PART II Molecular and Phenotypic Studies of Peutz-Jeghers Syndrome

Chapter 9

COX-2 expression and molecular alterations in Peutz-Jeghers hamartomas and carcinomas


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Abstract

Peutz-Jeghers syndrome (PJS) is a hamartomatous polyposis disorder with a high cancer risk. Debate exists about the pre-malignant potential of hamartomas. Also, treatment options are not available other than surveillance. Therefore, molecular alterations in hamartomas and PJS carcinomas were studied. The objective was (i) to evaluate expression of COX-2 as target for chemopreventive treatment and (ii) to define the neoplastic potential of hamartomas at the molecular level. Paraffin-embedded samples of 24 PJS hamartomas, including 2 hamartomas with dysplastic changes, and 11 PJS carcinomas were available. Slides were stained with antibodies against COX-2, β-catenin, cyclin-D1, p21waf1/cip1, Ki67, and p53. DNA was studied for LOH at 19p (STK11), 5q (APC), and 17p (TP53); mutations in β-catenin, APC and K-RAS; and MSI. Moderate or strong epithelial COX-2 was present in 25% of hamartomas, including two hamartomas with dysplastic changes, and 64% of carcinomas. Several hamartomas showed focal nuclear β-catenin (18%) and cyclin-D1 overexpression (29%), both unrelated to dysplasia at histological examination. Disturbed topographical expression of Ki-67 in relation to p21waf1/cip1 was focally present in 27% of hamartomas, including those with dysplastic changes. Most carcinomas showed nuclear β-catenin (71%), cyclin-D1 overexpression (71%) and aberrant Ki-67 staining (100%). There was LOH at 19p in 32% of hamartomas and 82% of carcinomas. p53 staining was present in 4 (36%) carcinomas, one of which showed LOH at 17p. No β-catenin mutations were found. APC mutations were present in 2 carcinomas, but LOH at 5q was not found. Two carcinomas had K-RAS mutations, and one carcinoma had MSI. The presence of COX-2 expression in PJS carcinomas and dysplastic hamartomas provides a rationale for chemoprevention with NSAIDs or COX-2 inhibitors. Focal immunohistochemical changes, which may indicate a pre-malignant potential, were present in some non-dysplastic PJS hamartomas. Molecular changes in carcinomas and dysplastic hamartomas in PJS are distinct from the usual adenoma-carcinoma sequence.

Introduction

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant disorder, caused by a germline mutation in the STK11/LKB1 gene on chromosome 19p13.3 1,2. PJS is characterized by hamartomatous polyps throughout the gastrointestinal tract and melanin pigmentation of the skin and mucous membranes 3-6. PJS patients have an increased risk of cancer at relatively young age 7. Malignancies occur in the gastrointestinal (GI) tract and a variety of extra-gastrointestinal sites.

To date, carcinogenesis in the PJS is poorly understood. Malignant transformation of hamartomas appears rare 6,8,9, and PJS related carcinomas do not necessarily develop through a hamartoma-adenoma-carcinoma sequence similar to the adenoma-carcinoma sequence in sporadic colorectal carcinoma (CRC). The latter is driven by an accumulation of well characterized genetic alterations in tumor suppressor genes and oncogenes, including APC, K-RAS and TP53 10.
Inactivation of APC or activation of β-catenin prevents the degradation of β-catenin, which then accumulates in the nucleus of tumor cells, resulting in β-catenin/TCF mediated transcription of target genes, amongst which is cyclin-D1. Oncogenic activation of this pathway is considered necessary for the initiation of colorectal dysplasia. At the cell kinetic level, dysplasia in the gastrointestinal tract is characterized by disturbed topographical expression of the proliferation marker Ki-67, normally restricted to the crypt base, and p21waf1/cip1, a cell cycle inhibitor normally expressed by non-replicative upper crypt and surface cells. In patients with hereditary non-polyposis colorectal cancer (HNPCC) and in a minority of sporadic CRCs, carcinogenesis is driven by mismatch repair (MMR) deficiency, reflected by microsatellite instability ( MSI) throughout the genome.

