Expression of p21*ras*-related protein in the plasma and tissue of patients with adenomas and carcinomas of the colon

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Over-expression of p21*ras*-related protein was determined in the plasma by immunoblotting and in the tissue by immunohistochemistry in a cohort of patients undergoing colonoscopy. In the plasma samples, p21*ras* over-expression was detected in: 9% (4/47) of normal controls; 21% (13/61) of individuals with normal colonoscopies but with a prior history of colonic neoplasia; 12% (4/33) of small adenoma patients; 29% (6/21) of large adenoma patients; 63% (5/8) of carcinoma-in-adenoma patients; 50% (2/4) of Dukes' A carcinoma patients; and 20% (2/10) of Dukes' B-D carcinoma patients. In the tissue samples, p21*ras* over-expression was detected in: 25% (2/8) of small adenoma patients; 44% (4/9) of large adenoma patients; 100% (4/4) of carcinoma-in-adenoma patients; and 33% (1/3) of Dukes' B-C carcinoma patients. For matched plasma-tissue pairs, there was a statistically significant correlation for p21*ras* over-expression ($R = 0.47$, $p = 0.02$).

Keywords: p21ras, plasma biomarker, immunohistochemistry, colonic adenomas, colonic carcinomas.

Introduction

The *ras* family of oncogenes is frequently found to be activated in many common human malignancies (Barbacid 1987). This activation causing cellular transformation can occur by one of two mechanisms: point mutations at selected codons of the gene leading to the expression of mutant forms of the encoded p21 protein product in cells, or over-expression of the non-mutated gene leading to the accumulation of increased amounts of the normal p21 protein in cells. In colonic carcinogenesis, for example, intracellular increases of p21*ras* have been frequently identified in the tumour tissue of adenomas and carcinomas (Thor *et al.* 1984, Gallick *et al.* 1985, Lanza 1988, Jansson *et al.* 1990, Miller *et al.* 1992).

Using monoclonal antibodies directed against the p21*ras* proteins, it has been demonstrated that cells in culture that over-express the *ras* gene produce increased amounts of the p21*ras* protein not only inside the cells but also in the extracellular supernatant (Brandt-Rauf 1991). Thus, if such cells are inoculated into mice and allowed to grow into tumours, increased amounts of the p21*ras* protein can be detected immunologically in the blood of the animals (Kakkanas and Spandidos 1990). Similarly, increased amounts of the p21*ras* protein have been detected immunologically in the blood of a number of cancer patients with different types of malignancies that could be associated with increased intracellular expression of p21*ras*, although this has not been directly demonstrated in the tissue in these cases (Epelbaum *et al.* 1989, Kakkanas and Spandidos 1990, Brandt-Rauf *et al.* 1992, Perera *et al.* 1992, Weissfeld *et al.* 1994).

As noted, p21*ras* has been shown to accumulate in some colonic tumours. We therefore examined the expression of p21*ras* in the plasma and tissue of colonoscopy patients with newly diagnosed adenomas and carcinomas to determine if plasma over-expression is detectable in such patients and whether plasma over-expression is correlated with tissue over-expression.

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METHODS

The study population was drawn from a previously described cohort of patients who underwent colonoscopy at Columbia-Presbyterian Medical Center between May, 1988 and September, 1991 (Luo *et al.* 1995). All colon carcinoma cases diagnosed at colonoscopy during this time period were included ($n = 22$). A random selection of subjects from the remainder yielded the following two groups: individuals with newly diagnosed colonic adenomas ($n = 54$) and individuals with normal colonoscopic examinations ($n = 108$). The latter were further subdivided into individuals with normal colonoscopic examinations but with a prior history of colonic carcinoma or colonic adenoma ($n = 61$) and individuals with normal colonoscopic examinations and no prior history of neoplasia ($n = 47$); this was done since individuals with a prior history of colonic neoplasia are believed to be at higher risk for a second neoplasm (Wanebo 1993), and, thus, they might be different in terms of their p21 expression.

Clinical data collected on each of these patients included age, sex and race. The subgroups of patients were similar in terms of age, sex and race, except that the adenoma patients were predominantly male (61%) while the reverse was true for the carcinoma patients (41% male) and the normal controls (38% male), as previously described (Luo *et al.* 1995). For individuals with adenomas, the size of the lesion (diameter in mm) and the site of the lesion (right colon vs left colon vs rectum), and for individuals with carcinomas, the clinical stage (carcinoma-in-adenoma or invasive carcinoma by Dukes' classification), the histological grade of the lesion (well-differentiated vs moderate-poorly differentiated), and the site of the lesion (right colon vs left colon vs rectum) were also determined.

