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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–cancer 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 locus), 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 hamartomas 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–canceroma sequence and PJS related tumours suggest the presence of a distinct pathway of carcinogenesis.

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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 inactivating 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 19p13.3 loci of the STK11/LKB1 gene, suggesting genetic heterogeneity.

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 FJS 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
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 haematoxilin and eosin. Polyp epithelium and cancer tissue 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 proteinase K), depending on the size of the tissue fragments, and incubated overnight at 96°C. The 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 of 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 system (Microchem, Denmark) at a 1:100 magnification was performed. The samples were submerging in citrate buffer (0.01 M, pH 6.0) for 20 minutes. Slides were submerged in citrate buffer 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 with the primary antibody to detect mutant p53 expression. The monoclonal mouse antibody and rabbit antihuman IgG antibody (Dako, Glostrup, Denmark) at a 1/200 dilution in PBS was used at a 1/100 dilution in PBS. For Smad4 staining, the monoclonal mouse antihuman antibody B-8 (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used at a 1/100 dilution in PBS. Metal-fixed, paraffin-embedded tissue blocks were cut at 5 μm and mounted on to glass slides. Endogenous peroxidase activity was blocked in 0.3% H₂O₂ in methanol for 20 minutes. Sections were dewaxed and rehydrated in a series of graded alcohols. The sections were then autoclaved in citrate buffer (pH 6.0) and allowed to cool. The sections were then incubated overnight with a primary antibody to p53 or Smad4. The sections were then incubated with a secondary antibody (rabbit antiserum) and an appropriate streptavidin-biotin complex (Dako) at a 1/200 dilution in PBS.
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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 ORGS

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 same DNA. The primer used in this reaction added EcoRI and BamHI recognition sequences to the amplified product. The fragments were digested with EcoRI and BamHI 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 amplified from 20% of the DNA was 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, Waltham, Massachusetts, USA) in a final volume of 20 μl, containing 2.0 mM of MgCl₂, 250 μM of dNTPs, 200 μM 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 for the amplification were: the upstream primer for fragment A, 5'-CGCTCCTGAAGAAATTCAC-3' downstream; fragment S2 (nucleotides 4026-4229) using primer 5'-ACTGCAAGGGTTCTAGTTTATC-3' upstream and the downstream primer for fragment A; fragment S3 (nucleotides 4179-4383) using primers 5'-TACGTCTGACTTGGTATA-3' upstream and 5'-TTTGGATCCTCCTTGGTTT-3' downstream; and fragment S4 (nucleotides 4328-4594) using primers 5'-AAACCTCACCCACCTCC-3' upstream and 5'-GCATTATTCTTATTTCCCATC-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 with a temperature gradient of 20-70°C for 20/20-70% urea/20-70% formamide, using the DCode system (Biorad, Hercules, California, USA). Electrophoresis conditions were 120 V for four hours at 56°C. The gels were stained with ultraviolet sensitive silver staining.

In addition, in the one fresh frozen carcinoma, APC gene mutational status was studied using an in vitro synthesized protein assay as described previously, which is capable of detecting mutations in genes that alter the resultant in vitro synthesized protein product. The APC gene was divided into five overlapping segments; segment 1 was isolated from complementary DNA templates prepared with reverse transcription of mRNA, 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 sulphate polyacrylamide gel with a gradient from 10% to 20%. The (translation-coupled) proteins 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 polyp from the patient without a germline mutation in the STK11/LKB1 gene (p = 0.01, Fisher's exact
Carcinogenesis in Peutz-Jeghers Syndrome (1)

Figure 1 (A) Loss of heterozygosity (LOH) at the STK11/LKB1 locus 19p in a hamartomatous polyp (P) and a colonic carcinoma (C) from patients with Peutz-Jeghers syndrome. DNA extracted from normal tissue (N) is displayed next to tumour DNA. (B) LOH at 18q in a colonic carcinoma (C) compared with normal tissue (N).

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>1/1</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Small bowel</td>
<td>7/16</td>
<td>0/1</td>
<td>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>8/22</td>
</tr>
<tr>
<td>Total</td>
<td>8/22</td>
<td>4/10</td>
<td>4/10</td>
<td>15/59</td>
</tr>
</tbody>
</table>

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</th>
<th>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 overexpression</th>
<th>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>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Small bowel carcinoma</td>
<td>+</td>
<td>+</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>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>+</td>
<td>+</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>+ (transcription)</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–carcinoma 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. 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. 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) 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, 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–carcinoma sequence, similar to the role of APC in the adenoma–carcinoma 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, 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–carcinoma sequence and PJS related tumours involve the APC locus at 5q and K-ras codon 12 mutations. 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. 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, are very rare in PJS hamartomas. 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. 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. Only one of the five PJS carcinomas showed immunohistochemical expression of p53, which suggests the presence of mutant p53 gene product; and no mutations in the commonly affected exons 5–8 were detected in the carcinomas. Taken together, these results suggest that the molecular genetic alterations 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 neoplastic 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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