Effect of *Helicobacter pylori* on *NFKB1*, *p38α* and *TNF-α* mRNA expression levels in human gastric mucosa

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Abstract. *Helicobacter pylori* infects ~50% of the world population, causing chronic gastritis and other forms of cellular damage. The present study assessed the influence of *H. pylori* on the mRNA expression levels of nuclear factor-κB1 (*NFKB1*), *p38α* and tumor necrosis factor-α (*TNF-α*) in human gastric mucosa in a southern Brazilian population. Human gastric tissue was collected by upper endoscopy and *H. pylori* diagnosis was performed using a rapid urease test and histological analysis. Total RNA was extracted and purified for subsequent cDNA synthesis and analysis by quantitative polymerase chain reaction (qPCR). The gastric tissue samples were divided into four groups as follows: Normal, inactive chronic gastritis, active chronic gastritis and intestinal metaplasia. The *SDHA* gene was classified as the most stable when compared with *ACTB*, *GAPDH*, *B2M* and *HPRT1* genes, and was therefore selected as the reference gene for qPCR data normalization. TNF-α mRNA expression was significantly higher in samples that were positive for *H. pylori* and with active chronic gastritis. However, no difference was detected in the mRNA expression levels of *NFKB1* and *p38α* between the groups. The present study concluded that the presence of *H. pylori* is associated with *TNF-α* upregulation in human gastric mucosa, but had no effect on *NFKB1* and *p38α* mRNA expression levels.

Introduction

Helicobacter pylori (*H. pylori*) are Gram-negative bacteria that adhere to the surface of gastric mucosa and cause inflammation, but do not invade gastric epithelial cells (1). *H. pylori* infect ~50% of the world population and the main risk factors include age, ethnicity, gender, geographic location and socioeconomic status (2). Infection with *H. pylori* is considered the major cause of active chronic gastritis and also serves an notable role in peptic ulcers (3).

Since the discovery of *H. pylori*, it has been strongly associated with the development of gastric cancer (4). *H. pylori* was the first bacterial species to be recognized by the International Agency for Research on Cancer as a group I carcinogen (5). The development of cancer in *H. pylori* infected individuals may be through the following possible mechanisms: i) The production of mutagenic radicals as an inflammatory response to *H. pylori* infection; ii) the reduction of antioxidants in mucosa; and iii) the induction of a hyperproliferative state (6,7). However, only a small percentage of infected individuals will develop neoplasia (1-3%) due to specific interactions between the host and pathogen, which are dependent on specific bacteriological factors and/or inflammatory responses regulated by host genes (8,9).

H. pylori-induced gastritis can lead to other types of cellular damage (10). The most prevalent form of gastric neoplasia is intestinal-type adenocarcinoma, which evolves through a series of events initiated by the transition of normal mucosa to superficial chronic gastritis, which subsequently results in atrophic gastritis, intestinal metaplasia, and eventually to dysplasia and neoplasia (11). Various inflammatory biomarkers, including the tumor necrosis factor-α (TNF-α), nuclear factor-κB (NF-κB) and interleukins (ILs), can be used to track the progress of diseases, as well as assist in the development of novel anti-inflammatory drugs for the treatment and prevention of cancer (8). Pathogenic stimuli induce the expression of *TNF-α*, which in turn induces proteases and other mediators responsible for inflammatory responses. TNF-α has been associated with the various steps involved in tumorigenesis, including cell transformation, invasion, proliferation, survival, promotion, angiogenesis and metastasis (8). The activation of TNF receptor 1 (TNFR1) by TNF-α can result in the activation of NF-κB as a result of cellular responses (12).