Importantly, the adenoma-carcinoma sequence can be inhibited or reversed by NSAIDs and selective cyclooxygenase-2 (COX-2) inhibitors, which are considered promising chemopreventive agents. The main target of these drugs is cyclooxygenase-2 (COX-2), involved in the conversion of arachidonic acid into prostaglandins, which interfere with processes such as apoptosis and angiogenesis. COX-2 is not expressed under physiological conditions, but is induced by inflammation and neoplasia. Expression occurs early in the adenoma-carcinoma sequence, and is found in both epithelial and stromal cells within tumors. Recently, COX-2 expression was also reported in hamartomas from PJ S patients and Stk11/Lkb1 heterozygous mice, which provide a murine model for PJ S. Whether COX-2 expression occurs in carcinomas has not been evaluated.

Insights in the molecular pathogenesis of PJ S may help to define the neoplastic potential of hamartomas and guide chemopreventive treatment strategies. Previous studies reported loss of heterozygosity (LOH) at the STK11/LKB1 locus 19p13.3 in hamartomas and carcinomas from Peutz-Jeghers patients. Also, somatic mutations in STK11/LKB1 were found in hamartomas without LOH at 19p13.3. However, alterations in APC, K-RAS and TP53 were relatively rare when compared to the adenoma-carcinoma sequence, suggesting an alternative pathway of carcinogenesis. Miyaki et al. reported β-catenin mutations in large hamartomas, implicating a possible neoplastic potential of these lesions. The present study further explores the molecular pathogenesis of PJ S hamartomas and carcinomas, extending our previous work. Expression of COX-2, a possible therapeutic target, β-catenin, cyclin-D1, p21waf1/cip1, Ki67 and p53 was evaluated. In addition, DNA from hamartomas and carcinomas was investigated for mutations in APC, β-catenin (CTNNB1), and K-ras; LOH at 19p (STK11-locus), 5q (APC-locus), and 17p (TP53-locus); and MSI.

Materials and methods

Patients and tissue samples:

The study population consisted of 19 PJ S patients from 15 different families. The diagnosis Peutz-Jeghers syndrome was confirmed by histological examination of hamartomas by an experienced pathologist (GJAO). Mutational analysis revealed STK11/LKB1 germline mutations in 16 patients from 12 families, most of which have been published previously. Twenty-four hamartomas (stomach, n=1; small bowel, n=16; colorectal, n=7), including 2
hamartomas with adenomatous (dysplastic) changes, and 11 carcinomas were available for study. Samples were formalin-fixed and paraffin-embedded, except the hamartomas with adenomatous changes (n=2), which were fixed in Hollande's fixative, precluding the assessment with PCR-based techniques.

**Microdissection and isolation of DNA:**

Paraffin-embedded tissue was cut into 5 μm sections, and mounted onto PALM, or glass slides. Slides were deparaffinized and stained with hematoxylin. Epithelium from hamartomas and carcinomas was microdissected with the PALM® Laser Microbeam System, or by hand if large fields of tumor cells were present. Wild type DNA was obtained from surrounding stromal cells. DNA was isolated from microdissected tissue with the Puregene DNA purification system (Genta Systems, Minneapolis, MN).

**Immunohistochemistry:**

Hamartomas and carcinomas were studied with immunohistochemistry as described previously 26, using monoclonal antibodies against: COX-2, #160112 (Cayman Chemical, Ann Arbor, MI) at a dilution of 1:100; β-catenin, clone 14 (Transduction Laboratories, Lexington, KY) at a dilution of 1:1000; cyclin D1, ab-1 (Neomarkers, Union City, CA), at a dilution of 1:1000; p21^waf1, ab-1 (Oncogene Research Products, San Diego, CA) at a dilution of 1:25; Ki-67, MIB-1 (Dako, Glostrup, Denmark) at a dilution of 1:200; and p53, DO7 (Dako, Glostrup, Denmark) at a dilution of 1:200. A known p53 positive colorectal carcinoma was used as a positive control for p53 staining. The staining pattern in the adjacent normal mucosa was used as a marker for specific staining of β-catenin, cyclin-D1, p21, and Ki67. To assess the specificity of the COX-2 antibody, a subset of adenomas was stained with the primary antibody after pre-adsorption of a human COX-2 control peptide (Cayman Chemical, Ann Arbor, MI) for 1 hour at room temperature. This resulted in blocking of COX-2 staining of samples fixed in formalin or Hollande's fixative. Staining of normal mucosa served as control for the specificity of immunohistochemistry in Hollande's fixed samples; the staining pattern appeared reliable for all antibodies tested.

