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Genetic Alterations and Epithelial Dysplasia in Juvenile Polyposis Syndrome and Sporadic Juvenile Polyps

Tsung-Teh Wu,* Banafsheh Rezai,* Asif Rashid,* Michael C. Luce,† Matt C. Cayouette,† Christopher Kim,‡ Nirmal Sani,‡ Lopa Mishra,‡ Christopher A. Moskaluk,* John H. Yardley,* and Stanley R. Hamilton*§

From the Department of Pathology and Oncology Center,§ The Johns Hopkins University School of Medicine and Hospital, Baltimore, Maryland; LabCorp,† Research Triangle Park, North Carolina; and the Department of Veteran Affairs and Georgetown University,‡ Washington DC*

Juvenile polyps are regarded as hamartomatous polyps and occur in sporadic and familial syndromic settings. There is increased risk of gastrointestinal neoplasia in patients with juvenile polyposis syndrome, but the molecular mechanisms are not known. We therefore studied 78 colorectal juvenile polyps from 12 patients with juvenile polyposis syndrome and 34 sporadic juvenile polyps for epithelial dysplasia and genetic changes associated with colorectal neoplasia. Dysplasia occurred in 31% of syndromic juvenile polyps but not in sporadic juvenile polyps (P < 0.0001). Topographic control of proliferation and expression of the cyclin-dependent kinase inhibitor p21(WAF1/CIP1) seen in native colorectal epithelium was lost in 79% of dysplastic juvenile polyps and in 8% of nondysplastic juvenile polyps (P < 0.000001). Somatic mutations in the adenomatous polyposis coli (APC) gene were demonstrated in 50% of dysplastic juvenile polyps (3 of 6) but not in any of 16 juvenile polyps without dysplasia (P = 0.01). Both sporadic and syndromic juvenile polyps had K-ras mutations (14%) and there was no relationship to dysplasia. p53 gene product overexpression identified by immunohistochemical staining occurred rarely in dysplastic juvenile polyps (2 of 24, 8%). Our results indicate that the multiple genetic alterations involved in usual colorectal neoplasia also play a role in neoplastic transformation of juvenile polyps, predominantly in juvenile polyposis syndrome. (Am J Pathol 1997, 150:939–947)

Juvenile polyps (JPs) are regarded as hamartomatous polyps and commonly occur in the colon and rectum, predominantly of young people. JPs usually occur sporadically but can be familial in juvenile polyposis syndrome (JPS), which is transmitted as an autosomal dominant trait.[1] Dysplastic (adenomatous) change has been reported in up to 9% of syndromic JPs[2–10] and occasionally in sporadic JPs.[8,11–13] There is also increased risk of colorectal adenocarcinoma[2,10,14–19] and gastric carcinoma[20] in JPS, with gastrointestinal carcinoma reported in up to 17% of patients.[21]

In colorectal tumorigenesis, the adenoma-adenocarcinoma sequence and its associated accumulation of multiple genetic alterations involving the *APC* (adenomatous polyposis coli), K-*ras*, *p53*, and *DCC* (deleted in colorectal carcinoma) genes are well established.[22–24] *APC* and K-*ras* gene mutations occur early during colorectal tumorigenesis and can be identified in 50 to 60% of large colorectal adenomas.[25–28] Studies of aberrant crypt foci, the earliest identified lesions in colorectal neoplasia, suggest *APC* mutation is the initial event in the development of dysplasia.[29] Deletion of the long arm of chromosome 18, where *DCC* is located, and *p53* gene mu-

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Address reprint requests to Dr. Stanley R. Hamilton, Division of Gastrointestinal/Liver Pathology, Department of Pathology, Ross Research Building Rm 632, The Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, MD 21205-2196.

