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Chapter 8

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

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Molecular genetic alterations in hamartomatous polyps and carcinomas of patients with Peutz-Jeghers syndrome


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Abstract

**Aim**—To investigate whether mutations in the STK11/LKB1 gene and genes implicated in the colorectal adenoma–carcinoma sequence are involved in Peutz-Jeghers syndrome (PJS) related tumorigenesis.

**Methods**—Thirty nine polyps and five carcinomas from 17 patients (from 13 families) with PJS were analysed for loss of heterozygosity (LOH) at 19p13.3 (STK11/LKB1 gene locus), 5q21 (APC gene locus), 18q21–22 (Smad4 and Smad2 gene loci), and 17p13 (p53 gene locus), and evaluated for immunohistochemical staining of p53.

In addition, mutational analysis of K-ras codon 12, APC, and p53 and immunohistochemistry for Smad4 expression were performed on all carcinomas.

**Results**—LOH at 19p was seen in 15 of the 39 polyps and in all carcinomas (n = 5). Interestingly, six of the seven polyps from patients with cancer had LOH, compared with nine of the 31 polyps from the remaining patients (p = 0.01). In one polyp from a patient without a germline 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 APC mutation in four of the five carcinomas. LOH at 17p was not seen in any polyps or carcinomas. Immunohistochemistry showed expression of p53 in one carcinoma and focal expression in three polyps. At subsequent sequence analysis, no 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 STK11/LKB1 acts as a tumour suppressor gene, and may be involved in the early stages of PJS tumorigenesis. Further research is needed to see whether LOH in PJS polyps could be used as a biomarker to predict cancer. Differences in molecular genetic alterations noted between the adenoma–carcinoma sequence and PJS related tumours suggest the presence of a distinct pathway of carcinogenesis.

Keywords: Peutz-Jeghers syndrome; carcinogenesis; STK11/LKB1; hamartoma

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant condition characterised by hamartomatous polyps, which can occur throughout the gastrointestinal tract, and melanin spots found on the lips and buccal mucosa. Patients with PJS have an increased risk of developing cancer at a relatively young age. Malignancies occur in the gastrointestinal tract but also in non-gastrointestinal sites including the pancreas, breast, ovary, and testis. Recently, a gene defect leading to PJS has been identified. The STK11/LKB1 gene encodes a serine/threonine kinase that is expressed ubiquitously in human tissues and might be involved in G1 cell cycle arrest. Although the STK11/LKB1 gene appears to play a crucial role in tumour development in patients with PJS, a low frequency of mutation is found in similar sporadic cancers. To date, more than 60 patients with PJS and inactive germline mutations in the STK11/LKB1 gene have been reported. Possible hotspots of mutation are exons 1 and 6, which together account for one half of the currently described germline alterations. However, not all families affected by PJS are linked to the 19p13.3 locus of the STK11/LKB1 gene, suggesting genetic heterogeneity. Alternative loci are 19q and the breakpoints at a pericentric inversion on chromosome 11.

Investigation of the genetic alterations in premalignant and malignant lesions from patients with familial adenomatous polyposis (FAP) has been instrumental in the discovery of the sequential genetic events in colorectal carcinogenesis. These studies have found a progressive accumulation of mutations in the APC, K-ras, Smad2, Smad4, and p53 genes, which correlate with the subsequent stages of the adenoma–carcinoma sequence. The possible malignant potential of hamartomatous polyps in patients with PJS is not well understood. Although very rare, malignant transformation of PJS polyps has been reported, indicating that a hamartoma–carcinoma sequence might occur in PJS. Mutational analysis of the genes involved in colon carcinogenesis has been performed in hamartomas and carcinomas from patients with PJS, and has shown that K-ras mutations are very rare in PJS tumours compared with FAP and sporadic adenomas or carcinomas. In addition, no loss of heterozygosity (LOH) at 5q (close to the APC gene) was found in PJS tumours. In contrast, inactivating mutations of the wild-type allele of the
STK11/LKB1 gene are common in PJS tumours, indicating that the PJS gene might be involved in PJS related tumour development, and that it might act as a tumour suppressor gene.

In our present study, we extended our previous work by investigating the 19p LOH status in polyps and carcinomas from 17 patients with PJS, including one without a germline mutation in the STK11/LKB1 gene. In addition, we analysed these tissues for the specific genetic alterations known to occur in the adenoma-carcinoma sequence to clarify the molecular pathogenesis of PJS tumours.

