Akceptuję

Analysis of the doctoral thesis presented by Mgr Salwador Cyranowski
With the title: „***The role of chitinase-3-like protein 1 in the pathobiology of gliomas***"
In Medicine and Health Sciences
Defended at the Warsaw University of Medicine.
Research performed at : Pracownia Neurobiologii Molekularnej, Instytut Biologii Doświadczalnej im. M. Nenckiego Polskiej Akademii Nauk w Warszawie
Studium Medycyny Molekularnej, Wydział Lekarski, Warszawski Uniwersytet Medyczny

The aim of the study is to determine the role of the CHI3-L1, a protein that is differently expressed in distinct types of gliomas and its possible involvement in the invasiveness ability of the malignant glioma making it a valuable target for the efficacy of treatment.
The plan and setting of the manuscript are clearly summarized in Polish and English.
This abstract is concise and clear enough, to make logically appear the link between the main findings about the lectin CHI3L1 and glioma aggressiveness through its effect on the MMP2/osteopontin/vascularization axis. Further on, the strategy described by Mgr Salwador Cyranowski consists in identifying the effects of the activity of the CHI3L1 on the glioma properties building an orthotopic model of implantation to study the development of the human brain cancer cells in athymic mice. The selected glioma is first genetically engineered not to express the molecule of interest in order to further find out the differentially expressed genes and their effects on the glioma biology.
The protein of interest: Chitinase-3-like protein 1, is first described in the introduction pointing out its lectin properties as it recognizes glycosides, mainly the chitobiose derivatives. It is called a chitinase enzyme, as such it should recognize the N-acetyl glucosamine oligomers rather than the oligomers of glucosamine. Although the name "Glycoside hydrolase" is commonly accepted, the term "Glycosyl hydrolase" is better describing an enzyme that cuts a glycosidic bond rather than a oligosaccharide as the term glycoside hydrolase would mean (for glyco"fans" only).
But as evolution caused the loss of enzymatic activity, the CHI is a Lectin (CHI3L1). It recognizes a large spectrum of sequences of glycosylated molecules, mainly high MW polymers. It also recognizes proteic ligands in a mode of action frequently encountered in glyco-specific molecules that are acting as important co-receptors with multiple modulatory mechanisms. For example, the recognition of interleukins, and receptors and the over regulation by other Lectins as Gal3, is evidencing the importance of the inter-regulation of the immune control by glycosylation-dependent recognitions and signaling.
This is described in the *introduction*. Moreover, the complexity of CHI3L1 activities in relation with various receptors and its effects at several levels of tumor development are summarized graphically.
The introduction presents nicely a logical progression describing the involvement of CHI3L1 in immunomodulatory cells in brain pathologies up to a clear explanation showing why it is an important molecule in cancer cells themselves, especially in glioma. At the molecular level, the relation with the immune checkpoint molecules is pointed out.
Logical progression leads to the description of glioblastoma, the hallmarks of the pathology, the characteristics of the invasiveness at the cellular and molecular levels involving matrix degradation enzymes as MMPs, and ADAMs, ADAMTS.
The cellular composition of the glioblastoma site is analyzed with a special attention to the infiltration by myeloid cells that participate to the tumor microenvironment which in turn, is permissive for tumor expansion. But this is orchestrated by the angiogenic switch response to the tumor cell growth. Here

pathologic angiogenesis is approached, as well as the interest of normalization of the newly developed vasculature (Fig. 1.4). In the present work it is mainly considered for its consequence on tumor cells accessibility to treatments.

The state of the art summarizing the known roles of CHI3L1 in glioblastoma shows the need for more information about the mechanism of its modulation and regulation. Additional examples of such need are: implications of CHI3L1-Gal3-Gal3BP protein complexes on the deficits of T cell–mediated responses in GBM progression; the CHI3L1-increased VEGF expression by signaling through syndecan-1, integrin αVβ5 and phosphorylated FAK; the CHI3L1 implication in GBM resistance to bevacizumab (anti-angiogenic as VEGF inhibitor) directly affecting the GBM vasculature and invasiveness.

Minor remarks

Several references are missing; for example: on Page 12, the sentence "CHI3L1 up-regulates VEGF (vascular endothelial growth factor) expression in glioma U87-MG and SNB-75 cells, and both proteins synergistically promote endothelial cell angiogenesis in vitro" should mention a reference. This is true also on pages 16 and 17.

Speaking of "cell death 1 (PD-1) and PD-1 ligand 1 (PD-L1)": are proteins not names.

It is worth noticing that writing should use either the present or the preterit, but not both in the same sentence as in: "An increase in the expression of collagen-coding genes, especially COL3A1, COL4A1 and COL5A2, is associated with a malignant growth in the brain as the tumor utilized collagen scaffolding to pave its invasion."