Table 1. *Demographic Summary of Patients with Juvenile Polyposis Syndrome and Sporadic Juvenile Polyps*

	Age (years)		Sex	
	Mean ± SD	Range	Female	Male
JPS (n = 12)	18 ± 11	7–50	10	2
Sporadic JPs (n = 33)	10 ± 9*	2–45	10†	23

*P = 0.08 *versus* JPS.
†P = 0.02 *versus* JPS.

tations usually occur at a later stage and can be demonstrated in 70 to 75% of colorectal carcinomas but much less frequently in adenomas.[22,30]

These genetic alterations are also closely linked to cell cycle control. The cyclin-dependent kinase inhibitor p21$^{(WAF1/CIP1)}$ is regulated by p53[31] and complexes with proliferating cell nuclear antigen.[32,33] In normal colorectal mucosa, p21$^{(WAF1/CIP1)}$ expression is confined to the epithelium in the nonreplicative upper crypts and luminal surface, and it is not expressed in the proliferative lower crypt epithelium.[34] Recently, this normal topographical relationship between proliferation and p21$^{(WAF1/CIP1)}$ expression was found to be lost in colorectal adenomas and dysplastic aberrant crypt foci.[34,35]

The association between JPS, dysplasia, and adenocarcinoma is clear, but the molecular mechanisms have not been studied previously. We therefore evaluated in syndromic and sporadic JPs the topographic profile of Ki-67 and p21$^{(WAF1/CIP1)}$, mutations in the *APC* and K-*ras* genes, and *p53* gene product overexpression, which is commonly associated with *p53* gene mutations.[36–39] We relate the findings to the pathogenesis of dysplasia and the clinical utility of the alterations as markers for neoplasia.

Materials and Methods

Patient Population and Specimens

A total of 78 colorectal JPs from 12 patients with JPS and 34 sporadic JPs from 33 patients were included in this study. Patients with JPS were identified based on either family history of at least one first-degree relative who also had a JP and/or the presence of 3 or more colorectal JPs in a patient, as in our previous studies.[2] The specimens were identified in the surgical pathology files of The Johns Hopkins Hospital and/or the tumor bank of the Bowel Tumor Working Group of The Johns Hopkins University School of Medicine from 1986 through 1995. Demographic data are summarized in Table 1. The patients with JPS tended to be older (P = 0.08) and were more frequently female (P = 0.02).

Grading of Epithelial Dysplasia

Coded slides were evaluated independently by five gastrointestinal pathologists (T. T. Wu, A. Rashid, C. A. Moskaluk, J. H. Yardley, and S. R. Hamilton). Each JP was classified as negative, indefinite, or positive for dysplasia (low grade or high grade) with the criteria of idiopathic inflammatory bowel disease.[40] When analyzed by calculation of Kappa statistics, the inter-observer agreement on classification of dysplasia was moderate (Kappa statistic, 0.33 ± 0.02; Kendall's coefficient of concordance, 0.68; $P <$ 0.000001). Agreement was strongest for negative for dysplasia (Kappa, 0.44 ± 0.03) and positive for dysplasia (low grade, 0.45 ± 0.03; high grade, 0.42 ± 0.03) but weakest for indefinite dysplasia (Kappa, 0.08 ± 0.03). The majority grade was used for comparison of dysplasia with genetic alterations.

Immunohistochemical Stains

Immunoperoxidase staining using diaminobenzidine as chromogen was performed with the Techmate 1000 automatic staining system (BioTek Solutions, Tucson, AZ). Ki-67 proliferating cell marker (mouse monoclonal antibody MIB-1, Immunotech, Westbrook, ME) at 1:150 dilution, p21$^{(WAF1/CIP1)}$ cyclin-dependent kinase inhibitor protein (mouse monoclonal antibody WAF-1, Oncogene Research Products, Cambridge, MA) at 1:25 dilution, and p53 suppressor gene product (mouse monoclonal antibody DO7, Dako, Nutley, NJ) at 1:100 dilution were stained in deparaffinized serial sections of formalin-fixed tissue after antigen retrieval using a heat-induced epitope retrieval method.[41]

Abnormal coexpression of Ki-67 and p21$^{(WAF1/CIP1)}$ was judged by expression of nuclear Ki-67 staining and p21$^{(WAF1/CIP1)}$ staining in the same areas of superficial epithelium and upper glands of serial stained sections. For p53 immunohistochemistry, the criterion for diffuse nuclear p53 staining was the presence of positive staining in more than 50% of epithelial nuclei. Focal nuclear p53 staining represented clustering of positive staining in less than 5% of epithelial nuclei.

