Author's response to reviews

Title: MYC, FBXW7 and TP53 copy number variation and expression in Gastric Cancer

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Author's response to reviews: see over
Dear Editor,

Please find enclosed an electronic copy of the review manuscript in BMC Gastroenterology format.

Title: MYC, FBXW7 and TP53 copy number variation and expression in Gastric Cancer

Author: Danielle Queiroz Calcagno, Vanessa Morais Freitas, Mariana Ferreira Leal, Carolina Rosal Texeira de Souza, Samia Demachki, Raquel Montenegro, Paulo Pimentel Assumpção, Marília de Arruda Cardoso Smith, Andrea Kely Campos Ribeiro dos Santos, Rommel Rodrigues Burbano.

We would like to submit this manuscript to the BMC Gastroenterology, entitled: “MYC, FBXW7 and TP53 copy number variation and expression in Gastric Cancer”.

The aim of this article was investigated MYC, FBXW7 and TP53 gene copy number variations, mRNA and protein expression in GC samples and gastric adenocarcinoma cell lines. Possible associations between these findings and clinicopathological features or invasion and migration ability of cell lines were also evaluated. The expression level of MYC mRNA in tumor tissue samples was significantly higher than non-neoplastic tissue and the expression level of FBXW7 and TP53 mRNA in tumor tissue specimens was markedly lower than that of correspondent non-tumor tissue. Moreover, MYC and FBXW7 mRNA were associated with the presence of lymph nodes metastasis and III-IV tumor stage. Yokobori et al. (2009) showed decreased FBXW7 mRNA levels in gastric tumor associated with presence of lymph nodes metastasis and poor prognostic. However, to our knowledge, this is the first study to report association between the presence of lymph node metastasis and III-IV CG stage with an increase of MYC mRNA expression, ensuring the novelty and impact of the paper.

Additionally, we also observed increased MYC and reduced FBXW7 mRNA and protein expression in ACP02 compared to ACP03 cells. Furthermore, ACP02 cells had higher invasive ability than ACP03 cells.
These findings reinforce that MYC and FBXW7 deregulation may be crucial for invasive ability of GC cells in agreement with the observed in gastric tumors.

*In vitro*, increased *MYC* and reduced *FBXW7* expression was associated with a more invasive phenotype of gastric cancer cell lines. This result encouraged us to investigate the activity of gelatinases MMP-2 and MMP-9 in both cell lines. Both proteases activity investigated are synthesized predominantly by stromal cells rather than cancer cells, and it has been proposed that both contribute to cancer progression. We observed a significant increase of MMP-9 activity in ACP02 compared to ACP03 cell line. These results confirm that ACP02 have more invasion ability compared to ACP03.

In conclusion, our findings show that *FBXW7* and *MYC* mRNA levels reflect the potential for aggressive biologic behavior of gastric tumors and maybe used as an indicator of poor prognosis in GC patients. Furthermore, cell cycle regulation by ubiquitin ligase has potential in developing new targets in GC therapy.

The authors assert that manuscript has not been previously published or submitted for publication elsewhere.

This study received the approval of the Ethics Committee.

All authors have contributed significantly, and they are in agreement with the entire content of this manuscript.

Thank you for your attention.

Yours sincerely,

Danielle Queiroz Calcagno, PhD
Reviewer's report

Title: Clinical significance of MYC, FBXW7 and TP53 copy number variation and expression in Gastric Cancer

Version: 2 Date: 16 October 2012
Reviewer: Yueyong Liu

Reviewer's report:

The manuscript by Calcagno et al. reported the copy number variation and expression levels of MYC, FBXW7 and TP53 in gastric cancer. The aim of this paper is to find the correlation among three genes in the clinical samples. The authors made efforts towards to find a useful indicator of poor prognosis by investigating the FBXW7 levels in gastric cancers associated with MYC levels. Due to the complexity of FBXW7 regulation, either in a p53 dependent transcriptional regulation or post-transcriptional regulation, for instance self-ubquitination, so authors only focusing on mRNA level may mislead the finding. And the poor quality of FBXW7 antibodies may hamper the authors’ attempt to test the change of FBXW7 in protein level.

Major concerns and suggestions:

1. The author didn't show significant correlation between FBXW7 and myc, probably because FBXW7 is subject to post transcriptional regulation. FBXW7 IHC results on clinical samples are necessary in this study to substantiate the finding. Authors may try different antibodies against FBXW7 for either IHC or western blot.

   - We used two different antibodies against FBXW7 protein, Abnova Corporation (Taiwan) and Abcam (Hong Kong), in three dilutions (1:50; 1:100; 1:200) to analysis the expression of FBXW7 protein in gastric adenocarcinoma samples and positive control of breast cancer. Both antibodies showed the same results and the best dilution and recommended is 1:50. All the samples of gastric adenocarcinoma samples were negative for FBXW7 expression. Thus, no association with clinicopathological data could be performed.

2. The authors should specify each sample in the both mRNA and protein levels of MYC and FBXW7 instead of presenting the abundance in total, in which the decreased FBXW7 and increased MYC levels are extensively reported by researchers.