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NF-κB serves an essential role in inflammation and innate immunity, and it is becoming increasingly recognized for its crucial role in the initiation and progression of cancer, operating in numerous cell signaling pathways (13). NF-κB regulates the expression of various substances associated with inflammation and is inactive in the majority of cells, but it exists in an activated form in cancer cells (14). This activation is induced by a wide variety of carcinogens and inflammatory stimuli (14,15). In the classic NF-κB signaling pathway, lipopolysaccharides, TNF-α or IL-1 activate TNFR and IL-1 receptors (13).

p38 is a mitogen-activated protein kinase (MAPK) involved in the regulation of inflammatory cytokine biosynthesis (16) and stress responses, such as heat shock or infection (17,18). Protein kinases are the main regulators in pathways of embryogenesis, cell differentiation, proliferation and cell death (19). The p38 MAPK family consists of four identified isoforms, including p38α, p38β, p38γ and p38δ, encoded by the genes *MAPK14*, *MAPK11*, *MAPK12* and *MAPK13*, respectively. In human tissues, the most abundant MAPK is p38α (16,20). Although the activation of p38α is normally associated with anti-proliferative functions, studies indicate that it has the ability to upregulate cell proliferation in human tumors and cancer cell lines (20,21).

The use of quantitative polymerase chain reaction (qPCR) to compare mRNA levels between biopsies from different individuals and disease states requires meticulous normalization. For the correction of results from different samples and experimental conditions, the use of an endogenous reference is essential (22). In a number of studies, it has been demonstrated that although common reference genes are considered to be stable and secure in various tissues, this is often not the case (23,24). Only one previous study has validated reference genes for gastric samples in a Western population of patients with adenocarcinoma (25).

Currently, the association of inflammation, innate immunity and cancer is widely accepted (26); however, the cellular and molecular mechanisms that mediate these processes remain unknown. The present study aimed to evaluate the mRNA levels of *TNF-α*, *NFKB1* and *p38α* in human gastric mucosa samples, and to investigate the influence of *H. pylori* on the expression of these genes in a southern Brazilian population. In order to perform gene expression analysis, the suitability of five reference genes were assessed to determine their suitability for use in qPCR normalization. Although the five genes have been widely studied (27,28), there are presently no studies evaluating their association with *H. pylori* infection and human gastric inflammation.

Materials and methods

Sample acquisition. Human gastric tissues were obtained by upper endoscopy from 79 adult patients, including 49 women and 30 men (aged 47.33±14.31 years), who were admitted to the Endoscopy Service at Hospital Bruno Born (Lajeado, Brazil) between October 2013 and April 2014 with symptoms of gastritis, including epigastric pain, nausea, vomiting, bloating, belching, heartburn, halitosis and flatulence (29). The present study was approved by the local ethics committee (Univates, Lajeado, Brazil; CEP 353.624) and written informed consent was obtained from each patient prior to sample collection. Exclusion criteria included coagulation disorders (including patients with problems preventing gastric biopsy), and the use of anti-inflammatory drugs, antibiotics or proton pump inhibitors.

Two biopsy samples (~3 mm) were obtained from the lesser curvature of the gastric antrum, one of which was used for the rapid urease test (RNA Laboratórios, Cascavel, Brazil) and the other was placed in RNAlater Stabilization Solution (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for posterior RNA extraction, cDNA synthesis and gene expression analysis. In addition, two further samples of ~3 mm were collected by the gastroenterologist for routine histological analyses. Briefly, the samples were formalin-fixed, paraffin-embedded (both Allkimia, Campinas, SP, Brazil) and stained with Giemsa (Quimica Especializada Erich Ltda, São Paulo, SP, Brazil) for *H. pylori* detection under the Leica DM500 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

H. pylori infection diagnosis and histological analysis. The rapid urease test is based on the principle that the enzyme urease produced by *H. pylori* hydrolyses urea into ammonia (30). A biopsy sample was introduced immediately after collection into a tube containing the rapid urease test. The consequent rise in the pH of the medium in the tube as a result of ammonia production was detected by a phenol red indicator, changing the medium color and indicating the presence of *H. pylori*. When the color change occurred during the first 24 h, the test was considered positive (31). *H. pylori* diagnosis was performed based on the results of the rapid urease, and the samples were considered positive following confirmation with the modified Giemsa staining procedure (32). The samples were classified according to a histological analysis and observation under the Leica DM500 microscope, using the Sydney classification system (33). The samples were divided into groups according to the severity of changes observed in the histological examination, as follows: i) normal (absence of inflammation); ii) inactive chronic gastritis (inflammation without neutrophils); iii) chronic gastritis (inflammation with neutrophils); and iv) the presence of intestinal metaplasia (a precancerous lesion).

