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Title: Characterization and strong risk association of *TLR2* del -196 to -174 polymorphism and *Helicobacter pylori* and their influence on mRNA expression in gastric cancer

Authors: Caroline de Matos Lourenço, Manoela Dias Susi, Mariah Cristina Antunes do Nascimento, Vilson Serafim Junior, Ana Paula Simedan Vila, Gabriela Helena Rodrigues-Fleming, Eny Maria Goloni-Bertollo, Ana Elizabete Silva and Juliana Garcia de Oliveira-Cucolo.

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To the Ya-Juan Ma, Science Editor, Editorial Office

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Dear Editor,

Due to our interest in publishing this manuscript (Manuscript NO: 53292) for free charges in *World Journal of Gastrointestinal Oncology (WJGO)*, we are now resubmitting a revised version of the manuscript and our response to the reviewers' comments. The changes made to the manuscript have been highlighted in yellow. We are very thankful for the comprehensive review performed to our manuscript referred above. Thus, acknowledge that the reviewers' critiques were pertinent and contributed to improve our study.

Reviewer 1:**Comments**

The authors present that two polymorphisms in toll-like receptor-2 (TLR2 -196 to -174 ins/del and TLR2 19216 T/C) are strongly associated with gastric cancer and *H. pylori* infection in the Brazilian population; and that TLR2 mRNA expression levels are upregulated in gastric cancer tissues and modulated by both *H. pylori* infection and the presence of variant genotypes. That is interesting and significant for understanding the development of gastric cancer. However, there are some small issues in this manuscript.

1. In Figure 1, it is better to add the data of C group.

Answer: In order to clarify the reviewer comment, and as shown in our manuscript text, in the section "*Subjects and Samples*" (page 7), we used 14 samples from patients with histologically normal gastric mucosa and *H. pylori*-negative (Hp-) as calibrator for gene expression. Thus, C group in the Figures 1 and 2 is represented by the dashed line (Median of RQ=1.0).

2. In "*Subjects and Samples*", the number of pylori- positive cases and pylori-negative cases in the CG group and GC group used to quantify TLR2 mRNA levels should be clear.

Answer: This information was added in the manuscript, session: "*Subjects and Samples*" (page 7). Like this, stands out the group of GC with 36 patients (25 men and 11 women; mean age, 62.32±14.66 years; 21 *H. pylori*- positive and 15 *H. pylori*- negative) and CG group with 48 patients (29 men and 19 women; mean age, 53.10±9.41 years; 16 *H. pylori*- positive and 32 *H. pylori*- negative).

3. There are some spelling or grammatical errors throughout the manuscript. For e.g., in Table 2. TLR2 -196 to -174 ins/del(rs111200466)- Codominant - "in/ins" should be corrected. Please revise carefully.

Answer: We thank the reviewer for their comment and the manuscript has been carefully revised as suggested.

Reviewer 2:**Comments**

The authors examined whether TLR2 19216T/C (rs3804099) and TLR2 -196 to -174 ins/del (rs111200466) polymorphisms contribute to gastric carcinogenesis in the Brazilian population. They found that two polymorphisms in toll-like receptor-2 (TLR2) are strongly associated with gastric cancer and *H. pylori* infection in the Brazilian population. In addition, TLR2 mRNA expression levels were upregulated in gastric cancer in the presence of the TLR2 -196 to -174 del variant allele or the wild-type TLR2 19216 T allele and in the presence of *H. pylori*. They conclude that The TLR2 -196 to -174 ins/del and TLR2 19216 T/C polymorphisms are strongly associated with GC. Although the findings are interesting, major revision is needed to better support this conclusion.

1. The case groups include 269 patients with chronic gastritis, 202 patients with gastric cancer and 381 patients without any gastric disease and *H. pylori* infection. Total case number must be 852, not 854.

Answer: We thank the reviewer for the observation. It was a typo in the text. The correct total number of samples is 852 and has already been corrected in the manuscript (Session "*Subjects and Samples*", page 7).

2. They included gastric disease-free control group (mean age = 51.26). The authors should explain how they confirmed histopathology of gastric lesion in this gastric disease-free group. In addition, because the mean age of this group is 51.26 and many people over age 50 have chronic gastritis; it is likely that gastric disease-free group has chronic gastritis.

Answer: We understand the issues raised by the reviewer, but the differential of our study is that the Control Group comprises patients who underwent endoscopy for medical indication. However, in the histopathological exam was evidenced absence of gastric lesions. Therefore, only individuals with histologically normal gastric mucosa without *H. pylori* infection were included in the present study, after histopathological confirmation performed by a

trained professional of Sacred Heart University -Bauru-SP following hospital standard. This information was included in the manuscript "*Subjects and Samples*" section (page 7).