   - The correlation among the different targets mRNA expression was analyzed by Spearman test, in which a value below 0.30 was determined as a weak correlation; 0.30-0.70 as a medium correlation; and above 0.70 as a strong correlation. We observed a tendency of correlation between the increase of MYC mRNA expression and decrease of FBXW7 mRNA expression was detected. This assay takes in consideration the individual values of each sample
if to just show is correlation in higher MYC and reduced FBXW7 mRNA expression.

3. Statistical data is not clearly presented. For example, Table 2 contains p-value numbers which are differently claimed in the second paragraph on RESULTS.
   • Sorry, we corrected for the right value.

4. In vitro experiment does not tightly relate to and support the previous clinical results, making the manuscript not logic and organized in general.
   • In our previous cytogenetic studies, we described gain MYC copies and TP53 deletion in ACP02 and ACP03 gastric adenocarcinoma cell lines and gastric adenocarcinoma tissue samples. Since ACP02 and ACP03 present genetic alterations similar to those of gastric tumors, these cell lines may be used as an experimental model of gastric carcinogenesis and help change the landscape of gastric cancer in our region.
   In present study, we also observed increased MYC and reduced FBXW7 mRNA and protein expression in ACP02 compared to ACP03 cells. Furthermore, ACP02 cells had higher invasive ability than ACP03 cells. These findings reinforce that MYC and FBXW7 deregulation are crucial for invasive ability of GC cells in agreement with the observed in gastric tumors and supported the results that the increased MYC and reduced FBXW7 mRNA expression was associated with the presence of lymph node metastasis and III-IV GC stage in gastric adenocarcinoma.

5. Fluorescence Image data is lack of quality.
   • We improved the quality of the fluorescence image.

Minor points:
1. Line 5 “MYC activation by FBXW7 loss triggers p53 activation” to the bottom in BACKGROUND, authors should refer to the reference.
   • The reference was added.
2. Line 8 in “Comparison between ACP02 and ACP03 cell lines” refers to figure 2 instead of figure 1.
   • The change was made.
3. Authors should double check the whole text for typos and grammar mistakes.
   • The review was made.

Reviewer's report
Title: Clinical significance of MYC, FBXW7 and TP53 copy number variation and expression in Gastric Cancer
The study reports the potential clinical significance of three molecular factors in gastric cancer patients. The copy number variations of the genes MYC, FBXW7 and TP53, as well as expression levels of the corresponding mRNA transcripts and proteins are studied in a cohort of 33 gastric cancer patients and in two gastric cancer cell lines. The results are combined with clinicopathologic features as morphologic classification (Lauren), TNM stage, age and gender. One of the main results of the study is that deregulation of MYC (amplification) and FBXW7 (LOH) were associated with lymph node metastasis and stage III-IV disease. No significant relationship was found between the expression or copy number changes of TP53 and the clinicopathologic variables belonging to the patient samples.

Furthermore, the in vitro invasiveness and migratory capability of two gastric cancer cell lines was examined as well as the descriptive analysis of protein levels (western blots) of the MYC, FBXW7 and p53 proteins.

1. The study reports copy number variations and expression levels of the three studied genes, which also is reflected in the title. However, data of the mutational status of these genes would have strengthen the study, in particular for the analysis of the tumor suppressor genes. Loss of p53 function may not be related to gene expression levels or immunohistochemistry expression. Thus, there might be a clear relationship between p53 function and the clinicopathical variables examined here that the authors are not aware of. It would also be interesting to display such results together with copy number and expression data. At least, this should be thoroughly discussed in the discussion section.

- The authors agree with the comments, however all tissue samples were microdissected and the resulting tissue was not enought to perform DNA sequencing, since TP53 gene encodes 11 exons. Furthermore, is wellknown that TP53 mutations lead to an abnormal proteins which, in turns, increase its half-life what makes it suitable for IHC.
2. The study is all over descriptive, all though some functional data of tumor cell invasiveness and migration are coupled to the levels of protein expression of MYC and FBXW7 in the two gastric cancer cell lines studied.

3. The IHC analysis revealed that MYC were more highly expressed in intestinal type than diffuse type gastric cancer in the patient samples. Diffuse type gastric cancer is a highly invasive phenotype where the tumor cells respect fewer tissue boundaries. The cell line ACP02 with the highest in vitro invasiveness through Matrigel, was however found to have higher MYC expression than the comparator ACP03 with a less invasive phenotype, all though ACP03 had a higher migratory capacity. How do the author explain these apparently contradictory results regarding invasiveness?

- IHC analysis revealed that MYC protein expression is more frequently found in intestinal-type GC than in the diffuse-type GC specimens. These alterations could lead to an abnormal MYC protein, which are not recognized by both antibodies used in the present study. This aberrant protein might result of chromosomal translocations involving MYC locus (8q24) in diffuse-type CG from individuals of Northern Brazil.

4. It is hypothesized that ACP02 is secreting more proteolytic enzymes than ACP03. In stead of launching this possible explanation for the higher invasiveness of ACP02 cells, the authors could examine proteolytic activity by for instance performing zymographies of the two particular cell lines.

- The zymography was performed and added to the manuscript.

Minor Essential Revisions
1. The lines 2-3 p.10 should be moved to discussion section as protease activity is not actually analyzed in the two cell lines, but is stated as an expanation for the results found.

- The change was made

2. Check the p-value of CNV TP53 versus Age (Table 1). The p value is non-significant, but might be of an even higher value
• The change was made

Discretionary Revisions
None
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests