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ORIGINAL ARTICLE

Differential expression of MYC in *H. pylori*-related intestinal and diffuse gastric tumors

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Abstract Evidence suggests that the carcinogenic process guided by *Helicobacter pylori* is related to the expression of cell cycle and apoptosis proteins as BCL-2, BAX, and MYC. However, the literature is conflicting regarding the expression frequency in the histological subtypes and did not consider *cag*A gene presence. To investigate the expression of these proteins considering the histological subtypes of gastric cancer associated with *H. pylori* (*cag*A), a total of 89 cases were used. *H. pylori* infection and *cag*A status were determined by PCR. Immunodetection was performed for MYC, BCL-2, and BAX proteins. *H. pylori* was found in 95.5% of the patients, among them, 65.8% were *cag*A(+). Nuclear MYC was detected in 36.4%, BAX in 55.7%, while BCl-2 in just 5%. Nuclear MYC staining was significantly lower in the intestinal than diffuse subtype ($p=0.008$) and was related with the presence of *H. pylori* *cag*A(+). Additionally, most of the few cases cytoplasmic MYC positive were in the intestinal subtype. In diffuse tumors, although most nuclear MYC positive cases were *cag*A(+), it was not significant. No difference was observed between BCL-2 or BAX expression considering the presence of *cag*A gene in the histological subtypes. It seems that MYC could be relevant for the diffuse tumorigenic pathway associated with *H. pylori* and possibly influenced by the presence of *cag*A gene, while in intestinal tumors, the tumorigenic pathway does not occur through the MYC expression.

Keywords Gastric cancer · *H. pylori* · *cag*A · MYC

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Introduction

Gastric carcinoma is the fourth most common cancer worldwide, and in Brazil, it is a relevant cause of cancer mortality [1, 2]. Adenocarcinomas, the most frequent gastric tumors, are classified by Laurén [3] into intestinal and diffuse subtypes, which differ regarding their epidemiological and prognostic features [4] and develop through distinct genetic pathways [5, 6].

Gastric cancer has a multifactorial etiology [7] and *Helicobacter pylori* is the major etiologic risk [8]. Evidence suggests that the presence of virulence factors could be responsible for the organism's ability to cause different diseases. In fact, only 1–2% of the population infected with *H. pylori* will develop gastric cancer [9]. This bacterium has a great genetic diversity, and strains carrying the *cag*A gene are strongly associated with the development of gastric cancer [10]. However, despite some advances, the pathway associated with *H. pylori* in gastric pathogenesis remains poorly understood. For a better understanding of this process, several studies have investigated specific patterns in the expression of apoptosis or cell cycle-related proteins such as BCL-2, BAX, and MYC, associated with the carcinogenic process guided by *H. pylori.*

BCL-2 and BAX are members of the BCL-2 family. BAX is a cytosolic monomer in viable cells, but during apoptosis it changes its conformation, integrates into the outer mitochondrial membrane, and oligomerizes. In contrast, antiapoptotic BCL-2 prevents BAX activation/oligomerization

and consequently inhibits mitochondrial proapoptotic events [11, 12].

Overexpression of BCL-2 suppresses cell proliferative activity in human gastric carcinomas and is correlated with less aggressive biological behavior [13]. The difference in BCL-2 protein expression in the Lauren subtypes is controversial. Xu et al. demonstrated that BCL-2 expression was higher in the diffuse subtype [14], but Liu et al. showed that it was higher in the intestinal subtype [15]. As for BCL-2, there is no consensus on the relevance of BAX expression in any of the Lauren histological [12]. Besides, Ashktorab et al. observed that BAX is translocated from the cytoplasm to the mitochondria, triggering apoptosis in cells infected with *H. pylori* [11]. However, the relationship of these proteins and *H. pylori* infection in gastric cancer is still controversial, and the majority of reports have not considered the bacterial genotype.