Blood samples for all the subjects were obtained immediately prior to colonoscopy by routine venipuncture techniques, and the plasma was separated by centrifugation and stored frozen at –70 °C until the time of analysis. Formalin-fixed, paraffin-embedded tumour tissue samples were available for 17 of these patients (7 carcinoma cases and 17 adenoma cases).

Plasma samples were analysed blind to the clinical status of the individual for the presence of p21*ras* protein by immunoblotting with the mouse monoclonal antibody 142-24E05 raised against a synthetic peptide corresponding to amino acid residues 96–118 of human H-, K- and N-*ras* (Perera *et al.* 1990, Brandt-Rauf *et al.* 1992). The 21 kDa bands were quantitated by volumetric integration of optical density using a laser densitometer, and positive elevations of p21*ras* were defined as any integrated optical density greater than the mean plus two standard deviations of the integrated optical densities of such bands in the plasma of 50

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normal, healthy, non-smoking volunteers, as described previously (Perera *et al.* 1992).

Five-micron tissue sections were analysed blind to the plasma status of the individual for the presence of p21*ras* protein by immunohistochemistry with the same mouse monoclonal antibody 142-24E05. After routine deparaffinization and graded rehydration, tissue sections were equilibrated with phosphate buffered saline (PBS), pH 7.4, and then blocked (with 0.25% bovine serum albumin, 1.5% normal horse serum in PBS) for 1 h at room temperature. The sections were then incubated overnight with the primary antibody at 1:4000 dilution at 4 °C. After washing with PBS, biotinylated goat anti-mouse immunoglobulin (Vectastain; Vector Laboratories, Burlingame, CA) was applied for 30 min at room temperature. The sections were washed with PBS and incubated with a streptavidin–alkaline phosphatase complex solution (Vestastain) for 1 h at room temperature. The sections were washed again with PBS and then incubated with CAS Red Chromogen solution (Cell Analysis Systems, Elmhurst, IL) for 20 min at room temperature. The sections were counter-stained with Feulgen's (Cell Analysis Systems), cover-slipped and analysed for p21*ras* immunostaining using the CAS 200 computerized imaging system (Cell Analysis Systems). For the analysis, a similarly stained NIH 3T3 cell line with a known constant amount of normal p21*ras* protein per cell (2.37 fg per cell) (Hand *et al.* 1987) was used for calibration. Each tissue section was analysed in 10 randomly selected fields with p21 content calculated in comparison to the calibration cell line corrected for DNA content based on the Feulgen staining. Positive elevations of p21*ras* were defined as any tissue section with average p21 content from 10 fields greater than the control cell line.

