Peer Review File

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Reviewer A

Comment 1: line 16: NUT midline carcinoma is also known as NUT carcinoma, and the WHO has changed the official name to NUT carcinoma.

Response 1: Thanks for this comment. We changed all the "nut midline carcinoma" to "nut carcinoma", and changed the abbreviation NMC to NC in the text, we also added a new reference to explain the revision made by WHO.
Changes in the text: (page 8/line 7-8) WHO redefined NUT midline carcinoma as NUT carcinoma (NC) in 2015 (8).

Comment 2: throughout the entire document: all genes need to be italicized according to standard convention. The official name for the NUT gene is NUTM1. The protein it encodes can be called NUT or NUTM1.

Response 2: Thanks for this comment. We have revised these descriptions in the text according to this comment, and annotated the official name of the NUTM1 gene in the introduction section.
Changes in the text: (page 4/line 12-14) The pathogenesis of NC is complex and related to the acquired chromosomal rearrangements involving NUTM1 (NUT Carcinoma Family Member 1) and other causes leading to the differentiation blocking.

Comment 3: line 40: provide a reference for the median survival time of 6.7 mo: it is Bauer et al..

Response 3: Thanks for this comment. We have added the reference to the text.
Changes in the text: (page 4/line 8-10) Bauer et al. investigated the clinical characteristics of 57 patients with NC found that the median survival time was 6.7 months.

Comment 4: lines 42-43: the statement that gene mutations leading to abnormal proteins are the main cause of NUT midline carcinoma is confusing. The only abnormal protein known in this disease is the NUT-fusion oncoprotein. Otherwise, there are no other known oncogenic drivers or tumor suppressors that are mutated.

Response 4: Thanks for this comment. We have revised the inappropriate description in text.

Changes in the text: (page 4/line 12-14) The pathogenesis of NC is complex and related to the acquired chromosomal rearrangements involving NUTM1 (NUT Carcinoma Family Member 1) and other causes leading to the differentiation blocking

Comment 5: lines 55-58: the "catastrophic" event that leads to BRD4-NUT or BRD3-NUT is a single chromosomal chromoplexy event. This paragraph suggests that "it has been reported" that environmental factors may cause this event. First, I don't believe this has been reported, and second, even if it has, it is pure speculation and there is no data to support this statement. It is misleading and should be removed, like other subsequent statements in this review that are pure speculation.

Response 5: Thanks for this comment. We have removed the sentences in the revised paper.

Changes in the text: The sentences “It has been reported that the pathogenesis of NMC is related to the catastrophic genetic rearrangements which may be caused by most common factors causing tumors, such as genetic factors, environmental exposure, chemical agents, radiation exposure, and others.” has been removed.

Comment 6: line 64-65: I don't believe that NUT is the only testes-specific factor expressed in post-meiotic germ cells. There are many factors expressed in post-meiotic spermatids.

Response 6: Thanks for this comment. We corrected the sentences in the text.

Changes in the text: (page 5/line 10-11) Shiota et al. found that Nut is expressed in post-meiotic spermatogenic germ cells.

Comment 7: line 69: reference 6 is inappropriate here. It is best to always use the original reference

for data presented. Reference 6 should be replaced with the original report: French CA et al,. 2008 PMID: 17934517

Response 7: Thanks for this comment. We have replaced the reference according to this comment.
Changes in the text: (page 5/line 15) reference 18.

Comment 8: lines 69-70: ZNF532 does not bind directly to NUT and in fact has not been shown to associate with NUT at all. The paper referenced (ref. 7, and also incorrectly referenced as "Artyom et al" - should be Alekseyenko A et al.,) states that ZNF532 interacts with BRD4-NUT. It does not state that it interacts with NUT or that it does so directly.

Response 8: Thanks for this comment. We have revised the descriptions in the text according to this comment. However, we speculated that Alekseyenko et al. also found another fusion oncogene, ZNF532–NUT.
Changes in the text: (page 5/line 15-17) Alekseyenko et al. found that ZNF532 as a chromatin factor could interact with BRD4–NUT complexes, and they also identified another fusion oncogene, ZNF532–NUT.

