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Dysregulation of Expression of the FOXO3 Transcription Factors Pathway in *Helicobacter pylori*-Associated Gastric Cancer

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Abstract

The transcription factor FOXO3 acts as a tumor suppressor and is the major target of AKT kinases pathway. The protein encoded by FOXO3, prevents the activation of genes that are important for proliferation, differentiation and death cell. In addition, FOXO3 interacts with BCL-2 family proteins; and among them, BBC3 (PUMA) and BCL2L (BIM) are responsible for regulating apoptosis through the BAX and BAK chains. This study investigated the expression of FOXO3a, BBC3 and BCL2L in gastric cancer considering the presence of *Helicobacter pylori* and its involvement in the modulation of gene and protein expression. A total of 225 gastric biopsy samples were evaluated from individuals with normal gastric mucosa (n=54), gastritis (n=108), and gastric cancer (n=63), of both genders and over 18 years old. Samples were subclassified according to the presence or absence of *Helicobacter pylori,* detected by PCR. Real-time quantitative RT-PCR (qRT-PCR) and western blotting, were used to detect the expression. FOXO3 showed increased gene ($p<0.0001$) and protein ($p=0.036$) expression in patients with gastric cancer, who also exhibited a *boost* in mRNA expression through *H. pylori infection***.** BCL2L had a subtle increase in its expression independent of the presence of *H. pylori* ($p<0.0001$)*,* while the BBC3 gene showed no alterations*.* Our results suggested that increase FOXO3 expression may play an important role in carcinogenesis by performing positive *feedback* in an attempt to eliminate neoplastic cells. FOXO3 could be suggested a prognostic marker as well as a potential molecular therapy target for gastric cancer patients.

Keywords: Apoptosis; BBC3; BCL2L; FOXO3; Gastric Cancer

Introduction

Gastric cancer has become one of the most common neoplasms in the world. In Brazil, it is the third most frequent type of cancer among men and the fifth among women with approximately 13,360 new cases in men and 7,870 in women in 2020 [1]. Gastric cancer is considered a multifactorial neoplasm and its development depends on the participation of different factors, which may be genetic and/or environmental. Numerous studies have sought to explain the development/etiology of gastric cancer and suggested a series of genetic and epigenetic alterations, mainly in tumor suppressor genes [2,3,4].

One of the factors that can precede gastric cancer is atrophic gastritis, usually caused by *Helicobacter pylori* (*H. pylori) infection.* It is believed that half of the world population is already infected by this bacterium; however, only 15% of those infected have any symptoms or complications [23, 24]. In 1994, *H. pylori* was recognized as a Type I carcinogen by the WHO, and its chronic infection is the best-known risk factor for gastric cancer [5].

Considering the genetic components associated with the etiology of gastric cancer, the genes of the Forkhead box family stand out, as they are responsible for encoding proteins that control the transcription of several genes that act in the

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regulation of numerous biological processes. The processes include apoptosis, DNA repair, cell cycle regulation, longevity, aging, cancer, and neurogenesis, all of which are necessary to maintain homeostasis and development [6].

Of the genes present in the FOX family, researchers have reported a possible relationship between gastric cancer and the Forkhead box O3 (FOXO3) gene. FOXO3 is a tumor suppressor gene mapped to chromosome 6 (q21), which has three exons and two introns, and an intron is located within the coding sequence of the forkhead domain [7-10]. The transcription factor FOXO3 acts as a tumor suppressor and is the major target of Serine/Threonine kinase (AKT). Some genes are directly affected by the action of the FOXO3 protein, including p130(RB2), cyclin D, BBC 3, and BCL2L11, Fas ligand, and Bcl-XL. The genes of the Bcl-2 family (Bcl-XL, BCL2L11, and BBC3) are responsible for managing apoptosis through pro-apoptotic proteins, such as PUMA, BIM, and BAX/BAK [7-10].

