Author’s response to reviews

Title: Co-mutations of TP53 and KRAS Serve as Potential Biomarkers for Immune Checkpoint Blockade in Squamous-Cell Non-small Cell Lung Cancer: A Case Report

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Author’s response to reviews:

Dear editor,

Thank you for giving us the opportunity to improve my manuscript further. We have carefully read the comments of reviewers and made the revisions according to every reviewer’s comments. The revised portions were highlighted in red in the text.

To reviewer #1:

1. Why it was stated that "immunohistochemistry was used to analyze PD-L1 related genes"? Immunohistochemistry detects PD-L1 expression at protein level, not at gene level. The abstract mentioned that this study used NGS to evaluate PD-L1 expression, which should be the gene level PD-L1 expression. I suggest authors do more literature review about bioinformatics profiling platforms, improve accuracy of terms and add references.

Response: We apologize for the mistake, which causes you cannot understand the whole manuscript. We have corrected this mistake and highlighted in red color in the revised manuscript.
2. "a gene panel involving 416 genes associated with cancer, was utilized to analyze the postoperative tumor samples from patients in January 2018". Why was this mentioned? How was it linked to the reported case?

Response: Previous immunohistochemical techniques for the detection of classical immunotherapeutic markers were negative, so the use of large panel detection to see TMB and other potential targets. Next-generation sequencing (NGS) with a gene panel covering 416 cancer-related genes was performed on the postoperative tumor tissue. Libraries were prepared with Hyper Prep Kit (Kapa) and then sequenced on Hiseq 4000 NGS platforms (Illumina). All of the standard drive genes such as EGFR, KRAS, ALK, ROS1, RET, MET, ERBB2 were covered in this panel. None of the other common driving mutations found except TP53 and KRAS mutation.

3. It was mentioned in Discussion and conclusion that 'the rationale for combined immunotherapy with chemotherapy depends on the hypothesis that cytotoxic chemotherapy will indiscriminately kill normal and cancer cells, while immunotherapy can "rev up" the immune system against cancer cells". This statement does not explain why the reported case may benefit more from this combined treatment compared to chemotherapy only. What are the potential results for not combining immunotherapy after chemotherapy?

Response: Thanks for the critical comment. Chemotherapy kills tumor cells, which in turn exposes new antigens of tumor cells, thereby activating the immune system. Chemotherapy favor the maturation of antigen-presenting cells and augmented tumor antigen presentation through the MHC class I, increased PD-L1 expression on tumor cells, downregulation of immunosuppressive cells at the tumor site (such as CD4+CD25+FOXP3+Tregs or MDSCs), and the increase of CD8+ T-cells and macrophages. All of these effects augment the efficacy of immune checkpoint inhibitors on the reduction of tumor size. In third-line treatment, chemotherapy yielded modest efficacy results. Hence, both polychemotherapy and single-agent regimens are limited by a high incidence of side effects. Median survival was only 4.0 months in case of progression after first- and/or second-line treatment ( J Thorac Oncol 2009, 4(12):1544-1549). The overall response rates (RR) reported in third-line treatment were approximately 17–18%( Future Oncol 2014, 10(13):2081-2096). Pembrolizumab combined with chemotherapy has become the standard first-line treatment for patients with advanced non-small cell lung cancer (PD-L1>1%) based on KEYNOTE-407 and KEYNOTE-189 clinical trials. At that time, considering the rapid progress of patients and tumor burden, we chose immunotherapy combined with chemotherapy. As for the results of not combining immunotherapy after chemotherapy, we believe that the effect of chemotherapy alone is worse than that of combining immunotherapy with chemotherapy.
4. This manuscript did not explain why co-mutations of TP53/KRAS was more important than other markers (i.e. low PD-L1 expression, MSI and TMB-low) in validating PD-1 blockade immunotherapy for the reported case. I suggest to review and reference more details in literature to support the rationale. For example, Dong et al (2016) reported that co-mutations of TP53/KRAS was associated with strong/high PD-L1 and prolonged PFS. Summarizing the cases having co-mutations of TP53/KRAS but weak PD-L1 expression(from discovery and validation sets in Dong et al can validate PD-1 blockade immunotherapy for the reported case in this manuscript. There are other papers reporting the impact of TP53/KRAS on immune response in squamous-cell NSCLC too.

Response: Thank you very much for your kind suggestion. Studies have reported the interplay between tumor mutations in the lung adenocarcinoma with the immune microenvironment. KRAS/TP53 co-mutated lung adenocarcinoma patient have high PD-L1 expression, high TMB, and thus the response to anti-PD1 treatment. It seems neither one is the answer since our patient (squamous-cell NSCLC) has weak PD-L1 expression in the tumor and low TMB of 3.2 mutations/Mb. It may be the first case of TMB-L/PD-L1 low expression and TP53 and KRAS mutant lung squamous cell NSCLC patients have a long-term benefit of immunotherapy. This makes this case study even more interesting in terms of the underlining mechanism. So we say that co-mutations of TP53 and KRAS was more critical than other markers (i.e. low PD-L1 expression, MSI and TMB-low) in validating PD-1 blockade immunotherapy for the reported case.