Total RNA extraction. Samples were processed in a Turrax-like tissue homogenizer (Ultra Stirrer; Stanhope-Seta, Chertsey, Surrey, UK) with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The total RNA extraction method by TRIzol was an adaptation from the original method described by Chomczynski and Sacchi (34). Following extraction, the RNA was purified using the RNAspin Mini kit (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's instructions and stored at -80˚C. The concentration and purity of the RNA was assessed by L-Quant spectrophotometer (Loccus Biotecnologia, Cotia, Brazil) at 260 and 280 nm wavelengths. A ratio of ~2.0 was considered appropriate for RNA purity.

Synthesis of cDNA. cDNA was synthesized from 1 μg total RNA using the Superscript III First Strand Synthesis System SuperMix (Invitrogen; Thermo Fisher Scientific, Inc.) and

Table I. Primer sequences, product lengths and mean Tm of the studied genes.

Gene symbol	Primer sequence (5'-3')	NCBI no.	Product length (bp)	Mean Tm (˚C)
NFKB1				
Sense	ACACCGTGTAAACCAAAGCC	NM_003998.3	209	82.71
Antisense	CAGCCAGTGTTGTGATTGCT			
p38α				
Sense	CAGTGGGATGCATAATGGCC	NM_001315.2	243	82.12
Antisense	GCATCTTCTCCAGCAAGTCG			
TNF-α				
Sense	CCCTGGTATGAGCCCATCTATC	NM_000594.3	120	84.82
Antisense	AAAGTAGACCTGCCCAGACTCG			
SDHA				
Sense	TGGTTGTCTTTGGTCGGG	NM_004168.2	85	81.69
Antisense	GCGTTTGGTTTAATTGGAGGG			
ACTB				
Sense	CTGGAACGGTGAAGGTGACA	NM_001101.3	140	82.31
Antisense	AAGGGACTTCCTGTAACAATGCA			
B2M				
Sense	CTATCCAGCGTACTCCAAAG	NM_004048.2	165	78.88
Antisense	ACAAGTCTGAATGCTCCACT			
GAPDH				
Sense	CTTTGTCAAGCTCATTTCCTGG	NM_002046.3	133	84.70
Antisense	TCTTCCTCTTGTGCTCTTGC			
HPRT1				
Sense	AGATGGTCAAGGTCGCAAG	NM_000194.2	128	79.78
Antisense	GTATTCATTATAGTCAAGGGCATATCC			

NFKB1, nuclear factor-κB1; *TNF-α*, tumor necrosis factor-α; *SDHA*, succinate dehydrogenase complex, subunit A, flavoprotein; *ACTB*, β-actin; *B2M*, β_2-microglobulin; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; NCBI, National Center for Biotechnology Information; Tm, melting temperature.

oligo-dT primers, according to the manufacturer's instructions, and stored at -20˚C.

Primer design. The primers used for the amplification of cDNA fragments specific for *p38α*, *TNF-α* and *NFKB1* were designed using the published sequence of each gene and the online tool Primer3 (35). To select a reference gene, five reference genes commonly used in gene expression studies were examined to identify the most appropriate gene for the current experiment. The primers were supplied by Laboratory of Molecular Biology, Endocrinology and Tumors (Federal University of Rio Grande do Sul, Porto Alegre, Brazil) (27). Each primer was synthesized by Invitrogen Brazil, Ltd. (São Paulo, Brazil). A dissociation curve was created for each pair of primers in order to confirm reaction specificity. The melting temperature (Tm) is specific to each amplicon. The primer sequences, product length and mean Tm of each gene are presented in Table I.

qPCR conditions. The amplification of cDNA and relative quantification was performed by qPCR with the StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). A Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen; Thermo Fisher Scientific, Inc.) with a total volume of 25 µl, including 12.5 µl SuperMix, 0.5 µl 50 µmol/l Rox reference dye, 0.3 µl of each primer (10 µmol/l forward and 10 µmol/l reverse), 9.4 µl H_2O and 2.0 µl 1:20-diluted template cDNA. Duplicate measurements were recorded according to the following protocol: Initial incubation for 3 min at 94˚C, followed

Table II. Main characteristics of the study population (n=79) according to HP infection.

