Enhanced Expression of c-Ha-ras p21 in Human Stomach Adenocarcinomas Defined by Immunoassays Using Monoclonal Antibodies and in Situ Hybridization

Noriaki Ohuchi, Patricia Horan Hand, Giorgio Merlo, Jun Fujita, Renato Mariani-Costantini, Ann Thor, Masato Nose, Robert Callahan, and Jeffrey Schlom

ABSTRACT

Using c-Ha-, c-Ki-, and c-N-ras-specific probes in a RNA-RNA hybridization assay we found enhanced expression of c-Ha-ras protooncogene in stomach adenocarcinomas relative to nonneoplastic epithelium, whereas little or no transcription of either c-Ki- or c-N-ras was detected. Enhanced levels of c-Ha-ras RNA expression were detected in all of the adenocarcinomas examined. Hybridization with c-Ha-ras was also detected in nonneoplastic gastric epithelium adjacent to carcinoma, although the labeling was less intense than that of carcinoma cells. More extensive analysis of the c-Ha-ras p21 expression was then carried out in formalin-fixed, paraffin-embedded tissue sections and extracts from surgically resected stomach tissues using monoclonal antibodies (MAbs) RAP-5 and Y13-259. The data obtained from the immunohistochemical studies were consistent with the results of in situ hybridization assay. Adenocarcinomas were much more reactive with MAb RAP-5 than benign and normal tissues, and the majority of carcinomas demonstrated increased expression of c-Ha-ras p21. Quantitative liquid competition radioimmunoassays using MAb Y13-259 also demonstrated significantly higher levels of c-Ha-ras p21 in extracts from stomach adenocarcinomas than those from normal mucosa. No strict correlation was found between ras p21 expression and the degree of tumor differentiation or histological type. Although advanced carcinomas generally demonstrated higher levels of ras p21 than early carcinomas, no correlation among advanced carcinomas and ras p21 levels was observed in relation to depth of tumor invasion to the muscularis propria, subserosa, or serosa. Benign lesions, in comparison, were much less reactive with MAB RAP-5 than carcinomas. Among the benign lesions tested, dysplastic lesions were more reactive than nondysplastic lesions. Normal stomach mucosa was generally nonreactive with the exception of parietal cells. Our results indicate that transformation of the stomach mucosa from benign to malignant phenotype is associated with an increase in c-Ha-ras p21 expression.

INTRODUCTION

Members of the ras oncogene family (Ha-ras, Ki-ras, and N-ras) have been identified as normal cellular components (protooncogenes) in a variety of species, including human. Two possible mechanisms for the ability of ras genes to transform cells have been identified. The first involves a qualitative change in the ras gene by a point mutation, causing an alteration at amino acid positions 12, 13, or 61 of the M, 21,000 ras gene product (ras p21) in certain types of human tumors (1, 2). However, analyses of human tumors for point mutation using either the NIH 3T3 cell transfection assay or restriction enzyme analyses have shown that only a small percentage of carcinomas contain point mutated ras (3–5). Increased levels of the proto-ras gene have also been associated with cellular transformation; e.g., when expressed at high levels a proto-ras gene is able to transform NIH 3T3 cells (6, 7).

An activated human oncogene homologous to v-raf has been found in a primary stomach carcinoma by DNA transfection in NIH 3T3 cells (8). Sakamoto et al. found another transforming gene, hst, in a primary stomach carcinoma and a noncancerous portion of the same patient, and in a lymph node metastasis of another patient (9). However, no transforming genes in these transformants were homologous to Ha-, Ki-, or N-ras of human origin (9). In contrast, Bos et al. have recently reported a mutated c-Ki-ras allele at position 12 as well as an amplified normal allele in one stomach carcinoma using NIH 3T3 transfection assay and synthetic oligonucleotide probes (10). A recent study showed enhanced ras p21 expression in stomach carcinomas using immunohistochemical techniques (11). However, no attempt was made to identify which ras gene was involved or to use quantitative assays such as radioimmunoassay.

The in situ hybridization technique (12–16) employed here complements studies performed with MAbs because it allows us to determine which of the three ras protooncogenes is transcriptionally active. Such precise identification is not possible at the protein level using currently available immunological techniques.

MAbs reactive with ras p21 have been utilized with formalin-fixed, paraffin-embedded tissues and immunohistochemical techniques (17–19). The advantages of this approach include: (a) evaluation of a variety of malignant, potentially premalignant, and benign surgically resected human stomach tissues; (b) determination of ras p21 expression at the single cell level; and (c) correlation of clinical, surgical, and histological data with ras p21 expression. Immunohistochemical methods, however, provide only qualitative and semiquantitative data. Therefore, we have also utilized quantitative liquid competition RIAs with MAb Y13-259, directed against ras p21, for the detection and quantitative analysis of ras p21 in extracts from surgically resected and snap frozen human stomach tissues.