5. The conclusion that "TP53/KRAS co-mutations in squamous NSCLC can serve as a predicting factor" is too strong. TP53/KRAS co-mutations can be a potential factor to assess possible response to PD-1 blockade immunotherapy, but further studies with more cases are needed to confirm the prediction power.

Response: Thanks for the critical comment. We have corrected this sentence and highlighted it in red color in the revised manuscript.

6. I suggest the authors use English writing and editing resources to improve the language. There are also copyedit errors in this manuscript, e.g. "P53" should be "TP53" in abstract conclusion, terms are not consistent "squamous NSCLC, squamous-cell NSCLC", "co-mutations of TP53/KRAS, TP53 and KRAS".

Response: We apologize for the mistake. We have corrected this mistake and highlighted in red color in the revised manuscript. We have tried our best to correct some grammatical and stylistic errors with the help of a native English speaker. All changes have been marked in red.
To reviewer #2:

1. The authors claimed this patient has weakly stained PD-L1 (line 31), and MMR-related genes were strongly stained by IHC. This is critical data for the study and should be shown in the figures.

Response: Thank you very much for your kind suggestion. The pathological examination of postoperative tumor tissue revealed of weakly stained PD-L1 (2%) and strongly stained MMR-related proteins by immunohistochemistry. The figures have been shown in the revised manuscript.

2. For the TMB analysis, more detailed description and criteria of how the TMB number was culled. The method and standard for the TMB analysis is critical for interpretation of the result.

Response: Details of the TMB analysis were provided as follows and also briefly described in the revised manuscript and highlighted in red color.

1) DNA Extraction and Library Construction. For WES, genomic DNAs from FFPE sections were extracted with QIAamp DNA FFPE Tissue Kit (Qiagen), and quantified by Qubit 3.0 using the dsDNA HS Assay Kit (ThermoFisher Scientific). Library preparations were performed with KAPA Hyper Prep Kit (KAPA Biosystems). Target enrichment was performed using the xGen Exome Research Panel and Hybridization and Wash Reagents Kit (Integrated DNA Technology) according to the manufacturer's protocol.

2) Next Generation Sequencing of DNA. Sequencing was performed on Illumina HiSeq4000 platform using PE150 sequencing chemistry (Illumina). For targeted-panel, customized xGen lockdown probes (Integrated DNA Technologies) targeting 416 cancer-relevant genes were used for hybridization enrichment. The capture reaction was performed with Dynabeads M-270 (Life Technologies) and xGen Lockdown hybridization and wash kit (Integrated DNA Technologies) according to the manufacturers' protocols. Captured libraries were on-beads PCR amplified with Illumina p5 (50 AAT GATACG GCG ACC ACC GA 30) and p7 primers (50 CAA GCA GAAGAC GGC ATA CGA GAT 30) in KAPA HiFi HotStart ReadyMix (KAPA Biosystems), followed by purification using Agencourt AMPure XP beads. Libraries were quantified by qPCR using KAPA Library Quantification kit (KAPA Biosystems). Library fragment size was determined by Bioanalyzer 2100 (Agilent Technologies). The target-enriched library was then sequenced on HiSeq4000 or HiSeq4000 NGS platforms (Illumina) according to the manufacturer's instructions. The average coverage depth was 140X and 1341X for tumors using
WES and Panel, respectively. The average coverage size of WES and Panel for TMB estimation was 32 Mb and 1.4 Mb, respectively.

3) Sequence Alignment and Processing. Base calling was performed using bcl2fastq v2.16.0.10 (Illumina, Inc.) to generate sequence reads in FASTQ format (Illumina 1.8+ encoding). Quality control (QC) was applied with Trimmomatic[1]. High quality reads were mapped to the human genome (hg19, GRCh37 Genome Reference Consortium Human Reference 37) using modified BWA aligner 0.7.12[2] with BWA-MEM algorithm and default parameters to create SAM files. Picard 1.119 (http://picard.sourceforge.net/) was used to convert SAM files to compressed BAM files which were then sorted according to chromosome coordinates. The Genome Analysis Toolkit[3] (GATK, version 3.4-0) was modified and used to locally realign the BAMs files at intervals with indel mismatches and recalibrate base quality scores of reads in BAM files[4].

4) SNVs/Indels/CNVs Detections. Single nucleotide variants (SNVs) and short insertions/deletions (indels) were identified using VarScan2 2.3.9[5] with minimum variant allele frequency threshold set at 0.01 and p-value threshold for calling variants set at 0.05 to generate Variant Call Format (VCF) files. All SNVs/indels were annotated with ANNOVAR, and each SNV/indel was manually checked with the Integrative Genomics Viewer [6] (IGV). Copy number variations (CNVs) were identified using ADTEx 1.0.4 [7].

