Author's response to reviews

Title: Prognostic relevance of ALT-associated markers in liposarcomas: a comparative analysis

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Author's response to reviews: see over
Dear Dr. Marshall,

We thank you for the opportunity you gave us to resubmit our paper which has been improved by the changes requested by the reviewers. We are now submitting a revised version of the manuscript entitled “Prognostic relevance of ALT-associated markers in liposarcoma: a comparative analysis” (MS: 1022926618351914). The paper has been revised taking into account all of your and the reviewer's suggestions, and the changes have been highlighted through the main text. The replies to every comment, along with the indication on which pages of the manuscript changes have been made, are enclosed.

I ensure that the revised manuscript conforms to the journal style and I also declare that no authors have competing interests to disclose.

Yours sincerely,

Maria Grazia Daidone

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Replies to Reviewers’ comments.

Reviewer #1

Minor essential revisions:

1. Abstract. In accord with the reviewer’s suggestion, we pointed out that the prognostic relevance of ALT mechanisms in liposarcoma depends on the marker used. ALT-associated promielocytic bodies was a prognostic discriminant of increased mortality at 10 years of follow-up, whereas TRF length distribution was prognostic only after 15 years.

2. Methods. In accord with the reviewer’s suggestion, we provided more detail on the APB assay (page 6).

3. Results. We added a table listing the patients data and clinicopathologic characteristics (Table 1 additional file) as requested also by reviewer#2.

4. Discussion. Liposarcomas are heterogeneous as regards morphology and malignancy level and are classified according to karyotypic complexity. Our case series includes different histological subtypes (well differentiated, de-differentiated, myxoid and round cell, see Table 1 additional file) and the reviewer’s comment about the heterogeneity of this cohort of tumor is right and can be a reason for the incomplete overlapping of the TRF and APB results. We mentioned such a comment in the Conclusion section (page 11). The complete concordance reported for APB and TRF methodologies in Henson et al (Henson JD et al. A robust assay for alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res 2005; 11:217-25) was described in 40 astrocytomas (including 33 glioblastoma multiforme and 7 astrocytomas).

As far as other comments is concerned (point #4) the rewiever observed that only 3 samples were APB+ but TRF-ve, the observation is incorrect because the APB+/TRF- samples are 11/85 (13%).
Minor essential revisions:

1. Page 7. The sentence “to avoid false positive” has been restated.

2. Page 7. “1,000 Kb2” has been modified in “1,000 kb^2”, that represents the variance cut-off value used to discriminate ALT positivity. For further details see Telometric 1.2 User’s Guide. Briefly, Telometric Software obtains telomere length data by first obtaining the average grayscale intensity for each row of pixels in an outlined lane. These intensities are then used to obtain a relative frequency for each telomere length. Since the telomere length spacing may be non-uniform, a second data set of relative frequencies is generated using linear interpolation at uniformly spaced telomere length intervals. The statistics are then calculated as follows:

\[
\text{Mean} = \frac{\sum_{i=1}^{n} \gamma_i L_i}{\sum_{i=1}^{n} \gamma_i} \\
\text{Median} = L_j \text{ such that } \sum_{i=1}^{j} \frac{\gamma_i}{\sum_{i=1}^{n} \gamma_i} = \frac{1}{2} \\
\text{Mode} = \max_i \frac{\gamma_i L_i}{\sum_{i=1}^{n} \gamma_i} \\
\text{Variance} = \frac{\sum_{i=1}^{n} \gamma_i (L_i - \text{Mean})^2}{\sum_{i=1}^{n} \gamma_i} \\
\text{SIR} = \frac{P_{75} - P_{25}}{2}
\]

where \( n \) is the total number of uniformly spaced telomere length points, \( L_i \) is the \( i \)th telomere length, \( \gamma_i \) is the relative frequency of \( L_i \), \( P_{75} \) is the telomere length at the 75th percentile, and \( P_{25} \) is the telomere length at 25th percentile.

Discretionary Revisions

1. We included a table listing patients data and clinicopathologic characteristics (tab. 1 additional).
2. In accord with the reviewer’s suggestion, we cited the papers by Schneider-Stock and Johnson.

3. As other groups already reported (Yeager TR et al. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res 1999; 59:4175-9.; Henson JD et al. A robust assay for alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res 2005; 11:217-25.) the presence of APBs correlates precisely with the presence of ALT (determined by TRF analysis) in ALT positive cell lines. In the latter paper the authors tested 26 frozen soft tissue sarcomas specimens for ALT by both TRF analysis and APB assay and scored as ALT positive tumours with more than 0.5% of APB-positive cells in the sample. Using this threshold they were able to objectively classify tumors as ALT positive even when TRF analysis was equivocal. The 0.5% cutoff was an arbitrary choice for the purpose of avoiding false positive results due to possible artefactual colocalizations. However this criterion will need to be reassessed in the light of further data and at this stage we cannot exclude the possibility that tumors with less than 0.5% APB+ cells may be ALT+ (Jeyapalan JN et al. Evidence for alternative lengthening of telomeres in liposarcomas in the absence of ALT-associated PML bodies. Int J Cancer 2008; 1122(11):2414-21).

4. In accord with the reviewer’s suggestion we included white arrows to point out APBs in Figure 1A.

5. As the reviewer commented, the cases which have been characterized as ALT+ by APB criteria but do not show telomere length heterogeneity on TRF Southern blot showed a limited percentage of APBs. As we pointed out on page 8, TRF analysis could potentially miss ALT due to admixed tumor and normal cell components and, as the reviewer suggested, ALT positivity missed by TRF is likely due to a low proportion of tumor cells in the samples, in fact, as Henson et al demonstrated (A robust assay for alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res 2005; 11:217-25) TRF analysis is univocally positive only when more than 40% of the cells in a sample are ALT positive.

6. Page 10. As the reviewer suggested, we cited Johnson et al (ref.# 18).