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Advances in Brief

Frequent Somatic Mutations in *Serine/Threonine Kinase 11*/Peutz-Jeghers Syndrome Gene in Left-sided Colon Cancer[1]

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Abstract

We analyzed somatic mutation and loss of heterozygosity (LOH) in the *serine/threonine kinase 11* (*STK11*)/Peutz-Jeghers syndrome gene in 49 colorectal tumors in three different stages of a dysplasia-carcinoma sequence. We detected LOH in 10 of 19 (52.6%) informative colorectal cancers at loci *D19S886* and/or *D19S883*, but no LOH was observed in 25 informative adenomas. We detected a total of 9 somatic mutations [7 of 13 (53.8%) left-sided colon cancers and 2 of 7 (28.6%) left-sided adenomas with high-grade dysplasia], but no mutations were detected in right-sided colon tumors. Of the nine mutations, one was a frameshift mutation (the same mutation detected in Peutz-Jeghers syndrome family previously), and the other eight were missense mutations. This results indicate that *STK11* is a tumor suppressor gene and that genetic changes of *STK11* play an important role in left-sided colon cancer carcinogenesis.

Introduction

PJS[4] is a rare autosomal dominant disorder with varying degrees of penetrance, characterized by gastrointestinal hamartomatous polyps and mucocutaneous melanin pigmentation; these polyps are thought to be nonmalignant disturbances of superfluous tissue (1, 2). Such patients are, however, at an increased risk of developing both gastrointestinal and nongastrointestinal cancers. Giardiello *et al.* (3) estimated that these patients have an 18-fold higher risk of malignancy than the general population. Recently, the PJS gene, *STK11*, encoding a novel serine/threonine kinase and residing on chromosome 19p13.3 at a distance of 190 kb proximal to *D19S886*, was identified. Numerous germ-line mutations were detected in individuals affected by PJS (4–7). These findings suggest that genetic changes of *STK11* might also be associated with the development of a sporadic form of colorectal cancer.

Colorectal tumors provide an excellent opportunity to study tumor progression because most carcinomas appear to arise from adenomas, and tumors at various stages in the development of the adenoma-carcinoma sequence can be easily obtained for analysis (8). Thus, we collected colorectal tumors in three different stages: adenomas with low-grade dysplasia (defined as mild and moderate dysplasia), adenomas with high-grade dysplasia (defined as severe dysplasia or carcinoma *in situ*), and invasive cancers, characterized according to the guidelines of the National Polyp Study Group (9).

Here, we performed a PCR-based LOH and mutation analysis of the *STK11* gene in a series of 49 colorectal tumors in three different stages of the dysplasia-carcinoma sequence to determine whether *STK11* genetic alterations could be involved in colorectal tumor development and, if so, determine to which stage it is linked.

Materials and Methods

Materials. Paraffin-embedded histological sections of 26 colorectal adenomas (14 low-grade dysplasias, 6 left-sided and 8 right-sided; 12 high-grade dysplasias, 7 left-sided and 5 right-sided) and 23 invasive colorectal cancers (13 left-sided and 10 right-sided) were obtained from the Catholic University Medical College-affiliated hospital (Seoul, Korea). The term "invasive cancer" means, strictly, a cancer that has invaded beyond the muscularis mucosa. The line of demarcation between the right and left colon has been defined by the embryonic division line at the junction of the proximal two-thirds and the distal one-third of the transverse colon. None of the patients had a family history of PJS, familial adenomatous polyposis, or HNPCC.

Microdissection. Tumor cells were selectively procured from H&E-stained slides using a 30G1/2 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator, as described previously (10, 11). We also obtained inflammatory cells or normal mucosal epithelium for corresponding normal DNA from the same slides in all cases.

DNA Extraction. DNA extraction was performed by a modified single-step DNA extraction method, as described previously (10–12).

LOH Analysis. Tumor and corresponding normal DNA from each slide were amplified by thermal cycler (MJ Research Institute, Watertown, MA) with three microsatellite markers (Research Genetics, Huntsville, AL), *D19S886*, *D19S883*, and *D19S565*, in the 19p13.3 region. Each PCR was generally performed under standard conditions in a 10 μl of reaction mixture containing 1 μl of template DNA, 0.4 μM each primer, 125 μM each dNTP, 1.5 mM $MgCl_2$, 0.4 units of Taq polymerase, 0.5 mCi [^{32}P]dCTP (Amersham, Buckinghamshire, United Kingdom), and 1 μl of 10× buffer. The reaction mixture was denatured for 5 min at 95°C and incubated for 35 cycles (denaturing at 95°C for 50 s, annealing at 57°C for 90 s, and extending at 72°C for 90 s), with some variations in the annealing temperature. Final extension was continued for 10 min. Reaction products (2 μl) were then denatured and electrophoresed in 6% polyacrylamide gel containing 7 M urea. After electrophoresis, the gels were transferred to 3-MM Whatman paper, dried, and subjected to autoradiography using Kodak-OMAT film (Eastman Kodak, Rochester, NY).

