Cyclooxygenase 2 Expression and Molecular Alterations in Peutz-Jeghers Hamartomas and Carcinomas

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ABSTRACT

Purpose: Peutz-Jeghers syndrome (PJS) is a hamartomatous polyposis disorder with a high cancer risk. Debate exists about the premalignant potential of hamartomas. Also, treatment options other than surveillance are not available. Therefore, molecular alterations in hamartomas and PJS carcinomas were studied. The objective was (a) to evaluate expression of cyclooxygenase (COX)-2 as target for chemopreventive treatment and (b) to define the neoplastic potential of hamartomas at the molecular level.

Experimental Design: 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, p21waft/cip1, Ki-67, and p53. DNA was studied for loss of heterozygosity (LOH) at 19p (STK11), 5q (APC), and 17p (TP53); mutations in β-catenin, APC, and K-RAS; and microsatellite instability.

Results: 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 p21waft/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 four (36%) carcinomas, but LOH at 5q was not found. Two carcinomas had K-RAS mutations, and one carcinoma had microsatellite instability.

Conclusions: The presence of COX-2 expression in PJS carcinomas and dysplastic hamartomas provides a rationale for chemoprevention with nonsteroidal anti-inflammatory drugs or COX-2 inhibitors. Focal immunohistochemical changes, which may indicate a premalignant potential, were present in some nondysplastic PJS hamartomas. Molecular changes in carcinomas and dysplastic hamartomas in PJS are distinct from the usual adenoma-carcinoma sequence.

INTRODUCTION

PJS is a rare autosomal dominant disorder, caused by a germ-line mutation in the STK11/LKB1 gene on chromosome 19p13.3 (1, 2). PJS is characterized by hamartomatous polyps throughout the GI tract and melanin pigmentation of the skin and mucous membranes (3–6). PJS patients have an increased risk of cancer at a relatively young age (7). Malignancies occur in the GI tract, pancreas, biliary tree, and a variety of extra-GI 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 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, among which is cyclin D1 (11). Oncogenic activation of this pathway is considered necessary for the initiation of colorectal dysplasia. At the

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3 The abbreviations used are: PJS, Peutz-Jeghers syndrome; CRC, colorectal carcinoma; COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; MSI, microsatellite instability; GI, gastrointestinal; LOH, loss of heterozygosity; DGGE, denaturing gradient gel electrophoresis; MCR, mutation cluster region; FAP, familial adenomatous polyposis.
COX-2 Expression in Peutz-Jeghers Tumors

STK11/LKB1 locus), β-catenin (CTNNB1), and K-APC carcinogenesis (20, 21). Miyaki et al. (22) reported β-catenin mutations in large hamartomas, implicating a possible neoplastic potential of these lesions. The present study further explores the molecular pathogenesis of PJS hamartomas and carcinomas, suggesting an alternative pathway of carcinogenesis (20, 21). Importantly, the adenoma-carcinoma sequence can be inhibited or reversed by NSAIDs and selective COX-2 inhibitors, which are considered promising chemopreventive agents (13, 14). The main target of these drugs is COX-2, which is involved in the conversion of arachidonic acid into prostaglandins, which interfere with processes such as apoptosis and angiogenesis (15, 16). 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 (17). Recently, COX-2 expression was also reported in hamartomas from PJS patients and Stk11/Lkb1 heterozygous mice, which provide a murine model for PJS (18). Whether COX-2 expression occurs in carcinomas has not been evaluated.