Table 2. *Classification of Dysplasia, Immunohistochemistry, and Mutations in Juvenile Polyps from Juvenile Polyposis Syndrome*

Alteration	Percentage with alteration (%)		
	Negative* (n = 40)	Indefinite* (n = 14)	Positive* (n = 24)
Loss of normal topographical relationship of Ki-67 and p21	5	29	79†
Truncating *APC* mutation (segments 2 and 3)‡	0	0	50
K-*ras* mutation (codons 12 and 13)	9§	0	25†
Focal nuclear p53 staining	0	7	50
Diffuse nuclear p53 staining	0	0	8

*Classification of dysplasia. In the positive category, there were 23 with low-grade dysplasia and 1 with high-grade dysplasia.
†Includes 1 JP with high-grade dysplasia.
‡n = 19 for *APC* gene mutation analysis; 10 negative for dysplasia, 3 indefinite for dysplasia, and 6 positive for dysplasia.
§n = 32 for K-*ras* mutation analysis.

There were no JPs that had 5 to 50% of epithelial nuclei with p53 staining.

In Vitro *Synthesized-Protein (IVSP) Assay for* APC *Gene Mutation*

Frozen tissue was available from 22 JPs (19 from 12 patients with JPS and 3 sporadic JPs). The presence of JP and grade of dysplasia were evaluated with hematoxylin and eosin (H&E)-stained sections flanking the sections for DNA extraction. Mutations of the *APC* gene in two overlapping segments (segments 2 and 3) between codons 686 and 1693 were analyzed by IVSP assay.[25,42] In brief, *APC* gene segment 2 (codons 686 to 1217) and segment 3 (codons 1099 to 1693) were amplified from DNA by polymerase chain reaction (PCR) using primers as previously described.[29] The PCR products were used directly without purification as templates in a coupled transcription-translation assay with 1.75 μl of Express ^{35}S protein labeling mix (New England Nuclear, Boston, MA). The translation products were analyzed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein bands were visualized by fluorography after impregnation with Entensify (New England Nuclear). This assay detects 41 to 52% of germline *APC* mutations identified by IVSP assay of segments 1 to 5 (M. Luce, personal observation).[25]

K-ras *Mutation Assay*

DNA from epithelium of 104 JPs was prepared from eosin-stained tissue sections of microdissected routine formalin-fixed, paraffin-embedded specimens. The first exon of K-*ras* was amplified and used as the template for three separate allele-specific ligation assays[43] for all possible mutations of the first and second nucleotides of codon 12 and the second nucleotide of codon 13. The oligonucleotides used were as previously described.[29] For each ligation assay, 2 μl of PCR product (one-twenty-fifth of a 50-μl PCR reaction volume) was used in the reaction conditions described previously.[29] Templates of known K-*ras* mutations were used as positive controls.

Statistical Analysis

Differences in prevalence were evaluated by χ^2 test and differences in means by two-tailed *t*-test.

Results

Epithelial dysplasia (23 low grade and 1 high grade; Figure 1) occurred in 31% of JPS polyps from 4 of 12 patients (Table 2) but not in any sporadic JPs (Table 3; $P < 0.0001$). By contrast, the frequency of JPs graded as indefinite for dysplasia tended to be higher in sporadic JPs, although the difference was not statistically significant ($P = 0.08$)

Most JPs without dysplasia retained the normal topographic relationship between proliferation as indicated by Ki-67 expression and p21$^{(WAF1/CIP1)}$ expression (89% of JPS and 97% of sporadic JPs; Figure 1 and Tables 2 and 3). By contrast, abnormal coexpression of Ki-67 and p21$^{(WAF1/CIP1)}$ in surface and upper glandular epithelium occurred in 32% of JPs from JPS and 3% of sporadic JPs ($P < 0.002$). There was a strong correlation between abnormal coexpression and dysplasia; 79% of JPs with dysplasia had abnormal expression, but only 8% of non-dysplastic JPs (19% of JPs indefinite for dysplasia and 3% of JPs negative for dysplasia; $P < 0.000001$).

