohistochemistry; NA, not available; ND, not detected; LOH, loss of heterozygosity.

then screened peripheral blood DNA from the other 50 probands for promoter methylation of *MLH1* only. In this group, we identified a second individual (VT) with *MLH1* methylation (**Fig. 1b**). Analysis of the tissue distribution of methylation in individuals TT and VT showed that regions A and C of the *MLH1* promoter (as assessed by COBRA) were methylated in all available normal tissue, which included buccal mucosa, hair follicles and peripheral blood collected two years before this study.

The medical histories of these two unrelated individuals are remarkable: during a 20-year period, they were successfully treated for six (individual TT) and four (individual VT) primary malignancies (**Table 1**). Their first colorectal cancers occurred at 43 and 46 years of age, respectively, and their family histories fulfill the Amsterdam II (individual TT) or modified Amsterdam (individual VT) criteria for the clinical diagnosis of hereditary nonpolyposis colorectal cancer[15] (HNPCC; see pedigrees in **Supplementary Fig. 1** online). In both individuals, extensive screening did not detect germline mutations in *MLH1*. The adenocarcinomas in the colorectum, small bowel and endometrium were pathologically similar to those seen in HNPCC[16]. All of the carcinomas available for testing, including the infiltrating ductal carcinoma of the breast, showed microsatellite instability (MSI) and complete loss of MLH1 protein expression (**Fig. 2**). Allelic loss of *MLH1* was evident in all tumors tested, except the endometrial cancer (**Table 1**).

To determine the distribution of methylation in the *MLH1* promoter, and to establish whether one or both alleles were methylated, we used bisulfite sequencing[17] of an *MLH1* promoter fragment that contains a G→A polymorphism (at nucleotide –33) for which both individuals TT and VT are heterozygous. We amplified bisulfite-modified DNA with primers that anneal to both methylated and unmethylated templates. Sequencing of individual alleles showed that the *MLH1* promoter was hypermethylated in peripheral blood, hair follicles and buccal mucosa of both individuals, but that methylation was restricted to the G allele (**Fig. 3**). Although we never observed a hypermethylated A allele in either individual, we occasionally observed hypomethylated G alleles.

The presence of the epimutation in normal somatic tissue derived from all three germ cell lineages (endoderm, buccal mucosa; mesoderm, blood; ectoderm, hair follicles) implies that it occurred as a germline event, and that it might be passed on to offspring. To assess germline tissue directly for the presence of the epimutation, we isolated spermatozoa from semen of individual TT by fluorescence-activated cell sorting. Spermatozoa from individual TT were negative with respect to *MLH1* methylation by COBRA. As a more sensitive means of detection, we cloned and sequenced products of PCR amplification with one MSP primer and one of the methylation-unbiased primers used in the studies of somatic methylation (**Fig. 4a**). Both A and G alleles were recovered, but as in somatic cells, hypermethylation was evident only on the G allele (**Fig. 4b**,**c**). We found no evidence of *MLH1* methylation in the spermatozoa of 14 anonymous unaffected donors (**Supplementary Fig. 2** online).

We then used the methylation-unbiased primers (**Fig. 3b**) to amplify spermatozoa DNA from individual TT and, after cloning into a plasmid vector, screened it for methylated alleles by hybridization with a methylation-specific probe. This method identified 5 of 526 screened colonies, which we confirmed by sequencing to be hypermethylated G alleles (data not shown). The presence of the epimutation in a low proportion of germ cells from individual TT is consistent with an incomplete resetting of the epigenetic mark on *MLH1*, as has been reported with examples of epigenetic inheritance in mice[18,19]. Tissues from the parents of individuals TT and VT were not available for study. Methylation was not detected by