Insights into the molecular pathogenesis of PJS may help to define the neoplastic potential of hamartomas and guide chemopreventive treatment strategies. Previous studies reported LOH at the STK11/LKB1 locus 19p13.3 in hamartomas and carcinomas from Peutz-Jeghers patients (19–23). Also, somatic mutations in STK11/LKB1 were found in hamartomas without LOH at 19p13.3 (22). However, alterations in APC, K-RAS, and TP53 were relatively rare when compared with the adenoma-carcinoma sequence, suggesting an alternative pathway of carcinogenesis (20, 21). Miyaki et al. (22) reported β-catenin mutations in large hamartomas, implicating a possible neoplastic potential of these lesions. The present study further explores the molecular pathogenesis of PJS hamartomas and carcinomas, extending our previous work (20, 21). Expression of COX-2, a possible therapeutic target, β-catenin, cyclin D1, p21(waf1/cip1), Ki-67, 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 PJS patients from 15 different families. The diagnosis of PJS was confirmed by histological examination of hamartomas by an experienced pathologist (G. J. A. O.). Mutational analysis revealed STK11/LKB1 germ-line mutations in 16 patients from 12 families, most of which have been published previously (20, 24, 25). Twenty-four hamartomas (stomach, n = 1; small bowel, n = 16; colorectal, n = 7), including 2 hamartomas with adenomatous (dysplastic) changes, and 11 carcinomas (Table 3) were available for study. Samples were formalin fixed and paraffin embedded, except for the hamartomas with adenomatous changes (n = 2), which were fixed in Holland’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 briefly 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 inflammatory and stromal cells. DNA was isolated from microdissected tissue with the Puregene DNA purification system (Genuta 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; 1:100 dilution), β-catenin (clone 14; Transduction Laboratories, Lexington, KY; 1:1000 dilution), cyclin D1 (ab-1; Neomarkers, Union City, CA; 1:1000 dilution), p21(waf1) (ab-1; Oncogene Research Products, San Diego, CA; 1:25 dilution), Ki-67 (MIB-1; Dako, Glostrup, Denmark; 1:200 dilution), and p53 (DO7; Dako; 1:200 dilution). A known p53-positive CRC 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 Ki-67. To assess the specificity of the COX-2 antibody, a subset of adenomas was stained with the primary antibody after preadsorption of a human COX-2 control peptide (Cayman Chemical) for 1 h at room temperature. This resulted in blocking of COX-2 staining of samples fixed in formalin or Holland’s fixative. Staining of normal mucosa served as control for the specificity of immunohistochemistry in Holland’s fixed samples; the staining pattern appeared reliable for all antibodies tested.

Slides were scored by an experienced GI pathologist (G. J. A. O.). Epithelial COX-2 staining and staining of stromal cells underlying the epithelium were assessed separately in a semiquantitative manner on a scale ranging from 0 to +++ (0, no expression; +, weak staining; ++, moderate staining; ++++, strong staining). Nuclear accumulation of β-catenin was scored semiquantitatively, using a scale ranging from 0 to +++ (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 staining and cyclin D1 staining were considered positive if >10% of nuclei stained positive (27, 28). The compartmentalization of p21(waf1/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 evaluated previously for LOH at 19p13.3 (STK11/LKB1), 17p (TP53), and 5q21 (APC). The remaining carcinomas were investigated for LOH as described previously (26), using the following markers (for primer sequences, see The Genome Database4): D19S886, D19S565, D19S883, and D19S814 at 19p13.3; D5S346, D5S107, and D5S82 at 5q21; and p53alu, TP53, and D17S796 at 17p. Hamartomas and carcinomas were screened for MSI using the BAT26 marker. PCR

4 www.gdb.org.
products were analyzed using an automated ABI377 sequencer and Genescan 2.1 software (PE Biosystems, Foster City, CA). If MSI was found with the BAT26 marker or a shift was noted with any of the other markers tested for LOH, MSI analysis was performed according to the Bethesda criteria (29), and immuno-histochemical staining for hMLH1, hMSH2 and hMSH6 was performed as described previously (30).

**Analysis of Mutations in APC, β-Catenin (CTNNB1), and K-RAS.** DNA from hamartomas was screened for mutations in the APC MCR (31) with DGGE (32). 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'GAAATAGGATGTAATACGACGAA-3' and 5'CGCTCTCFAAATTTACTTCT-3' for fragment I; 5'-ACTGAGGTCTCAGGT'TTAT-3' and 5'-GACATGGCAATCGAGACACT-3' for fragment II; 5'-TACTCTGTCACGATCTTATGAT-3' and 5'-ATTITTAAGTA-CTTCTGCCTTG-3' for fragment III; and 5'-AAACACCTCACCACCTC-3' and 5'-GACATTATTTAATCCACACT-3' for fragment IV (33). PCR reactions were performed in a final volume of 20 μl with 1.5 units of Taq polymerase and 2.5 mM MgCl₂. 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 h at 150 V 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).

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 (34). Sequencing was performed as described above.

K-RAS mutations in hamartomas and six PJS carcinomas were reported previously. A modified method was used for K-RAS mutation analysis in the remaining carcinomas. DNA was amplified with primer A (5'-TCAAGAATATAAACTTGTTTGAAGATAACCT-C3') and primer D (5'-CGAAATATATGGTTCGAGAAAAC-3'). First-round PCR products were digested with BsrNI (New England Biolabs, Beverly, MA) at 60°C for 1 h. Digested (mutant-enriched) DNA was amplified in a second-round PCR using primer B (5'-TCAAGAATATGGTTGACCT-GA-3') and a modified primer A, to which a 5'-CTCGCAACT-GAATTATAAACCTTGTG-3' and primer B.

Mutations were always confirmed by a second experiment with DNA from a separate PCR as template.