Among 6 JPs with low-grade dysplasia in H&E-stained frozen sections from four patients with JPS, 3 polyps from two patients had mutations identified in the *APC* gene (2 in segment 2 and 1 in segment 3) by

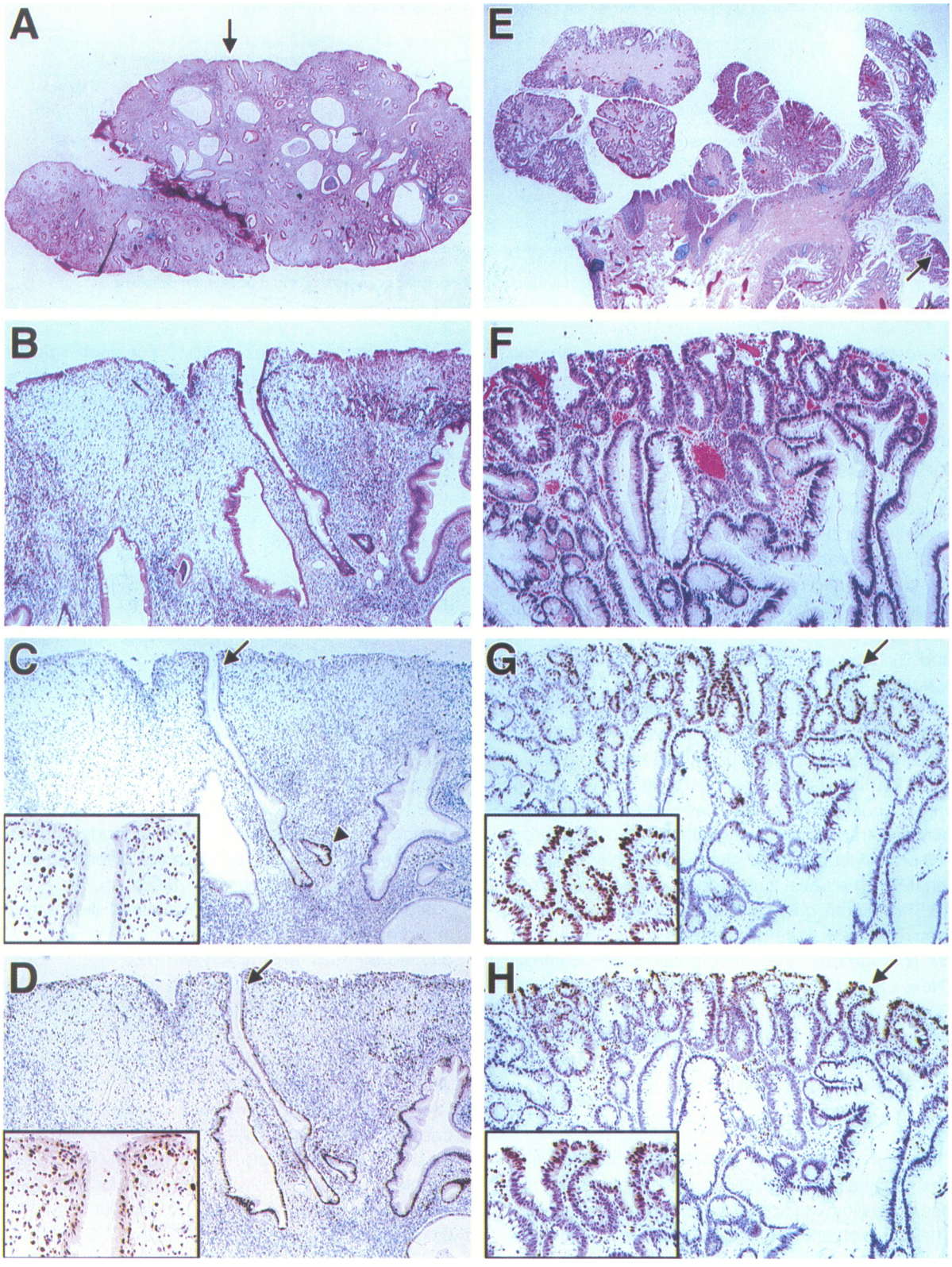

Figure 1. *Topographic control of Ki-67 and p21 (WAF1/CIP1) in juvenile polyps.* A *and* B: *Histopathology of classic sporadic juvenile polyp negative for dysplasia. Typical dilated crypts, inflamed granulation tissue, surface erosions, and reactive epithelial change are present. The* arrow *in* A *indicates area shown in* B *to* D. C: *Immunohistochemical stain for Ki-67. Staining of scattered epithelial nuclei in the superficial glands* (arrow *and* inset) *indicates low proliferation rate with more frequent staining in deeper glands* (arrowhead). D: *Immunohistochemical stain for p21. In comparison with* C, *more diffuse nuclear staining is evident in superficial areas* (arrow *and* inset), *which lacked Ki-67 staining, indicating normal topographical*

Table 3. *Classification of Dysplasia, Immunohistochemistry, and Mutations in Sporadic Juvenile Polyps*

Alteration	Percentage with alteration (%)		
	Negative* (n = 22)	Indefinite* (n = 12)	Positive* (n = 0)
Loss of normal topographical relationship of Ki-67 and p21	0	8	NA
Truncating *APC* mutation (segments 2 and 3)†	0	0	NA
K-*ras* mutation (codons 12 and 13)	18	25	NA
Focal nuclear p53 statining	9	33	NA
Diffuse nuclear p53 staining	0	0	NA

*Classification of dysplasia.
†n = 3 for *APC* gene mutation analysis; 2 negative for dysplasia and 1 indefinite for dysplasia.

IVSP assay (Figure 2 and Tables 2 and 3). By contrast, no *APC* gene mutations were present in 4 JPs that were indefinite for dysplasia or 12 negative for dysplasia ($P = 0.01$).

K-*ras* mutation (Figure 3) was identified in 11% of JPs from JPS and 21% of sporadic JPs (*P* value was nonsignificant). There was no statistical association between epithelial dysplasia and the presence of K-*ras* mutation, which was found in 25% of JPs with dysplasia, 12% of those indefinite for dysplasia, and 11% of those negative for dysplasia. No K-*ras* mutations were identified in the three dysplastic JPs with *APC* gene mutation.