Our objectives were (a) to define whether c-Ha-, c-Ki-, or c-N-ras p21 was expressed in stomach adenocarcinomas using in situ hybridization; (b) to qualitatively detect ras p21 expression at the single cell level in formalin-fixed, paraffin-embedded stomach tissues using immunohistochemical assays; and (c) to quantitatively detect the levels of ras p21 expression in cellular extracts from stomach tissues using direct-binding liquid competition RIAs. Our aim was to determine if ras oncogenes were involved in, or associated with stomach adenocarcinomas. Further, we sought to define if ras p21 expression correlated with degree of tumor differentiation, histological subtype, or depth of tumor invasion.
MATERIALS AND METHODS

Human Stomach Tissues. Eight primary stomach adenocarcinomas and three normal tissues adjacent to stomach carcinomas used for in situ hybridization were frozen in liquid nitrogen immediately after surgery, and preserved at −70°C. The histopathological diagnoses of these tissues, based on Diff Quick (American Scientific Products, McGraw Park, IL) staining of frozen sections and pathology records, are listed under Table 1.

Formalin-fixed, paraffin-embedded tissues used for immunohistochemical analyses were obtained from 44 patients with gastric carcinoma, 14 with benign gastric lesions (seven with hyperplastic polyps, three with adenomatous polyps, and four with dysplastic lesions), and 11 with normal histology. The 11 specimens containing normal gastric mucosa were taken from two patients with gastritis, one with reflux esophagitis, two with hyperplastic polyps, and six patients who died from nonneoplastic diseases (confirmed at autopsy). Among 14 specimens with benign lesions, four specimens were histologically diagnosed as dysplasia because of cellular atypia, abnormal glandular differentiation, and disorganized mucosal architecture (20, 21). Stomach carcinomas were subdivided into four groups based on their histological patterns including well-differentiated tubular adenocarcinomas (N = 12), moderately differentiated tubular adenocarcinomas (N = 11), poorly differentiated adenocarcinomas (N = 13), and signet ring cell carcinomas (N = 8). Sections (5 μm) of paraffin-embedded tissue were cut and mounted on gelatin-coated glass slides. One section from each specimen was stained with hematoxylin and eosin for cellular visualization.

Snap frozen tissues used for direct-binding liquid competition RIA and immunohistochemistry included adenocarcinomas (N = 9) and normal mucosa from patients with gastric carcinoma (N = 5). Three of five normal mucosas were obtained from the same patients whose malignant lesions were also studied, and the other two normal mucosae were from patients with early gastric carcinoma whose malignant lesions were not large enough to be used in this study. Samples of normal mucosa were resected from the stomach apart from foci of carcinoma. Immediately after collection, the tissues were cut into pieces of approximately 0.5 g, quick frozen in liquid nitrogen, and stored at −70°C for direct-binding liquid competition RIA analysis. An adjacent piece of tissue was fixed in 10% buffered formalin and embedded in paraffin for histopathological and immunohistochemical analyses.

In Situ Hybridization. The in situ hybridization was performed according to Harper et al. (22) with some modifications. Frozen sections were cut at 5 μm, thawed, and mounted onto acid precleaved and poly-L-lysine (100 μg/ml)-coated microscope slides, and immediately fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at 4°C. The sections were then washed in cold PBS for 1 min, and dehydrated in graded ethanol. Pretreatment consisted of two 1-min rinses in two changes of 2× SSC followed by acetylation in 0.025% (v/v) acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min. The slides were rinsed in 2× SSC and PBS, 1 min each, incubated in 0.1 M Tris-HCl, pH 7.0/0.1 M glycine (30 min), rinsed in 2× SSC, and transferred in 2× SSC-50% formamide at 50°C prior to hybridization. The SP6 transcription vector containing human c-Ha-ras open reading frame sequences in reverse orientation was obtained from Oncogene Science, Inc. (Minoa, NY). The human c-N-ras and c-Ki-ras probes, also inserted in SP6 transcription vectors, were acquired from Amersham (Arlington Heights, IL). A 1.3-kilobase BamHI-EcoR1 murine ribosomal DNA (gift of Dr. J. Fetherston, University of Kentucky, KY) was subcloned into the transcription vector pSP65 (Promega Biotech., Madison, WI) in the 3′ to 5′ orientation. This probe was used as positive control. The pSP64 transcription vector (Promega Biotech.) with no insert was used as negative control probe. RNA complementary to cellular transcripts was transcribed using the SP6 system according to the manufacturers specifications and labeled with [35S]UTP (400 Ci/mmol; Amersham, Arlington Heights, IL). Two μl of labeled RNA probe per slide (2 × 10⁶ cpm) was added to 18 μl of hybridization solution to give a concentration of 50% formamide, 2× SSC, 0.01 M dithiothreitol, 1 mg/ml Escherichia coli tRNA, 1 mg/ml-sonicated salmon sperm DNA, and 2 μg/ml BSA. The probes were heated at 65°C for 5 min in the hybridization solution and transferred to a multiblock heater at 50°C. Prior to hybridization, the slides were retrieved from the 2× SSC-50% formamide incubation, blotted with lint-free paper, and transferred to a slide warmer. Twenty μl of hybridization solution was applied to the sections, which were then covered with acid-precleaned, siliconized coverslips and sealed with rubber cement. The hybridization was carried out for 4 h at 50°C. The sections were then washed in 2× SSC and subsequently transferred to two sequential washes in 2× SSC-50% formamide at 55°C, rinsed in 2× SSC, and blotted with lint-free paper. Thirty μl containing RNase A (Boehringer Mannheim, FRG), 100 μg/ml, and RNase T1 (Boehringer Mannheim), 400 units/ml, in 0.2 μm sodium phosphate, pH 6.5, were gently applied to the tissue section. The sections were covered with acid-precleaned coverslips, sealed, and incubated for 30 min at 37°C. After RNase treatment, the sections were rinsed in 2× SSC, washed at 55°C in 2× SSC-50% formamide (5 min) and in 2× SSC overnight. The sections were dehydrated, dried, and dipped in Kodak NTB2 emulsion, melted at 45°C, and diluted 1:1 with distilled water. After 6 days of exposure in light-proof boxes at 4°C, the autoradiographs were developed in Kodak D-19 developer for 5 min, washed in tap water, fixed in Kodak rapid fixer for 4 min, rinsed in tap water, and counterstained with

