5'-CpG island methylation of the *LKB1/STK11* promoter and allelic loss at chromosome 19p13.3 in sporadic colorectal cancer

J Trojan, A Brieger, J Raedle, M Esteller, S Zeuzem

Abstract

***Background*—In patients with Peutz-Jeghers syndrome (PJS), causative germline mutations in the *LKB1/STK11* gene on chromosome 19p13.3 have been identified. Because of the loss of heterozygosity (LOH) at 19p13.3 in hamartomas and the cancer susceptibility of patients with PJS, LKB1/STK11 is suggested to act as a tumour suppressor. However, the frequency of genetic and epigenetic inactivation of *LKB1/STK11* in sporadic tumours is unclear.**

***Aims*—To investigate the *LKB1/STK11* gene for promoter hypermethylation and allelic loss in tumour specimens of patients with sporadic colorectal cancer.**

***Methods*—DNA from 50 consecutive paraffin embedded sporadic colorectal adenocarcinomas and corresponding normal epithelium was extracted. After bisulphite treatment, specimens were analysed for methylation of the *LKB1/STK11* promoter 5'-CpG island by methylation specific polymerase chain reaction (MSP). In addition, tumours were analysed for LOH of chromosome 19p13.3. In tumours exhibiting LOH, *LKB1/STK11* was sequenced.**

***Results*—MSP was successful in 48 of 50 tumour specimens. Of those, four (8%) demonstrated hypermethylation of the *LKB1/STK11* promoter 5'-CpG island. Moreover, LOH at either D19S886 or D19S878 was observed in five of 38 (13%) informative tumours. All five tumours showing LOH at 19p13.3 were advanced and four of five were located in the left sided colon. There was no correlation between LOH and *LKB1/STK11* promoter hypermethylation or somatic mutation.**

***Conclusions*—In sporadic colorectal cancer, hypermethylation of the *LKB1/STK11* promoter and allelic loss at the *STK 11* gene locus are rare events. LOH at 19p13.3 was associated with advanced tumour stage and left sided location but not with *LKB1/STK11* promoter hypermethylation or somatic mutation.**

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Keywords: sporadic colorectal adenocarcinoma; tumour suppressor genes; protein-serine-threonine kinase gene *LKB1/STK11*; promoter hypermethylation; loss of heterozygosity

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Several tumour suppressor genes, which are inactivated through germline mutations in the most commonly inherited colorectal cancer susceptibility syndromes, such as the *APC* gene in familial adenomatous polyposis and the DNA mismatch repair genes *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6/GTBP* in hereditary non-polyposis colorectal cancer, are involved in the development and progression of sporadic colorectal cancer.[1 2] Moreover, loss of transcription of tumour suppressor genes, including *p16*, *MGMT*, and *MLH1*, by epigenetic changes such as hypermethylation of 5'-CpG islands in the promoter region have been demonstrated in colorectal cancer.[3 4]

Recently, a gene mutation in patients with Peutz-Jeghers syndrome (PJS), an autosomal dominant disorder characterised by mucocutaneous pigmentation, intestinal hamartomas, and an increased risk of cancers of the gastrointestinal tract, breast, testis, and ovary, has been identified by genetic linkage studies and positional cloning.[5 6] This gene, named *LKB1*, *STK11*, or *LKB1/STK11*, is located on chromosome 19p13.3[7] and encodes for a serine-threonine kinase, a human homologue of *Xenopus* early embryonic kinase 1.[8] LKB1/STK11 is suggested to act as a tumour suppressor gene in PJS because hamartoma formation in PJS patients with inactivating *LKB1/STK11* germline mutations is associated with somatic loss of the wild-type *LKB1/STK11* allele.[7 9 10] The development of cancer in patients with PJS does not exclusively arise in association with hamartomas[11] but dysplasia with consecutive neoplastic transformation within hamartomatous polyps accounts for at least some malignancies in this syndrome.[12 13] In contrast with the pathogenesis of sporadic colorectal cancer, frequently involving *APC, K-ras, DCC, MCC*, and *p53*,[1 2 14] the molecular mechanisms leading to cancer in patients with PJS remain unclear. Because patients with PJS are at increased risk of colorectal cancer, *LKB1/STK11* may also be a target during the carcinogenesis of sporadic colorectal cancer. Although some reports revealed a low frequency of somatic mutations of the *LKB1/STK11* gene in colorectal tumour specimens,[15–17] conflicting data were most recently reported by Dong and colleagues.[18] This group identified somatic *LKB1/STK11* mutations in one third of left sided colorectal cancers and in two colonic adenomas. In con-