Nuclear staining for p53 was diffuse in two JPs with low-grade dysplasia from two patients with JPS. Focal p53 staining, however, was common and unrelated to the sporadic or syndromic setting of the JPs or to the presence of dysplasia; 17% of JPs from JPS and 18% of sporadic JPs had focal staining, including 63% of those with dysplasia, 26% of those indefinite for dysplasia, and 11% of those negative for dysplasia (Tables 2 and 3).

Discussion

Although the pathogenesis of JPs is not well understood, they are generally classified as hamartomatous polyps.[44] Recently, a putative tumor suppressor gene locus termed JP1 was reported to be deleted in sub-epithelial cells of both familial and sporadic JPs.[45] The increased risk of developing adenocarcinoma in patients with JPS but not sporadic JPs[46] makes the diagnosis of dysplasia in JPs an important issue, although some cancers in JPS appear to develop in mucosa unassociated with JPs. We chose the classification schema for dysplasia in inflammatory bowel disease[40] to evaluate JPs because of the frequent background of inflamed granulation tissue with erosion in JPs. Histopathological classification of dysplasia in JPs, however, as evidenced by this study, revealed only moderate agreement among individual gastrointestinal pathologists, especially for the category of indefinite for dysplasia. Nonetheless, in this study using coded slides, dysplasia as classified by at least three of five gastrointestinal pathologists occurred only in JPs from patients with JPS and not in sporadic JPs. This finding supports the previous concept that patients with JPS but not sporadic JPs have increased risk of neoplastic transformation.[46] The diagnosis of JPs as indefinite for dysplasia can be problematic for patient management, especially in JPS. This dilemma will not be solved until reliable markers can be used to distinguish between dysplasia and reactive changes.