MYC plays a key role in the regulation of cell cycle progression and apoptosis. The *MYC* gene, located on human chromosome 8q24, encodes a transcriptional factor involved in proliferation, growth, and apoptosis [16, 17]. It is tightly regulated in normal cells and is only expressed during embryogenesis and in tissue compartments that possess high proliferative capacity, while having a short half-life. In human tumors, its expression is frequently enhanced. Deregulated expression of MYC occurs in a broad range of human cancers, indicating a key role in tumor progression [18, 19]. Several studies have demonstrated *MYC* gene amplification and increased protein expression in precancerous gastric lesions and in gastric cancer [17, 20]. The increase in MYC expression has also been associated with *H. pylori* infection in adenocarcinomas [5].

Therefore, the aim of this study was to investigate whether there are differences in the expression of BCL-2, BAX, and MYC proteins in adenocarcinomas in association with the *cagA* genotype of *H. pylori*, also considering the histological subtypes. The identification of a characteristic genetic pattern in gastric tumors may help predict prognosis and may be helpful in therapeutic research.

Materials and methods

Patients and specimens

This study was approved by the ethics committee of the Federal University of Ceará. A total of 89 surgically resected adenocarcinoma specimens were obtained from two hospitals in Fortaleza, Ceará State, Brazil: Walter Cantideo Hospital at Federal University of Ceará and Santa Casa de Misericórdia Hospital. Fragments of tumor were collected during the gastrectomies and frozen at −80°C. Representative formalin-fixed tumor specimens were selected and histological sections (5 μm) were made for immunohistochemical assays. The histological diagnoses and tumor classification were based on Lauren's criteria.

DNA extraction, *H. pylori* urease C and *cag*A gene detection

Genomic DNA was extracted from frozen tumor tissue samples consisting mainly (>80%) of tumor cells using the cetyltrimethyl ammonium bromide method adapted from Foster and Twell [21]. *H. pylori* infection was detected by amplification of the *urease*C gene using specific primers and conditions, as previously described by Lage et al. [22]. The *cag*A gene was identified using the primers described by Domingo et al. [23]. Negative (water) and positive (case *H. pylori/cag*A positive) controls were assayed in each run. PCR products were separated on 6% polyacrylamide electrophoretic gels, silver stained, and considered *H. pylori*(+) and *cag*A(+) when fragments of 294 and 297 bp, respectively, were present.

Immunohistochemistry

MYC protein expression was determined in all 85 cases while expression of BCL-2 and BAX was analyzed in only 70 cases due to the unavailability of material for such analysis. Immunostaining was performed according to the protocol previously described by Lima et al. using the following primary antibodies: Bcl-2 clone 124, mouse, dilution 1:80; Bax, polyclonal, rabbit, dilution 1:400 (both from DakoCytomation); and MYC, clone 9E10, mouse, dilution 1:80, from Labvision [24]. The reaction was developed with the LSAB + system (DakoCytomation) according to the manufacturer's recommendation.

Histopathological and immunostaining analysis

The histological classification was obtained from the medical reports and was confirmed by a pathologist on our team. The cases with mixed pattern were re-evaluated and the prevalent tumor pattern was considered. The slides were independently evaluated by three experienced technicians using light microscopy at ×400 magnification. Specimens were considered positive for related proteins if they demonstrated ≥5% stained tumor cells.

Statistical analysis

All statistical analyses were conducted using the SPSS® 15.0 version statistical software program (SPSS, Chicago, IL, USA) using the χ^2 and Fisher's exact tests. Correlations were analyzed using Spearman's correlation coefficient and considered statistically significant at $p<0.05$.

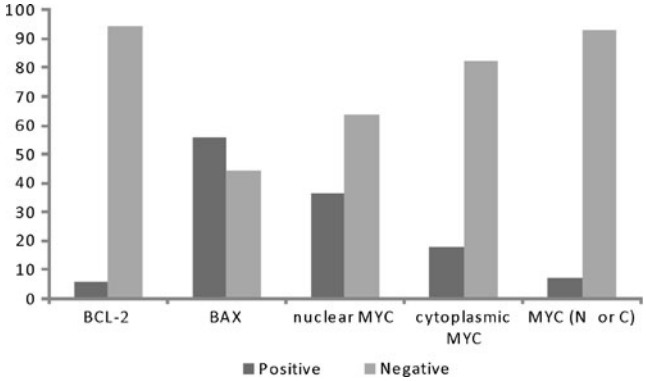

Fig. 1 Frequency of immunostaining of MYC, BCL-2, and BAX in gastric adenocarcinomas. (*N*) nuclear MYC; (*C*) cytoplasmic MYC