**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 GI 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 p21⁰⁰⁰ stopwords were assessed. LOH analysis was performed for the STKI1, 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.

**COX-2 Expression.** Epithelial and stromal COX-2 expression was assessed semiquantitatively in hamartomas and carcinomas (Table 1). COX-2 expression was present in 10 of 22 (45%) hamartomas without dysplasia. However, only 4 (18%) nondysplastic hamartomas had focally moderate or strong COX-2 staining, which was diffusely observed in two hamartomas with dysplastic changes, and 7 of 11 (64%) carcinomas (Tables 1 and 3; Fig. 1, A–D). In hamartomas without dysplastic changes, moderate or strong epithelial COX-2 expression was only found in colorectal cases and not in small intestinal cases, whereas a duodenal hamartoma with dysplasia and one 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 of 22 (55%) hamartomas without dysplasia. Six of these (27%) showed moderate or strong staining. Both dysplastic hamartomas had strong stromal COX-2 expression (Fig. 1C). In contrast, one of three GI carcinomas, one (14%) had weak stromal COX-2 staining, 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.

**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; Fig. 1, E and F), but not in extraintestinal 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 (Fig. 1, G
Table 2: Alterations in hamartomas with and without dysplastic changes and carcinomas from patients with PJS

Immunohistochemical assessment of p53; the analysis of LOH at 19p, 5q, and 17p; and K-RAS mutational analysis of hamartomas and six carcinomas were published previously (20, 21).

<table>
<thead>
<tr>
<th></th>
<th>Hamartomas, no dysplasia n = 22 (%)</th>
<th>Hamartomas dysplasia n = 2 (%)</th>
<th>Carcinomas(^a) n = 11 (%)</th>
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</thead>
<tbody>
<tr>
<td>Nuclear β-catenin (IHC)(^b)</td>
<td>4/22 (18%)</td>
<td>0/2</td>
<td>5/11 (45%)</td>
</tr>
<tr>
<td>Cyclin D1 (IHC)</td>
<td>7/22 (32%)</td>
<td>0/2</td>
<td>6/11 (55%)</td>
</tr>
<tr>
<td>Loss of topographical control of Ki-67 and p21(^c)</td>
<td>4/20 (20%)</td>
<td>2/2 (100%)</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>p53 IHC</td>
<td>0/22</td>
<td>0/2</td>
<td>4/11 (36%)</td>
</tr>
<tr>
<td>LOH 19p (STK11 locus)</td>
<td>7/22 (32%)</td>
<td>nt</td>
<td>8/11 (73%)</td>
</tr>
<tr>
<td>LOH 5q (APC locus)</td>
<td>0/22</td>
<td>nt</td>
<td>0/11</td>
</tr>
<tr>
<td>LOH 17p (TP53 locus)</td>
<td>0/22</td>
<td>nt</td>
<td>1/10 (10%)</td>
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<tr>
<td>β-Catenin mutation</td>
<td>0/22</td>
<td>nt</td>
<td>0/11</td>
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<tr>
<td>APC mutation</td>
<td>0/22</td>
<td>nt</td>
<td>2/11 (18%)</td>
</tr>
<tr>
<td>K-RAS mutations</td>
<td>0/22</td>
<td>nt</td>
<td>2/11 (18%)</td>
</tr>
<tr>
<td>MSI</td>
<td>0/22</td>
<td>nt</td>
<td>1/11 (9%)</td>
</tr>
</tbody>
</table>

\(^a\) Both hamartomas with dysplastic changes were fixed in Hollande’s fixative, precluding the use of PCR-based assays; nt, not tested.

\(^b\) See also table 3.

\(^c\) IHC, immunohistochemistry.

Assessed in hamartomas and GI carcinomas. There was no reliable Ki-67 staining in two hamartomas and one carcinoma, presumably due to technical reasons.

<table>
<thead>
<tr>
<th>Site</th>
<th>COX-2 IHC</th>
<th>β-Catenin IHC/mutations</th>
<th>Cyclin D1 IHC</th>
<th>Loss of compar.</th>
<th>p53 IHC/17p LOH</th>
<th>5q LOH/ APC mutations</th>
<th>K-RAS mutations</th>
<th>MSI</th>
</tr>
</thead>
<tbody>
<tr>
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<td>+/−</td>
<td>+</td>
<td>+</td>
<td>−/−</td>
<td>−/−</td>
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<td>+/−</td>
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<td>+</td>
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<td>Small bowel(^*)</td>
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<td>+/−</td>
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<tr>
<td>Small bowel</td>
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<td>+</td>
<td>+/−</td>
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<td>−</td>
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</table>

\(^*\) Assessed in hamartomas and GI carcinomas. Previously reported carcinomas (20, 21) are marked as *, ns = no reliable staining, ni = noninformative.

