Peer Review File

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Reviewer A

Proficient in English. The article is without a scoop.

Reply: We thank the reviewer for the overall comment.

1) The cohort is incorrectly described in the abstract. eg. retrospectively analyzed 35 Chinese CRC and 125 Western CRC, but the results show 35 (10.32%) patients and 41 on page10 line 173.

Reply: We apologize for this mistake. We have now corrected and thoroughly checked the analysis and statistics related to *BRAF* mutated in the Chinese cohort, including **Page 11 lines 199-200** ("In Chinese cohort, 35 patients carried 39 *BRAF*-mutated. 43.59% (17/39), 12.82% (5/39), 23.08% (9/39) and 20.51%% (8/39) patients had class 1, 2, 3 and NA (unknow) *BRAF*-mutated, respectively."), **lines 204-205** ("Of the patients with class 3 *BRAF*-mutated, 6 missense were found, including p.D594G (3), p.N581S (2), p.G466V (1), p.N581Y (1), p.F595L (1), p.D594N (1)."), **Page 12 line 215** (deletion the description of "p.G466V" due to reduced mutation frequency.) and Supplemental table 2 (Correct the number of Class 3). we make sure no such errors in the revised manuscript.

2) The inclusion criteria of the Chinese may be biased as most patients are stage 4. What about the western cohort

Reply: We thank the reviewer for this critical comment. we added the description of the Western cohort TNM stage in **Page 10 lines 185-186**, the result was consistent with the inclusion criteria of the Chinese cohort (Most patients were diagnosed at stage IV disease (72/125, 57.60%). We thank the reviewer for this advice again.

3) Underwent all patients get the same treatment?

Reply: We thank the reviewer for this constructive advice. In this study, we focused on clinical features, mutational characteristics, and overall survival (OS) from database. It is not possible to obtain neat treatment data. Treatment-related was the limitation of this study. Therefore, this study only conducted prognostic correlation analysis for *BRAF*-mutated types. We added the description of above as shown in **Page 17 lines 326-328** (The limit of this study is that it is retrospective, it is not possible to obtain neat treatment and PFS/OS data. Therefore, this study only conducted prognostic correlation analysis for *BRAF*-mutated types. Furtherly, prospective clinical trials are needed to explore therapeutic benefits.).

4) The MSI status is not defined, what is the applied cout-off for MSI and MSS. The cut-off value of TMB is not described.

Reply: We thank the reviewer for this constructive suggestion. We have now added

the tumor mutation burden and microsatellite instability status analysis in **Pages 8-9 lines 144-154** to make this clear in the revised manuscript, which now reads as the following.

The tumor mutation burden (TMB) in the Chinese cohort was defined as the number of non-silent somatic mutations (non-synonymous SNV, indel, and splice ± 2) per mega-base (1 Mb) of coding genomic regions sequenced (1.03 Mb for this 1021 panel) [29]. Western cohort from MSKCC was included frameshift additional. In the present study, the upper quartile of TMB was deemed as TMB-high (TMB-H) [22, 23]. The threshold values of the Chinese cohort and Western cohort were 9 muts/Mb and 11.74 muts/Mb, respectively. The microsatellite instability status of NGS data in Chinese cohort were inferred using MSIsensor (v0.2), which reported the percentage of unstable somatic microsatellites through Chi-square test on predefined microsatellite regions covered by 1021- panel. Default parameters were used [30]. The Western cohort of MSI status was also calculated by MSI sensor [22, 31], the data was download as described above.

5) Needs to be addressed

Reply: We thank the reviewer for this constructive advice and we agree with the efforts to address potential sources of bias should be mention. In the revised manuscript, we added this content in the method section as shown as **Page 7 lines 118-120** and **Page 8 lines 140-143**, which now reads as.

(Page 7 lines 118-120)

MSKCC data sets focus on metastasis CRC were selected, on the one hand, to match the Chinese cohort TNM staging, and on the other hand, batch data sets could eliminate the bias of artificial selection of data, thus making the analysis results more credible.

(Page 8 lines 141-143)

The 1021-panel has corrected coverage data for GC content and sequencing bias resulting from probe design, which can eliminate bias in mutation analysis.

