No. 5 Volume 37 October 1990 Page 439–532

Hepato-Gastroenterology

Current Medical and Surgical Trends

Official Organ of the International Gastro-Surgical-Club

Georg Thieme Verlag, Rüdigerstraße 14, D-7000 Stuttgart 30, Postfach 10 48 53, D-7000 Stuttgart 10
Thieme Medical Publishers, Inc., 381 Park Avenue South, New York, NY 10016

Relationship between Ras Oncogene Expression and Clinical and Pathological Features of Colonic Carcinoma

F. Michelassi[1], *G. Grad*[1], *F. Erroi*[2], *M. Roncella*[3], *J. Romagnoli*[3], *M. Handcock*[4]

[1]Departments of Surgery, and [4]Statistics of the University of Chicago, Chicago, Illinois; [2]Clinica Chirurgica II, University of Padova, Padova, Italy; and [3]Patologia Chirurgica II, University of Pisa, Pisa, Italy

Summary

In order to investigate the value of *ras* oncogene expression as a prognostic indicator in colonic adenocarcinoma, we evaluated the level of *ras* gene protein product (p21) in the available material of 109 surgical specimens resected at our institution between 1978 and 1981. Pathology slides and archived paraffin blocks were retrieved for confirmation of the original diagnosis, determination of stage, and measurement of p21 content. P21 titers were obtained using the RAP-5 monoclonal antibody in a semiquantitative immunohistochemical assay. Titer was expressed as the highest dilution of antibody given definitive staining using the Avidin-Biotin peroxidase method. The analysis indicated that tumors with high ($\geq$ 1:40,000) p21 titer had a lower five-year survival rate than tumors with low ($<$ 1:40,000) titers (34.3% vs 60.8%, $p < 0.02$). When a logistic regression analysis was used with the dependent variable being five-year survival and the independent variables being age, sex, location of tumor, Dukes' stage, mucin production, p21 titer, differentiation degree and tumor size, the statistically significant relationship of the level of *ras* gene protein product to long-term survival was negated by the concomitant knowledge of Dukes' stage. On the other hand, when only the variables available in the pre-operative period were entered in the multivariate analysis, p21 titers retained a significant relationship with long-term survival ($p < 0.05$). We conclude that *ras* oncogene determination in colonic carcinomas may have clinical importance for the pre-operative identification of a group of colonic tumors with a more aggressive behavior and a poorer prognosis

Key words

Colon – Carcinoma – Prognosis – Rasoncogene expression

Supported in part by Award 87 – 100 of the American Cancer Society

Hepato-gastroenterol. 37 (1990) 513 – 516

Introduction

The ability to predict the long-term prognosis of an individual patient with a colorectal carcinoma has been the aim of many studies performed since the introduction of Dukes' classification. An improved prognostic capability would enable surgeons to identify subgroups at high or low risk, could considerably influence the type of operative procedure performed, and help to determine the need for adjuvant therapy. Furthermore, a more precise prognosis would also be helpful in evaluating the results of different therapies and different series, as well as in allocating follow-up resources more effectively and efficiently.

In the past, pathological and clinical characteristics have been analyzed with limited success in an attempt to identify prognostic indicators. In recent years, much effort has been directed toward understanding the biological reasons for different tumor behavior. In this process, supportive evidence has been accumulated indicating that genes, collectively known as oncogenes, may play an important role. Of all known oncogenes, *ras* oncogene has been by far the most frequently studied (1–4). *Ras* oncogene codes for a 21-kilodalton protein referred to as p21 protein product (5). Increased p21 has been found in human colorectal adenocarcinoma with immunohistochemical assays (6–9), spot hybridization (10), Western immunoblotting (11), and quantitative liquid competition radioimmunoassay (12). Recently, data have been produced indicating that alterations of carbohydrates at cell surface level are functionally related to tumor invasiveness (13–14) and that human *ras* oncogene is implicated in contributing to the alteration of this surface carbohydrate (15). In view of this recent work, this investigation was carried out to establish the value of *ras* oncogene expression as a prognostic indicator in colorectal carcinomas.

Materials and methods

A retrospective review of 109 colon adenocarcinomas resected at the University of Chicago Medical Center between 1978 and 1981 was carried out. The clinical records of all these patients were reviewed, and a complete follow-up to December, 1986 was obtained through the Registry of Neoplastic Diseases of the University of Chicago in all cases.

