Advances in Brief

Antibody to ras Proteins in Patients with Colon Cancer


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Abstract

The current study examined sera from 160 colon cancer patients and 60 normal individuals to determine whether antibody to mutated p21 ras protein was present. Studies focused on the aspartic acid substitution at amino acid position 12 (denoted D12), one of the most common mutations in colon adenocarcinoma. IgA antibodies directed against mutated p21 ras-D12 peptide were detected in 5% (32%) of 160 colon cancer patients, but only in 1 (2.5%) of 40 normal individuals. The greater incidence of antibody in cancer patients provides presumptive evidence that immunization to the ras proteins occurred as a result of the malignancy. Examination of sera for antibody reactivity to wild-type p21 ras protein (denoted p21 ras-G12) as well as p21 ras proteins bearing the D12, V12, S12, or L61 mutations showed that antibody detected was largely to normal segments of the p21 ras protein. Epitope mapping, using peptide neutralization assays with mutated or normal ras peptides as competitors, demonstrated that in 10 (67%) of 15 sera examined the antibody reactivity to p21 ras-G12 protein was neutralized by peptides near the carboxyl terminus of p21 ras protein, but not by peptides spanning the specific point mutation region. Antibody reactivities correlated with peripheral blood lymphocyte count, but did not correlate with patient age, sex, histology, stage, tumor locus, lymph node metastasis, or serum carcinoembryonic antigen.

Introduction

The ras oncogenes are cancer-related genes that become activated by specific point mutations and can be identified in a wide variety of human malignancies (1, 2). Three ras genes have been defined in the human genome, H-ras-1, K-ras-2, and N-ras (3). These genes encode a highly conserved group of M, 21,000 proteins, denoted as p21 ras. Activation of the ras proto-oncogene occurs most commonly at codon 12 or codon 61 and results in corresponding single amino acid substitutions within the p21 protein. The activating amino acid substitutions impair the GTPase activity of the ras protein and generate constitutively activated signaling complexes with transforming activity (4, 5). Mutated ras proteins are implicated in malignant transformation of many human cancers including approximately 45% of colon adenocarcinomas. The most common specific amino acid substitutions replacing glycine at amino acid position 12 of the wild-type p21 ras protein in colon adenocarcinoma are aspartic acid (13%), valine (15%), cysteine (6%), or serine (4%; Ref. 6). Only a few cases with leucine replacing glutamine at amino acid position 61 have been reported.

Mutated p21 ras proteins are not expressed by normal tissue and thus represent cancer-specific proteins. Mice immunized to whole mutated ras proteins are able to mount a T-cell response that is specific for the mutated segment of the molecule (7, 8). Conversely, immunizing animals with peptides that span the mutated segment can result in the generation of both helper/inducer and cytolitic T-cell responses that recognize mutated protein (9, 10). Recently, it has been demonstrated that T cells from normal individuals can recognize peptides that span the mutated segment of the molecule (11–13). Furthermore, memory T cells, isolated from a patient with a follicular thyroid carcinoma, were found to specifically recognize a ras peptide with a leucine substitution at residue 61 (14), a common mutation in that tumor. Studies in our laboratory examining peripheral blood lymphocytes from patients with pancreatic cancer showed that CD4+ T-cell immunity to the mutated segment of ras protein was present in some patients with pancreatic cancer. Studies focused on the aspartic acid substitution (denoted D12) as the most common mutation. Patients were found in whom T cells responded to both ras D12 peptides and p21 ras-D12 protein (15). Existent specific T-cell reactivity to mutated ras peptides and proteins imply that the patient’s own tumor acts as a source of immunizing protein and that mutated ras proteins can elicit immune responses in humans. Often, helper/inducer T cells and antibodies can respond to the same protein. The current study examined whether antibodies specific to ras proteins exist in sera of patients with colon cancer. Sera from 160 patients with colon cancer and 60 normal donors were examined to evaluate antibody reactivity to p21 ras proteins using ELISA. Studies focused on the aspartic acid substitution (D12). IgA antibodies directed against p21 ras-D12 protein could be detected in colon cancer patients to a substantially greater extent than in normal individuals. However, the majority of antibody detected was to the normal but not the mutated portion of p21 ras proteins. The greater incidence of antibody in cancer patients provides evidence that immunization to the ras proteins occurred as a result of the malignancy. Antibody correlated to peripheral blood lymphocyte counts.
Antibody to ras Proteins in Colon Adenocarcinoma

Whether or not the presence of antibody correlates with outcome has not yet been evaluated.

