

Characterization of oncogene suppressor marker expression in patients with submucosal gastric carcinoma

ROBERSON A. MORON¹, CARLOS EDUARDO JACOB¹, CLAUDIO JOSÉ CALDAS BRESCIANI¹, KLEBER SIMÕES², VENÂNCIO AVANCINI FERREIRA ALVES², KYOSHI IRYA², JOAQUIM GAMA-RODRIGUES¹, IVAN CECCONELLO¹, ADHEMAR LONGATTO-FILHO²⁻⁴ and BRUNO ZILBERSTEIN¹

Departments of ¹Gastroenterology and ²Pathology, and ³Laboratory of Medical Investigation (LIM) 14, Department of Pathology, University of São Paulo School of Medicine, São Paulo, SP 14784-400, Brazil; ⁴Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga; ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal

Received October 14, 2017; Accepted December 6, 2017

DOI: 10.3892/mco.2017.1545

Abstract. The aim of the present study was to determine the clinical significance of p53 and p21^{ras}, p21^{waf1}, p27^{kip1} and p16^{ink4a} expression in cases of early gastric cancer. A total of 81 patients who had undergone gastrectomy with D2 lymphadenectomy between 1971 and 2004 were retrospectively investigated. The immunohistochemical expression of p21^{ras}, p53, p21^{waf1/cip1}, p27^{kip1} and p16^{ink4a} in the tissues was evaluated. In normal, metaplastic and tumoral mucosa, p53 was positive in 53, 87.3, and 87.1% of the cases, respectively. In the same tissues, p21^{ras} was positive in 85.3, 86 and 96.8%, respectively. Positivity for p16^{ink4a} was detected in 46.3, 91.1 and 86% of the cases, respectively, whereas p27^{kip1} was positive in 60, 94.7 and 95.3%, and p21^{waf1/cip1} was positive in 32.4, 72.7 and 71.4% of the cases, respectively. All the tumors were positive for p53. Tumors with lymph node invasion presented with overexpression (+4) of p53 in 47% of the cases vs. 17% of patients who did not have lymph node involvement. Therefore, higher expression of p53, p21^{ras} and p21^{waf1/cip1} in the tumor exhibited a statistically significant association with lymph node involvement.

Introduction

The incidence and mortality rates of gastric cancer are high, mainly in Western countries, with an estimated 989,600 new gastric cancer cases and 738,000 deaths in 2008, accounting for 8% of the total cancer cases and 10% of total cancer-related deaths (1). Asia, Eastern Europe and South America are the principal regions of gastric cancer occurrence, which may be associated with dietary variables and the high prevalence

of *Helicobacter pylori* (*H. pylori*) infection (2). However, the gastric carcinoma rates worldwide have decreased markedly (3).

The biology of gastric cancer is associated with the activity of several molecular markers, which are correlated with the risk of lymph node invasion and metastasis; among these markers, p21^{ras}, p53, p21^{waf1/cip1}, p27^{kip1} and p16^{ink4a} currently appear to be inherently correlated with gastric cancer behavior (4-8). These markers are differentially expressed, depending on the gastric carcinoma type (9). Well-documented series of tumors with specific immunohistochemical antibodies reported an association between protein expression and clinical outcome (10). Patients with gastric cancer merely invading the mucosal space have a very low incidence of lymph node metastasis, whereas up to 20% of cases with submucosal invasion had nodal metastasis, requiring lymphadenectomy for complete disease removal (11). As the majority of reports have focused on invasive gastric cancer, the aim of the present study was to investigate a series of early gastric cancers and examine the expression of the p53, p16^{ink4a}, p21^{waf1/cip1}, p21^{ras} and p27^{kip1/cip1} proteins in normal, metaplastic and mucosal tumor tissues, and determine the positive and negative correlations with the risk of lymph node metastasis in early gastric cancer patients with submucosal invasion submitted to gastrectomy with D2 lymphadenectomy.

