Author's response to reviews

Title: ADAR2 editing activity in newly diagnosed versus relapsed pediatric high-grade astrocytomas

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Author's response to reviews: see over
Point-by-point

Referee 1

Minor essential revisions
1. A major drawback of this study is the limited number of paired diagnosis and relapse patient
tumor samples that was used, which can be explained by the rarity of the disease and the fact that
surgery at relapse is infrequently performed. It is therefore not possible to draw statistically valid
conclusions on the role of RNA editing at diagnosis and relapse. The authors acknowledge this in
their abstract (‘Despite the low number…’), but not in their manuscript. They should explain the
scarcity of material.

As correctly suggested by the Referee, we have now added the following phrase in the Discussion at
page 12, line 6:
“due to the rarity of this pediatric disease and the fact that surgery at relapse is infrequently
performed in these patients”.

Would there be a possibility to extend the number of samples, for example in collaboration with
other groups?

Unfortunately, we were unable to enlarge our cohort for this study, due to the rarity of these
pediatric tumors (biopsy at diagnosis and relapse in the same patient) and because we needed
tumor tissues with similar diagnosis (we included only high-grade astrocytomas excluding, for
eexample mixed lineage astrocytomas) and from a specific brain area (supratentorial), considering
that RNA editing varies among different regions of the brain (Cenci et al, 2008).
However, due to the intriguing results obtained from the present study, we will try to enlarge our
cohort in collaboration with other Research Institutes for a future multicentric study.

2. The authors state that only limited information is available on genetic and molecular alterations
in pediatric high grade gliomas. They however should refer to an important paper in this field by
Paugh et al: ‘Integrated molecular genetic profiling of pediatric high-grade gliomas reveals key
differences with the adult disease’ (JCO 2010; 28(18):3061-8).

Thanks to this Referee, we have now added this important reference at page 3, line 15.

The distinction between GBM (case 1, 2 and 4) and supratentorial PNET with glial differentiation
can be challenging. Has pathology been reviewed by two independent neuropathologists and has
PNET been ruled out?

In order to rule out any possible ambiguity in the diagnosis, each sample in our Institute (including
the ones involved in this study) is analyzed by two independent experienced neuropathologists
(usually, a resident pathologist and an external one, that is the national pathologist referee for
pediatric brain tumors). Specifically, both of them have confirmed the diagnosis reported in the
manuscript (Table 1).

4. Are the authors able to biologically explain the discrepancy of further ADAR2 reduction in case
1-3 at relapse (Fig. 3) and no substantial changes in RNA editing in this material?
As reported in the Results and Discussion, we detected a statistically significant further drop of editing in the recurrences when compared to the original tumors at some specific sites: GluR-6 Y/C and GluR-5 Q/R sites in Case 1; GluR-B R/G site in Case 2; GluR-B Q/R site in Case 3 (Figure 1 and Table 2), and this correlates with the ADAR2 mRNA reduction observed in relapses (Figure 3). Despite the few sites mentioned above, no substantial changes were found at the remaining sites. However, this might be explained by a previous report showing that there is not a direct correlation between ADAR expression and activity (Wahlstedt et al., 2009). Indeed, additional studies pointed out the possible existence of “mediators” (i.e. proteins) that can modulate ADAR efficiency at specific sites by protein-protein contact (Marcucci et al., 2011).

One possibility could be that in the evolution of cancer cells from the newly diagnosed tumor to its recurrence some “mediators” are expressed in order to support ADAR (despite its further decrease) in “maintaining” a basal/minimum editing level necessary for cell survival, as a total ADAR2 ablation is lethal (Higuchi et al., 2000).

Still, the correlation between ADAR2 expression and its activity remains to be fully elucidated.

Following the Reviewer’s comment, we have decided to include the following text in Discussion (page 12, line 18), to provide additional hints that can partially explain the discrepancy between ADAR2 expression and activity:

“Interestingly a recent study showed the existence of “mediators” (i.e. proteins) that can modulate ADAR2 efficiency (Marcucci et al., 2011).”

Do the different editing sites have different sensitivity to ADAR2 activity?

