Ras Oncogene p21 Levels Parallel Malignant Potential of Different Human Colonic Benign Conditions

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• **Ras oncogenes are a specific family of genes believed to play a role in malignant transformation and tumor growth in humans. To gain a better understanding of the role these oncogenes may play in malignant transformation, we evaluated the levels of a ras gene protein product (p21) in formaldehyde-fixed, paraffin-embedded specimens of normal human colonic mucosa, hyperplastic polyps, tubular adenomas, villous adenomas, and epithelium from a patient with ulcerative colitis. The p21 protein content was measured using the RAP-5 monoclonal antibody in a semiquantitative immunohistochemical assay. The titer value was expressed as the highest dilution of antibody giving definite staining using the avidin-biotin peroxidase method. Differences in p21 titer values among all classes of polyps were significant (hyperplastic polyps values were less than tubular adenomas values, which were less than villous adenoma values). The p21 titers obtained from ulcerative colitis specimens were similar to those obtained from villous adenomas. We conclude that the levels of ras oncogene protein product increase with the malignant potential of benign human colonic conditions. These findings suggest that the ras oncogene protein product may play an important role in the malignant transformation of benign lesions of the human colon. If these findings are confirmed, as technology progresses to allow molecular probes to measure gene products in biopsy specimens, high-risk patients could be monitored and treated before actual malignant transformation occurs.**

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By means of DNA transfection technology, the ras oncogene family has been identified as a group of specific genes capable of inducing malignant transformation of NIH 3T3 cell line.[1-4] The family codes for a 21-kilodalton protein referred to as the p21 protein product.[5] Increased ras oncogene expression has been found in a variety of solid human tumors,[6-8] and it has been correlated with increased cell proliferation,[9] histologic grade, nuclear anaplasia, and degree of undifferentiation.[10] There are only limited data about the relationship between ras oncogene expression and both benign and premalignant lesions, and about the role the ras oncogene may play clinically in malignant transformation. Therefore, we evaluated the levels of ras oncogene p21 protein product in histologic sections of patients with various benign and premalignant colonic epithelia. The use of colonic lesions appeared well suited for this study since they provide a wide spectrum of malignant potentials.

MATERIALS AND METHODS

Archival formaldehyde-fixed, paraffin-embedded material was retrieved from the Department of Surgical Pathology files of the University of Chicago Medical Center. The specimens included normal colonic mucosa (n = 5), hyperplastic polyps (n = 14), tubular adenomas (n = 14), villous adenomas (n = 9), and epithelium from a patient with ulcerative colitis (n = 9). Specimens of normal colonic mucosa were obtained from patients who had undergone partial colectomy because of trauma, while epithelial specimens of ulcerative colitis were obtained from patients who had undergone colectomy for active disease. Pathologic classifications of the polyps was performed according to the method of Morson and Sobin.[11]

The RAP-5 mouse IgG2a monoclonal antibody to the ras p21 protein product was obtained from the National Institutes of Health, Bethesda, Md. A semiquantitative immunohistochemical assay was performed as previously reported.[12] Briefly, 4-μm-thick paraffin sections were deparaffinized in xylene and rinsed in absolute ethanol. To block endogenous peroxidase activity, tissue sections were placed for ten minutes in methanol containing 0.3% hydrogen peroxide. After rinsing in phosphate-buffered saline (PBS) adjusted to a pH of 7.4, nonspecific binding was blocked by preincubating tissue sections with 10% normal horse serum. The normal horse solution was then blotted off the slide and the primary antibody was applied. Primary incubation (30 minutes at 25°C) was carried out with serial dilutions of RAP-5 antibody from 1:5000 to 1:160 000 in PBS, 0.1% bovine serum albumin, 0.05% sodium azide in TRIS buffer. Biotinylated antimouse IgG was used