3. In Fig. 3, carriers of the TLR2 -196 to -174 ins/del + del/del genotypes in gastric cancer patients had higher TLR2 mRNA expression levels than carriers of the ins/ins genotype. In addition, TLR2 19216 T/C+C/C variant carriers in gastric cancer patients showed reduced TLR2 expression levels with respect to TT wild-type genotype carriers. In chronic gastritis, there was no significant difference in TLR2 mRNA expression levels between TLR2 -196 to -174 ins/del + del/del genotypes and ins/ins genotype and between TLR2 19216 T/C+C/C and TT genotype. Thus, The authors need to explain why TLR2 expression in carriers of the TLR2 -196 to -174 ins/del + del/del genotypes and TLR2 19216 T/C+C/C variant significantly increased and reduced only in gastric cancer patients.

Answer: For the *TLR2 del -196 to -174* polymorphism (rs111200466) the genotypes associated with gastric cancer risk were *TLR2 -196 to -174 ins / del + del / del*, so it can be seen that TLR2 receptor expression was increased to carrier of these risk genotypes. While for the *TLR2 19216 T/C* polymorphism (rs3804099) the polymorphic allele presence, TC+CC, was considered a protective factor for the gastric cancer development. Thus, for this polymorphism there is an increased expression of TLR2 receptor for wild-type carrier (*TLR2 19216 T/T*). In the literature, West et al. (2017) indicates that the TLR2 mRNA expression levels were significantly increased in tumor tissues compared to either adjacent non-tumor tissues or normal tissues from GC-free individuals, regardless of risk factors or *H. pylori* presence [1]. Previous study of our research group evaluated also TLR2 receptor expression in different premalignant lesions (chronic gastritis, gastric atrophy, metaplasia) and observed a slightly increased of the gene and protein TLR2 expression in relation to normal gastric tissues [2]. Therefore, these studies show that the expression of TLR2 is mainly increased in tumor tissue, such as gastric cancer,

while in normal tissue gastric lesion its expression is not accentuated. This could justify this increase in TLR2 receptor expression only in the gastric cancer group, specifically for those with risk-associated genotypes. We believe that this increased expression was only possible in the gastric cancer group and not in the chronic gastritis group, as this is a benign lesion and probably do not yet present significant alterations in genes associated with carcinogenesis. Now this information has been included in the discussion (page 13).

Reviewer 3:

The author(s) studies genetic polymorphisms profile and genetic expression of specific gene, TLR2. There is a few studies and correlation of H. pylori prevalence. Brazilian H.pylori infection prevalence is quite low when compare to those in other Asian or other high prevalence countries.

1. Title: Characterization and risk association of TLR2 polymorphisms and Helicobacter pylori with mRNA expression in gastric carcinogenesis. Comment: I think the author can arrange for better new heading to attract the audience by putting some words that specify the interesting result of this study. Example; is it dominantly found on which genotype? Do you find the strong correlation?

Answer: To answer the reviewer's comment the title of manuscript has been changed for: "**Characterization and strong risk association of TLR2 del -196 to -174 polymorphism and *Helicobacter pylori* and their influence on mRNA expression in gastric cancer.**"

2. Abstract: In part of conclusion - I've not yet agree with the solution of gene modulate by H.pylori infection shown in mechanism in this study. Please, change wording or phrase.

Answer: The conclusion of abstract has been changed by replacing the word modulated for "influenced" (Section, Abstract; page 4). We believe that the word "influence" is more appropriate in this case, since the TLR2 expression may be influenced by the presence of the bacteria, as a consequence of the host inflammatory response and also by the presence of variant genotypes.

3. Method: I understood that the author used relative quantitation method on gene expression results. I suggest the author to re-check statistic result with mean RQ that if it's non-normal distribution of population, you may use relative quantitation on log10 value to adjust the value distribution and then use median.

Answer: We understand the reviewer's suggestion regarding the use of parametric tests and the mean of RQ as opposed to the median. However, in the present study, all data were first analyzed by the normality test (D'Agostino-Pearson omnibus test) and after checking the non-normal distribution of population, the Mann-Whitney statistical test was applied for comparison between groups (GC, CG and C), as well as to analyze the influence of *H. pylori* infection and polymorphisms on *TLR2* mRNA expression. Thus, the expression results are presented in the form of Median RQ and not Mean RQ. Regarding the use of log 10, we have not used this transformation, as it can change the values of RQ that present positive expression to negative expression. Therefore, our research group has used non-parametric tests and the median of RQ, as can be seen in our recent publications [2-5]. So in order not to compromise all our results and other reviewers' suggestions we chose to keep this results presentation.