Materials and Methods

Sera. Sera were collected from 155 patients with newly diagnosed colon cancer, 5 patients with recurrent colon cancer, and 60 healthy normal individuals. One hundred sixteen patients from Yokohama City University Hospital (Yokohama, Japan), and KiK University Hospital (Osaka, Japan), and 44 patients from the University of Washington Medical Center (Seattle, WA), Loyola University Medical Center (Chicago, IL), and Mayo Clinic (Rochester, MN) were studied. Blood (10 ml) from each colon cancer patient or normal individual was obtained with informed consent. Sera were stored at −20°C prior to testing.

p21 ras Proteins. Purified wild-type p21 K-ras-G12 protein and mutated K-ras-D12 protein were purchased from Oncogene Science (Manhasset, NY). These were purified without SDS and BSA. Endotoxin was removed using the endotoxin removal device (END-X B15; Cape Cod, Woods Hole, MA). Mutated H-ras-V12 protein, H-ras-S12 protein, and H-ras-L61 protein were expressed in Escherichia coli and purified in our laboratory. In brief, plasmids capable of expressing p21 ras protein in a eukaryotic host were constructed by cloning a synthetic C-Ha-ras gene into the pGH-L9 plasmid (16, 17). The synthetic gene was altered by cassette mutagenesis to encode one of the three activated p21 ras proteins (18). The transfected bacteria were expanded, and the detergent lysates of transfected bacteria were purified by size on a Sephadex G75 (SG75) column. The fractions containing M, 21,000 ras protein were concentrated by Amicon filtration (PM10). The ras protein was further purified by HPLC (Waters 625 LC System; Millipore, Milford, MA) on a DEAE ion exchange column (Bio-Rad Laboratories, Richmond, CA). Fractions containing ras protein were analyzed using SDS-PAGE (Pharmacia PhastSystem; Pharmacia LKB Biotechnology, Uppsala, Sweden) and Western blotting. The primary antibody was a monoclonal mouse IgG pan-ras antibody (pan-ras Ab3) specific for both mutated and wild-type proteins (Oncogene Science). The fractions containing p21 ras were pooled, dialyzed, sterile filtered, and dried, and endotoxin was removed as previously described. Protein concentration was determined by spectrophotometer (Bio-Rad Protein Assay; Bio-Rad Laboratories). Recombinant myoglobin (M, 19,000; Sigma) was used as negative controls.

Synthetic ras Peptides. Five peptides derived from the amino acid sequence of the wild-type p21 K-ras-G12 protein were constructed and used in the current studies. All peptides are 18 amino acids in length. Two ras peptides, YKLVVGAG-GVGSALT (p125–142), and GETCLLDLDTAGQEYY (p147–164), were constructed to span the specific point mutation region at codon 12 or 61. The other three peptides, DTKQAQDLARSY-GIPFIE (p125–142), KTRQVAFYTLVREIR (p147–164), and EIRTQRLFKSKEEKTG (p162–179) with an α-helix constructing protein molecule surface, are from the carboxyl terminus amino acid sequence of the wild-type p21 K-ras-G12 protein. Peptides were synthesized by Dr. Patrick S. H. Chou (Biopolymer Facility, Department of Immunology, University of Washington) using 9-fluorenylmethoxycarbonyl chemistry in an automated peptide synthesizer (Model 433A; Applied Biosystems, Inc., Foster City, CA) and purified by HPLC.

