Peer Review File

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Review Comments:

In this work authors have analyzed a large cohort of testicular seminomas and assessed the immunoexpression of MMR proteins. They demonstrate that true loss of expression of MMR and MSI is a rare event in seminomas, and so not responsible for the typical lymphocytic infiltrate.
The work is well written and organized. Figures are ok, and the description of possible false negatives related to fixation issues is important for the reader to apprehend.

Comment 1: *There are two very recent (2019) studies that authors should include in their Discussion, since they very similarly assess this exact same issue ("Detailed Characterization of Immune Cell Infiltrate and Expression of Immune Checkpoint Molecules PD-L1/CTLA-4 and MMR Proteins in Testicular Germ Cell Tumors Disclose Novel Disease Biomarkers", PMID 31614500; and "Widening the spectrum of Lynch syndrome: first report of testicular seminoma attributable to MSH2 loss", PMID 31442315). Authors too disclose MMR immunoexpression patterns in germ cell tumors and describe a single case of Lynch syndrome associated seminoma with MSH2 loss and MSI-high. These should be commented on and discussed.*

Reply 2: We kindly apologize for not having addressed the two recent publications mentioned by the reviewer, which indeed provide recent and novel data on MMR deficiency in testicular germ cell tumors.

The paper by Lobo et al. published in *Cancers* thoroughly correlates MMR protein levels of testicular germ cell tumors with the immune cell infiltrate in these neoplasms. Interestingly, no correlation between the protein levels MLH1, PMS2, MSH2, or MSH6 and the infiltration of CD20-/CD3-positive lymphocytes was observed for seminomas, supporting our observation that lymphocyte influx is not driven by MMR deficiency in these tumors. We now discuss the work by Lobo et al. in the 2nd paragraph of the discussion section of the revised manuscript.

The correspondence letter by Lobo et al. published in Histopathology provides evidence that MMR deficiency in testicular germ cell tumors can be of hereditary cause (Lynch Syndrome). Over the recent years, there are vast reports about Lynch Syndrome associated Cancers beyond the "classical" CRC, endometrial and upper urological tract tumors. We now discuss the reported case (MSH2 deficient seminoma due to Lynch Syndrome) by Lobo et al. in the discussion section of the revised manuscript. Please read also our reply to the Comment #3 of the reviewer for this issue.

Changes in the text: 2nd paragraph discussion section: A recent work by Lobo et al. supports our observation that the inflammatory infiltrate in seminoma is unrelated to MMR status. By means of a thorough characterization of the immune cell infiltrate in 271 tumor samples of testicular germ cell tumors, the authors demonstrated that both CD20- and CD3-positive immune cells were not associated with the expression levels of any of the four MMR proteins MLH1, PMS2, MSH2, and MSH6 in the subset of seminomas.

Comment 2: *Please describe better how the "percentage of staining" was assessed and what was the definition of nuclear staining intensity 1+, 2+ and 3+ - how was this defined objectively?*

Reply 2: If no unequivocal nuclear staining in tumor cells was observed, nuclear staining was scored as negative (0). Clear-cut nuclear staining was scored as mild (1+), moderate (2+) or strong (3+) depending on the average staining intensity of all tumor cells with positive nuclear staining. The percentage of positive tumor cells was recorded estimating the fraction of tumor cells with positive nuclear staining (1+, 2+ or 3+) from all analyzable tumor cells on the respective TMA spot.
We have modified the second paragraph of the M&M section to describe the IHC scoring in more detail:

Changes in the text: 2nd paragraph M&M section: If no unequivocal nuclear staining in tumor cells was observed, staining was recorded as negative (0). Clear-cut nuclear staining in tumor cells was scored as mild (1+), moderate (2+) or strong (3+) depending on the average staining intensity of all tumor cells with positive nuclear staining. The percentage of positive tumor cells was recorded estimating the fraction of tumor cells with positive nuclear staining (1+, 2+ or 3+) from all analyzable tumor cells on the respective TMA spot.