In the figure: Gal3 is not mentioned.

More important remarks concern:

a few too restrictive criteria, as in the description of "The neovasculature of glioblastoma: "Aberrant vasculature is a hallmark of glioblastoma pathology." This is a hallmark of all solid tumors.

Moreover, it should be remarked that angiogenesis is necessary during the fetal development or during healing but in developed organisms, a proper vasculature is necessary.

The sentence: "This contributes to hypoxia, necrosis and immunosuppression, and creates a specific TME". It seems that the process should be understood the other way around. It should be considered that hypoxia is the first event that turns on the other events, simply because O2 availability is not sufficient when the tumor cells grow, as O2 diffusion is restricted (< 100 micrometers).

As Mgr Salwador Cyranowski writes "Despite the BBB being disrupted and more permeable, the delivery of anti-tumor drugs to the tumor remains a key challenge in the treatment of GBM" this is very true and has important consequences indeed, could this point be commented?

As the introduction is insisting on angiogenesis (induction of VEGF for example) it is surprising that hypoxia is not mentioned.

Similarly, and together with the above remark, the sentence: "Given the obstacles in the drug delivery to the site of GBM, the normalization of vasculature is desirable for an effective anti-tumor activity in GBM" would need to be more commented in view of the effects of normalization of the vasculature, the literature shows this point clearly in various cancer types.

Considering the molecular mechanisms of the CHI3L1 in complex with Gal3- Gal3BP protein complexes which regulate infiltration and reprograming tumor-associated myeloid cells and is resulting in deficits of T cell–mediated immune responses in GBM, it appears that this very interesting mechanism recalls the work by Gabriel Rabinovitch on Gal1-mediated activation of VEGF receptor in the absence of hypoxia. Could you comment on this possible similarity of action Gal1 to VEGFRec and CHI3L1 GAL3-

Gal3BP? This might be important as CHI3L1 signaling was also implicated in GBM resistance to bevacizumab (the VEGF inhibitor and anti-angiogenic drug) similarly to the Gal1 mediated resistance to bevacizumab.

After those points and remarks for discussion and requests for Mgr Salwador Cyranowski's opinion one can say that the 15 pages-introduction, is well built and clear. It shows the interest of studying the CHI3L1 lectin and its role in angiogenesis. The regret is the lack of attention to hypoxia a cancer hallmark, for its key and fundamental effects in glioblastoma, which is known to be highly responsive to the pO2 variations. One can also remark that the expression of the important molecules analyzed in this introduction is hypoxia-dependent.

The materials and methods section: from page 25 to 38, shows that the biochemical methods are familiar. The descriptions are adequately more detailed in the case of the newest methods as CRISP/R Cas-9 gene modification.

There are a few remarks concerning some descriptions which omit to show the complete understanding of the principle of the methods. This is surely due to the fact that the PhD student is very handy and more than familiar with all presented methods but still the manuscript remains a doctoral thesis and it is required to show that knowledge.

In a PhD it is adequate to express what is the key principle of a method and not write only "the manufacturer's protocol". For example, why electroporation is useful to introduce a plasmid in a cell? what is its advantage over a lipofection and why this can happen?

The description of the Fluorescence-activated cell sorting (FACS) and clonal selection is confusing as it is not first said that the aim is cloning U87-MG-RFP cells successfully transfected with pCMV-Cas9-GFP plasmid. This is necessary for the reader to expect the proper cytometer settings.

Similarly, in the ELISA section, a reader cannot learn nor understand what is an Enzyme linked immunoassay, although a PhD is the occasion to show that the student knows what is the mechanism of an experiment. Again, the description "according to the manufacturer's instructions" should be forbidden at this level.

This lack of accuracy is confirmed in the description of Fluorescence microscopy where the names of the antibodies are not proper. The names do not permit to control that the experiment was done correctly. Which immunoglobulin class was used at each step? Indeed, the name of a labeled antibody should be (for the second step antibody for example): the fluorophore, the species where the antibody comes from, its specificity (species and Ig class), and its class of immunoglobulin; for example, FTC-rabbit IgG anti mouse Fab. This is true at all steps of a scientific carreer.

Considering the Matrigel invasion assay, it is highly appreciated that it is done in the presence of an attractant (cells) in the lower compartment.

In general, and despite the above remarks, the reader sees that the methods are well controlled and understood.

The results section brings together the main findings *in vitro* and *in vivo* about the involvement of CHI3L1 in the biology of glioma.

The results of the strategy start by the determination of CHI3L1 expression in gliomas of various WHO grades, demonstrating its overexpression in GBM, but also in the benign PA tumors.

