Author's response to reviews

Title: Correlation of MLH1 and MGMT expression and promoter methylation with genomic instability in patients with thyroid carcinoma

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

Please find enclosed the revised version of the Manuscript 'Analysis of MLH1 and MGMT expression and promoter methylation on genomic instability in patients with thyroid carcinoma” by Santos et al.

Again, we thank you and the reviewers for the thoughtful and helpful review of our manuscript, which certainly improved the manuscript. We believe we have addressed all concerns raised by the reviewer.

We hope you will find the new version of our manuscript acceptable for publication in BMC Cancer in the present format.

Thank you very much for your attention.

Sincerely yours

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Response to Reviewer 1:

Major compulsory revisions

1. The objective of the paper has been clarified (study of the association between expression/methylation of MLH1 and BRAF V600E/MSI and expression/methylation of MGMT and IDH1/RAS mutation. Anyway, the following aspect remain still unclear:

   - Why the Authors did made a conclusion also on the association between MSI and histological pattern, which was not included in the aim of the paper?
   - Why did the Authors evaluate also RET/PTC mutation?

   That is an important point. We apologize if that was not clear in the previous version of this manuscript.

   First, RET/PTC and BRAF V600E mutations are genetic alteration specifically found in papillary thyroid carcinomas while IDH1 are mainly found in Follicular thyroid carcinomas and follicular variant of papillary thyroid carcinoma. RAS mutations are found in both. Secondly, in the literature, it has been suggested that BRAF V600E and RAS with MSI and hypermethylation of MLH1 while hypermethylation of MGMT is associated with IDH1 mutation. There is no information about RET/PTC rearrangement.

   Therefore, to identify a correlation between hypermethylation of MGMT and/or MLH1 and specific mutation, samples were subdivided according mutational status.

   In this new version of the manuscript we included information that may clarify why RET/PTC was evaluated. The information was included in the introduction section (page 4).

2. In the statistical analysis there is not reference to the comparison between histological types and this is counteracting with the conclusion made in the abstract and in the discussion.

   The reviewer is correct. We now include further information regarding statistical analysis.

3. The recruitment process is not described at all: this is a relevant concern of the study.
Biopsy was obtained from patients who underwent thyroid surgery for thyroid cancer. Final histological classification was obtained from paraffin-embedded sections. We have included this information in the new version of the manuscript.

4. The malignant samples are not 82 and not 92. I still do not understand why the Authors did include in the study also sample coming from benign lesion and normal tissue: in the aim the evaluation of differences between malignant and benign lesions was not listed.

We apologize for this mistake. We changed the number of malignant samples to 82 (page 9).

Regarding other question, a total of 96 thyroid tissue samples were used. The study included 70 PTCs, 12 FTCs, 7 benign follicular thyroid adenoma (FTA) and 7 adjacent normal thyroid tissues.

Normal and benign were used in MSI analysis (described in material and methods) as follow: To assess MSI, we compared the band pattern produced after gel electrophoresis of paired PCR reactions containing patient-matched normal and tumour DNA. If the normal and tumour (benign or malignant) PCR amplification products displayed different electrophoretic motilities, were scored the case as positive for MSI. Normal thyroids were used to Benign lesions were used as control for MIS (Figure 2).

**This information was included in the new version of the manuscript.**

Additionally, normal thyroids were also used in the expression assays (real time PCR analysis). In this assay, the relative expression levels were calculated according to the 2−ΔΔCT (ddCt formula) as described [8, 17]. In this formula the expression is relative an endogenpur control (RPS8) and a normalization group (ddCt). The normal thyroids (n=7) were used to normalize for the expression of MLH1 and MGMT.

5. Table 2 has to be amended: if a comparison between histological types has been made, I would expect to see only one p value for each of the two genes. In particular, I would like to see a unique value of p followed by a post-hoc
analysis with a correction for multiple comparisons. I suggested doing it also in the previous review with no results (even though in the point to point answer Authors have told to do it). Furthermore, in the objective and in the methods the Authors did not mention the comparison between histological patterns as an aim.

We apologize if that was not clear. However, we had not performed the comparison between histological subtypes. So multiple comparisons were not performed. We, in fact, performed the comparison in each histological subtype group. As these subgroups are clinically different, to group all samples is not correctly. To better clarify this issue, we have included additional information in the statistical analysis section

6. Numbers in table 3 are still inconsistent with those in number 2 and each other.

As stated in the Table 3, we performed the analysis omitting some samples. As example, in the negative group were included only samples without any of the alterations investigated here. That is important to identify and effect of a specific mutation. We would introduce bias if we included in the BRAF negative group samples that are positive for other mutations (IDH1, RET/PTC or RAS). Additionally we excluded “Samples harboring more than one mutation from statistical analysis”, as stated in the manuscript. That is the reason why numbers are different, but not inconsistent.

7. I cannot understand totals reported in table 5 and 6: according to me numbers are still inconsistent.

The difference observed in the table 5, is similar to that observed in Table 3 (point #6). Briefly, the numbers reported (as total) are the same totals reported in table 2 (total of MLH1 and MGMT methylated samples in each subgroup). The total of
samples in each subgroup (independent of methylation status) were omitted to avoid any misunderstandings.

8. Limits of the study are not discussed still in the amended version.

The reviewer is correct. Because we believe that one of the limitations of our study is the sample size, we included that in the discussion section. We also included that other CpG regions could also be evaluated.

9. English has to be revised and sentence construction is sometime meaningless.

I agree with the Reviewer, and it is corrected.