Comment 3: *Related to the single seminoma with MLH1 and PMS2 loss, is the patient belonging to a Lynch syndrome family? What is the family history of this patient? Please expand on that.*

Reply 3: Due to the retrospective nature of our study, cases between 2009 and 2018 were incorporated. The mentioned single seminoma with MMR deficiency due to MLH1 and PMS2 loss was diagnosed 2013 in a 35 year old patient. No history suggestive for any hereditary tumor origin was provided. Since then no subsequent resection has been performed in this patient. As there are no definite hints towards a hereditary origin of the MMR deficient seminoma, we assume that MMR deficiency in this tumor represents a sporadic hit. However, as germline testing is not possible in our study due to legal issues in Germany, we cannot totally rule out the possibility of a hereditary cause.
Indirect evidence for a sporadic tumor further comes from the fact that the germ cell neoplasia in situ (GCNIS) adjacent to the seminoma was MMR intact. This fact contrasts observations in Lynch syndrome-associated colorectal carcinomas where it was shown that MMR deficiency seems to be one of the initiating steps of tumor development, often being present at early stages of tubular adenomas or even in morphologically bland (non-neoplastic appearing) crypts (Staffa et al 2015, Yurgelun et la. 2012). We discuss this issue more thoroughly in the revised manuscript and have added the study by Lobo et al. as suggested above (see comment #1) by the reviewer.

Changes in the text: paragraph 5 of the discussion section: Lobo et al. have recently expanded the wide spectrum of Lynch syndrome-associated neoplasms by reporting a single case of a MMR-deficient seminoma due to a MSH2 germline mutation (38). Regarding the seminoma with MLH1 and PMS2 protein loss observed in our study, we cannot reliably distinguish between a sporadic and a hereditary cause for MMR deficiency in this case as germline testing was not performed. However, indirect evidence supports a sporadic origin for this tumor. Germ cell neoplasia in situ (GCNIS) adjacent to the seminoma exhibited intact MLH1 and PMS2 protein expression, indicating that MMR loss has occurred during tumor progression. For a hereditary cause, one may expect that MMR loss rather represents an

early step of tumor development, at least based on available data derived from Lynch syndrome-associated colorectal carcinomas, where MMR deficiency is already evident in precursor lesions such as very early tubular adenomas and may even be observed in morphologically non-neoplastic crypts (39, 40). Moreover, no clinical evidence for Lynch syndrome was reported clinically in our case, further arguing against a hereditary tumor.

Comment 4: *Was there any case which had a particular poor outcome, with disease recurrence and emergence of cisplatin resistant phenotype? If so, please discuss on the putative role of MMR in the context of cisplatin resistance in germ cell tumors – also relevant literature available.*

Reply 4: Due to the study design of our work, we have focused on including as many seminomas as possible for TMA-based MMR screening. All tumors were diagnosed at the Institute of Pathology of the University Medical Center Hamburg-Eppendorf, however, surgical resection itself was performed at various hospitals in the region over a period of 10 years, complicating the reliably gathering of outcome data of all patients. Furthermore, it was not the scope of our study to correlate MMR expression with disease recurrence and emergence of cisplatin resistant phenotype but rather to check for prevalence of MMR deficiency in primary resection specimen. For the one seminoma with MMR deficiency no tumor recurrence was reported.
We have no additional data to report for comment 4.

Changes in the text: - none -

Comment 5: The low-power view of the seminoma in Figure 1 can be a bit improved in terms of sharpness.

Reply 5: We agree that the quality of the provided figure 1 was suboptimal in the provided manuscript. Therefore, we have modified this figure which should now depict more clearly the technical issue of staining artifacts of MMR-IHC in seminoma.

Changes in the text: We have created a new version of Figure 1 in the revised version of the manuscript.

Comment 6: *There are minor English typos to correct.*

Reply 6: We have corrected the revised manuscript for several minor spelling and grammar mistakes in the revised version of the manuscript.