Serum Antibody Detection Using ELISA. The purified ras proteins were diluted in carbonate buffer (pH 9.6), and 50 μl of a 0.02-μg protein solution were added to Immulon II poly-styrene ELISA plate wells (Dynatech Laboratories Inc., Chantilly, VA) and incubated for 18 h at 4°C. Human sera were diluted to 1:25 in 0.2% casein buffer (1.58 g/liter Tris-HCl, 9 g/liter NaCl, 3.72 g/liter EDTA, 0.2 g/liter Thimerisol, and 0.05% NP40) and rocked for 18 h at 4°C. The monoclonal mouse IgG anti-ras antibody (pan-ras Ab3; Oncogene Science) specific for both mutated and wild-type ras proteins was diluted 1:500 in 0.2% casein buffer as a positive control and incubated in the same manner as the human sera. Excess ras protein was washed six times from each well. Two hundred μl of 0.2% casein buffer without NP40 were added to each well and incubated for 3 h at room temperature for blocking. After washing, 100 μl of the diluted human sera, diluted pan-ras antibody, or buffer alone were added to the appropriate wells and incubated overnight at 4°C. The next day the plates were washed six times. As the secondary antibody, antihuman IgA HRP (Accurate Chemicals, Westbury, NY) diluted 1:500 in 0.2% casein buffer with 0.05% NP40 and 1% mouse sera was added. Antimouse immunoglobulin HRP (Amersham, Arlington Heights, IL) diluted in 0.2% casein buffer with 0.05% NP40 was used for positive control wells. The plates were incubated for 2 h at room temperature. After the incubation the plates were washed six times and developed with equal parts tetramethylbenzidine peroxidase substrate and peroxidase Solution B (Kirkegaard and Perry Laboratory, Inc., Gaithersburg, MD). After 40 min, the development reaction was stopped by the addition of 1 N HCl. Absorbency was read at a wavelength of 450 nm. All evaluations were performed in quadruplicate. The positive antibody control was a monoclonal mouse IgG pan-ras antibody specific for both mutated and wild-type proteins.

Peptide Neutralization ELISA. Sera from 15 patients with detectable antibody to p21 ras-G12 in the previous ELISA study were selected by the level of absorbency, between 0.4 and 1.0, for this study. Patient sera as primary antibody were preincubated for 1 h with or without ras peptides (100 μg/ml) or p21 ras (1 μg/ml) as competitors. Other procedures for the ELISA were as follows as detailed above. The percentage of inhibition of the absorbency by the competitor was calculated.

Antibody Detection Using Western Blotting. Immunoblotting with chemoluminescence was used to verify antibody reactivity. Depending on the stock concentration of the recombinant protein, between 80 and 100 ng purified protein were analyzed in each lane. Briefly, protein was transferred to nitrocellulose (Hybond-C; Amersham) and then blocked in 5% BSA-0.05% NP40 in PBS for 1 h. The blot was incubated with human sera diluted 1:200–1:1000 in 1% BSA-0.1% NP40 in PBS.

The abbreviations used are: HRP, horseradish peroxidase; CEA, carcinoembryonic antigen.
normal p21 ras-D12 proteins. The above results show that serum antibodies were against the mutated D12 segment or responding to mutated p21 K-ras-D12 protein. Also responding to mutated p21 K-ras-D12 protein was examined for antibody reactivity to both mutated p21 K-ras-D12 and normal p21 K-ras-G12 proteins, the results showed that sera from these patients.

Statistical Analysis. The correlation between antibody and p21 ras proteins and clinical parameters were analyzed. Student’s t test for paired and unpaired data was used to compare the statistical significance of the differences between the percentage of patients with and without antibody in relation to age, sex, tumor locus (colon and rectum), histology (well differentiated and poorly differentiated), stage, peripheral blood lymphocyte count, lymph node metastasis, and serum CEA.