PUMA (p53 upregulated modulator of apoptosis) is a pro-apoptosis protein mainly regulated by the tumor suppressor p53. It rapidly induces apoptosis through a BAX and mitochondria-dependent pathway [10]. One of the functions of the PUMA protein is to repair apoptosis failures in neoplastic cells, by adjusting several apoptotic pathways through interaction with some anti-apoptotic members of the BCL-2 family, which are indispensable for apoptosis induced by exogenous and endogenous p53 [12].

BIM is a pro-apoptosis protein of the BCL-2 family, encoded by the BCL2L11 gene and located in the outer membrane of mitochondria. This protein acts as an important apoptosis inducer, mediating the translocation of the apoptosis-inducing factor and mitochondrial depolarization [13,14]. The expression of this gene can be induced by the nerve growth factor as well as by FOXO3. In addition to favoring apoptosis, like PUMA, BIM works as a double agent and can promote autophagy [15].

Thus, the objective of the present work was to analyze the expression of the FOXO3, BCL2L11, and BBC3 genes and to evaluate their correlation with the gastric carcinogenesis process.

Material and Methods

Patients and Samples

A total of 225 gastric biopsy samples from individuals of both genders and over 18 years old who had not received antiparasitic or antibiotic treatments in the last 30 days were evaluated. After collection, the samples were separated into groups: Control (patients with healthy gastric mucosa), Gastritis, and Cancer, according to histopathological analysis, following the criteria of the updated Sydney System and Lauren System. Subsequently, the groups were also divided according to the presence or absence of *Helicobacter pylori* (**Table 1**).

Table 1: *Frequency of Helicobacter pylori infection, subjects and group information.*

Groups	*H. pylori*	N	Age ± SD	Gender			
Control (N:54)	Positive	10	59 ± 15	♂	2	♀	8
	Negative	44	54 ± 17	♂	17	♀	27
Gastritis (N: 108)	Positive	51	51 ± 17	♂	23	♀	28
	Negative	57	56 ± 17	♂	21	♀	36
Gastric Cancer (N: 63)	Positive	41	62 ± 15	♂	18	♀	23
	Negative	22	65 ± 13	♂	9	♀	13
Total		**225**	**56 ± 17**	♂	**90**	♀	**135**

Legend: N: Number of individuals; SD Standard Deviation; ♀ female; ♂ Male

For molecular analysis, at the time of collection, the samples were stored in 2 mL tubes with RNALatter solution to ensure the integrity of the material. Then they were identified and stored in a -20 °C freezer. For protein analysis, after collection, the samples were immediately submerged in liquid nitrogen and stored at -80 °C until manipulation.

Gastric biopsy samples were collected at the Gastroenterology services of the Hospital Estadual de Bauru (HEB), the Hospital das Clínicas de Marília, and the Santa Casa de Marília. Samples from individuals with gastric cancer were obtained in collaboration with the Federal University of São Paulo (UNIFESP) and the Federal University of Goiás (UFG). This study was approved by the Research Ethics Committee of the Centro Universitário do Sagrado Coração (UNISAGRADO), Bauru, SP; under opinion number: 1,119,830.

DNA Extraction and *Helicobacter pylori* Detection

DNA extraction from the biopsies of the antrum region of the stomach was performed according to the protocol established by the QiAmp® DNA Mini Kit from QIAGEN (Cat No. 51304).

H. pylori was diagnosed using the Polymerase Chain Reaction (PCR) technique. The pair of oligonucleotides Hpx1 [(CTGGAGARACTAAGYCCTCC (R = purine; Y = pyrimidine)] and Hpx2 (GAGGAATACTCATTGCGAAGGCGA) was used, under conditions of 40 cycles: 94C°,1 min; 59°C, 1 min, and 72°C, 1 min. After PCR, the detection of H. pylori was verified by electrophoresis, through which a fragment of 150 bp was visualized by agarose gel electrophoresis 2.5%, stained with ethidium bromide, and viewed and photographed in a transilluminator on the Imager 2200 image capture system (Innotech Corporation)

RNA Extraction, cDNA Synthesis and Real-Time Quantitative PCR (qPCR) Gene Expression Analysis

To extract the RNAs, the tissue was first processed in a lysis solution using the Precellys 24 equipment. Subsequently, the to-

tal RNA was extracted using the miRNeasy® Mini Kit (QIAGEN - Cat No. 217004). The procedures were performed following the manufacturer's instructions. The RNA concentration of each sample and the absorbance ratio (A260/A280) were measured in the NanoDrop 2000 equipment (Spectrophotometer ND – 2000 – NANODROP, USA), and only samples with a ratio value between 1.85 and 2.2 were used.

