Peer Review File

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Reviewer A

In their study, "Molecular characterization of squamous cell carcinoma of the anal canal (SCCA)", the authors collect 311 anal squamous cell carcinoma and reports a landscape of PD-1, PD-L1, and HER2 IHC, HER2 ISH, and NGS results. They report that TP53-mutant tumors show a distinct molecular profile from TP53-WT tumors, with higher rates of CDKN2A, EWSR1, JAK1, FGFR1 and BRAF mutations.
This is a largely confirmatory study of previously reported mutational and immunohistochemical findings in SCCA, based here on reference lab testing; this manuscript is of limited novelty. Important limitations include lack of HPV status and treatment/outcome data. However, the current study is a large series of this rare tumor type. The following are several recommendations, requests for clarification, and issues to address.

1. Was tumor staging evaluated in this study? How was local specimen site defined? Did it include local lymph nodes? In addition, are the sites of distant metastasis sites available? A detailed description of specimen sites should be included. In addition, tumor staging, which has been correlated with survival in SCCA, should be included if available.

Thank you for your comments and suggestions. In this study, clinical or pathologic staging was not evaluated. Local versus metastatic specimen site was evaluated individually. Local specimen lesions include the anus, anal canal, anorectum, overlapping lesion of rectum/anus/anal canal, rectum, anorectal junction, skin of anus, perianal skin, cloacegenic zone, vagina/labia, urethra, skin of thigh, gluteal skin, and inguinal lymph node. Distant metastatic specimen sites include liver, small bowel, omental, ovary, brain, paraspinal, lung and pleural lesions as well as neck, cervical, supraclavicular, periaortic, paratracheal, subcarinal, scalene and retroperitoneal lymph nodes. We added these details to the text in baseline patient characteristics on page 6, lines 149-155.

2. What was the defined threshold for PD-L1 and PD-1 IHC positivity? Did multiple pathologists score each slide? This threshold by CPS or TPS score should be included in the Methods section. In addition, the specific antibiodies and methods for IHC should be included. Given that PD-L1 status is a focus of this paper, a supplemental table comparing all metrics available (demographics, specimen site, genomic profiling results) between PD-L1 positive and negative cases would also be helpful.

Thank you for this insightful comment; we have added the following to the methods section:

Immunohistochemistry (IHC) was performed on full formalin-fixed paraffin-embedded (FFPE) sections of glass slides. Slides were stained using automated staining techniques, per the manufacturer's instructions, and were optimized and validated per CLIA/CAO and ISO

requirements. Staining was scored for intensity (0 = no staining; 1+ = weak staining; 2+ = moderate staining; 3+ = strong staining) and staining percentage (0-100%). Results were categorized as positive or negative by defined thresholds specific to each marker based on published clinical literature that associates biomarker status with patient responses to therapeutic agents. A board-certified pathologist evaluated all IHC results independently. The primary antibody used against PD-L1 was SP142 (Spring Biosciences). The staining was regarded as positive if its intensity on the membrane of the tumor cells was >=2+ and the percentage of positively stained cells was >5%. The primary antibody used for PD-1 was MRQ-22 (Ventana) and staining was scored as positive if the number of PD-1+ TIL was>1 cell per high-power field. PD-1 TIL density was evaluated using a hotspot approach. The whole tumor sample was reviewed at a low power (4x objective), and the areas of highest density of TIL in direct contact with malignant cells of the tumor at 400x visual field (40x objective × 10x ocular) were enumerated.

3. Was histopathology reviewed in this study? Are there any differences in histopathologic findings seen in TP53-MT vs. wildtype tumors? If available, this study would benefit from a comprehensive comparison of the histopathologic features to evaluate for any genotype-phenotype correlations. The current WHO describes the histopathology of anal squamous cell carcinoma as keratinizing, basaloid, or occasionally cylindroma-like; evaluation based on these descriptors would be helpful. If the information on tumor histology is not available, it should be explicitly stated in the limitations.

Unfortunately, histopathology was unable to be reviewed for this study and we have added this to our limitations section on page 14, lines 349-351.