It has been shown that ADAR enzymes can edit different sites/substrates at different extent (Bass B., 2001). How ADARs can select the adenosine to be modified (selectivity) and at which percentage the deamination will be done (efficiency) is still under investigation (Kallman et al, 2003; Yi-Brunozzi et al., 1999; Stefl et al., 2006; Marcucci et al, 2011, Daniel et al., 2012).

Specifically, it has been shown that different RNA sequences/structures of diverse substrates may determine variations on selectivity and efficiency of ADAR2 (for example purines opposite to the editing site in the dsRNA structure necessary for ADAR2 binding has a negative effect on efficiency of editing) (Kallman et al, 2003).

5. Would the authors be able to speculate on potential shortcomings of the control tissue they have used? Does brain contusion result in altered RNA editing profiles or ADAR expression?

Finding the best compromise between ethical reasons and the necessity to collect brain tissues from healthy individuals matching for age (children) and brain area (sopratentorial) was really hard. Pediatric patients undergoing focal brain resection for head injury sequelae (e.g. brain contusion) have given us the possibility to match all these parameters.

Notably, we found that RNA editing profiles of head injury tissues and the editing profiles of brain control tissues reported in previous studies were comparable (Cenci et al., 2008, Galeano et al. 2010; Paz et al., 2007; Maas et al., 2001). Differently from the editing levels that are absolute values and, therefore, easily comparable among studies, RNA levels (qRT-PCR) of ADAR2 are arbitrary values highly dependent on different internal control genes, apparatus, protocols and specific primes. Considering the above, unfortunately, we cannot comment on ADAR2 mRNA levels in our control samples compared with other normal tissue samples.

6. The authors have mainly used US English. They therefore should change ‘favourable’ to ‘favorable’, ‘analysed’ to ‘analyzed’ and ‘summarised’ to ‘summarized’. Furthermore
‘immunohistochemistry’ (page ) should be ‘immunohistochemistry’ and on page 5 there is a typo in ‘reverse transcriptase-polymerase’.

Thanks to the Reviewer, we have now corrected all the points underlined and we have changed the text to US English accordingly.
Referee 2

Major compulsory revisions

1) The authors mention a relationship between grade and ADAR2 editing activity why did they focus on the comparison between diagnosis and relapse of high-grade gliomas only. They could have expanded easily the cohort with astrocytomas of lower grade and this could have strengthened their findings.

There is a connection between ADAR2 editing activity and astrocytoma grade of malignancy and, indeed, we and others have found that ADAR2 activity is progressively lost from astrocytoma grade I to grade IV (Cenci et al, 2008; Paz et al, 2007). However, in this case report we would take a close look on pediatric matched pairs of high-grade astrocytomas (that showed a clear drop of editing levels) at time of diagnosis and at their recurrence in the same patient, in order to see if ADAR2 activity is also connected with progression of disease. However, as properly suggested by the Referee, a comparative analysis of RNA editing profiles between pediatric low-grade astrocytomas (I and II grade) that show progression of disease and those that do not (Cavaliere et al., 2005; DeAngelis, 2001) could be an interesting issue to investigate in a future study.

2) The authors focus on the editing of five genes whose contribution to oncogenesis is not clear. Could they comment on that? Eventually test other genes susceptible to editing with a more direct effect on oncogenesis?

Since it has been shown that ADAR2 editing activity is strongly impaired in high-grade astrocytomas, we have selected specific sites edited mainly or exclusively by this enzyme, avoiding sites edited by ADAR1.

Regarding the contribution to oncogenesis of the substrates tested in this study, we would like to mention that Glutamate receptors (such as AMPA and kainates) are involved in cell proliferation and cancer (Luksch et al., 2011; Piao et al., 2009). Particularly, it has been shown that glutamate receptor antagonists can limit brain tumor proliferation (Rzeski et al., 2001) and that silencing of AMPA receptor subunits reduces glioma growth in vivo (de Groot JF et al.2008). Important studies have also demonstrated that editing events within GluR-B transcript (a subunit of AMPA channel) inhibits glioma cell migration in vivo (Ishiuchi et al, 2007; Ishiuchi et al, 2002). In addition, BLCAP and GABA(A) receptors have been found important for cell transformation (Zuo ZH et al., 2006; Li et al., 2012). Despite the above, there are not validated edited substrates that are also recognized as tumor suppressor genes or oncogenes.