Patients and methods

Patients. Cases were retrospectively assessed from the files of the Gastric Cancer Database of Hospital das Clínicas of the University of São Paulo School of Medicine (São Paulo, Brazil), between 1971 and 2004. Records from 1,989 patients with gastric cancer were selected. The inclusion criteria were as follows: Patients with primary gastric adenocarcinoma with submucosal invasion who had been submitted to gastrectomy with D2 lymphadenectomy, with at least 15 lymph nodes isolated from the surgical specimen. Patients with previous gastric surgery and gastric stump cancer were excluded. From the 120 patients with submucosal early gastric cancer (EGC), 81 patients fulfilled the inclusion criteria for the present study. Representative areas of tumoral, normal and metaplastic mucosa were selected from new paraffin sections stained with

Correspondence to: Dr Adhemar Longatto-Filho, Laboratory of Medical Investigation (LIM) 14, Department of Pathology, University of São Paulo School of Medicine, 455 Dr Arnaldo Avenue, Room 1147, São Paulo, SP 14784-400, Brazil
E-mail: longatto16@hotmail.com

Key words: gastric cancer, p21^{ras}, p53, p21^{waf1/cip1}, p27^{kip1}, p16^{ink4a}

hematoxylin and eosin. The Manual Tissue Microarray kit (Beecher®, Sun Prairie, WI, USA) was used for removing the samples from the previously selected areas of the donor blocks and create new paraffin blocks of tissue microarrays (TMA).

The gastric carcinomas were classified according to the guidelines of the Japanese Gastric Cancer Association and Union for International Cancer Control (UICC, 1997) and histologically classified according to the Lauren classification (12).

Immunohistochemical reactions. The TMA paraffin blocks were submitted to immunohistochemical examination for the following markers: p21^{ras} (monoclonal mouse antibody; clone NCC-RAS-001, DakoCytomation M0637, Carpinteria, CA, USA), p21^{waf1/cip1} (monoclonal mouse antibody; clone SX118, DakoCytomation M7202, Glostrup, Denmark), p27^{kip1} (monoclonal mouse antibody; clone SX53G8 DakoCytomation M7203), p16^{ink4a} (monoclonal mouse antibody; clone E6H4 DakoCytomation K5334), and p53 (monoclonal mouse antibody; clone DO-7 DakoCytomation, M7001). For positive reaction observation, 60 mg% 3,3'-diaminobenzidine tetrahydrochloride (D-5637, Sigma-Aldrich; Merck KGaA, USA), 1 ml dimethyl-sulfoxide, 1 ml H₂O₂ 6% and 100 ml phosphate-buffered saline were incubated for 5 min at 37°C in the dark. The specific details of the immunohistochemical reactions are presented in Table I. Positive controls for the anti-p21^{ras}, anti-p21^{waf1/cip1} and anti-p27^{kip1} antibodies were normal palatine tonsyl. For the anti-p53 antibody, the positive control was pulmonary adenocarcinoma. High-grade cervical intraepithelial neoplasia was used as control for anti-p16^{ink4a}. Nuclear brown staining was considered as a positive reaction for p53, p21^{waf1/cip1} and p27^{kip1}; for p21^{ras}, cytoplasmic staining alone was considered as a positive reaction; and for p16^{ink4a}, cytoplasmic or nuclear staining was accepted as a positive reaction. The negative control was obtained without the primary antibody incubation stage. The positive immunoreaction quantification was as follows: The samples were quantified in 5 categories: 0, no cells stained; 1+, ≤10% positive cells; 2+, 11-25% positive cells; 3+, 26-50% positive cells; and 4+, >51% positive cells. The significance of each category was correlated with the pathological variables, avoiding establishing an arbitrary cut-off point. The association analysis between the clinical data and marker positivity was performed using the Chi-squared test. The level of significance was set at 5% ($\alpha=0.05$).

Positive controls for the anti-p21^{ras}, anti-p21^{waf1/cip1} and anti-p27^{kip1} antibodies, were normal amygdale. For the anti-p53 antibody, the positive control was pulmonary adenocarcinoma. High-grade cervical intraepithelial neoplasia was used as control for anti-p16^{ink4a}.

Nuclear brown staining was considered as a positive reaction for p53, p21^{waf1/cip1} and p27^{kip1}; for p21^{ras}, cytoplasmic staining alone was considered as a positive reaction; and for p16^{ink4a}, cytoplasmic or nuclear staining was accepted as a positive reaction. The negative control was obtained without the primary antibody incubation stage.

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Results

Patient characteristics. A total of 81 complete cases were obtained, including tumoral, normal and metaplastic mucosa. There was lymph node invasion in 11 patients (13.6%). It was not possible to recover the lymph node samples with cancer invasion from the paraffin blocks in 8 cases. The epidemiological and clinical characteristics of the patients are listed in Table II. There were no significant differences between the tumor markers and patient sex (Pearson's Chi-squared test = 2.06 and P=0.55). There was no significant association between recurrence and expression of the markers (Pearson's Chi-squared test = 0.76 and P=0.99). The intestinal type was the most common according to the Lauren classification (45 patients, 55.5%). The diffuse type was observed in 25 (30.9%) patients and the mixed type in 11 (13.5%) patients.