5) TMB analysis SNVs and INDELs called were further filtered using the following criteria: (i) minimum ≥4 variant supporting reads and ≥2% variant allele frequency (VAF) supporting the variant, (ii) filtered if present in > 1% population frequency in the 1000g or ExAC database, (iii) filtered through an internally collected list of recurrent sequencing errors (≥3 variant reads and ≤20% VAF in at least 30 out of ~2,000 normal samples) on the same sequencing platform. Final list of mutations were annotated using vcf2maf (call VEP for annotation). Tumor mutation burden (TMB) was defined as the total number of missense mutations. In addition, we profiled TMB of these samples by a targeted next-generation sequencing (NGS) panel (Geneseeq) to evaluate its correlation with WES results. Panel TMB was counted by summing all base substitutions and indels in the coding region of targeted genes, including synonymous alterations to reduce sampling noise and excluding known driver mutations as they are over-represented in the panel, as previously described [8]. TMB was measured in mutations per megabase (mb).


3. There are only few oncogenic driver genes including KRAS, TP53, EGFR, STK11 were mentioned for the NGS sequencing of patient genome. What are the other genes included in this 416 genes panel? Any DNA repair related genes that related to tumor mutation burden included?

Response: Thanks for the critical comment. The gene panel was shown in supplement material of Table 1. All of the common drive genes such as EGFR, KRAS, ALK, ROS1, RET, MET, ERBB2 were covered in this panel. DNA repair-related genes such as HRR pathway (PTEN, BRCA1/2, RAD50, RAD51, etc.) and MMR (MLH1, MLH3, MSH2, MSH6, and PSM2) were included in this panel.

4. The authors need to clarify what is the similarity and differences of this patient sample comparing with previous reported cases that also have KRAS/TP53 mutation. More importantly, what could be the potential explanation that this patient responded to immune checkpoint blockade.

Response: This particular case shown the consistent result with what observed in the Dong et al. (Clin Cancer Res 2017, 23(12):3012-3024.) studies, based on the premise that KRAS/TP53 co-mutated lung adenocarcinoma patient tumors have high PD-L1 expression, high TMB and thus the response to anti-PD1 treatment. Of the 34 patients with NSCLC who were included in the Dong et al. only three patients were squamous cell-NSCLC, included one case of TMB-H/PD-L1 NA, TP53 mutation, and KRAS wild type; One case of TMB-L/PD-L1 N, TP53 mutation, KRAS wild type; One case of TMB-L/PD-L1 N, TP53 wild type, KRAS wild type; Both of them were different from this patient. It may be the first case of TMB-L/PD-L1 low expression and TP53/KRAS mutant lung squamous cell NSCLC patients have a long-term benefit of immunotherapy. In this squamous-cell NSCLC patient, the patient was resistant to paclitaxel, so he was treated with pembrolizumab combined with gemcitabine, which achieved significant tumor shrinkage. Chemotherapy kills tumor cells, which in turn exposes new antigens of tumor cells, thereby activating the immune system. The patient with co-mutations of TP53 and KRAS had low PD-L1 expression, but the tumors shrank significantly. This patient responded to gemcitabine when combined with pembrolizumab, squamous-cell NSCLC with co-mutations of TP53 and KRAS may more likely to expose new antigens, and the underlying mechanism should be further studied.

5. This patient responded to gemcitabine when combined with pembrolizumab, but not rh-endorstain combined with docetaxel. The authors should discuss the differences between these chemotherapy drugs and clinical usage. Furthermore, whether the effect observed is simply come from anti-PD1 treatment or due to the use of different chemotherapy drug.
Response: Thanks for the critical comment. In third-line treatment, chemotherapy yielded modest efficacy results. Hence, both polychemotherapy and single-agent regimens are limited by a high incidence of side effects. Median survival was only 4.0 months in case of progression after first- and/or second-line treatment (J Thorac Oncol 2009, 4(12):1544-1549). The overall response rates (RR) reported in third-line treatment were approximately 17–18% (Future Oncol 2014, 10(13):2081-2096). The differences between these chemotherapy drugs and clinical usage have been described in the revised manuscript and highlighted in red color. It is well known that immunotherapy has a slow onset of action. The patient is treated with gemcitabine combined with pembrolizumab, and the tumor shrinks obviously only after two cycles of therapy. We think that chemotherapy may play a significant role at the beginning, and the subsequent tumors further shanked. We think immunization definitely plays an vital role in subsequent treatment.

6. In the conclusion, the authors claimed KRAS/TP53 co-mutation to be predicting factor for responses to anti-PD1 treatment. Since the authors only reported one case, there is not enough evidence to confirm KRAS/TP53 co-mutation as response marker to immune checkpoint blockade.

Response: Thanks for the critical comment. We have corrected this sentence and highlighted in red color in the revised manuscript.

Sincerely Yours,

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