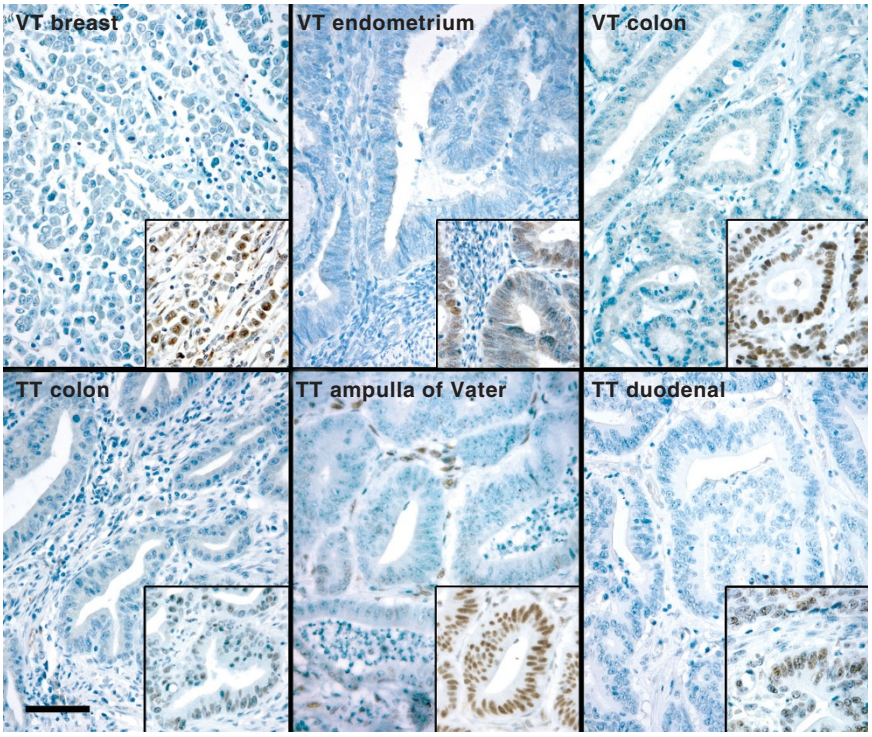

Figure 2 Immunohistochemical analysis of MLH1 expression in representative cancers (as indicated) from individuals TT and VT, using immunoperoxidase with hematoxylin counterstain. All cancers show complete loss of MLH1 expression. In each frame, the inset shows positive staining of the same tumor for MSH2. Scale bar (lower left), 100 m.

COBRA or MSP in peripheral blood from one of individual TT's two children and from two of individual VT's three children. All these offspring were heterozygous with respect to the G→A polymorphism. The genotype of the father of individual VT's children is unknown, and so we could not determine whether they inherited the G allele from individual VT. Individual TT's spouse has the genotype AA, indicating that their daughter must have inherited the G allele from individual TT, and that the epimutation had been cleared from the allele she received. Given the low proportion of spermatozoa carrying the epimutation, it is probably transmitted to offspring only occasionally.

Our results suggest that individuals TT and VT carry a germline epimutation in *MLH1*. The epimutation is confined to one of the two alleles of *MLH1*, is found in normal somatic tissues with distinct embryonic origins, and is present in spermatozoa of individual TT. In four of the five tumors in which we found allelic loss of *MLH1*, the A allele (the unmethylated allele) was lost; in the fifth, loss of heterozygosity (LOH) did not include the site of the G→A promoter polymorphism (**Table 1** and **Supplementary Table 1** online). The occurrence of multiple MSI tumors in individuals TT and VT implies that the epimutation in *MLH1* has predisposed them to develop malignancies by inactivating one allele, in essence acting as the first hit to *MLH1*. Hypermethylation of the *MLH1* promoter, as seen in these two individuals, is invariably associated with transcriptional silence of the gene[20]. Methylation of *MLH1* has also been observed in normal colonic tissue adjacent to MSI tumors[21], and although its relationship to tumor development is not clear, it may be a precursor event[22]. An individual with hypermethylation of *MLH1* in peripheral blood and LOH in his colon tumor has been reported[23]; our results support the speculation that this reflected a germline defect in one allele. Epimutation might also account for the unbalanced expression of *MLH1* alleles in some individuals with HNPCC[24]. The small proportion of hypomethylated G alleles in individuals TT and VT is consistent with mosaicism, which is typical of epigenetic silencing. Smaller proportions of epimutated alleles might produce different, but still substantial, risks of developing cancer.

