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Dear Editor

Please find enclosed the edited manuscript in Word format (file name: 14637-review.doc).

Title: *TLR2* and *TLR4* polymorphisms influences mRNA and protein expression and colorectal cancer

Authors: Marcela Alcântara Proença, Juliana Garcia de Oliveira, Aline Cristina Targa Cadamuro, Maysa Succi, João Gomes Netinho, Eny Maria Goloni-Bertolo, Érika Cristina Pavarino, Ana Elizabete Silva.

Name of Journal: *World Journal of Gastroenterology*

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We are very thankful for the comprehensive review performed to our manuscript referred above, and the request that we resubmit a revised version of it. As you will see from the resubmitted copy we have addressed the issues raised by the reviewers in a constructive manner and editor's suggestions, clarifying some issues. Below we list our responses to each individual point raised by the Referee.

We have uploaded our revised manuscript according hoping that it now reaches the standard for publication in the *World Journal of Gastroenterology*.

Comments 1

This study examined the effect of promoter region polymorphisms TLR2-196 to -174del and TLR4-1607T/C(rs10759932) on mRNA and protein expression in tumor tissue and also of TLR4+896A/G(rs4986790) on colorectal cancer(CRC) risk in a Brazilian population. The author identified TLR2-196 to -174del was associated with

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increased CRC risk. However, there are some limitations in the manuscript, which may greatly affect the quality of the paper.

1. Study populations: "The case group (CRC) consisted of 194 samples from patients with a confirmed diagnosis of sporadic CRC by clinical histopathological parameters, 160 of which were studied based on samples of peripheral blood and 40 on samples of biopsies or surgical fragments and their respective normal adjacent mucosa (105 men and 89 women; mean age: 62 ±12 years)", the total sample size (194 or 200?) is inconsistent according to the description.

Answer: The total of patients is 194, because of 6 patients we had both biopsy and blood samples. Therefore, for these 6 patients the genotyping study was performed with DNA extracted of peripheral blood and the gene expression of RNA extracted of biopsies. This information was added in METHODS - Study populations (page 6).

2. In page 10, line 22, "However, no association with gastric cancer was found in the Japanese population, nor did we find any studies reporting the presence of this polymorphism in CCR", "CCR" should be "CRC".

Answer: There was a typing error, the correct is CRC. We already corrected in the manuscript.

3. The sample size in this study is only 194 cases and 240 controls, I believe the sample size is relatively small, please provide the power of such sample size in discovering the differences between groups.

Answer: We agree with the reviewer that in association studies the sample size may limit the power of the statistical analysis, however other studies have also been published with sample size similar or smaller than the present study (Oliveira. JG et al., *World J Gatroenterol* 18: 1235-1242, 2012; Hong Fan et al. *World J Gastroenterol* 14(14):2230 -2234, 2008; Jia-Qi Li et al. *World J Gastroenterol* 17(16):2131 -2136, 2011). In addition, some studies have evaluated the same polymorphism with similar number of subjects, for example Khan AU, et al. 2014 (Int J Immunogenet. 41(2):105-11, 2014) studied 100

healthy and 87 case patients and Kalinderi K, et al., 2013 (Neurol Sci. 34(5):679-82, 2013) evaluated 215 patients and 118 control subjects. Also to be considered that the frequencies of polymorphic alleles for both TLR2-196 to -174del and TLR4-1607T/C observed in the present study are high (10%), and only for TLR4+896A/G is lower (3% and 5% for control and case groups). In addition, the power of the sample test was conducted and it was found that this is sufficient considering a one-sided test that showed $p < 0.05$ for the polymorphism in question.

4. PCR-RFLP is a relatively simple method for genotyping, but this method is not so reliable for the judgment of genotypes. Please discuss the limitation of such method in the manuscript.

Answer: This technique is still widely used for genotyping. We know it is a simple method, but it is still efficient. To avoid errors in genotyping we used ultrapure agarose to make a 3% gel, which permits the total separation of the bands. In addition we always used a positive and a negative control, and the gel was viewed by 2 observers. Samples with any doubts were repeated and at the end of the study 12% of the samples were randomly selected to be redone so that we could confirm the results. Several recent papers assessing the same polymorphisms support the efficiency of this technique (Oliveira. JG et al., 2012 *World J Gatroenterol* 18: 1235-1242; Khan AU, et al., 2014, Int J Immunogenet. 41(2):105-11, 2014; Kalinderi K, et al., 2013, Neurol Sci. 34(5):679-82, 2013; Tahara T, et al., 2008, *Dig Dis Sci* 53: 919-924.; Soares SC, et al., 2008, *Genet Mol Res* 7: 1011-1019; Huang H, et al., 2010, *J Biomed Res* 24: 100-106.)

5. What is the distributions of primary information, such as age and gender, between cases and controls? No any adjustment was conducted for the analysis and some bias might affect the results.

Answer: This information about age and gender in both case and control groups are reported in Methods - Study populations (pages 6-7). For calculations of Odds ratios (ORs) and 95% confidence intervals (CIs) for the polymorphisms under study the multiple logistic regression models were adjusted for age, gender, smoking and

drinking in both groups. Now this information was added in Methods - Statistical analysis (page 9).

Comments 2

The study by Proença et al. explores the role of TRL2 and TRL4 polymorphism and colorectal cancer risk. The article is meaningful and well written. I would suggest to accept, pending minor changes:

1. Methods: nucleic acid extraction, please specify the modifications to the quoted protocol.

Answer: The modification of protocol consisted of using Ficoll-Paque™ PLUS to separation of the blood phases. This information was added in Methods - Nucleic acid extraction (page 7).