Materials and methods

Patients and Tissue Samples
Thirty nine hamartomatous polyps and five adenocarcinomas of 17 patients with PJS from 15 families formed the study population. The clinical diagnosis of PJS was confirmed by histopathological review of the hamartomas by an experienced pathologist (GJAÖ). The hamartomas were from the gastrointestinal tract (stomach, two; small bowel, 27; colon, 10); the carcinomas were from the stomach (one), pancreas (one), small bowel (one), and colon (two). Eleven patients came from seven different families with a germline STK11/LKB1 mutation; in one patient from another family, no germline mutation was found. In the five remaining patients from five other unre- related families, the mutational status was unknown (table 1).

DNA isolation
Formalin fixed and paraffin wax 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 to glass slides, and stained with haematoxylin and eosin. Polyp epithelium and cancer tissues were carefully microdissected. The microdissected tissue was collected into microcentrifuge tubes containing 50-200 μl DNA isolation buffer (50 mM Tris-HCl, pH 8.0, 0.2% Tween-20, and 100 mg/ml protease K), depending on the size of the tissue fragments, and incubated overnight at 96°C. Samples were heated to 96°C for 10 minutes to inactivate the protease 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 the epithelium using a laser capture microdissection (Dako, Glostrup, Denmark) at a 1/200 dilution was used to avoid negative results caused by wild-type contamination.

Table 1 Number of patients with Peutz-Jeghers syndrome (PJS), affected families, and studied hamartomatous PJS polyps, and the accompanying germline STK11/LKB1 mutational status

<table>
<thead>
<tr>
<th>Number of patients/families</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>Number of polyps</td>
<td>11 patients from 7 families</td>
<td>1 patient</td>
<td>17 patients from 13 families</td>
<td></td>
</tr>
<tr>
<td>Number of carcinomas</td>
<td>22</td>
<td>1</td>
<td>6</td>
<td>33</td>
</tr>
</tbody>
</table>

LOH analysis

Two polymorphic microsatellite markers were used for 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 and within the p53 gene on 17p13, respectively. For analysis of the alternative PJS loci, the markers D19S891 for 19q, D6S5257 for 6p, and D6S311 for 6q were used. Primer sequences were obtained from the genome database (www.gdb.org), except for p53-Alu. One primer for each marker was end labelled with [32P]-dTMP. AmpliTaq Gold polymerase was used for amplification of the genomic DNA fragments. The PCR conditions for these markers were: 94°C for 10 minutes, followed by 33 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for one minute; and then a final extension step of 72°C for 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 from the five carcinomas were used for amplification of K-ras specific sequences by the polymerase chain reaction (PCR). Mutations were identified by allele specific hybridisation using a previously described protocol.

Immunohistochemistry for p53 and Smad4

Immunohistochemistry for p53 and Smad4 was performed as described previously. 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 alcohols. Endogenous peroxidase activity was blocked in 0.3% H2O2 in methanol for 20 minutes. Slides were submersed 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 one hour with the primary antibody. To detect mutant p53 expression, the monoclonal mouse antibody 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, California, USA) was used at a 1/100 dilution. After washing, biotinylated rabbit anti-mouse IgG antibody (Dako, Glostrup, Denmark) at a 1/200 dilution in PBS with 10% normal AB serum was applied for 30 minutes, followed by streptavidin–biotin

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peroxidase in PBS with 10% normal AB serum (1/200) for 30 minutes. The peroxidase activity was visualized using diaminobenzidine (DAB; 1/20) in 0.05 M Tris-HCl/0.1% H₂O₂ for 10 minutes. The nuclei were counterstained with haematoxylin. A known p53 positive colorectal carcinoma was used as a positive control; the same sample was used as a negative control by replacing the primary antibody with PBS. p53 staining was considered positive when more than 10% of the cells showed nuclear p53 expression.

**SEQUENCE ANALYSIS OF 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 three polyps as described previously. Briefly, exons 5–8 were amplified by PCR. Template DNA was obtained by PCR, using an aliquot of the genomic DNA. The PCR products were run 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, California, USA). Bacterial clones with inserts were picked. DNA was isolated, followed by bidirectional DNA sequencing using Sequenase version 2.0 (United States Biochemicals, Cleveland, Ohio, USA).