Comment 9: line 77: the MGA fusion was also described in Diolaiti D et al., PMID: 30552129.

Response 9: Thanks for this comment. We have added the new reference to the text.
Changes in the text: (page 5/line 20) reference 22.

Comment 10: lines 79-82: The statement that NUT carcinoma is heterogenous is true, however the only reference which actually subcateogorizes NUT carcinoma into prognostic groups based on anatomic location and gene fusion is that by Chau et al., 2019: PMID: 32328562.

Response 10: Thanks for this comment. We revised the descriptions and added the reference to the text.
Changes in the text: (page 6/line 4-6) Chau et al. grouped 124 out of 141 NC patients by anatomic

location and fusion type, and nonthoracic primary NC group with non-BRD4-NUT fusion had the best outcome (26).

Comment 11: line 83: the first report of the gene fusion and exonic structures was by French CA et al., 2003 PMID: 12543779 . This would be an appropriate reference to add to

Response 11: Thanks for this comment. We added the reference to the text according to this comment.

Changes in the text: (page 5/line 25-page 6/line 1) French CA et al. for the first time reported the fusion oncogene, BRD4-NUT in 2003 (24).

Comment 12: lines 83-88: The significance of the BRD4-NUT structural variants is unknown. The reports referenced have a very small number of cases from which conclusions cannot be drawn, especially with regards to sensitivity to BET inhibitors. This information is misleading and should be removed.

Response 12: Thanks for this comment. We have removed the sentences in the text according to this comment.

Changes in the text: The sentences "The major carcinogenic variants in NMC have been reported as the fusion of BRD4 exon 11 to NUT exon 2. Stirnweiss et al. found two other BRD4-NUT subtypes (BRD4-NUT14:ex2 and ex15:ex2), and alternative splicing was found to cause the pathogenic complexity of this new subtype NMC cell lines expressing the BRD4-NUTM1 (86 exon11:exon2) variant are more responsive to BET, on average, than those with other BRD4-NUTM1 translocation variants." have been removed.

Comment 13: lines 91-95: The interpretation of the paper by Lee is misleading. The numerous mutations found in the tumors sequenced by Lee were stated to be clocklike somatic mutations not unlike those that would be expected in a benign, dividing somatic cell. One main point of the Lee paper was that NUT carcinomas are genomically stable and do not harbor additional oncogenic

mutations other than the NUT-fusion oncogene.

Response 13: Thanks for this comment. We have revised the inappropriate descriptions in text according to this comment.

Changes in the text: (page 5/line 22-24) Lee et al. sequenced the whole genome and transcriptome of three NC patients and found that except for BRD3 / 4 –NUT oncogene rearrangement, no canonical oncogenes or tumor suppressor genes were affected, but it caused NC, a fatal disease.

Comment 14: lines 98-100: These sentences are misleading. NUT is not a histone acetyltransferase, nor does can it read chromatin. It does not confer specificity for acetylating H2A and H4. p300 is the acetyltransferase that NUT recruits and other complex members confer histone specificity.

Response 14: Thanks for this comment. We revised the inappropriate descriptions in the text according to this comment.

Changes in the text: (page 6/line 11-13) NUT is exclusively expressed in the testis of human and mice, and p300 and/or CBP are the only acetyltransferases present in the Nut interactome. Nut recruits p300 and/or CBP to control histone H3 and H4 acetylation.

Comment 15: Line 105: reference 14 here is inappropriate.

Response 15: Thanks for this comment. We removed the reference.

Changes in the text: (page 6/line 18) The original reference 14 has been removed.

Comment 16: lines 108-110: Misleading. NUT carcinoma does not arise within germ cells. These lines should be deleted.

Response 16: Thanks for this comment. We have removed the inappropriate descriptions in the text.

Changes in the text: The sentences “Specific chromatin modifications, such as H4 hyperacetylation in spermatogenic cells after meiosis, and ectopic activation of these factors may initiate a oncogenic

molecular circuit." have been removed.

Comment 17: line 113-114: BRD4 does not necessarily dysfunction in non-NMC cancers. It is more accurate to say that non-NMC cancers are non-oncogenitically addicted to BRD4; they are dependent on BRD4, but it is unmutated and as far as we can tell functions normally.