Immunohistochemical reactions. All the immunohistochemical reactions are summarized in Table III. Intestinal type tumors did not express p21^{waf1} in 29.4% of the cases. In 67.6% of the samples, an intermediate positivity (<25% of the cells stained) was observed. In only 2.9% of the cases there was overexpression (>25% of positive cells). Among the diffuse type tumors, there was no expression of p21^{waf1/cip1} in 40% of the cases; intermediate positivity was observed in 45% and overexpression in 15% of the cases. Mixed tumors were all positive for p21^{waf1/cip1} staining; however, 77.8% exhibited intermediate positivity and 22.2% exhibited overexpression. (Pearson's Chi-squared test = 16.6 and P=0.034).

Positive reaction for p21^{ras} was observed in the normal mucosa in 81.1% of the Lauren intestinal type tumors, followed by 86.4% of the diffuse type and 100% of the mixed type. A statistically significant difference was observed among the three histological types (Pearson's Chi-squared test = 9.54 and P=0.048). p27^{kip1} was positive in all cases with diffuse and mixed tumor types. A total of 8.8% of the intestinal type patients did not exhibit p27^{kip1} expression. Higher immunohistochemical expression of p27^{kip1} in the tumor was noted in 66.6, 50 and 33.3% of the diffuse, intestinal and mixed type tumors, respectively. Statistically significant differences were observed among the three histological types (Pearson's Chi-squared test = 16.29 and P=0.038). A total of 34 (42%) patients exhibited invasion limited to the superficial part of the submucosal layer (Sm1) and 47 (58%) had invasion to a depth of >0.5 mm (Sm2). Among the markers, there was a higher positivity only for p27^{kip1} in the normal mucosa adjacent to Sm1 tumors (Pearson's Chi-squared test = 12.81 and P=0.012).

Perineural invasion occurred in 5 (6.17%) cases. A greater overexpression of p16^{ink4a} was observed in normal mucosa in cases with perineural invasion (Pearson's Chi-squared test = 13.42 and P=0.009). Vascular invasion was observed in 4 (4.9%) cases. There was a statistically significant correlation between positivity for p16^{ink4a} in normal mucosa and vascular invasion (Pearson's Chi-squared test = 33.42 and P<0.001).

Lymphovascular invasion was observed in 16 cases (19.7%). A total of 19 (23.4%) patients exhibited lymph node metastasis

Table I. Summary of the immunohistochemical procedures utilized in the study.

Antibody	Clone	Dilution	Antigen retrieval	Source
p21 ^{ras}	NCC-RAS-001	1:100	Pressure cooker: Citric acid 10 mM, pH 6.0, 3' and 30'	DakoCytomation M0637, Carpinteria, CA, USA
p21 ^{waf1/cip1}	SX118	1:100	Pressure cooker: Tris-EDTA 1 mM, pH 8.0, 2' and 30'	DakoCytomation M7202, Glostrup, Denmark
p27 ^{kip1}	SX53G8	1:400	Pressure cooker: Tris-EDTA 1 mM, pH 8.0, 2' and 30'	DakoCytomation M7203, Glostrup, Denmark
p16 ^{ink4a}	E6H4	1:400	Pressure cooker: Citric acid 10 mM, pH 6.0, 3' and 30'	DakoCytomation K5334, Glostrup, Denmark
p53	DO-7	1:200	Pressure cooker: Citric acid 10 mM, pH 6.0, 3' and 30'	DakoCytomation, M7001, Glostrup, Denmark

EDTA, ethylenediaminetetraacetic acid.

Table II. Clinical characteristics of patients with early gastric carcinoma (n=81).

