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ORIGINAL ARTICLE

p27^{KIP1} expression in gastric cancer: Differential pathways in the histological subtypes associated with *Helicobacter pylori* infection

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Abstract

Objective. Decreases in p27^{KIP1} and C-MYC expression have been associated with *Helicobacter pylori* infection. Furthermore, C-MYC seems to be a transcriptional repressor of p27^{KIP1}. Therefore, in a series of gastric adenocarcinomas we studied the association of p27^{KIP1} expression with *H. pylori* genotype (*vac*A, *cag*A, *cag*E and *vir*B11) and the involvement of C-MYC in this process. ***Material and methods.*** Expression of p27^{KIP1} and C-MYC was determined by immunohistochemistry in 84 gastric adenocarcinoma samples and *H. pylori* infection and genotype were determined by polymerase chain reaction. ***Results.*** Most p27^{KIP1}-negative cases (94.0%) were *H. pylori*-positive and 44.8% were C-MYC-positive. In the diffuse gastric cancer subtype, p27-negative-C-MYC-positive was the most frequent combination (cluster II), and was associated with the more pathogenic *H. pylori* strains. Although an association with p27^{KIP1} and *H. pylori* strain was found in the intestinal gastric cancer subtype, negativity for p27^{KIP1} and C-MYC markers was the most frequent cluster, followed by cluster II, and both were present, independent of the *H. pylori* genotype. ***Conclusions.*** Reduced expression of p27^{KIP1} was closely linked to *H. pylori* infection, and was dependent on the more pathogenic strains. Moreover, intestinal and diffuse subtypes showed distinct carcinogenic pathways influenced by *H. pylori* strains. These data add insight to the differential influence and relevance of *H. pylori* genotype in gastric cancer development.

Key Words: *C-MYC, gastric cancer, Helicobacter pylori genotypes, histological subtypes, p27^{KIP1}*

Introduction

Gastric cancer is one of the most frequent malignancies worldwide and one of the leading causes of cancer mortality in Brazil [1,2]. Adenocarcinoma, the most common type, has been classified by Laurèn [3], according to its clinical and histological features, into two main types, diffuse and intestinal, with distinct carcinogenetic pathways. Intestinal gastric adenocarcinoma, which is well differentiated, is the most frequent type and has a more favorable prognosis. Its etiology depends on environmental factors. Diffuse-type adenocarcinoma is poorly differentiated, has a poor prognosis, and its relationship with environmental factors is controversial [4–6].

Helicobacter pylori infection is the major environmental factor that contributes to the development of human gastric cancer [7,8]. The ability of *H. pylori* to promote gastric carcinogenesis is related to the host's genetic susceptibility and to specific bacterial virulence factors [9,10]. *H. pylori* is highly prevalent in human populations and has remarkable genetic diversity [11,12]. This diversity has been found to affect the bacterial virulence and consequently the disease outcome [13–15]. Two well-established virulence factors of *H. pylori* are vacuolating cytotoxin A (VacA) and the cytotoxin-associated gene A (CagA) [16,17]. The VacA protein has potential to influence gastric epithelial cell cycling and epithelial cell-signal transduction. The *vac*A gene is present in all strains and

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comprises two variable regions: the signal sequence (*s1* and *s2*) and the middle region (*m1* and *m2*), which combine mosaicism. Strains bearing *s1* and *m1* alleles have long been noted as being more virulent than *s2m2* strains [5,18,19]. The *cag*A gene is considered a marker for the presence of the *cag* pathogenicity island (*cag*-PAI). It is located within the right portion of the *cag*-PAI and induces intense inflammatory responses and alterations of the gastric epithelium [20,21].

cag-PAI also includes the *cagE* and *vir*B11genes, located in the right and left regions of *cag*-PAI, respectively. The *vir*B11 gene encodes the homonymous protein, which has a key role due to its structural position in the formation of the type IV secretion system, through which CagA is injected into host cells, and because it exhibits adenosine triphosphate synthase activity [16,22,23]. *cag*E is also considered to play a role in constructing the type IV secretion system and some authors consider it to be a marker of the integrity of *cag*-PAI, like the *cag*A gene [15,24,25].

Despite the association of *H. pylori* infection with the development and progression of gastric cancer, the precise molecular mechanisms responsible for the promotion of gastric cancer by *H. pylori* remain poorly understood. One of the proposed mechanisms is modulation of gastric epithelial cell-cycle kinetics by alterations of cell-cycle regulators, like $p27^{KIP1}$, p53, and $p21^{WAF1/CIP1}$ [26–29]. Although some studies have already described an association between *H. pylori* and p53 mutation [30–32], knowledge of the relationship between *H. pylori* and $p27^{KIP1}$ expression is still limited. $p27^{KIP1}$ is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CDKI). It binds to a wide variety of cyclin/CDK complexes, including CDK2 and -4, inhibiting kinase activity and blocking the G1/S transition necessary for cell-cycle progression [33–36]. In addition, $p27^{KIP1}$ has been implicated in the regulation of apoptosis and cell differentiation, and in the response to inflammatory stimuli [37,38]. The $p27^{KIP1}$ protein level is mainly regulated through degradation by ubiquitin-dependent proteolysis [38,39]. However, some studies have demonstrated a decreased expression of $p27^{KIP1}$ due to transcriptional repression by C-MYC, a helix–loop–helix zipper transcriptional factor [40,41]. The presence of the C-MYC protein has also been shown to block the nuclear transport of $p27^{KIP1}$ from the cytoplasm, mediated by increase in synthesis of cyclins D1 and D2 [40,42].

