

RAPID COMMUNICATION

## Expression of altered retinoblastoma protein inversely correlates with tumor invasion in gastric carcinoma

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### Abstract

**AIM:** To investigate the clinical and pathological significance of altered retinoblastoma (Rb) encoding protein (pRb) in gastric carcinoma.

**METHODS:** Expression of altered pRb was analyzed in 91 patients with gastric adenocarcinoma by immunohistochemistry.

**RESULTS:** Sixty-five percent (59/91) of the tumors were positively stained and the staining in tumor nuclei of gastric carcinoma ranged 0%-90%. Moreover, strong expression of altered pRb was found in 35% (6/17), 24% (5/21), 17% (8/46) and 0% (0/7) of T1, T2, T3 and T4 gastric carcinomas, respectively. Altered pRb expression was inversely correlated with the depth of tumor invasion ( $P = 0.047$ ). Degree of immunoreactivity had no significant correlation with tumor grade, node metastasis and distant metastasis. In terms of prognostic significance, univariate analysis showed that poor differentiation [41 (66.1%) vs 34 (42.5%)  $P = 0.051$ ], advanced tumor stage ( $P < 0.001$ ) and weakly altered pRb expression [17 (80.5%) vs 58 (49.6%)  $P = 0.044$ ] were associated with worse prognosis in these patients. However, multivariate analysis revealed that advanced tumor stage was the only independent poor prognostic factor ( $P < 0.001$ ).

**CONCLUSION:** The mutation of Rb gene is frequent in gastric carcinoma. The expression of altered pRb inversely correlates with tumor invasion and is not an independent prognostic marker in gastric adenocarcinoma.

**Key words:** Gastric adenocarcinoma; Altered retinoblastoma protein; Immunohistochemistry

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### INTRODUCTION

Cellular proliferation normally follows an orderly progression through the cell cycle, which is governed by protein complexes encoding various genes. Malignant transformation results from a series of genetic alterations that lead to aberrant regulation of cell division (cell-cycle control), cell death (apoptosis), and maintenance of genomic integrity (DNA repair).

The retinoblastoma gene (Rb) encoding the 105kd nuclear phosphoprotein, maps to chromosome 13, band 13q14, and is a prototypical tumor-suppressor gene<sup>[1,2]</sup>. The retinoblastoma protein plays an important role in regulating the ability of cells to enter S phase, during which DNA is synthesized<sup>[3]</sup>. Although the alteration of the Rb gene has been shown to be critical for the development of retinoblastoma<sup>[4-6]</sup>, dysregulation of the Rb gene is important in a wide array of human cancers, such as sarcoma<sup>[7]</sup>, hepatoma<sup>[8]</sup>, leukemia<sup>[9]</sup>, and esophagus<sup>[10]</sup>, breast<sup>[11]</sup>, bladder<sup>[12]</sup>, prostate<sup>[13]</sup> and lung cancers<sup>[14]</sup>. But studies concerning Rb gene change in gastric carcinoma are limited.

This study was to examine the correlation of altered pRb expression with the clinicopathological status and prognostic value of altered protein expression in patients with gastric carcinoma.

### MATERIALS AND METHODS

#### Patients

The study population consisted of 91 patients (80 men, 11 women; mean age: 68 years; range: 32-86 years) who underwent surgery for primary gastric adenocarcinoma at our institute between 1992 and 2000. Eighteen (19.8%) tumors were located in the upper third, 19 (20.9%) in the middle third, and 54 (59.3%) in the lower third of the stomach, respectively. Following the international tumor node metastasis (TNM) staging system, 23 (25.3%)

tumors were considered stage I, 20 (22.0%) stage II, 28 (30.8%) stage III, and 20 (22.0%) stage IV, respectively. Histological grade was assessed following the World Health Organization (WHO) criteria. Forty-nine (53.8%) tumors were well- or moderately- differentiated, and forty-two (46.2%) poorly- or undifferentiated.