Response 17: Thanks for this comment. We have revised these descriptions in the text according to the comment.

Changes in the text: (page 6/line 23-25) BRD4 has been implicated in the pathogenesis of a variety of cancers, including hematological malignancies and solid tumors.

Comment 18: Line 117: again it is appropriate to reference the original discovery of the BRD4-NUT fusion: French CA et al., PMID: 12543779.

Response 18: Thanks for this comment. We have added the reference according to this comment.

Changes in the text: (page 7/line 2) reference 24

Comment 19: line 129-130: This is misleading. NMC mimics poorly differentiated cancers of various types, most commonly squamous cell carcinoma. 30% of NMCs exhibit squamous differentiation morphologically, and at least 70% express squamous markers, such as p40 or CK5/6. There is no mention in this review that the diagnosis of NMC is actually quite easy. It can be diagnosed by positive staining with the NUT antibody, clone C52B1 with 100% specificity. Haack H et al., PMID: 19363441.

Response 19: Thanks for this comment. Since the reference was not closely related to the pathogenesis of NC, we removed the sentence and reference here.

Changes in the text: The sentence "NMC mimics stem cell tumors, which brings difficulty to its diagnosis (26)." has been removed.

Comment 20: line 134: reference 27 in inappropriate.

Response 20: Thanks for this comment. We removed reference 27.

Changes in the text: The original reference 27 has been removed.

Comment 21: line 137: the statement that it is not known whether BRD4-NUT directly regulates MYC is not correct. It was shown to bind to the promoter and regulatory regions and to lead to MYC protein stabilization in references Alekseyenko 2015 and Grayson 2014.

Response 21: Thanks for this comment. We have revised the descriptions in the text.

Changes in the text: (page 7/line 17-19) BRD4-NUT is located in the promoter of MYC gene. Grayson et al. found that BRD4-NUT prevented the differentiation of NC by maintaining MYC expression.

Comment 22: line 142: AFP is detectable only in a subset of patients. The statement here is misleading.

Response 22: Thanks for this comment. Since the relationship between AFP and the pathogenesis of NC is not confirmed at present, we deleted this paragraph.

Changes in the text: The original paragraph have been removed.

Comment 23: lines 159-164: The data do not support any of the statements in this paragraph. Indeed, Stirnweiss identified mutations in RECQL5, however they did not demonstrate that it functioned abnormally, nor did they demonstrate that its mutation led to genomic instability in NMC cells. In fact, a more robust analysis by Lee indicated that NMC cells are very genomically stable and this contradicts the statement that RECQL5 has anything to do with the pathogenesis of NMC. This paragraph should either be re-written or removed.

Response 23: Thanks for this comment. We have deleted this paragraph according to this comment.

Changes in the text: The original paragraph has been deleted.

Comment 24: lines166-172: The role of autophagy in NMC is completely unknown and thus this paragraph is irrelevant.

Response 24: Thanks for this comment. We have deleted this paragraph.

Changes in the text: The original paragraph has been deleted.

Comment 25: lines 195-210: The cytomorphology described is inaccurate, misleading, and disorganized. NMC is a poorly differentiated squamous carcinoma. I suggest that the author read the morphologic description provided in the following WHO chapters, or else just delete the paragraph:

a. WHO (2015) Classification of Tumors of Lung, Pleura, Thymus and Heart, 4th edition. Thymus : NUT carcinoma. 229-231.

b. WHO (2017) Classification of Tumors of Head and Neck. Tumors of the nasal cavity, paranasal sinuses and skull base : NUT carcinoma. 20-21.

Response 25: Thanks for this comment. We read the recommended articles above in detail. Because this paragraph is irrelevant to the pathogenesis of NC, so we deleted this paragraph.

Changes in the text: The original paragraph has been deleted.

Comment 26: lines 220-221: Type of radiation was not analyzed, just the outcome of initial radiation.

Response 26: Thanks for this comment. We have revised the inappropriate descriptions in the text.

Changes in the text: (page 9/line 13-15) Bauer et al found that factors affecting the clinical response included age younger than 18 years, initial radiotherapy, and presence of metastasis.

