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ras p21 Expression in Relation to DNA Ploidy, S-Phase Fraction and Prognosis in Colorectal Adenocarcinoma

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***ras* p21 expression, as indicated by the monoclonal antibody ras 11, was estimated using immunohistochemistry on 69 primary colorectal adenocarcinomas. Also, DNA ploidy and S-phase fraction (SPF) were analysed with flow cytometry. Positive staining for ras 11 tended to be more common in DNA non-diploid tumours ($P = 0.11$), but was significantly correlated with high SPF ($P = 0.038$). Positive ras 11 staining, Dukes' stage, DNA ploidy and SPF were related to the recurrence-free interval of patients with Dukes' A–C tumours ($P = 0.0014$, $P = 0.023$, $P = 0.035$ and $P = 0.040$, respectively). ras 11 staining was a prognostic factor independent of both Dukes' stage and DNA ploidy ($P = 0.011$). The results indicate that pan *ras* p21 expression is associated with proliferative activity and has an independent prognostic value in colorectal adenocarcinoma.**
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INTRODUCTION

RECENT ADVANCES in molecular genetics suggest that the proto-oncogenes may be activated and contribute to tumour transformation through rearrangements, point mutations, amplification and possibly other genetic mechanisms [1, 2]. The oncogenes most frequently detected in human tumours belong to the *ras* gene family, which code for a group of 21 000 dalton proteins, designated p21s. ras p21 is located on the inner surface of the plasma membrane, binds guanine nucleotide and has GTPase activity. Similarly with other GTP-binding proteins, it may serve as a transducer molecule for signals affecting cell proliferation and thus be involved in the events of the cell cycle [1–7]. *ras* p21 expression, DNA content and S-phase fraction (SPF) indicate the existance of chromosomal abnormalities. They can also give us an insight into DNA replication and the biological behaviour of the tumours. Previous analyses of colorectal tumours using flow cytometry [8–10] and immunohistochemistry [11] have suggested that DNA ploidy, SPF and high levels of pan ras p21 indicated by ras 11 are prognostic factors. However, relations between DNA ploidy, SPF and ras 11 immunostaining, to our knowledge, have not been reported.

In the present work, ras 11 staining was investigated by using immunohistochemistry, DNA ploidy and SPF were analysed by flow cytometry in a series of colorectal adenocarcinomas in order to assess the relationships between these factors and their prognostic value.

MATERIALS AND METHODS

Material was collected from the Linköping health care region for 69 patients with primary colorectal adenocarcinomas diagnosed between 1972 and 1986. None of the patients had received preoperative treatment. At diagnosis, 5 cases were stages as Dukes' A, 35 as Dukes' B, 17 as Dukes' C, and 7 as Dukes' D. The stage was not recorded in 5 cases. Among the 57 patients with limited disease, 18 recurrences were observed during a follow-up period ranging from 5 to 161 months.

Immunohistochemistry

ras 11 is a mouse IgG_{2b} monoclonal antibody which detects both normal and mutated forms of ras p21. This antibody is commercially available.

Immunohistochemical staining was performed on paraffin-embedded tumour tissue using a modification of the method of Sternberger [12], this is described in detail elsewhere [11]. Briefly, serial 5 μm sections were deparaffinised in xylene and rehydrated through a series of ethanols. 1% hydrogen peroxide in methanol was added to block endogenous peroxidase activity. After rinsing in phosphate-buffered saline (PBS) adjusted to pH 7.4, non-specific binding was blocked by preincubating the tissue sections with a 10% normal rabbit serum. After the removal of the blocking solution, sections were incubated with the primary mouse monoclonal antibody ras 11 (Du Pont, Scandinavia AB, Stockholm, Sweden) for 90 min at room temperature. Subsequent incubation with peoxidase-conjugated rabbit anti-mouse immunoglobuline and PAP (Dakopatts, Glostrup, Denmark), each lasted for 30 min. The slides were washed in PBS between each incubation step. The peroxidase reaction was then performed using 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma) and 0.02% hydrogen peroxide for 8 min. The sections were counterstained with haematoxylin for 1 min, dehydrated in a series of ethanols, cleared in xylene and then mounted under a coverslip. In each run, control tissue sections were included where isotype identical monoclonal antibody MOPC-141 (Sigma) and PBS replaced the primary antibody, respectively. None of the negative controls showed positive staining. The immunohistochemical staining in each case was graded as follows; negative reaction (−) if less than 5% of the tumour cells were positive, moderate reaction (+) if 5–50% of the tumour cells were positive, and marked reaction (++) if