Sixteen of the 20 stage IV patients had distant metastasis (5 peritoneal, 5 hepatic, 1 lung, 3 peritoneal and T4 lesion, 1 peritoneal and hepatic, 1 peritoneal and N3 lymph node status), while the other cases were classified as stage IV because of N3 lymph node status ( $n = 3$ ) and/or T4 lesions ( $n = 1$ ). Sixteen patients underwent palliative surgery (16 distant metastases). The other 75 patients underwent radical D2 resection. Of the patients 12 were treated with postoperative systemic chemotherapy (5 for 6 cycles of FEM, and 7 for 6 cycles of 5-FU). To rule out any confounding effect of adjuvant chemotherapy, this variable was entered in the multivariate analysis of survival. Follow-up evaluation consisted of physical examination, chest radiography, and tumor marker (CEA, and CA19.9) assay every 6 mo for the first 3 years and once a year thereafter, abdominal computed tomography (CT), and endoscopy once a year.

### Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor samples taken during surgery were used for the study. pRb expression was detected immunochemically using the monoclonal antibody (M7131, DAKO, Denmark) generated from mouse anti-human Rb product. The 4- $\mu$ m thick sections from their original histology blocks were eparaffinized in xylene and treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase. For immunohistochemical detection of the pRb protein, the sections were boiled for 30 min in 10 mmol/L citrate buffer solution (pH6.0) using a microwave heater for antigen retrieval. Mouse monoclonal anti-Rb (Clone Rb1, DAKO, Glostrup, Denmark) was used as primary antibody. The clone Rb1, which can be used on (archival) paraffin-embedded material, is a mouse monoclonal antibody against recombinant human retinoblastoma gene product representing amino acid 330-612 and reacts with the retinoblastoma gene product as indicated by immunoblotting SV40 transformed human tumor cell lines<sup>[13]</sup>. The sections were first incubated for 60 min at room temperature with the primary antibody. Biotinylated anti-mouse IgG was then applied as secondary antibody, followed by peroxidase-cojugated streptavidin. Demonstration of binding sites with the peroxidase reaction was achieved with 3, 3'-diaminobenzidine tetrahydrochloride (0.25 mg dissolved in 1 mL 0.02% hydrogen peroxide). Phosphate-buffered saline was used for rinsing between each step. Faint nuclear staining, sufficient to aid in orientation but not to influence the judgement of positivity, was performed with Mayer hematoxylin solution.

### Evaluation of pRb protein expression

For each section, 10 high-power fields were chosen randomly, and a total of 1000 cells were evaluated by a pathologist (Tseng HH), who had no prior knowledge

of the patients' outcomes or tumor characteristics. The pattern of immunoreactivity was scored on the basis of the percentage of tumor cell nuclei with positive staining. The parameter obtained was the mean relative nuclear positive area evaluated on at least 10 fields observed at a magnification of  $\times 40$ .

Immunoreactivity was semiquantitatively graded. The grade scheme was: 0+ (no immunoreactive cells present), 1+ (1%-24%), 2+ (25%-49%), 3+ (50%-74%), 4+ (75%-100%) immunoreactivity cells, respectively.

In evaluation of the clinical significance of pRb expression in gastric carcinoma, tumors with grade "0+ - 2+" immunostaining were grouped as weak staining, and tumors with grade "3+ and 4+" immunostaining as strong staining.

### Statistical analysis

Associations between categorical variables were analyzed using Fisher's exact test,  $\chi^2$  test, or Mann-Whitney *U* test. Univariate analysis of survival was carried out with a Kaplan-Meier estimator, and the difference between curves of subgroups was determined using the log-rank test. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test. Independent survival variables were studied by Cox regression analysis. All data were calculated with SPSS for windows with  $P < 0.05$  as the level of significance.