Abbreviations used in this paper: *LKB1/STK11*, serine-threonine kinase gene *LKB1/STK11*; LOH, loss of heterozygosity; PJS, Peutz-Jeghers syndrome; UICC, International Union Against Cancer; PCR, polymerase chain reaction; MSP, methylation specific polymerase chain reaction; bp, base pairs.

trast, *LKB1/STK11* promoter hypermethylation leading to transcriptional inactivation was found in a few cancer cell lines and a subset of primary tumours.[19]

To further analyse the role of *LKB1/STK11* on chromosome 19p13.3 in the pathogenesis of sporadic colorectal cancer, we investigated the frequency of genetic and epigenetic alterations at the *LKB1/STK11* gene locus in tumour specimens from 50 consecutive patients with colorectal cancer.

Methods

TUMOUR SPECIMENS

Tumour specimens from 50 consecutive patients (21 females, 29 males) with sporadic colorectal adenocarcinoma (International Union Against Cancer (UICC) stage I, n=9; stage II, n=10; stage III, n=17; stage IV, n=14) were analysed. At the time of diagnosis, patients were aged 35–95 years (mean 55 (17) years). In 38 patients tumours were left sided (descending colon, sigmoid colon, or rectum) whereas 12 patients had a right sided colon carcinoma (transverse colon, ascending colon, or caecum). Histologically, two tumours were characterised as well differentiated, 39 as moderately differentiated, six as poorly differentiated, and three as undifferentiated. None of the patients had a family history of PJS, familial adenomatous polyposis, or hereditary non-polyposis colorectal cancer and none of the tumours exhibited microsatellite instability (data not shown).

For molecular analysis, representative 5 µm sections of paraffin embedded normal and tumour tissue were mounted onto slides and dried for 60 minutes at 50°C. After microdissection DNA was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany).

METHYLATION SPECIFIC PCR OF THE *LKB1/STK11* PROMOTER

Analysis of methylation patterns within the 5'-CpG island of the LKB1/STK11 gene were carried out using chemical modification of 1 µg of genomic DNA from colorectal cancer specimens with sodium bisulphite and methylation specific polymerase chain reaction (MSP) using sense primers for methylated and unmethylated polymerase chain reactions (PCR) beginning at base pairs (bp) 15 and 17, respectively, from GeneBank sequence AF 035625, as previously described.[19] Primers used for the unmethylated reaction were 5'-GGATGA AGTTGATTTTGATTGGGTT-3' (sense) and 5'-ACCCAATACAAAATCTACAAA CC AACA-3' (antisense) and for the methylated reaction 5'-ACGAAGTTGATTTTGA TCGGGTC-3' (sense) and 5'-CGATAC AAAATCTACGAACCGACG-3' (antisense). PCR was carried out in a final volume of 50 µl containing 3.5 mM magnesium chloride, 15 mM ammonium sulphate, 60 mM Tris HCl (pH 8.5), 250 µM each of dATP, dTTP, dCTP, and dGTP (Invitrogen, Leek, Netherlands), 0.1 µM forward and reverse primers (Biospring, Frankfurt, Germany), and 2.5 U of Ampli*Taq* Gold DNA polymerase (Perkin Elmer, Weiterstadt, Germany) for 10 minutes at 95°C followed by 55 cycles of 30 seconds at 95°C, 30 seconds at 55°C, one minute at 72°C, and a final extension of 10 minutes at 72°C. PCR products were electrophoresed on non-denaturating polyacrylamide gels (8%) and visualised by silver staining.