6) The molecular parts needs to be described

Reply: We thank the reviewer for this critical comment. We agree that molecular parts are necessary. However, we feel confused about this question. We try to explain it as follows. We have added two modifications based on the checklist. First, We have added biomarkers description in **Pages 5-6 lines 79-85** that can provide reference for this study in the revised manuscript, which now reads as the following.

Potential prognostic markers in this field have been discovered in recent years. High BRAF allele fraction (≥ 2%, allele fraction) showed worse PFS/OS than low-BRAF AF patients (< 2%), suggesting that AF is an independent prognostic factor[17]. RNF43-mutated could predict response to anti-BRAF/EGFR combinatory therapies in BRAF V600E mCRC[18]. Whole transcriptome sequencing (WTS) suggests that a subset of patients with specific molecular features may derive greater clinical benefit from triplet than doublet therapy[19]. This biomarker can help tailor patients' treatments.

Second, We have added a molecular parts description of the analytical grouping as shown as **Page 11 line 193-198**, which now reads as the following.

We first analyzed the types and distribution of BRAF-mutated, as well as the differences in the incidence of BRAF-mutated in Chinese and Western cohort, pathway enrichment analysis was also performed. Then, we analyzed the mutation spectrum and con-mutation differences between group Class 1 BRAF-mutated and group other types of BRAF-mutated (based on classification system) [20], and the KEGG pathway enrichment was also performed. We also compared BRAF-mutated and BRAF wild-type populations as described in the analysis above.

7) BRAF mutated har a lower concomitant mutation frequency, data presented for BRAF non mutated is not shown.

The data are not well presented when it comes to BRAF co-mutated with KRAS. Must clarify which BRAF class is included and also KRAS mutations.

Reply: We thank the reviewer for this insightful advice. We firstly added a comparison of *BRAF* wild type and *BRAF*-mutated co-mutation descriptions in a Chinese cohort. The results are as expected, *BRAF* wild-type is associated with higher frequencies of concomitant mutation, mainly manifested in the tumor suppressor genes *TP53* (77% vs. 66%) and *APC* (67% vs. 43%), as well as tumor driver genes *KRAS* (49% vs. 29%) in the Chinese cohort. However, there was no statistically significant difference (see **Page 13-14, line 252-258**).

The top 10 concomitant mutation of BRAF-mutated in Chinese population were in TP53 (66%), APC (43%), LRP1B (31%), KRAS (29%), FBXW7 (26%), NOTCH1 (26%), PIK3CA (26%), FAT2 (20%), MLL2 (20%), MLL3 (20%). In BRAF wild-type (Supplemental table 6), TP53 (77%), APC (67%), KRAS (49%), PIK3CA (18%), SMAD4 (18%), TCF7L2 (18%), FBXW7 (17%), MYC (17%), LRP1B (16%), PTEN (11%). We can find that BRAF wild-type is associated with higher frequencies of concomitant mutation, mainly manifested in the tumor suppressor genes TP53 and APC, as well as tumor driver genes KRAS in the Chinese cohort.

A detailed mutation map can be seen in Supplement figure 1. The clinical and pathological features of 304 *BRAF wild-type* Chinese CRC patients were added in **Supplemental table 6**.

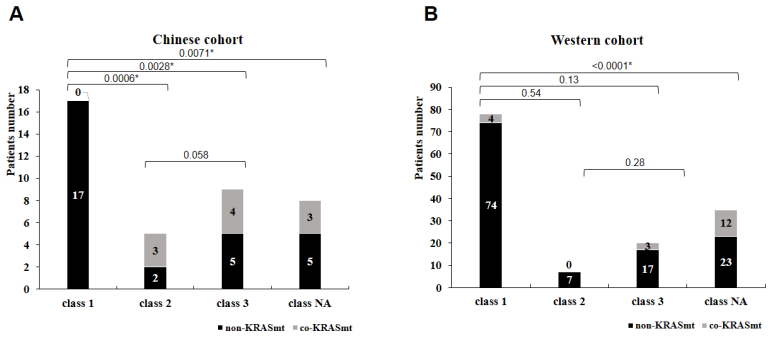
Next, as the reviewer suggested, we performed additional analysis and description of which *BRAF* class is included and also *KRAS* mutations. We found that *KRAS*-mutated were mutually exclusive with class 1 *BRAF*-mutated in the Chinese cohort, but 4 patients in the Western cohort carried both *KRAS* and class 1 *BRAF*-mutated. In addition, we found that there was no class 2 *BRAF*-mutated in the Western cohort, which may be related to the absence of fusion mutation in the MSKCC, because there were 3 Class 2 co-mutation patients in the Chinese cohort, all of which were *BRAF* fusion with co-*KRAS*mt. We added this section of analysis to **Page 14 Line 264-272**, which is described below.