Table 1 ras p21 RNA expression in adenocarcinomas and normal mucosae adjacent to carcinoma using RNA-RNA in situ hybridization

<table>
<thead>
<tr>
<th>Histopathological diagnosis</th>
<th>c-Ha-ras EXPRESSION IN STOMACH ADENOCARCINOMAS</th>
<th>c-Ki-ras EXPRESSION IN STOMACH ADENOCARCINOMAS</th>
<th>c-Na-ras EXPRESSION IN STOMACH ADENOCARCINOMAS</th>
<th>Ribosomal control</th>
<th>pSP64 control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>4.4 (40)</td>
<td>0.8 (7)</td>
<td>1.2 (11)</td>
<td>7.9 (71)</td>
<td>1.0 (9)</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>9.3 (27)</td>
<td>1.0 (4)</td>
<td>1.0 (4)</td>
<td>10.8 (24)</td>
<td>1.0 (4)</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-differentiated tubular</td>
<td>17.8 (338)</td>
<td>0.7 (13)</td>
<td>0.5 (9)</td>
<td>13.7 (261)</td>
<td>1.0 (19)</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>2.0 (83)</td>
<td>1.5 (33)</td>
<td>1.2 (49)</td>
<td>4.6 (187)</td>
<td>1.0 (41)</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>7.5 (105)</td>
<td>1.1 (15)</td>
<td>1.3 (19)</td>
<td>16.3 (228)</td>
<td>1.0 (14)</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>13.1 (367)</td>
<td>0.8 (22)</td>
<td>0.4 (10)</td>
<td>5.0 (139)</td>
<td>1.0 (28)</td>
</tr>
<tr>
<td>tubular adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well-differentiated tubular</td>
<td>18.4 (306)</td>
<td>0.7 (12)</td>
<td>0.7 (12)</td>
<td>6.5 (109)</td>
<td>1.0 (17)</td>
</tr>
<tr>
<td>adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal mucosa adjacent to</td>
<td>17.2 (309)</td>
<td>0.6 (10)</td>
<td>0.4 (8)</td>
<td>12.7 (229)</td>
<td>1.0 (18)</td>
</tr>
<tr>
<td>carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>1.7 (15)</td>
<td>0.6 (5)</td>
<td>1.2 (11)</td>
<td>8.2 (74)</td>
<td>1.0 (9)</td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>5.4 (92)</td>
<td>1.2 (20)</td>
<td>0.8 (14)</td>
<td>10.2 (174)</td>
<td>1.0 (17)</td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>1.9 (43)</td>
<td>0.4 (9)</td>
<td>0.7 (15)</td>
<td>7.7 (172)</td>
<td>1.0 (23)</td>
</tr>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-MuSV-3T3 (DT)</td>
<td>ND</td>
<td>12.0 (96)</td>
<td>ND</td>
<td>17.2 (138)</td>
<td>1.0 (8)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>ND</td>
<td>ND</td>
<td>17.5 (70)</td>
<td>16.7 (267)</td>
<td>1.0 (4)</td>
</tr>
</tbody>
</table>

* A quantitation of labeling was attempted by counting silver grains using an ocular micrometer under light microscope at magnification of 1000×.

1 Labeling index: a relative ratio of (number of silver grains in each section) to (number of silver grains in pSP64-treated section).

2 Number of silver grains in the area of 625 μm². In each section three different areas of carcinomas or normal epithelial cells were evaluated by the counting of grains, and the mean number was utilized.

3 ND, not determined.

Downloaded from cancerres.aacrjournals.org on January 27, 2018. © 1987 American Association for Cancer Research.
hematoxylin and eosin. Stained sections were examined for silver grains.

Monoclonal Antibodies. Murine IgG2a MAb RAP-5 was generated using a synthetic peptide reflecting amino acid positions 10–17 (valine substituted for glycine at position 12) of the human ras gene product p21, for the T24 bladder carcinoma cell line m21 (17). This antibody has demonstrated reactivity with both the point-mutated and proto-onc forms of ras p21. MAB Y13-259, generated from clone Y13-259 derived by fusion of Y3 rat myeloma cells with rat spleen cells following immunization with Ha-MuSV-transformed NRK cells by Furth et al. (23), is a monoclonal antibody which immunoprecipitates both the point-mutated and proto-onc forms of human, rat, and mouse. MABS UPC-10 (a purified murine myeloma IgG2a protein), and MOPC-21 (a purified murine myeloma IgG1 protein) (24) were obtained from Litton Bionetics, Charleston, SC.

