Molecular genetic alterations in gastrointestinal polyposis syndromes: with emphasis on the Peutz-Jeghers syndrome

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Chapter 10

Molecular Genetic Alterations in Hamartomatous Polyps and Carcinomas of Peutz-Jeghers Patients

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ABSTRACT

Aim: To investigate whether mutations in the \textit{STK11/LKB1} gene and genes implicated in the colorectal adenoma-carcinoma sequence are involved in Peutz-Jeghers syndrome (PJS) related tumorigenesis.

Methods: We analysed 39 polyps and 5 carcinomas of 17 PJS patients from 13 families for loss of heterozygosity (LOH) at 19p13.3 (STK11/LKB1 gene locus), 5q21 (APC gene locus), 18q21-22 (Smad4 and Smad2 gene locus) and 17p13 (p53 gene locus) and evaluated immunohistochemical staining for p53. Also, mutational analysis of K-ras codon 12, APC and p53 and immunohistochemistry for Smad4 expression was performed on all carcinomas.

Results: LOH at 19p was observed in 38\% (15/39) of the polyps and in all carcinomas (n=5). Interestingly, 86\% (6/7) of polyps from patients with cancer had LOH, versus 29\% (9/31) of polyps from the remaining patients (p=0.01). In 1 polyp from a patient without a germline \textit{STK11/LKB1} mutation, no LOH at 19p, or at three alternative PJS candidate loci (19q, 6p and 6q) was found. No LOH at 5q was observed. However, mutational analysis revealed an \textit{APC} mutation in 4 out of 5 carcinomas. LOH at 17p was not observed in polyps and carcinomas; immunohistochemistry showed expression of p53 in 1 carcinoma and focal expression in 3 polyps. At subsequent sequence analysis, no \textit{p53} mutation was found. One carcinoma had an activating K-ras codon 12 mutation and another carcinoma showed 18q LOH, however, no loss of Smad4 expression was seen.

Conclusions: These results provide further evidence that \textit{STK11/LKB1} acts as a tumor-suppressor gene, and may be involved in early stages of PJS-tumorigenesis. Further research is needed to investigate whether LOH in PJS-polyps can serve as a biomarker to predict cancer. Differences in molecular genetic alterations noted between the adenoma-carcinoma sequence and PJS-related tumors suggest the presence of a distinct pathway of carcinogenesis.
Molecular Alterations in Peutz-Jeghers Syndrome

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant condition characterized by hamartomatous polyps, which can occur throughout the gastrointestinal tract, and melanin spots found on the lips and buccal mucosa [1;2]. Patients with PJS have an increased risk for developing cancer at a relatively young age [3-5]. Malignancies occur in the gastrointestinal tract but also in non-gastrointestinal sites including the pancreas, breast, ovary and testis [6].

Recently, a gene defect leading to PJS has been identified. The $\text{STK11/LKB1}$ gene encodes a serine/threonine kinase that is ubiquitously expressed in human tissues [7;8] and might be involved in G1 cell cycle arrest [9]. Although the $\text{STK11/LKB1}$ gene appears to play a crucial role in tumor development in PJS patients [10-12], a low frequency of mutation is found in similar sporadic cancers [13-18]. To date, more than 60 PJS patients with inactivating germline mutations in the $\text{STK11/LKB1}$ gene have been reported [19]. Possible hotspots of mutation are exon 1 and exon 6 which together account for one half of the currently described germline alterations. However, not all PJS-families are linked to the 19p13.3 locus of the $\text{STK11/LKB1}$ gene, suggesting genetic heterogeneity [20-22]. Alternative loci are 19q [20] and the breakpoints at a pericentric inversion at chromosome 6, as was found in a PJS patient [23].

Investigation of the genetic alterations in pre-malignant and malignant lesions from familial adenomatous polyposis (FAP) patients has been essential in the discovery of the sequential genetic events in colorectal carcinogenesis [24]. These studies have elucidated an accumulating mutational progression order of the $\text{APC}$, $\text{K-ras}$, $\text{Smad2}$ and $\text{Smad4}$ and $\text{p53}$ gene, which correlate with subsequent stages of the adenoma-carcinoma sequence. The possible malignant potential of hamartomatous polyps in PJS patients is not well understood. Although very rarely, malignant transformation of PJS polyps has been reported [5;25], possibly indicating that a hamartoma-carcinoma sequence might occur in PJS. Mutational analysis of genes involved in colon carcinogenesis has been performed in hamartomas and carcinomas from PJS patients, showing that $\text{K-ras}$ mutations are very rare in PJS tumors compared to FAP and sporadic adenomas or carcinomas [10;26]. In addition, no LOH at 5q (near $\text{APC}$) was found in PJS tumors [10]. In contrast, inactivating mutations of the wild type allele of the $\text{STK11/LKB1}$ gene are common in PJS tumors, indicating that the PJS gene may be involved in PJS related tumor development [10], and may act as a tumor suppressor gene.