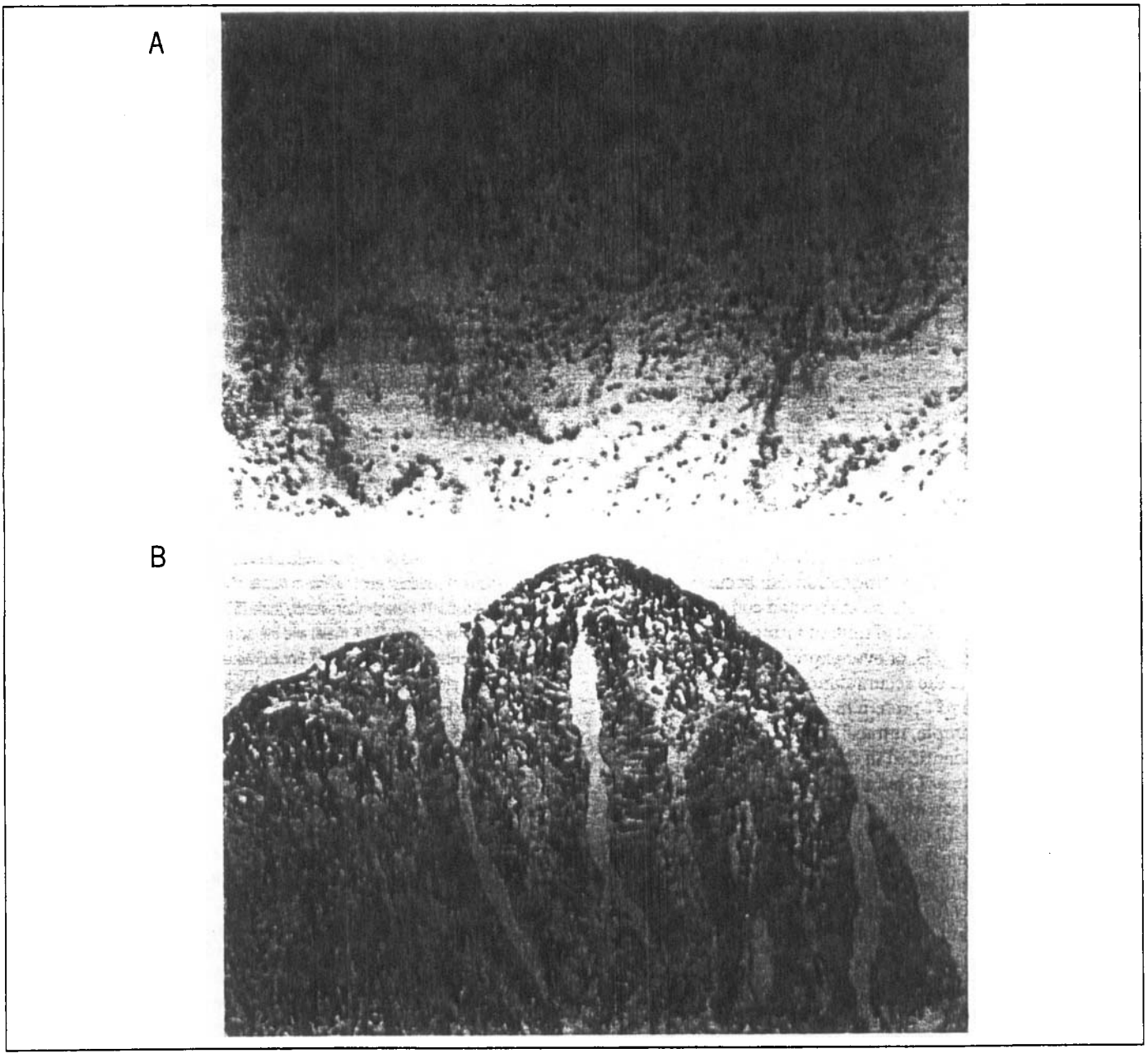

Figure 1. Representative immunohistochemistry of p21*ras*-negative (A) and p21*ras*-positive (B) colonic tumours probed with mouse monoclonal antibody 142-24E05 raised against a synthetic peptide corresponding to p21 residues 96–118 and with a secondary antibody (goat anti-mouse)–streptavidin–alkaline phosphatase detection system (200×; Feulgen's counterstain).

For the proportion of positive plasma elevations of p21*ras* in each subgroup of patients, the odds ratio and 95% confidence intervals were calculated (adjusting for age, sex and race and using the normal controls as reference), and a χ^2 for linear trend was determined, with a *p* value less than 0.05 considered to be significant. Tissue p21*ras* expression (positive or negative) was compared with plasma p21*ras* expression (positive or negative) by calculating the correlation coefficient for concordance for the 24 patients with matched pairs of tissue and plasma samples, with a *p* value less than 0.05 considered to be significant.

Results

The results are presented in Figures 1 and 2 and Table 1. The percentage of samples positive for plasma p21*ras* over-expression was 40.9% in the carcinoma cases, 18.5% in the adenoma cases, 21.3% in the cases with normal colonoscopies but a prior history of colonic neoplasia and 8.5% in the normal controls; the percentage of positives among the carcinoma cases was statistically significantly greater than the normal controls ($p = 0.003$). Among the patients with lesions, the percentage of positive samples increased from 12.1% in small adenoma patients to 28.6% in large adenoma patients to 62.5% in carcinoma-in-adenoma patients and then decreased in the Dukes' A (50%) and Dukes' B-D (20%) patients; compared with the normal controls, the percentage of positives was statistically significantly greater in the large adenoma patients ($p = 0.014$), the carcinoma-in-adenoma patients ($p = 0.016$) and the Dukes' A patients ($p = 0.005$). Using the normal controls as the comparison group with an assigned odds ratio of 1, the adjusted odds ratios (and 95% confidence intervals) for the other groups were: normal colonoscopies with history of prior colonic neoplasia, 3.11 (0.91, 10.6); small adenomas, 0.83 (0.13, 5.17); large adenomas, 4.58 (1.36, 15.42); carcinoma-in-adenoma, 11.3 (1.58, 80.8); Dukes' A, 22 (2.53, 191.3); Dukes' B-D, 3.75 (0.47, 29.56). The χ^2 for linear trend from small adenoma to large adenoma to carcinoma-in-adenoma to Dukes' A to Dukes' B-D was statistically significant ($\chi^2 = 6.4$, $p = 0.011$). Among the carcinoma patients, there was a higher percentage of positive plasma p21*ras* over-expression in the moderate/poorly differentiated cases (37.5%) compared with the well differentiated cases (16.7%), but this difference was not statistically significant. Also, no significant differences in plasma p21*ras* over-expression were noted in relation to the site of the carcinoma or adenoma (right colon vs left colon vs rectum).