Results

Of the 89 tumor samples analyzed, 61 (71%) were from males and 24 (28%) from females. The average age was 56.5 years (range 23–90 years old). *H. pylori* was found in 95.5% (85/89) of the patients. Among them, 65.8% (56/85) were *cag*A(+). *H. pylori*-negative tumors were not considered for the *H. pylori* association because this was not the subject of this study and also because of the small number of cases. Of the *H. pylori* positive cases, the intestinal subtype was more frequent (63.5%, 54/85) than the diffuse (36.4%, 31/85). Most of the cases (60%) were in advanced grade (III–IV). BAX protein was detected in 55.7% of the cases (39/70), while BCL-2 was detected in just 5.7% (4/70) (Fig. 1). MYC staining was observed in the nucleus (36.4%; 31/85) or in the cytoplasm (17.6%; 15/85) and co-localization occurred in 7% (6/85) of the cases with a total of 47% (40/85) of MYC staining.

No correlation was found between BCL-2, BAX, and MYC in the total samples. However, considering each histological subtype, there was a negative correlation in the diffuse tumors between nuclear MYC and BAX positivity ($r=-0.395$; $p=0.038$). Conversely, despite the few cases that were BCL-2 positive, a probable association between BCL-2 and cytoplasmic MYC was demonstrated by a significant positive correlation between them ($r=0.801$; $p=0.000$). No significant correlation was observed in the intestinal subtype.

Table 1 shows the frequencies of positive cases for BCL-2, BAX, and MYC distributed according to histological subtype. There was a difference between these tumors only with respect to nuclear MYC immunostaining, where a significantly lower detection ($p=0.008$) was observed in the intestinal subtype (74%, 40/54) than the diffuse subtype (45%, 14/31). When the positivity of MYC was considered independent of the stain location (cytoplasmic/nuclear), no statistically significant difference was found.

To verify if the frequency of BCL-2, BAX, and MYC protein expression is influenced by the presence of *H. pylori cag*A(+), we distributed the data considering this aspect. Table 2 shows that most cases infected with *H. pylori* cagA(+) were negative for nuclear MYC (83%, 31/37) in the intestinal subtype, contrasting with a substantial nuclear MYC positivity (63%) in the diffuse tumors (Fig. 2a, b). Indeed, among the intestinal tumors, there were significantly fewer cases of *H. pylori cag*A(+) with nuclear MYC immunoreactivity ($p=0.023$) than cases of *H. pylori cag*A(−). This difference was not observed in the diffuse tumors.

Considering only the cases of *H. pylori cag*A(+), regarding to MYC expression (Fig. 3, Table 3), a significant difference between the intestinal and diffuse tumors seen before (shown in Table 2) was confirmed ($p\leq0.001$), and also the absence of either MYC cytoplasmic or nuclear MYC expression remained associated with the intestinal tumors ($p=0.028$). Positivity was similar in both histological subtypes regardless of the pattern of MYC staining (C + N), and no difference was observed when only the cytoplasm staining was analyzed (intestinal $p=0.876$; diffuse $p=0.672$). However, the few cases (15) with only MYC cytoplasmic staining, most of them (12/15; 80%) were in the intestinal subtype as seen in Table 1.

Table 1 Frequency of positive cases of markers in histological subtypes

		Intestinal *n* (%)	Diffuse *n* (%)	Total *n* (%)	*p* Value
MYC (N)	+	14/54 (26)	17/31 (55)	31/85 (36.4)	0.008*
	−	40/54 (74)	14/31 (45)	54/85 (63)	
MYC (C)	+	12/54 (22.2)	03/31 (9.6)	15/85 (17.6)	0.144
	−	42/54 (77)	28/31 (90)	70/85 (82)	
MYC (N and C)	+	22/54 (40.7)	18/31 (58)	40/85 (47)	0.123
	−	32/54 (59.2)	13/31 (42)	45/85 (53)	
BCL-2	+	01/41 (2.4)	03/29 (10)	4/70 (5.7)	0.160
	−	40/41 (97)	26/29 (89)	66/70 (94)	
BAX	+	21/41 (51)	18/29 (62)	39/70 (55.7)	0.368
	−	20/41 (48)	11/29 (37)	31/70 (44)	