We suppose that the epimutations of *MLH1* in individuals TT and VT arose either in the parental germ line or very early in embryogenesis. Epigenetic states can be maintained in the germ line to produce epigenetic inheritance[18,19], and the presence of the epimutation in a small proportion of spermatozoa from individual TT indicates that it has the potential to be transmitted to offspring. Epimutation of *MLH1* resembles aberrations of parental imprinting[25] and the silencing of expanded triplet repeats[26], but *MLH1* is not an imprinted gene, and the clearing of the epimutation in individual TT's daughter militates against an underlying defect in the affected locus as a cause of silencing. More similar is the occurrence of facioscapulohumeral muscular dystrophy in individuals and kindreds who have hypomethylation of the D4Z4 repeat array[27]. The occurrence of germline epimutation at *MLH1* suggests that normally active loci can become silent chromatin in the germ line, perhaps through association with constitutively silent sequences. Regardless of the mechanism, our finding indicates that an epimutation can predispose individuals to develop a common disease. The maintenance of epigenetic states has a complex molecular basis that is quite unlike the stable transmission of sequence that produces mendelian traits[1,2]. Within an organism, epigenetic states are frequently mosaic, and when inheritance occurs, it is often weak. Consequently, epimutations may masquerade as polygenic traits by producing patterns of disease that are inconsistent with single gene mutations.