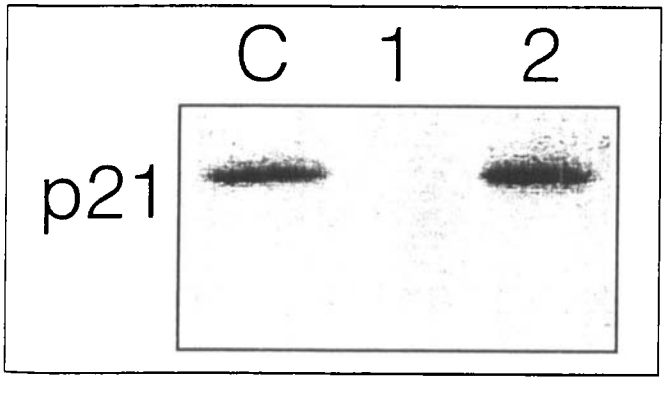

Figure 2. Representative immunoblots of p21*ras*-negative plasma (Lane 1), p21*ras*-positive plasma (Lane 2), and positive control (5 ng of purified, human, recombinant H-*ras* p21; Lane C) probed with mouse monoclonal antibody 142-24E05 and with a secondary antibody (horse anti-mouse)–avidin–biotin–peroxidase detection system.

Diagnosis	Plasma p21*ras* positives (%)	Tissue p21*ras* positives (%)
Carcinoma	9/22 (40.9%)*	5/7 (71.4%)
In Adenoma	5/8 (62.5%)*	4/4 (100.0%)
Dukes' A	2/4 (50.0%)*	–
Dukes' B-D	2/10 (20.0%)	1/3 (33.3%)
Adenoma	10/54 (18.5%)	6/17 (35.3%)
Small (< 10 mm)	4/33 (12.1%)	2/8 (25.0%)
Large (≥ 10 mm)	6/21 (28.6%)*	4/9 (44.4%)
Normal with prior neoplasia	13/61 (21.3%)	–
Normal controls	4/47 (8.5%)	–

Table 1. Plasma and tissue p21*ras* over-expression in colonoscopy patients.
Key: Statistically significantly elevated compared with normal controls ($p < 0.05$).

From the immunohistochemistry results, the mean tissue p21*ras* level in 17 adenoma patients was 2.14±0.63 fg per cell (range = 1.06–3.54 fg per cell) with 6 of 17 (35.3%) considered positive for over-expression. Among these adenoma patients, the p21*ras* levels were lower (2.06±0.82 fg per cell; 25% positive) in eight patients with small adenomas compared with levels (2.22±0.44 fg per cell; 44.4% positive) in nine patients with large adenomas, but the differences were not statistically significant. The mean tissue p21*ras* level in seven carcinoma patients was 3.92±1.83 fg per cell (range = 1.67–6.24 fg per cell) with five of seven (71.4%) considered positive for over-expression. Among these carcinoma patients, the p21*ras* levels were higher (4.92±1.40 fg per cell; 100% positive) in four carcinoma-in-adenoma patients compared with levels (2.58±1.55 fg per cell; 33.3% positive) in three patients with Dukes' B-C stage carcinomas, but the differences were not statistically significant. As a group, these carcinoma patients did have statistically significantly higher tissue p21*ras* levels than the adenoma patients ($p = 0.043$), and the percentage of samples positive for p21*ras* over-expression was higher in the carcinoma patients compared with the adenoma patients but not to a statistically significant degree ($p = 0.1$).

The percentage of samples positive for p21*ras* tissue over-expression increased from 25% in small adenoma patients to 44.4% in large adenoma patients to 100% in carcinoma-in-adenoma patients and then decreased to 33.3% in the Dukes' B-C stage patients in a pattern quite similar to the percentage positive for plasma over-expression in these categories. Furthermore, 17 of 24 (71%) plasma-tissue pairs were concordant for p21*ras* over-expression (positive in both or negative in both) and only 7 of 24 (29%) were discordant (positive in one and negative in the other). This resulted in a statistically significant correlation between positive p21*ras* over-expression in tissue and plasma ($R = 0.47$, $p = 0.02$).