N nuclear staining, *C* cytoplasmic staining

* $p<0.05$

Table 2 Frequency of detection of markers between the histological subtypes

		Intestinal				Diffuse			
		*cag*A+*n* (%)	*cag*A−*n* (%)	Total *n* (%)	*p* Value	*cag*A+*n* (%)	*cag*A−*n* (%)	Total *n* (%)	*p* Value
MYC (*N*)	+	6/37 (16)	8/17 (47)	14/54 (25)	0.023*	12/19 (63)	05/12 (41)	17/31 (54)	0.242
	−	31/37 (83)	9/17 (52)	40/54 (74)		07/19 (36)	07/12 (58)	14/31 (45)	
MYC (*C*)	+	08/37 (21)	04/17 (23)	12/54 (22)	0.876	02/19 (10)	01/12 (8.3)	03/31 (9)	0.672
	−	29/37 (78)	13/17 (76)	42/54 (77)		17/19 (89)	11/12 (91)	28/31 (90)	
BCL-2	+	1/27 (3)	0/14 (0)	1/41 (2)	0.659	02/17 (11)	01/12 (8.3)	03/29 (10)	0.633
	−	26/27 (96)	14/14 (100)	40/41 (97)		15/17 (88)	11/12 (91)	26/29 (89)	
BAX	+	13/27 (48)	8/14 (57)	21/41 (51)	0.585	09/17 (52)	09/12 (75)	18/29 (62)	0.208
	−	14/27 (51)	06/14 (42)	20/41 (4)		08/17 (47)	03/12 (25)	11/29 (37)	

N nuclear staining, *C* cytoplasmic staining

* $p<0.05$

Discussion

In this work, almost all (95.5%, 85/89) of the cases were from *H. pylori*-infected patients. This high incidence is in agreement with other studies of gastric cancer either using PCR, in which *H. pylori* was detected in 95% [25] and 100% [26] of cases, or using serology [27] where *H. pylori* was identified in 94% of specimens.

Currently, a research challenge is to identify the pathways by which *H. pylori* can contribute to gastric carcinogenesis. Several studies have focused on markers related to cell proliferation control and apoptosis. In fact, there are consistent data regarding an association between the presence of the bacterium and *TP53* mutation and a decrease in p27 protein expression influenced by *H. pylori* genotype [28–31]. However, the data are controversial regarding the influence of *H. pylori* or its genotype on the expression of key regulatory genes of apoptosis, such as BAX and BCL-2.

Konturek et al. showed that *H. pylori* infection is associated with increased BAX and decreased BCL-2 protein expression in duodenal ulcer patients [32]. Conversely, another study by the same author in gastric cancer patients demonstrated an increased protein expression of BCL-2 and decreased expression of BAX with no association with *H. pylori* infection [33]. In a cell culture study, Yang et al. observed that the presence of *H. pylori* has suppressed the BCL-2 protein expression [34]. Considering the presence of *cag*A(+), Cabral et al. showed that the infection caused by *H. pylori cag*A(+) was related to the increased expression of BAX in patients with *H. pylori*-related gastritis [35]. However, Zhu et al. using cultured cells, showed that *H. pylori cag*A(+) did not alter BCL-2 or BAX expression [36].

In the present study, few cases were BCL-2 positive. As all the cases were *H. pylori*(+), this low frequency agrees with the findings of Yang et al. in which *H. pylori* downregulates BCL-2 [34]. Additionally, the cases in the present study were mainly represented by more malignant tumors, and according to Xu et al. the peak of BCL-2 expression occurs in the early stages and lowers in progressive gastric cancer [14]. However, in our study we intended to evaluate the importance of genotype comparing the difference with strains carrying the *cag*A gene, since this gene is associated with more aggressive lesions and risk for gastric cancer development [37]. Therefore, we classified the data according to the presence of *H. pylori*