The synthesis of complementary DNA (cDNA) from total RNA was performed using the kit: High-CapacitycDNA Reverse Transcription Kits, according to the protocol established by the manufacturer.

The qPCR was performed in the ABI Prism 7500 Fast Sequence Detection System equipment, using TaqMan gene expression assay and specific probes. The relative quantification of expression was calculated using the 2-ΔΔ Ct method according to Livak and Schmittgen (2001). To analyze the mRNA expression, the following assays were used: FOXO3a (Hs00818121_m1), BCL2L11 (Hs00708019_s1), and BBC3 (Hs00248075_m1). The TBP gene (Hs00187332_m1) was used as an endogenous control (Applied Biosystems).

Protein expression analyzed by Western Blotting

Gastric biopsy fragments from the three groups analyzed (Control, Gastritis, and Cancer) were homogenized in 300µl of RIPA buffer containing inhibitors (NaF 1M, Complete Protease Inhibitor Cocktail – Roche Diagnostics, and PMSF 0.1M). Subsequently, the homogenate was centrifuged at 14.000rpm for 15min at 4°C. After centrifugation, the supernatant was transferred to new tubes. The total proteins extracted was quantified by the Bradford method, using the NanoDrop 2000 spectrophotometer (Thermo Scientific®).

The proteins were diluted in RIPA buffer with inhibitors for homogenization and stored at -80 °C until use. To perform the immunoblot, the proteins were normalized to 25 ug, separated in a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane employing electroblotting. Then, the membranes were stained with ponceau, washed with 1X PBST Buffer Solution, and incubated in 5% skim milk/PBST for two hours at room temperature to block nonspecific staining. After incubation, the membranes were washed again for three minutes, twice in 1X PBST, and incubated at 4 °C overnight in the primary antibodies: anti-FOXO3a (Ab10962a) and anti-α-tubulin (Ab7291), diluted in BSA (albumin bovine serum) 5%/PBST.

Subsequently, the membranes were washed for five min, six times, in 1X PBST and incubated in the anti-mouse HRP Rabbit secondary antibodies (Abcam: 616520) on the membrane labeled with anti-α-tubulin and HRP Goat anti-rabbit IGG (Santa Cruz Biotechnology: 2004) on the membrane labeled with anti-FOXO3a, diluted in 5% BSA/PBST, for 90 min at room temperature. Finally, the membranes were washed for five min, six times, in 1X PBST and revealed using photographic films. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Statistical Analysis

Data were first analyzed using the graphical box-plot method to detect outliers. When necessary, the distribution was tested using the D'Agostino & Pearson tests. For expression and association analysis, the Wilcoxon Signed Rank, Kruskal-Wallis, Brown-Forsythe, Fisher's exact, and Chi-square tests were used, depending on the groups analyzed. Values of $p<0.05$ were considered significant. The analyses were performed using GraphPad Prism 5 (USA).

Results

Helicobacter Pylori Detection

H. pylori was detected in 102/225 (45%) gastric biopsy samples analyzed. The results indicated a higher incidence of *H. pylori* in the Gastritis and Cancer groups than the Control group (Table 2). The analysis revealed a significant association between the *H. pylori* and the development of chronic gastritis and gastric cancer. Were founded a p=0.0005; OR=3.937; CI=1.808–8.296 when comparing the Control to the Gastritis group, showing an approximately fourfold increase in the risk of developing gastritis in patients with the presence of *H. pylori.* When comparing the Control vs Cancer group, the result p<0.0001; OR=8,200; CI=3.442–19.800 was obtained, showing again that *H. pylori* increased the risk of developing gastric cancer. When comparing the Gastritis vs Cancer group, the result was p<0.0001; OR=2.083; CI=1.118–3.850.