Characteristics	No. (%)
Sex	
Female	31 (38.3)
Male	50 (61.7)
Ethnic groups	
Caucasian	59 (72.2)
Asian	7 (8.6)
Afro-Brazilian	14 (17.3)
Others	1 (1.2)
Mean age, years \pm SD	61.5 \pm 12.5
Median age, years \pm SD	62 \pm 15.8
Familial history of gastric cancer	12 (14.8)
Main symptom	
Dyspepsia	58 (71.6)
Asymptomatic	18 (22.2)
Upper digestive bleeding	5 (6.2)
Hospital mortality (<30 days)	5 (6.2)
Brain stroke	1 (1.2)
Sepsis	2 (2.5)
Respiratory complications	2 (2.5)
Follow-up mortality (>30 days)	2 (2.5)
Pleural metastasis	1 (1.25)
Pulmonary metastasis	1 (1.25)
Histological type (Lauren)	
Intestinal	45 (55.5)
Diffuse	25 (30.8)
Mixed	11 (13.5)
Mean number of removed lymph nodes	32
Involved lymph nodes	19 (23.4)
Mean follow-up time, months	88

SD, standard deviation.

from adenocarcinoma, of whom 4 (21%) exhibited Sm2 submucosal invasion and 15 (78.9%) Sm1 type invasion. According to the Lauren classification, 10 (52.6%) were intestinal, 7 (36.8%) were diffuse and 2 (10.5%) were mixed type.

There was a correlation between the higher expression of p53 in the tumor and the presence of lymph node involvement (Pearson's Chi-squared test = 8.18 and P=0.042). Patients with lymph node involvement also exhibited a higher expression of p21^{waf1/cip1} (Pearson's Chi-squared test = 15.86 and P=0.003). Lymph node invasion was correlated with positive expression of p21^{ras} (Pearson's Chi-squared test = 9.533 and P=0.023), but not of p16^{ink4a} (Pearson's Chi-squared test = 1.27 and P=0.86) or p27^{kip1} (Pearson's Chi-squared test = 2.55 and P=0.63). All the markers were significantly enhanced when the tumor was compared with normal mucosa. Comparison of normal and metaplastic mucosa revealed an increased expression of p53, p21^{waf1/cip1} and p27^{kip1}, but not p16^{ink4a} and p21^{ras}. The comparison of metaplastic and tumoral mucosa revealed increased expression of p21^{ras} in cancer cells.

Discussion

The results herein observed endorse, in part, the data available in the literature. Despite the excellent prognosis of early gastric adenocarcinoma when there is no lymph node metastasis, the majority of the markers correlated with cancer aggressiveness are associated with early stages of carcinoma invasion. Studies using immunohistochemical markers have the limitation inherent to the semi-quantification of the positive reactions (5-7).

There is robust experimental evidence that genetic alterations and risk factors differ according to the histological type (13). Our results revealed significant differences between neoplastic and non-neoplastic protein expression, which corroborate, in part, the evidence observed in experimental settings. Normal mucosa exhibited p21^{ras} positivity in 81.1% of the patients with intestinal type carcinoma, 86.4% with diffuse type, and 100% with mixed type, as previously reported (14). The prognostic value of p21^{ras} in intestinal type carcinomas remains disputable (15).

Table III. Immunohistochemical characteristics of the 81 early gastric carcinoma cases.