Comment 27: line 227: Reference 59 is a meta-analysis which includes the cohorts from references 57 and 58. Tox my knowledge, they do not distinguish initial complete surgical resection from surgery at any point during treatment. That distinction is critical as there is no benefit to surgery that is not performed initially. The statement is therefore misleading.

Response 27: Thanks for this comment. We removed the inappropriate descriptions and the original reference 59, according to the results in the present reference 59 (the original reference 58), the extent of surgical resection with negative margins is also significant to PFS and OS, which was added in the revised paper.

Changes in the text: (page 9/line 15-18) A recent study on 48 patients with head and neck NC reported that the initial surgical resection and extent of surgical resection with negative margins were significantly associated with the improvement of progression-free survival (PFS) rate and overall survival (OS) rate.

Comment 28: line 245: I don't know what flavone piperidol is, but the compound used in reference 60 was flavopiridol, a CDK9 inhibitor.

Response 28: Thanks for this comment. We have corrected this mistake, and it should be flavopiridol.

Changes in the text: (page 10/line 11) Vincristine, doxorubicin, and flavopiridol (CDK9 inhibitor) show significantly better activity than etoposide and vorinostat;

Comment 29: line 249: references 61 and 62 include three patients, not two, all of whom had a complete response to the Ewing regimen, which in my opinion is more promising than any other therapeutic regimen to date, though still not curative in most patients.

Response 29: Thanks for this comment. We have revised the inappropriate descriptions in the text.

Changes in the text: (page 10/line 15-18) In two case reports, three pediatric NC patients were treated with a comprehensive protocol for sarcoma (SSG IX), involving surgery, chemotherapy and focal radiotherapy. These three patients experienced remission for 6 years, 14 years, and 13 years, respectively.

Comment 30: line 249-250: the statement that chemotherapy has no effect on lesions beyond the

primary site is incorrect, misleading, and should be removed.

Response 30: Thanks for this comment. We have deleted these sentences.

Changes in the text: The sentences "As a part of the initial treatment, chemotherapy improves the OS for mediastinal primary but has no influence on tumors at other sites (59)." have been deleted.

Comment 31: lines 252-254: There is no data to suggest that stem cell transplant is effective. The patient in reference 63 died. This sentence is misleading and should be removed.

Response 31: Thanks for this comment. We deleted the sentence according to this comment.

Changes in the text: The sentences "Clinically, dose-intensive treatment regimens may stably benefit NMC patients and high-dose chemotherapy may work in combination with stem cell transplantation as a treatment option for NMC (63)." have been removed.

Comment 32: line 270: none of the references have NMC patients. A more appropriate reference is author's reference 66, Lewin et al., PMID:29733771, and Piha-Paul et al.,PMID:32328561. Also note that JQ1 is a tool compound, not a drug that will ever be used in humans.

Response 32: Thanks for this comment. We have replaced the inappropriate reference in the text, and read the recommended articles above in detail. References were added according to this comment.

Changes in the text: (page 11/line 17-19) A study shows that BET inhibitor JQ1 can induce the differentiation and growth arrest in NC cell lines, and also exert antitumor effect in xenograft models of NC.

Comment 33: lines 275-277: This sentence is misleading: Differentiation of NMC cells indicates a therapeutic response of the tumor. It is not a feature of relapsing tumor.

Response 33: Thanks for this comment. We have revised the inappropriate description in the text.

Changes in the text: (page 11/line 8-11) Pathological examination after tumor biopsy revealed a

decreased NUT expression in areas of differentiation in a NC patient after BETis treatment. This indicates that NC cells may switch to a more differentiated squamous cell phenotype after BETis treatment is initiated.

Comment 34: line 290: 74 is the incorrect reference. It should be PMID: 2973377.

Response 34: Thanks for this comment. We have replaced the inappropriate reference in the text.
Changes in the text: (page 11/line 25) reference 69.

Comment 35: line 301: reference 65 is inappropriate and should be replaced with reference 14.