Table 3: Results of immunohistochemistry (IHC) and the analysis of LOH and somatic mutations in Peutz-Jeghers carcinomas (n = 11)

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 or in DNA from laser-assisted microdissected hamartomatous crypts from five different areas with nuclear β-catenin staining within three hamartomas. Mutations in the APC MCR were only present in two carcinomas. Previously, DGGE suggested the presence of four APC mutations in PJS carcinomas (21). However, sequencing revealed two carcinomas with a frameshift 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 p21\(^{wat/cp1}\), expressed at the upper parts of the crypts and epithelial surface (Fig. 2, A and B). However, four hamartomas without dysplasia (20%) had abnormal co-expression of Ki-67 and p21\(^{wat/cp1}\) at the surface and upper crypt epithelium (Fig. 2, C–F). Altered expression was not related to polyp size. Re-examination of crypts with altered expression patterns showed reactive changes (Fig. 2D). Furthermore, superficial Ki-67 staining, intermingled with p21\(^{wat/cp1}\)-positive cells, was present in both hamartomas with dysplastic changes (Fig. 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 (Fig. 2, J–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 of 11 carcinomas, 1 of which showed LOH at the TP53 locus 17p. LOH at the STK11 locus 19p13.3 was detected in both hamartomas (32%) and carcinomas (73%). K-RAS codon 12 mutations were found in two carcinomas. One carcinoma was MSI positive according to the Bethesda criteria (29) and showed loss of hMLH1 staining, presumably due to somatic inactivation.
DISCUSSION

PJS is a hereditary cancer syndrome, in which the pathogenesis is not well understood. To date, a premalignant 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 premalignant nature of hamartomatous polyps at the molecular level. Moderate or strong epithelial COX-2 expression was found in 6 of 24 hamartomas, including 2 hamartomas with dysplastic changes, and in 7 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 p21wat/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 (35). Also, the NSAID sulindac causes adenoma regression in patients with FAP (36). The main target for NSAIDs appears to be COX-2, which is overexpressed in many malignant and premalignant GI tumors (37). The mechanism of COX-2 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 polyg development was decreased in ApcΔ716 mice cross-bred 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. (18) reported COX-2 overexpression in 16 of 23 (70%) PJS polyps. We found epithelial COX-2 expression in 10 of 22 (45%) nondysplastic 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 2 hamartomas with dysplastic changes particularly justifies further investigation of the chemopreventive value of NSAIDs and COX-2 inhibitors against intestinal and extraintestinal carcinomas in PJS patients. The discrepancy in the number of hamartomas with COX-2 up-regulation in our study compared with the report of Rossi et al. (18) may relate to demographic differences, subtle differences in fixation or staining protocols, or the site of hamartomas, because 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 premalignant potential of PJS hamartomas. LOH of the STK11 locus 19p13.3 was reported previously in ~40% of PJS hamartomas (20–22). Few studies have addressed additional alterations in hamartomas. Miyaki et al. (22) found β-catenin mutations in 19% of hamartomas. They noted that mutations were related to large polyg 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 or 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 promoter 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 p21wd1/cip1. Furthermore, crypts staining positive with one of the immunohistochemical markers remained negative for dysplasia after re-examination of H&E-stained slides.

Fig. 2 Immunohistochemical staining of p21 (A, F, I, and L) and Ki-67 (B, C, H, and K) in normal mucosa, hamartomas, and a carcinoma from PJS patients. A and B, p21 expression at the upper crypt and surface epithelium of normal colonic crypts (A); Ki-67-positive cells are restricted to the proliferative zone at the crypt base (B). C–F, hamartoma with focal disturbance of the topographically controlled expression of Ki-67 and p21, with loss of mutuality. The frame in C marks the high-power fields presented in D–F. Ki-67 is restricted to the proliferative crypt epithelium 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 (the frame in G covers high-power fields shown in H and I) showing superficial Ki-67 positivity (H) intermingled with p21 expression (I). J–L, dysplasia continues with infiltrating carcinoma (Dys/ca) and adjacent hamartomatous crypts (HA; frame in J covers high-power fields shown in K and L). Aberrant Ki-67 expression marks the transition from hamartomatous to dysplastic crypts (K), whereas p21 is expressed in both components (L).
but they 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 p21\textsuperscript{wildtype} and Ki-67 is suggestive of a benign growth pattern (12). However, the finding of \(\beta\)-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 previously (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 a 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 were found in a minority of the hamartomas. The significance of these changes remains uncertain, and additional studies are needed to define the neoplastic potential of hamartomas at the molecular level.

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