Results

Detection of Serum Antibody to p21 ras-D12 Protein in Colon Cancer Patients and Normal Individuals. Normal wild-type ras protein contains glycine at amino acid position 12 (denoted G12). Initial studies focused on detection of antibody reactivity to the aspartic acid substitution (D12), as one of the most common mutations in colon cancer. Sera from 160 colon cancer patients and 40 normal individuals were tested for IgA antibody reactivity to p21 K-ras-D12 proteins using an ELISA. IgA antibodies directed against p21 ras-D12 protein were detected in 51 (32%) of 160 colon cancer patients, but only in 1 (2.5%) of 40 normal individuals using a cutoff of 3 SDs above the mean of normal individuals as the definition of a positive response (Fig. 1). The greater incidence of IgA antibody to p21 K-ras-D12 protein in sera of colon cancer patients provides evidence that immunization to the ras proteins occurred as a result of the malignancy.

ELISA results were verified by Western blotting analysis in a selected subset of patients. Sera from five colon cancer patients who had the antibody reactivity to the p21 K-ras-D12 proteins (absorbency from 0.66 to 1.62) in ELISA were compared to sera from five colon cancer patients and five normal individuals who had no detectable antibody reactivity (absorbency less than 0.03). The results of immunoblot showed that the serum antibody reactivity to p21 ras-D12 in the cancer patients was as marked as that of a pan-ras mAb, whereas colon cancer patients with no antibody in ELISA and the normal individual showed no detectable evidence of antibodies to p21 ras-D12 (data not shown).

For Most Colon Cancer Patients, Serum Antibodies Responding to Mutated p21 ras-D12 Protein Also Respond to Normal p21 ras-G12 Protein. The above results show that serum antibody to mutated p21 ras-D12 protein can be detected in sera of colon cancer patients. Whether or not the detected serum antibodies were against the mutated D12 segment or nonmutated segments of the p21 ras-D12 protein was examined. Sera from the same colon cancer patient population were examined for antibody reactivity to both mutated p21 K-ras-D12 proteins and to normal wild-type p21 K-ras-G12 proteins using an ELISA. Results (Table 1) show that 41 (26%) of 160 patients exhibited detectable antibody reactivity to both the p21 K-ras-D12 and p21 K-ras-G12 proteins. Sera from 10 (6%) of 160 colon cancer patients responded to mutated p21 K-ras-D12 protein, but not to the normal p21 K-ras-G12 protein. Antibody response to the normal wild-type p21 K-ras-D12 protein but not to the mutated p21 K-ras-D12 protein was detected in only 7 (4%) of 160 colon cancer patients, and responses were low by absorbancies (Table 1).

Antibody from a Small Percentage of Patients Responds Only to Mutated p21 ras Proteins. The above data show that antibody in the majority of colon cancer patients recognizes normal p21 K-ras-G12 protein. In only a small number of colon cancer patients was the serum antibody reactivity against only the mutated p21 ras-D12 protein (Table 2). To determine specific antibody reactivity to other mutated p21 ras proteins, we retested sera from 142 of the 160 colon cancer patients (adequate sera was not available from 18 patients) as well as from 60 normal individuals. ELISAs were performed with a larger panel of proteins, including four additional ras proteins (p21 H-ras-G12, ras-V12, ras-S12, and ras-L61 proteins) and myoglobin as negative controls. Sera from 9 (6%) of 142 patients who had antibody reactivity to only mutated p21 K-ras-D12 responded specifically to p21 ras-D12 protein, but not to normal p21 ras-G12 protein or other mutated p21 ras-V12, S12, and L61 proteins as well as myoglobin. A representative patient is shown in Fig. 2A. Thus, the epitopes recognized by the p21 ras-D12 protein-reactive antibody are most likely directed against the mutated D12 segment of the p21 ras-D12 proteins. In sera from 41 colon cancer patients who had antibody reactivity to both mutated p21 K-ras-D12 and normal p21 K-ras-D12 proteins, the results showed that sera from these patients...
Antibody to ras Proteins in Colon Adenocarcinoma

Table 1 For most patients, antibody that responds to mutated p21 K-ras-D12 protein also responds to normal p21 K-ras-G12 protein

<table>
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<th>Antibodies to p21 K-ras protein</th>
<th>% of positive patients</th>
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<td>D12 mutations</td>
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<tr>
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*a* Sera from 160 colon cancer patients were examined for antibody responses to both mutated p21 K-ras-D12 proteins and to normal wild-type p21 K-ras-G12 protein using an ELISA, as described in "Materials and Methods." The definition of a positive antibody response was determined using a cutoff of 3 SD above the mean of normal individuals.