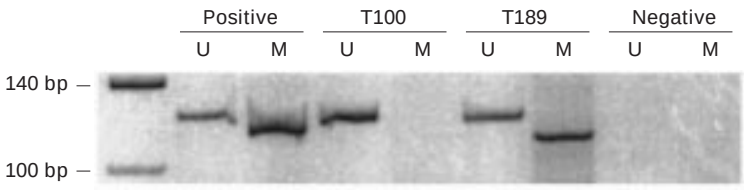

Figure 1 Methylation specific polymerase chain reaction (PCR) of the LKB1/STK11 promoter 5'-CpG island in sporadic colorectal cancer. In the left lane the 140 bp and 100 bp bands of a molecular weight standard are shown. The presence of a visible PCR product in those lanes marked U indicates unmethylated LKB1/STK11 promoter 5'-CpG islands; a visible PCR product in those lanes marked M indicates the presence of methylated LKB1/STK11 promoter 5'-CpG islands. Corresponding lanes are: positive controls for PCR reactions (for the unmethylated reaction DNA extracted from the colorectal cancer cell line HT29 and for the methylated reaction from the colorectal cancer cell line H6), primary colorectal cancers (T100 and T189), and negative controls for PCR reactions.

LOSS OF HETEROZYGOSITY ANALYSIS OF 19p13.3

After PCR amplification of DNA extracted from normal and tumour tissue the microsatellite markers D19S886, located telomeric to the *LKB1/STK11* gene locus on chromosome 19p13.3, and D19S878, located 6.5 cM proximal to D19S886,[20] were analysed for loss of heterozygosity (LOH). Primer sequences for D19S886 were 5'-TGGATCTACACTCC GGC-3' (sense) and 5'-ATTTTACTGGC TGGCACTTG-3' (antisense), and for D19S878 were 5'-GCCTGGGCGACA GAGAAT-3' (sense) and 5'-GGTTGC CCGCAGAGTG-3' (antisense). PCR was carried out in a final volume of 50 µl containing 2.5 mM magnesium chloride, 15 mM ammonium sulphate, 60 mM Tris HCl (pH 8.5), 250 µM each of dATP, dTTP, dCTP, and dGTP (Invitrogen), 0.1 µM of 6-carboxy-fluorescein labelled forward and 0.1 µM reverse primers (Biospring), and 2.5 U of Ampli*Taq* Gold DNA polymerase (Perkin Elmer) for 10 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C, one minute at 72°C, and a final extension of 10 minutes at 72°C. Electrophoresis was carried out in an ABI 310 DNA sequencer (Perkin Elmer) and the final analysis was performed using the Gene Scan 2.1 software (Perkin Elmer).

GENOMIC PCR AMPLIFICATION AND SEQUENCE ANALYSIS OF THE *LKB1/STK11* GENE

Genomic PCR amplification of the coding region of the *LKB1/STK11* gene was carried out using published primer sets.[5 6 15] PCR reactions were carried out in a total volume of 50 µl, consisting of 1.5–3.5 mM magnesium chloride, 15 mmol/l ammonium sulphate, 60 mM Tris HCl (pH 8.5), 250 µM each of dATP, dTTP, dCTP, and dGTP (Invitrogen), 1 µM of forward and reverse primers (Biospring), and 2.5 U of Ampli*Taq* Gold DNA polymerase (Perkin Elmer). The following amplification conditions were used: 12 minutes at 95°C; 40 cycles of 45 seconds at 95°C, one minute annealing at 55°C, and two minutes at 72°C; and a final extension of 10 minutes at 72°C.

