Peer Review File

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Reviewer #1

The authors provide a comprehensive review on the possible application of new microRNA-based biomarkers in diagnostic scenarios of testicular germ cell cancer. The issue is timely and in view of the high number of papers published during last few years, a review article to update the reader is welcome.

However, before this article can be published a number of corrections and changes should be made.

Introduction:

Comment 1: This part of the paper is far too lengthy. Most of the issues discussed here are in fact issues belonging to general reviews of testicular germ cell tumors, not to a review on new biomarkers. This part of the manuscript could be shortened by 20-30% without losing track to the particular goal of the article.

This part has been shortened

Diagnosis of metastatic GCT:

Comment 2: The same critique applies to this part of the manuscript. It is certainly justified to mention functional imaging (FDG-PET scan) but it is clearly inappropriate to spend more than 15 lines with commenting on one single imaging study and criticizing a possibly suboptimal treatment used in that study (ref#39 of the manuscript). The authors are giving more space to radiology in their manuscript than to the classical serum tumor markers. This part of the manuscript needs to be shortened, too.

This part has been shortened. However, we do think that PET is unfortunately still overused in GCT management and that it is considered a surrogate biomarker of active germ cell tumors. Therefore, we think it is appropriate to spend some words about it.

Serum tumor markers:

Comment 3: By contrast, in this part of the manuscript some more details would be welcome. The authors just state that the detection of tumor markers depends on histology and tumor burden. This is certainly correct, but why not being precisely? You could state that less than 30% seminoma patients have expression of beta HCG while nonseminomas have expression of AFP and beta HCG in no more than roughly 50-60%. A very good and recent reference with original data would be Dieckmann et al. (BioMed Res Int 2019).

Serum tumor markers section expanded. Reference added as suggested.

New biomarkers in GCT: miRNAs

Comment 4: In the first paragraph of this section (nineth line) the authors state that the miRNAs of the miR371 cluster are undetectable in both benign testicular tissue

and in teratoma. This statement is not correct, since two recent studies showed that miR-371a-3p is detectable in benign testicular tissue though to a lesser degree than in GCT (Boellaard; Andrology 2019; Belge, Oncotarget 2020). The presence of this miR also in ejaculate fluid could be worth mentioning in this section.
Thanks for this comment. The sentence has been changed

miRNAs in the pre-orchiectomy setting:
Comment 5: The pioneering work of the Murray group is correctly stated. But the Gillis study (ref #54 of manuscript) is definitely not the first study providing a proof of principle as indicated in the first sentence of this paragraph. The Gillis study was published in 2013 but the German group had reported on 11 patients even one year earlier (Belge, Eur Urol, 2012). To be fair, the credit of being the first should be given to the latter group.
Reference added

Comment 6: Further, in the last paragraph of this section, the authors refer to the largest study on miR-371 testing in GCT patients (ref #58) and they state that 669 patients were involved. However, a quick glance to the abstract of that study shows that only 616 patients were included.
Thanks for pointing this out. The number has been corrected

Patients with metastatic disease:
Comment 7: In the last sentence of this section, it is said that miR371 was overexpressed in all of the 46 patients with relapses of the study referred to (refe#58). However, this is not true, since only 38 of the 46 patients had detectable serum levels of this miR.
Corrected

Equivocal clinical scenarios and miRNAs utility
Comment 8: Other clinical scenarios where the employment of the miR-371 test could be helpful would be the primary diagnosis of small testicular tumors which is an ever growing problem in the urologic community. However, as the present review focuses on new biomarkers in advanced disease (head-line of the article), this rather urologic problem might be spared. Another possible application of the test could probably be the CUP syndrome where GCTs could be ruled out with the test.
CUP section added

Postchemotherapy residual disease
Comment 9: The Leao-study (ref#73) is certainly the pivotal study regarding the utility of miR-371 in the assessment of postchemotherapy masses. However, Rosas-Plaza (Cells 2019) also report data with respect to this issue. That study needs to quoted, too.
Study added

Limitations of miR371

Comment 10: The utility of the miR371 test in the postchemotherapy setting is not only limited by the presence of teratoma but also by the size of the residual vital cancer. As shown in article #58, the expression of miR371 decreases with decreasing size in primary tumors. It is rational to assume that the sizes of vital residual cancer masses do likewise matter.
we agree and we have added a sentence to reinforce this concept

Status of miRNAs validation in GCT
Comment 11: The authors correctly point to the need for further validation studies and they refer to two prospective trials launched in North America. However, it would be fair to note that several other studies (most of them very similar to the tow trials reported herein, e.g. DRKS00019223) are ongoing in Europe where several active groups in several countries have adopted miR-371 and other miRs as a novel diagnostic aid and as a subject of clinical research, likewise.
There is no mention of other studies in GCT for serum miRNAs in clinicaltrial.gov. DRKS00019223 has not been registered.

References
Comment 12: As a review article has the goal to summarize important studies reported in the literature, references are always a critical issue in review articles. The total number of references of the present paper of n=85 is certainly appropriate. However, the selection of references could certainly be improved. In the eyes of the reviewer there are too many references relating to general or radiological issues of GCT.

Among the references specifically relating to miR-371, a number of important papers are missing:
Syring J. Urol. 2015 (one of the first clinical studies on the utility of miR371),
added
Mego (J Cell Mol Med 2019) one of the early studies on mi371 in metastatic disease)

Radtke (Urol. Int. 2018) The first study pointing to the extremely short half-life of miR371
added
Vilela-Salgueiro (Philos. Trans. R. Soc . B, 2018) a thorough study on microRNA-371 in tissue of GCT
Myklebust (Front. Genet. 2019) pointing to possible interference of miR- measurement with hemolysis
added
Morup (Cancers, 2020) the first series from Denmark

In all, a number of the references to general and radiological issues could probably be deleted in favor of more references to classical markers and studies of miR-371a-3p.

Thanks for the suggestions. Some of these references add important information and have been added.

Comment 13: The following references are incomplete (mainly page numbers missing)
Ref #1, #38, #39, #45, #80.
Those are special issues/abstracts

Furthermore,
Comment 14: a short note to the methodology of measuring microRNAs in serum should be given specifically the principle of measuring the target miR in relation to an endogenous miR.
A short note has been added in the limitation paragraph

Reviewer #2
Excellent and thorough review of these promising micro RNA markers. This a good synopsis of where the data currently stands and it will be exciting to see what comes of the trials planned for the future in this domain. Pending results from the ongoing multicenter trials, this has the potential to be practice changing and it's important to have a clear presentation of current state of affairs as was done in this paper.

Minor edits:
Introduction
Comment 1: First paragraph- Not necessarily true, chemo can be used in stage I with BEP X1 in non seminoma for example without absolute evidence of disease.

Thanks. That sentence has been modified

Page 8
Comment 2:…patients with GCT, Murray et al. demonstrated that miR371 and miR302/367 clusters are detectable in the cerebrospinal fluid as well as the serum of these pa6ents with a sensi6vity and specificity higher than the classic tumor markers.
It would be helpful to add the percentages for sensitivity / specificity here to compare to classic tumor markers.

The cited paper described miRNAs vs tumor markers in few cases and on a single case base. Therefore the operating characteristics of miRNAs vs TM were not reported in the paper.

Page 11
Comment 3: Unlikely miR371, should be changed to unlike miR371

Thanks. This has been changed as suggested

Page 12
Comment 4: Fifteen/36 patients presented teratoma… à 15/36 patients presented with teratoma…
Thanks. This has been changed as suggested