Antibody Responses to p21 ras Proteins in Colon Cancer Patients Can Be Blocked by Peptides from the Carboxyl Terminus of p21 ras Protein. The above data show that the antibody reactivity to p21 ras proteins in the majority of responding patients was directed against normal common segments rather than mutated segments. Sera from 15 patients who had the antibody reactivity to both the wild-type and mutated p21 ras proteins were further tested using the peptide neutralization assay. Five 18-mer ras peptides (p4-21, p48–65, p125–142, p147–164, and p162–179) were used. The results (Fig. 3) demonstrated that in 10 (67%) of 15 antibodies, reactivity to p21 ras protein was inhibited more than 25% by either peptide p147–164 or p162–179 near the carboxyl terminus of the p21 ras protein. None of the reactivity in these patients was inhibited more than 25% by peptide p4–21 or p48–65, which span the hot point mutation region at codons 12 or 61. Thus, epitopes of antibodies to the normal portion of p21 ras protein in some colon cancer patients were localized near the carboxyl terminus of the p21 ras protein.

Analysis of the Correlation between Serum Antibody and the Patient Characteristics and Clinical Parameters. The existence of antibody to p21 ras protein was analyzed in relation to a variety of clinical parameters available retrospectively. Antibody responses did not correlate with patient age, sex, locus, histology, stage, lymph node metastasis, or serum CEA (P > 0.05). Detection of antibody reactivity to p21 ras proteins correlated with the peripheral blood lymphocyte count. Antibody was detected in 2 (16%) of 13 patients with a lymphocyte count of <1000, and in 4 (55%) of 7 patients with a lymphocyte count >3000 (P < 0.05). However, data were not available for all patients. Prospective studies with more patients are needed to validate the conclusions concerning clinical correlations. Data concerning the presence or absence of specific ras mutations in tumors and clinical outcome were not available for analysis, but should be studied prospectively.

Discussion

ras oncogenes are activated by point mutations that result in the expression of p21 ras proteins with single-substituted amino acids, altered tertiary structure, and transforming activity (1, 2, 4, 5). ras proteins have been studied as a potential tumor-specific antigen because ras mutations are common in human malignancy, and the common mutations are limited in number and location in the molecule. Importantly, the common mutations present in human malignancy are also commonly observed in murine malignancies, so that results in murine models can be extrapolated to humans. Immunity to ras can be elicited in animal models by immunization to ras peptides and protein. Our previous animal studies have shown that under well-defined circumstances, murine CD8+ CTLs and CD4+ helper T cells specific for the mutated segment of p21 ras protein can be generated. Immunizing animals with peptides that span the mutated segment can result in the generation of helper/inducer and cytolytic T-cell responses that recognize intact mutated protein (9, 10). CTLs specifically immune to mutated ras peptides can lyse cells transformed by the ras protein with the same mutation (10). Thus, ras protein represents a tumor-specific antigen.