In the present study, we extend our previous work [10], investigating the 19p LOH status in polyps and carcinomas from 17 PJS patients, including 1 without a germline mutation in the $\text{STK11/LKB1}$ gene. In addition, we analysed these tissues for the specific genetic alterations known from the adenoma-carcinoma sequence to further clarify the molecular pathogenesis of PJS tumors.
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MATERIALS AND METHODS

Patients and tissue samples:
Thirty-nine hamartomatous polyps and 5 adenocarcinomas of 17 PJS patients from 13 families formed the study population. The clinical diagnosis of PJS was confirmed by histopathologic review of the hamartomas by an experienced pathologist (GJAO). The hamartomas were from the gastrointestinal tract (stomach \(n=2\), small bowel \(n=27\), colon \(n=10\)); the carcinomas were from the stomach \((n=1)\), pancreas \((n=1)\), small bowel \((n=1)\), and colon \((n=2)\). Eleven patients came from 7 different families with a germline \(STK11/LKB1\) mutation \([10;12;22;27]\); in 1 patient from another family, no germline mutation was found. In the 5 remaining patients from 5 other unrelated families, the mutational status was unknown.

Table 1: Number of PJS-patients, PJS-families and studied hamartomatous PJS-polyps, and the accompanying germline \(STK11/LKB1\) mutational status.

<table>
<thead>
<tr>
<th>Number of patients/families</th>
<th>Number of polyps</th>
<th>Number of carcinomas</th>
<th>(STK11/LKB1) germline mutation</th>
<th>no (STK11/LKB1) germline mutation</th>
<th>Unknown mutational status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 patients from 7 families</td>
<td>22</td>
<td>3</td>
<td>11</td>
<td>1</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>5 patients from 5 families</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>5 patients from 5 families</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>39</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

DNA isolation:
Formalin-fixed and paraffin-embedded tissue was available for study from all the hamartomas and four carcinomas; one colonic carcinoma was a fresh frozen specimen. The samples were cut into 5 μm sections, mounted on glass slides and H&E stained. Polyp epithelium and cancer tissue was carefully microdissected. The microdissected tissue was collected in microcentrifuge tubes containing 50-200 μl DNA isolation buffer (50 mM Tris-HCl pH 8.0, 0.2% Tween-20 and 100mg/ml proteinase K) depending on size of the tissue fragments and incubated overnight at 56 °C. Samples were heated to 96 °C for 10 minutes to inactivate the proteinase K. For normal control wild type DNA, lymphocyte DNA or DNA isolated from the muscularis propria from the same sample was used. In 10 polyps, in which no LOH was found in the above fashion, more accurate microdissection of epithelium using a Laser Capture Microscope was done, to further enrich the sample and to avoid that negative results were caused by wild type contamination.
LOH-analysis:

Two polymorphic microsatellite markers were used for loss of heterozygosity (LOH) analysis of each studied locus: D19S886 and D19S565 flanking the STK11/LKB1 gene on 19p13.3; D5S346 and D5S122 near the APC gene on 5q21; D18S474 and D18S487 on 18q21-22 (Smad2 and Smad4); D17S513 and p53-Alu near respectively within the p53 gene on 17p13. For analysis of the alternative PJS loci, the markers D19S891 for 19q; D6S257 for 6p; and D6S311 for 6q were used [20;23]. Primer sequences were obtained from The Genome Database (www.gdb.org), except p53-Alu [28]. One primer of each marker was end-labelled with $^{32}$P-$\gamma$ATP. AmpliTaq Gold polymerase was used for amplification of the genomic DNA fragments. The PCR conditions for these markers were 94 °C 10 minutes; followed by 33 cycles of 94 °C 30 seconds; 55 °C 30 seconds; 72 °C 1 minute; followed by 72 °C 10 minutes. The samples were run on a 6% polyacrylamide gel. The gels were dried and exposed overnight to a Kodak Biomax™ XR film.