Immunohistochemical Assays. Immunohistochemical methods for formalin-fixed, paraffin-embedded tissue sections and MAB RAP-5 purified from ascites have been described previously (19). For this study we used ascites fluids of MABS RAP-5 and Y13-259. Tissue sections were deparaffinized and treated with methanol containing 0.3% H2O2 for 10 min to block endogenous peroxidase activity. After rinsing in PBS (pH 7.4), the sections were incubated in 10% normal horse serum for 15 min. The pretreatment was removed and primary MAB RAP-5 (ascites fluid at 1:15,000 dilution) was added, and the slides were incubated for 30 min at room temperature. MAB UPC-10 (5 µg/ml) was used as an isotype identical (IgG2a) control for MAB RAP-5 on a serial section from each tissue. The primary MABs were removed and the slides were washed in PBS and then incubated with biotinylated horse anti-mouse IgG (Vector Labs., Inc., Burlingame, CA) at 1:500 dilution for 30 min. Following a PBS rinse, the sections were incubated with Vectastain ABC kit for 30 min. The slides were rinsed in PBS, and the peroxidase reaction was initiated using 0.06% diaminobenzidine (Sigma Chemical Co., St. Louis, MO) with 0.01% H2O2 for 5 min. The sections were briefly counterstained with hematoxylin.

Immunohistochemical assays with MAB Y13-259 were performed using the same techniques described for MAB RAP-5, except for pretreatment with 10% rabbit serum, primary antibody incubation (ascites fluid of MAB Y13-259 at 1:750 dilution), and second antibody incubation (biotinylated rabbit anti-rat IgG at 1:500 dilution; Vector) MAB MOPC-21 was used at 40 µl/ml as a control for nonspecific binding of immunoperoxidase reagents to the tissues. In the assays with MAB MOPC-21, 10% normal horse serum was used as a pretreatment of tissue sections, and biotinylated horse anti-mouse IgG was used as a secondary antibody.

Scoring Methods for Immunohistochemical Evaluation. Each section was evaluated for the presence of cell-associated diaminobenzidine precipitate indicative of primary MAB binding using the following scoring system: (−) negative; (+) clearly positive; (++) strongly positive. A percentage score was assigned which represents the sum (as a percentage of total cells) of cytoplasmic (+ and ++) reactivity. This scoring procedure should be considered "semiquantitative." The approximate percentage of reactive cells for malignant stomach tissues was scored according to the number of carcinoma cells positive, divided by the total percentage of reactive cells for benign lesions was scored according to the number of reactive cells for benign lesions divided by the total number of normal epithelial cells and multiplied by 100. For each malignant, benign, or normal tissue more than 200 epithelial cells were required for immunohistochemical analyses.

Statistics for Immunohistochemical Evaluation. The average percentage of reactivity of MAB RAP-5 with gastric tissues is presented as mean ± SD. Results are compared by Wilcoxon rank sum test for nonparametric methods (25).

Protein Extracts of Stomach Tissues. Tissues were homogenized for 3 min on ice in 10 mM Tris-HCl (pH 7.2) with 0.2 mM CaCl2 (10 g/100 ml). The homogenate was subjected to pressure homogenization using a cell disruption bomb (Parr Instrument Co., Moline, IL) for 10 min at 1000 lb/in² and then clarified at 2800 × g for 5 min. The supernatant was sonicated on ice for 2 min with 15-s intervals (Branson sonifier). The sonicate was then centrifuged at 9500 × g for 10 min. The supernatant was assayed for ras p21 by solid-phase liquid competition RIA. Protein concentration was determined by the method of Lowry et al. (26).

Cells. v-Ha-PT3, line 568, is a Ha-MuSV-transformed NIH 3T3 cell line (27). T24 human bladder carcinoma cell line, Hu-ras-Ha-T24, contains the point-mutated T24 human Ha-ras gene (28). The T24 cell line was kindly provided by Dr. S. A. Aaronson, National Cancer Institute, NIH, Bethesda, MD. ras p21 was purified from 90–95% homogeneity from Escherichia coli strain (AR548) expressing the full-length T24 mutant human Ha-ras p21 (recombinant ras p21) (29). This pure recombinant ras p21 was kindly provided by Drs. T. Chao, M. Gross, and R. Sweet (Smith Kline and French Laboratories, Philadelphia, PA). Kis-MuSV-transformed NIH 3T3 (DT) cell line is known to express Ki-ras p21 (30). The 568 and DT cell lines were kindly provided by Dr. R. Bassin (National Cancer Institute, NIH, Bethesda, MD). Human mammary carcinoma cell line MCF-7 has been shown to express N-ras p21 (31).