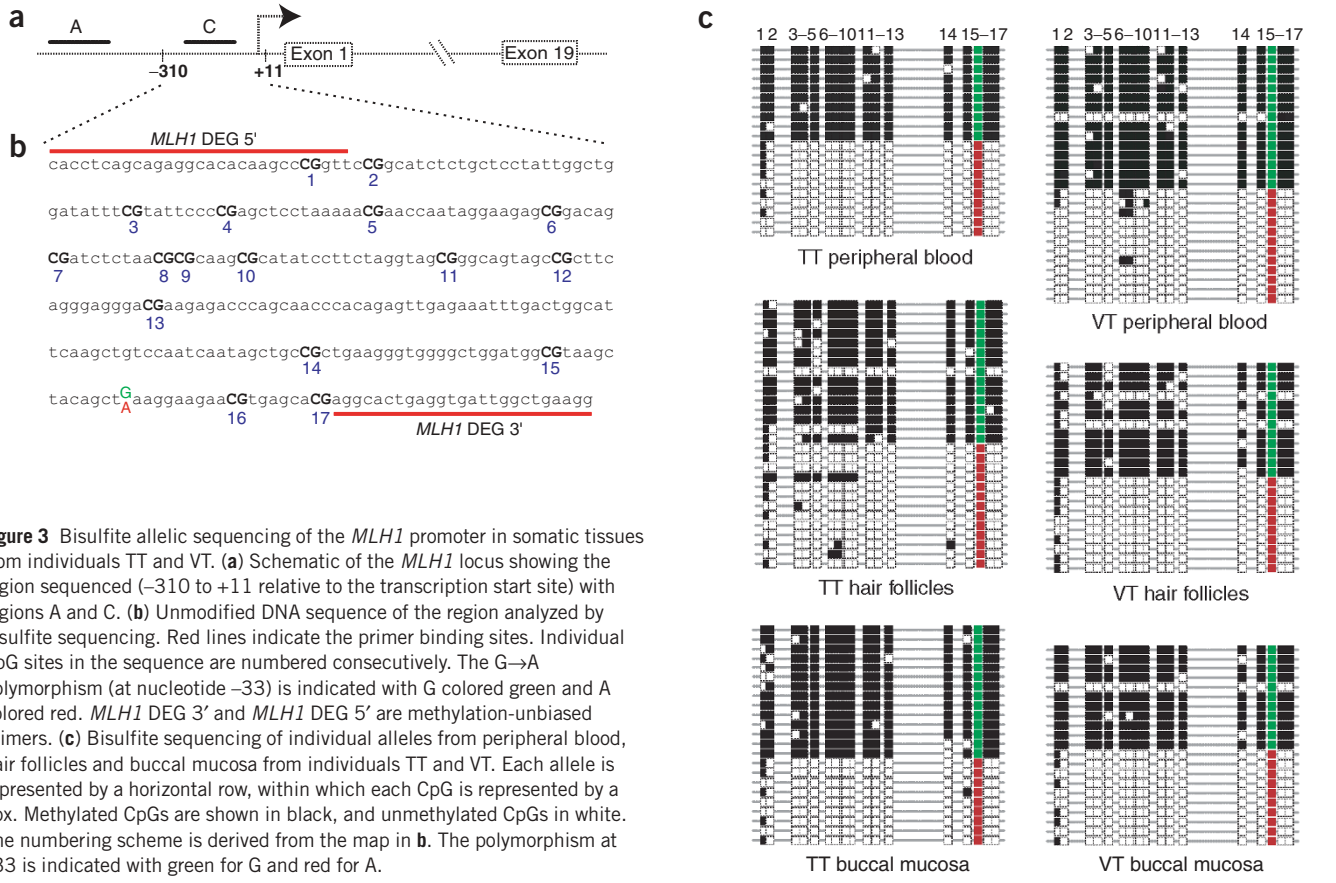

Figure 3 Bisulfite allelic sequencing of the *MLH1* promoter in somatic tissues from individuals TT and VT. (**a**) Schematic of the *MLH1* locus showing the region sequenced (–310 to +11 relative to the transcription start site) with regions A and C. (**b**) Unmodified DNA sequence of the region analyzed by bisulfite sequencing. Red lines indicate the primer binding sites. Individual CpG sites in the sequence are numbered consecutively. The G→A polymorphism (at nucleotide –33) is indicated with G colored green and A colored red. *MLH1* DEG 3′ and *MLH1* DEG 5′ are methylation-unbiased primers. (**c**) Bisulfite sequencing of individual alleles from peripheral blood, hair follicles and buccal mucosa from individuals TT and VT. Each allele is represented by a horizontal row, within which each CpG is represented by a box. Methylated CpGs are shown in black, and unmethylated CpGs in white. The numbering scheme is derived from the map in **b**. The polymorphism at –33 is indicated with green for G and red for A.

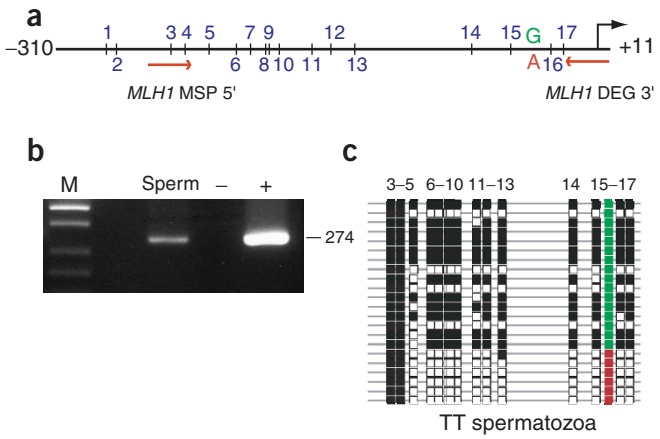

Figure 4 Analysis of *MLH1* methylation in spermatozoa from individual TT. (**a**) The *MLH1* promoter with CpG sites 1–17 as in **Figure 3b**, the G→A polymorphism and the sites of primers used to amplify bisulfite-modified DNA. *MLH1* MSP 5′ is specific for methylated sequence; *MLH1* DEG 3′ is the same methylation-unbiased primer shown in **Figure 3b**. (**b**) PCR products from bisulfite-modified spermatozoa DNA. Amplification of spermatozoa DNA (sperm) was weak in relation to the positive control (+, RKO cancer cell line; –, no template control). M, pUC19/*Msp*I DNA ladder. (**c**) Bisulfite sequencing of individual alleles from the sperm PCR product shown in **b**. Methylated CpGs are shown as black boxes, and unmethylated CpGs as white boxes. CpGs are numbered as in **Figure 3b**. The polymorphism at –33 is indicated with green for G and red for A.