K-ras codon 12 mutational analysis:

The genomic DNA samples of the 5 carcinomas were used for amplification of K-ras specific sequences by PCR. Mutations were identified by allele specific hybridization using a previously described protocol [26].

Immunohistochemistry for p53 and Smad4:

Immunohistochemistry for p53 and Smad4 was performed as described previously [29;30]. p53 staining was performed on both polyps and carcinomas; Smad4 staining was performed on carcinomas only. Unstained 5 μm sections were cut. Sections were dewaxed and rehydrated in xylene and a series of graded alcohol. Endogenous peroxidase activity was blocked in 0.3% H$_2$O$_2$ in methanol for 20 minutes. Slides were submerged in citrate buffer (0.01 M; pH 6.0) and heated in a temperature-probe controlled microwave oven for 10 minutes at 100°C. After cooling for 20 minutes, 10% normal goat serum in phosphate-buffered saline (PBS) was applied for 20 minutes. The sections were subsequently incubated for 1 hour with the primary antibody. To detect mutant p53 expression, the monoclonal mouse anti-human antibody D07 (Dako, Glostrup, Denmark) was used at a 1:200 dilution in PBS. For Smad4-staining, the monoclonal mouse anti-human antibody B-8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:100 dilution. After washing, biotinylated rabbit-anti-mouse IgG antibody (Dakopatts, Denmark) at a 1:200 dilution in PBS with 10% normal AB serum was applied for 30 minutes, followed by streptavidin-biotin peroxidase in PBS with 10% normal AB serum (1:200) for 30 minutes. The peroxidase activity was visualised using dianminobenzidine (DAB) (1:20) in Tris-HCl 0.05 M/0.1% H$_2$O$_2$ for 10 minutes. Counterstaining of the nuclei was done with hematoxilin. A known p53 positive CRC was used as a positive control; the same sample was used as a negative control.
by replacing the primary antibody by PBS. p53-staining was considered positive when more then 10% of the cells showed nuclear p53 expression.

Sequence analysis of the exons 5-8 in the p53 gene:

Sequence analysis was performed to detect mutations in the commonly affected exons 5-8 of the p53 gene in all carcinomas and from microdissected areas with p53 expression in 3 polyps as described previously [31]. In brief, exons 5-8 were amplified by PCR. Template DNA was obtained by PCR, using an aliquot of the first PCR as input DNA. The primers used in this reaction added EcoR1 and BamH1 recognition sequences to the amplified product. The fragments were digested with EcoR1 and BamH1 and cloned into the plasmid vector pBluescript (Stratagene, La Jolla, CA, USA). Bacterial clones with inserts were pooled, DNA was isolated, followed by bidirectional DNA sequencing using Sequenase version 2.0 (United States Biochemicals, Cleveland, OH, USA).

APC-mutational analysis:

APC mutational analysis in DNA from the 5 carcinomas was performed using a denaturating gradient gel electroforesis (DGGE) technique as has been described previously [32]. This technique focuses on mutations occurring in the APC mutation cluster region (MCR) in which 65% of the somatic mutations of the APC gene occur [32;33]. The DNA was extracted from tissue sections as described above. Amplification of the MCR was done in two steps as described previously [34]. Initially, two overlapping fragments spanning the MCR (fragment A: nucleotide 3874-4229 and fragment B: nucleotide 4114-4624) were amplified in a PTC-200 (MJ Research Inc, Waltham, MA, USA) in a final volume of 20 μl containing 2 mM of MgCl2, 250 μM of dNTPs, 200 nM of each primer and 1 unit of Taq DNA polymerase (Perkin Elmer) in the buffer supplied by the manufacturer. Cycling was performed during 30 cycles at 94 °C 40 seconds; 54 °C 1 minute; and 72 °C 1 minute; followed by a final extension at 72 °C 10 minutes. The respective primers used for the first amplification round were 5'-GAAATAGGATGTAATCAGACG-3' upstream; 5'-GAGCTGGCAATCGAACGACT-3' downstream for fragment A and 5'-GCTCAGACACCCAAAAGTCC-3' upstream; 5'-CATTCCCATTGTCATTTCC-3' downstream for fragment B. Subsequently, four smaller fragments were amplified in the second amplification round, with 1 μl of the PCR product of the first round as a template: fragment S1 (nucleotide 3874-4092) using the upstream primer for fragment A and 5'-CGCTCCTGAAGAAAAATTCAAC-3' downstream, fragment S2 (nucleotide 4026-4229) using primer 5'-ACTGCAGGGTTCTAGTTATC-3' upstream and the downstream primer for fragment A, fragment S3 (nucleotide 4179-4383) using primers 5'-TACTTCTGTCAGTCTTCGAT-3' upstream and 5'-ATTTTAGGTACTTCTCGCTTG-3' downstream for fragment A, fragment S4 (nucleotide 4328-4594) using primers 5'-AAACACCTCCACCACCTCC-3' upstream and 5'-GCATTATTCTAAATTCCACATC-3' downstream. A GC-clamp was attached to one of the primers for each fragment. PCR was
performed in a final volume of 50 μl with 2,0 mM MgCl₂ / 58 °C annealing temperature for
fragment S1; 2,0 mM MgCl₂ / 58 °C for fragment S2; 2,25 mM MgCl₂ /58 °C for fragment
S3; and 1,75 mM MgCl₂ / 58 °C for fragment S4, during 30 cycles. Subsequently 3-5 μl of
nested PCR product of each fragment was used for DGGE. DGGE was performed on 10%
polyacrylamide gels with a preformed gradient of 20-70% urea / 20-70% formamide, using
the DCode system (Biorad, Hercules, CA, USA). Electrophoresis conditions were 120 volts
during 4 hours at 56 °C. The gels were stained with ultrasensitive silver staining.

In addition, in the one fresh frozen carcinoma, APC gene mutational status was
studied using an in vitro synthesised protein assay as described previously [35], which is
capable of detecting mutations in genes that alter the resultant in vitro synthesized protein
product. The APC gene was divided in 5 overlapping segments; segment 1 was isolated from
complementary DNA templates prepared with reverse transcription of messenger RNA,
segments 2 to 5 were isolated directly from genomic DNA. PCR products were used directly
as templates in coupled transcription-translation reactions. Samples were analysed by
electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel with a gradient from 10 to 20
percent. The (truncated) proteins were visualized by fluorography after the gel had been
impregnated with ENHANCE (New England Nuclear, Boston, MA, USA).

RESULTS

LOH at 19p13.3:

In the 39 hamartomatous polyps from 17 patients, LOH at 19p near the STK11/LKB1
gene was found in 15 cases (38%)(figure1, table 2). Fine microdissection of the epithelium,
using a Laser Capture Microscope did not reveal additional polyps with LOH. In the polyp of
the patient without a germline mutation in the STK11/LKB1 gene, no LOH at 19p was found.
The 5 carcinomas (3 from patients with a known germline mutation in STK11/LKB1; 2
from patients with unknown mutational status) all showed LOH at 19p (figure 1, table 3).
Interestingly, 6 out of the 7 polyps (86%) of patients with a carcinoma did show LOH at 19p,
compared to only 9 out of 31 polyps (29%) of the remaining patients, excluding the polyp
from the patient without a germline mutation in the STK11/LKB1 gene (p=0.01, Fisher's exact
test). All 5 patients with a carcinoma had at least one polyp with LOH, whereas only 5 from
the 11 patients without cancer had polyps with LOH (p=0.06, Fisher's exact test).

LOH at the possible alternative PJS loci 19q; 6p; and 6q:

In 1 polyp of a patient without a germline STK11/LKB1 mutation, LOH at 19q, 6p and
6q was studied to investigate whether these loci are involved in a possible STK11/LKB1
independent PJS pathogenesis. However, no LOH at 19q, 6p and 6q was found.
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Table 2: Number of polyps with LOH observed in PJS patients according to polyp site and mutational status of the STK11/LKB1 gene.

