GASTRIC CANCER

p21 and p27 immunoexpression in gastric well differentiated endocrine tumors (ECL-cell carcinoids)

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Abstract

AIM: To investigate the expression of cyclin-dependent kinase inhibitors p21 and p27 in gastric well differentiated endocrine tumors (GWDET) (ECL-cell carcinoids).

METHODS: The expressions of p21 and p27 were examined immunhistochemically in endoscopic biopsy specimens from 16 patients matching the diagnostic criteria of GWDET. Percentage of positive nuclear staining either weak or strong was noted. The association of immunoexpressions with age, gender, tumor localization, multifocality and accompanying chronic atrophic gastritis, neuroendocrine cell hyperplasia (NEH), neuroendocrine dysplasia (NED), intestinal metaplasia (IM), Ki-67 proliferation index and clinical outcome were also evaluated.

RESULTS: All cases expressed p27 with a mean expression score of 43.6%, while 31.3% of the cases showed any p21 expression. p21 and p27 immunoexpressions were significantly correlated with each other ($P < 0.01$), and the p21-expressing group had higher p27 expression scores (68% *vs* 22%). p21 and p27 expressions were lower in women, in non-atrophic mucosa and cases whose tumors were located somewhere other than fundus without submucosal extension. On contrary, p21 and p27 expressions were higher in males and the patients with submucosal extension and atrophic gastritis. Cases presenting lower p27 scores had solitary tumors showing neither NEH-NED nor IM. Despite, cases with lower p21 expression presented multifocal tumors accompanied by NEH-NED. However, no correlation of p21 and p27 expressions was found with age and Ki-67 expression.

CONCLUSION: p27 is widely expressed in GWDETs, while p21 expression is sparse and observed in two thirds of the cases. Loss of p21 and p27 expressions may be correlated with different carcinoid tumor subtypes; however, more studies are needed to assess the role of these prospective markers in gastrointestinal endocrine tumors.

INTRODUCTION

Carcinoid tumors arise from proliferating enterochromaffin-like cells (ECL) of fundus. Elevated plasma gastrin levels are responsible of the neoplastic changes in these cells, but neoplastic transformation can also be observed in absence of hypergastrinemia as well. The overall incidence rates for carcinoid tumors have increased significantly over the past 25 years[1]. Recent studies also suggest an apparent increase in incidence of gastric carcinoids which accounted about 2% of the total gastrointestinal (GI) carcinoids in the past but increased to a frequency of 7.2% to 30% in recent years[2-5]. Increased awareness of the existence of these lesions as well as the improvements in upper GI endoscopy and more frequent application of immunohistochemical methods to biopsies are probably related to this trend. The causes of this inclinement need further investigations, but the conclusion is clear that we have to cope with more endoscopic biopsies with neuroendocrine cell hyperplasia and tumors. The role of the pathologist is not only to diagnose the lesion but also provide the histopathologic data that will help to determine the management strategies.

Well differentiated endocrine tumor (WDET) is proposed as synonymous with the time-honoured term "carcinoid" according to the last WHO classification of the endocrine tumors of the GI tract (Table 1) and defined as "an epithelial tumor of usually monomorphous endocrine cells showing mild or no atypia and growing in the solid nests and trabeculae or pseudoglandulae, restricted to the mucosa or submucosa"[6]. Further WDET's of stomach are ECL-cell carcinoid, EC-cell, serotonin-

producing carcinoid, G-cell gastrin-producing tumors and others[6]. ECL-cell carcinoids clinically has three distinctive types: Type 1 which is associated with chronic atrophic gastritis with or without hypergastrinemia; Type 2 with hypertrophic gastropathy with high gastrin levels; and Type 3, sporadic form, associated with neither atrophy nor hypergastrinemia[7]. Gastric well differentiated endocrine tumors (GWDET) are benign, non-angioinvasive tumors, measuring $\leqslant$ 1 cm in size and showing $\leqslant$ 2 mitoses per 10 high power field (HPF). The preferred therapy is endoscopic removal, followed by regular endoscopic surveillance and antrectomy in hypergastrinemic cases[8].

