Peer Review File

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Reviewer A

Comment 1: BRAF mutations were detected by immunohistochemical BRAF V600E (VE1) antibodies and confirmed by three pathologists-method of BRAF mutational status identification should be added to Methodology section, not only as an addition to the Table.

Reply 1: Thank you for your advice. We have added the immunohistochemical of BRAF V600E mutational identification in the Method section (see Page 4, line 12 to 25).

Changes in the text: See on Page 4, line 12 to 25.

Comment 2: The number of patients with different TNM staging should be added to the groups description.

Reply 2: Thank you for your advice. We have added the TNM staging information of PTC patients in Table 1.

Changes in the text: See in Table 1.

Comment 3: Of course, it would be interesting to verify the serum NRG1 status in patients with non-malignant thyroid nodules - to check whether it may be a possible marker of malignancy - which is intensively searched for the thyroid cancer.

Reply 3: Thank you for your kind and meaningful advice. It is meaningful to test serum NRG1 status in patients with non-malignant thyroid nodules. This result will enrich the clinical significance of NRG1. However, our study failed to collect enough blood samples from healthy people for further exploration. Our purpose is to get some novel glycobiomarkers in PTC patiens with different BRAF V600E status. We will take your suggestion and collect more blood from healthy people for the follow-up study.

Changes in the text: None.

Comment 4: The discussion section is rather short. Authors should speculate on a possible link of serum NRG1 and BRAF V600E status.

Reply 4: Thank you for your kind and meaningful advice. We have added a speculation on the relationship of serum NRG1 and BRAF V600E status in Discussion section (see Page 9, line 7 to 8).

Changes in the text: See Page 9, line 7 to 8.

Reviewer B

Comment 1: Authors did not explain in Introduction why they analyzed NRG 1 in PTC samples although there are the papers showing the role of NRG1 in the predisposition

to PTC, e.g. He et al. (2018).
Reply 1: Thank you for your meaningful advice. We have added the explanation about why we analyzed NRG1 in PTC samples in Introduction section (see Page 3, line 15 to 16).
Changes in the text: See Page 3, line 15 to 16.

Comment 2: Page 2, lines: 1-3: The reference Moremen et al. (2012) is not adequate to the sentence "Some tumor biological features such as metastasis and cell survival depend on the presence of specific cellular glycoforms(3)."
Reply 2: Thank you for your kind advice. The reference Moremen et al. (2012) mainly introduced the protein domain-specific glycosylation in facilitating or modulating biological recognition events. We thought it was appropriate to refer to this paper. We also added a new reference at your suggestion (see Page 3, line 6, Reference 4).
Changes in the text: See Page 3, line 6, Reference 4.

Comment 3: Page 2, line 11: A lack of a reference to the sentence "NRG1 can be secreted to serum as described". What does it mean "as described"?
Reply 3: Thank you for your advice. We have added the reference in the manuscript (see Page 3, line 14, Reference 9).
Changes in the text: See Page 3, line 14, Reference 9.

Comment 4: Page 3: A lack of information about antibody anti-NRG1 used to immunoprecipitation of neuregulin 1 (lines 3-5) and detection of NRG1 in antibody overlay lection microarray (lines: 10-11): catalog number, mono-/polyclonal, clone number.
Reply 4: Thank you for your suggestion. We have added the antibody information of NRG1 in the Method and Material section (see Page 4, line 28 to 29).
Changes in the text: See Page 4, line 28 to 29.

Comment 5: Page 4: Secondary antibody was omitted in the description of Western blotting in the section Lectin blot assay.
Reply 5: Thank you for your suggestion. It was our mistake for missing the secondary antibody information. We have added the secondary antibody information in the Method and Material section (see Page 6, line 1 to 2).
Changes in the text: See Page 6, line 1 to 2.

Comment 6: Page 4, line 25-27: Why the samples from non-PTC patients (not mentioned in the section Serum specimens and clinicopatholigical data and Table 1) were used in ELISA assays?
Reply 6: Thank you for your meaningful advice. This was a wrong expression in our manuscript. We did not use samples from non-PTC patients. It should be samples from BRAF(-) and BRAF(+) PTC patients. We have corrected the mistake in Page 6, line 25.
Changes in the text: See Page 6, line 25.

Comment 7: Page 5, lines 11 and 19: It should it be GSL2 (lectin from Griffonia simplicifolia) instead of GLS2.
Reply 7: Thank you for your meaningful advice. We have changed all “GLS2” into “GSL2” in our manuscript (see Page 7, line 14, 15, 22, Page 11, line 15). The expression in the figures were correct.
Changes in the text: See Page 7, line 14, 15, 22, Page 11, line 15.