Slides were scored by an experienced gastrointestinal pathologist (GJAO). Epithelial COX-2 staining, and staining of stromal cells underlying the epithelium was assessed separately in a semiquantitative manner on a scale from 0-+++ (0: no expression; +: weak staining; ++: moderate staining; +++: strong staining). Nuclear accumulation of β-catenin was scored semiquantitatively, using a scale from 0-+++ (0: no expression; +: <5% positive nuclei; ++: <25% positive nuclei; +++: >25% positive nuclei). Nuclear β-catenin staining which was restricted to the proliferative compartment, as found in the normal small intestinal mucosa, was not scored. p53 and cyclin-D1 staining were considered positive if >10% of nuclei stained positive 27,28. Compartmentalization of p21waf1/cip1 and Ki-67 was judged by comparing the two staining patterns. Loss of normal topographical control was considered positive if expression of p21 and Ki-67 occurred in the same area of epithelium 12.

**Analysis of LOH and MSI:**

Hamartomas and 6 of the 11 carcinomas were previously evaluated for LOH at 19p13.3 (STK11/LKB1), 17p (TP53) and 5q21 (APC). The remaining carcinomas were investigated for
LOH as described previously \(^2\), using the following markers (for primer sequences see www.gdb.org): D19S886, D19S565, D19S883 and D19S814 at 19p13.3; D5S346, D5S107 and D5S82 at 5q21; and p53au, TP53 and D17S796 at 17p. Hamartomas and carcinomas were screened for MSI using the BAT26 marker. PCR products were analyzed using an automated ABI377 sequencer and the Genescan 2.1 software (PE Biosystems, Foster City, CA). If MSI was found with the BAT-26 marker, or a shift was noted with any of the other markers tested for LOH, MSI analysis was performed according to the Bethesda-criteria \(^9\) and immunohistochemical staining for hMLH1, hMSH2 and hMSH6 was performed as described previously \(^8\).

**Analysis of mutations in APC, β-catenin (CTNNB1), and K-RAS:**

DNA from hamartomas was screened for mutations in the **APC** mutation cluster region (MCR) \(^3\) with denaturing gradient gel electrophoresis (DGGE) \(^4\). In addition, DNA from several hamartomas was directly sequenced for confirmation of DGGE results. DNA from carcinomas was directly sequenced. Four overlapping fragments spanning the MCR were amplified using the following primers: 5'-GAAATAGGATGTAATCAGACGA3' & 5'-CGCTCTFAAAAMTTCAAAC-3' for fragment I; 5'-ACTGAGGGTCTATGTGTAT-3' & 5'-GAGCTGCAATGAAAGACT-3' for fragment II; 5'TACTTCTGTACCTGACTGAT3' & 5'-ATTTTATAGGTACTTCTCGCTTG-3' for fragment III; and 5'-AAACACCTCCACCACCCCTC-3' & 5'-GCATTATTTTCAATTTCCACATC-3' for fragment IV \(^5\). PCR reactions were performed in a final volume of 20 μl with 1.5 unit platinum taq polymerase and 2.5 mM MgCl\(_2\). The annealing temperature was 52° C for fragments I and II, and 56° C for fragments III and IV. For DGGE, a GC-clamp was attached to one of the primers. PCR products were run for 7 hours at 150 volts on a 10% polyacrylamide gel with a denaturing gradient from 20% (8.4 % urea; 8% formamide) to 70% (29.4% urea; 28% formamide). DNA was visualized using a silver staining protocol. Sequencing of PCR products was done in both directions using the above mentioned primers with the Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Foster City, CA) in a final volume of 10 μl. Sequences were analyzed using the ABI377 automatic sequencer and Genescan 2.1 software (PE Biosystems, Foster City, CA). Activating mutations in the **APC**-binding domain of β-catenin were assessed in DNA from hamartomas and carcinomas, using primers for exon 3 of β-catenin \(^6\). Sequencing was performed as described above. **K-RAS** mutations in hamartomas and 6 PJS carcinomas were previously reported. A modified method was used for **K-RAS** mutation analysis in the remaining carcinomas. DNA was amplified with primer A (5'-ACTGAAATATAAA CTTGTGTTAGTTGGACCT-3') and D (5'-CATGAAAATGTCAGAGAAACC-3'). First round PCR products were digested with BstN1 (New England Biolabs, Beverly, MA) at 60°C during 1 hour. Digested (mutant enriched) DNA was amplified in a second round PCR using primer B (5'-TCAAGAATGTGCCTGGACC-3') and a modified primer A, to which a 5'-CTCGCA-tail was added. **K-RAS** codon 12, which lies immediately downstream of primer A, was then sequenced in two directions, using forward sequence primer 5'-CTCGCAACTGAATATAACTTGTG-3', and primer B. Mutations were always confirmed by a second experiment with DNA from a separate PCR as template.
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Results