In gastric cancer, decreased $p27^{KIP1}$ protein expression is an indicator of poor prognosis, and is associated with more aggressive characteristics and tumor proliferation [28,35,43]. The association between *H. pylori* and reduced $p27^{KIP1}$ expression has been indicated by some *in vitro* studies and others involving eradication of this microorganism [39,44,45]. However, only a few papers have analyzed *in vivo* the association between *H. pylori* infection and the expression of the $p27^{KIP1}$ protein and they were restricted to gastritis and intestinal metaplasia [27,44]. Moreover, there are no *in vivo* studies which relate *H. pylori* genotypes with $p27^{KIP1}$ and C-MYC expression in gastric cancer. Thus we examined, in a series of gastric adenocarcinomas, the relationship between the *H. pylori* genotype and $p27^{KIP1}$ protein expression and also the involvement of C-MYC in this process. Since the intestinal and diffuse tumors are different entities, the data were also analyzed considering these histological subtypes.

Material and methods

Clinical specimens

The study was approved by the Ethics Committee of the Hospital Complex of the Federal University of Ceará and all subjects signed an informed consent form before inclusion. Samples from 84 patients with gastric adenocarcinoma who had undergone gastrectomy were collected from two hospitals in Ceará State, Brazil: Walter Cantídeo Hospital at the Federal University of Ceará and Santa Casa de Misericórdia Hospital, both located in Fortaleza, the state capital. The histological classification was made according to the Laurèn classification [3] by the team's pathologists.

DNA extraction

Genomic DNA was extracted from frozen tumor tissue using the cetyltrimethyl ammonium bromide technique, adapted from the method of Foster and Twell [46]. DNA extraction was done only in fragments that showed > 80% tumor cells, and the quality was assessed by 1% agarose gel electrophoresis with ethidium bromide staining. Also, the amount of DNA was determined using a NanoDrop™ 3300 fluorospectrometer.

Detection of H. pylori and the presence of vacA, cagA, cagE, and virB11 genes

The *H. pylori* infection was detected by amplification of the *ure*C gene using primers for polymerase chain reaction (PCR), as described by Lage et al. [47]. For the *H. pylori*-positive samples, the presence of the

alleles *vac*A, and the *cag*A, *cag*E, and *vir*B11 genes was identified using primer sets shown in the published literature. These are shown in Table I. PCR mixtures, for amplification of *cag*E and *vir*B11 genes, were prepared in a volume of 20 µl using MasterMix® (Taq DNA Polymerase, dNTPs and $MgCl_2$) according to the manufacturer's instructions (Promega, Madison, WI), with addition of 0.8% Tween 20, 0.3 µM (*vir*B11) or 1 µM (*cag*E) of each primer and 1 µl of the DNA sample.

The *cag*A, *vac*A *s1/s2*, and *vac*A *m1* genes were amplified in a 25-µl volume containing 2.5 µl of 10× PCR buffer (Invitrogen, Cergy Pontoise, France), 1% Tween 20, 1.5 mM $MgCl_2$ (Invitrogen), 200 µM (each) of dNTPs (Invitrogen), 1 U of Platinum *Taq* polymerase (Invitrogen), 0.4 µM (*ure*C, *cag*A, *vac*A *s1/s2*, and *vac*A *m1*) or 0.3 µM (*vac*A *m2*) for each primer and 1 µl of *H. pylori* DNA.

The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. The size of the amplification product was used to confirm the identity of the PCR product. The sample was considered *H. pylori*-positive when an *ure*C fragment of 294 bp was amplified. For confirmation of the specificity of reaction, PCR products from *ure*C gene were cloned with a TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) and sequenced using the ABI PRISM® BigDye™ Terminator v.3.0 cycle-sequencing kit (Applied Biosystems, Foster City, CA) and ABI PRISM® 3100 DNA Sequencer (Applied Biosystems). *vac*A, *cag*A, *cage*, and *vir*B11 genes were considered positive when a specific fragment was detected (Table I). DNAse-free water was used as a negative control. DNA preservation was also confirmed by amplification of different genes by other approaches under study in the laboratory. Random samples were re-analyzed for confirmation of the results.

Immunohistochemistry

Immunostaining was performed according to a previously described protocol of Hsu et al. [49]. For antigen retrieval, deparaffinized sections were pretreated by heating in a microwave oven in 10 mM citrate buffer, pH 6.0, for 20 min. After cooling, sections were then immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Sections were then incubated in a moist chamber overnight at 4°C with primary antibody. The primary antibodies used were $p27^{KIP1}$ (clone SX53G8; dilution 1:50; DakoCytomation; Glostrup, Zealand, Denmark) and C-MYC (clone 9E11; dilution 1:100; Novocastra; Newcastle upon Tyne, UK). After rinsing with phosphate-buffered saline (PBS), the slides were incubated with secondary antibody followed by streptavidin–biotin–peroxidase complex (LSAB+ system; DakoCytomation), both for 30 min at room temperature with a PBS wash between each step. The reaction was revealed with diaminobenzidine–H_2O_2 (DAB+ system; DakoCytomation), counterstained with Harry's hematoxylin and mounted. A confirmed case of nuclear C-MYC-positive and

Table I. PCR primer sets used for genotyping *H. pylori*.