Parameter	HP^+ (n=27)	HP^- (n=52)
Age (years)		
Mean ± standard deviation	47.93±12.17	47.02±15.40
Range	22-64	19-81
Gender[a]		
Female (n=49)	15 (30.61)	34 (69.39)
Male (n=30)	12 (40.00)	18 (60.00)
Previous HP treatment[a] (n=29)	6 (20.69)	23 (79.31)

[a]Values expressed as case numbers (% of the row total). HP, *H. pylori*.

Table III. Main characteristics of the study population according to histological analysis.

Parameter	Normal (n=17)	ICG (n=29)	ACG (n=25)	IM (n=8)
Age (years)				
Mean ± standard deviation	45.00±15.47	46.90±15.95	46.80±11.93	55.50±11.67
Range	21-75	19-81	22-64	35-70
Gender[a]				
Female	13 (76.47)	19 (65.52)	15 (60.00)	2 (25.00)
Male	4 (23.53)	10 (34.48)	10 (40.00)	6 (75.00)
Previous HP treatment[a]	5 (29.41)	8 (27.59)	6 (24.00)	5 (62.50)
HP^+	0 (0.00)	0 (0.00)	25 (100.0)	2 (25.00)

[a]Values expressed as case numbers and (% of group total). ICG, inactive chronic gastritis; ACG, active chronic gastritis; IM, intestinal metaplasia; HP, *Helicobacter pylori*.

Table IV. Candidate reference genes for normalization of quantitative polymerase chain reaction in human gastric non-neoplastic tissues, according to their stability, as calculated by NormFinder software.

Rank	Gene	Stability value
1	*SDHA*	0.149
2	*ACTB*	0.180
3	*HPRT1*	0.231
4	*GAPDH*	0.265
5	*B2M*	0.270

ACTB, β-actin; *B2M*, β_2-microglobulin; *SDHA*, succinate dehydrogenase complex, subunit A, flavoprotein; *HPRT1*, hypoxanthine phosphoribosyltransferase 1.

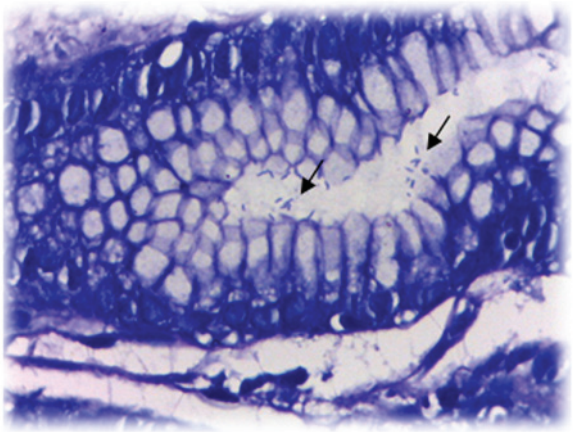

Figure 1. Representative image of histological examination of human gastric tissue positive for *H. pylori*, identified by Giemsa staining, highlighted by the black arrows.

by 45 cycles of 30 sec denaturation at 94˚C, 30 sec annealing at 55˚C and 30 sec extension at 60˚C. Standard curves were constructed by plotting the cycle threshold values of the qPCR performed on a five-fold dilution series of cDNA standards.

