Reviewer’s report

Title: Assessment of piRNA biogenesis and function in testicular germ cell tumors and their precursor germ cell neoplasia in situ

Version: 1 Date: 15 Oct 2017

Reviewer: Keyan Salari

Reviewer's report:

The revised version of the manuscript reads much more clearly and the authors have adequately addressed some of the concerns raised by both reviewers. However, upon review of the revised manuscript, the following issues persist that should be addressed prior to publication.

Major:

1. As pointed out in the prior review, the central conclusion of the manuscript that PIWI/piRNA pathway genes expressed exclusively in normal germ cells and not in GCNIS cells adjacent to TGCT is based entirely on gene expression correlation between piRNA genes and markers of germ cells (DDX4, DAZL) vs. GCNIS (NANOG, POU5F1). This level of evidence is insufficient to make such strong claims and conclusions as:

Line 134-136: "However, their expression is also highly correlated between each other and with the germline markers DAZL and DDX4 (Pearson's r=0.80-0.99, Supplementary table S1), which confirms the fact that PIWI/piRNA pathway genes are expressed exclusively in germ cells in normal adult testis."

Line 140-142: "This finding strongly suggests that PIWI/piRNA pathway genes are expressed only in germ cells present in the testis samples adjacent to TGCTs, but not in the GCNIS cells."

Line 236-241: "Surprisingly, piRNA biogenesis genes are only expressed in normal germ cells present in either healthy adult testis or testis tissues adjacent to TGCTs. Moreover, piRNA biogenesis in germ cells residing next to TGCTs exhibits conventional features characteristic of germline piRNAs, which suggests that PIWI/piRNA pathway is not altered in these germ cells. Conversely, neither GCNIS cells nor TGCT cells appear to express PIWI/piRNA pathway genes."

The study's results are based on RNA extractions performed on healthy testis (clearly containing normal germ cells), TGCT specimens (clearly containing malignant germ cell tumor cells), and testis tissue adjacent to TGCTs (containing an undefined mix of normal germ cells and possibly GCNIS cells). The concern here is that, from the data presented, there is no way to know the
proportion of normal germ cells vs. GCNIS cells in the specimens of tissue adjacent to TGCT. The authors assume that specimens with low levels of NANOG/POU5F1 (and high levels of DDX4/DAZL) have a low proportion of GCNIS cells (and high proportion of normal germ cells) and vice versa. While that may be a reasonable assumption, it is an unsupported leap to assert that because the levels of these markers correlate with piRNA pathway genes, the genes are "exclusively expressed" in normal germ cells and not in GCNIS cells. There needs to be some form of evidence showing localization of piRNA gene expression to normal germ cells and absence of such expression in GCNIS cells in order to substantiate the authors conclusions. The authors could start by providing H&E analysis of all the testis tissue specimens from adjacent GCT to estimate the proportion of normal germ cells vs. GCNIS. Immunohistochemistry could then be used to confirm normal vs. GCNIS cells as well as perform co-staining with the relevant PIWI/piRNA related proteins. The authors stated that there are some issues with the commercially available antibodies for these proteins. If no suitable antibody can be obtained for IHC, the same co-localization experiments could be performed at the RNA level using RNA in situ hydbridization (RISH). Alternatively, laser capture microdissection could be used to separately isolate the normal germ cells from the GCNIS cells and the RNA expression analyses could be repeated on these more purified cell populations. Without some form of evidence that provides resolution at the cellular level, the authors cannot comment on the expression of piRNA genes in germ cells vs. GCNIS cells.

2. In the results section named "Small RNA profiling reveals normal piRNA biogenesis in germ cells present in testis tissues adjacent to TGCTs", the authors refer to the testis tissue adjacent to nonseminomas as "GCNIS NS" and the testis tissue adjacent to seminomas as "GCNIS SE". As elaborated in point #1 above, the authors have not demonstrated whether GCNIS cells exist in any of the specimens adjacent to either type of TGCTs (nonseminomas or seminomas). It is misleading to refer to these samples as GCNIS NS or GCNIS SE without demonstrating by H&E/IHC, or another similar method, that GCNIS cells exist in these specimens and what their approximate proportion is in each sample.

Minor:

1. The new version of the Background/Introduction is unnecessarily lengthy. In particular, the first 3-4 paragraphs can be summarized in 1-2 paragraphs (e.g. description of Testicular Dysgenesis Syndrome can be removed). The end of this section describing the results of the authors' prior study can also be made more concise.

2. The authors have now appropriately mentioned the possible tumor suppressor role that PIWI/piRNA genes may have in TGCT development. Acknowledging this possibility, I would suggest the authors modify the conclusion in lines 244-245 that "PIWI/piRNA
pathway is unlikely to be one of the drivers of TGCT development" to read that they are "unlikely to be an oncogenic driver of TGCT development", as they may still function as a driver via loss of tumor suppressor activity.

3. Finally, the manuscript needs to be edited for to correct several English grammatical mistakes.

Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

Yes

Does the work include the necessary controls?
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