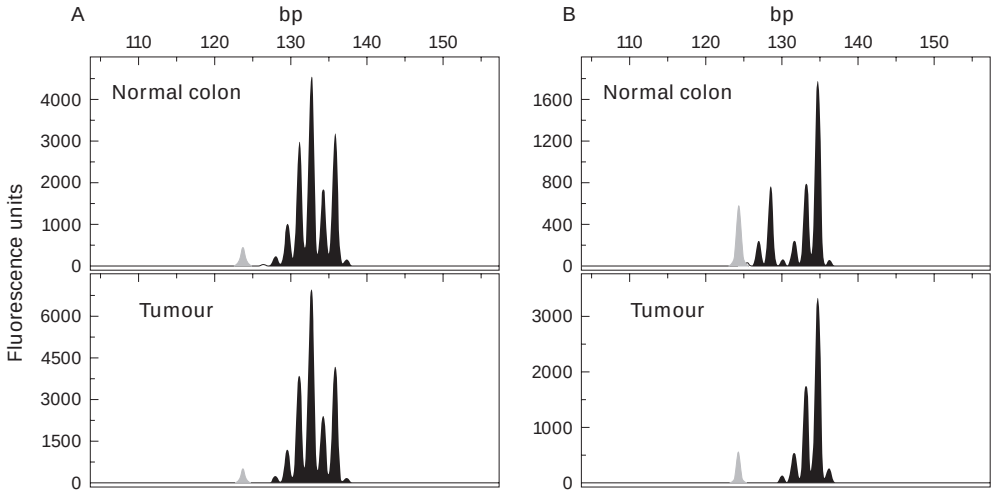

Figure 2 Fluorescent analysis of the microsatellite marker D19S878 (centromeric to the LKB1/STK11 gene locus) on chromosome 19p13.3. The 123 bp peak of the size standard is plotted in light grey in all electrophoretic profiles. The polymerase chain reaction (PCR) products of normal colon tissue (upper panels) and corresponding tumour tissue (lower panels) in patient No 31 (A) and No 3 (B) with sporadic colorectal adenocarcinoma were electrophoretically analysed on an automated ABI 310 DNA sequencer (Perkin Elmer). (A) Tumour without allelic loss at D19S878. (B) Tumour exhibiting loss of the 127 bp allele of D19S878 whereas the 134 bp allele is conserved.

PCR products of the *LKB1*/*STK11* gene were purified and bidirectionally sequenced according to the instructions of the Dye Deoxy Terminator protocol (Perkin Elmer). Automated sequence analysis was carried out on an ABI 310 DNA sequencer (Perkin Elmer).

Results

LKB1/*STK11*5'-CpG ISLAND METHYLATION

Methylation specific PCR (MSP) for analysis of the *LKB1*/*STK11* promoter 5'-CpG island was carried out with DNA extracted from 50 paraffin embedded colorectal cancer specimens. DNA extracted from the colorectal cancer cell lines HT29 and H6 served as positive controls for the unmethylated and methylated reactions, respectively (fig 1). In two specimens there was insufficient DNA for MSP. In the remaining 48 tumour specimens MSP was successful. Of those, four (8%) tumours demonstrated both methylated and unmethylated *LKB1*/*STK11* promoter 5'-CpG islands whereas the remaining 44 specimens displayed only unmethylated promoter islands. The presence of methylated *LKB1*/*STK11* promoter 5'-CpG islands was not associated with tumour stage, location, or histological grading.

LOH ANALYSIS OF 19p13.3

To investigate the *LKB1*/*STK11* gene locus in sporadic colorectal cancer for allelic loss at chromosome 19p13.3, we analysed paraffin embedded normal DNA and corresponding tumour DNA. Thirty eight of 50 tumour specimens were considered informative for at least one microsatellite marker. LOH at either D19S886 or D19S878 was observed in five of 38 (13%) informative sporadic colorectal cancer specimens. Allelic loss at D19S878 occurred in four tumours (fig 2) whereas one tumour exhibited LOH at D19S886. Three of five tumours with LOH at 19p13.3 were classified as UICC stage IV whereas the other two were UICC stage III tumours. Four of the five tumours with LOH at 19p13.3 were located in the sigmoid colon or rectum. There was no association between LOH and histological grade. None of the tumours displaying *LKB1*/*STK11* promoter 5'-CpG island hypermethylation was found to exhibit LOH at chromosome 19p13.3.

LKB1/*STK11* SEQUENCE ANALYSIS

To test if the *LKB1*/*STK11* gene in five tumours exhibiting LOH at 19p13.3 was inactivated because of a somatic mutation of the remaining allele, genomic sequencing of the coding region and splicing sites of the complete *LKB1*/*STK11* gene was performed. In none of those tumours was a somatic *LKB1*/*STK11* mutation detected, suggesting that LOH occurred independently of mutational events of the remaining *LKB1*/*STK11* allele.

Discussion

Inactivating *LKB1*/*STK11* germline mutations in combination with loss of the wild-type allele are responsible for the development of hamartomatous polyps in patients with Peutz-Jeghers syndrome (PJS).[7 9 10] To address the pathogenesis of intestinal hamartomas and adenocarcinomas, Gruber *et al* studied specimens of PJS patients carrying a heterozygote *LKB1*/*STK11* germline mutation.[9] Their data further supported the hypothesis that hamartomas and adenocarcinomas in patients with PJS develop through allelic loss of the wild-type *LKB1*/*STK11* allele whereas alterations of genes affected in the majority of sporadic colorectal carcinomas (*APC*, *K-ras*, p*53*) occur at later stages of tumour progression. Thus *LKB1*/*STK11* is considered a tumour suppressor gene in PJS.