Thanks to the Referee's comment, we have now included the following in the Introduction at page 4, line 13:

“Interestingly, it has been shown that glutamate receptor antagonists inhibit in vitro proliferation of several human tumor cells, including brain gliomas (Rzeski et al., 2001) and that silencing of a specific AMPA receptor subunit reduces glioma growth in vivo (de Groot et al., 2008). Furthermore, editing events within GluR-B inhibits glioma cell migration in vivo (Ishiuchi et al, 2002).”

In Results page 8, line 9:

“because these sites are mainly, if not exclusively, edited by ADAR2 (Higuchi et al., 2000).”

3) It is said in the manuscript that the relationship between editing and ADAR2 expression is not necessarily linear? Is ADAR2 activity equivalent for any gene susceptible to be edited this way?
According to a previous report, editing activity does not necessarily correlate with mRNA or protein expression of ADAR enzymes (Wahlstedt et al, 2009). Additional studies pointed out the possible existence of “mediators” (i.e. proteins) that can modulate ADAR efficiency by protein-protein interaction (Marcucci R. et al., 2011). However, to the best of our knowledge, literature does not report the existence of a clear/unequivocal correlation between ADAR expression and editing at a specific site. It has been reported that ADAR enzymes edit different sites/substrates at different extent (Bass 2001). How ADARs can select the adenosine to be modified (selectivity) and at which percentage the deamination will be done (efficiency) is still under investigation (Kallman et al, 2003; Yi-Brunozzi et al., 1999; Stefl et al., 2006; Marcucci et al, 2011, Daniel et al., 2012). Specifically, it has been shown that different RNA sequences/structures of diverse substrates may determine variations on selectivity and efficiency of ADAR2 (for example purines opposite to the editing site in the dsRNA structure necessary for ADAR2 binding has a negative effect on efficiency of editing) (Kallman et al, 2003).

Considering the reviewer question we have now added the following in Introduction at page 4, line 1:

“RNA editing levels depend on the different substrates/sites, cell types, tissues and developmental stage (Bass BL, 2001; Wahlstedt et al., 2009).”

Minor Essential Revisions

1) How do the authors explain why RNA editing of the target genes is similar at diagnosis and at relapse while ADAR2 levels are decreasing in 3 out of 4 cases?

As reported in the Results and Discussion, we detected a statistically significant further drop of editing in the recurrences when compared to the original tumors at some specific editing sites, GluR-6 Y/C and GluR-5 Q/R sites in case 1; GluR-B R/G site in case 2; GluR-B Q/R site in case 3 (Figure 1 and Table 2), and this correlate with ADAR2 mRNA reduction observed in relapses (Figure 3). Despite the few sites mentioned above, no substantial changes were found at the remaining sites; however, this is in agreement with a previous report showing that there is not a direct correlation between ADAR expression and activity (Wahlstedt et al. 2009). Indeed, additional studies pointed out the possible existence of “mediators” (i.e. proteins) that can modulate ADAR efficiency by protein-protein interaction (Marcucci et al., 2011). One possibility should be that in the evolution of cancer cells from the newly diagnosed tumor to its recurrence some “mediator” could be expressed in order to support ADAR (despite its further decrease) in “maintaining” a basal/minimum editing level necessary for cell survival (as a total ADAR2 ablation is lethal (Higuchi et al., 2000).

Following the Reviewer’s comment, we have decided to include the following text in the Discussion (page 12 line 18), to provide additional hints that can partially explain the discrepancy between ADAR2 expression and activity:

“Interestingly, a recent study pointed out the existence of “mediators” (i.e. proteins) that can modulate ADAR2 efficiency (Marcucci R. et al., 2011).”
2) Is ADAR2 level prognostic in pediatric astrocytomas, especially high-grade ones? There is plenty of gene-expression data out-there to test this issue.

