Author's response to reviews

Title: Downregulation of programmed cell death 10 is associated with tumor cell proliferation, hyperangiogenesis and peritumoral edema in human glioblastoma

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Author’s response to reviews: see over
Response to Reviewer Dr. Gwenola Boulday

Reviewer's report:
In the article entitled “Downregulation of programmed cell death 10 is associated with tumor cell proliferation, hyperangiogenesis and peritumoral edema in human glioblastoma”, Lambertz et al. have investigated the pattern of PDCD10 expression in a cohort of 27 human primary glioblastomas. They show that PDCD10 mRNA and protein expression is downregulated in glioblastomas compared to control samples. PDCD10 expression is absent in proliferative tumor cells as well as in tumoral microvessels. In contrast, PDCD10 expression colocalizes with caspase 3 positive apoptotic cells in pseudopalisades. The authors also show that level of PDCD10 expression is inversely associated with tumoral microvessel density, MGMT methylation and peritumoral edema.
Altogether, the results presented in this interesting descriptive paper suggest that PDCD10 could be involved in the fate of tumoral cells. Level of PDCD10 expression could therefore modulate the severity of GBM.
Several points should be clarified to improve the quality of the paper especially within the results section. A clarification is requested concerning the status of the RhoA signaling in GBM samples.

Major compulsory revisions
1-Paragraph 5 of the result section and Fig5C: RhoA activation.
A clarification is required to be able to conclude concerning activation of the RhoA signaling pathway. It is an important point since in the CCM field, it is now established that loss of any of the CCM genes leads to RhoA activation even though it was controversial at first for CCM3. RhoA activation is usually revealed by analyzing level of GTP-RhoA compared to total RhoA. In the material section, authors report the use of a rabbit anti-phosphoRhoA antibody from Calbiochem. It is the anti-RhoA pSer188 pAb? The authors show in Fig 5 an enhanced pRhoA in GBM samples compared to controls but conclude in the main text that RhoA signaling is activated in GBM samples. This is very confusing since to my knowledge, an upregulation of RhoA phosphorylation at Ser 188 leads to inactivation of this signaling. Can the authors confirm the material used to perform this experiment? Total RhoA should be also provided as a control. Other classical read outs of the RhoA signaling pathway (GTP pulldown assays, staining of pMLC…) could be helpful to conclude on this point.

Response: Many years ago we ordered pSer188-RhoA antibody (Calbiochem) (the company “Calbiochem” no longer exists since long time) and total RhoA antibody for study of RhoA signaling after silencing CCM1-3 in endothelial cells, and the antibody pSer188-RhoA never work well in Western blot in our lab. Since the Western blot showed in Fig. 5D of last version of our manuscript was done years ago, the original blots are no longer available and so we are unable to confirm this result in original Western blot membranes. We deeply apologize for the wrong description of the antibody and the data in last version of the manuscript. Now we have accepted the reviewer’s suggestion and have detected p-MLC2 (Cell signaling) and total RhoA...
(Santa Cruz Technology) on newly prepared Western blot membranes. The new Western blot data have been shown in revised version of Fig. 5C and 5D. Meanwhile, we have changed the text accordingly in revised manuscript (underlined text on page 6 and page 10). Here, we appreciate again the reviewer for her carefully reading and for giving us the opportunity to correct this mistake.

Discretionary Revisions
2-Throughout the Results section, short introduction/conclusion sentences explaining the purpose/conclusion of experiments found in each paragraph would improve the manuscript (i.e. why analyzing pAkt? What is the conclusion of seeing CD68+ cells also positive for PDCD10? Why analyzing methylation status of MGMT? Is it expected to find about 37% of GBM samples with MGMT promoter methylation?).

Response: Thanks for reviewer’s suggestions. We have added related background information for detection of p-Akt and for the promotor methylation of MGMT (line 180 on page 7; line 182-183 on page 17 and line 243-247 on page 10).

PDCD10 is a ubiquitously expressed protein. So if it is absent in a specified cell population, it implies a possible alteration of the cellular function. The expression of PDCD10 in CD68-positive cells suggests that macrophage is not the cell type accounting for the PDCD10 deficiency in GBM tissue.