Response 35: Thanks for this comment. We have replaced the inappropriate reference in the text.
Changes in the text: (page 12/line 14) see reference 77

Comment 36: line 328: The original report of the use of vorinostat, which reprograms the epigenome of NMC cells was by Schwartz et al., 2011 PMID: 21447744. This was reported 4 years prior to the stated reference and should be added.

Response 36: Thanks for this comment. We added the reference according to this comment.
Changes in the text: (page 13/line 16) reference 81 was added.

Comment 37: lines 333-334: CDK9 inhibitors are not that potent in vitro in NMC cells. See report by Bragelmann et al., PMID:28930680.

Response 37: Thanks for this comment. We read the recommended literature in detail, and removed the inappropriate descriptions.
Changes in the text: The statement "Studies have revealed that the CDK9 inhibitor FP is one of the most cytotoxic drugs against NMC cell lines in vitro" has been deleted.

Comment 38: line 341: reference 80 is truly interesting, however it is a single case report and so

the information cannot be generalized.

Response 38: Thanks for this comment. We have revised the inappropriate descriptions in the text.
Changes in the text: (page 14/line 1-3) A patient with NC was relieved after radiotherapy combined with anlotinib hydrochloride. However, more evidence is needed to confirm the therapeutic effect of anlotinib hydrochloride on NC.

Reviewer B

Comment 1: Pathologically, NMC is characterized by the very low differentiation of squamous cell carcinoma ... improve this definition? see WHO definition?

Response 1: Thanks for this comment. We added descriptions to help define the poor differentiation of NC and modify the inappropriate statement in the text.
Changes in the text: (page 4/line 10-12) Pathologically, NC is characterized by variable degrees of squamous differentiation with a predominance of the poorly or undifferentiated component.

Comment 2: Describe the affected locations by NUT carcinoma? the term midline is no longer recommended because there are cases outside this location. Please, discuss this topic.

Response 2: Thanks for this comment. We added the descriptions and references about NC of non-midline structure, and explain WHO's redefinition of NMC in 2015 (See references 2-8).
Changes in the text: (page 4/line 3-8) NUT midline carcinoma (NMC) is a malignant tumor with involvement of testicular nuclear gene rearrangement, which has the tendency to arise from midline anatomical sites, and it was initially described as a mediastinal tumor in 1991. with increasing cases reported in non-midline structure such as renal pelvis, pancreas, parotid gland, bladder, sublingual gland and femur. WHO redefined NUT midline carcinoma as NUT carcinoma (NC) in 2015.

Comment 3: Emphasize treatment modalities and their impact on pediatric vs adult patients with NUT carcinoma? Recommended

Response 3: Thanks for this comment. A study shows that the prognosis of children with salivary gland NUT carcinomas may be better than that in adults. However, there is no accepted evidence supporting that the prognosis of children and adults is different after the treatment of NC currently, and comprehensive protocol for sarcoma (SSG IX) and targeted therapy may be promising therapeutic regimens.

Changes in the text: (page 14/line 4-11) NC can develop of all ages without gender preference. Only a few patients responded to the treatment, including pediatric cases (53, 62, 63, 66, 83). Wang et al. found that children with salivary gland NUT carcinomas represented a distinct subset with male predilection and better overall survival (84). However, due to the small number of cases responding to the treatment, it is not enough to explain the difference in the therapeutic effect between children and adults, and more evidence is needed to confirm this result. As the treatment strategy mentioned above, comprehensive protocol for sarcoma (SSG IX) and targeted therapy may be promising strategies.

Comment 4: Emphasize treatment modalities and their impact on midline and non-midline NUT carcinomas? if possible

Response 4: Thanks for this comment. Some malignant tumor patients can achieve good therapeutic effect on conventional treatments like surgical resection combined with adjuvant radiotherapy and chemotherapy. Other targeted therapies for NC have also been proven to be effective in several tumors. However, there is no established treatment for NC. Even with targeted therapy, only a few patients have achieved long-term remission. For the treatment of NC, clinicians may take targeted therapy and SSG IX into consideration besides the conventional treatments.

Changes in the text: (page 14/line 10-11) As the treatment strategy mentioned above, comprehensive protocol for sarcoma (SSG IX) and targeted therapy may be promising strategies.