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more than 50% of the tumour cells were positive. The slides were examined independently by two pathologists, without knowledge of the clinical and flow cytometric data; there was minimal disagreement in their scoring.

Flow cytometry

50 μm sections from the same paraffin-embedded tissue used for the peroxidase antiperoxidase (PAP) method were deparaffinised with xylene, then dehydrated stepwise in 99.5, 95, 70 and 40% ethanol. Finally, they were washed twice in distilled water. The samples were treated with 0.4% trypsin (Sigma) in a citrate buffer for 24 h in a 37°C shaking waterbath. After filtration through a nylon mesh, the suspension was stained with propidium iodide [13, 14].

The cell suspensions were analysed with a FACScan flow cytometer (Becton-Dickinson). Histograms including 10000 cells, and DNA peaks with a mean coefficient of variation of 6.5% were recorded. Normal diploid cells from the same specimens were used as internal controls. Diploid tumour populations were defined as having a single $G_{0/1}$ peak. Tumours were considered aneuploid if there was evidence of more than one distinct $G_{0/1}$ peak. SPF was estimated by using a rectangular model. The number of S-phase cells was calculated by multiplying the number of channels between the $G_{0/1}$ and G_2/M peaks by the mean number of cells per channel in a part of the S-phase interval judged as representative by the operator. Small disturbing peaks in the S-phase region could be excluded when the SPF was calculated. The SPF was divided into three categories: <5%, 5–10% and >10%. A simple method for background correction was used by subtraction of a constant estimated in each of the histograms. The mean number of registrations per channel in an area well separated and to the right of the G_2/M peak was calculated and substracted from the mean number of registrations per channel in the S-phase interval.

Statistical analysis

Relationships between *ras* p21 expression and DNA ploidy were tested by means of a χ^2 test for contingency tables with ordered categories [15]. The association between ras 11 immunostaining and SPF was tested using linear regression analysis [15]. Cox's proportional hazards model was used to estimate and test the influence of *ras* p21 expression, Dukes' stage, DNA ploidy and SPF on prognosis [16]. In the linear regression models as well as in the Cox regression models, SPF was treated as a continuous variable. However, when illustrating the relationships, SPF was classified into three categories, i.e. <5%, 5–10 % and >10%. In the multivariate survival analyses, Dukes' stage was also adjusted for using the stratified Cox model [17]. The curves describing recurrence-free interval were computed according to a method of Kaplan and Meier [18]. All *P* values cited are two-sided and those less than 0.05 were judged as statistically significant.

RESULTS

Of the 69 tumours, ras 11 immunohistochemical reactivity with the tumours was negative in 29 cases (42%) and positive in 40 cases (58%). Figure 1 illustrates a tumour with marked positive staining of ras 11. Although the strongest staining was located along the cell membrane, the positive staining for ras 11 was distributed throughout the cytoplasm. Some positive staining was also observed on smooth muscle and stromal cells.

33 cases (48%) were DNA diploid and 36 (52%) were non-diploid. Excluding 18 cases in which SPF could not be estimated, the mean SPF of the remaining tumours was 9.8% (S.D. 6.2). 15 (29%) tumours had a SPF of less than 5%, 17 (33%) had a SPF of between 5 and 10%, and 19 (37%) had a SPF of greater than 10%. The non-diploid tumours had a significantly higher mean SPF (14.6%) than that of diploid tumours (6.2%, $P<0.0001$).