## RESULTS

A total of 59 out of 91 (65%) tumors were positive for altered pRb, with 16 cases graded as "1+", 24 as "2+", 11 as "3+" and 8 as 4+". In 91 unselected primary gastric carcinomas, we compared the common prognostic markers, such as age, sex, tumor location, Lauren type, tumor grading, TNM system, and altered pRb expression. On examining the distribution of tumors with strong or weak altered pRb expressions (Table 1), no significant differences were found in sex, age, tumor site, Lauren type, and histologic grading. However, statistical analysis demonstrated a significant association between altered pRb status and tumor stage. The altered pRb protein-high tumors were more in earlier stage (stages I and II) than in more advanced stage (stages III and IV) ( $P = 0.010$ ). Moreover, the altered pRb status significantly correlated with depth of invasion (T status), but not with lymph node metastasis (N status), and distant metastasis (M status). The altered pRb expression was weak in 11 of the 17 (65%) T1 tumors, 16 of the 21 (76%) T2 tumors, 38 of the 46 (83%) T3 tumors, and 7 of the 7 (100%) T4 tumors. The altered pRb was inversely correlated with the depth of tumor invasion ( $P = 0.047$ ).

Analysis of survival was carried out in 75 patients undergoing curative resection. The results of univariate analysis of survival in relation to clinicopathological findings are reported in Table 2. Tumor staging and altered pRb expression were correlated with 5-year survival. Kaplan-Meier curves showed that the 5-year survival was 80.5% in patients with strong altered pRb expression ( $n = 17$ ) compared with 49.6% in patients with weak altered pRb expression ( $n = 58$ ) ( $P = 0.0436$ ). Only TNM

**Table 1** Altered pRb expression and clinicopathological findings in 91 patients with gastric adenocarcinoma

Variables	n	Altered pRb expression, n (%)		P
		Strong	Weak	
Age				
< 70 yr	43	7 (16)	36 (84)	0.307
≥ 70 yr	48	12 (25)	36 (75)	
Sex				
Male	80	17 (21)	63 (79)	1.000
Female	11	2 (18)	9 (82)	
Tumor location				
Upper third	18	2 (11)	16 (89)	0.553
Middle third	19	5 (26)	14 (74)	
Lower third	54	12 (22)	42 (78)	
Lauren type				
Intestinal	67	12 (18)	55 (82)	0.304
Diffuse	20	4 (20)	16 (80)	
Mixed	4	2 (50)	2 (50)	
Differentiation				
High or moderately	49	10 (20)	39 (80)	0.905
Low	42	9 (21)	33 (79)	
Tumor depth				
T1	17	6 (35)	11 (65)	0.047
T2	21	5 (24)	16 (76)	
T3	46	8 (17)	38 (83)	
T4	7	0 (0)	7 (100)	
Lymph node metastasis				
N0	28	9 (32)	19 (68)	0.109
N1	35	6 (17)	29 (83)	
N2	22	3 (14)	19 (86)	
N3	6	1 (17)	5 (83)	
Distant metastasis				
No	75	17 (23)	58 (77)	0.366
Yes	16	2 (13)	14 (87)	
TNM stage				
I and II	43	14 (33)	29 (67)	0.010
III and IV	48	5 (10)	43 (90)	

**Table 2** Univariate analysis of survival in 75 patients with curative resection for gastric adenocarcinoma

Variables	5-year survival, n (%)	P
Age		
< 70 yr	36 (54.0)	0.8036
≥ 70 yr	39 (58.8)	
Sex		
Male	69 (57.2)	0.5069
Female	6 (50.0)	
Tumor location		
Upper third	14 (51.9)	0.4312
Middle third	14 (64.8)	
Lower third	47 (54.6)	
Differentiation		
Well + moderate	41 (66.1)	0.0509
Low	34 (42.5)	
TNM stage		
I and II	43 (88.6)	< 0.0001
III and IV	32 (14.7)	
Altered pRb expression		
strong	17 (80.5)	0.0436
weak	58 (49.6)	

**Table 3** Multivariate analysis of survival in 75 patients with curative resection for gastric adenocarcinoma

Variables	Category	Relative risk	95% CI	P
Histologic grade	Poorly vs well and moderately-differentiated	1.073	0.640-1.800	0.788
Altered pRb expression	Weak vs strong	0.891	0.443-1.791	0.746
TNM stages	III & IV vs I & II	5.067	2.764-9.289	0.000

stage was found to be an independent prognostic factor for survival (Table 3) on multivariate analysis by Cox regression.