4. Was there any bias in this study towards cases that progressed? Any bias should be included in the formal discussion of limitations.

Given the limited clinical data provided on each deidentified patient we are unaware of any bias towards testing patients who have progressed.

5. Was germline DNA evaluated? If not, this needs to be emphasized in a formal discussion of limitations.

Unfortunately, germline DNA testing was unable to be reviewed for this study and we have added this to our limitations section on page 14, lines 349-351.

6. The clinical significance of TP53-MT vs. TP53-WT SCCA is lacking in the current study. In addition, the clinical significance of PD-1/PD-L1 expression is also lacking. This is commented on in the current limitations. Of note, the study by Zhu et al 2020 (PMID 33483624) included survival data based on TP53 mutation and PD-L1 IHC status, and should be referenced and discussed.

We have modified our text and included this article within our discussion section on page 10 lines 243-246, and page 17 lines 377-380.

7. The limitations also include that HPV status was not evaluated. This is a key limitation and should be emphasized. Is it possible for HPV status to be obtained from off target reads, at least as a surrogate marker of HPV infection? In addition, in the limitations section, multiple sentences are devoted to the discussion of the literature on p16 IHC as a prognostic marker in HPV-related cancers (lines 333-338). This literature is not a limitation of the study and should be moved to a different section.

We have removed lines 333-338 from the limitations section and elaborated this key limitation as well as the known literature regarding p16 IHC as a prognostic marker in HPV-related cancers into the discussion section. Page 9, lines 218-225.

8. The comment in the introduction that "little is known about the molecular characteristics of the difference between TP53-WT versus TP53-MT tumors" (lines 107-108) should be modified to include the multiple prior genomic studies that evaluate this difference. Chung et al 2016 (PMID 27052656) is one such genomic profiling study that noted genomic differences in anal squamous cell carcinoma based on TP53 mutation status and should be cited. In addition, Zhu et al 2020 (PMID 33483624) included genomic profiling and survival data stratified based on TP53 mutation.

We have modified this statement to include both suggested references on page 5 lines 107-110.

9. The authors state in the abstract and introduction that this is the "largest known molecularly-profiled cohort of SCCA" (line 35 and 105) and "most extensive analysis of genetic mutations in SCCA" (line 228). The cohort studied in Williams et al 2020 (PMID 32461623) included 574 molecularly-profiled SCCA. The statements should be corrected, and the citation added. In addition, the statement in the introduction that "no large comprehensive molecular profiling of SCCA has been published" (line 102) should be removed, given that several other large molecular profiling studies have been published (including PMIDs 33483624, 27052656, 28784613). Lastly, the statement that "novel mutations noted in this study include HRAS,…" (line 230) is incorrect as the authors cite a manuscript that previously reported this mutation in SCCA.

We have modified all of these statements noted above on their respective pages and lines, as well as included the Williams et al data/reference within our introduction, pages 4-5, lines 105-108.

10. For completeness, in a supplemental table, variants should be included one per row and perhaps limit the variants to the pathogenic and likely pathogenic variants. It would also be helpful if columns for VAF and total read depth were included.

While we agree with this statement, we do not have the details of each variant.

Reviewer B

I would like to congratulate the authors for developing such a comprehensive and useful data in anal cancer. The article reads well, it is quite interesting and I have just a few comments/suggestions:

-- curious to know what was the TMB of the few MSI tumors? interesting to mention
Unfortunately, we do not have the TMB of the few MSI tumors.

-- there is a typo in line 247
Thank you, we have corrected the typo in line 247, which is now line 248 with the additional edits.
-- there was a few BRCAmut tumors. do you have the VAF of these mutations?
Unfortunately, we do not have the VAF of these mutations.

-- a recent study showed that deep sequencing tecnique is able to detect HPV in cervical cancers which were HPV negative by PCR. So it is assumed that cervical cancer (and anal, likewise) are all HPV+. please comment on this issue when you classify TP53 WT as a proxy of HPV+ anal cancer. this is the study https://www.nature.com/articles/s41416-020-01111-0

We have modified our text to include this reference within our introduction, pages 5, lines 104-106.