**APC MUTATIONAL ANALYSIS**

APC mutational analysis in DNA from the five carcinomas was performed using a denaturing gradient gel electrophoresis (DGGE) technique, as has been described previously. 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. The DNA was extracted from tissue sections as described above. The MCR was amplified by PCR using primers described previously. Initially, two overlapping fragments spanning the MCR (fragment A: nucleotides 3874–4229 and fragment B: nucleotides 4114–4624) were amplified in a PTC-200 machine (MJ Research Inc, Watertown, Massachusetts, USA) in a final volume of 20 μl containing 2 mM MgCl₂, 250 μM of dNTPs, 200 nM of each primer, and 1 U of Taq DNA polymerase (Perkin Elmer) in the buffer supplied by the manufacturer. Cycling was performed during 30 cycles at 94°C for 40 seconds, 54°C for one minute, and 72°C for one minute, followed by a final extension step at 72°C for 10 minutes. The respective primers used were: fragment A, 5'-GAAGATTTAGGATGTAATCAG ACG-3' upstream and 5'-GAGCTCGGCAA TCGAAGCAGT-3' downstream; fragment B, 5'-GCTACGCACCCAAAAGTC G-3' upstream and 5'-CATTTCCCATGTC ATTTCCG-3' downstream. 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 (nucleotides 3874–4092) using the upstream primer for fragment A and 5'-CGCTCCTGAAGAAATTCAC-3' downstream; fragment S2 (nucleotides 4026–4229) using primer 5'-ACTGAGGGTGCT CTA22ATC-3' upstream and the downstream primer for fragment A; fragment S3 (nucleotides 4179–4383) using primers 5'-TAC2TTGTCGACCTGGATA-3' upstream and 5'-ATTTGACCTGCCT CGTG-3' downstream; and fragment S4 (nucleotides 4328–4594) using primers 5'-AAAGCTCCACGACCTCC-3' upstream and 5'-GCATTATGGATTACACATC-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 fragments S1 and 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, which was performed on 10% polyacrylamide gels. The temperature of the buffer used for DGGE was 65°C for fragment S1 and S2; 60°C for fragment S3; and 45°C for fragment S4. The DGGE conditions were as described previously. The bands were visualized by fluorography after the gel had been impregnated with ENHANCE (New England Nuclear, Boston, Massachusetts, USA).

Results

**LOH AT 1p13.3**

In the 39 hamartomatous polyps from 17 patients, LOH at 19p, near the STK11/LKB1 gene, was found in 15 cases (fig 1; 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 in five carcinomas (three from patients with a known germline mutation in STK11/LKB1; two from patients with unknown mutational status) all showed LOH at 19p (fig 1; table 3). Interestingly, six of the seven polyps from patients with carcinoma showed LOH at 19p, compared with only nine of 31 polyps from the remaining patients, excluding the polyps from the patient without a germline mutation in the STK11/LKB1 gene (p = 0.01, Fisher's exact
Carcinogenesis in Peutz-Jeghers Syndrome (1)

Table 2 Number of polyps with loss of heterozygosity (LOH) at 19p seen in patients with Peutz-Jeghers syndrome 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 from patients with a germline STK11/LKB1 mutation</th>
<th>Number of polyps with LOH from patients without a germline STK11/LKB1 mutation</th>
<th>Number of polyps with LOH from 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>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Small bowel</td>
<td>7/16</td>
<td>0/1</td>
<td>11/27</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>4/10</td>
<td>15/29</td>
<td>15/29</td>
</tr>
</tbody>
</table>

LOH at the possible alternative PJ5 loci 19q, 6p, and 6q

In one polyp from a patient without a germline STK11/LKB1 mutation, LOH at 19q, 6p, and 6q was studied to see whether these loci were involved in STK11/LKB1 independent PJ5 pathogenesis. However, no LOH at 19q, 6p, and 6q was found.

LOH at 5q and APC mutational analysis

No LOH at 5q (APC gene) was found in either polyps or carcinomas. Mutational analysis of the APC gene was carried out in the five carcinomas by DGGE, and revealed an APC mutation in four of five cases (fig 2; table 3). In one carcinoma, no mutations were found in fragments S1–S3, and fragment S4 was non-informative. The APC mutation was confirmed by an in vitro synthesised protein assay in the one carcinoma (stop codon 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 three polyps and unequivocal expression in one carcinoma (fig 3), suggesting the presence of mutant p53 protein product. Subsequent sequence analysis of p53 exons 5–8 in all carcinomas and in microdissected tissue from the areas with p53 expression in the three polyps did not detect a mutation.

LOH at 18q and immunohistochemistry for Smad4

LOH at 18q (Smad2 and Smad4 locus) was found in one carcinoma (fig 1; table 3) but not in 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

Table 3 Molecular changes and immunohistochemical (IHC) expression of p53 and Smad4 in the five studied Peutz-Jeghers syndrome related carcinomas

<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</th>
<th>Smad4 expression</th>
<th>17p LOH/IHC p53 mutation exons 5–8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach carcinoma</td>
<td>Unknown</td>
<td>+</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>
<td>–</td>
</tr>
<tr>
<td>Pancreas carcinoma</td>
<td>+</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>
<td>–</td>
</tr>
<tr>
<td>Colon carcinoma (fresh frozen)</td>
<td>Unknown</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Fragment S4 from the APC mutation cluster region is non-informative. DGGE, denaturing gradient gel electrophoresis; LOH, loss of heterozygosity.
one colon carcinoma (table 3), caused by a base pair substitution from wild-type glycine (GGT) to aspartic acid (GAT).