## DISCUSSION

The half-life of mutant Rb protein is even longer than that of the wild Rb protein. This feature results in immunohistochemically detectable expression of the mutant Rb protein. It has been established that altered Rb protein expression pattern gives a fairly good estimate of the mutation frequency in the Rb gene<sup>[16,17]</sup>.

In this study, 65% (59/91) of the tumors were positively stained and the staining in tumor nuclei of gastric carcinoma ranged 0%-90%. Constancia *et al*<sup>[18]</sup> reported that alterations affecting the Rb gene are rather infrequent in human gastric carcinomas, but our data indicate the altered pRb protein expression is frequent and widely variable in gastric carcinoma. We directly detected the altered Rb protein in tumor cells, which is different from the Constancia's study detecting the normal Rb protein<sup>[18]</sup>. The Rb gene mutation is frequent in various human malignancies including sarcoma<sup>[7]</sup>, hepatoma<sup>[8]</sup>, leukemia<sup>[9]</sup>, and esophagus<sup>[10]</sup>, breast<sup>[11]</sup>, bladder<sup>[12]</sup>, prostate<sup>[13]</sup> and lung cancers<sup>[14]</sup>, being similar with that in gastric carcinoma in our study.

Rb mutations resulting in the loss of Rb function play a role in the initiation of retinoblastoma and other tumors that develop as second malignancies in individuals with the hereditary form of retinoblastoma<sup>[19]</sup>. The functional loss of the Rb gene has been implicated in a diverse group of human malignancies, including carcinoma of the breast, urinary bladder, liver, esophagus, prostate, and colon<sup>[8,10-12,20,21]</sup>. The significance of these mutations in other tumors is a potentially critical issue. A key issue is whether the changes reported within the Rb gene in other various cancers are causally related to the initiation of tumors or whether they are involved in tumor progression. Phillips *et al*<sup>[20]</sup> reported that loss of Rb gene function is an early event in prostatic tumorigenesis. Our findings demonstrate that the altered pRb protein expression is more frequent in gastric carcinomas with superficial invasion than in those with advanced invasion. This feature means that the Rb gene mutation is also an early event in the development of gastric carcinoma and then gradually gets lost during its progression. However, only 35% (6/17) of T1 lesions had strong staining in our study. The T1 lesions occurred earlier than T2, T3, and T4 lesions and might be a "late" event in the development of gastric carcinoma. Therefore, in-depth studies should be undertaken to clarify whether the Rb gene mutation is causally related to the initiation of gastric adenocarcinoma

as in the hereditary retinoblastoma.

The prognostic value of pRb protein expression in various carcinomas has been investigated. Some studies found that pRb protein expression is an important prognostic indicator of cancers, such as bladder cancer<sup>[22]</sup> and non-small-cell lung cancer<sup>[23]</sup>. Low pRb protein expression is associated with significantly poorer survival among them. In contrast, in colorectal cancer no prognostic significance of pRb protein expression has been reported<sup>[14]</sup>. Our findings demonstrate that there is a negative correlation between pRb protein expression and survival in gastric carcinoma at univariate analysis, but not significantly correlated between them at multivariate analysis. In the present study, however, the pRb protein-high tumors were more frequent in earlier stage (stages I and II) than in advanced stage (stages III and IV), suggesting that the pRb protein expression is not an independent prognostic marker of gastric adenocarcinoma.

In conclusion, our data indicate that the mutation of Rb gene is frequent in gastric carcinoma. The expression of altered pRb protein inversely correlates with tumor invasion and is not an independent prognostic marker in gastric adenocarcinoma

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