From 19 PJS patients, 24 hamartomas, ranging in size from 6 to 65 mm (median 20 mm), and 11 carcinomas were studied. Two hamartomas had dysplastic changes. Seven of the carcinomas originated in the gastrointestinal tract. Hamartomas and carcinomas were investigated for molecular alterations involved in the adenoma-carcinoma sequence. Changes in the expression patterns of COX-2, β-catenin, cyclin-D1, Ki-67 and p21waf1/cip1 were assessed. LOH analysis was performed for the STK11, APC, and TP53 loci; mutations in β-catenin, APC, and K-RAS were investigated; and MSI was tested. The results are listed in Tables 1-3.

Table 1: Results of epithelial and stromal COX-2 staining in PJS-related hamartomas, dysplastic hamartomas and carcinomas. COX-2 expression was assessed semiquantitatively as negative, weak, moderate or strong. Stromal staining was only assessed in GI carcinomas.

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<th>weak (%)</th>
<th>moderate (%)</th>
<th>strong (%)</th>
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<td>12 (55)</td>
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<td>1 (9)</td>
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<td>3 (27)</td>
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<td>Carcinomas, n=11</td>
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<th>Stromal COX-2</th>
<th>negative (%)</th>
<th>weak (%)</th>
<th>moderate (%)</th>
<th>strong (%)</th>
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<td>Hamartomas, n=22</td>
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<td>6 (27)</td>
<td>5 (23)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Dysplastic hamartomas, n=2</td>
<td>5 (71)</td>
<td>1 (14)</td>
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<td>2 (100)</td>
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<td>GI-carcinomas, n=7</td>
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COX-2 expression:

Epithelial and stromal COX-2 expression were assessed semiquantitatively in hamartomas and carcinomas (Table 1). COX-2 expression was present in 10 out of 22 (45%) hamartomas without dysplasia. However, only 4 (18%) non-dysplastic hamartomas had focally moderate or strong COX-2 staining, which was diffusely observed in two hamartomas with dysplastic changes, and 7 out of 11 (64%) carcinomas (Table 1 and 3)(Figure 1A-D). In hamartomas without dysplastic changes, moderate or strong epithelial COX-2 expression was only found in colorectal and not in small intestinal cases, whereas a duodenal hamartoma with dysplasia and one out of three small intestinal carcinomas showed moderate or strong COX-2 staining. Stromal COX-2 expression was focally present beneath the luminal surface epithelium in 12 out of 22 (55%) hamartomas without dysplasia. Six of these (27%) showed moderate or strong staining. Both dysplastic hamartomas had strong stromal COX-2 expression (Figure 1C). In contrast, out of 7 GI-carcinomas one (14%) had weak and one (14%) had moderate stromal COX-2 staining.
Stromal COX-2 expression was not related to epithelial COX-2 expression. Also, COX-2 staining in hamartomas was not related to polyp size.

Figure 1. Immunohistochemical staining of COX-2, β-catenin and cyclin-D1 in hamartomas and carcinomas from PJS patients. A: Epithelial COX-2 expression in non-dysplastic hamartoma. B and C: Hamartoma with dysplasia (B, H&E staining), showing epithelial and stromal COX-2 expression (C). Frame in B covers high power field shown in C. D: COX-2 expression in a colon carcinoma. E and F: Nuclear accumulation of β-catenin and loss of membranous β-catenin in a hamartoma (E) and a gastric carcinoma (F). G and H: Nuclear over-expression of cyclin-D1 in a hamartoma (G) and a small intestinal carcinoma (H).