SSCP Analysis and DNA Sequencing. Twelve sets of Primers (Table 1) covering nine exons of *STK11* gene were designed by using the OLIGO software program (Version 5.0; National Bioscience Inc., Plymouth, MN) according to the genomic sequence of *STK11*, which was obtained from GenBank accession nos. AF032984, AF032985, and AF032986. PCR amplifications were performed under exactly the same conditions as described above, with the exception of the annealing temperature (Table 1). The amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cya-

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[4] The abbreviations used are: PJS, Peutz-Jeghers syndrome; *STK11*, *serine/threonine kinase 11*; LOH, loss of heterozygosity; HNPCC, hereditary nonpolyposis colon cancer; SSCP, single-strand conformation polymorphism.

Table 1 *Primers for amplification and sequencing of STK11*

Twelve sets of primers covering nine exons of the *STK11* gene were designed by using the OLIGO software program.

Primer	Sequence	Fragment size (bp)	Annealing temperature (°C)
STK1A-F	5′-CTCAGGGCTGGCGGCGGGACT-3′	163	64
STK1A-R	5′-CTTGCGGCGCGGCTGGTAGATGA-3′		
STK1B-F	5′-ACGTTCATCCACCGCATCGAC-3′	150	60
STK1B-R	5′-GCACAGCGTCTCCGAGTCCAG-3′		
STK1C-F	5′-TCTTACGGCAAGGTGAAGGAGGTG-3′	140	60
STK1C-R	5′-CCGACCCCAGCAAGCCATACTTA-3′		
STK2-F	5′-CTGACGTTGGGTCGGCTGATA-3′	151	58
STK2-R	5′-GGTCCCAACACCGGAAAGGATAT-3′		
STK3-F	5′-CCCCCGTGCTCCCTGGGCCTGT-3′	173	66
STK3-R	5′-CCCTGCCCCGCGCACGCA-3′		
STK4-F	5′-CGGCCCCAGGACGGGTGT-3′	218	64
STK4-R	5′-CTCAGGGAGTGCCCGGGAGG-3′		
STK5-F	5′-CCTGAGGGCTGCACGGCACC-3′	213	66
STK5-R	5′-CCCCTCGGAGTGTGCGTGTGGT-3′		
STK6-F	5′-GACCACGCCTTTCTTCCCTCCC-3′	213	62
STK6-R	5′-CACAAAAGCCCCGCCTCCCT-3′		
STK7-F	5′-TCACCCAGGGCCTGACAACAGAG-3′	193	64
STK7-R	5′-GCAGCCTCGGCCCCACTG-3′		
STK8-F	5′-CCTGACAGGCGCCACTGCTTC-3′	240	64
STK8-R	5′-GGCCCCCGCCAGACTCAC-3′		
STK9A-F	5′-TGTAAGTGCGTCCCCGTGGTG-3′	204	64
STK9A-R	5′-CGCCCTGGATTTGGTGCTA-3′		
STK9B-F	5′-GTGTATGAACGGCACAGAGGC-3′	195	64
STK9B-R	5′-CAGGCGTTGTCCCCACAT-3′		

nol). The samples were denatured for 5 min at 95°C and loaded onto a mutation detection enhancement gel (AT Biochem, Malvern, PA) with 10% glycerol. After electrophoresis, the gels were transferred to 3-MM Whatmann paper, and autoradiography was performed using X-OMAT film. After detection of abnormal bands in SSCP analysis, PCR was performed using DNA eluted from dried gels, and sequencing was performed using Amplicycle Sequencing Kit (Perkin-Elmer Corp., Branchburg, NJ).

Results

LOH of 19p13.3. The fixed marker order and composite map information for three marker loci were obtained from the genetic location database (http://cedar.genetics.soton.ac.uk.public_html) at the University of Southampton (Southampton, United Kingdom; Ref. 13).