Gene	Primer sequence	Reference	Annealing temperature (°C)	Size (bp) of PCR product
*ure*C	F: -5′AAGCTTTTAGGGGTGTTAGGGGTTT3′; R: -5′AAGCTTACTTTCTAACACTAACGC3′	47	55	294
*vac*A				
s1/s2	F: -5′ATGGAAATACAACAAACACAC3′; R: -5′CTGCTTGAATGCGCCAAAC3′	48	55	259/286
m1	F: -5′GGTCAAAATGCGGTCATGG3′; R: -5′CCATTGGTACCTGTAGAAAC3′		55	290
m2	F: -5′GGAGCCCCAGGAAACATTG3′; R: -5′CATAACTAGCGCCTTGCAC3′		52	192
*cag*A		48		
	F: -5′ATAATGCTAAATTAGACAACTTGAGCGA3′; R: -5′TTAGAATAATCAACAAACATAACGCCAT3′		56	297
*cag*E	F: -5′TTGAAAACTTCAAGGATAGGATAGAGC3′; R: -5′GCCTAGCGTAATATCACCATTACCC3′	15	50	509
*vir*B11	F: -5′TTAAATCCTCTAAGGCATGCTAC3′; R: -5′GATATAAGTCGTTTTACCGCTTC3′	15	49	491

F = forward; R = reverse.

p27-positive human breast carcinoma was used as a positive control. Controls of primary antibody specificity included omission or substitution of primary antiserum by normal bovine serum.

Immunostaining analyses

The immunohistochemical evaluation was performed by two experienced analysts independently using direct light microscopy. Any conflicting results were jointly considered to give a consensual determination. Protein expression was quantified by manual counting of at least 1000 tumor cells in 10 different fields at a magnification of $\times 400$. The labeling index (LI) was expressed as the percentage of tumor-positive cells for nuclear or cytoplasmic staining in each sample [50]. Only cases with scores $\geq 5\%$ were considered positive. Also, the pattern of staining (focal, multifocal, or diffuse) was observed. In the diffuse pattern, the staining of tumor cells occurs uniformly, distributed throughout the whole sample. In the focal staining pattern, staining is restricted to the same region and there can be one to five staining foci. Finally, in the multifocal pattern, more than five foci are observed, distributed non-uniformly.

Statistical analyses

The analyses were carried out using the statistical software SPSS version 15.0 (SPSS Inc., Chicago, IL). Statistically significant differences were evaluated by the chi-square test or Fisher's exact test. Correlations between $p27^{KIP1}$ expression and the *H. pylori* genotype group were analyzed by Spearman's rank correlation coefficient. The results were considered statistically significant when *P*-values were < 0.05.

Results

Of the 84 analyzed cases, 59 were males and 25 were females. The median age was 64.5 years (range 23–90 years). The classification of the gastric tumors was as follows: 2.4% (2/84) were stage IA, 10.7% (9/84) were IB, 23.8% (20/84) were II, 19.0% (16/84) were IIIA, 10.7% (9/84) were IIIB, and 33.3% (28/84) were IV.

Detection of $p27^{KIP1}$, H. pylori infection, and vacA, cagA, cagE, and virB11 genes

Of the analyzed cases, $p27^{KIP1}$ expression was detected in only 20.2% (17/84) and *H. pylori* infection was present in 95.2% (80/84). All $p27^{KIP1}$-positive cases were *H. pylori*-positive, with a diffuse stain pattern observed in 76.5% of the cases (13/17), focal in 17.6% (3/17) and multifocal in 5.9% (1/17). The LI ranged from 5.6% to 41%. Three cases with exclusive cytoplasmic $p27^{KIP1}$ staining and two cases with concomitant nuclear and cytoplasmic $p27^{KIP1}$ staining were also *H. pylori*-positive. On the other hand, among the 79.8% (67/84) of $p27^{KIP1}$-negative cases, 94.0% (63/67) were *H. pylori*-positive. All *H. pylori*-negative cases were also $p27^{KIP1}$-negative.

The *H. pylori vac*A *s1m1* combination was the most frequently observed (72.5%; 58/80), with *s1m2*, *s2m1*, and *s2m2* being detected in 15.0% (12/80), 5.0% (4/80) and 7.5% (6/80) of the cases, respectively. The *H. pylori cag*-PAI genes studied had similar frequencies: 65.0% (52/80) for *cag*A, 53.8% for *cag*E (43/80), and 61.2% (49/80) for the *vir*B11 gene.

