Author’s response to reviews

Title: DNA Methylation Alterations in Grade II- and Anaplastic Pleomorphic Xanthoastrocytoma

Authors:

Ramón Martínez (ramon.martinez@med.uni-goettingen.de)
Francisco Javier Carmona Sanz (carmonasfj@gmail.com)
Miguel Vizoso (mvizoso@idibell.cat)
Veit Rohde (nchisekr@med.uni-goettingen.de)
Matthias Kirsch (matthias.kirsch@zfn.uni-freiburg.de)
Gabriele Schackert (gabriele.schackert@uniklinikum-dresden.de)
Santiago Ropero (santiago.ropero@uah.es)
Werner Paulus (werner.paulus@uni-muenster.de)
Alonso Barrantes (alonso.barrantes@med.uni-goettingen.de)
Antonio Gomez (agomezm@idibell.cat)
Manel Esteller (mesteller@idibell.cat)

Version: 4 Date: 26 January 2014

Author’s response to reviews: see over
Re: Follow-up on decision of MS: 1770274471113469 "DNA Methylation Alterations in Grade II- and Anaplastic Pleomorphic Xanthoastrocytoma"

To:

Prof Stefan Imreh and Ms Roselyn Remoto.
BMC Cancer

We are pleased to resubmit a revised version of the manuscript "DNA Methylation Alterations in Grade II- and Anaplastic Pleomorphic Xanthoastrocytoma", which we would like you to consider for publication in BMC Cancer as a Research Article.

We thank reviewers for the comments raised that have helped us improving the manuscript, and hope that our responses to the suggestions raised are satisfactory. We hope that the results are more accurately described and interpreted. Following your request, we have included on the Acknowledgements section, the details regarding each author contribution on the manuscript, as well as the funding body. All authors of this research paper have directly participated in the planning, execution, or analysis of the study, and have approved the final, submitted version.

Being fully conscious of the sample size constraints, we have tried to deeply characterize the tumors investigated, aiming to extract the maximum information of the data obtained, while being aware that the conclusions drawn should be taken with care and further confirmed in larger studies.

Following this letter you can find detailed answers to the reviewers.

Sincerely yours,

PD Dr. med. R. Martinez-Olivera
Reviewer's report

**Title:** DNA Methylation Alterations in Grade II- and Anaplastic Pleomorphic Xanthoastrocytoma  
**Version:** 3  
**Date:** 12 December 2013  
**Reviewer:** Gianluca Marucci

**Reviewer's report:** The Authors have analyzed the DNA methylation profiles of PXA.

This is a well-written, potentially interesting paper, which details previous literature in a comprehensive manner. However I have some reservations about this manuscript.

**Major Compulsory Revisions**

1. Frequently in the paper PXA is defined as a benign tumor! PXA is not a grade I tumor (like pilocytic astrocytoma) and correctly the Authors assert that it shows a 10-years survival rate of 70%. Thus it is more appropriate to define it as a low grade glioma.

We fully agree with the reviewer that PXA are from a histological point of view a grade II (i.e. low-grade) tumor type. PXA has been considered in several neuropathological series as a benign tumor associated with a favorable prognosis (such as “Tumors of the Central Nervous system” by Peter Burger and Bernd Scheithauer, Armed Forces Institute of Pathology and Universities Associated for Research and Education in Pathology, Bethesda, 1994, pages 96-102).

Nevertheless, we deeply appreciate the insight of the reviewer since one major dilemma in PXA remains on the difficulty to assess which PXA showing higher MIB-1, and probably higher mitotic index, in the absence of necrosis and microvascular proliferation, is going to rapidly recur or even though, to relapse as a more malignant anaplastic lesion. These considerations are of capital importance after initial resection of those PXA in order to decide whether adjuvant therapy (radio / chemotherapy) is necessary. This situation is basically influenced by the rarity and incomplete understanding of the pathobiology of PXA, which makes difficult to draw a tight parallel between histological findings and clinical behavior.