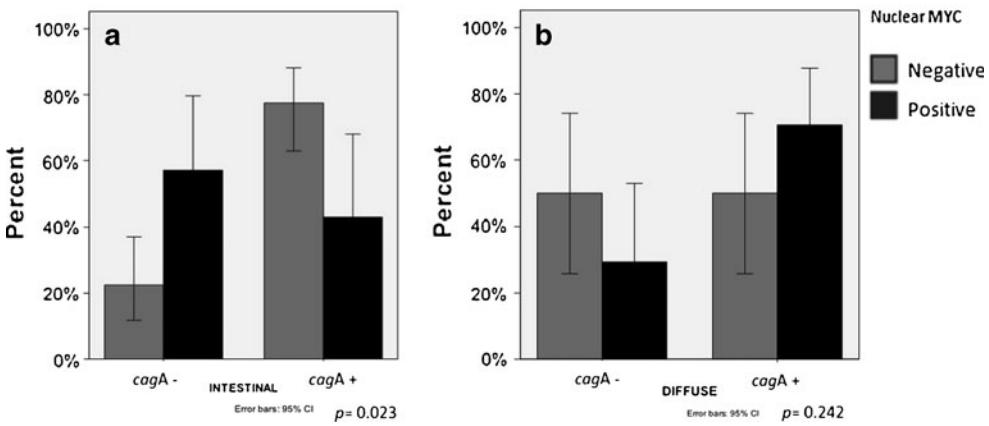

Fig. 2 Nuclear MYC immunostaining in intestinal and diffuse subtypes, considering the presence of the *H. pylori* cagA gene. *$p<0.05$

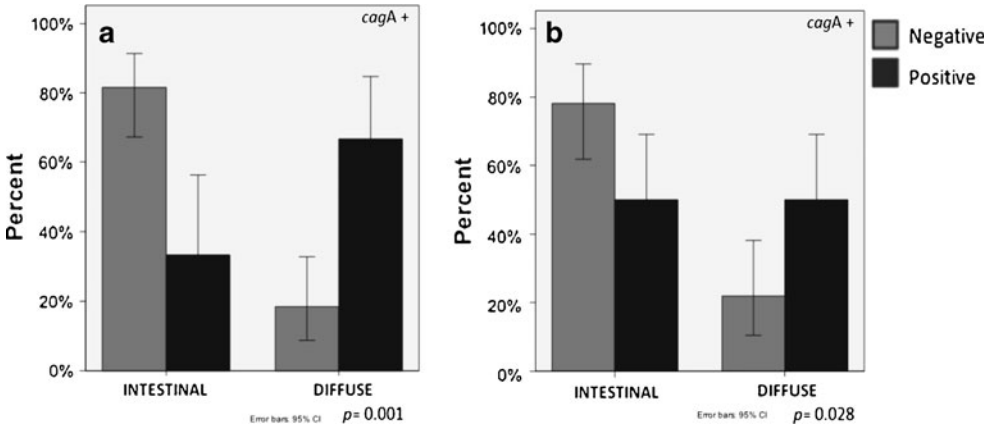

Fig. 3 Analysis of the difference in detection of MYC in cases *H. pylori cag*A (+) strains. **a** Nuclear staining; **b** nuclear and cytoplasmic staining. *$p<0.05$; **$p<0.01$

*cag*A. From this analysis, we did not detect any significant difference between BCL-2 and BAX expression according to *H. pylori cag*A status, which is in agreement with the findings of Zhu et al. [36].

Few papers have analyzed BCL-2 and BAX protein expression patterns considering the histological subtypes. As in this study, van der Woude et al. and Lee et al. did not observe any difference in BCL-2 or BAX immunostaining between intestinal and diffuse subtypes [12, 13]. Alternatively, Xu et al. found that BCL-2 positivity was higher in diffuse tumors [14]. Although none of these studies considered *H. pylori* infection, in our study, we found no association between *cag*A status and BAX or BCL-2 positivity according to the histological subtype. Thus, divergence in BCL-2 and BAX protein expression does not depend on the presence of the *cag*A gene and is not specific for a histological subtype.

Yang et al. in a cell culture study suggested the involvement of *H. pylori*-induced apoptosis by increasing BAX and MYC expression and decreasing BCL-2 expression [34]. However, in diffuse tumors, we observed a significant negative association between nuclear MYC and BAX and a positive correlation between BCL-2 and cytoplasmic MYC. Considering these results, it seems that the MYC protein expression in diffuse tumors is not influenced by *cag*A presence.