APC/β-catenin pathway:

Activation of the APC/β-catenin pathway (Wnt-pathway), reflected by nuclear accumulation of β-catenin (scored as ++ or +++), was found in 17% of hamartomas, focally clustered in areas of several crypts and in 71% of the GI-carcinomas (n=7)(Figure 1.E-F), but not in extra-intestinal carcinomas (Table 3). Nuclear overexpression of cyclin-D1, which may be a transcriptional target of the APC/β-catenin pathway, was found focally in 29% of hamartomas, and in 71% of GI-carcinomas (Figure 1.G-H). In hamartomas, there was no correlation between cyclin-D1 overexpression and nuclear β-catenin staining (P>0.05). Dysplasia was absent in crypts with nuclear β-catenin or cyclin-D1, which showed reactive changes. Positive staining did not relate to polyp size. Mutations in β-catenin were not found in hamartomas or carcinomas, nor in DNA from laser-assisted micro-dissected hamartomatous crypts from 5 different areas with nuclear β-catenin staining within 3 hamartomas. Mutations in the APC-MCR were only present in two carcinomas. Previously, DGGE suggested the presence of 4 APC mutations in PJS carcinomas 21. However, sequencing revealed two carcinomas with a frame-shift mutation in addition to two cases with a polymorphism at codon 1493 (ACG>ACA). LOH at the APC-locus 5q was not found.

Epithelial kinetics:

Most hamartomas retained the normal topographical relationship of Ki-67, restricted to the crypt base, and p21waf1/cip1, expressed at the upper parts of the crypts and epithelial surface (Figure 2.A-B). However, 4 hamartomas without dysplasia (20%) had abnormal co-expression of Ki-67 and p21waf1/cip1 at the surface and upper crypt epithelium (Figure 2.C-F). Altered expression was not related to polyp size. Re-examination of crypts with altered expression
patterns showed reactive changes (Figure 2.D). Superficial Ki-67 staining, intermingled with p21waf1/cip1 positive cells, was furthermore present in both hamartomas with dysplastic changes (Figure 2.G-I) and aberrant Ki-67 staining was present in all GI carcinomas. Only one carcinoma with certified adjacent hamartomatous epithelium was available, showing that Ki-67 staining sharply demarcated the transition between hamartomatous and dysplastic cells (Figure 2.J-L).

Figure 2: Immunohistochemical staining of p21 (A, F, I, L) and Ki67 (B, C, E, H, K) in normal mucosa, hamartomas, and carcinoma from PJ5 patients. A, B: p21 expression at the upper crypt and surface epithelium of normal colonic crypts (A), and Ki-67 positive cells restricted to the crypt base (B). C-F: hamartoma with focal disturbance of topographically controlled expression of Ki-67 and p21, with loss of mutuality. The frame in C marks fields presented in D-F. Ki-67 is restricted to the crypt base outside the frame (C). However focally there are crypts with superficial Ki-67 positive cells (E), which also express p21 (F). Those crypts show reactive changes (D). G-I: dysplastic crypts within a hamartoma (G, frame covers high power fields shown in H and I) with superficial Ki-67 positivity (H) and intermingled p21 expression (I). J-L: Dysplasia continues with infiltrating carcinoma (dys/ca) and adjacent hamartomatous crypts (Ha) (J, frame covers fields shown in K and L). Aberrant Ki-67 expression marks the transition to dysplasia (K); p21 is expressed in both components (L).

p53, K-RAS mutations and MSI:

Positive p53 staining, indicating the presence of mutant p53 protein, was not observed in hamartomas, but occurred in 4 out of 11 carcinomas, one of which showed LOH at the TP53-locus 17p. LOH at the STK11-locus 19p13.3 was (previously) detected in both hamartomas (32%) and carcinomas (73%). K-RAS codon 12 mutations were found in 2 carcinomas. One carcinoma was MSI-positive according to the Bethesda-criteria and showed loss of hMLH-1 staining, presumably due to somatic inactivation.
Carcinogenesis in Peutz-Jeghers Syndrome (2)

Table 2: Alterations in hamartomas with and without dysplastic changes and carcinomas from patients with Peutz-Jeghers syndrome. Immunohistochemical assessment of p53, the analysis of LOH at 19p, 5q and 17p, and K-RAS mutational analysis of hamartomas and 6 carcinomas was published previously.