None of the 35 patients with class 1 BRAF-mutated had con-KRASmt. However, 3 patients with class 2, 4 patients with class 3 and 3 patients with class NA BRAF-mutated had concurrent oncogenic KRAS mutation (Most of the sites were p.A146X,

*and no p.G12C appeared) (P < 0.001) in Chinese cohort. At the same time, we analyzed the Western cohort data, and we found 4 class1 BRAF-mutated patients co-occurred with KRAS (p.G12A, p.G13D, p.I171Nfs*14, p.G12D). The number of class 2, class 3 and class NA BRAF-mutated patients with co-KRASmt were 0, 3 and 12, respectively. In addition, we found that there was no class 2 BRAF-mutated in the Western cohort, which may be related to the absence of fusion mutation in the MSKCC, because there were 3 Class 2 co-mutation patients in the Chinese cohort, all of which were BRAF fusion with co-KRASmt. The result is shown in Figure 4.*

Finally, we updated the relevant results as the following **Figure 4.**

Figure 4. Concurrent *KRAS* mutations in different *BRAF*-mutated. Class 1 BRAF-mutated were mutually exclusive from *KRAS* in Chinese cohort and Western cohort. X-axis denotes the BRAF class. Y-axis denotes the patients number.



We thank the reviewer for the whole constructive suggestion.

Reviewer B

I have read your article regarding clinical features and mutational analysis of type 1, 2, and 3 BRAF mutations in mCRC.

Overall, the article is easy to read; however, authors should carefully review some minor but persistent grammatical issues (capital letters, some sentences do not make sense...).

Reply: We thank the reviewer for the overall favorable comment. We have thorough read the whole text and integrated the statements that do not make sense.

Specific comments:

1. Throughout the text, please italicize mutation names when appropriate.

Reply: We apologize for this mistake. We have thoroughly corrected the italicize mutation. we make sure no such errors in the revised manuscript.

2. Abstract: "BRAF-mutated CRC had a relative poor prognosis" should be changed to "BRAF mutated still have poor prognostic."
Reply: We thank the reviewer for this constructive advice. In the revised manuscript, we modified the description of "*BRAF*-mutated CRC had a relative poor prognosis" to "*BRAF*-mutated colorectal cancer (CRC) still have poor prognostic." as suggested as shown in **Page 2 line 21**.

3. At the end of the abstract, there is a mistake: OS for class 1 is not 47.57 months but 19.43; please correct this.
Reply: We apologize for this type mistake. We have now corrected this in revised manuscript (**Page 3 line 46-47**).

4. Abstract, conclusions: "prognioses" or prognostic?
Reply: We apologize for this mistake. We have now corrected this in revised manuscript (**Page 3 line 52**).

5. In the introduction, on page 5, lines 70-74, please add the following reference:
Tabernero J, Ros J, Élez E. The Evolving Treatment Landscape in BRAF-V600E-Mutated Metastatic Colorectal Cancer. Am Soc Clin Oncol Educ Book. 2022 Apr;42:1-10. doi: 10.1200/EDBK_349561. PMID: 35503983.
Reply: We thank the reviewer for this critical supplementary reference. We agree that this was inadequate. We have added the following reference as ref. 6 in **Page 5 line 70.**