It has been shown that epigenetic silencing of the O(6)-methylguanine-DNA-methyltransferase (MGMT) gene by promoter methylation is correlated with improved progression-free survival (PFS) and overall survival (OS) in adult GBM. Therefore, promoter methylation of MGMT gene is a favorable prognostic factor for GBM patients. According to literature, methylation of the MGMT promoter is found in 35%–45% of malignant gliomas (WHO grades III and IV) (Ohgaki H et al., Am J Pathol. 2007;170(5):1445–1453). The present study showed 37% of GBM patients with MGMT promoter methylation, which is in agreement with the statistical data published in literature.

3-The genotypic/phenotypic heterogenicity of GBM described by the authors in the introduction as well as in the last sentence of the abstract is not clear to me. What about the 27 samples of GBM used in this study? Is the cohort representative of this heterogeneity? Can authors comment that point?

Response: Mutation on several genes such as PTEN, p53, IDH1 and LOH 1p/19q and epigenic mutation of MGMT have been often detected in GBM patients. Therefore, it is commonly accepted that GBM displays the genotypic heterogeneity. Glioblastoma is also undoubtedly the most polymorphic of any known neoplasm, which is the reason for its previous name as “Glioblastoma multiforme”.

We have accepted the advice of the reviewer and modified the related sentences (underlined text in abstract on page 2 and in background on page 4).
Twenty-seven GBM were consecutively collected from adult patients with primary GBM who were treated in our department. The inclusion criterion was the histopathological diagnosis of primary GBM according to WHO classification. So the cohort is representative of the heterogeneity of GBM.

4-2nd paragraph of the result section: Features of GBM and Fig2 Description of the histopathologic features of GBM in the main part of the text could be more precise. Indeed, it is difficult for non specialist of the field to understand the cellular organization of the tumor (only astrocytes? Oligodendrocytes? Same cell type(s) in areas, in pseudopalisading area, in densed proliferative tumor cell areas, in areas surrounding the thrombosed vessels? Where is the “infiltrating area”? ). Only GFAP staining is performed on GBM sections together with PDCD10. What about other normal neuro-glial markers? Tumoral neuro-glial markers? PDCD10 positive cells are negative for GFAP so what do the authors conclude about the nature of those cells? In the figure 2 there is no comparison possible between control and GBM samples. The Panel G shows PDCD10 positive cells in a control section that the authors claim to be neuronal cells. This is a high magnification, without any other neuronal-specific staining. What about glial cells in control samples? In panel L is it really ECs that are PCNA positive? A high magnification would be helpful.

**Response:** We have accepted the suggestion of the reviewer and described histopathological features of GBM in a more comprehensible way in revised version of manuscript (underlined text on page 7-8).

**Cellular organization of GBM** is a complicated issue. As described in the book “WHO classification of tumors of central nervous system” (edited by Louis et al.), “few human neoplasms are heterogeneous in composition as it glioblastoma”. Microscopically, GBM is a densely cellular and poorly differentiated neoplasm that is composed of pleomorphic astrocytic elements. The individual cells can be various forms including cells having granular or lipidized cytoplasm, multinucleated giant cells (giant cell GBM), and small cells with dense nuclei (small cell GBM). The inclusion criterion of present study was the histopathological diagnosis of primary GBM (according to WHO classification, without the oligodendrogial component). As a research paper focused on PDCD10, we presented the typical histopathological features of GBM, i.e., microvascular hyperplasia and necrosis (Fig. 2A-C).

**Pseudopalisading area** is formed by accumulated tumor cells. Due to hypoxia, many tumor cells in pseudopalisading underwent apoptosis (Fig. 3C-F), and part of these tumor cells attempt to escape from the hypoxic area migrating toward to distant area where neo-angiogenesis is active; this distant area contains dense proliferating tumor cells and microvessels, which is defined as “infiltration area” in the present study.

As mentioned above, GBM is composed of pleomorphic astrocytic elements. Thus, immunostaining of GFAP can confirm the astrocytic lineage of GBM. The GBM slides used in the present study contained only tumor core and infiltration area without peritumoral components, and therefore, there is principally no neuronal cells in these tumor materials. Staining with other glial markers is rarely used in GBM. GFAP is a marker of astrocytic differentiation. Our double
staining showed absence of PDCD10 immunoreactivity in GFAP-positive cells (Fig. 2I), suggesting lack of PDCD10 expression in differentiated astrocytic tumor cells. It needs to be further studied in the future what is the functional meaning of PDCD10 absence in differentiated astrocytic tumor cells.