Relationship between H. pylori vacA, cagA, cagE, and virB11 genes and $p27^{KIP1}$ detection

To investigate the relationship between *H. pylori* *cag*A, *cag*E, *vir*B11, and *vac*A genes and $p27^{KIP1}$ expression in the pathogenesis of gastric cancer, the cases were divided into groups according to the presence of the studied *H. pylori* genes. The division criteria utilized were *cag*-PAI integrity and presence of the *vac*A alleles, as described in Table II. This Table also shows that the highest frequency of lack of $p27^{KIP1}$ expression (76.7%; 23/30) occurred in cases with *H. pylori* subgroup A1 strain (*vac*A s1m1, *cag*A, *cag*E, and *vir*B11), followed by subgroup A2 strains (*vac*A s1m1 and *virB*11- as left-side marker of *cag*-PAI and *cag*E or *cag*A- as right-side marker of *cag*-PAI). Statistical analysis was carried out only in the A group due to insufficient numbers of cases in the B and C groups. Although the A1 subgroup had the highest number of $p27^{KIP1}$-negative cases, a representative number of $p27^{KIP1}$-positive cases were observed in this subgroup. In subgroup A2, only $p27^{KIP1}$-negative cases were found. However, this was not statistically significant ($P = 0.064$). It is interesting to note that in group B (*vac*A s1m2 or s2m1) and group C (*vac*A s2m2) the majority of $p27^{KIP1}$-negative cases were in strains with only one or no *cag*-PAI gene.

Association of $p27^{KIP1}$ negativity with the A1 subgroup was observed only when the 63 $p27^{KIP1}$-negative cases were analyzed. From this analysis, there was a statistical difference between the A1 subgroup and the A2 and A3 subgroups ($P = 0.006$ and $P = 0.022$, respectively). Also, Spearman's rank correlation showed a negative correlation between

Table II. Comparison between the results of p27 nuclear expression in *H. pylori* genotype groups in the 80 cases of gastric adenocarcinoma analyzed.

Genotype group	*N*	p27(+) (*n* = 12)	p27(–) (*n* = 46)	*P*
Group A: *vac*A s1m1				
A1 – *ure*C(+), *cag*A(+), *cag*E(+) and *vir*B11(+)	30	7	23	0.724[a]
A2 – *ure*C(+), *cag*A(+) and *vir*B11(+) or *cag*E(+) and *vir*B11(+)	11	0	11	0.064[b]
A3 – *ure*C(+), *cag*A(+) or *cag*E(+) or *vir*B11(+) or *cag*A(+) and *cag*E(+)	11	3	8	0.427[c]
A4 – *ure*C(+)	6	2	4	0.376[d]
Group B: *vac*A s1m2 or *vac*A s2m1		*n* = 3	*n* = 13	
B1 – *ure*C(+), *cag*A(+), *cag*E(+) and *vir*B11(+)	2	0	2	–
B2 – *ure*C(+), *cag*A(+) and *vir*B11(+) or *cag*E(+) and *vir*B11(+)	1	0	1	–
B3 – *ure*C(+), *cag*A(+) or *cag*E(+) or *vir*B11(+) or *cag*A(+) and *cag*E(+)	7	1	6	–
B4 – *ure*C(+)	6	2	4	–
Group C: *vac*A s2m2		*n* = 2	*n* = 4	
C1 – *ure*C(+), *cag*A(+), *cag*E(+) and *vir*B11(+)	0	0	0	–
C2 – *ure*C(+), *cag*A(+) and *vir*B11(+) or *cag*E(+) and *vir*B11(+)	1	0	1	–
C3 – *ure*C(+); *cag*A(+) or *cag*E(+) or *vir*B11(+) or *cag*A(+) and *cag*E(+)	2	1	1	–
C4 – *ure*C(+)	3	1	2	–
Total	80	17	63	

[a]A1 versus other groups.
[b]A2 versus other groups.
[c]A3 versus other groups.
[d]A4 versus other groups.

p27^{KIP1}-negative cases of the A1 subgroup with the A2 (r_S = –0.349; P = 0.005) and A3 subgroups (r_S = –0.289; P = 0.022). Additionally, as the A group was the most representative, the 46 p27^{KIP1}-negative cases belonging to this group were analyzed separately (Figure 1) and there was also a statistical difference between the A1 and A2 (P < 0.001) and between the A1 and A3 (P = 0.004) subgroups. Spearman's rank correlation showed a negative correlation between p27^{KIP1}-negative cases of the A1 subgroup with the A2 (r_S = –0.561; P < 0.001) and A3 subgroups (r_S = –0.459; P = 0.001).

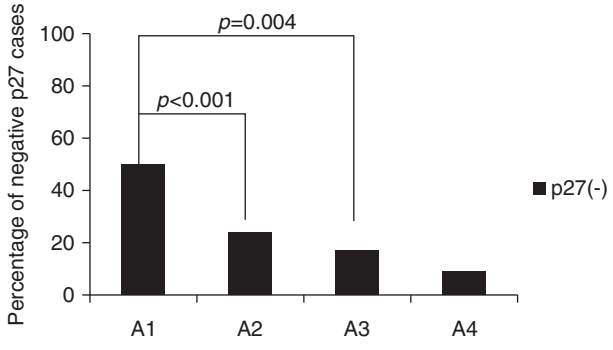

Figure 1. Comparison of 46 p27^{KIP1}-negative cases among *H. pylori* genotype groups A1, A2, A3, and A4.

Relationship between H. pylori genotype group and p27^{KIP1} detection and histological type

No statistical difference between intestinal and diffuse histological subtypes related to p27^{KIP1} expression was observed. However, when the cases were distributed according to the *H. pylori* genotype groups, a statistical difference between these histological subtypes in the A1 subgroup was found (P = 0.038) (Table III). In the A group, a tendency towards reduction of p27^{KIP1} negativity could be observed with loss of studied *cag*-PAI genes in both histological types (Table III). This tendency was not observed in cases from the B group. Most of the p27^{KIP1}-positive cases (13/17) were found in the intestinal subtype, but without a statistical difference (P = 0.39).