Since no direct correlation was reported between ADAR2 expression and editing activity (Wahlstedt H. et al., 2009), we did not screen ADAR2 mRNA levels at large scale, as properly suggested by the Referee. Now, considering the results obtained from Case 4, in which a rescue of ADAR2 expression and activity was found, it will be intriguing to correlate ADAR2 levels (available from several databases) with patient outcomes (information that is more difficult to obtain) in a future study.

3) If the only patient recovering editing activity has a good prognosis, this should stimulate the authors to study non recurring patients as well.

It has been previously shown that ADAR2 editing activity progressively decreases from low to high-grade astrocytomas (Cenci et al, 2008, Paz et al, 2007; Maas et al 2001). Herein we have described a recurrent GBM of a patient in remission showing high editing levels (recurrence of Case 4). Therefore, high editing levels seems to be linked with a less malignant feature of the cell. On this regard, as correctly suggested by the Referee, it will be attractive to study RNA editing in non recurring astrocytomas both low and high-grades (see also reply to the point 1).

Discretionary Revisions

Is there an antibody to detect ADAR2? Where is it found in the tumor?

At present we have not found a good antibody to detect ADAR2 in tissue sections by IHC. We have tried several ADAR2 antibodies but they did not show expected (nuclear/nucleolar localization) and consistent (similar results with different Abs) cellular patterns in control/normal brain tissues (personal data).
Referee 3:

Major compulsory revisions

1. Analysis of Ki67: In which manner were the immune-histochemically analyzed Ki67 proliferation indices on the paraffin-embedded tissues evaluated shown in Table 1? Did you use any computational evaluation or manually counting the number of positive stained cell nuclei per high power-field? Or is it based on the investigator’s experience? Please comment on that.

Ki67 proliferation index was evaluated by two independent experienced neuropathologists (a resident pathologist and an external one), counting the number of Ki67 positive stained cell nuclei per total number cells in at least 10 random fields by manual counting.

We have now included the following in Material and Methods at page 7 line 15:

“Ki-67 levels were also evaluated by IHC on the paraffin-embedded tissues by two independent experienced neuropathologists.”

To test the Ki67 expression levels (shown in figure 2B), also three independent RT-PCRs were performed? If not, why not? If yes, please add error bars to the graph. [Maybe it would be better to separate figure 2B into 2B (gel electrophoresis) and 2C (graph of mRNA expression levels).

The Ki67 expression levels were evaluated by three independent semi qRT-PCRs. As properly requested by the Referee, the new Figure 2 now includes panel B, with the gel electrophoresis, and panel C, showing the graph of mRNA expression levels (densitometric analysis) with the error bars of three independent experiments.

We have corrected the Figure 2 legend at page 18 accordingly:

“(B) a representative example of Ki-67 mRNA expression levels analyzed by semi-quantitative RT-PCRs in the control (normal white matter), newly diagnosed and recurrent tumor samples of Case 4. (C) Densitometric analysis of Ki-67 mRNA expression is represented in arbitrary units calculated as a relative-fold increase in expression compared to the control arbitrarily set to 1. Each sample was normalized to β-actin mRNA. Error bars indicate standard error of the mean (S.E.M.) (n=3).”

2. Because of its age at diagnosis, the child in case 4 underwent significantly different treatment with pre-surgical chemotherapy and a later onset of irradiation. The impact of these treatment differences should be discussed in reference to the more favorable outcome of this patient.

Thanks to the Referee’s comment, we have now realized that an error occurred in Table 1: Case 4 did not undergo pre-surgery CT treatment, but underwent pre-radiation CT due to the very young age at time of diagnosis (2.3 years). As soon as the patient reached the 3 years old, he was eligible for the RT treatment as reported in Table 1. Indeed, neither of the patients (1-4) described in this case-report underwent pre-surgery treatments.