6. Also on page 5, line 81, please include a brief paragraph (2-3 lines) about biomarkers, mentioning that RNF43 mutation, transcriptomic analysis, and the BRAF AF have demonstrated prognostic and predictive value and may help tailor patients' treatments:
- Elez E, Ros J, Fernández J, Villacampa G, Moreno-Cárdenas AB, Arenillas C, Bernatowicz K, Comas R, Li S, Kodack DP, Fasani R, Garcia A, Gonzalo-Ruiz J, Piris-Gimenez A, Nuciforo P, Kerr G, Intini R, Montagna A, Germani MM, Randon G, Vivancos A, Smits R, Graus D, Perez-Lopez R, Cremolini C, Lonardi S, Pietrantonio F, Dienstmann R, Tabernero J, Toledo RA. RNF43 mutations predict response to anti-BRAF/EGFR combinatory therapies in BRAFV600E metastatic colorectal cancer. Nat Med. 2022 Oct;28(10):2162-2170. doi: 10.1038/s41591-022-01976-z. Epub 2022 Sep 12. PMID: 36097219; PMCID: PMC9556333.
- Ros J, Matito J, Villacampa G, Comas R, Garcia A, Martini G, Baraibar I, Saoudi N, Salvà F, Martin Á, Antista M, Toledo R, Martinelli E, Pietrantonio F, Boccaccino A, Cremolini C, Dientsmann R, Tabernero J, Vivancos A, Elez E. Plasmatic BRAF-V600E allele fraction as a prognostic factor in metastatic colorectal cancer treated with BRAF combinatorial treatments. Ann Oncol. 2023 Jun;34(6):543-552. doi: 10.1016/j.annonc.2023.02.016. Epub 2023 Mar 14. PMID: 36921693.
- Kopetz S, Murphy DA, Pu J, et al. Molecular correlates of clinical benefit in previously treated patients (pts) with BRAF V600E-mutant metastatic colorectal

cancer (mCRC) from the BEACON study. J Clin Oncol. 2021 May 20;39(15_suppl):3513–3513

Reply: We thank the reviewer for this critical comment. We agree that such statements are necessary. We have added biomarkers description in **Page 5-6 line 79-85** that are important for precision therapy of mCRC in the revised manuscript, which now reads as the following.

Potential prognostic markers in this field have been discovered in recent years. High BRAF allele fraction (≥ 2%, allele fraction) showed worse PFS/OS than low-BRAF AF patients (< 2%), suggesting that AF is an independent prognostic factor[17]. RNF43-mutated could predict response to anti-BRAF/EGFR combinatory therapies in BRAF V600E mCRC[18]. Whole transcriptome sequencing (WTS) suggests that a subset of patients with specific molecular features may derive greater clinical benefit from triplet than doublet therapy[19]. This biomarker can help tailor patients' treatments.

7. Page 5, line 88: "Recently," this is not true as this paper was published in 2017.

Reply: We thank the reviewer for precise language guidance. We have now corrected this in revised manuscript (**Page 6 line 91**).

8. Page 9, line 155, "patients were BRAF..." patients are not BRAF; their tumors are. Please correct this.

Reply: We thank the reviewer for precise language guidance again. We have now corrected this in revised manuscript as shown as **page 10 line 173** [Thirty-five (10.32%) patients carried *BRAF* mutation, with a total of 17 patient tissues (48.57%) were $BRAF^{V600E}$].

9. In the text, it is mentioned several times the association of BRAF mutation with MSI and TMB-h. However, the high TMB is probably associated with the high rate of MSI among patients with BRAF mutated tumors. This should be mentioned.

Reply: We thank the reviewer for this constructive advice. In the revised manuscript, we have added the mentioned as suggested in **page 17 lines 330-332**, which now reads as the following.

However, TMB-H may be associated with MSI-H rates in patients with BRAF-mutated tumors, which is consistent with published literature[36] showing that a majority of MSI-H samples are also high TMB (83%), and 97% had TMB ≥10 mutations/Mb.

10. Page 11, line 198: the authors have included a variable name literally from the database "MSI_STATUS"; please correct this.

Reply: We thank the reviewer for these critical comments. As the reviewer suggested, we updated the description of "MSI_status" and "MSI" in the whole text as the following **line 32, page 12 line 221, Table 1, Table 2, and Table 3.**

11. Nice work with the co-mutation analysis.

Reply: We thank the reviewer for this favorable comment.