Among the intestinal subtype (Table III), the majority of the p27^{KIP1}-positive cases were observed in the A1 subgroup (53.8%; 7/13), besides a high number of p27^{KIP1}-negative cases in this subgroup. Six of these p27^{KIP1}-positive cases showed a predominant high LI and diffuse staining pattern. Two cases had exclusive cytoplasmic p27 staining and two cases had concomitant nuclear and cytoplasmic p27 staining, all belonging to the A group. On the other hand, in the diffuse subtype tumors, despite the small number of cases, the p27^{KIP1}-positive cases were present

Table III. Comparison of p27 nuclear positivity among the *H. pylori* genotype groups according to the histological subtypes.

	Intestinal (I)			Diffuse (D)			I×D
Genotype group	p27(+)	p27(–)	*P*	p27(+)	p27(–)	*P*	*P*
Group A							
A1	7	13	0.188[a]	0	10[e]	0.107[a]	0.038*
A2	0	8	0.076[b]	0	3[e]	0.618[b]	–
A3	1	7	0.375[c]	2	1	0.045[c]	0.152
A4	1	3	1.000[d]	1	1	0.134[d]	1.000
Subtotal	9	31	–	3	15	–	–
Group B							
B1	0	1	–	0	1	–	–
B2	0	0	–	0	1	–	–
B3	0	5	–	1	1	–	–
B4	2	2	–	0	2	–	–
Subtotal	2	8	–	1	5	–	–
Group C							
C1	0	0	–	0	0	–	–
C2	0	0	–	0	1	–	–
C3	1	0	–	0	1	–	–
C4	1	0	–	0	2	–	–
Subtotal	2	0	–	0	4	–	–
Total; *n* (%)	13 (25)	39 (75)	–	4 (14.3)	24 (85.7)	–	–

[a]A1 versus other groups.
[b]A2 versus other groups.
[c]A3 versus other groups.
[d]A4 versus other groups.
[e]A1+A2 versus other groups ($P = 0.067$).
*Histological subtype (intestinal, diffuse) versus $p27^{KIP1}$ detection.

in the less pathogenic A subgroups, with statistical significance ($P = 0.045$) for the A3 subgroup. Although all cases from the subgroups A1 and A2 were p27-negative (13/28), considering both subgroups there was no statistical significance when they were compared to the other groups ($P = 0.067$). Only one case in this group had focal cytoplasmic staining with low LI.

Figure 2 shows the distribution of the 46 $p27^{KIP1}$-negative cases according to the *H. pylori* A group and histological subtypes. From this Figure it is possible to observe that, in both intestinal and diffuse subtypes, there was a decrease in $p27^{KIP1}$ negativity according to the less pathogenic group. The intestinal subtype tumors showed statistical significance between subgroup A1 versus subgroups A2 and A3 ($P = 0.010$ and $P = 0.025$, respectively). Also, in the intestinal tumors, Spearman's rank correlation test showed that the presence of $p27^{KIP1}$-negative cases in the A1 subgroup was inversely correlated with the A2 ($r_S = -0.501$; $P = 0.004$) and A3 subgroups ($r_S = -0.459$; $P = 0.009$). In the diffuse subtype tumors, a statistical significance was only observed between subgroup A1 versus subgroup A2 ($P = 0.022$) and Spearman's rank correlation test showed that the presence of $p27^{KIP1}$-negative cases in the A1 subgroup was inversely correlated with the A2 subgroup ($r_S = -0.707$; $P = 0.003$).

Relationship between $p27^{KIP1}$ and C-MYC expression and association with H. pylori genotype group

To verify the involvement of C-MYC with $p27^{KIP1}$ negativity, both markers were analyzed together. C-MYC positivity was detected in 40.5% (34/84) of the cases, and 85.3% (29/34) of these were p27-negative. Considering the $p27^{KIP1}$-negative cases, 44.8% (30/67) were C-MYC-positive, 14 of which were intestinal and 16 diffuse subtype. Among the $p27^{KIP1}$ cytoplasmic staining cases, only one was C-MYC-positive and it belonged to the diffuse

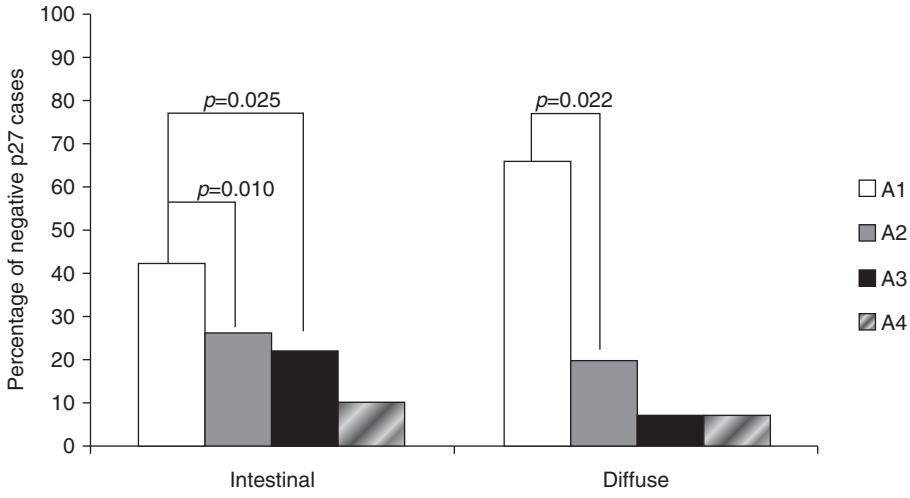

Figure 2. Comparison of 46 p27-negative cases among *H. pylori* genotype groups A1, A2, A3, and A4 according to histological type.

subtype. In histological subtypes, no statistical difference was observed between $p27^{KIP1}$ and C-MYC expression.