The observation that p21 ras protein can be immunogenic in mice stimulated the current studies to determine whether immunity to p21 ras protein is present in patients with colon cancer. The current study showed that 32% of a panel of colon cancer patients had IgA antibodies against ras proteins. The greater incidence of antibody in cancer patients provides strong evidence that immunization to the protein occurred as a result of the malignancy. Immunoglobulin class switching from IgM to IgA or IgG generally requires T cell help, which is usually cognate. Cognate help means that B cells and T cells recognize different epitopes, but that the epitopes are on the same molecule. The antibody response to p21 ras detected in colon cancer patients was an IgA response, therefore, T cell help was most probably involved. The major antibody response to p21 ras is to epitopes located in the nonmutated COOH terminus segment rather than to the mutated segments that are unique to the mutated ras protein. Only a small number of colon cancer patients had a detectable antibody response to only the mutated ras-D12 proteins as compared to a panel of mutated or wild-type ras-G12 proteins. One issue is whether the T cell help considered necessary for immunoglobulin class switch is to mutated or nonmutated segments. Helper T-cell responses specific for the mutated portion of ras proteins have been elicited in animal models. T-cell responses to nonmutated segments have not been studied in animal models. Human T-cell studies by our group and others have shown that human CD4+ T cells are capable of
Fig. 2  Antibody from a small percentage of colon cancer patients responds specifically to mutated p21 ras-D12 proteins. Sera from 142 colon cancer patients and 60 normal individuals were examined for specific IgA antibody responses to mutated p21 K-ras-D12 in an ELISA, as described in "Materials and Methods." A panel of normal or mutated p21 ras proteins and myoglobin was used as control proteins. Sera from 9 (6%) of 142 patients responded to only mutated p21 K-ras-D12 protein. A, representative patient. Sera from 41 of 142 colon cancer patients who had antibody responses to mutated p21 K-ras-D12 responded to all of the mutated or normal ras proteins. B, representative patient. The definition of a positive antibody response was determined using a cutoff of 3 SDs above the mean of normal individuals.

recognizing ras proteins in a mutation-specific manner (12-15). T cells specifically recognizing mutated ras peptides have been isolated from patients with follicular thyroid carcinoma and colorectal cancer (14, 19-22). Thus, although the majority of antibody detected was to nonmutated portions of ras protein, the T cell help may be to the mutated segment in some patients.

To elicit antibody responses and CD4+ T cell help for antibody responses, ras protein must access the immune system. Normally, p21 ras protein is affixed to the inner aspect of the cell membrane and is thus not considered to be a secreted protein. However, p21 ras can be readily detected in vivo under a variety of circumstances (23), including presence in the extracellular tumor environment (24). Thus, p21 ras protein at the site of tumor deposition is likely to be available in the external cell environment to be processed and presented by autologous APC expressing class II MHC antigens, including B cells with immunoglobulin receptors specific for ras protein.

Although human antibody reactivity to ras proteins in cancer patients has not been previously reported, the formation of antibodies directed against p53, HER-2/neu, and other onco- genic proteins have been described (24-33). Whether the detection of antibody to p21 ras could serve as a surrogate marker for tumors expressing ras mutations is still being evaluated. The ras mutations of individual patient tumors has not been deter-
Antibody to ras Proteins in Colon Adenocarcinoma

...of antibody specific for mutated ras has not been ruled out in a minority of patients. Although the majority of antibodies were to the nonmutated segment of ras, antibody responses to the nonmutated segment may provide a method to identify individuals who have been exposed to mutated p21 ras protein and might yet be shown to provide an early indication of incipient or occult malignancies undetectable by conventional methods.

The correlation of antibody formation to prognosis is not known and should be evaluated. Investigations to determine whether antibodies to other oncogenic proteins correlate to prognosis or other important disease parameters are still in their initial phases. Initial studies correlating antibody to p53 to prognosis of lung cancer have demonstrated that the presence of antibody to p53 did not correlate with survival (34). Other reports have suggested antibody formation to p53 is a poor prognostic indicator in breast cancer (35). However, it is not clear whether antibody to p53 or the presence of mutant p53 is the primary cause of a poor prognosis. The examination of antibody to ras might provide important information in similar studies. Mutant K-ras in colon cancer does not predict the patient’s response to chemotherapy or survival (36). Thus, antibody to ras might be more easily studied as an independent variable. In the current studies, the evaluation of the clinical correlation of antibody existence showed that antibody reactivity did not correlate with patient age, sex, histology, stage, or serum CEA, but did correlate with lymphocyte number in peripheral blood drawn before operation. Lymphocyte number and antibody reactivity were evaluated at only one point in time, and data were not available on all patients. Studies with larger numbers of patients are needed to validate the conclusions concerning clinical correlations. Whether changes occur serially and whether serial changes in antibody levels can be used as indicators of changes in disease status also should be evaluated in prospective studies.

Acknowledgments

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References


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