<table>
<thead>
<tr>
<th>Polyp site</th>
<th>Number of polyps with LOH of patients with a germline STK11/LKB1 mutation</th>
<th>Number of polyps with LOH of patients without a germline STK11/LKB1 mutation</th>
<th>Number of polyps with LOH of patients with unknown mutational status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>Small bowel</td>
<td>7/16</td>
<td>0/1</td>
<td>4/10</td>
<td>11/27</td>
</tr>
<tr>
<td>Colon</td>
<td>1/5</td>
<td>3/5</td>
<td>4/10</td>
<td>4/10</td>
</tr>
<tr>
<td>Total</td>
<td>8/22</td>
<td>0/1</td>
<td>7/16</td>
<td>15/39</td>
</tr>
</tbody>
</table>

LOH at 5q and APC-mutational analysis:
No LOH at 5q (APC gene) was found in both polyps and carcinomas. Mutational analysis for APC was done in the five carcinomas by DGGE, and revealed an APC mutation in 4 out of 5 cases (figure 2, table 3). In one carcinoma, no mutation was found in fragment S1-S3, whereas fragment S4 was non-informative. The APC-mutation was confirmed by an in vitro synthesized-protein assay in the one carcinoma (stopcodon truncation of segment 2), from which a fresh frozen specimen was available.

LOH at 17p; p53 immunohistochemistry; and sequence analysis of the p53 gene:
LOH at 17p (p53 gene) was not found in polyps or carcinomas. Immunohistochemistry showed focal expression of p53 in 3 polyps and unequivocal expression in 1 carcinoma (figure 3), suggesting the presence of mutant p53 protein product [29]. Subsequent sequence
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Figure 2: DGGE pattern of an APC gene mutation in fragment S3 of the APC mutation cluster region (MCR) in a Peutz-Jeghers syndrome (PJS) carcinoma (lane 1). Wild type fragment S3 from a different PJS carcinoma (lane 2). Wild type fragment S3 from the colon carcinoma cell line CaCo2 (lane 3).

analysis of the p53 exons 5-8 in all carcinomas and in microdissected tissue from the areas with p53 expression in the 3 polyps did not show a mutation in these exons of the gene.

LOH at 18q and immunohistochemistry for Smad4:

LOH at 18q (Smad2 and Smad4 locus) was found in one carcinoma (figure 1, table 3); no 18q-LOH was found in any of the polyps. Smad4 expression was present in all carcinomas, including the colon carcinoma with 18q LOH and the pancreatic carcinoma.

K-ras codon 12 mutational analysis:

Mutational analysis of the K-ras oncogene in codon 12 revealed an activating mutation in one colon carcinoma (table 3), caused by a basepair substitution from wild type glycine (GGT) to aspartic acid (GAT).

DISCUSSION

To increase insight in PJS-related carcinogenesis, we analysed LOH at the locus of the Peutz-Jeghers gene STK11/LKB1 on chromosome 19p13.3 in 39 polyps and 5 carcinomas of 17 PJS patients. In addition, we studied LOH at loci containing the genes known to be
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Table 3: Molecular changes and immunohistochemical (IHC) expression of p53 and Smad4 in the five studied PJS-related carcinomas. In the fresh frozen specimen, the APC mutation found with DGGE was confirmed by an in vitro synthesized-protein assay.

<table>
<thead>
<tr>
<th>Patient with STK11/LKB1 germline mutation</th>
<th>19p LOH</th>
<th>5q LOH / APC mutational analysis (DGGE)</th>
<th>K-ras codon 12 mutation</th>
<th>18q LOH / IHC Smad 4 expression</th>
<th>17p LOH / IHC p53 over-expression / p53 mutation exon 5-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach carcinoma</td>
<td>Unknown</td>
<td>-/+</td>
<td>-</td>
<td>-/+</td>
<td>-/-</td>
</tr>
<tr>
<td>Small bowel carcinoma</td>
<td>+</td>
<td>-/-</td>
<td>-</td>
<td>-/+</td>
<td>-/++</td>
</tr>
<tr>
<td>Pancreas carcinoma</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>-/+</td>
<td>-/-</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>+/+</td>
<td>-/-</td>
</tr>
<tr>
<td>Colon carcinoma (fresh frozen)</td>
<td>Unknown</td>
<td>-/+ (truncation of segment 2)</td>
<td>-</td>
<td>-/+</td>
<td>-/-</td>
</tr>
</tbody>
</table>

1Fragment S4 from the APC-MCR is non-informative.

involved in the adenoma-carcinoma sequence: APC, Smad4 and Smad2, and p53. In the 5 available carcinomas, mutational analysis of p53, APC and K-ras codon 12 was performed.

