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 germ-line 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 \(STK11\) 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

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 \(MSH2, MLH1, PM21, PM22, \) 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\(^3\) and encodes for a serine-threonine kinase, a human homologue of \textit{Xenopus} early embryonic kinase 1.\(^6\) \(LKB1/STK11\) 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.\(^7,8\) The development of cancer in patients with PJS does not exclusively arise in association with hamartomas\(^9\) 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 \(p53,\) 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,\(^15,16\) 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-
trast, LKB1/STK11 promoter hypermethylation leading to transcriptional inactivation was found in a few cancer cell lines and a subset of primary tumours. 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) and in 12 patients right sided colorectal cancer (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 was 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. Primers used for the unmethylated reaction were 5′-GGATGA AGTTGATTTGATGGTTGTT-3′ (sense) and 5′-ACCCAAATACAAAAATCTCACAACCCAAACA-3′ (antisense) and for the methylated reaction 5′-ACGAAGTGTATTGTTGATCGGTC-3′ (sense) and 5′-CGATAC AAAACATCAGAAACCAGCAG-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 AmpliTag Gold DNA polymerase (Perkin Elmer, Wetterstad, 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.

**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, were analysed for loss of heterozygosity (LOH). Primer sequences for D19S886 were 5′-TGGATCTACATTCCGGC-3′ (sense) and 5′-ATTTTACCTGGCTTGACATTGTTTTG-3′ (antisense), and for D19S878 were 5′-GCCTGGGGCCAGAGAAT-3′ (sense) and 5′-GGTTTGC CGCAGAGATTG-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 AmpliTag 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 13 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 AmpliTag 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 1](https://www.gutjnl.com)
Methylation specific PCR (MSP) for analysis of the LKB1/STK11 promoter 5'-CpG island was not associated with tumour chromosome 19p13.3, we analysed para
genetic sequence analysis was carried out on an ABI 310 DNA sequencer (Perkin Elmer). Automated sequence analysis was carried out on an
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
informative sporadic colorectal cancer specimens. 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.

LOKB1/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 hamar-
A

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.

B

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.

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In summary, promoter hypermethylation and allelic loss of the LKB1/STK11 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 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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