In daily practice, type 1 ECL-cell carcinoids are more frequently observed than other subtypes. Despite their benign nature, recurrence is one of the leading problems in management of these tumors[9]. Besides, lymph node metastases, although rare, are reported even in minute lesions[10]. Furthermore, the frequent recurrences lead to more aggressive surgical approaches. So the prediction of tumor behavior from endoscopic biopsies is very critical, but it is quite difficult, if not impossible. In most of the cases, biopsy specimens are very small and submucosa is rarely visualized. Even when the submucosa is present, it is generally not deep enough to rule out submucosal invasion. Cytomorphologic features are helpless since the tumors do not show nuclear pleomorphism, anaplasia and frequent mitosis. Ki-67 proliferation index (PI) and p53 has clinical implications to some extent but new markers are needed to understand the mechanisms of tumor development and differences in clinical behavior even in benign lesions.

p21^{waf1} (p21) and p27^{kip1} (p27) are nuclear proteins reported to have a role in development and progression of several organ tumors. They are members of the KIP subgroup of cyclin-dependent kinase inhibitory (CDKI) family, which prevents cell entry to S phase in response to DNA damage, thus act as tumor suppressors. Loss of p27 has a role in carcinogenesis of several organ tumors, including breast, prostate and colon, and is related with poor prognosis[11]. p27 expression has been reported to be valuable in differentiating parathyroid adenomas and carcinomas[12,13]. Altered patterns of p21 expression seem to have an association with clinical outcome in certain tumors but there are conflicting results whether low or high levels of p21 serve as a prognostic marker[13,14]. Although there are previous studies on the role of CDKIs in several types of endocrine carcinomas, the expression of these proteins in GI endocrine tumors, particularly GWDETs, is less well investigated[15,16].

In this study, we first investigated expressions of p21 and p27 in GWDETs, and then evaluated the association of p21 and p27 expressions with age, gender, tumor localization, multifocality and accompanying chronic atrophic gastritis, as well as neuroendocrine cell hyperplasia (NEH), neuroendocrine dysplasia (NED), intestinal metaplasia (IM), Ki-67 PI and clinical outcome.

MATERIALS AND METHODS
Subjects
Formalin-fixed, paraffin-embedded endoscopic biopsy

Table 1 Gastric endocrine tumor classification, WHO[6]

Classification	Diameter	Ki-67 PI[1]	Mitosis	Extension
Well differentiated endocrine tumor (carcinoid tumor)	$\leqslant$ 1 cm	$\leqslant$ 2%	$\leqslant$ 2/10 HPF[2]	Mucosa-submucosa
Well differentiated endocrine carcinoma (malignant carcinoid)	> 1 cm	> 2%	> 2/10 HPF[2]	Muscularis propria and beyond
Poorly differentiated endocrine carcinoma (small cell carcinoma)	Any diameter	> 15%	> 10/10 HPF[2]	Vascular and neural invasion

[1]Proliferation index; [2]High power field.

specimens from 16 patients, whose archival materials were avaliable for immunohistochemistry and matching the diagnostic criteria of GWDET[6], were included in the study. The biopsy samples were fixed in 100 mL/L neutral buffered formalin. Following routine tissue process, tissues were cut into 4-μm thickness and stained with hematoxylin & eosin and chromogranin A. Age, gender, tumor localization, multifocality (defined as tumors seen in more than one samples taken from different parts of stomach or same part of the stomach but described as separate nodules in endoscopy report) and accompanying chronic atrophic gastritis, NEH, NED and IM were recorded. Size of the tumors (although smaller than 1 cm) could not be measured properly because of the fragmented nature of the biopsies.