Previous studies provide evidence that STK11/LKB1 acts as a tumor-suppressor gene and that germline mutations cause PJS [7;10;36]. In this investigation, LOH of 19p13.3 was found in all five studied carcinomas and 38% of the 39 hamartomatous polyps, supporting the role of the STK11/LKB1 gene in PJS-related tumorigenesis. Literature reports suggest genetic heterogeneity in PJS [20-22]. In the polyp from a PJS patient without a germline STK11/LKB1 mutation, LOH analysis of three alternative candidate PJS-loci (6p, 6q and 19q) [20;23] did not show allelic losses. Different mechanisms abrogating functional expression of wild-type STK11/LKB1, such as point mutations or methylation of the promotor region of the STK11/LKB1 gene, as has been described in a cervical carcinoma cell line [9], cannot be excluded. Alternatively, the absence of LOH in the single polyp of a PJS patient without evidence of a germline STK11/LKB1 mutation could reflect a distinct pathway and may support the existence of genetic heterogeneity.

Of importance is whether loss of STK11/LKB1 induces malignant transformation of PJS tumors, and acts as the initiating event of a possible hamartoma-carcinoma sequence, similar to the role of APC in the adenoma-carcinoma sequence. On a microscopic level we
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were unable to detect differences between hamartomatous polyps with and without LOH. There were no hamartomas with neoplastic epithelial changes observed in this study, and a neoplastic potential thus remained at least obscure at the microscopic level. Since neoplastic change in PJS hamartomas is very rarely found and has only been reported occasionally [5,25], it remains unclear whether loss of wild type \textit{STK11/LKB1} is a key event in the initiation of PJS related carcinogenesis. It is of interest, however, that LOH at 19p13.3 occurred in 6 out 7 (86%) polyps of patients with a carcinoma (n=5), compared to 9 out 31 (29%) polyps of those without cancer (n=11)(p=0.01), excluding the patient without a \textit{STK11/LKB1} germline mutation. Further research is needed to investigate whether 19p13.3 LOH in polyps could serve as a biomarker to predict PJS-related carcinogenesis.

Previous reported differences between molecular alterations in the adenoma-carcinoma sequence and PJS related tumors involve the APC locus at 5q and \textit{K-ras} codon 12 mutations [10;26]. Inactivation of the \textit{APC}-tumor-suppressor pathway is considered to be an early and initiating event in colorectal neoplastic growth, and LOH at 5q is found in up to 50% of sporadic colorectal tumors [37]. In this study, LOH at 5q was not found in the PJS hamartomas and PJS related carcinomas. However, mutational analysis of the mutation cluster region (MCR) of the \textit{APC} gene revealed \textit{APC}-mutations in 4 out of 5 studied carcinomas (confirmed by a positive protein truncation test for \textit{APC} mutation in one fresh frozen specimen) indicating that nevertheless the \textit{APC} gene may play a role in PJS-related carcinogenesis. In earlier work, we reported that \textit{K-ras} codon 12 mutations, which are found in 50% of colorectal adenomas larger than 1 cm [37], are very rare in PJS-hamartomas [26]. In this study, only one \textit{K-ras} codon 12 mutation was found in five carcinomas. Of note, the pancreatic carcinoma analysed in this study did not have a \textit{K-ras} codon 12 mutation, a very common event in sporadic pancreatic carcinoma [38]. The \textit{p53} gene is considered to play an important role in the progression of premalignant lesions towards malignancy, being mutated in 85% of colorectal carcinomas and very rarely in benign tumors [37]. Only 1 out of 5 PJS-carcinomas showed immunohistochemical expression of \textit{p53}, which suggests the presence of mutant \textit{p53} gene product [29]; and no mutations in the commonly affected exons 5-8 were detected in any carcinoma. Taken together, these results suggest that the molecular genetic alterations in the 5 studied PJS related carcinomas differ from sporadic carcinomas, and may follow a distinct path of carcinogenesis. Whether such a PJS-related carcinogenesis follows a hamartoma-carcinoma sequence remains unclear, since neoplastic change in hamartomas is only rarely observed and it is therefore questionable whether hamartomas act as truly pre-neoplastic lesions.

In conclusion, the present study further establishes the role of the \textit{STK11/LKB1} gene in PJS-related tumorigenesis, although its function remains largely unknown. In addition, our results suggest that PJS-related carcinomas display different molecular genetic alterations compared to sporadic gastrointestinal tumors. Further research is needed to address the possible neoplastic potential of the PJS hamartomas and to unravel the role of the PJS gene \textit{STK11/LKB1} in tumor formation and carcinogenesis.
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