There was no statistical difference between C-MYC expression and *H. pylori* genotype group. However, when this association was examined considering the histological subtypes, there was a significant difference in C-MYC expression between the intestinal and diffuse tumors in the *H. pylori* subgroup A1 (P = 0.039), with a predominance of C-MYC negativity in the intestinal subtype.

For the combined C-MYC and $p27^{KIP1}$ analysis, considering the *H. pylori* genotype groups, the data were divided into four clusters (I–IV), according to the expression of these genes. This analysis showed that these clusters had different frequencies according to the histological subtype (Figure 3). In diffuse tumors, the most frequent cluster was the p27-negative-C-MYC-positive (cluster II). It was predominant in *H. pylori* strains A1 and A2, with a significantly higher frequency (P = 0.002) when compared to p27-negative-C-MYC-negative (IV). On the other hand, in the intestinal tumors, the cluster II had similar frequency among all subgroups, and there was a statistically significant difference between intestinal and diffuse tumors, related to the presence of this cluster, in the *H. pylori* A1 subgroup (P = 0.018). Additonally, in diffuse tumors, positivity for both markers (I) was more frequent in the A3 and A4 subgroups, especially in relation to the A3 subgroup (P = 0.055). The opposite was observed in the intestinal tumors, where positivity for both markers was restricted to the A1 *H. pylori* genotype. Negativity for both markers (IV) was not so frequent in the diffuse

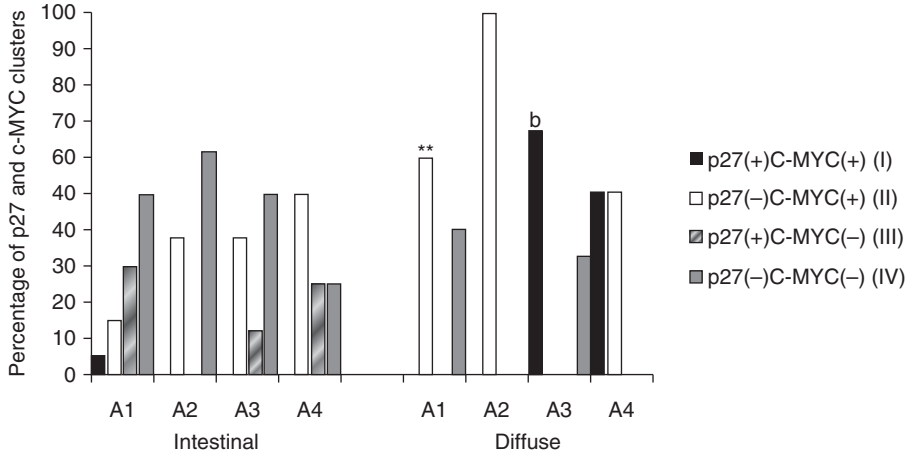

Figure 3. Percentage of clusters I (n = 4), II (n = 21), III (n = 8), and IV (n = 25) according to the *H. pylori* genotype subgroup and histological classification in 40 intestinal tumors and 18 diffuse tumors of group A (n = 58). **P = 0.002 (cluster II versus cluster IV), P = 0.018 (histological subtype versus cluster II). [b]P = 0.055.

tumors. Conversely, it was the most frequent cluster in the intestinal tumors, followed by cluster II, which was present regardless of the *H. pylori* genotype. In addition, the p27-positive-C-MYC-negative cases (III) were only observed in the intestinal tumors.

Discussion

The association of *H. pylori* infection with the development of gastric cancer is well accepted, but the mechanisms by which this bacterium acts are still unknown. Some evidence indicates that it is involved in altered expression of the tumor suppressor $p27^{KIP1}$. Shirin et al. [45] showed an inverse correlation between chronic *H. pylori* infection and $p27^{KIP1}$ protein expression in gastric epithelial cell culture. Another study conducted by Kuzushita et al. [51] suggested that *H. pylori* infection may contribute to the development of the gastric cancer $p27^{KIP1}$ pathway since *H. pylori*-infected rats with $p27^{KIP}$ deficiency are more likely to develop gastric cancer. *In vivo*, Yu et al. [44] observed reversion of $p27^{KIP1}$ protein expression after eradication of *H. pylori* in patients with intestinal metaplasia. Additionally, studies indicate the pathogenic nature of certain types of *H. pylori* strains [9,15,18], raising the hypothesis that the pathogenicity of bacteria is related to the presence of more virulent strains. In spite of the relevance of the absence of $p27^{KIP1}$ in gastric cancer pathogenesis associated with the presence of *H. pylori* infection, few *in vivo* studies to date have tried to elucidate the influence of this bacterium in $p27^{KIP1}$ expression. Therefore, in the present study, alterations in the expression of $p27^{KIP1}$ in samples from gastric adenocarcinoma patients were analyzed in association with the presence of particular *H. pylori* strains, taking into account the presence of genes considered as important virulence factors. Also, we examined the influence of C-MYC expression in this process, considering distinct subtypes of gastric adenocarcinoma.