Statistical analysis. Data were tabulated and analyzed with descriptive statistics using SPSS software version 20.0 (IBM SPSS, Armonk, NY, USA). To confirm the normality of gene expression values, the Kolmogorov-Smirnov test was performed. The Mann-Whitney U test was performed to compare two groups, and the Kruskal-Wallis test followed by Dunn's multiple comparison test were performed to compare more than two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Gene expression variability for reference gene selection was evaluated using NormFinder algorithm, which is a Visual Basic application for Microsoft Excel that calculates the average expression stability of each studied gene (36).

Results

Classification according to H. pylori infection. The samples were classified as positive (HP^+) or negative (HP^-) for *H. pylori* infection, according to the rapid urease test and histological exam results (Fig. 1). Table II presents the principal characteristics of the study population with regards to the *H. pylori* infection groups. The participants were from the region of Taquari Valley, State of Rio Grande do Sul (Brazil) and their mean age was 47.33±14.31 year.

Classification according to histological analysis. According to the severity of changes observed on histological examination, the samples were classified into the following groups: Normal; inactive chronic gastritis (ICG); active chronic gastritis (ACG); and intestinal metaplasia (IM). The group classification is presented in Table III.

All samples classified as ACG were diagnosed as HP^+. In the IM group, 62.5% (n=5) of patients received previous HP treatment, 25.0% (n=2) were HP^+, while 12.5% of this group (n=1) had no prior report of *H. pylori* infection. The groups classified as normal and ICG were diagnosed as HP^-.

Reference gene selection. For reference gene selection, qPCR was performed on 39 samples (this is the number of samples that had been collected at the time and was deemed sufficient

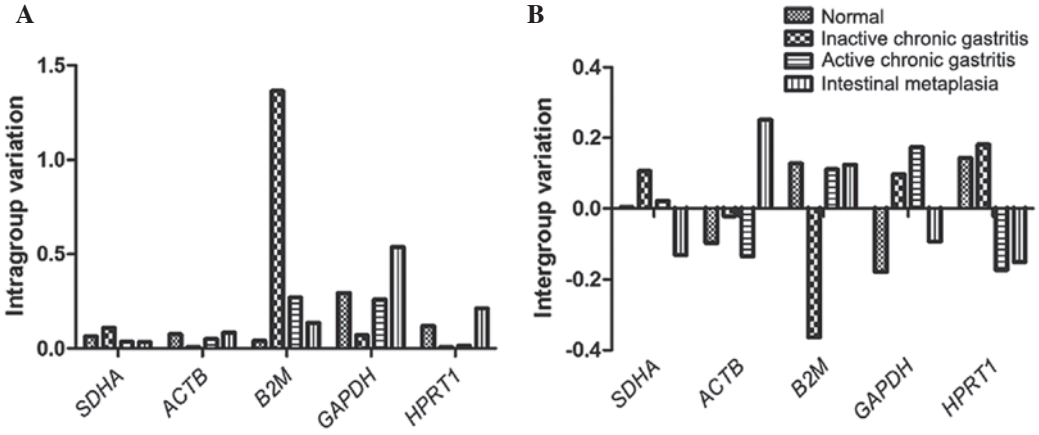

Figure 2. (A) Intra- and (B) intergroup variation of five reference genes in human gastric samples as calculated by NormFinder, identifying *SDHA* as the gene with the smallest, and *B2M* with the highest, variation. *SDHA*, succinate dehydrogenase complex, subunit A, flavoprotein; *ACTB*, β-actin; *B2M*, β_2-microglobulin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, hypoxanthine phosphoribosyltransferase 1.