Table 2: *Frequency of H. pylori infection in the patient groups.*

	Control	Gastritis	Gastric Cancer	Total
H. pylori +	10 (18,5%)	51 (47%)	41 (65%)	102 (45%)
H. pylori -	44 (81,5%)	57 (53%)	22 (35%)	123 (55%)
Total	54 (100%)	108 (100%)	63 (100%)	225 (100%)

Analysis of Gene Expression

The results were analyzed in two stages. First, we analyzed the values obtained between the Control, Gastritis, and Cancer groups, without considering the presence of *H. pylori.* Subsequently, the groups were divided into subgroups considering the presence of *H. pylori* and again tested statistically, always being compared to the Negative Control group.

FOXO3 Gene Expression

Disregarding the presence of *H. pylori,* a statistically significant difference was found when comparing the three groups (Control *vs* Gastritis *vs* Cancer) (p<0.0001). The analysis between the groups (post-test) revealed a statistically significant difference in the Cancer *vs* Control and Cancer *vs* Gastritis groups, both with p<0.0001, evidencing an increase in the expression of the FOXO3 (**Figure 1**).

Considering the presence of the bacterium, results similar to those mentioned above were found. There was a statistically significant difference between all groups (p<0.0001). The Positive Cancer group had increased expression when compared to all other groups, a result that may suggest the influence of *H. pylori* on the modulation of FOXO3 expression. The aforementioned increase in expression was not statistically significant when the Cancer Positive group was compared to the Cancer Negative group, suggesting that neoplastic transformation also influences the increase in FOXO3 gene expression regardless of the presence of *H. pylori*. However, the results

obtained in the present study indicate that the presence of bacteria associated with neoplastic transformation can potentiate the expression of FOXO3 (**Figure 1**).

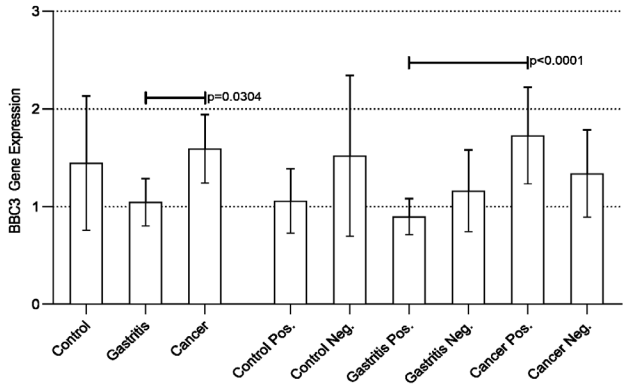

Figure 1: *Real-time quantitative RT-PCR analysis of FOXO3 expression in all groups studied.*

BCL2L11 Gene Expression

Following the same statistical procedure, we verified a statistically significant difference in the expression of the BCL2L11 gene, when comparing the groups (Control, Gastritis, and Cancer). A subsequent analyzes between pairs (post-test) found a statistically significant increase in the expression of the gene in the Cancer group compared to the Control and Gastritis groups. When considering the presence of *H. pylori,* we found a difference between all groups. However, when comparing the groups between pairs, no significant difference was observed in any analyses (**Figure 2**).

