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GS-2019-CATP-09(GS-20-388)
Title: An affordable immunohistochemical approach to estimate the prevalence of BRAFV600E in large cohort studies – Establishing the baseline rate of BRAF mutation in an institutional series of papillary thyroid carcinoma from Thailand

Reviewer #1
This manuscript focused on the BRAF mutation rate in Thailand was interesting and well written. It demonstrated that BRAFV600E mutation was detected in 60.9% of Thai PTC using immunohistochemical staining and sanger sequencing, and it was associated with several aggressive clinicopathological variables of thyroid cancer. However, there are still several points to be modified in your study:
Response: *We sincerely thank the reviewer for evaluating our manuscript and giving constructive criticism and valuable comments.*

Comment 1: As '0' is not acceptable to be included in the Chi-square test, those '0' in Table 1 should be deleted and combined with the other item for accurate data analysis.
Response: *Thank you for pointing out this error. We have combined the variables with value '0' with other items in Table 1, where possible. Otherwise we commented "n/a" where the test was not applicable.*

Comment 2: Cut margin correlates with the clinical behavior of thyroid carcinoma, but does not correlate with the molecular feature of the tumor. It will lead to misunderstanding and should be omitted from the data analysis and the result.
Response: *In fact, as shown in Table 1, the margin status correlated with VE1 status (69.8% margin+ in VE1/BRAF+ vs. 30.2% margin+ in VE1/BRAF-, p = 0.022), which remained significant on bivariate analysis (Table 2). Indeed, it turned to be nonsignificant on multivariate analysis, however we believe that correlation between positive margin and VE1/BRAF+ is well in line with our claim that BRAFV600E mutation was associated with several aggressive clinicopathological variables of thyroid cancer.*

Comment 3: Thyroid cancer with *BRAF*^{V600E} mutation has been reported to have significant association with the prognosis of the tumor, however, follow-up data of recurrence and death were not included in the current study. It will be perfect to add follow-up data in this manuscript.
Response: *We acknowledge the reviewer for this suggestion but our main objective was to estimate the prevalence of BRAF*^{V600E} *mutation in Thai PTC by using a novel affordable approach (VE1 IHC). We did not aim to study the prognostic significance of BRAF*^{V600E} *mutation in Thai PTC so did not collect these data in our study since the beginning. However, we appreciate the reviewer's comment and will keep this suggestion for another project.*

Reviewer #2
The authors have investigated BRAF VE1 IHC positivity in large scale cohort, using TMA. The IHC based evaluation, particularly in countries with limited-resource settings can be a useful tool. And by collecting the baseline data of Thai PTCs, the authors can contribute to establish the clinical

significance of VE1 IHC not only in diagnosing but also managing patients with PTC. The manuscript is well-written and the results are concise. There are some minor issues to be solved which would improve the manuscript.
Response: *We greatly appreciate a favorable evaluation of our manuscript by the reviewer.*

Comment 1: On page 8, under Analytical performance of VE1 in the pilot cohort section, the authors stated "Of 100 PTC cases employed for pilot study, 69/100 (69%) were positive for VE1 expression." Does the positivity for VE1 mean H score greater than 0?
Response: *Correct. To explain that, we added a sentence on p. 7, under "VE1 immunohistochemistry".*

Comment 2: What was the subtype of 5 discordant cases? Was there any trend among the subtypes with false positivity or negativity?
***Reply**: All of the 5 discordant cases were of classic variant of PTC. We added a sentence on p. 9 under "Analytical performance of VE1 in the pilot cohort".*

Comment 3: On page 9-10, the authors stated "Most of PTCs were of conventional variant 369/476 (77.5%), followed by follicular variant 59/476 (12.4%), and tall cell variant 14 /476 (2.9%). Can you give further information about subtypes of follicular variant? Was there any positive encapsulated follicular variant PTC case?
Response: *We added a sentence regarding subtypes of follicular variant on p. 10 under "Clinical and pathological characteristics" and also added the subtypes in Table 1. This study did not include noninvasive encapsulated follicular variant PTC because a current WHO classification considers such neoplasms as non-PTC but rather a separate entity, NIFTP. An institutional prevalence of NIFTP has been previously described in PMID 28486057.*

Comment 4: On page 10, under Correlation of BRAF mutation with clinicopathological variables section, "On univariate analysis, $BRAF^{V600E}$ was significantly associated with margin positivity (P = 0.022), extrathyroidal extension (P <0.0001), classic variant (P <0.001), and Hashimoto's thyroiditis (P = 0.009).". should be corrected to "On univariate analysis, $BRAFV^{600E}$ was significantly associated with margin positivity (P = 0.022), extrathyroidal extension (P <0.0001), classic variant (P <0.001), and absence of Hashimoto's thyroiditis (P = 0.009).".
Response: *Thank you for pointing out this issue. We have corrected the sentence as advised.*

Comment 5: On page 13, in the sentence "This is relatively lower than $BRAF^{V600E}$ prevalence in PTC reported by the close neighbors like Vietnam (83%) and the Philippines;", the percentage of BRAF positivity in Philippines should be inserted.
Response: *We have added the percentage of BRAF positivity in Philippines.*

Reviewer #3
This study examined immunohistochemical identification of $BRAF^{V600E}$ mutation with a monoclonal antibody VE1 in formalin-fixed paraffin-embedded papillary thyroid carcinomas. The VE1 immunohistochemistry and direct sequencing results for detecting $BRAF^{V600E}$ mutation in PTC tissue showed almost perfect agreement (κ=0.884) with an overall percentage agreement of 95.0 %. This study is the first report from Thailand, which demonstrated 60.9% of 476 Thai PTC cohort was positive for $BRAF^{V600E}$ mutation immunohistochemically.
Response: *We thank the reviewer for giving constructive criticism and needful advice.*

Major comments:

Comment 1: As the immunohistochemical evaluation using H score ranging from 0 to 300, please describe a cut off value dividing positive/negative for *BRAF^{V600E}* mutation in the Materials and Methods section on page 7.

Response: *In this study, any positive H-score was considered as indicative of mutation on immunostaining. We added a sentence on p. 7, under "VE1 immunohistochemistry". From our previous study with the similar approach (PMID 32150939: reference #16), where H-score of ≥ 10 was considered as positive for mutation, we found out that there was no significant association between cut-off point of H-score against the BRAF mutation. Any was found to be positive for BRAF mutation.*

Comment 2: Were there any statistical difference among different age groups, such as 1) <20, 2) between 20 and 45, 3) between 45 and 55, and 4) >55 years old? Although a comparison between 2 age groups (<55 vs. >55) in Table 1 and Table 2 did not show a statistical difference, RET/PTC rearrangements predominate in pediatric PTC patients, and the difference between <55 and >55 was marginal significance (0.058) in Table 1.

Response: *We have divided patients into four age groups as suggested by the reviewer; however, the data analysis showed no significant differences or correlation with age groups (p = 0.098). To keep Table 1 easy to read, we decided not to show these findings and left only cutoff 55 yo, as per AJCC 8e.*

Minor comments:

Comment 3: A description in the abstract on page 2 and conclusion on page 14, "A combination of mutation-specific IHC and TMA allows conducting large cohort studies in limited-resource settings," better be modified to "A combination of mutation-specific IHC and TMA allows conducting large cohort studies more labor-saving and cost-efficiently."

Response: *Thank you for the suggestion. We have modified the sentence.*

Comment 4: In Table 1 on page 21, age at diagnosis (yr) mean + SD should be in one line, and <55 and >55 must be adjusted to the same level of the other columns.

Response: *Thank you for pointing this out. We have fixed the issue as advised.*