In normal colorectal mucosa, there is topographic regulation of epithelial proliferation and $p21^{(WAF1/CIP1)}$ expression, with $p21^{(WAF1/CIP1)}$ normally expressed in the superficial compartment and Ki-67 in the proliferative zone of crypts. This topographic control is lost during the neoplastic process in the colorectum with intermixture of cells expressing Ki-67 and $p21^{(WAF1/CIP1)}$ in the surface and upper glandular epithelium of adenomatous glands.[34,35] Studies of proliferation using ornithine decarboxylase and tyrosine kinase enzymes have shown increased proliferation in JPs as compared with adjacent normal mucosa in patients with JPS.[47] We found abnormal coexpression of Ki-67 and $p21^{(WAF1/CIP1)}$ in 79% of JPs with dysplasia. The specificity of this abnormal coexpression as evidence of dysplasia remains open to question because we also found this abnormality in five JPs (four from JPS and one sporadic JP) that were indefinite for dysplasia

regulation. E *and* F: *Histopathology of juvenile polyp with low-grade dysplasia from patient with juvenile polyposis syndrome. The* arrow *in* E *indicates area shown in* F *to* H. *The histopathological features of the dysplastic epithelium are similar to those seen in colorectal adenomas and dysplasia in inflammatory bowel disease.* G: *Immunohistochemical stain for Ki-67. The dysplastic epithelium has a high proliferative rate* (arrow *and* inset). H: *Immunohistochemical stain for p21. The topographic control of Ki-67 and p21 is lost as evidenced by the dysplastic epithelium expressing p21* (arrow *and* inset) *in the same areas expressing Ki-67 in* G. *This area also showed diffuse nuclear staining for* p53 *gene product (not illustrated).*

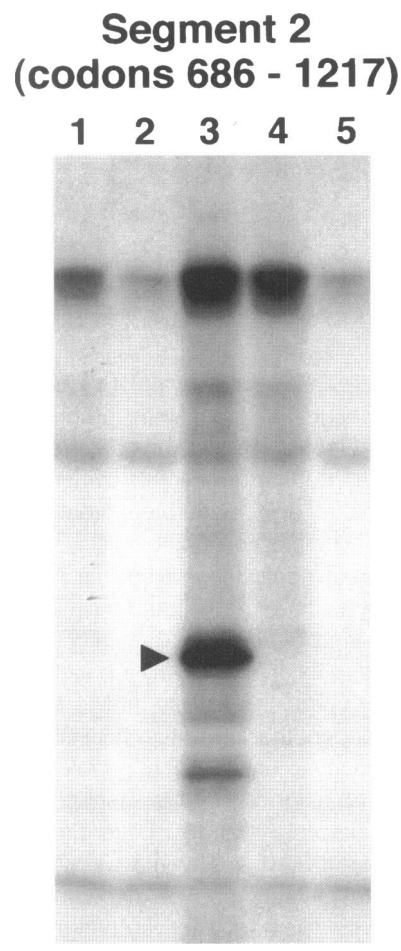

Figure 2. *In vitro synthesized-protein assay for truncating* APC *gene mutation in JPs. The assay of segment 2 is of DNA from five representative samples of JPs from patients with JPS.* Lanes 3 *and* 4 *are from different JPs of the same patient. The band corresponding to truncated protein from the mutated* APC *gene is evident in* lane 3 (arrowhead).

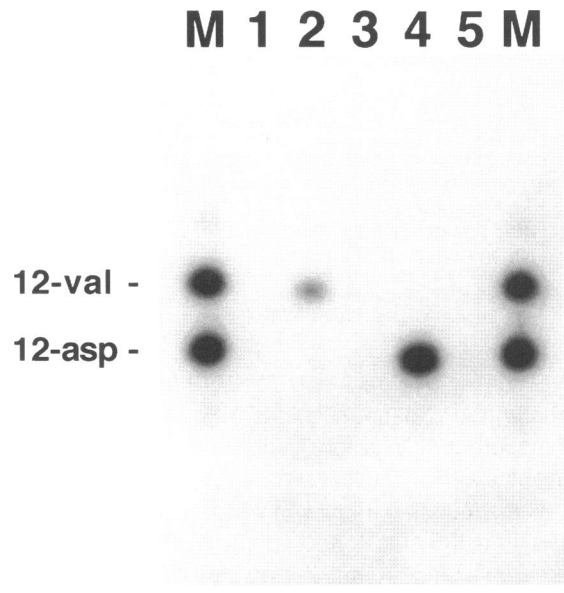

Figure 3. *K-*ras *mutation analysis using ligation assay of exon 1 at the second nucleotide of codon 12. In* lane 2, *the band indicates the presence of a valine (val) mutation, and in* lane 4, *an aspartic acid (asp) mutation. No mutation is identified in* lanes 1, 3, *and* 5. Lanes labeled M *have positive control DNAs from cell lines containing valine and aspartic acid mutations at the second nucleotide of codon 12.*