Considering those arguments we have decided to refer PXA along the manuscript as grade II tumors avoiding the term benign as much as possible.
2. Page 6, the Authors state “tissue obtained from epilepsy neurosurgical procedures was included in the study as normal control”: it is known that DNA methylation changes are observed in epileptic specimens [Kobow K, El-Osta A, Blümcke I. The methylation hypothesis of pharmacoresistance in epilepsy. Epilepsia. 2013 May;54 Suppl 2:41-7. / Kobow K, Blümcke I. The emerging role of DNA methylation in epileptogenesis. Epilepsia. 2012 Dec;53 Suppl 9:11-20]. Thus different control samples could be more useful.

We have measured the DNA methylation levels of the genes interrogated on additional samples obtained from DNA of healthy individuals. Specifically, we pyrosequenced 2 new samples from (a) white matter DNA derived from frontal cortex of a 64 yr females obtained after post-mortem interval of 2 hr, and (b) gray matter DNA derived from frontal cortex of a 73 yr female at post-mortem interval of 5 hr.

Although we agree with the reviewer that specific DNA methylation alterations are found in epileptic specimens, we did not observe any differences in the DNA methylation levels for the genes measured on this study.

3. Page 11, the Authors state “All PXA patients presented with a 4-8-week history of epileptic seizures”: PXA are tumors included in the group of so called LEATs (long-term epilepsy associated tumors), i.e. tumors observed in patients who have been investigated and treated for drug-resistant seizure episodes for 2 years or longer. Thus the Authors should provide some considerations to explain the unusual short history of seizures observed in this PXA series.

We fully agree the reviewer that patients displaying PXA are typically young adults usually presenting with a long-term history of seizures and less frequently with an expanding cerebral mass, mostly temporal or parietal located.

The reason why our patients were diagnosed more rapidly should be addressed on the German Public Health System, in which one seizure is always considered as emergency requiring hospital assistance in a neurological unit. In every case at least a CT scan will be performed on admission. Generally, even though at this stage a tumor mass will be visualized or suspected and MRI will follow. After diagnosis of a brain tumor the patient will be referred to a neurosurgical unit. The whole process will take no more than 3-4 days, even in the case that initial neurological assessment does not take place in a fourth-level center.
4. Table 1. PXA 2 should be removed from the list of studied cases. As the Authors state “no tumor tissue was available for characterization”, thus its presence in this series does not make sense.

We agree with the reviewer that this PXA case does provide less information than other in our series. Nevertheless, we had enough tissue to perform bead arrays and epigenetic assessment. Unfortunately we were not able to preserve further tissue for BRAF and CD34 characterization.

In order to clarify this point we have added in Table: “no tumor tissue available for BRAF and CD34 characterization”. We hope that Reviewer 1 also agrees in this with us.

5. Table 1, PXA 6. More details should be provided about anaplastic features: which was mitotic index? What about necrosis?

Additional details on immunohistological features, tumor localization and MIB index have been incorporated on Table 1.

6. Table 1 & Figure 1, PXA 4&5. This recurrent case, arisen in the oldest patient of the series (59y), represents the key case, determining a significant part of results. At page 4 the Authors assert that in PXA “MIB-1 labeling index is frequently <1%”; but in Figure 1 legend they state that MIB-1 was 10% in the grade II PXA no. 4. In my opinion a MIB-1 value of 10% is too high for a grade II tumor; a tumor frequently defined in this manuscript by the Authors as benign. Thus the Authors should provide strong evidences that case no. 4 was not a malignant glioma ab initio: if this was true, it would explain the overlapping with DNA methylation alterations found in GBM and thus it would impair the meaning of the study.

The review addresses in this point a capital issue concerning assessment of PXA cases showing higher MIB-1 indexes. We agree that in several PXA cases, only a precise and extensive analysis will allow differentiating them from GBM, mainly giant-cell GBM although the last one more typically presents with deep cerebral location, more extensive necrosis and brisk mitotic activity (Ohgaki et al., Pathology and Genetics of Tumors of the Nervous System. Lyon, 2000, McLendon et al, In: Bigner DD, McLendon RE, Bruner JM, eds. Pathology of Tumors of the Nervous System 1998)

We have performed two independent neuropathological analyses in PXA cases 4 and 5 because of the clinical- (a 59y. old patient) and pathological unfrequent (MIB-1 index) features, according to the 2007 WHO classification criteria. On the other hand cerebral location (parietal, cortical), clinical presentation
(seizures) and MRI aspect were typical for PXA. The question of patient’s age has been addressed in the manuscript.