Direct-binding Liquid Competition RIA for ras p21. Quantitative immunomassays have been described in detail elsewhere (32). Briefly, 50 µl (10 µg) of a protein extract of the v-Ha-transformed NIH 3T3 cell line 568) were added to each well of 96-well polyvinyl chloride microtiter plates and allowed to dry overnight at 37°C (reaction plate). To minimize nonspecific protein adsorption, microtiter wells of the detection plate and the reaction plate (a plate with no extract adsorbed to wells) were treated with 50 µl of 5% BSA in PBS and incubated for 1 h at 37°C. The BSA was removed and the wells were washed once with 1% BSA in PBS. To each well of reaction plate, either 40 µl of 125I-labeled MAB Y13-259 (75,000 cpm), or 40 µl of 125I-labeled MAB MOPC-21 (75,000 cpm) diluted in PBS containing 1% BSA, 0.05% Tween-20 (assay buffer) were added. 125I-labeled MAB MOPC-21 was used as a negative control in this assay system. Either 40 µl of competitor antigen (diluted in assay buffer containing 0.02% Empigen) or assay buffer containing 0.02% Empigen were then added to the reaction plates containing 125I-labeled MAB Y13-259, or MAB MOPC-21. The final detergent concentration in each well was 0.05% Tween-20 and 0.01% Empigen. The reaction plates were incubated for 1 h at room temperature. Aliquots (30 µl) were then transferred from each well of the reaction plate to the detection plate in duplicate. The detection plates were incubated 16–18 h at 4°C. The wells were then washed three times with assay buffer and cut from the plates. 125I-labeled MAB Y13-259 IgG bound to the wells of the detection plate was measured in a gamma counter. Percentage of IgG bound was determined by dividing the average of the cpm bound to wells of the detection plate in the presence of competitor antigen by the average of the cpm obtained in the absence of competitor antigen multiplied by 100.

RESULTS

In Situ Hybridization of Adenocarcinomas and Nonneoplastic Macrouses of the Stomach. The in situ hybridization analysis consistently demonstrated c-Ha-ras RNA expression in all of the eight stomach adenocarcinomas examined (Fig. 1, Table 1). The labeling indices obtained were determined by counting the number of silver grains in a 625-µm² tumor area, divided by the number of grains in a corresponding area of the negative control pSP64 hybridized slide. These values indicated a marked heterogeneous expression of c-Ha-ras DNA in different carcinomas examined (Table 1). The c-Ha-ras labeling indices in adenocarcinomas varied from 2.0 to 18.4 (the average index was 11.2 ± 6.0). Four of eight tumors were strongly labeled with c-Ha-ras RNA probe (labeling index, >10). There was no apparent correlation between the degree of differentiation or histological subtype and the level of c-Ha-ras RNA expression, since tumors with a high labeling index (>10) included both well and poorly differentiated adenocarcinomas. These differences in c-Ha-ras hybridization intensity were not related to differential
Fig. 1. A–E, RNA-RNA in situ hybridizations of a stomach adenocarcinoma using human c-Ha-ras probe (A and B), c-Ki-ras probe (C), c-N-ras probe (D), and pSP64 negative control probe (E). Note that the carcinoma cells are strongly labeled with c-Ha-ras probe, while no specific labeling with either c-Ki- or c-N-ras probe is detected. (Original magnifications: A, ×430; B–E, ×860). F, serial section stained with Diff Quick. (Original magnification: ×330). G, RNA-RNA in situ hybridization of a normal gastric mucosa adjacent to carcinoma using c-Ha-ras probe. Nonneoplastic cells are labeled with c-Ha-ras probe, although the intensity is less than carcinoma cells. (Original magnification: ×450).

preservation of cellular RNAs in the tumor samples as demonstrated by the similar labeling indices obtained with the ribosomal probe in cases having different c-Ha-ras labeling indices (e.g., cases 4 and 6, Table 1). Epithelial cells from the lower two thirds of nonneoplastic gastric glands also demonstrated slightly elevated c-Ha-ras RNA expression in the three cases examined. Their labeling indices, ranging from 1.7 to 5.4 (the average index was 3.0 ± 1.7), were lower than those of carcinomas except for one normal mucosa (i.e., case 10, Table 1), which was in the range of carcinomas with low labeling. No evidence of expression of either c-Ki-ras or c-N-ras RNA was detected in any adenocarcinoma or nonneoplastic mucosa specimen. The labeling indices obtained with either the c-Ki-ras or the c-N-ras probe corresponded with or were less than those obtained with the negative control pSP64 (Table 1).

To eliminate the possibility of a technical failure to obtain hybridization with Ki-ras and N-ras probes, two human cell lines (DT and MCF-7) were examined for positive controls for Ki-ras and N-ras, respectively, using the same probes as described in “Materials and Methods.” The DT cell line, a Ki-MuSV-transformed derivative of the NIH 3T3 cell (30), demonstrated an elevated expression of c-Ki-ras p21. On the other hand, MCF-7, a human mammary carcinoma cell line, demonstrated an elevated level of c-N-ras p21 (Table 1).

Immunohistochemical Reactivity of MAb RAP-5 with Adenocarcinomas of the Stomach. A variety of formalin-fixed, paraffin-embedded gastric tumors were analyzed for expression of ras p21 using MAb RAP-5 and immunohistochemical methods (Fig. 2). As shown in Fig. 2, adenocarcinomas of the stomach were reactive with MAB RAP-5, demonstrating a heterogeneous expression of ras p21 among the tumors examined, as well as within each individual tumor mass. Only one adenocarcinoma was completely negative, and 36/44 (82%) demonstrated ≥25% of carcinoma cells reactive with MAB RAP-5. Tubular adenocarcinoma generally demonstrated cytoplasmic ras p21 expression within carcinoma cells (Fig. 3A). MAB RAP-5 was also reactive with signet ring cell carcinoma in the cytoplasm (which is eccentrically over-distended by mucus), although it was not reactive with the mucinous component (Fig. 3B).