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<tbody>
<tr>
<td></td>
<td>no dysplasia</td>
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<tr>
<td>Nuclear β-catenin (IHC)</td>
<td>4/22 (18%)</td>
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<tr>
<td>Cyclin-D1 (IHC)</td>
<td>7/22 (32%)</td>
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<tr>
<td>Loss of topographical control of Ki-67 and p21</td>
<td>4/20 (20%)</td>
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<td>p53 IHC</td>
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<tr>
<td>LOH 19p (STK11-locus)</td>
<td>7/22 (32%)</td>
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<td>LOH 5q (APC-locus)</td>
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<td>LOH 17p (TP53-locus)</td>
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<td>β-catenin mutation</td>
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<tr>
<td>MSI</td>
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1. Both hamartomas with dysplastic changes were fixed in Hollande's fixative, precluding the use of PCR-based assays, nt=not tested
2. Assessed in hamartomas and GI-carcinomas. No reliable staining in 2 hamartomas and 1 carcinoma presumably due to technical reasons

Discussion

PJS is a hereditary cancer syndrome, in which the pathogenesis is not well understood. As yet, a pre-malignant potential of hamartomas has not been established, and no treatment options are available, other than surveillance to detect carcinomas at an early stage. The objective of the present investigation was to evaluate the expression of COX-2 in PJS tumors as a potential target for chemopreventive treatment, and to further define a pre-malignant nature of hamartomatous polyps at the molecular level. Moderate or strong epithelial COX-2 expression was found in a 6 out of 24 hamartomas, including two hamartomas with dysplastic changes, and in 7 out of 11 carcinomas, providing a rationale for chemopreventive treatment with NSAIDs or COX-2 inhibitors. Furthermore, few molecular alterations associated with the adenoma-carcinoma sequence were found in hamartomas, although nuclear β-catenin, overexpression of cyclin-D1, and a disturbed topographical relationship between p21Waf1/cip1 and Ki-67 were focally present in a subset of cases. These alterations may reflect early progression of a hamartoma-adenoma-carcinoma sequence. However, no underlying genetic alteration was found.

Epidemiological studies have shown a protective effect of NSAIDs against colorectal adenocarcinoma, and to a lesser degree against cancer of the esophagus, stomach, breast, lung, prostate, urinary bladder and ovary. Also, the NSAID sulindac causes adenoma regression in patients with FAP. The main target for NSAIDs appears to be COX-2, which is overexpressed in many malignant and pre-malignant gastrointestinal tumors. The mechanism of COX-2
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Table 3: Results of immunohistochemistry (IHC) and the analysis of LOH and somatic mutations in Peutz-Jeghers carcinomas (n=11). Previously reported carcinomas are marked as * 26,21, ns = no reliable staining, ni = non-informative.