Since a high frequency (95%) of *H. pylori* infection was found in the study, with only four cases negative for *H. pylori*, we decided to evaluate the data according to the bacterial genotype. However, since we identified different genotypes of *H. pylori* strains, we divided the data into *H. pylori* genotype groups, based on the presence of right (*cag*A and *cag*E) and left (*vir*B11) *cag*-PAI markers and the *vac*A mosaicism. Strains bearing *cag*A, *vac*A *s1m1*, and/or *cag*E and *vir*B11 genes (A1 and A2 subgroups) were the most frequent and were considered more pathogenic since these genes were already related in the other studies to more aggressive lesions and to the integrity of the pathogenicity island (*cag*A, *cag*E, and *vir*B11), another important virulence factor of *H. pylori* strains [7,14,15,52–54]. In the general analysis, the majority of the $p27^{KIP1}$-negative cases were found in the more pathogenic *H. pylori* groups (A1 and A2), but without a statistical difference. In fact, when we carried out the analysis considering only the $p27^{KIP1}$-negative cases, there was a statistical difference between the frequencies in the A1 subgroup and the A2 and A3 subgroups. Also, there was a negative correlation between $p27^{KIP1}$-negative cases in the A1 versus the A2 and A3 subgroups. These data indicate that strains considered more pathogenic could be responsible for decreasing $p27^{KIP1}$ protein, leading to development/promotion of gastric carcinoma. These findings contradict the study by Eguchi et al. [39], which demonstrated, *in vitro*, that *H. pylori* virulence-associated genes, including the *cag*A, *cag*E, and *vac*A genes, may play a minor role in decreasing the level of $p27^{KIP1}$ protein in gastric epithelial cells.

A relevant aspect of this study is that the data were analyzed considering the adenocarcinoma subtypes. Currently, based on the molecular changes involved, intestinal and diffuse subtypes are considered distinct tumors, having different tumorigenic pathways [6]. Most studies, especially those that consider the influence of *H. pylori*, do not make this distinction, instead considering gastric adenocarcinoma as a single disease. This may explain some conflicting data [39,45]. Wiksten et al. [55] and Sgambato et al. [56] carried out studies involving $p27^{KIP1}$ expression in both histological subtypes, but without considering *H. pylori* infection and, like in the present study, they did not observe a correlation between $p27^{KIP1}$ expression and intestinal and diffuse tumors. On the other hand, Saragoni et al. [57] showed a correlation between decreased $p27^{KIP1}$ expression and diffuse mixed histotype, but they did not evaluate the influence of *H. pylori* strains in that process. Thus, the present study is the first to explore the influence of different strains of *H. pylori* in $p27^{KIP1}$ expression in both histological subtypes. In this analysis, there was a clear interference of *H. pylori* strain with $p27^{KIP1}$ expression, since in the general analysis, without considering *H. pylori* groups, no difference relating to $p27^{KIP1}$ expression was found between intestinal and diffuse subtypes. However, when these groups were considered, some differences were observed.

In fact, in the present study, *H. pylori* strains seemed to act in distinct ways, according to histological subtype. Both subtypes had a common pathway involving $p27^{KIP1}$ inactivation in association with the more pathogenic strain (A1). $p27^{KIP1}$ positivity in this group was only observed in the intestinal subtype, indicating the existence of two pathways in this histological subtype, one where blockade of $p27^{KIP1}$ is

not involved, while in the other it is an important step. On the other hand, in the diffuse tumors, p27 negativity was statistically related to more pathogenic strains (A1 and A2 subgroups) and, conversely, despite the small number of cases in this group, $p27^{KIP1}$ positivity was related to the less pathogenic strains, indicating that, in these tumors, blockade of the $p27^{KIP1}$ pathway, related to more pathogenic strains, was unique and important.

The reduction in $p27^{KIP1}$ expression in gastric cancer, associated with *H. pylori* infection, can be explained by the transcriptional and post-translational mechanisms. In an acute exposure model, *H. pylori* increased the degradation of $p27^{KIP1}$ protein via a proteasome-dependent pathway [39]. On the other hand, in a long-term co-culture model during which *H. pylori* selects for apoptosis-resistant gastric epithelial cells, the expression of $p27^{KIP1}$ mRNA, detected by Northern blotting, was reduced by about 30% [45]. Similar results were obtained by cDNA microarray analysis [58]. Additionally, there are indications that *H. pylori* increases C-MYC expression in patients with gastric cancer [59] and since there is evidence that C-MYC decreases transcription and the nuclear transport of $p27^{KIP1}$ from the cytoplasm [40,42], or sequestration of $p27^{KIP1}$ in cyclin D/cdk4 complexes [60], the increase in C-MYC expression is suggested to be another way of $p27^{KIP1}$ inactivation by *H. pylori* [27].