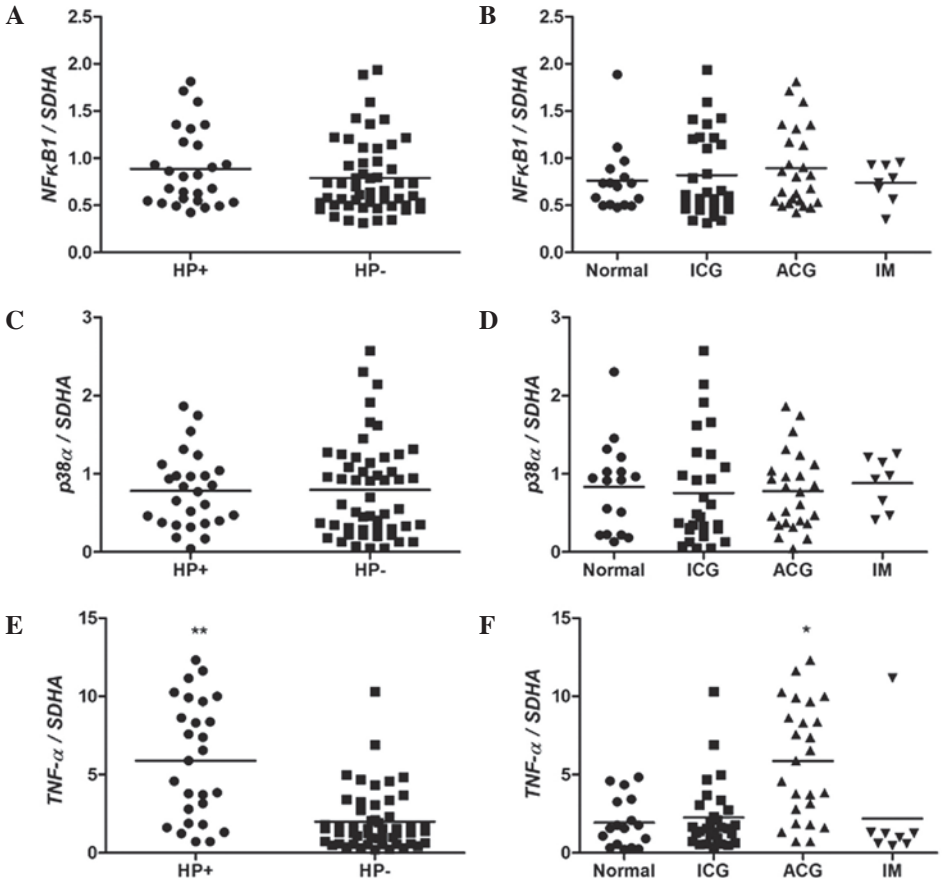

Figure 3. mRNA expression levels of (A and B) *NFKB1*, (C and D) *p38α* and (E and F) *TNF-α* in human gastric mucosa, according to HP infection and histological analysis statuses. *P<0.01 vs. normal (Kruskal-Wallis followed by Duncan's multiple comparisons test), **P<0.0001 vs. HP^{-} (Mann-Whitney U test). ICG, inactive chronic gastritis; ACG, active chronic gastritis; IM, intestinal metaplasia; *SDHA*, succinate dehydrogenase complex, subunit A, flavoprotein; HP, *H. pylori*.

for this analysis), divided into the following groups: Normal (n=11), ICG (n=11), ACG (n=12) and IM (n=5).

The expression stability of a candidate gene was indicated by its stability value, with a smaller value indicating a more stable gene. The stability values of the candidate genes obtained using NormFinder software are presented in Table IV. NormFinder analysis demonstrated that the succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*) was the most stable gene, with the lowest stability value and smallest intergroup variation (Fig. 2), and thus *SDHA* was selected for normalization of the subsequent data. The most stable combination of the two genes was *SDHA* plus *ACTB* (stability value, 0.112). The qPCR assay of each gene was analyzed in the linear phase, and a linear function of the relative fluorescence vs. cycle number was fitted with a typical R^2 value of >9.

Expression of NFKB1, p38α and TNF-α. As presented in Fig. 3, no significant differences in *NFKB1* and *p38α* levels were observed among the groups with regards to the *H. pylori* infection status (P>0.05; Mann-Whitney test; Fig. 3A and C) and histological analysis (P>0.05; Kruskal-Wallis followed by Duncan's multiple comparisons test; Fig. 3B and D). However, a statistically significant difference was observed between the expression of *TNF-α* in groups with and without *H. pylori* infection (P<0.0001; Fig. 3E). Considering the results of histological analysis, the ACG group demonstrated higher expression levels of *TNF-α* when compared with the other groups (P<0.01; Fig. 3F).