Patients' slides and archived paraffin blocks were retrieved for confirmation of pathology and determination of tumor stage. Each tumor was staged in accordance with the Astler-Coller modification of Dukes' classification (16). *Ras* oncogene expression was determined by a semiquantitative immunohistochemical assay, using RAP-5 mouse IgGa monoclonal antibody (17) against a synthe-

Table 1 *Ras* oncogene expression and long-term survival

	< 5 years	≥ 5 years
< 1:40,000	29 (39.2%)	45 (60.8%)
≥ 1:40,000	23 (65.7%)	12 (34.3%)

$p < 0.02$

Table 2 *Ras* oncogene expression, local depth of tumor and occurrence of metachronous metastasis

	Specimens (n) with low titer	Specimens (n) with high titer	
Dukes' A & B	48 (64.9%)	12 (34.3%)	N. S.
Dukes' C & D	26 (35.1%)	23 (65.7%)	
No metachronous metastasis*	43 (66.2%)	12 (48%)	
Metachronous metastasis*	22 (33.8%)	13 (52%)	N. S.

* Only Dukes' A, B and C considered
N. S. = not significant

tic peptide corresponding to position 10–17 of the Hu-ras protein product from the T-24 bladder carcinoma (18).

Briefly, 4 micron-thick paraffin sections were deparaffinized in xylene and rinsed in absolute ethanol. To block endogenous peroxidase activity, tissue sections were placed in methanol containing 0.3% H_2O_2 for 10 minutes. After rinsing in phosphate-buffered saline (PBS), nonspecific binding was blocked by preincubating tissue sections with 10% normal horse serum (Vector Laboratories, Burlingame, CA). Primary incubation (30 min at 25°C) was carried out with serial dilutions of RAP-5 antibody from 1:5,000 to 1:160,000 in TRIS buffer (0.1% BSA, 0.05% sodium azide). Biotinylated antimouse IgG (Vector Laboratories) was employed as the secondary antibody (30 min at 25°C), followed by application of the avidin-biotin complex (Vector Laboratories) for 30 min. The peroxidase reaction was then initiated by the addition of 0.06% diaminobenzidine (Sigma Laboratories, St. Louis, MO) and 0.01% H_2O_2 for 5 min, and sections were counterstained with Gills hematoxylin No. 3 for 15 sec. Negative control sections were incubated with leukocyte common antigen as primary antibody (Dako Corp., Santa Barbara, CA). A known positive human colon carcinoma served as a positive control for p21 immunoreactivity. A semiquantitative estimate of p21 expression was obtained by determining the highest dilution of antibody which elicited definitive cytoplasmic staining in at least 10% of all epithelial cells. Subtle uniform brown blushes were considered negative. The slides were examined independently by two investigators, with minimal disagreement on scoring.

Statistical univariate comparison between p21 levels and five-year survival was based on Fisher's exact test (19). Moreover, a multivariate logistic regression analysis (20) was also performed, considering five-year survival as the dependent variable and age (both as a continuous variable and dichotomized at 40), sex, location of tumor along the colon (cecum, ascending with hepatic flexure, transverse with splenic flexure, descending, sigmoid, rectosigmoid), Dukes' stage, p 21 titer, mucin production, differentiation degree and tumor size as the independent variables.

Results

Staging of the 109 colonic adenocarcinomas in accordance with the Astler-Coller classification revealed that there were seven Dukes' A carcinomas, 50 Dukes' B, 33 Dukes' C and 19 Dukes' D. None of the patients with Dukes' A adenocarcinoma, 10 (20%) out of 50 with Dukes' B, 22 (66%) out of 33 with Dukes' C and all with Dukes' D adenocarcinoma died of recurrent disease before five years had passed. The five-year survival for the four stages was, therefore, 100%, 80%, 33% and 0%, respectively.

The immunoperoxidase reaction for p21 protein appeared as a diffuse brown cytoplasmic stain. At low dilutions all epithelial and stromal elements displayed some degree of staining. However, at higher dilutions, normal stromal fibroplasts and smooth muscle cells did not stain, leaving epithelium exhibiting different degrees of staining.

On the basis of our previous work (17) demonstrating that titers of *ras* oncogene protein product in normal colonic epithelial cells were always less than 1:40,000, the population in this study was dichotomized into specimens with "low" p21 titers (< 1:40,000) and those with "high" titers (≥ 1:40,000). The difference in long-term survival in these two groups was statistically significant. Table 1 displays the data on which this analysis was based. Of the 109 cases in which it was possible to determine *ras* oncogene p21, 74 patients had low titers and 35 had high titers. Of the 35 patients with high p21 titers only 12 (34.3%) survived more than five years. On the other hand, 45 (60.8%) of the 74 patients with low p21 titers survived more than five years ($p < 0.02$).

Forty-eight specimens (64.9%) with low p21 titers were classified as Dukes' stage A or B, while only one-third of specimens with high p21 titers were confined to the intestinal wall. Of the 90 patients with Dukes' A, B and C adenocarcinoma, 52% with high p21 titers developed distant metastasis in contrast to only 33.8% with low p21 titers (Table 2). Although the presence of high p21 titers suggests a more advanced tumor stage and a more likely occurrence of metachronous distant metastasis, these differences were not statistically significant.