Patients who were heterozygous for a given marker were considered informative. Twenty-five of 26 cases of adenomas and all 23 cases of invasive carcinomas were informative for at least one of the markers studied, and the results are summarized in Table 2. We observed no LOH at all in 25 informative adenomas with low- or high-grade dysplasia. In invasive carcinomas, 9 of 17 (52.9%), 6 of 15 (40%), and 6 of 22 (27.3%) informative cases showed allelic loss at the markers *D19S886*, *D19S883*, and *D19S565*, respectively. The autoradiograms of two selected cases showing LOH are displayed in Fig. 1. Six cases, including cases 10 and 16, both of which had one noninformative marker, showed allelic loss at all three markers (Fig. 1*b*). Three cases (cases 8, 14, and 19) revealed allelic loss at only one marker, *D19S886* (Fig. 1*a*); this single region showed allelic loss in all left-sided colon cancers with *STK11* mutations, except in cases 20 and 23. Case 20 revealed retention of heterozygosity at *D19S886* but showed allelic loss at *D19S883* and *D19S565*. The location of the *STK11* gene relative to these markers is between *D19S886* and *D19S883*. The composite map distance between the two markers *D19S886* and *D19S883* is only 0.54 Mb, and *STK11* resides at a distance of 0.19 Mb proximal to *D19S886*, spanning over 23 kb. Therefore, we concluded that case 20 might also contain one allele deletion of the *STK11* gene. There was no significant difference in allelic loss frequencies between the left-sided and right-sided colon cancers (Fisher's exact test, $P = 1.000$).

Mutation Analysis of the *STK11* Gene. We detected 9 somatic mutations of the *STK11* gene in 26 adenomas and 23 invasive carcinomas, as summarized in Table 2, and two representative cases with aberrant bands and mutations are shown in Fig. 2. No adenomas with low-grade dysplasia had mutations of the *STK11* gene (0 of 14), whereas 2 of 12 (16.7%) adenomas with high-grade dysplasia and 7 of 23 (30.4%) invasive carcinomas had mutations. There was a signifi-

Table 2 *STK11 mutations and LOH in histopathological subtypes of colorectal tumor*[a]

Case no.	Site	Histological type	19p13.3 LOH			*STK11* mutation
			D19S886	*D19S883*	*D19S565*	
Adenoma						
AD12	Transverse (distal one-third)	High-grade dysplasia	□	□	□	gGGC → AGC (Gly171Ser)
AD22	Rectum	High-grade dysplasia	▨	□	□	*CCG → CTG (Pro281Leu)
Adenocarcinoma, right-sided colon						
1	Cecum	Mucinous	□	□	□	
2	Cecum	WD	▨	▨	□	
3	Cecum	PD	▨	▨	□	
4	Cecum	Mucinous	▨	▨	□	
5	Cecum	PD	▨	▨	□	
6	Ascending	MD	□	□	□	
7	Ascending	MD	□	▨	□	
8	Ascending	MD	■	□	□	
9	Ascending	MD	■	■	■	
10	Transverse (proximal two-thirds)	MD	■	▨	■	
Adenocarcinoma, left-sided colon						
11	Sigmoid	WD	□	□	□	
12	Sigmoid	MD	□	□	□	
13	Sigmoid	MD	▨	□	□	
14	Sigmoid	WD	■	□	□	*cGAC → AAC (Asp208Asn)
15	Sigmoid	MD	■	■	■	842C DEL, FS, stop at codon 286
16	Sigmoid	MD	■	■	▨	*GGC → GAC (Gly215Asp)
17	Sigmoid	MD	■	■	■	*ACG → ATG (Thr367Met)
18	Rectum	MD	■	■	■	
19	Rectum	WD	■	□	□	*cGAG → AAG (Glu199Lys)
20	Rectum	MD	□	■	■	gGGC → AGC (Gly171Ser)
21	Rectum	MD	□	▨	□	
22	Rectum	WD	□	▨	□	
23	Rectum	WD	▨	□	□	TTC → TTG (Phe354Leu)

[a] MD, moderately differentiated; WD, well differentiated; PD, poorly differentiated; DEL, deletion; FS, frameshift; □, retention of heterozygosity; ■, LOH; ▨, noninformative; *, CpG island.