Recently, the expression of MYC has been regarded as an important alteration in gastric tumorigenesis, also, Kim et al. showed that the combined expression of MYC with EGFR and FGFR2 could predict overall survival and time to progression of chemotherapy-treated gastric cancer patients [38]. However, the exact role of MYC in gastric cancer is not well-established. Our study agree with Calcagno et al. and Zhang et al. that showed that MYC expression was higher in *H. pylori*-associated cases of gastric cancer than without infection [17, 20]. Additionally, our findings showed that the lack of MYC detection was associated with the presence of the *cag*A gene. This finding is in agreement with that of Bach et al. whose study in AGS cells showed that *MYC* was downregulated when coincubated with *cag*A(+) and upregulated by up to 6.8-fold when coincubated with mutant *cagA*(−) [39].

There is still controversy regarding the histological type that prevails in the expression of MYC. In the present study, we found a significantly higher number of negative cases more associated with intestinal tumors than with diffuse tumors. This result is in contrast to the findings of Xu et al., where MYC levels were higher in the intestinal than in the diffuse subtype [14], and of Calcagno et al. where MYC protein was present in both subtypes with all intestinal tumors showing nuclear MYC staining [17]. However, only seven cases were analyzed in the latter

Table 3 MYC, BCL-2 and BAX immunostaining frequencies in both histological subtypes considering the presence of the *cag*A gene

		*cag*A+strains			
		Intestinal *n* (%)	Diffuse *n* (%)	Total *n* (%)	*p* Value
MYC (N)	+	06/37 (16)	12/19 (63)	18/56 (32)	0.001*
	−	31/37 (83)	7/19 (36)	38/56 (67)	
MYC (C)	+	8/37 (21)	2/19 (10)	10/56 (17)	0.262
	−	29/37 (78)	17/19 (89)	46/56 (82)	
BCL-2	+	01/27 (3)	02/17 (11)	3/44 (6)	0.329
	−	26/27 (96)	15/17 (88)	41/44 (93)	
BAX	+	13/27 (48)	09/17 (52)	22/44 (50)	0.757
	−	14/27 (51)	08/17 (47)	22/44 (50)	

N nuclear staining, *C* cytoplasmic staining

*$p<0.05$

study. Although these studies indicated that the deregulation of MYC expression is a characteristic of intestinal tumors, the discrepancy can be explained by the fact that they did not consider the presence of *H. pylori* or *cag*A or differentiate between nuclear and cytoplasmic MYC staining.

In fact, in the *cag*A + cases, nuclear positivity of MYC was more associated with the diffuse subtype (63%), although with no statistical difference, while the negativity was significantly associated with the intestinal subtype (83%). Previously, our research group showed the relevance of MYC in diffuse type tumors where it was associated with p27 negativity [6]. Since the histological subtypes act through different carcinogenic pathways, our findings indicate that in a relevant number of cases of the intestinal tumors, the carcinogenesis process *H. pylori*-related does not occur through the MYC protein expression. Interestingly, the observation that most of the few cytoplasmic MYC positive cases were in the intestinal subtype and were associated with the presence of *cag*A + (8/12, 66.7%), suggest that in these few tumors there is a blockage in the transport of MYC protein through the nuclear membrane. Additional studies of the molecular events (transcriptional or post-transcriptional level) are required to better understand this and other molecular markers involved in the gastric cancer carcinogenesis process considering the *H. pylori* and *cag*A gene.

In this report, we emphasize the difference between intestinal and diffuse tumors. The data indicate that in diffuse tumors, the *H. pylori*-associated tumorigenic pathway occurs preferentially through the MYC protein but not in the intestinal tumors. Also, nuclear and cytoplasmic MYC detection was categorized, since MYC protein plays a role as a transcription factor only in the nucleus. Thus, the expression of MYC related to a specific subtype of gastric tumor may help to elucidate the distinct carcinogenic pathways of these tumors and could work as therapeutic target.

Conflicts of interest The authors declare that they have no conflicts of interest.

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