Comment 8: Six lectins with significantly increased binding affinity in BRAF+ PTC were indicated (page 5 and Fig. 2) based on the lectin assay performed for 50 lectins (Fig. 1). Are the images on Fig. 1B representative? The significant difference between BRAF+ and BRAF- samples for HHL lectin is not visible on Fig. 1B. While the substantial differences are visible in case of e.g. ConA, PWA, and PHA-E lectins.
Reply 8: Thank you for your meaningful advice. Figure 1B was representative. However, it was our mistake to provide wrong 50 lectin spots information in overlay lectin microarray. We have provided the correct information of lectin spots (see Figure 1A). HHL lectin still had significant difference between BRAF+ and BRAF- samples.
Changes in the text: See Figure 1A.

Comment 9: I did not find GSL2 lectin in the table on Fig. 1A.
Reply 9: Thank you for your meaningful advice. It was our mistake to provide wrong 50 lectin spots information in overlay lectin microarray. We have provided the correct information of lectin spots. (see Figure 1A). GSL2 was in line 12 in the lectin spots.
Changes in the text: See Figure 1A.

Comment 10: Page 8: The graph on Fig. 2A does not match the images on Fig. 1B. The fluorescence intensity is the highest for CAL and BPL among the lectins presented on the graph (Fig. 2A). But this is not reflected on Fig. 1B, e.g. PWA with the lowest intensity on Fig. 2A is more intensive than CAL on Fig. 1B.
Reply 10: Thank you for your meaningful advice. It was our mistake to provide wrong 50 lectin spots information in overlay lectin microarray. We have provided the correct information of lectin spots.
Changes in the text: See Figure 1A.

Comment 11: Page 8: "A" and "B" should be added to Fig. 1.
Reply 11: Thank you for your meaningful advice. We have added A and B labels in Figure 1.
Changes in the text: See Figure 1.

Comment 12: Authors concluded that "BRAF (+) PTC patients had significantly higher fucosylation compared with BRAF (-) PTC patients." (page 6), but none of the lectins specific for fucose (AAL, UEA, LCA) did show significant differences in lectin microarray (Fig. 2A).
Reply 12: Thank you for your meaningful advice. The AAL ELISA results were shown in Table 2 and Table 3. Although the AAL, UEA, LCA lectins for fucose showed no

significant differences in Figure 2A, which was probably due to the small sample bias, the AAL ELISA results in Table 2 and Table 3 indicated significant differences between BRAF (+) and BRAF(-) PTC patients.
Changes in the text: None.

Comment 13: The numerous punctuation and letter errors should be corrected. And other small mistakes are quite common, e.g. PBST concentration (0.05 %Tween20 in PBS, pH7.2–7.4) was given twice in Elisa Index of NRG1 (page 4), the numbers of references should be given instead of the full data of the papers by Li et al. (2015) and Adamczyk et al. (2012) (page 7) which in turn are not included in the section References.
Reply 13: Thank you for your meaningful advice. We have checked and corrected the punctuation and letter errors in our manuscript. We have deleted the repeated PBST concentration in the Method section (see Page 6, line 22). We have corrected the reference style (see Page 9, line 18 to 22, Reference 17 and 18).
Changes in the text: See Page 6, line 22, Page 9, line 18 to 22, Reference 17 and 18.

Reviewer C
Comment 1: In materials and methods, immunoprecipitation and antibody overlay lectin microarray section (page 3, line 5), the authors state “an equal amount of purified NRG1 protein” were used. How did they assess that? Were proteins quantified? Which method did they use? I think it is a crucial point, and all the following results rely upon it. In fact, before testing for differences in glycosylation patters, eventual differences of NRG1 protein expression between BRAF mutant and wild-type must be verified. Otherwise, the differences observed could be due to the relative abundance of NRG1 rather than to actual glycosylation dissimilarities. In addition, different baseline concentration could lead to different binding performance. Although, the western blot is not crystal clear (figure 3), baseline differences in NRG1 expression do exist. It is true that in lectin/western blot ratio were used thus softening the doubts, but they still stand if the above-mentioned questions are not addressed.
Reply 1: Thank you for your meaningful advice. We used BCA assay to quantify the purified proteins to ensure the equal amount of proteins. Briefly, the amount of total protein was quantified using the ThermoScientific™ Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, MA, USA). It is true that there was baseline differences in NRG1 expression between BRAF mutant and wild-type from Figure 3. Therefore, we defined lectin/NRG1 ratio as band intensity in lectin blot to minimize the baseline differences.
Changes in the text: None.