and in two JPs that were negative for dysplasia, both from patients with JPS. Alternatively, the frequent occurrence of abnormal coexpression in JPS but not in sporadic JPs suggests that abnormal coexpression could be a very early event in the development of colorectal neoplasia, occurring before dysplasia.

The development of dysplasia in JPS provides an opportunity for study in another context of alterations in genes that are known to be involved in usual colorectal tumorigenesis. Unlike familial adenomatous polyposis patients, no germline *APC* mutations are present in patients with JPS.[48] We found somatic *APC* mutations, however, in three of six dysplastic JPs (95% confidence interval, 12 to 88%) from JPS patients using IVSP assay, similar to the prevalence of somatic *APC* gene mutations identified in sporadic adenomas or adenomas from familial adenomatous polyposis patients (48 to 62%).[26,49] This prevalence is an underestimate of the frequency of *APC* mutations in dysplasia in JPs because the assay evaluated only codons 686 to 1693 of the 2843 codons in the gene. Thus, *APC* mutation appears to play an important role in the development of dysplasia in JPS as well as in usual colorectal neoplasms[29] and idiopathic inflammatory bowel disease.[50]

We found that K-*ras* mutation occurred in 25% of dysplastic JPs, comparable to the rate of K-*ras* mutation in colonic adenomas.[29,51–53] The presence of K-*ras* mutations in JPs negative for dysplasia or indefinite for dysplasia in the sporadic setting as well as JPS was an unexpected finding. K-*ras* mutation has been identified in 20% of hyperplastic polyps[29] and in essentially all aberrant crypt foci without dysplasia.[29,54] The marked reactive epithelial changes that occur commonly in JPs may predispose to K-*ras* mutation; nondysplastic epithelial lesions (16% of six examples of villous regeneration and 29% of seven examples of active colitis) in patients with ulcerative colitis, which usually has marked reactive epithelial changes, have also been found to have K-*ras* mutations.[55] By contrast, K-*ras* mutation was seen in 8 to 25% of the carcinomas arising from ulcerative colitis[55–58] as compared with 50 to 60% frequency of K-*ras* mutation in sporadic colorectal carcinoma.[27,28] Our findings provide additional evidence that K-*ras* mutation may not be crucial in the initial phase of neoplasia, in contrast to *APC* mutation.

We found diffusely positive nuclear p53 immunohistochemical staining in two dysplastic JPs. We and

others have found that mutation of the *p53* gene in colorectal neoplasms is usually associated with this pattern.[36–39] The low prevalence of p53 abnormalities in dysplastic JPs fits the model of late occurrence of *p53* mutation during colorectal tumorigenesis.[22,30] By contrast, *p53* mutation was identified more frequently in dysplasia and carcinoma arising in ulcerative colitis than in sporadic colorectal carcinoma.[59–62] Focal clusters of nuclear p53 staining were common in JPs, but their significance is unclear.

Our results provide evidence that multiple genetic alterations are involved in the development of dysplasia in JPs, but the clinical implications are unknown. *APC* mutation is a highly specific marker for dysplasia and was detected frequently in dysplastic JPs, but frozen tissue is required with current methods. Loss of topographic regulation of proliferation and $p21^{(WAF1/CIP1)}$ expression is a sensitive marker but less specific for dysplasia, whereas diffuse *p53* overexpression is highly specific but has very low sensitivity. K-*ras* mutations were not related to dysplasia. Thus, although our findings help to clarify the pathogenesis of colorectal neoplasia in patients with JPS, it is unclear that these alterations can serve as clinically useful markers. Rather, the clinical context of the JPs (syndromic or sporadic) should influence decisions about surveillance and prophylactic colectomy.

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