Recently, promoter hypermethylation was established as a new concept of tumour suppressor gene inactivation.[3 21] In particular, hypermethylation of 5'-CpG islands, usually located in the promoter region of widely expressed genes, has been shown to correlate with decreased or loss of expression. In this study using the published primer sets for the

5'-CpG island of the *LKB1/STK11* promoter, we showed the feasibility of *LKB1/STK11* promoter methylation analysis in a large series of paraffin embedded colorectal cancer specimens, and confirmed the data of Esteller *et al* who found one of 43 primary colorectal cancer specimens methylated at the *LKB1/STK11* promoter.[19]

Furthermore, allelic loss at chromosome 19p13.3 was analysed. Using microsatellite markers flanking the *LKB1/STK11* gene locus[20] we found LOH on chromosome 19p13.3 in five of 38 (13%) informative tumour specimens of patients with sporadic colorectal cancer. To investigate if allelic loss at 19p13.3 occurred in combination with alterations of the remaining allele—according to a "two-hit" inactivation mechanism characteristic of tumour suppressor genes[22]—we sequenced the entire coding region of the *LKB1/STK11* gene in all five tumours with LOH at 19p13.3. No somatic *LKB1/STK11* gene mutations were found in these tumours. Our data are in accordance with the studies of Aviziente *et al* and Resta *et al* who also did not detect somatic *LKB1/STK11* gene mutations in 13 and 10 sporadic colorectal adenocarcinomas with LOH at 19p13.3, respectively.[15 17] The data are further confirmed by Wang *et al* who could not detect any bandshifts in the nine exons of the *LKB1/STK11* gene in 72 sporadic colorectal adenocarcinomas by single strand conformational polymorphism.[16] In contrast, Dong *et al* detected somatic *LKB1/STK11* gene mutations in 7/23 (30%) Korean patients with invasive adenocarcinomas of the sigmoid colon or rectum. Allelic loss at chromosome 19p13.3 was present in six of seven cancers with a somatic *LKB1/STK11* mutation.[18] Moreover, this study reported *LKB1/STK11* mutations in two of 12 (17%) colonic adenomas with high grade dysplasia. Eight of nine mutations reported by Dong *et al* were missense whereas only one was a frameshift mutation causing a premature stop codon. It remains unclear if these geographical differences in *LKB1/STK11* mutation frequency during colorectal tumorigenesis are caused by environmental factors such as dietary exposure to certain carcinogens.

To date, absence or a low frequency of somatic *LKB1/STK11* mutations has been reported in sporadic breast cancer (0/62),[23] testicular tumour (1/28),[15] malignant melanoma (4/85),[24–26] biliary (1/16), periampullary (0/19),[27] and pancreatic adenocarcinoma (5/112),[24 27] carcinoma of the stomach (3/36),[24 28] carcinoma of the uterine cervix (1/26), ovarian adenocarcinoma (0/37),[29] ovarian granulosa cell tumour (1/24),[24 29] renal cell cancer (0/19), lung cancer (1/28), and sarcoma (0/24).[24] In contrast with the predominance of truncating frameshift germline *LKB1/STK11* mutations in patients with PJS, the vast majority of somatic *LKB1/STK11* mutations detected so far are missense mutations. Recently, Mehenni *et al* and Ylikorkala *et al* reported functional in vitro assays to assess the effects of *LKB1/STK11* germline mutations detected in patients with PJS.[30 31] Mutant LKB1/STK11 proteins resulting from frameshift or missense *LKB1/STK11* mutations, including a single amino acid change (G163D) reported in a sporadic testicular tumour, resulted in severely impaired or complete loss of kinase activity. Nevertheless, the consequences of somatic missense *LKB1/STK11* mutations have not yet been studied in vivo.

In summary, promoter hypermethylation and allelic loss of the *LKB1/STK 11* gene are rare events in sporadic colorectal cancer in Caucasian patients. LOH at 19p13.3 is associated with advanced tumour stage and left sided location but not with *LKB1/STK11* promoter hypermethylation or somatic mutation. Because epigenetic or genetic inactivation of *LKB1/STK11* in sporadic colorectal cancer is a rare event, it is unlikely that *LKB1/STK11* alterations are key players in the molecular pathogenesis of this tumour entity.

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