4. Results: - Please, add table: I wish I could see all demographic profile of population, but I can't see any table. Example; If the population of positive *H.pylori* distribution on each gene study group should be demonstrated that is clearer by writing in text. - Figure 1: Please, correct to clear comparative group. For the figure legend: I think the word "control" is not control group but standard samples for relative quantitation RT-PCR. - Figure 2: If you could arrange *TLR-2* results in additional figure. Please, show 2 groups of positive *H.pylori* GC and CG VS negative *H.pylori* GC and CG. or A) GC - positive *H.pylori* VS negative *H.pylori*. B) CG - positive *H.pylori* VS negative *H.pylori*. Figure legend should be clear to explain either. - Figure 3: showed the change

between group of polymorphisms related expression. If the author can add figures of GC with each polymorphism profile with positive *H.pylori*/ negative *H.pylori*, it may explain related mechanism.

Answer: The table with the epidemiological data of individuals with gastric disease-free control group (C), chronic gastritis (CG) and gastric cancer (GC) patients was added as requested by the reviewer (Session, Material and Methods, Subjects and Samples, page 7). In order to clarify the reviewer comment, we used 14 samples from patients with histologically normal gastric mucosa and *H. pylori*-negative (Hp-) as calibrator for gene expression. Thus, C group in the Figure 1 and 2 is represented by the dashed line (Median RQ=1.0). In addition, graphs were added to the figures with the relative gene expression of each sample from the gastric cancer and gastritis groups (Figure 1, C and D) and *H. pylori* positive and *H. pylori* negative (Figure 2, C and D). In Figure 3, the main objective was to evaluate influence the polymorphic variants in relation to TLR2 gene expression, so due to the limited sample number we did not separate according by *H. pylori* infection.

5) Biostatistical results: I wish I could see the error bar and stat label in the grafts of all figures between or among the comparative groups.

Answer: The changes were made according to the reviewer's recommendation.

6) Conclusion: I've not yet agree with strong conclusion on *H. pylori* manipulation on TLR-2 gene expression and in each polymorphism types. Please, better show the detail of results for this correlation. This research is very interesting; however, more details of result should be demonstrated and revised.

Answer: We understand the reviewer's comment on the conclusions of our study, which were based on association analyzes. However, our results clearly show higher expression of *TLR2* mRNA in the *H. pylori*-positive group (RQ= 6.38) in comparison with *H. pylori*-negative group (RQ= 0.79). To better demonstrate these results we added in Figures 1 and 2 the histograms showing the expression of *TLR2* for each case of gastric cancer (36 samples) and chronic

gastritis (48 samples) groups. Despite interindividual variation it is possible to observe the highest expression of *TLR2* mRNA in the Hp + group. In addition, a previous study of our research group showed increased expression of both *TLR2* and *TLR4* mRNA and protein expression in *H. pylori*-positive-chronic gastritis patients in relation to Hp-negative normal gastric mucosa [2]. In another study, our results have shown the influence of *H. pylori* infection on the expression pattern of several genes and miRNAs, as well as changes of this expression pattern after eradication of the bacteria[3]. Moreover, we recently evaluated some *TLR9* gene polymorphisms in samples of gastric cancer and chronic gastritis and also observed this association of *H. pylori* infection with higher *TLR9* mRNA expression levels [4]. Therefore, together these results indicate that *H. pylori* infection may alter the expression of genes involved with inflammatory and immune response.

Reviewer 4:

Caroline de Matos Lourenço demonstrated that TLR polymorphisms are associated with gastric cancer in this manuscript.

1. I doubt statistical methods. For example, it is strange to compare three groups with three comparisons of two groups.

Answer: For the evaluation of risk association between the polymorphisms *TLR2* -196 to -174 ins/del and *TLR2* 19216 T/C and gastric lesions evaluated, we performed a logistic regression analysis in the models: (1) codominant (heterozygous *vs.* wild-type homozygous and polymorphic homozygous *vs.* wild-type homozygous), (2) dominant (heterozygous + polymorphic homozygous *vs.* wild-type homozygous), (3) recessive (polymorphic homozygous *vs.* wild-type homozygous + heterozygous), (4) overdominant (heterozygous *vs.* wild-type homozygous + polymorphic homozygous), or (5) log-additive (polymorphic homozygous with 2 + heterozygous *vs.* wild-type homozygous) by SNPstats software (<https://www.snpstats.net/start.htm>), in this type of analysis is expected to treat the binary variables. For this reason three comparisons of two groups were made. This type of analysis is common

in polymorphic studies and many studies in literature, in high impact magazines, bring comparisons in this way [5-10]. Recently, an article from our group with the same type of statistical analysis was published in World Journal Gastrointestinal Oncology^[4].

2. *H. pylori* infection is the most well-known risk of gastric cancer. TLR2 analysis should be performed after stratification for *H. pylori* infection.