The cellular reactivity of MAB RAP-5 with gastric adenocarcinomas was further analyzed in relation to the degree of histological differentiation (Table 2). There was a slight indication that signet ring cell carcinomas were less reactive with MAB RAP-5 than moderately differentiated tubular adenocarcinomas (P = 0.1, by Wilcoxon rank sum test). However, no strict correlation between the cellular reactivity with MAB RAP-5 and the degree of differentiation or histological subtype was
Fig. 3. Immunohistochemical staining of MAb RAP-5 with formalin-fixed, paraffin-embedded tissue sections of the stomach. Counterstained with hematoxylin. A, well-differentiated adenocarcinoma showing cytoplasmic staining (×330); B, signet ring cell carcinoma. The cytoplasm eccentrically over-distended by mucin is reactive with MAb RAP-5, but the mucin component is not reactive (×430); C, dysplasia. Note that atypical glands characterized by abnormal differentiation of tubules and disorganized mucosal architecture are reactive with MAb RAP-5 (×80); D, normal gastric mucosa. Parietal cells (arrows) are occasionally reactive with MAb RAP-5 (×130).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>MAb RAP-5 reactivity with adenocarcinomas of the stomach in relation to the degree of differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation of carcinoma</td>
<td>Number of specimens examined</td>
</tr>
<tr>
<td>Well-differentiated tubular adenocarcinomas</td>
<td>12*</td>
</tr>
<tr>
<td>Moderately differentiated tubular adenocarcinomas</td>
<td>11</td>
</tr>
<tr>
<td>Poorly differentiated adenocarcinomas</td>
<td>13</td>
</tr>
<tr>
<td>Signet ring cell carcinomas</td>
<td>8</td>
</tr>
</tbody>
</table>

* Specimens from different individuals.  
* Mean ± SD.  
* Differs from moderately differentiated tubular adenocarcinomas (P = 0.1).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>MAb RAP-5 reactivity with adenocarcinomas of the stomach in relation to the degree of invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of carcinoma invasion</td>
<td>Number of specimens examined</td>
</tr>
<tr>
<td>Mucosa</td>
<td>11*</td>
</tr>
<tr>
<td>Submucosa</td>
<td>7</td>
</tr>
<tr>
<td>Muscularis propria</td>
<td>10</td>
</tr>
<tr>
<td>Subserosa</td>
<td>9</td>
</tr>
<tr>
<td>Serosa</td>
<td>7</td>
</tr>
</tbody>
</table>

* Specimens from different individuals.  
* Mean ± SD.  
* Differs from carcinoma cells involving mucosa (P < 0.02).  
* Differs from carcinoma cells involving submucoa (P < 0.1).  
* Differs from carcinoma cells involving submucoa (P = 0.02).  
* Differs from carcinoma cells involving submucoa (P = 0.1).

The reactivity of MAb RAP-5 with carcinomas was also analyzed in relation to the degree of tumor invasion (Table 3). Carcinomas with invasion to the muscularis propria, subserosa, and serosa were more reactive with MAB RAP-5 than superficial carcinomas involving the mucosa (P ≤ 0.01), or carcinomas involving the submucosa (P ≤ 0.1). There was no striking difference in the average percentage of reactivity, however, between carcinomas infiltrating to the muscularis propria, subserosa, and serosa (P > 0.1).

Immunohistochemical Reactivity of MAb RAP-5 with Benign Lesions of the Stomach. The percentage of cellular reactivities of MAB RAP-5 with benign gastric lesions are shown in Fig. 2B. The average reactivity with these benign lesions was significantly lower than that with carcinomas (P < 0.01). Five of 14 tissues (35%) demonstrated ≥25% of epithelial cells reactive with MAB RAP-5. Of these tissues, four were histologically diagnosed as dysplastic lesions, while the other was a hyperplastic polyp with chronic inflammation. The reactivity of MAB RAP-5 with dysplastic tissue was significantly (P < 0.01) higher than that of nondysplastic lesions (Fig. 2B). Dysplastic epithelium reactive with MAB RAP-5 was characterized by cellular atypia, abnormal differentiation, and disorganized mucosal architecture (Fig. 3C).

Immunohistochemical Reactivity of MAB RAP-5 with Normal Mucosae of the Stomach. Normal mucosae of the stomach from 11 patients without evidence of carcinoma (Fig. 2C) and from 11 patients with adenocarcinoma of the stomach (Fig. 2D) were examined for their reactivity with MAB RAP-5. Although none of the normal mucosae from patients without carcinoma demonstrated ≥ 25% epithelial cells reactive, seven tissues were reactive with MAB RAP-5 (at least one cell positive). Generally, parietal cells showed reactivity with MAB RAP-5, but chief cells and mucous cells did not (Fig. 3D). Parietal cells of normal mucosa were also reactive with MAB Y13-259. Normal appearing mucosa taken from patients with carcinoma, in contrast, were more reactive with MAB RAP-5 than those from patients without carcinoma (P < 0.02). Parietal cells as well as chief cells adjacent to carcinomas occasionally showed positive cytoplasmic staining. Nonepithelial cells such as smooth muscle, macrophages, and neutrophils were occasionally weakly reactive with MABs RAP-5 and Y13-259.