Immunohistochemistry
Mouse monoclonal antibodies (mAb) anti-p21 (Neomarkers, MS 230-P, 200 mg/L, 1:40 dilution), anti-p27 (Neomarkers, MS 256-P, 200 mg/L, 1:50 dilution) and Ki-67 (Dako, M 7240, 1:150 dilution) were used for immunohistochemistry by streptavidin-biotin peroxidase method. Briefly, sections were incubated at 50℃ overnight, deparaffinized in xylol and rehydrated through graded alcohol rinses. To enhance antigen retrieval, slides prepared for p21 staining were heated in 0.1 mmol/L EDTA buffer (pH 8) in a microwave oven (650 W for 5 min plus 450 W for 20 min) and those for p27 were immersed in citrate buffer (10 mmol/L, pH 6) and heated in a vacuum-pan for 2.5 min and cooled for 20 min. Endogenous peroxidase activity was blocked with 30 mL/L H_2O_2 for 5 min. The incubation time with monoclonal antibodies p27 and p21 was optimized as 30 min. Immunostaining of p27 and p21 was performed with the streptavidin-peroxidase method (Labvision, TM-125-HL), and alkaline phosphate-based visualization system. 3, 3'-diaminobenzidine (Dako, TA-125 HD) was used as a chromogen. Carcinomatous breast and colon tissue served as a positive control, whereas primary mAb was omitted for negative control sections. Stained lymphocytes were used as an internal control. Scoring was performed separately by two pathologists (BD, BS) who were blinded to the patient characteristics as well as each others scores. In each case, a total of 200 cells were counted in two randomly selected representative HPF areas. Percentage of positive nuclear staining either weak or strong was noted. The mean of the scores counted by each observer was accepted as expression score.
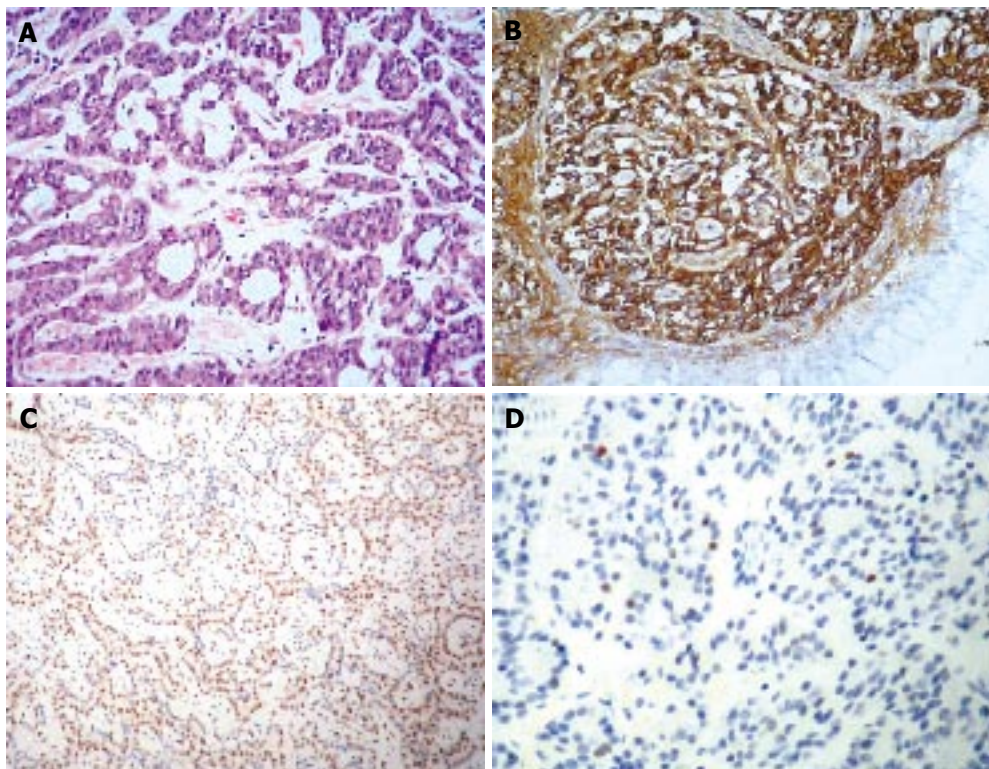
Figure 1 Histochemical staining. **A**: Tumor cells arranged in ribbons, tubular structures or small nests (HE x 20); **B**: Chromogranin immunoexpression (x 10); **C**: p27 immunoexpression (x 20); **D**: p21 immunoexpression (x 20).

Table 2 Accompanying histopathologic features		
Pathology	**Patients (*n*)**	**Rate (%)**
Atrophy	8	50.0
Neuroendocrine hyperplasia	7	43.8
Intestinal metaplasia	9	56.3
Neuroendocrine dysplasia	3	18.8
Multifocality	5	31.4
Submucosal extension	8	50.0

Table 3 p21, p27, Ki-67 staining ratios (%)		
Gene	**Mean ± SD**	**Range**
p21	1.09 ± 1.44	0-4
p27	43.58 ± 31.27	10-95
Ki-67	0.7 ± 0.94	0-3

Statistical analysis

Statistical analysis was carried on a PC-based analysis program, SPSS 10.0. Pearson correlation analysis was carried out for univariate associations between p21 and p27 scores and age. Mann-Whitney U test was performed to investigate the relationship of p21 and p27 scores with gender, localization (fundus), multifocality, NEH, neuroendocrine dysplasia and presence of atrophy and IM as well as clinical outcome. Submucosal invasion, although enclosed in the definition of GWDET, was also noted when present and evaluated for its statistical significance by using Mann Whitney U test. Chi-square test and Fisher's exact test were applied for dichotomized data. Student's t-test was used for associations with age. $P < 0.05$ was considered statistically significant.