12. In the discussion, I would appreciate some thoughts about biomarkers, particularly now that several papers (that must be included) have demonstrated the predictive value of RNF43 mutations and the transcriptomic profiling as well as the prognostic value of BRAF allele fraction in plasma. 2-3 lines will be enough but really appreciated:

- Elez E, Ros J, Fernández J, Villacampa G, Moreno-Cárdenas AB, Arenillas C, Bernatowicz K, Comas R, Li S, Kodack DP, Fasani R, Garcia A, Gonzalo-Ruiz J, Piris-Gimenez A, Nuciforo P, Kerr G, Intini R, Montagna A, Germani MM, Randon G, Vivancos A, Smits R, Graus D, Perez-Lopez R, Cremolini C, Lonardi S, Pietrantonio F, Dienstmann R, Tabernero J, Toledo RA. RNF43 mutations predict response to anti-BRAF/EGFR combinatory therapies in BRAFV600E metastatic colorectal cancer. Nat Med. 2022 Oct;28(10):2162-2170. doi: 10.1038/s41591-022-01976-z. Epub 2022 Sep 12. PMID: 36097219; PMCID: PMC9556333.
- Ros J, Matito J, Villacampa G, Comas R, Garcia A, Martini G, Baraibar I, Saoudi N, Salvà F, Martin Á, Antista M, Toledo R, Martinelli E, Pietrantonio F, Boccaccino A, Cremolini C, Dientsmann R, Tabernero J, Vivancos A, Elez E. Plasmatic BRAF-V600E allele fraction as a prognostic factor in metastatic colorectal cancer treated with BRAF combinatorial treatments. Ann Oncol. 2023 Jun;34(6):543-552. doi: 10.1016/j.annonc.2023.02.016. Epub 2023 Mar 14. PMID: 36921693.
- Kopetz S, Murphy DA, Pu J, et al. Molecular correlates of clinical benefit in previously treated patients (pts) with BRAF V600E-mutant metastatic colorectal cancer (mCRC) from the BEACON study. J Clin Oncol. 2021 May 20;39(15_suppl):3513–3513

Reply: We thank the reviewer for these critical comments and constructive advices. As the reviewer suggested, we have added biomarkers description in **page 16 lines 317-320** that are important for precision therapy of mCRC in the revised manuscript, which now reads as the following.

Several studies have shown that potential biomarkers including high BRAF allele scores (≥2%)[17], RNF43-mutated[18], Consensus Molecular Subtypes (CMS)[19], and POLD1/POLE-mutated[32] might bring clinical benefits from different treatment modalities.

13. Table 1: the results of BRAF mut-type should be moved to the "BRAF mutation" column instead of in the "total" column.

Reply: We apologize for this mistake. In the revised manuscript, we have moved it to correct column **as shown as Table 1.**

14. Throughout the text, authors should define the high TMB cut-off: perhaps 10 Mut/Mb as per FDA?

Reply: We thank the reviewer for this critical comment and we agree with the reviewer's advice. At present, it is a generally accepted method to divide the tumor mutational burden (TMB) threshold using the upper quartile. This study is divided into Chinese cohort and Western cohort. The Chinese population TMB was calculated using the 1021-panel method, which was also divided using the upper

quartile, and the result was 9 Muts/Mb. The TMB threshold of the Western population cohort was 11.74 Muts/Mb. It was divided into the upper quartile of the entire mCRC cohort, which was higher than that of the Chinese population, possibly because the MSKCC TMB calculation added additional frameshift mut-type. The above is described in the revised version, **pages 8-9, lines 145-150**, which now reads as the following.

The tumor mutation burden (TMB) in the Chinese cohort was defined as the number of non-silent somatic mutations (non-synonymous SNV, indel, and splice ± 2) per mega-base (1 Mb) of coding genomic regions sequenced (1.03 Mb for this 1021 panel) [29]. Western cohort from MSKCC was included frameshift additional. In the present study, the upper quartile of TMB was deemed as TMB-high (TMB-H) [22, 23]. The threshold values of the Chinese cohort and Western cohort were 9 muts/Mb and 11.74 muts/Mb, respectively.

Thank you for your attention to these details.

Reply: We thank the reviewer for this favorable comment again.