The surgical specimens of patients who underwent anterior temporal lobe resections due to temporal lobe epilepsy are commonly used control materials for the experimental research of neurosurgical diseases. Such control materials have been also used in our previous studies such as recent paper published in NeuroOncology (El. Hindy et al., NeuroOncology 2013;15(10):1366-78). It is known that this type of control tissue contains mainly neurons; glial cells are normally under rest status if any. Presenting PDCD10-staining from the control section (Fig. 2G) is just to confirm PDCD10 as a ubiquitously expressed protein. To convince the reviewer for expression of PDCD10 in control section, we have replaced the high magnification photo with a photo made with low-magnification, in which PDCD10 immunoreactivity is clearly detected in nearly all cells in the control. We have also removed the description of “neuronal expression” of PDCD10 in control in revised manuscript.

We have accepted the reviewer’s suggestion, and added high-magnification view of an endothelial cell in white box inserted in Fig. 2L in revised manuscript, in which PDCD10-negative but PCNA-positive staining in nuclear of EC could be clearly seen.

5- Peritumoral edema analysis: MRI should be mentioned in the methods together with the cohort description. How many patients underwent MRI? Who are the patients showing a edema Grade 0 in Fig5B-D (controls)? Do Astro II patients show any peritumoral edema?

**Response:** As suggested by the reviewer, MRI is now mentioned together with the cohort description (underlined text on page 6 in revised manuscript). All 27 GBM and 13 astrocytoma grade II patients included in our study underwent MRI. No edema could be detected in six astrocytoma grade II patients and one GBM patient. All other patients showed a peritumoral edema ranging from grade I to III.

6- Main text, 4th paragraph of the results section and Fig4: Description of the results concerning PDCD10 mRNA and protein expression in GBM versus control samples as “fold of change” in panel A and “fold” in B is confusing. It would also be interesting to know how many GBM samples are composing the 2 subgroups.

**Response:** To avoid confusion, we have improved the result description (underlined text on page 9 in revised manuscript) and figure presentation (revised Fig. 4B). The number of samples for these two subgroups is now indicated in the figure legend of Fig. 4 (underlined text on page 23).

7-In the discussion section, authors might be more cautious with the use of “hyper-angiogenesis” as a “phenomena” occurring in the human CCM pathology even though knock down strategies in vitro in endothelial cells have demonstrated enhanced angiogenesis. Mechanisms involved in the CCM pathogenesis are still to be clarified.
**Response:** We agree with the reviewer’s comment and the mentioned sentence has been deleted.

8- In the background section “Moreover, much attention…Pdcd10 heterozygous mice.” In the paper from Chan et al. used as a reference in this sentence, Pdcd10 heterozygous mice do not develop CCM lesions. The sentence should be modified.

**Response:** We are sorry for improper description. The sentence has been modified according to the paper by Chan et al. (line 84-85 on page 5).

Minor essential revisions
9-Scale bars should be added to Fig2, A-C Panels.
**Response:** We have indicated the magnification in Fig.2A-C.

10- Fig Legend Fig1, the following sentence is not clear : “The level of GFAP elevated … grade II”
**Response:** The sentence has been changed as underlined in the Fig.1 legend (page 21).

11- Main text, 2nd paragraph of the results section, the sentence “Necrotic foci… are composing pseudopalisades” has to be clarified
**Response:** Description of the histopathological feature has been overall improved (underlined text on page 7-8).

12- In the discussion, the conclusion sentence in paragraph 1 has to be clarified.
**Response:** The sentence has been modified as underlined on page 14.

13- last sentence of the background section: “The expression of PDCD10 was associated with tumor cell survival signaling… genotype of GBM” has to be clarified.
**Response:** The sentence has been modified as underlined on page 4.

14- Last sentence of the Abstract background needs to be clarified “We attempted…its expression with genotype and clinic phenotype… »
**Response:** The sentence has been modified as underlined in abstract (page 2).

15- Typos
Fluorescence/ fluorescent instead of “fluorescent” throughout the manuscript Signaling instead of “signalling” in the abstract In results section second paragraph: Severely instead of “severally” 5th paragraph: “the association analysis… (p>0.05)”, is probably "<”

**Response:** spelling for “Fluorescence/fluorescent has been proved throughout the text. We have changed British to America spelling standard in revised manuscript. So “signaling” is used throughout the text.
Sorry for misspelling. “Severely” has been replaced with “severally”.
Since there is no statistical significance in association of PDCD10 expression and MGMT promoter methylation, p > 0.05 is correct.