Discussion

In the present study, the expression levels of *TNF-α* were increased in *H. pylori*-positive samples, regardless of the patient gender. The group classified as ACG, when compared with other groups, also demonstrated an increase in *TNF-α* expression levels. This may be explained by the fact that all samples in this group tested positive for *H. pylori* infection. These results are in accordance with the study conducted by Pimentel-Nunes *et al* (37), who observed increased levels of *TNF-α* mRNA in gastric biopsies infected with *H. pylori*. It is known that *H. pylori* infection leads to inflammation of the stomach, associated with the production of inflammatory cytokines, such as TNF-α, as demonstrated by studies observing increased levels of TNF-α in infected patients (38,39). However, a study by Abbas *et al* (40) conducted on patients with liver cirrhosis observed no statistically significant difference in the levels of *TNF-α* expression when comparing positive and negative *H. pylori* groups, or groups with moderate and severe gastropathy (41).

TNF-α is a key cytokine in tumor promotion; therefore, it is important to clarify how the pro-inflammatory cytokines induced by *H. pylori* are involved in the development of gastric cancer (42). Previous studies have demonstrated the presence of the gene encoder of the TNF-α inducer protein (Tipα) in the *H. pylori* genome, which functions as a carcinogenic factor by inducing the gene expression of *TNF-α* and activating NF-κB (41,42).

In the current study, no significant difference was observed in *NF-κB* expression levels between groups, which is similar to the results observed in the study of Pimentel-Nunes *et al* (37). Furthermore, Naito and Yoshikawa (43) reported that *H. pylori* activates the *NF-κB* gene in epithelial cells of the gastric mucosa *in vitro* and *in vivo*. In addition, Huang *et al* (44) demonstrated that *H. pylori* induces phosphorylation of the proteins Iκβα (an NK-κB inhibitor) and RELA (also known as p65) that activate RELA nuclear translocation, making *H. pylori* an activator of the NF-κB signaling pathway. Considering the results of the present study, it is not possible to determine whether the NF-κB pathway is associated with increased levels of *TNF-α*, since the methods used could not verify the phosphorylation levels and, consequently, the protein activation.

Ferrand *et al* (45) demonstrated an increase in *TNF-α* expression levels in response to *H. pylori* infection in mouse gastric epithelial cells *in vitro*. This increase was associated with the activation of NF-κB and nuclear translocation of p65. The same study observed the influence of *H. pylori* on the NF-κB pathway and its involvement in the migration of mesenchymal stem cells, which may be associated with gastric pathophysiology and carcinogenesis (45). In the current study, a total of 87.5% of the IM samples, which is considered a precancerous lesion, had a history of infection with *H. pylori* (previous HP treatment or HP^+ status), suggesting the involvement of *H. pylori* in the development of these lesions.

Kim *et al* (46) demonstrated that *H. pylori* induces the activation of p38 MAPK *in vitro*, which reduces the expression of the *MucA* gene, responsible for the production of mucus, by promoting apoptosis in gastric epithelial cells. Seo *et al* (47) concluded that MAPKs, such as p38 and ERK, can control the activation of NF-κB in gastric epithelial cells infected by *H. pylori*. However, these studies were analyzed at the protein level, not the mRNA level. In the present study, no differences in *p38* expression were observed. For the activation of p38 translocation to the nucleus and the stimulation of transcription factors, p38 must be phosphorylated. Thus, protein analysis is required to detect this phosphorylation.

Among the p38 isoforms, the *p38α* gene was selected for analysis, since it is the most abundant isoform in tissues and also the most widely studied. However, the isoform p38δ is detected primarily in endocrine tissue (48), which may form the subject of further studies due to its presence in gastric epithelium endocrine cells. O'Callaghan *et al* (49) reported the importance of studying p38δ as a result of evidence demonstrating that it may act as a promoter and as a tumor suppressor.