Discussion

To increase insight into PJS related carcinogenesis, we analysed LOH at the locus of the PJS gene, STK11/LKB1, on chromosome 19p13.3 in 39 polyps and five carcinomas from 17 patients with PJS. In addition, we studied LOH at loci containing the genes known to be involved in the adenoma–cancer sequence: APC, Smad4 and Smad2, and p53. In the five available carcinomas, mutational analysis of p53, APC, and K-ras codon 12 was performed. Previous studies provide evidence that STK11/LKB1 acts as a tumour suppressor gene and that germline mutations cause PJS.10 20 In our present study, LOH of 19p13.3 was found in all five carcinomas and over a third of the 39 hamartomatous polyps, supporting the role of the STK11/LKB1 gene in PJS related tumorigenesis. Literature reports suggest genetic heterogeneity in PJS.10 22 In the polyp from the patient with PJS but without a germline STK11/LKB1 mutation, LOH analysis of three alternative candidate PJS loci (6p, 6q, and 19q)15 23 did not show allelic losses. Different mechanisms abrogating functional expression of wild-type STK11/LKB1, such as point mutations or methylation of the promoter region of the STK11/LKB1 gene, as has been described in a cervical carcinoma cell line,2 cannot be excluded. Alternatively, the absence of LOH in the single polyp of a patient with PJS without evidence of a germline STK11/LKB1 mutation could reflect a distinct pathway and might support the existence of genetic heterogeneity.

Of importance is whether loss of STK11/LKB1 induces the malignant transformation of PJS tumours, and acts as the initiating event of a possible hamartoma–cancer sequence, similar to the role of APC in the adenoma–cancer sequence. On a microscopic level, we were unable to detect differences between hamartomatous polyps with and without LOH. We saw no hamartomas with neoplastic epithelial changes, so that a neoplastic potential remained obscure at least at the microscopic level. Because neoplastic change is very rarely found in PJS hamartomas and has only been reported occasionally,7 25 it remains unclear whether the loss of wild-type 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 six of the seven polyps from patients with carcinoma (n = 5), compared with nine of the 31 polyps from those without cancer (n = 11; p = 0.01), excluding the patient without an STK11/LKB1 germline mutation. Further research is needed to find out whether 19p13.3 LOH in polyps could be used as a biomarker to predict PJS related carcinogenesis.

Previous reported differences between molecular alterations in the adenoma–cancer sequence and PJS related tumours involve the APC locus at 5q and K-ras codon 12 mutations.26 30 Inactivation of the APC tumour suppressor pathway is thought to be an early and initiating event in colorectal neoplastic growth, and LOH at 5q is found in up to 50% of sporadic colorectal tumours.31 In our study, LOH at 5q was not found in the PJS hamartomas and PJS related carcinomas. However, mutational analysis of the MCR of the APC gene revealed APC mutations in four of five carcinomas studied (confirmed by a positive protein truncation test for APC mutation in one fresh frozen specimen), indicating that nevertheless the APC gene might play a role in PJS related carcinogenesis. In earlier work, we reported that K-ras codon 12 mutations, which are found in 50% of colorectal adenomas larger than 1 cm,32 are very rare in PJS hamartomas.33 In our present study, only one K-ras codon 12 mutation was found in five carcinomas. Of note, the pancreatic carcinoma analysed did not have a K-ras codon 12 mutation, a very common event in sporadic pancreatic carcinoma.34 The p53 gene is thought 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 tumours.35 Only one of the five PJS carcinomas showed immunohistochemical expression of p53, which suggests the presence of mutant p53 gene product36; and no mutations in the commonly affected exons 5–8 were detected in the carcinomas. Taken together, these results suggest that the oncogenic differences in the five PJS related carcinomas differ from those seen in sporadic carcinomas, and may follow a distinct path of carcinogenesis. Whether such a PJS related carcinogenesis follows a hamartoma-carcinoma sequence remains unclear, because neoplastic change in hamartomas is only rarely seen and it is therefore questionable whether hamartomas act as truly preneoplastic lesions.

In conclusion, our present study further establishes the role of the STK11/LKB1 gene in PJS related tumorigenesis, although its function remains largely unknown. In addition, our results suggest that PJS related carcinomas have different molecular genetic alterations compared with those found in sporadic gastrointestinal tumours. Further research is needed to investigate the possible neoplastic potential of PJS hamartomas and to unravel the role of the PJS gene, STK11/LKB1, in tumour formation and carcinogenesis.
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