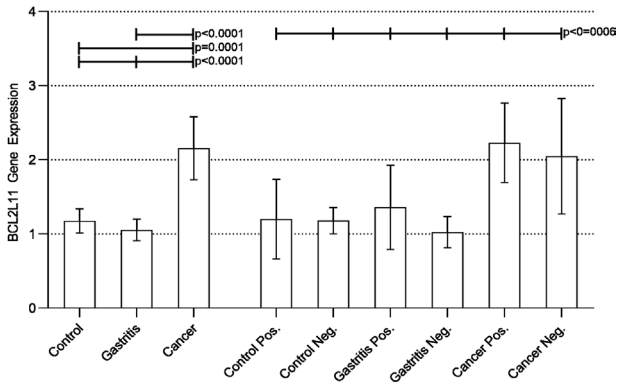

Figure 2: *Real-time quantitative RT-PCR analysis of BCL2L11 expression in all groups studied.*

BBC3 Gene Expression

Interestingly, the BBC3 gene showed a statistically significant difference only when comparing the Cancer *vs* Gastritis group (**Figure 3**). Considering the presence of the bacterium, only the comparison between the Positive Gastritis and Positive Cancer groups was statistically significant (**Figure 3**).

Protein Expression Analysis

For protein analysis, the same statistical treatment was performed, initially disregarding the presence of *H. pylori* with later analysis considering the presence of the bacterium.

The protein expression of FOXO3 did not have statistically significant differences in any analysis considering the Gastritis group. The results were p=0.0601 when comparing the Normal Control group *vs.* Gastritis and ep=0.1529 and ep= 0.0970, when analyzing the Normal *vs.* Control group and Positive Gastritis and Normal Control *vs.* Negative gastritis, respectively.

When comparing the Normal Control group *vs.* Cancer, without taking into account the presence of *H. pylori*, the test resulted in p=0.0360, demonstrating an increased expression of FOXO3 protein in patients with gastric cancer. When comparing Normal Control with the Cancer Positive and Cancer Negative groups, we found statistically significant results, with p=0.0001 in all tests. When comparing Gastritis *vs.* Cancer, the statistically significant difference was p=0.0360, also indicating an increased protein expression in patients with gastric cancer. Thus, the results show that FOXO3 independent of *H. pylori* is more expressed in gastric cancer tissues and does not seem to influence cases of gastritis.

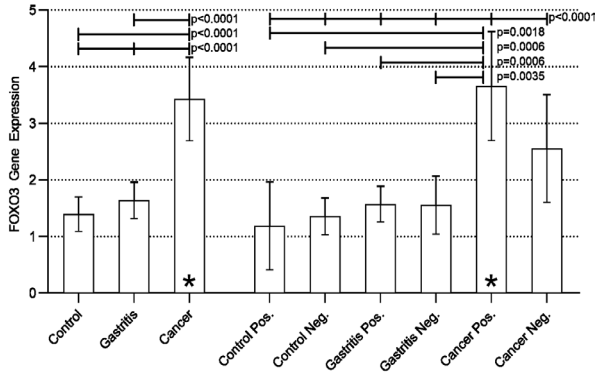

Figure 3: *Real-time quantitative RT-PCR analysis of BBC3 expression in all groups studied.*

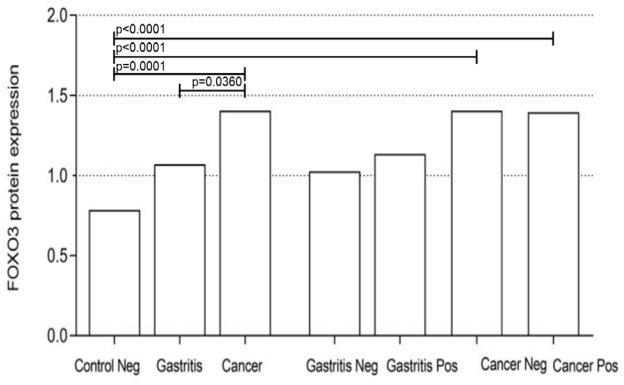

Figure 4: *Protein expression of FOXO3 as evaluated by western blotting. (A) Representative result of FOXO3 protein expression (C+: Positive Cancer; C-: Negative Cancer; G+: Positive Gastritis; G-: Negative Gastritis; NC: Normal Control, H. pylori negative; TUB: Tubulin.). (B) Relative FOXO3 protein expression levels was remarkably increase in gastric tumor tissues compared with other groups.*

Discussion

Several researchers have studied the bacterium *H. pylori* and its association with gastric diseases since the studies initiated by Marshall and Warren in 1983, with a worldwide consensus that *H. pylori* infection influences the development of pathologies that mainly affect the gastric tissue. [16] demonstrated a significant difference in the risk of developing gastric diseases with the presence of *H. pylori*, which agrees with the present study [16].