The results of the present study demonstrated that infection with *H. pylori* is associated with active inflammation of the gastric tissue, and 100% of the *H. pylori*-positive samples were also positive for active gastritis. The most severe type of lesion observed in the samples from the current study was intestinal metaplasia. Of these, 25% tested positive for *H. pylori*, and only one sample (12.5%) had no history of infection by *H pylori*. Although *H. pylori* is recognized as the main cause of chronic gastritis, other factors such as smoking, alcoholism, anxiety, stress, poor diet and lifestyle may contribute to the onset of clinical manifestations (50).

In the present study, five candidate genes were analyzed for their potential to be used as the reference gene in qPCR with human non-neoplastic gastric samples obtained by upper endoscopy. NormFinder was selected to compare candidate genes due to its ability to estimate inter and intragroup variation and consequently calculate a stability value (36). A value closer to zero indicates greater stability of gene expression. Considering that an arbitrary cut-off value of 0.15 indicates acceptable stability of the reference gene (51), the present study concluded that *SDHA* was the most appropriate gene for qPCR normalization compared with *ACTB*, *B2M*, *HPRT1* and *GAPDH*. Furthermore, the combination of *SDHA* and *ACTB* demonstrated a lower stability value, suggesting that it is a more stable combination of genes for the normalization of qPCR sample results.

Although *ACTB* has a low intragroup variation (similar to *SDHA*), its higher intergroup variation raises the stability value, thus it can not be suggested as a reference gene for the experiments of the present study. *ACTB* has been traditionally regarded as a reference gene in quantifying expression levels

in tumors (23). However, accumulating evidence indicates that *ACTB* is dysregulated in gastric and a number of other types of cancer (52-54). In the present study, the analysis was focused on non-neoplastic tissues; however, *ACTB* intergroup variation was observed to be higher in the samples with intestinal metaplasia, which is considered a precancerous lesion (55), thus affecting the gene stability value. The study by Wisnieski *et al* (25) analyzing normal and adenocarcinoma samples of gastric tissue and cell lines, determined that *ACTB* was the most appropriate reference gene for all tissues; however, *SDHA* was not included in their analysis.

GAPDH is one of the most commonly used reference genes and it is considered a 'classical' reference gene in the majority of scientific studies (51,56,57). Numerous studies of gene expression in human gastric mucosa use *GAPDH* as the reference gene (58,59), despite the knowledge that *GAPDH* is upregulated in stomach cancer (24). The present study demonstrated that *GAPDH* was highly variable between groups, and was therefore not recommended as a reference gene.

SDHA has been previously investigated as a reference gene in numerous studies with different experimental conditions. These studies determined that *SDHA* was the best reference gene compared with other frequently used reference genes (27,60,61). According to the results of the present and previous studies, *SDHA* may be used as a reference gene for qPCR in the conditions described, as a result of its stability. Therefore, the inclusion of *SDHA* as a candidate gene for further studies of reference gene selection with different conditions and samples must be considered.

Chronic gastritis is a prevalent disease in the world population, and its association with *H. pylori* infection is well-described (5). The study of *H. pylori*, its virulence factors and resistance to therapy are crucial in order to improve treatments aiming to eradicate infection with these bacteria. Understanding the genes associated with the inflammatory and proliferative pathways in different populations may facilitate the development of effective treatments and prevention of gastric disease, aid in the reduction of side effects and increase the efficacy of current treatments.

In conclusion, the present study demonstrated that *H. pylori* infection increases the expression of *TNF-α* mRNA expression levels in human gastric mucosa, but does not have an effect on the expression of *p38α* and *NFKB1*. It also demonstrated that *H pylori* infection is associated with chronic active gastritis and the presence of intestinal metaplasia in a southern Brazilian population. The *SDHA* was observed to be the most appropriate reference gene for qPCR in the current study, as a result of its stability. Therefore, the results support the inclusion of *SDHA* as a candidate gene for further studies of reference gene selection with different conditions and samples. Future studies are required to elucidate the association of *NFKB1* and *p38α* with *H. pylori* infection.

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