Antibody and Lauren classification	Mucosal region, immunohistochemical score [n (%)]											
	Normal mucosa						Metaplastic mucosa					
	0	1+	2+	3+	4+	P-value	0	1+	2+	3+	4+	P-value
p16^{ink}						0.66						0.13
Intestinal	19 (51.4)	13 (35.1)	4 (10.8)	1 (2.7)	0 (0.0)	(n=67)	2 (6.1)	13 (39.4)	2 (6.1)	7 (21.2)	9 (27.3)	(n=56)
Diffuse	12 (57.1)	5 (23.8)	3 (14.3)	0 (0.0)	1 (4.8)		1 (6.7)	5 (33.3)	4 (26.7)	3 (20.0)	2 (13.3)	
Mixed	5 (55.6)	2 (22.2)	1 (11.1)	1 (11.1)	0 (0.0)		2 (25.0)	0 (0.0)	3 (37.5)	1 (12.5)	2 (25.0)	
p53						0.77						0.35
Intestinal	15 (40.5)	16 (43.2)	5 (13.5)	1 (2.7)	0 (0.0)	(n=66)	3 (9.7)	13 (41.9)	12 (38.7)	3 (9.7)	0 (0.0)	(n=55)
Diffuse	5 (23.8)	11 (52.4)	4 (19.0)	1 (4.8)	0 (0.0)		3 (20.0)	9 (60.0)	1 (6.7)	1 (6.7)	1 (6.7)	
Mixed	2 (25.0)	4 (50.0)	1 (12.5)	1 (12.5)	0 (0.0)		1 (11.1)	5 (55.6)	3 (33.3)	0 (0.0)	0 (0.0)	
p27^{ras}						0.048						0.26
Intestinal	7 (18.9)	23 (62.2)	7 (18.9)	0 (0.0)	0 (0.0)	(n=68)	3 (9.5)	27 (84.4)	2 (6.3)	0 (0.0)	0 (0.0)	(n=57)
Diffuse	3 (13.6)	19 (86.4)	0 (0.0)	0 (0.0)	0 (0.0)		2 (12.5)	14 (87.5)	0 (0.0)	0 (0.0)	0 (0.0)	
Mixed	0 (0.0)	9 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)		3 (33.3)	5 (55.6)	1 (11.1)	0 (0.0)	0 (0.0)	
p27^{kip1}						0.28						0.3
Intestinal	13 (37.1)	14 (40.0)	7 (20.0)	1 (2.9)	0 (0.0)	(n=68)	1 (3.1)	6 (18.8)	14 (43.8)	9 (28.1)	2 (6.3)	(n=57)
Diffuse	10 (45.5)	8 (36.4)	4 (18.2)	0 (0.0)	0 (0.0)		0 (0.0)	4 (25.0)	9 (56.3)	2 (12.5)	1 (6.3)	
Mixed	3 (33.3)	3 (33.0)	1 (11.1)	1 (11.1)	1 (11.1)		2 (22.2)	1 (11.1)	4 (44.4)	2 (22.2)	0 (0.0)	
p21^{waf1/cip1}						0.6						0.5
Intestinal	28 (76.0)	6 (16.0)	3 (8.0)	0 (0.0)	0 (0.0)	(n=68)	9 (28.0)	6 (19.0)	7 (22.0)	6 (19.0)	4 (12.0)	(n=55)
Diffuse	13 (59.0)	07 (32.0)	2 (9.0)	0 (0.0)	0 (0.0)		2 (13.0)	4 (27.0)	4 (27.0)	3 (20.0)	2 (13.0)	
Mixed	5 (56.0)	3 (33.0)	1 (11.0)	0 (0.0)	0 (0.0)		4 (50.0)	0 (0.0)	3 (37.0)	1 (12.0)	0 (0.0)	

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Hyperexpression of p21^{waf1/cip1} was observed in diffuse and mixed type tumors, and the diffuse type included more cases with loss of expression of p21^{waf1/cip1}. These results were quite different from other studies that did not observe differences in p21^{waf1/cip1} expression among the histological types of gastric carcinoma (16-18). The incidence of lymph node metastasis is considered to be relatively higher in tumors with a superficially spreading growth pattern compared with the vertically infiltrating type, and the expression of p21^{waf1/cip1} was lower in the superficial spreading tumors (19).

However, we observed a strong positivity for p27^{kip1} in all tumors and a greater positivity in the diffuse type cases, as previously reported (4-6,14). The statistical analysis revealed greater positivity in the expression of p27^{kip1} in normal mucosa adjacent to Sm1 tumors. Although there was expression of p27^{kip1} in normal mucosa of Sm2 cases, there were fewer positive cells. We observed a progressive increase in the expression of p27^{kip1} in normal, metaplastic and tumoral mucosa. *H. pylori* infection was found to induce AKT/PI3K-mediated phosphorylation of p27, leading to cytoplasmic p27 mislocalization in gastric cancer, which is an adverse prognostic feature in gastric cancer. This is important, as p27 has both tumor-suppressive and oncogenic properties related to its subcellular localization. Cytoplasmic mislocalization of p27 induced by *H. pylori* may be an important mechanistic link between *H. pylori* infection and gastric carcinogenesis (20).

An association between hyperexpression of p16^{ink4a} in normal mucosa and perineural and vascular invasion was observed. Similar results were not reported in the literature (8,14). However, there was no correlation between increased expression of p16^{ink4a} (and p27^{kip1}) and lymph node invasion. Lymph node involvement was more frequent in p21^{ras}-positive cases ($P=0.023$); inversely, cases with negative p21^{ras} expression in the tumoral mucosa, did not exhibit lymph node metastasis. However, this result contradicts the findings of Kim *et al* (21), who did not identify a significant correlation between p21^{ras} positivity and lymph node metastasis in advanced gastric cancer. p21^{ras} positivity was not exclusively identified in tumor tissues, but was also described in non-neoplastic and normal tissues (22). The expression of p21^{ras} is generally augmented in gastric adenocarcinoma and may be associated with worse prognosis. The correlation between lymph node invasion and hyperexpression of p21^{ras} may be useful for selecting therapeutic alternatives for patients with p21^{ras}-negative tumors (23,24).