RESULTS

Patient and tumor characteristics

Mean age of the patients was 52.4 ± 7.0 (range: 41-66) years, and 56.3% of them were female. All cases were classified as type 1 ECL-cell tumors clinically. Nine (56.3%) of the tumors were located in corpus and 2 (12.6%) of them were in fundus. Most of them showed more than one histologic pattern (Figure 1A). Chromogranin A was identified in all tumors (Figure 1B). None of the cases showed mitosis in 10 HPF except one case (1/10 HPF). Multifocality was observed in 5 (31.4%) cases. Accompanying histopathologic features are shown in Table 2. Eleven (68.8%) of the cases were cured by endoscopic excision, followed by regular endoscopic surveillance and endoscopic ultrasound when necessary and did not show recurrences during the follow-up. Among the rest of 5 (31.4%) cases, 3 cases needed re-excision and 2 were conveyed to surgery because of multiple recurrences. No additional histopathologic features were found in the operation materials of these 2 cases. None of the cases showed distant organ or lymph node metastasis at the time of diagnosis nor developed any during a mean follow-up period of 34.12 ± 25.63 (range: 9-69) mo.

p21 and p27 expressions

Descriptive statistics of p21, p27 and Ki-67 nuclear immunreactivity are shown in Table 3, and Figure 1C, 1D. We observed a significant correlation between p21 and p27 expressions ($P < 0.01$, Pearson correlation analysis). All cases expressed p27, while 5 (31.3%) of the cases showed no expression for p21 in tumor areas. The p21-expressing group had higher p27 scores of 68% vs 22%, although statistically insignificant ($P = 0.07$) (Table 4). High p27-

expressing tumors (over the cut-off level of mean p27 expression of 43.6%) had higher p21 scores as well ($P = 0.022$, Mann Whitney U-test).

p21 and p27 expressions were higher in 3 tumors located in fundus either as a single focus ($n = 2$) or part of a multifocal spread ($n = 1$) ($P < 0.05$, Mann Whitney U-test) (Table 4). In addition, p21 and p27 expressions were higher in males and the patients with submucosal extension and atrophic gastritis. The patients with multifocal tumors, NEH and NED showed higher p27 and lower p21 immunoexpression. But no correlation of p21 and p27 expressions was found with the age and Ki-67 PI. Also, the p21-expressing tumors did not show any particular difference in respect to presence of atrophy, NEH, NED, IM, multifocality, submucosal extension, Ki-67 PI and age. Cases, who needed surgery or re-excision because of recurrence had lower p27 expression scores (26.7% ± 4.2% *vs* 50% ± 34.6% in recurrent cases) and p21 immunoexpression (0% *vs* 1.5% ± 0.53% in recurrent cases), however, data did not research statistically significant. These cases did not differ in respect to presence of NEH, NED, atrophy, submucosal extension, IM, multifocality, gender and age.

DISCUSSION

The overall incidence rates of gastric carcinoids and related endoscopic biopsies have increased significantly over the last few years. Although a better prognosis can be predicted for most cases, the clinical outcome and management of these tumors vary widely[2,4,17]. Determination of the most appropriate treatment modality and follow-up strategy depends on the associated or underlying disease process as well as the histologic findings in endoscopic biopsies.

Among the histologic parameters, Ki-67 PI is of diagnostic and prognostic value but it can be quite low in either benign or malignant neoplasms[6,15]. In this study, the expression of two prospective prognostic markers, CDKI's p21 and p27, and their relationship with clinicopathologic features were investigated.