Answer: This study aimed investigated the association of both polymorphisms with *H. pylori* infection; for this all the samples, including those in the case and control groups, were divided into *H. pylori*-negative cases and *H. pylori*-positive cases and compared (Results, pages 10 and 11; Table 4). We also sought to show the influence of the bacterium infection on the TLR2 gene expression, like this the samples from the GC and CG groups were separated according to the presence or absence of *H. pylori* infection and compared (Results, page 11; Figure 2).

Yours sincerely,



Juliana Garcia de Oliveira-Cucolo

Responsible author

References

- 1 West AC, Tang K, Tye H, Yu L, Deng N, Najdovska M, Lin SJ, Balic JJ, Okochi-Takada E, McGuirk P, Keogh B, McCormack W, Bhathal PS, Reilly M, Oshima M, Ushijima T, Tan P, Jenkins BJ. Identification of a TLR2-regulated gene

signature associated with tumor cell growth in gastric cancer. *Oncogene* 2017; **36**(36): 5134-5144 [PMID: 28481875 DOI: 10.1038/onc.2017.121]

2 Cadamuro AC, Rossi AF, Matos Biselli-Périco J, Fucuta Pereira P, Do Vale EP, Acayaba R, Leite KR, Goloni-Bertollo EM, Silva AE. Effect of *Helicobacter pylori* eradication on TLR2 and TLR4 expression in patients with gastric lesions. *Mediators Inflamm* 2015; **2015**: 481972 [PMID: 25873761 PMCID: PMC4385704 DOI: 10.1155/2015/481972]

3 Rossi AF, Cadamuro AC, Biselli-Périco JM, Leite KR, Severino FE, Reis PP, Cordeiro JA, Silva AE. Interaction between inflammatory mediators and miRNAs in *Helicobacter pylori* infection. *Cell Microbiol* 2016; **18**(10): 1444-1458 [PMID: 26945693 PMCID: PMC5074252 DOI: 10.1111/cmi.12587]

4 Susi MD, Lourenço Caroline M, Rasmussen LT, Payão SLM, Rossi AFT, Silva AE, de Oliveira-Cucolo JG. Toll-like receptor 9 polymorphisms and. *World J Gastrointest Oncol* 2019; **11**(11): 998-1010 [PMID: 31798780 PMCID: PMC6883180 DOI: 10.4251/wjgo.v11.i11.998]

5 Zabaglia LM, Ferraz MA, Pereira WN, Orcini WA, de Labio RW, Neto AC, Wisnieski F, de Oliveira JG, de Arruda Cardoso Smith M, Payão SL, Rasmussen LT. Lack of association among TNF- α gene expression, -308 polymorphism (G > A) and virulence markers of *Helicobacter pylori*. *J Venom Anim Toxins Incl Trop Dis* 2015; **21**: 54 [PMID: 26719751 PMCID: PMC4696262 DOI: 10.1186/s40409-015-0054-3]

6 de Oliveira JG, Silva AE. Polymorphisms of the TLR2 and TLR4 genes are associated with risk of gastric cancer in a Brazilian population. *World J Gastroenterol* 2012; **18**(11): 1235-1242 [PMID: 22468087 PMCID: PMC3309913 DOI: 10.3748/wjg.v18.i11.1235]

7 de Oliveira JG, Rossi AF, Nizato DM, Miyasaki K, Silva AE. Profiles of gene polymorphisms in cytokines and Toll-like receptors with higher risk for gastric cancer. *Dig Dis Sci* 2013; **58**(4): 978-988 [PMID: 23086128 DOI: 10.1007/s10620-012-2460-5]

8 Proença MA, de Oliveira JG, Cadamuro AC, Succi M, Netinho JG, Goloni-Bertollo EM, Pavarino É, Silva AE. TLR2 and TLR4 polymorphisms influence mRNA and protein expression in colorectal cancer. *World J Gastroenterol* 2015; **21**(25): 7730-7741 [PMID: 26167073 PMCID: PMC4491960 DOI: 10.3748/wjg.v21.i25.7730]

9 de Oliveira JG, Rossi AF, Nizato DM, Cadamuro AC, Jorge YC, Valsechi MC, Venâncio LP, Rahal P, Pavarino É, Goloni-Bertollo EM, Silva AE. Influence of functional polymorphisms in TNF- α , IL-8, and IL-10 cytokine genes on mRNA expression levels and risk of gastric cancer. *Tumour Biol* 2015; **36**(12): 9159-9170 [PMID: 26088449 DOI: 10.1007/s13277-015-3593-x]

10 Dos Santos MP, Sallas ML, Zapparoli D, Orcini WA, Chen E, Smith MAC, Payão SLM, Rasmussen LT. Lack of Association between IL-6 Polymorphisms and Haplotypes with Gastric Cancer. *J Cell Biochem* 2019; **120**(6): 9448-9454 [PMID: 30525242 DOI: 10.1002/jcb.28220]