A significant increase in the incidence of lymph node invasion was observed in cases with marked immunohistochemical positivity for p53 in tumor samples ($P=0.042$), as previously documented (4,5).

Lymph node involvement was also statistically related to tumor cell positivity for p21^{waf1}. None of the gastric carcinomas with negative immunoreaction for p21^{waf1} exhibited lymph node metastasis, and there was a higher frequency of lymph node involvement in cases with greater positivity for p21^{waf1}, as previously documented (17,25). It was observed that 65.4% of the cases were positive for p21^{waf1}, and a higher incidence of lymph node involvement was also found in patients with increased proliferative activity and loss of expression of p21^{waf1} in tumor tissue (26). The loss of p21^{waf1} expression and the relapse and mortality rates of gastric cancer indicate that the expression of p21^{waf1} is of prognostic value (25-27). Despite

strong evidence supporting the role of p21^{waf1} as a prognostic marker, this finding remains debatable (28).

There was no correlation observed between the expression of p27^{kip1} in the tumor and the presence of lymph node involvement in advanced cancer cases (29). We did not identify a statistical correlation between the presence of lymph node involvement and the immunohistochemical expression of p16^{ink4a}. This is a conflicting result compared with previous reports (30,31). Although the expression of p16^{ink4a} is altered in gastric tumors, this alteration is considered to be secondary to p53 mutations (5). In normal mucosa, the expression of p16^{ink4a} has been reported to be low (32). In gastric tumors, the p16^{ink4a} expression is expected to be increased in intestinal and diffuse type tumors, but no significant difference in patients' life expectancy was reported (32).

The p16 protein was expressed in >95% of the normal gastric mucosa, 92% of dysplastic gastric mucosa and in almost 45% of gastric carcinoma samples (32). In addition, the expression of p16 in carcinoma metastatic to the lymph nodes was significantly reduced compared with the expression in the primary tumor (32).

A gradual decrease of p21^{waf1}, p27^{kip1} and p16^{ink4a} immunoreactions was expected from the normal to metaplastic and tumor tissue; however, increased rates of immune positive reactions for all markers were observed. It was hypothesized that, in the early stages of cancer development, these mechanisms of cell regulation remain functional and, eventually become overexpressed in an attempt to prevent abnormal cellular proliferation. The progressive increase of the positivity for the protein markers in normal, metaplastic and tumoral mucosa occurred in parallel to the increase of the expression of p21^{ras} (which stimulates cell division), as well as p53 (which reflects the loss of the main pathway of cell maintenance control), the most commonly affected by mutations (33). The increased p53 expression detected by immunohistochemistry is frequently indicative of mutations in the p53 gene (34). This rationale may apply to p21^{ras} as well, since mutations are present in ~15% of the tumors, and, specifically in gastric cancer, direct mutations of p21^{ras} are less frequent (35,36). Mutations of the p16 gene in gastric cancer, however, appear to be uncommon, and the decrease in the expression of this protein in gastric cancer is considered to be secondary to other, still unknown mechanisms (37). Additionally, mutations of the p27^{kip1} and p21^{waf1} genes are also reported to be infrequent (38). The increased expression of these markers in early tumors is hypothesized to be an indirect consequence of an unknown mutation and/or abnormal mechanism of controlling cell division.

In summary, the analysis of the clinical characteristics and the immune expression of p53, p21^{ras}, p27^{kip1}, p21^{waf1} and p16^{ink4a} in patients with early gastric adenocarcinoma with submucosal invasion led to the conclusion that there is an important increase in the immunohistochemical expression of p53, p21^{ras}, p27^{kip1}, p21^{waf1/cip1} and p16^{ink4a} when normal tissue is compared with neoplastic parenchyma. There is also a positive correlation between higher tumor expression of p21^{ras}, p53 and p21^{waf1/cip1} and the presence of lymph node metastases, which was not observed for p27^{kip1} and p16^{ink4a}. Finally, tumors with Sm1 invasion exhibited a higher positivity for p21^{waf1/cip1}, whilst overexpression of p16^{ink4a} in the normal mucosa was associated with perineural and vascular invasion.

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