METHODS

Affected individuals and samples. This study was approved by the Human Research Ethics Committee of St. Vincent's Hospital, Sydney, Australia. All individuals in this study are of European descent. We obtained tissue samples with informed consent from individuals at St. Vincent's Hospital, Sydney, Australia, and the Victorian Clinical Genetics Service, Melbourne, Australia. Included in this study were 18 individuals with hyperplastic polyposis (mean age 69 y, range 62–78 y) and 11 individuals with sporadic colorectal cancer (mean age 71 y, range 48–91 y); the remainder all met clinical criteria for HNPCC (Amsterdam I, II or modified Amsterdam) or familial adenomatous polyposis. We extracted DNA from peripheral blood, buccal smears, hair follicles and sperm using a standard phenol-chloroform method. To exclude the possibility of contaminating somatic cells in the sperm, we sorted semen on a FACSVantage DiVa (Becton Dickinson) before extracting DNA. We identified sperm on the basis of DNA content after propidium iodide staining as described[28]. Flow cytometry and microscopy verified that we used a pure population of spermatozoa for DNA extraction.

Methylation analyses. We treated DNA (2 g) with sodium bisulfite and subjected it to COBRA for regions A and C of the *MLH1* promoter as previously described[29]. For bisulfite sequencing of peripheral blood, hair follicles and buccal mucosa, we amplified bisulfite-modified DNA with a degenerate primer set, *MLH1* DEG 5′ and *MLH1* DEG 3′, designed to be unbiased for the methylation status of the template. For bisulfite sequencing of spermatozoa, we replaced the degenerate forward primer with the methylation-specific primer *MLH1* MSP 5 (in some experiments we used the unbiased primers shown above). Primer sequences are available on request. We cloned PCR products into the pGEM-T vector (Promega) and sequenced individual clones using BigDyes (ABI). Sequences with non-CpG methylation, which may indicate partial nonconversion during the bisulfite reaction, were discarded from the analysis.

Colony hybridization assay. We used a standard colony hybridization assay to estimate the frequency of methylated alleles in spermatozoa. We probed bacterial colonies carrying individual plasmids with a ^{32}P-labeled oligonucleotide specific for methylated alleles (sequence available on request). We then sequenced clones with a positive signal to confirm methylation.

MSI analysis. Before extracting DNA from paraffin-embedded tumors, we examined an adjacent section histologically to ensure that it contained more than 80% tumor tissue. If this was not the case, we microdissected foci of the tumor. We determined the microsatellite status of each tumor as previously described using the following primer sets: Bat 25, Bat 26, Bat 40, *D5S346*, *D2S123* and *D17S250* (ref. 30). We classified tumors as MSI only if two or more markers showed instability.

LOH analysis. To detect allelic loss of *MLH1*, we used microsatellite markers *D3S1447* and *D3S3685* and single-nucleotide polymorphisms in *MLH1* (rs1800734 and rs4647277). We amplified genomic DNA with the primers P-SNP-F and P-SNP-R for rs1800734, and intron10-F and intron10-R for rs4647277. Primer sequences are available on request. Alleles at these markers were distinguished by restriction digestion of PCR amplicons with *Pvu*II and *Hha*I, respectively (with both enzymes, the G allele is sensitive and the A allele is resistant). Only heterozygous loci were considered informative, and LOH was scored when there was a reduction (ratio <0.5 or >2.0) or total loss of one allele in tumor DNA relative to peripheral blood DNA.

Immunohistochemical staining for MSH2 and MLH1. Immunohistochemical analysis of MLH1 and MSH2 was carried out as previously described[30]. The immunostaining analysis was reported independently by two histopathologists without knowledge of MSI status, germline or methylation results. Expression of MLH1 or MSH2 was considered to be absent where there was no staining of tumor cells in the presence of nuclear staining in nearby stromal lymphocytes or in epithelial cells in non-neoplastic tissue.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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