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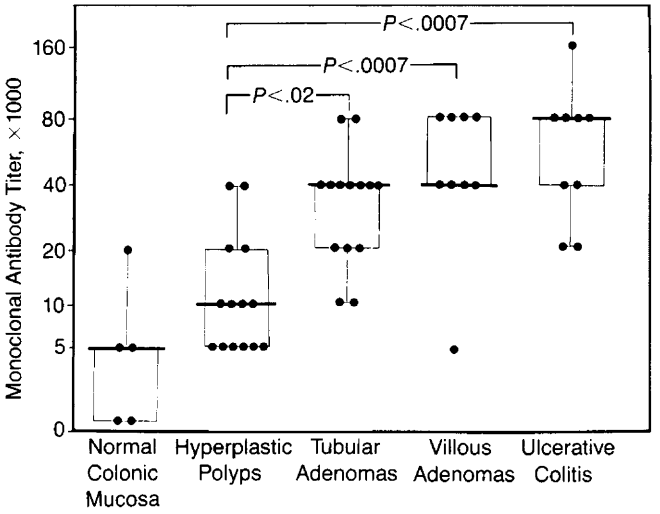
Cellular Ras Oncogene Protein Content in Specimens of Normal Colonic Mucosa, Polyps, and Epithelium From Patient With Ulcerative Colitis

	No. of Cases by Dilution						
	<1: 5000	1: 5000	1: 10 000	1: 20 000	1: 40 000	1: 80 000	1: 160 000
Normal colonic mucosa (n=5)	2	2	0	1	0	0	0
Hyperplastic polyps (n=14)	0	6	4	2	2	0	0
Tubular adenomas (n=14)	0	0	2	3	7	2	0
Villous adenomas (n=9)	0	1	0	0	4	4	0
Epithelium from ulcerative colitis (n=9)	0	0	0	2	2	4	1



Vertical scale indicates logarithm of highest dilution of monoclonal antibody RAP-5 staining ras oncogene protein product in normal colonic mucosa, benign colonic polyps, and epithelium from patient with ulcerative colitis. Boxes represent middle 50% of distribution of values obtained from each class of specimens. Each individual observation is marked with dot. Thickened line represents median.

as the secondary antibody (30 minutes at 25°C). Following a PBS wash, the avidin-biotin complex was applied to the sections for 30 minutes. The peroxidase reaction was then initiated by the addition of 0.06% diaminobenzidine and 0.01% hydrogen peroxide for five minutes. Counterstaining was obtained with Gills' hematoxylin No. 3 for 15 s. One slide per case was used as a negative control by using leukocyte common antigen as the primary antibody. Human colon carcinoma served as a positive control for ras p21 immunoreactivity.

A semiquantitative estimate of ras p21 expression was obtained by determining the highest dilution of antibody that elicited definitive cytoplasmic staining. Subtle, uniform, brown blushes were considered negative. The slides were examined independently by two investigators, with minimal disagreement on scoring. Statistical comparisons were based on Fisher's exact test and an appropriate ordinal loglinear model.[13,14]

RESULTS

The immunoperoxidase reaction for p21 protein appeared as a diffuse, brown cytoplasmic stain. At low dilutions, all epithelial and stromal elements within polyps and normal mucosa stained to a degree. At higher dilutions, normal stromal fibroblasts and smooth muscle cells did not stain, leaving the normal colonic epithelium and the benign colonic lesions exhibiting different degrees of stain. Specimens in each group exhibited a wide range of ras p21 expression. However, 83% of hyperplastic polyps and all normal colonic epithelia had a titer of less than 1:40 000, while adenomatous polyps, villous polyps, and dysplastic epithelial specimens of ulcerative colitis had a wider titer range, with 75% of specimens with titers equal to or greater than 1:40 000 (Table). The tendency of individual groups to stain at titers greater than or equal to 1:40 000 was compared using Fisher's exact test. There was a significant increase in the titer of ras p21 protein found in tubular adenomas ($P<.02$), villous adenomas ($P<.0007$), and epithelial specimens of ulcerative colitis ($P<.0007$) compared with hyperplastic polyps. Ulcerative colitis and tubular and villous adenomas as a group were also compared with hyperplastic polyps, with a significant difference in their p21 titer ($P<.0006$).

Applying the ordinal loglinear model, the groups of specimens were organized into three distinct statistical groups based on ras p21 expression: normal colonic mucosa and hyperplastic polyps exhibited the lowest ras p21 expression; tubular adenomas had intermediate expression; and villous adenomas, as well as epithelial specimens of ulcerative colitis, exhibited the highest expression of ras p21 protein (Figure).