The case PXA 4 showed indeed a higher MIB-1 index between 5-10% dependent on the tumor region analyzed. We have decided to ascribe it the higher score in order not to oversee the possibility of anaplasia. Since neither necrosis nor microvascular proliferation were observed and the mitotic index was <5 mitoses per 10 high power fields (HPF), the tumor was not considered as a PXA with anaplastic features. Further histological and immunohistochemical features were typical for PXA (giant pleomorphic tumor cells mono- and multinucleated, sparse foci of lymphocytic infiltrates, lipidized atypical astrocytes and reticulin rich areas).

In the case of PXA 5, additional atypical mitoses and foci of necrosis were observed, thus making the diagnosis of PXA with anaplastic features obligated.

In our investigation, clinical, radiological neuropathological and immunohistological evidences allowed us to make the diagnosis of PXA, ruling out the possibility of misinterpretation.

7. Table 1. At page 17 the Authors assert “high frequency of BRAF V600E mutations in WHO grade II PXAs and PXAs with anaplastic features (65 and 66% of cases, respectively)”. This statement is completely correct and these percentages have been confirmed by other studies (for example Chappé C et al, Brain Pathol. 2013). However in the present PXA series only 1 out of 10 cases (10%) presented BRAF V600E mutation. This huge difference in comparison to what usually observed should be explained by the Authors; otherwise it could be hypothesized that in this series have been included cases which are not PXA.

Analyses of PXA from the recent literature provide evidences of the implications of mutations of BRAF in brain tumors. Thus, Dias-Santagata (from David Louis group, Dias-Santagata et al. PLOS One, 2011) observed an incidence of mutations of BRAF of 60% from 20 investigated PXA. Similarly, they report a very low rate of BRAF mutations in glioblastoma multiforme (2.8% of 71 analyzed GBM), which may represent one valuable parameter to make the diagnosis glioblastoma less probably.

Schindler (from the group of Andreas von Deimling, Schindler et al, Acta Neuropathol., 2011) reported an incidence of 66% of mutated BRAF analyzing 64 PXA specimens. A rate of 2% of BRAF mutations were found in a collective of 133 GBM.
As the reviewer mentioned, Chappé et al. have also recently reported a rate of BRAF mutations in PXA of 60%, although in this study only 5 PXA cases were investigated.

Concerning CD34, Reifenberger et al. (Acta Neuropathol, 2003) reported a high rate of 84% immunoreactivity (investigating 44 grade II PXA). In anaplastic PXA the immunopositivity for CD34 was 44% (16 specimens were analyzed). These data is further confirmed by other groups (Koelsche et al., Brain Pathol. 2013, Fu et al., Neuropathology, 2010).

The low rate of BRAF mutations in the present series is rather interpreted as underestimation derived of the collective size. Nevertheless, the neuropathological profile of tumors in the present analysis is concordant with the data from the literature since we have observed a high rate of immunoreactivity for CD34.

**Minor Essential Revisions**

1. In the abstract the number of studied PXA should be added.
2. The location of tumors should be added (how many were temporal cases?).
3. Page 17, line 1: the first ref. is wrong and must be replaced with [41].
4. Page 18 “This precocious intervention would probably revert on a better prognosis and increased life expectancy”: the statement is completely speculative and should be removed.

All minor revisions have been addressed, and the corresponding corrections have been made throughout the manuscript.
Reviewer's report

Title: DNA Methylation Alterations in Grade II- and Anaplastic Pleomorphic Xanthoastrocytoma

Version: 3
Date: 24 December 2013
Reviewer: Çigir Avci

Reviewer's report: The article is well designed and may have important results in the field. But firstly, the number of the samples should be increased (especially PXA n=11 tumors, 10 patients) cases) to promote the accuracy of the work.

Level of interest: An article of limited interest

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests

Authors response to Referee 2

We fully agree with reviewer that the limited number of cases included on the study, and that it would be highly interesting to examine additional PXA samples, either in the discovery or in the validation phases. Due to the difficulty in getting such samples we could only perform pyrosequencing analysis of two additional cases. The clinical information has been included on Table 1, and pyrosequencing data was incorporated along with that of the previous cases examined.