There was a tendency of increased positive staining for ras 11 in the non-diploid cases compared to diploid cases. However, the difference was not statistically significant (Table 1). ras 11 staining was significantly correlated with SPF (Table 1). As SPF increased, the proportion of cases with positive ras 11 staining increased gradually (Fig. 2).

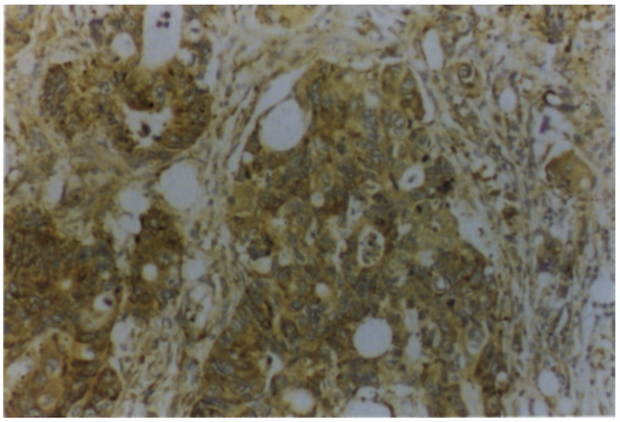

Fig. 1. Tumour stained with ras 11 showing a marked reaction (++) (×200).

Table 1. *ras* 11 *immunohistochemical reactivity with colorectal adenocarcinoma in relation to DNA ploidy and SPF*

	DNA ploidy (%)				SPF (%)		
Antibody staining	*n*	Diploid	Non-diploid	*P**	*n*	Mean (S.D.)	*P**
Ras 11							
–	29	59	41		20	7.7 (6.0)	
+	18	44	56		15	10.4 (5.6)	
++	22	36	64	0.11	16	11.9 (6.4)	0.038

*Test for trend.

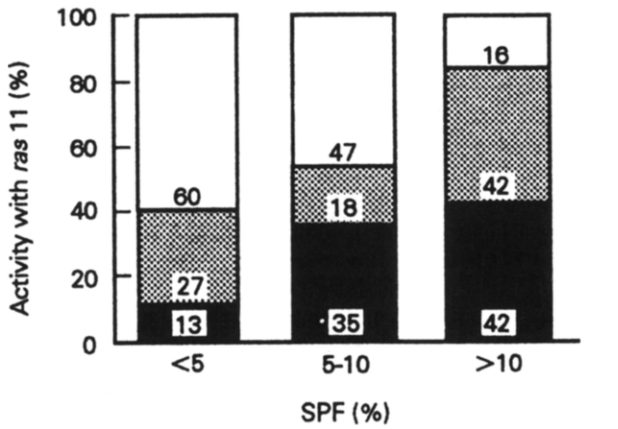

Fig. 2. Correlation between ras 11 immunohistochemical reactivity and S-phase fraction (SPF). □ = negative (−), ▨ = moderate (+) and ■ = marked (++) reactions.

In univariate Cox analyses, ras 11 staining, Dukes' stage, DNA ploidy and SPF were all significantly related to the recurrence-free interval of patients with Dukes' A–C tumours ($P = 0.0014$, $P = 0.023$, $P = 0.035$ and $P = 0.040$, respectively). These relationships are illustrated in Figs 3a–d. The prognostic significance of these variables remained even after adjustment for Dukes' stage. The prognostic value of ras 11 staining remained significant even after adjustment for both Dukes' stage and DNA ploidy ($P = 0.011$). Dukes' stage was not significantly related to either DNA ploidy or SPF ($P = 0.79$ and $P = 0.62$, respectively). However, DNA ploidy was not a significant prognostic factor ($P = 0.11$) after adjusting for both Dukes' stage and ras 11 staining (Table 2). No multivariate analysis including SPF could be performed since there were only 13 recurrences among the 41 patients with reliable SPF measurements.