<table>
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<tr>
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<th>COX2 /mutation</th>
<th>β-catenin IHC</th>
<th>cyclin-D1 IHC</th>
<th>Loss of compartment p21 / KI-67</th>
<th>19p LOH</th>
<th>p53 IHC/17p LOH</th>
<th>5q LOH/APC mutation</th>
<th>K-ras mutation</th>
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Overexpression during carcinogenesis is unknown. Overexpression may lead to resistance against apoptosis, disturbed adhesion and increased angiogenesis 15,16,38. The role of COX-2 in colorectal carcinogenesis was established by the observation that polypt development was decreased in Apc<sup>6716</sup> mice crossbred with Cox-2 knockout mice 38,39. Also selective COX-2 inhibitors decrease the number of adenomas in patients with FAP and mouse models of FAP 39-41. Therefore, sulindac and COX-2 inhibitors are considered promising chemopreventive agents, although clinical use is still limited to patients with FAP 14. The high cancer risk in PJS, however, points to the need for chemopreventive strategies for those patients as well. Recently, Rossi et al. reported COX-2 overexpression in 16 of 23 (70%) PJS polypt 18. We found epithelial COX-2 expression in 10 out of 22 (45%) of non-dysplastic hamartomas, although moderate or strong expression was only found in 4 (18%) cases. However, the finding of moderate or strong COX-2 staining in 7 of 11 PJS carcinomas and in the two hamartomas with dysplastic changes particularly justifies further investigation of the chemopreventive value of NSAIDs and COX-2 inhibitors against intestinal and extra-intestinal carcinomas in PJS patients. The discrepancy in the number of hamartomas with COX-2 up-regulation in our study compared to the report of Rossi et al. may relate to demographic differences, subtle differences in fixation or staining protocols, or to the site of hamartomas, since we only found COX-2 overexpression in colorectal hamartomas. In adenomas, COX-2 expression has been described in epithelial cells, and in stromal cells. Stromal COX-2 expression may be found in activated macrophages 17 or endothelial and stromal cells 42. Stromal COX-2 expression was present in 55% of PJS hamartomas, corresponding to colorectal adenomas 17,42, whereas it was only found in two PJS-carcinomas. Stromal COX-2 expression may be induced by a local inflammatory response, triggered by tumor cells or mechanical stress in protruding tumors. A role in angiogenesis has been proposed 38.
Molecular studies may help to define the pre-malignant potential of PJS hamartomas. LOH of the STK11 locus 19p13.3 was previously reported in ~40% of PJS hamartomas.20-22 Few studies addressed additional alterations in hamartomas. Miyaki et al. found β-catenin mutations in 19% of hamartomas. He noted that mutations were related to large polyp size, and restricted to a subset of cells within a hamartoma, suggesting a role for β-catenin in progression of the hamartoma-adenoma-carcinoma sequence. The present study failed to detect mutations in β-catenin in hamartomas or carcinomas from PJS patients. Still, nuclear β-catenin was found in hamartomas and GI-carcinomas from PJS patients, suggesting activation of the APC/β-catenin pathway. The lack of β-catenin mutations in our study may reflect activation of this pathway by other mechanisms, such as inactivation of APC or mutations in other genes involved, such as AXIN1.43 We did not find LOH of the APC-locus in hamartomas nor carcinomas, and a mutation in the APC-MCR was only present in two carcinomas from PJS patients. However, this finding does not exclude inactivation of APC by mutations outside the MCR or by CpG-island hypermethylation of the promotor region, as has been described in a PJS tumor by Esteller et al.44.

In the absence of underlying genetic alterations, the significance of focal immunohistochemical changes associated with the adenoma-carcinoma sequence in hamartomas remains uncertain. It is noteworthy that nuclear β-catenin was not associated with focal expression of cyclin-D1 or disturbed topographical control of p21waf1/cip1. Furthermore, crypts staining positive with one of the immunohistochemical markers remained negative for dysplasia after re-examination of H&E stained slides, but showed reactive changes, possibly explaining alterations in cell cycle regulating markers. Finally, polyp size did not correlate to positive staining of any of the immunohistochemical markers described in our study. Taken together, the present study could not define a neoplastic potential of hamartomas at the molecular level, besides the previously reported finding of LOH at the STK11 locus 19p13.3 in a subset of hamartomas. The grossly normal topographical control of the expression of p21waf1/cip1 and Ki-67 is suggestive of a benign growth pattern.12 However, the finding of β-catenin mutations in large hamartomas by Miyaki et al.22 could represent a changed growth pattern in such lesions. Most hamartomas in PJS patients occur in the small intestine,6,45 whereas carcinomas are more often found in the colon,7 suggesting that colorectal hamartomas carry more potential for progression than small intestinal hamartomas. We did not find molecular support for such a difference, although COX-2 overexpression was only found in colorectal hamartomas. Malignant transformation of hamartomas has been described.6,8,46 One carcinoma with certain adjacent hamartomatous epithelium was available for our study. In this case, aberrant Ki-67 staining sharply demarcated the transition to dysplasia. Also, Ki-67 positive cells were found at the surface and upper crypt epithelium in two hamartomas with adenomatous dysplastic changes. Thus, the localization of Ki-67 expression could be useful as marker to discriminate between dysplastic and hamartomatous crypts.

In conclusion, we show COX-2 expression in PJS tumors, pointing to the potential value of chemopreventive treatment with NSAIDs or COX-2 inhibitors against PJS carcinomas. The molecular alterations in hamartomas and PJS carcinomas appear distinct from the adenoma-carcinoma sequence. In particular, alterations related to dysplastic growth were only focally present, and found in a minority of the hamartomas. The significance of these changes remains
uncertain, and further studies are needed to define the neoplastic potential of hamartomas at the molecular level.

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References

Carcinogenesis in Peutz-Jeghers Syndrome (2)