Comment 2: The antibody clone specific for NRG1 protein is never mentioned, please report it to allow reproducibility. Also, NRG1 isoforms detected should be reported and discussed due to the complexity of the gene.
Reply 2: Thank you for your meaningful advice. The antibody used in our study was specific to HRG-α isoform of NRG1. We have discussed NRG1 isoforms in our

Discussion section (see Page 8, line 14 to 16).
Changes in the text: see Page 8, line 14 to 16.

Comment 3: Since collection of sera is of pivotal importance, authors should either cite the standard procedures to which they refer to or fully explain the procedures.
Reply 3: Thank you for your meaningful advice. We have added the procedures of collecting sera in the Method section (see Page 4, line 4 to 6).
Changes in the text: See Page 4, line 4 to 6.
Comment 4: As per the antibody clone, please report the manufacturer of the ELISA kit.
Reply 4: Thank you for your meaningful advice. We have introduced the ELISA kit in our Method section that the ELISA kits were purchased from Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences (see Page 6, line 7 to 8).
Changes in the text: None.

Comment 5: The description of statistical analysis is confused. The authors stated "Spearman's rho test or chi-square test was used to analyze non-parametric variants while the Student's test (two-tailed) was performed to compare two groups of parametric variants." However, Spearman' and chi-square test are not the non-parametric counterpart of Student's test. Maybe the authors refer to categorical and continuous variables. Please clarify this issue. Moreover, for continuous variables, before using the Student's test, the normality distribution of data should be assessed, especially for small sample size.
Reply 5: Thank you for your meaningful advice. We have modified the description of statistical analysis (see Page 7, line 1 to 5). We assessed the normality distribution of data before using Student's test (two-tailed) to compare two groups of continuous variables. Chi-square and Fisher's exact tests were used for categorical variables.
Changes in the text: See Page 7, line 1 to 5.

Comment 6: The authors ascribe a diagnostic value to glycosylation pattern of NRG1 as a surrogate of BRAF mutations. However, there are no specific analyses (i.e. expression cutoff, sensitivity, specificity, concordance); consequently, they should either perform them or simply describe the findings and their implications. In this regard, they should mention why it is important to detect BRAF mutation and the eventual clinical implications since BRAF wild-type PTC is still cancer.
Reply 6: Thank you for your meaningful advice. We have added description of the significance of our findings and empathized the importance of BRAF detection in the Discussion section (see Page 8, line 29 to Page 9, line 4).
Changes in the text: See Page 8, line 29 to Page 9, line 4.

Comment 7: Herein NRG1 glycosylation patterns are suggested as "supplementary biomarkers". Since the detection of BRAF mutation can be performed with different and sensitive assays (even rather cheap ones), what could this potential biomarker add? Does the glycosylation pattern correlate with aggressive features or clinical behaviour?

Reply 7: Thank you very much for your helpful advice. BRAF mutation is indeed helpful and sensitive for quick preoperative diagnosis of PTC from fine needle aspiration. Our research showed that the NRG1 glycosylation patterns in serum of PTC patients had different levels between BRAF mutation and BRAF wild type. Therefore, we may get BRAF information by blood sample in the future, which perhaps do less harm to patients and increase its sensitivity.
Changes in the text: None.

Comment 8: There are several conventional and unconventional abbreviations. Maybe, the authors should provide a list of abbreviations or fully report the unconventional ones on first mention to allow an easier understanding.
Reply 8: Thank you for your meaningful advice. We have provided full expressions of the unconventional ones on first mention in our revised manuscript, such as NRG1, PTC, AAL ELISA.
Changes in the text: See Page 2, line 2,5,8.

Comment 9: In the conclusion, the findings are described as a "better understanding of the function of NRG1 glycosylation". Herein, differences in the expression pattern are reported, and there are no mechanistic insights, thus the statement should be modified accordingly.
Reply 9: Thank you for your meaningful suggestion. In the Abstract section, we have modified our conclusion into "This study sheds a new light on the role of NRG1 glycosylation in PTC." (see Page 2, line 13 to 14). In the Discussion section, we have modified our conclusion into "…providing us a potential role of NRG1 glycosylation in PTC." (see Page 9, line 26).
Changes in the text: See Page 2, line 13 to 14 and Page 9, line 26.

Comment 10: Although there are not gross English mistakes, a careful language revision is suggested.
Reply 10: Thank you very much for your advice. We have now carefully revised our language of our manuscript.
Changes in the text: None.

Comment 11: In addition to point #10, the manuscript should be carefully revised for incongruences (e.g. cases are sometimes 60, then 54; BRAF + and -, then PTC, non-PTC, or even "thyroid nodular" in figure 2) and formatting as well.
Reply 11: Thank you very much for your advice. We have corrected the description in Figure 2 and the number of cases (see Page 6, line 23)
Changes in the text: See Page 6, line 25 and Figure 2.