COMMENT

The ras oncogene family has been identified as a group of specific genes associated with tumor growth and malignant transformation. Recent work[15-20] suggests that both quantitative changes (elevations) in the level of expression of c-ras genes and qualitative changes (mutations) affecting the protein product of those genes may be important in the process of carcinogenesis. However, a controversy regarding the methodology used to detect ras p21 protein has arisen: the specificity of the monoclonal antibody RAP-5 used to react with and to quantify human ras p21 protein has been questioned by Robinson et al,[21] who demonstrated that RAP-5 binds to proteins of a variety of molecular weights. The controversy is far from being settled, but Hand and coworkers[22] have recently shown that relative levels of a specific ras p21 protein product (Ha-ras) measured by competition radioimmunoassay, a very specific method, correlated well with the percentage of Ha-ras p21 positive cells detected by the immunohistochemical assays using the RAP-5 antibody. This finding suggests that although truly quantitative analysis of ras oncogene protein products may be obtained only with direct-binding liquid competition radioimmunoassay, relative levels may be detected with the immunohistochemical assay used in the present study and may be useful in determining the clinical importance of the ras oncogene family and its p21 protein product.

Using the aforementioned immunohistochemical assay, we have found that ras p21 expression correlates significantly with the malignant potential of different human colonic benign conditions. For example, adenomatous polyps of the large bowel are thought to be premalignant, with a potential for malignant change from 5% for tubular adenoma to 40% for villous adenoma when followed up for up to 15 years[23]; our analysis demonstrated that there was a significant increase in the titer of ras p21 protein found in tubular adenomas and an even greater increase in the titer in villous adenomas compared with hyperplastic polyps, lesions with a very low malignant potential, and normal colonic epithelium. These results are in agreement with those of Viola et al,[10] who found an increased ras oncogene

expression in premalignant lesions of the bladder compared with normal bladder epithelium, and with the findings of Hand et al[7] and Williams,[24] who have shown an increased ras p21 expression in benign colonic lesions. On the other hand, Thor et al[6] have found benign colonic tumors to be unreactive to antibody RAP-5. In that study, however, only a single concentration of antibody was used, and it may have been too low to demonstrate staining.

This study does not attempt to clarify whether the increased expression of ras oncogene is a necessary condition for the malignant transformation of benign colonic lesions. Nevertheless, it is tempting to suggest that activation of oncogenes represents the final common pathway of many different carcinogenetic stimuli and that, in the future, determination of high levels of oncogene protein products in normal or premalignant colonic epithelia may identify patients at greatest risk for developing colon cancer and in need of immediate therapy.

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Discussion

Q What levels of ras p21 protein are present in cancer of the colon?

A *Dr Michelassi:* The human colonic cancer that was used as a positive control was specifically a specimen in which the level of ras p21 protein was very high. Nevertheless, to answer your question probably in a more complete way: In the last nine months I have evaluated the level of ras p21 protein product in 149 consecutive specimens of rectal adenocarcinoma resected at the University of Chicago between 1965 and 1981 and, indeed, although there is some overlapping of p21 titer values with the villous adenomas and the ulcerative colitis specimens in the present study, usually the level of adenocarcinoma specimens is higher.

Q Is this product present in blood at all? Do you see a potential use in mass screening?

A The p21 protein product does not circulate in blood. The concentration of this product could be measured in bioptic tissue during screening colonoscopies once it is demonstrated that the level predicts the evolution of malignant lesions.

Q What about the mapping on specimens from normal colon? Is this an initiation or a promotion marker? Have you studied this product in any fetal tissues? Have you seen a gradation in the same specimen?

A Regarding the distribution of ras p21 protein in normal and abnormal colonic specimens, we are now evaluating ras p21 titer values in colonic cancer, within the 5 cm of distal margin and along the remaining colon. The work is on-going and I cannot tell you much right now. Regarding the importance of this product as an initiator, it is now thought that there are two important boosts of ras p21 production. The normal cell produces ras p21 protein. Along the line of a normal cell, an initial boost of ras p21 production may be important for the transformation from benign to malignant cell, while a second boost may be important for the transformation of the more favorable lesions, such as Dukes' A and B tumors, to the invasive Dukes' C and D tumors.