We have now corrected the Table 1 accordingly. Furthermore, we have slightly modified the sentence in Discussion at page 13, line 16:

“The hypothesis that younger patients (as in the Case 4 reported here) might be able to recover ADAR2 expression/activity, due to still unknown endogenous cellular factors or induced by specific treatments/drugs, and that this recovery may contribute to a more favorable outcome of these patients deserves additional investigations.”
3. Figure 2A shows no evaluation of statistical significances. While it is clear for GluR-B, GluR-6, GluR-5 and Kv1.1 from figure 1, it is not shown for Gabra-3 and BLCAP. Please add p-values or the asterisks, respectively.

We have now added the statistical significance for all the sites shown in the new Figure 2A, including data regarding Gabra-3 and BLCAP substrates. We have corrected the Figure 2 legend accordingly.

Additionally we have now included a comment on this figure in Result section page 9, line 12:
“Of note, the only site of BLCAP transcript showing a significant editing rescue was the K/R site, which is the only one mainly modified by ADAR2 (Galeano et al, 2010).”

4. Why do you focus on transcripts that translate into brain membrane receptors and ion channels, respectively? Could you briefly comment on that?

Since it has been shown that the RNA editing activity mediated by ADAR2 is strongly impaired in GBMs, we have selected specific sites mainly edited by this enzyme, avoiding sites edited by ADAR1. Indeed, validated ADAR2 substrates are transcripts coding for brain membrane receptors and ion channels as analyzed in this study.

Moreover, glutamate receptors (such as AMPA and kainate channels) are involved in cell proliferation and cancer (Luksch et al., 2011; Piao et al., 2009). Particularly, it has been shown that glutamate receptor antagonists can limit brain tumor proliferation (Rzeski et al., 2001) and that silencing of AMPA receptor subunits reduces glioma growth in vivo (de Groot JF et al.2008). Important studies have also demonstrated that editing events within GluR-B transcript inhibits glioma cell migration in vivo (Ishiuchi et al, 2007; Ishiuchi et al, 2002). GABA(A) receptors have been found important for cell transformation as well (Li et al., 2012).

Following the Referee’s question a new sentence, clarifying this point, was added to the Result section, page 8, line 9:
“because these sites are mainly, if not exclusively, edited by ADAR2 (Higuchi et al., 2000).”

Please introduce and explain also Gabra-3 and BLCAP in the “Background” text.

As suggested by the Referee, we have now introduced a short paragraph about these two edited proteins in the Result section at page 9, line 4:

“We performed RNA editing analysis of the Gabra-3 I/M site (edited by both ADAR1 and ADAR2) [19], the BLCAP Y/C, Q/R sites (edited by both ADAR enzymes) and the K/R site (edited mainly by ADAR2) [20, 21] in the tumor tissues of this patient, comparing the results with the controls. Editing within the Gabra-3 transcript controls trafficking of α3-containing receptors to the cell membrane (Daniel et al, 2011). Despite the fact that the role of editing events within BLCAP are still unknown, it has been proposed that this protein is a novel prognostic biomarker in bladder cancer and it is associated with cell proliferation (Moreira et al 2010).”

Minor essential revisions

1. Typographical errors:
a. Material and Methods, Patients and samples collection: lane 4: immunohistochemistry instead of immunohistochemistry.
b. Materials and Methods, Editing analysis: lane 2: Carlsbad instead of Carlsband.

Thanks to the Referee, we have corrected the above points.
2. Abbreviation: The abbreviation RMN in table 1 should be explained in the text below.

We have now changed Table 1 trying to simplify the data reported, specifically the abbreviation RMN has been now removed as we believe it is redundant.

Discretionary revisions:

1. Maybe it would be helpful to introduce the term “recoding” in the “Background” part.

We have included the following in Introduction at page 4, line 9: “editing events that change amino acid sequence (recoding editing)”

2. To highlight the techniques of editing analyses a typical sequence chromatogram with nucleotides that undergo editing and thereby create a double peak could be included.

As suggested by the Referee, we have created a new figure as Supplementary material (Figure S1), if the Editor agrees. The Figure S1 shows one edited site (GluR-5 Q/R site in control vs Case 4) as example of the typical double peak.
Additional Reference for the Referee