Quantification of ras p21 in Adenocarcinomas and Normal Mucosae by Liquid Competition RIA. To determine the presence
and quantitative levels of ras p21 in stomach tissues, protein extracts of malignant and normal stomach biopsy materials were used as competitor antigens in the liquid competition RIA. Using as a standard the competition curve generated by pure recombinant ras p21, it was possible to quantitate ras p21 levels in terms of picograms of ras p21 per microgram of protein. Fig. 4 shows reactivity of a lysate of E. coli containing recombinant Hu-ras T24 p21, Ha-transformed NIH 3T3 cells (568), and some of the gastric tissue extracts, in a liquid competition RIA using MAb Y13-259. Absolute amounts of ras p21 (pg p21/μg protein) were determined based on the amount of extract protein required for 50% competition. As shown in Table 4, extracts of the adenocarcinomas contained ras p21 ranging from 38.8 to 90.5 pg p21/μg protein, while extracts of the normal mucosa taken from patients with carcinoma expressed ras p21 ranging from 13.8 to 36.2 pg p21/μg protein. There was a significant difference in levels of ras p21 expression between tissue extracts of adenocarcinomas and normal mucosae (P < 0.01). The levels of ras p21 from three tissue extracts of adenocarcinomas were compared with those of normal mucosa from the same patients (Table 4; patient numbers 1, 2, and 3). Approximately 2–3-fold more ras p21 was detected in these adenocarcinomas than in the respective normal mucosa.

Comparison of ras p21 Evaluation by Immunohistochemistry and Liquid Competition RIA. To determine if a correlation existed between ras p21 levels determined by liquid competition RIA and immunohistochemical analyses, both methods were utilized to evaluate ras p21 expression in the same tissue specimens. Immunohistochemical assays using both MAbs RAP-5 and Y13-259 were performed on formalin-fixed, paraffin-embedded tissue sections from four adenocarcinomas and one normal mucosa of the stomach which were also analyzed for quantitative levels of ras p21 by liquid competition RIA (Table 5). Although the percentage of positive cells as detected by MAb RAP-5 with these tissues was different somewhat (MAb RAP-5 was more reactive) from that obtained using MAb Y13-259, the staining patterns and anatomical location of immunoreactivity were almost identical at the single cell level. As shown in Table 5, the quantitative levels of ras p21 generated by RIA generally correlated with the percentage of reactive cells obtained by immunohistochemical assays.

**DISCUSSION**

Enhanced expression of c-ras genes has been demonstrated in certain human tumors utilizing immunohistochemical techniques (17–19), and Western blotting analyses (33, 34), suggesting that increased amounts of c-ras p21 may alter the regulatory controls of cell transformation. Furthermore, elevation of c-Ha-ras RNA in a variety of human tumors has been demonstrated using dot-blot hybridization techniques (35, 36). We have examined expression of c-ras RNA in adenocarcinomas and normal mucosa of the stomach by *in situ* hybridization technique using RNA probes specific for c-Ha-, c-Ki-, or c-N-ras RNA. In fact, under the conditions of assay used, *in situ* hybridization allows a precise differential identification of transcripts of the three c-ras protooncogenes, thus answering a question which cannot be addressed by immunoassays using any anti-ras p21 MAbs currently available. The results obtained by *in situ* hybridization clearly indicate that c-Ha-ras is the c-ras actively transcribed in stomach adenocarcinomas. Therefore the identity of the immunoreactivity detected in this study is c-Ha-ras p21.

**Table 5 Comparison of ras p21 expression by immunohistochemical assays and liquid competition RIA**

<table>
<thead>
<tr>
<th>Adenocarcinomas of the stomach</th>
<th>Immunohistochemical assays (percentage of reactive cells)</th>
<th>Liquid competition RIA (pg p21/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb RAP-5</td>
<td>Poorly differentiated adenocarcinoma with signet ring cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>MAb Y13-259</td>
<td>Well-differentiated tubular adenocarcinoma (2)</td>
<td></td>
</tr>
<tr>
<td>MAb Y13-259</td>
<td>Signet ring cell carcinoma with poorly differentiated adenocarcinoma (3)</td>
<td></td>
</tr>
<tr>
<td>MAb Y13-259</td>
<td>Moderately differentiated tubular adenocarcinoma (4)</td>
<td></td>
</tr>
<tr>
<td>MAb Y13-259</td>
<td>Normal mucosa adjacent to carcinoma (5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5 presents the results obtained by *in situ* hybridization clearly indicate that c-Ha-ras is the c-ras actively transcribed in stomach adenocarcinomas. Therefore the identity of the immunoreactivity detected in this study is c-Ha-ras p21.
The use of MAb RAP-5 on formalin-fixed, paraffin-embedded tissue sections with immunohistochemical techniques allows the qualitative definition of levels of ras p21 expression in a spectrum of malignant, benign and normal tissues at the single cell level. MAB Y13-259 has been utilized in liquid competition RIA using tissue extracts to quantitate levels of c-Ha-ras p21 in malignant and normal tissues of the stomach. Our immunohistochemical results have shown that adenocarcinomas of the stomach are more reactive with MAB RAP-5 than benign and normal tissues, with the majority of carcinomas demonstrating enhanced expression of ras p21. The RIA results also showed that levels of ras p21 in extracts of stomach adenocarcinomas were higher than those from extracts of their normal counterparts. These results, which demonstrate significantly higher levels of ras p21 and c-Ha-ras transcripts in adenocarcinomas of the stomach in comparison to normal and/or benign gastric tissues, suggest that an increase in the expression of c-Ha-ras p21 may be associated with gastric adenocarcinoma.