Discussion

The observation of elevated levels of p21*ras* protein in the plasma and tissue of patients with colonic adenomas and carcinomas is consistent with prior studies of p21*ras* accumulation in colonic tumour tissues. For example, immunoblot analysis of tissue lysates from colon cancers demonstrated elevated levels of p21*ras* in 9 of 17 (53%) cases in one study (Gallick *et al.* 1985). Increased p21*ras* expression detected immunohistochemically has also been reported in a number of studies of colon tumours including: 47 of 145 (32%) adenomas and 54 of 70 (77%) carcinomas (Lanza 1988); 0 of 4 (0%) adenomas and 23 of 47 (49%) carcinomas (Thor *et al.* 1984); and 4–6 of 6 (67–100%) adenomas and 38–64 of 83 (45–77%) carcinomas, depending on the particular antibody employed (Jansson *et al.* 1990). In a more recent study, increased p21*ras* expression was determined immunohistochemically with the monoclonal antibody 142-24E05 (the same antibody as used in this study) in 82 of 118 (69%) colon carcinomas (Miller *et al.* 1992). These results are in excellent agreement with the 71% tissue-positives and 41% plasma-positives among carcinomas and 35% tissue-positives and 19% plasma-positives among adenomas in the present study. In addition, in the present study, the positive percentage rate for tissue and plasma tended to increase from small adenomas to large adenomas to carcinoma-in-adenomas and then to decrease in more invasive carcinomas. This is also consistent with prior tissue studies in which p21*ras* over-expression was felt to be a relatively early phenomenon in colonic carcinogenesis that tended to decrease with invasion and metastatic progression (Gallick *et al.* 1985, Lanza 1988, Jansson *et al.* 1990).

Prior studies have also reported elevated levels of p21*ras* by immunoblotting in the serum and plasma of various cancer patients, including those with colon cancer, in up to 67.5% of cases (Weissfeld *et al.* 1994). Furthermore, we have previously reported the discovery of increased p21*ras* in serum in an individual up to 18 months prior to the clinical detection of a colonic adenoma, and serum levels of p21*ras* returned to normal following surgical resection of the lesion in this patient, suggesting that the tumour was the source of the elevation of the serum protein (Brandt-Rauf *et al.* 1990). The high tissue-positive and plasma-positive rates and the statistically significant correlation between tissue and plasma results in the present study support the contention that increased p21*ras* expression is a relatively common occurrence in colonic neoplasia (even at the adenomatous stage) that can be detected by analysis for the protein in the blood in many patients. It should be noted, however, that in this study for each category plasma-positive rates tended to be lower than tissue-positive rates and several patients had discordant results between tissue and plasma over-expression. It is possible that not all tumours that over-express p21*ras* release increased amounts into the extra-cellular environment and that factors other than immunohistochemical staining intensity may be important in determining plasma levels in some cases. For example, one small (3 mm) tissue-positive adenoma case may have been plasma-negative because the overall tissue burden of p21*ras* contributed by such a small lesion may have been insufficient to cause a detectable increase in the plasma level. Further study will be necessary to define these and other potentially confounding factors.

Finally, although not statistically significant, the increased plasma-positive rate among individuals with normal colonoscopies but a prior history of colonic neoplasia compared with individuals with normal colonoscopies but no such history is intriguing. This is consistent with the previous report of enhanced p21*ras* expression in the non-neoplastic tissue adjacent to colonic tumours, suggesting that p21*ras* over-expression might be involved in a 'field cancerization' effect (Jansson *et al.* 1990). As noted, individuals with a prior history of colonic neoplasia are believed to be at higher risk for a second colonic neoplasm. It is tempting to hypothesize that the individuals in this subgroup in this study who are presumably subject to the 'field cancerization' effect of p21*ras* over-expression (as evidenced by their increased plasma levels) are the patients most likely to develop a second colonic neoplasm. On-going follow-up of this cohort will help to resolve this issue and to demonstrate the potential predictive value of such plasma biomarkers. Studies of p21*ras* over-expression in the banked serum samples of individuals who subsequently developed lung cancer in which samples were positive years prior to the clinical diagnosis of disease (Brandt-Rauf *et al.* 1992) provide further support for this hypothesis and indicate that further research is warranted.

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