Cell cycle regulation is influenced by nuclear proteins that enhance cell division, cyclin-dependent kinases (CDK) or disrupt cell division, CDKIs. The induction of CDKI inhibits the activities of CDK2 and completely arrests cells at G1 phase of the cell cycle, thus acts as tumor supressors[18]. Loss of CDKIs has a role in carcinogenesis of several organ tumors as well as endocrine tumors. Canavese *et al*[15] performed study on 109 endocrine tumors of the pancreas and GI tract, and found that p27 was highly expressed especially in differentiated tumors, while expression of p21 was very sparse in midgut (ileum-right colon) carcinoids but significantly higher in insulinomas[16]. Zirbes *et al*[19] stated that primary pulmonary carcinoid tumors failed to show a staining for p21 and p27.

We found that all cases expressed p27 with a mean expression score of 43.6%, while 31.3% of the cases showed p21 expression. p21 and p27 expressions were significantly correlated with each other and the p21-expressing group had a higher p27 expression score as 68% *versus* 22%. p27 has a 42% amino acid homology with p21, at the region that mediates inhibition of CDK[12],

but recently it was suggested that p21, supposed to be functionally similar to p27, plays a lesser role in tumor suppression[20]. But some studies suggest a co-regulation between p27 and p21 in some tumors like colorectal carcinoma and renal cell carcinoma[21,22], but it is not yet clear how these two CDKIs relate to each other in carcinogenesis sequences.

In the present study, cases presenting clinical progression had lower p21 and p27 immunoexpression. But no particular difference was found in respect to presence of atrophy, NEH, NED, IM, multifocality, submucosal extension, and age. Ki-67 PI was very low in most of the cases and no correlation was found between p21, p27 expressions and the other clinicopathologic features. When we examined the general features of all cases, we found that both p21 and p27 expressions were lower in women, in non-atrophic mucosa and cases whose tumors were located somewhere other than fundus without submucosal extension. Cases presenting lower p27 scores were generally solitary tumors showing neither NEH-NED nor atrophy and intestinal metaplasia. Despite, in cases with lower p21 expression, tumors were multifocal and accompanied by neuroendocrin cell hyperplasia and dysplasia. These observations may suggest a probable association of p27 loss with sporadic form of the disease (Type Ⅲ) and p21 loss with hypergastrinemic cases (Type Ⅰ or Ⅱ) although do not cover the whole picture.

Loss of p27 expression is reported to be correlated with aggressive behaviour of various organ tumors[12,14]. But although Lloyd *et al*[23] reported a marked decrease of 27 expression in benign and malignant endocrine tumors when compared with normal tissue and Erickson *et al*[12] suggested the use of p27 in the differential diagnosis between parathyroid hyperplasia and adenoma, Canavese *et al*[15] objected the use of high p27 expression as a marker of benign behaviour since it can be observed in differentiated

Table 4 p21 and p27 expressions and clinicopathologic features (%)

Clinicopathologic feature		p21	P^3	p27	P^3
Gender	Male	1.5	0.60	58.0	0.34
	Female	0.85		36.4	
Localization	Fundus	3.0	< 0.05	90.5	< 0.05
	Other	0.7		34.2	
Multifocality	Multifocal	0.7	0.57	45.7	0.92
	Single focus	1.25		42.9	
NEH[1]	Present	1.0	0.842	50.8	0.64
	Absent	1.16		37.5	
NED[1]	Present	0.7	0.57	45.7	0.80
	Absent	1.25		42.8	
Atrophy	Atrophic	1.3	0.47	46.4	0.50
	Non-atrophic	0.8		32.5	
Intestinal metaplasia	IM[2] (+)	χ^4	χ^4	49.6	0.09
	IM[2] (-)	X^4		16.5	
Submucosal extension	Present	1.0	0.893	49.3	0.67
	Absent	0.8		30.5	
p27 immunoexpression	p[21] expressing	χ^4	χ^4	68.0	< 0.05
	p[21] (-)	χ^4		21.8	
Follow-up	Recurrent	0.0	0.094	26.7	0.51
	Non-recurrent	1.5		49.9	

[1]Neuroendocrine cell hyperplasia-diysplasia; [2]Intestinal metaplasia; [3]Mann-Whitney U test; [4]Result is omitted since p21 value was constant in IM (-) cases.

malignant tumors. Observation on p21 is more limited and needs further studies. To our best of knowledge, there is no previous study dedicated only to p21 and p27 immuno-expressions in gastric well differentiated endocrine tumors. Although we appreciate that our study has limitations, the observations presented above can help to enlighten the pathogenesis of gastric carcinoid tumors. Larger series with long-term results are needed to assess the role of p21 and p27 in gastrointestinal endocrine tumors.

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