In a recent study, it was observed that cells of poorly differentiated carcinomas showed a tendency to react less frequently and less intensely with anti-ras p21 MAB (11). The expression of ras p21 has also been compared in the study reported here to the degree of tumor differentiation and depth of invasion. Although carcinomas of the signet ring cell type tended to be less reactive with MAB RAP-5, there was no statistically significant relationship between the degree of tumor differentiation or histological cell type and the levels of ras p21 expression using either immunohistochemical or RIA analysis. This is consistent with the result obtained by in situ hybridization; the labeling indices of c-Ha-ras RNA varied in different tumors, and strongly elevated expression of c-Ha-ras RNA was detected in both poorly and well-differentiated carcinomas.

The immunohistochemical methods reported here reveal that tumors which invaded into the muscularis propria, subserosa, and serosa generally demonstrated higher levels of ras p21 than tumors which were confined within the mucosa or submucosa. Carcinomas of the stomach involving the mucosa and submucosa have been considered early carcinomas, and carcinomas infiltrating to the muscularis propria, subserosa, and serosa have been considered advanced carcinomas with a statistically worse prognosis (37, 38). The data reported here suggest that c-Ha-ras p21 expression may be enhanced in advanced carcinomas of the stomach. There was no difference in ras p21 expression, however, among the advanced carcinomas in relation to tumor invasion to the muscularis propria, subserosa, or serosa, suggesting that in the advanced carcinomas the continuous expression of c-Ha-ras p21 is not necessary for the transformed phenotype of gastric epithelium.

Benign lesions and normal mucosae of the stomach were also examined for ras p21 expression. Although some of the benign lesions were reactive with MAB RAP-5, the percentage of cellular reactivity was much less than that observed in carcinomas. It is of particular interest that dysplastic lesions generally demonstrated stronger reactivity with MAB RAP-5 than non-dysplastic tissues. Stomach dysplasia has been considered pre-cancerous from several histopathological studies (39-41). Takahashi and Iwama (41) demonstrated that the three-dimensional architecture of atypical tubules in gastric adenoma was similar to that of well-differentiated adenocarcinoma. All of three normal mucosal adjacent to carcinoma demonstrated slightly enhanced expression of c-Ha-ras RNA by in situ hybridization. However, the quantitative level (labeling index) of c-Ha-ras RNA was lower than that obtained from carcinoma.

Immunohistochemistry using anti-ras p21 antibodies also demonstrated much less reactivity of normal mucosa compared to reactivity of carcinomas. It is also interesting to note that normal mucosa from patients with stomach adenocarcinoma demonstrated higher levels of ras p21 than those from patients without evidence of carcinoma by immunohistochemical assay. In previous studies, we have also found elevated levels of ras p21 in normal-appearing mammmary glands adjacent to invasive ductal carcinomas (19).

Utilizing liquid competition RIA with MAB Y13-259, we obtained quantitative levels of ras p21 in extracts of normal gastric mucosa ranging from 13.8 to 36.2 pg p21/μg protein. Because parietal cells of normal mucosa reacted with MBs RAP-5 and Y13-259 using immunohistochemistry, one must consider that the 125I-labeled MAB Y13-259 was also recognizing the ras p21 in parietal cells in tissue extracts from normal mucosa. It is also possible that normal stomach epithelium adjacent to carcinoma may be more reactive with 125I-labeled MAB Y13-259, since normal epithelium from patients with adenocarcinoma of the stomach demonstrated slightly enhanced expression of c-Ha-ras p21 using in situ hybridization and immunohistochemical assays.

In conclusion, advanced carcinomas of the stomach demonstrated elevated levels of c-Ha-ras p21 expression, with generally decreasing expression in early stage of stomach carcinomas, dysplastic lesions, and nonneoplastic lesions adjacent to carcinoma, respectively. Our results demonstrate the complementarity of two types of immunoassays using anti-ras p21 MBs and in situ hybridization to define that transformation of the stomach mucosa from the benign to the malignant phenotype is associated with an increase in c-Ha-ras p21 expression. Future studies, most likely defining the levels of expression of other protooncogenes and oncogenes, will obviously be necessary to further define the etiology of stomach carcinoma.

ACKNOWLEDGMENTS

We thank E. Smith and K. Siler for technical assistance, and L. Otby for photographic assistance.

REFERENCES


Enhanced Expression of c-Ha-ras p21 in Human Stomach Adenocarcinomas Defined by Immunoassays Using Monoclonal Antibodies and \textit{in Situ} Hybridization

Noriaki Ohuchi, Patricia Horan Hand, Giorgio Merlo, et al.


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/5/1413

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/47/5/1413.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.