2. Figures: please clearly indicate in each figure which comparison is statistitically significant (e.g. with a star) Are TRL2 mRNA and protein levels significantly correlated?

Answer: The star was added in each figure. Correlation analysis between *TRL2* mRNA and protein levels, as well as *TLR4* were performed, but we did not find significant correlations (TLR2: r= -0.009; *P*=0.971, TLR4: r= -0.174; *P*=0.504). Thus we do not add these results in the manuscript, because it is often no correlation between these two parameters as observed in other studies (Guo et al., Acta Biochimica et Biophysica Sinita, vol. 40, pp. 426-436, 2008; Jorge et al., Mediators of Inflammation, vol. 2013, pp. 1-11, 2013). The agreement or correlation between mRNA and protein expression is not absolute, but this type of divergence is expected. Firstly should be considered the sensibility of different techniques (PCR real time and immunohistochemistry) and also should be considered other pathways allow higher stability of protein in relation to mRNA or additional post-transcriptional mechanisms, including protein translation, post-translational modification, and degradation.

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Comments 3

Major concerns:

1. *In introduction, the chronic inflammation part should be expanded.*

Answer: We have expanded the first paragraph of introduction on the relationship between chronic inflammation and cancer as the reviewer's suggestion.

2. In methods, approval and consent, clinical findings and demographic characteristics of study population should be given as a table, also statistical compressions need for these subgroups. For example; between smokers and non-smokers.

Answer: We find appropriate the reviewer's suggestion. The table and the information was added. Regarding risk factors, statistically significant differences were found between groups of age younger than 60 years and greater than or equal to 60 years, non-smokers and smokers and non-alcoholics and alcoholics. Only for the variable gender it was not found difference between groups, according to Fisher's exact test. (Table 1). Is well established that advanced age, smoking and alcohol intake are the major risk factors for the development of cancer, so these data were not considered for discussion.

3. In methods, polymorphisms genotyping part, allele specific PCR is not mentioned. Should be mentioned. Also, table 3 should be numbered as table 1 and table title should be changed.

Answer: The conditions for both allele specific and PCR-RFLP techniques were the same. We added this information in Methods - Polymorphism genotyping (page 7). Tables numerations were corrected.

4. What is the power of your study? As you mentioned, your study included 434 people for genotyping, 40 for mRNA analysis and 20 for the immunohistochemistry. You should include power analysis.

Answer: About the sample size for the polymorhisms association study we already justify above (Comments 1). For mRNA and immunohistochemistry analysis due to difficulties obtaining colorectal cancer samples it was not possible to obtain a higher number, but

considering the specificity and efficiency of both qPCR and immunohistochemistry techniques this number of samples has been shown adequate for this type of analysis (Jorge et al. 2013, Mediators of Inflammation, v. 2013; Rossi et al 2014, Mediators of Inflammation, v. 2014).

5. Haplotype analysis was mentioned in results. However, there is no results or table about the haplotyping. Authors should focus on this also.

Answer: Considering that was not observed statistical difference in the frequencies of allele combinations between CRC and C groups we have chosen not to put the table. However, now added in results both tables about haplotype analysis and combination of the three polymorhisms (Tables 5 and 4)

6. In results, mRNA and protein expression part, " so, these results are concordant with the findings regarding...." As we review the figures there is no consistency between mRNA and protein expressions regarding TRL4.

Answer: For TLR4, both gene and protein expression were found in basal levels, with no statistically significant difference compared to adjacent normal tissue. Thus, according these results we can affirm that there was agreement between mRNA and protein expression for TLR4. In Figure 2, A and B correspond to normal and tumor tissue respectively, for TLR2 protein we can observe an intense immunostaining in tumor, while D and E correspond to normal and tumor tissue respectively, for TLR4 protein a similar pattern of immunostaining is observed.

7. In discussion part, " you mentioned earlier studies about these polymorphisms, however, no association is found between TRL4 +896 A/G and cancer in different populations. Why you choose to include in your study so?

Answer: Although some studies have not reported any association of this polymorphism with other types of cancer, in a recent study of our research group we observed an association of *TLR4 +896A/G* with risk of gastric cancer and chronic gastritis in the Brazilian population (Ref. 26). In addition, other studies had reported an association of

this polymorphism with CRC in some populations (Refs. 22, 52). Therefore, these studies provided us interest in studying this rare polymorphism in CRC.

8. In tables, title of Table 1 indicates information about the gender, age, smoking. However, in table there is no presentation for these information. Should be corrected and indicated if exists so?

Answer: The Table 1 (new Table 3) presents the OR values, which the data of polymorphisms for multiple logistic regression analysis were adjusted considering the risk factors as gender, age, smoking and drinking, so this information was described as a footnote. However we added another table (new Table 1) with this information in Methods section - Study populations.

Minor concerns:

1. In methods, mRNA relative quantification by qPCR part, which reference gene was used should be indicated clearly either GAPDH or ACTB. Should be indicated in figures also.

Answer: Both reference genes (*GAPDH* and *ACTB*) were used in all analyzes of qPCR for *TLR2* and *TLR4* relative quantification. We added this information in the figure as well.

2. Table numerals should be arranged throughout the text.

Answer: The table numerals were corrected.

3. In table 1, dominant is written by double tt should be corrected.

Answer: The table was corrected.

Thanks again for the attention to our manuscript.

Yours sincerely,

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