In our study, a correlation between $p27^{KIP1}$ and C-MYC was not found, although most (85.3%) C-MYC positive cases were $p27^{KIP1}$-negative. However, most of the diffuse tumors were C-MYC-positive-p27-negative and statistically associated with the more pathogenic *H. pylori* strains (A1 and A2). Additionally, positivity for both markers was observed in less pathogenic strains, indicating that in this histological subtype the mechanism by which *H. pylori* causes the decrease in $p27^{KIP1}$ is C-MYC activation, as suggested by Kim et al. [27], related to the most pathogenic strains. C-MYC is a transcriptional factor of the helix–loop–helix/leucine zipper family of proteins, involved in several cellular processes, such as proliferation and apoptosis [61]. In those tumors, it is plausible to suggest that cellular proliferation and tumoral progression occur through transcription blockade of $p27^{KIP1}$ by C-MYC. Interestingly, in these tumors, only one of the cases with $p27^{KIP1}$ cytoplasmatic labeling was C-MYC-positive, contradicting studies of Yang et al. [40] and Perez-Roger et al. [42].

In the intestinal subtype, in spite of a decrease in $p27^{KIP1}$ expression associated with the presence of C-MYC, the predominant finding was negativity for both markers, and neither of them was associated with a specific *H. pylori* group, which justifies the contradictory data from Eguchi et al. [39]. Therefore, the influence of more pathogenic *H. pylori* strains on the decrease in $p27^{KIP1}$ expression, which has been statistically confirmed, could be caused by protein degradation in these tumors, as demonstrated by Eguchi et al. [39], instead of by C-MYC blocking $p27^{KIP1}$ expression, as suggested by Kim et al. [27]. Furthermore, it seems that sequestration in the cytoplasm, related to the most pathogenic *H. pylori* strains, might be another alternative way to inactivate $p27^{KIP1}$-associated inhibitory activity, since all cytoplasmic $p27^{KIP1}$ cases were found to be associated with strains of the A group in those tumors. The cytoplasmic positivity for $p27^{KIP1}$ protein corresponds to the accumulation of this protein in the cytoplasm, caused by its oncogenetically activated protein-kinase B-mediated phosphorylation, which blocks the recognition of its sites by nuclear importation factors [62]. However, this study included a fairly small number of cases with $p27^{KIP1}$ cytoplasmic

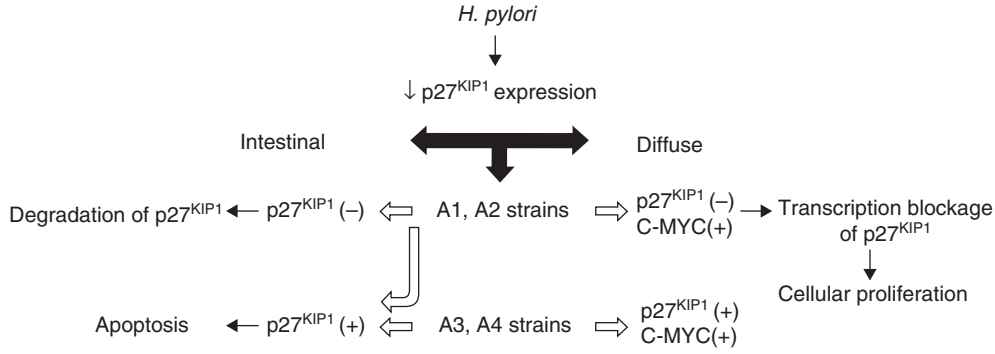

Figure 4. Schematic presentation of the different pathways for the two histological subtypes of gastric adenocarcinoma. For intestinal tumors, a decrease in $p27^{KIP1}$ expression occurs independent of C-MYC and is associated with the most pathogenic strains of *H. pylori*, probably by degradation of that protein. These strains are also related in some cases to $p27^{KIP1}$ positivity, leading to tumor cell apoptosis. In diffuse tumors, C-MYC blocks transcription of $p27^{KIP1}$, which occurs dependent on the most pathogenic strains, causing disordered cell proliferation and tumor progression.

expression, and this may have had an impact on our conclusions.

Additionally, it was observed, exclusively in intestinal tumors, that there is an association of $p27^{KIP1}$ positivity with the absence of C-MYC. Wu et al. [63] showed that inhibition of C-MYC leads to induction of increased $p27^{KIP1}$ levels. The upregulated $p27^{KIP1}$ level is caused by an increase in protein stability caused by the reduction of Skp2, a key molecule related to $p27^{KIP1}$ ubiquitination and degradation, and *de novo* protein synthesis [64]. The overexpression of $p27^{KIP1}$ protein in mammalian cells induces G1 arrest of the cell cycle, leading to inhibition of proliferation and induction of apoptosis [65]. Lee et al. [43] demonstrated that positive expression of $p27^{KIP1}$ correlated significantly with a favorable prognosis in gastric cancer.

Figure 4 summarizes the distinct pathways for intestinal and diffuse tumors.

In conclusion, our data indicate that reduced expression of $p27^{KIP1}$ is closely linked to *H. pylori* infection-associated gastric cancer and is dependent on the most pathogenic strains. Besides this, it seems that the two gastric adenocarcinoma histological subtypes have distinct carcinogenic pathways, with different influence of *H. pylori* strains. Eradication of *H. pylori* reverses the decreased expression of $p27^{KIP1}$ in gastric cancer and may possibly halt the gastric carcinogenesis cascade. Further understanding of regulators of the cell cycle, such as $p27^{KIP1}$, and the role of *H. pylori* in gastric cancer pathogenesis may provide not only valuable prognostic information but perhaps also a new dimension regarding its treatment.

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