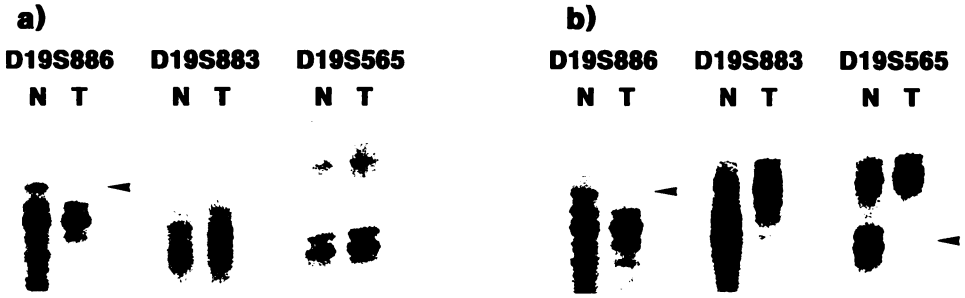

Fig. 1. For each of the three polymorphic markers, autoradiograms of LOH analysis for two selected cases are shown. Case 19 (*a*) exhibits allelic loss at only one marker but case 15 (*b*) shows allelic loss at all three markers. The location of the *STK11* gene relative to these markers is between *D19S886* and *D19S883*. *Lanes N*, normal sample; *Lanes T*, tumor.

cant increase in the frequency of mutations that paralleled the dysplasia-carcinoma sequence in tumor development (Fisher's exact test, $P = 0.047$). All nine mutations were detected exclusively in left-sided colon tumors, including two adenomas with high-grade dysplasia. Case 15 (Fig. 2, *a* and *c*) revealed a deletion of nucleotide 842 (C) in exon 6, which changed the proline (CCG) at codon 281 to an arginine (CGC) and led to a reading frameshift and a premature stop codon (TGA) at position 286. The other eight mutations were missense mutations (Fig. 2, *b* and *d*). Of these missense mutations, seven mutations were of the C:G → T:A transitional type, and of these, six were located at the dipyrimidine sequences and five were located at the CpG site.

Discussion

Colorectal cancer occurs both as a hereditary disorder and as a sporadic case. Familial disorders that cause susceptibility to colorectal cancer include familial adenomatous polyposis and HNPCC (14). PJS is another autosomal dominant disorder and is also associated with an increased risk of gastrointestinal carcinomas (3). Recently, the gene responsible for PJS was identified as *STK11*, which encodes a novel serine/threonine kinase of 433 amino acids (6, 7). Because most colorectal carcinomas appear to arise from adenomas, studies of different stages of colorectal neoplasia may shed light on the genetic alterations involved in tumor progression (9, 15). These facts led us to examine the genetic alterations of *STK11* gene in a sporadic form of colorectal tumors undergoing three different stages of a dysplasia-carcinoma sequence: adenoma with low-grade dysplasia, adenoma with high-grade dysplasia, and invasive carcinoma.

We have analyzed allelic deletion in 23 colorectal cancers, most frequently at loci *D19S886* (52.9%) and *D19S883* (40%) and less commonly at *D19S565* (27.3%). But we observed no LOH at all in 25 informative adenomas. *STK11* resided at a distance 0.19 Mb proximal to *D19S886*. The composite map distance between *D19S886* and proximal marker *D19S883* is only 0.54 Mb (11, 13). Therefore, we suspect that one allele of the *STK11* gene must also have been deleted in case 20, despite retention of heterozygosity at *D19S886*. Because these two markers lie in close proximity to the *STK11* gene, these two markers were beneficial for analyzing allelic loss of this gene. When we compared the frequencies of LOH by tumor site in invasive cancers, there was no significant difference between left- and right-sided colon cancer (Fisher's exact test, $P = 1.000$).

In observing the dysplasia-carcinoma sequence in its tumor development stages, mutations in *STK11* were first detected in adenomas with high-grade dysplasia at low frequency (16.7%), but they were detected in invasive carcinomas with increasing frequency (30.4%). However, no mutation was detected in 14 adenomas with low-grade dysplasia. Because the mutations were first detected in adenoma with high-grade dysplasia, *STK11* might be involved in tumor promotion and/or progression rather than initiation in the process of tumorigenesis. Interestingly, all nine mutations were detected exclusively in left-sided colon tumors, including two adenoma cases. Therefore, calculating the mutation rate values of *STK11* obtained from left-sided colon tumors results in a cancer mutation rate of 53.8% (7 of 13) and an adenoma with high-grade dysplasia rate of 28.6% (2 of 7). With the exception of case 23, all seven left-sided colon cancers with mutation have allelic loss at *D19S886* and/or *D19S883*; unfortunately, the marker at *D19S886* was noninformative in case 23. These results strongly support the notion of earlier work showing that the *STK11* is a tumor suppressor gene (6, 7) and also suggest that the genetic changes of both alleles of *STK11* play an important role in the