DISCUSSION

Previous analyses using bivariate flow cytofluorometry showed that patients with an active multiple myeloma had a higher p21 fluorescence in aneuploid tumour cells than in diploid tumour cells, normal donor and myeloma remission bone marrows. The high levels of p21 protein in aneuploid plasma cells could suggest the involvement of the H-*ras* oncogene in the malignant plasma cell transformation and the pathophysiology of multiple myeloma, which is further supported by advanced tumour stages and a shorter survival among patients with high p21 levels [3, 19]. We found that non-diploid tumours had higher expression of pan *ras* p21 compared to that of diploid tumours. However, this relation was not statistically significant.

It has been postulated that *ras* p21 expression may have a role in cell cycle events. Campisi *et al.* [4] have reported that the level of *ras* transcripts rises in mid to late $G_{0/1}$ and remains at a high level until the next G_1 in mouse fibroblasts stimulated by the serum growth factors to initiate synchronous proliferation. Czerniak *et al.* [5] used multiparameter flow cytometry to measure the p21 content in relation to the cell cycle of colonic cancer cell lines and obtained similar results. The injection of purified Ha-ras protein into NIH 3T3 cells stimulated quiescent cells to enter the S-phase and induced a transformed morphology [6]. In contrast, NIH 3T3 cells which were induced to divide by the addition of serum were unable to enter S-phase if ras p21 was neutralised by an anti-p21ras monoclonal antibody [7]. Our results show that increased levels of pan ras p21 were significantly correlated with a higher SPF. These findings, together with p21's membrane location and known biochemical properties, such as its GTP-binding property and GTPase activity, lead to the postulate that p21 may serve as an important transducing molecule from the cell membrane to its interior for signals affecting cell proliferation [1, 3–7]. Analyses of combining an immunohistochemical method, which reflects morphological characteristics and ras 11 staining, to rapid flow cytometry may provide an important insight into the biological behavior of colorectal adenocarcinoma.

Flow cytometric analysis has shown that a significant relationship exists between DNA ploidy, proliferative activity and prognosis in colorectal tumours. Patients with DNA aneuploid and high SPF tumours have a considerably poorer prognosis than those with diploid and low SPF tumours, independent of Dukes' stage and histological grade [8–10]. However, the prognostic value of SPF is somewhat controversial [20]. Our results confirm that DNA ploidy and SPF may be prognostic