When a multivariate logistic regression analysis was performed to evaluate significant relationships between all the clinicopathological features listed in the "Methods" section and patient survival, only one variable – the Dukes' stage – was found to be statistically significantly related to patient survival ($p < 0.01$). When the pathological variables available only in the pre-operative period (i. e., age, sex, location of tumor in the colon, p21 titer, degree of differentiation and mucin production) were included in the multivariate analysis, there was a significant relationship between p21 titers and long-term survival ($p < 0.05$).

Discussion

Cancer is the result of a progressive accumulation of successive mutations which cause the cell to acquire transformed characteristics such as the ability to grow in an unregulated fashion, or to metastasize. In colorectal carcinoma, several mutations have been identified which occur rather consistently, including increased expression of *ras* oncogene. In an attempt to develop a hypothesis of colorectal tumorigenesis, *Vogelstein* et al. (21) examined a large number of specimens for *ras* gene mutations, and other genetic changes. He found that these changes accumulated in a fashion that paralleled the clinical progression of tumors, and pro-

posed the successive accumulation of these mutations as a mechanism of tumor progression.

The biological consequences of increased expression of *ras* oncogene are poorly understood. In spite of these limitations, several authors have in the past investigated the question as to whether the level of p21 protein product correlates with the biological and clinical behavior of colon cancer. Correlation of *ras* expression with clinical outcome in patients with colorectal adenocarcinomas was initially attempted by *Kerr* et al. (22). *Kerr* did not find any relationship between *ras* oncogene expression, development of metastatic disease, and clinical outcome, but studied only 12 patients with a short follow-up period (average of 17 months). *Ravikumar* (23) attempted the same correlation with 18 patients. He concluded that 85% of specimens revealed enhanced *ras* oncogene expression, but that there was no correlation with metastatic behavior. However, by using dilutions up to only 1:15.000, *Ravikumar* failed to differentiate between colorectal cancers with high and low levels of p21 and so could not appreciate a possible correlation between the degree of *ras* oncogene expression and clinical behavior.

This investigation was, therefore, designed to avoid the problems of insufficient clinical material, short follow-up and narrow spectrum of dilutions, which have handicapped previous attempts to establish whether there is a correlation between *ras* oncogene expression and clinical outcome. The results of our study confirm the well-established prognostic value of Dukes' staging classification for carcinoma of the colon. Further, the analysis reveals that *ras* oncogene expression has a statistically significant association with long-term survival when Dukes' stage is not known. This finding highlights the potential value of preoperative *ras* oncogene expression as an aid in the biological staging of the adenocarcinoma, and in helping the surgeon to choose the type and extent of an operative procedure.

A controversy regarding the methodology used to detect *ras* p21 protein has recently 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. (24) who demonstrated that RAP-5 binds to proteins of a variety of molecular weights. The controversy is far from being settled, but *Hand* and his co-workers (12) have recently shown that relative levels of specific *ras* p21 protein product measured by competition radioimmunoassay, a very specific method, correlated well with the percentage of positive cells detected by the immunohistochemical assay using RAP-5 antibody. These findings suggest that, although truly quantitative anaylsis 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.

Although p21 is a membrane-bound protein, the immunoperoxidase reaction appeared as a diffused brown cytoplasmic stain. Although we believe that this is secondary to RAP-5 reacting with cytoplasmic peptide precursors of the final p21 protein, this aspect of the controversy has still not been settled. We occasionally noted that tumors had heterogeneous staining patterns in the same histological section. It is uncertain whether this heterogeneity is due to technical artifacts, areas of reduced tumor cell viability, the development of autonomous populations of cells no longer requiring elevated *ras* expression for growth, or to a variation in *ras* p21 levels at different times throughout the cell cycle. Finally, subjective interpretation of the results has been mentioned as a limitation common to all semiquantitative immunohistochemical methods. Our procedure of using two independent investigators to examine all slides minimizes this problem. If these problems are accepted and taken into account, the results of this study correlating the relative levels of *ras* oncogene expression detected with immunohistochemical assay and long-term survival retain their significance.

In summary, we believe that high levels of p21 protein characterize a group of biologically more aggressive colonic tumors with a poorer long-term prognosis. This finding may become clinically important in the pre-operative evaluation when pathological staging is not available.

Acknowledgments

We thank Dr. J. Schlom, (NIH, Bethesda, MD) for his generous gift of monoclonal antibody RAP-5, Miss Valerie Spiro, Registry of Neoplastic Diseases of the University of Chicago Medical Center, for her assistance in gathering complete follow-up data on all patients, and Mrs. Roberta Carden for her expert secretarial help.

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Fabrizio Michelassi, M. D. FACS

Department of Surgery (Box 168)
The University of Chicago Medical Center
5841 South Maryland Avenue
Chicago, Illinois 60637, USA