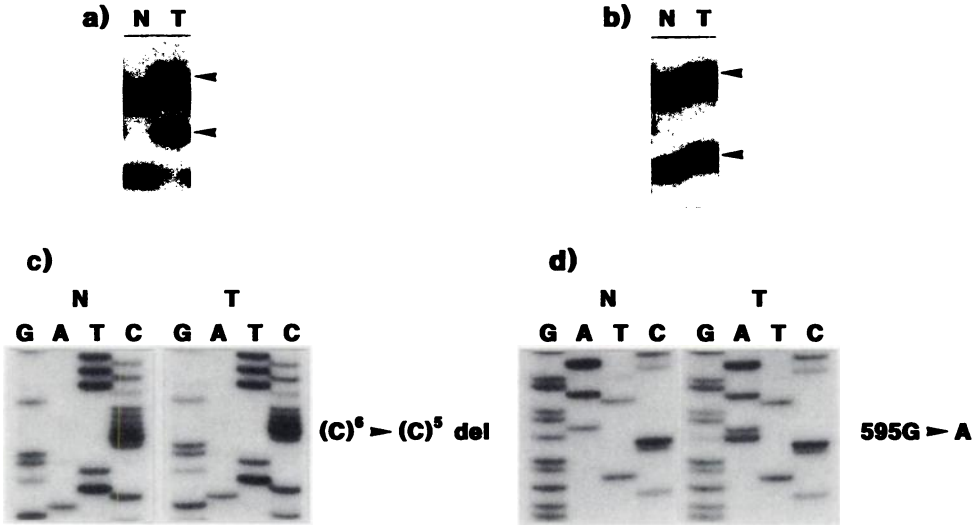

Fig. 2. *STK11* gene mutations in two colorectal carcinomas (cases 15 and 19). *a* and *b*, SSCP analysis of DNA from tumors (*Lanes T*) and normal samples (*Lanes N*) from cases 15 (*a*) and 19 (*b*). *Arrowheads*, abnormal electrophoresis bands. *c* and *d*, cyclic sequencing analyses were performed using DNA eluted from each of abnormal bands. Case 15 (*c*) revealed a deletion of nucleotide 842 (C) in exon 6, which led to a reading frameshift and a premature stop codon at position 286. In case 19 (*d*), there was a G → A transition at nucleotide 595 in tumor tissue as compared to normal control tissue.

conversion of high-grade dysplasia into invasive carcinoma, especially in the carcinogenesis of left-sided colon cancer. The large majority of mutations in *STK11* detected in PJS families are frameshift mutations that result in the truncation of the protein (6, 7); this also strongly supports the suggestion that *STK11* is a tumor suppressor gene. Here, we found one frameshift mutation. Case 15 carried a deletion of nucleotide 842 (C) in exon 6 (Fig. 2c), which changed the proline (CCG) at codon 281 to a arginine (CGC) and led to a frameshift and a subsequent stop codon (TGA) at codon 286. This is the same frameshift mutation that was detected in PJS family by Jenne *et al.* (7). The same nucleotide 842 (C) was substituted by a T in case AD22, resulting in a change from proline (codon 281) to leucine. Last, in both cases AD12 and 20, the same mutation of glycine at 171 into serine was observed. To our knowledge, this is the first report documenting the observation of somatic mutation of *STK11* in colorectal cancer as a tumor suppressor gene, and this result suggests the possibility of *STK11* mutations in the other sporadic form of tumors that are associated with PJS, such as breast and ovary cancers.

Recent studies in a subset of colorectal cancer and HNPCC have shown that microsatellite sequences are genetically unstable and susceptible to replication errors (16). This microsatellite instability was significantly correlated with the tumor's location in the proximal colon (16, 17), and inversely, genetic alteration of *APC*, K-*ras*, *DCC*, and *p53* were more than twice as frequent in distal colon cancer as in proximal cancer (18). Taken together, these data indicate that *STK11* is involved in left-sided colon carcinogenesis, together with *APC*, K-*ras*, *DCC*, and *p53*.

Even with a small number of cases, because we frequently observed genetic changes in both alleles of *STK11* gene in left-sided colon cancer, we concluded that the *STK11* gene is a tumor suppressor gene and that genetic alterations of *STK11* play an important role in tumor promotion and progression in left-sided colon cancer carcinogenesis. However, further studies on a large patient population will be important to verify these initial observations, and identification of the biological function of *STK11* will certainly broaden our understanding of the pathogenesis of not only hereditary PJS but also of relevant sporadic forms of cancer.

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