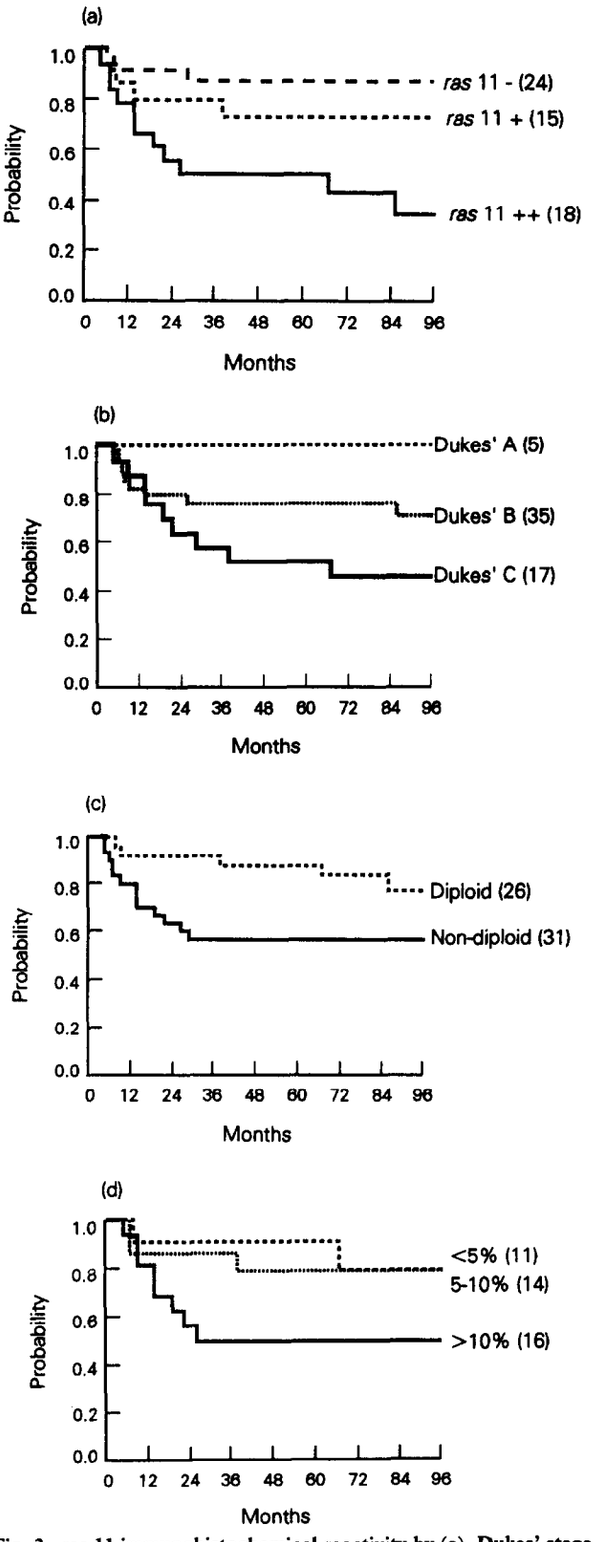

Fig. 3. ras 11 immunohistochemical reactivity by (a), Dukes' stage, (b), DNA ploidy, (c) and SPF (d) in relation to the recurrence-free interval of patients with Dukes' A–C tumours. The number of patients is noted in parenthesis.

Table 2. Multivariate analysis of the relationship between DNA ploidy, ras 11 *staining and recurrence-free interval using Cox's proportional hazards model**

	n	Recurrence rate ratio	95% CI	*P*†
DNA ploidy				0.11
Diploid	26	1.0	–	
Non-diploid	31	2.3	0.8–6.6	
Ras 11				0.011
–	24	1.0	–	
+	15	1.7	0.4–7.7	
++	18	4.4	1.2–16.3	

CI = confidence interval.
*Also adjusted for Dukes' stage.
†Test for trend.

indicators, independent of Dukes' stage, in colorectal adenocarcinoma.

Enhanced expression of pan *ras* p21 has been found to be related to poor prognosis in gastric and breast tumours [21, 22]. Our previous findings have indicated that higher levels of pan ras p21, as indicated by ras 11, predict a significantly shorter recurrence-free interval for colorectal adenocarcinoma independent of both Dukes' stage and the grade of differentiation [11]. In the present study, further analysis showed that an increased expression of pan *ras* p21 indicated by ras 11 is a biological marker for determining prognosis in colorectal adenocarcinoma, independent of both Dukes' stage and DNA ploidy.

Extended studies are needed to investigate whether expression of pan *ras* p21 and SPF add prognostic information independent of each other, and whether DNA ploidy has a prognostic value independent of ras 11 staining.

ras genes are expressed on the inner surface of the cell membrane [23]. Similar to findings of Radosevich *et al.* [24], the positive staining for ras 11 in our study was distributed throughout the cytoplasm, although the strongest staining was located along the cell membrane. This suggests that these cells are either metabolically very active or rich in hydrolytic enzymes, resulting in an increased amount of p21 precursors [25, 26] or/and products of p21 degradation, all of which ras 11 may react with. In addition, positive staining was observed on smooth muscle and stromal cells as also found by other investigators [27, 28]. We have come to the conclusion that the ras 11 immunohistochemical reactivity with colorectal adenocarcinoma is of value for evaluating proliferative activity and determining prognosis.

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