Recently [16], reported that FOXO3 is downregulated in gastric cancer tissues, a result that agrees with the results obtained

in the present study. Note that Li M. et al. used gastric paracarcinoma as a control, which may have directly influenced the results obtained [17]. On the other hand [18], conducted a study involving cells infected and not infected by *H. pylori* and suggested that the bacterium acts in the phosphorylation of the FOXO3 protein, leading to its inactivation and translocation from the nucleus to the cytoplasm. As it is a pro-apoptosis protein, when inactivated, FOXO3 ceases to exert the transcriptional activity of pro-apoptotic genes and manage the cell cycle, favoring tumor progression. The increased expression seen in the present study could be a response mechanism to the inactivation of the FOXO3 protein in patients who are failing in the apoptosis pathway; thus, the increase in FOXO3 mRNA could increase protein production in an attempt to compensate for its poor performance [17]. Hu et al., 2015 reported in their research that FOXO3 may serve as a prognostic marker and therapeutic target in gastric cancer. Furthermore, its activation reverses the phenotype of cancer cells, and protein silencing increases cancer cell motility [17].

The results obtained by Li et al. (2020) indicate that the FOXO3 protein is downregulated in carcinogenic tissues. However, the study used gastric paracarcinoma material from the same patients as a control, thus, FOXO3 would be more expressed at the edge of the tumor than at the center. The current study compared carcinogenic tissues with tissues from a healthy mucosa [17].

The results of the present study indicate that greater expression of FOXO3 could be a positive feedback mechanism attempting to reverse the neoplasm picture, as already suggested in the analysis of gene expression.

For mRNA expression of BCL2L11, the results agree with Zhang H, Duan J, Qu Y et al. 2016, who found no statistical difference in patients with gastric cancer [15]. When evaluating if *H. pylori has an influence,* Akazawa and colleagues evaluated in 2015 the expression of the BCL2L11 gene, and their results showed a significant increase in the expression of BCL2L11 in patients with the presence of *H. pylori,* noting that the degree of gastritis directly affects gene expression [20]. The results were partly replicated in the present study.

In a more recent analysis, in 2021, Mu J and collaborators performed an experiment using animals. Their results confirmed that PUMA is an apoptosis regulator, and its expression influences tumor progression [22]. A year earlier, in 2020, Yini Dang correlated the expression of BBC3 with the bacterium *H. pylori*, in gastritis samples. The resulting expression was four-times higher in the presence of the bacterium, and in the cell culture of cancer tissue, the expression was elevated in the presence of *H. pylori* [22].

Hu et al., 2015 reported in their research that FOXO3 may serve as a prognostic marker and therapeutic target in gastric cancer. Furthermore, its activation reverses the phenotype of cancer cells, and protein silencing increases cancer cell motility [19].

Li, et al., 2020 obtained results that indicate that the FOXO3 protein is downregulated in carcinogenic tissues. However, the study used gastric paracarcinoma material from the same patients as a control, thus, FOXO3 would be more expressed at the edge of the tumor than at the center. The current study compared carcinogenic tissues with tissues from a healthy mucosa [17].

Conclusion

The results obtained about the expression of FOXO3 mRNA and protein suggest that it could be resulting in positive *feedback* in response to changes caused by neoplasia. Furthermore, this work demonstrated that *Helicobacter pylori* plays an important role not only in the development of gastric cancer but also in boosting FOXO3 mRNA expression.

BCL2L11(BIM) and BBC3 (PUMA) do not seem to have significant effects on their expression due to the cascade of events triggered by FOXO3, *H. pylori,* and neoplasm. However, these findings may be related to the dual role of these molecules in both autophagy and cellular apoptosis.

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Conflicts of Interest: No conflicts of interest to declare.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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