It would be very interesting to study how the protein activity is controlled in PA. Is this studied?

The accurate determination of the cellular source of CHI3L1 in glioblastoma demonstrates, at the single cell resolution, that it is expressed predominantly by malignant cells, and in glioma-associated macrophages.

Moreover, an important finding concerns CHI3L1 expression that is positively correlating with the mesenchymal genes expression.

Those data obtained at single-cell resolution are very nice and convincing.

Using human glioma cell lines, the CHI3L1 expression study aimed to select the best candidate to use for the knocking down of the CH3L1 gene. The result is the selection of U87-MG glioma cells and patient-derived WG9 cells as highly expressing the gene and the protein as presented in figure 4.5. Although it might be obvious, data normalization for the cell number and density should be mentioned/explained.

CHI3L1 KO cells were obtained using CRISPR/Cas9 genome editing, selection of the U87-MG-RFP sgCHI3L1 transfectants was necessary and based on the fluorescence analysis of the doubly labelled cells. The figure 4.8 which shows those data would have been convincing by showing separately the green fluorescence not only the merge. But the cells are further "chosen" by flow cytometry selection by which the sort is clear and objective. Consequently, the data are totally validated. It would have been appreciated to see the cell fluorescence after sorting and of the further cultured clones.

Yet, altogether, the validation of the CHI3L1 depletion is quite convincing and consequently the conclusion that the extracellular CHI3L1 is not a factor governing glioma cell proliferation in vitro, is an important and solid point.

The transcriptome changes in CHI3L1 KO cells defined by analyzing the differentially expressed genes in the KO variants clearly pointing to MMP-2 and its action in the scaffolding of collagen, which is highly up-regulated in GBM.

Moreover, the immune check point genes involved in PD-1 and IL-10 signaling were downregulated in CHI3L1 KO cells. *It would be interesting to have a more precise explanation about the genes that are effectively involved.*

Importantly, the mesenchymal-associated genes in single-cell database are strongly downregulated in CHI3L1 KO compared to WT cells. Thus, the CHI3L1 KO cells loss of the mesenchymal transcriptomic prolife is a very important type of data to control dedifferentiation.

The data were further validated by a series of *in vivo* experiments conducted to show the impact of CHI3L1 depletion on tumor growth.

Indeed, depletion of CHI3L1 in U87-MG human glioblastoma cells reduces tumor growth in mice in a very convincing way which deserves congratulations.

CHI3L1 concentration is augmented in blood serum of tumor–bearing mice. This very interesting experiment which shows that the allogenic implantation is nuancing the effects of the xenogeneic situation (in athymic mice) where the immune response is impaired. This is in accordance with the limits of experiments performed in such mice which on the other hand, permits the allogenic model setting, and is better than the xenogeneic situation, until a proper syngeneic model will enrich the conclusions. Despite the above-mentioned limitations, the study is adequate to demonstrate the reduction of the myeloid cells' infiltration in CHI3L1 KO tumors.

Similarly, studying the mechanisms of reduced growth of CHI3L1 KO tumor, *in vivo*, do demonstrate that CHI3L1 KO cells have reduced ECM degradation capacity through the decreased

MMP-2 expression/activity, thus diminishing ECM degradation and glioma cell invasion, as well as the decrease in the tumor-derived SPP1 production reducing myeloid cell attraction.

What would be the best choice as syngeneic murine with similar characteristic?

Finally: study of the impact of CHI3L1 depletion on the tumor microenvironment is addressed to effect on the glioma vasculature development.

No direct correlation was found between the vessels and microvessels numbers and structure but a direct evidence was shown for the normalization characters as: laminin expression and distribution at the basement membrane of the endothelium, CD31 expression indicating the structure of the tubes formed in the CHI3L1 KO tumors, and the astrocyte covering of the vessels, with AQP4 proper expression and localization at the astrocytic endfeet and concomitant reduction of the GFAP.

At this point Fig 4 27 showing the correlation analysis for VEGFA and CHI3L1. It would be, necessary in the future, to determine from which murine cells both VEGFA and CHI3L1 are coming.

Altogether, presented results show that the expression of CHI3L1 promotes the deregulation of the vascular network in glioma with disruption of the structure of vascular walls, impaired coverage of blood vessels by astrocytes.

Those fundamental data are obtained with the use of the best modern methods which make them solid and highly interesting. Those data are fundamental for a future strategy aiming at vasculature normalization.

Discussion

It displays the same positive characters as the whole manuscript. Only few remarks should be added as on Page 67, noticing the importance of analyzing the phenotype of the recruited myeloid cells and insisting on the significance of other genes involved, as SPP1 being down-regulated when CHI3L1 is silenced and is, in addition, associated with the mesenchymal signature.

The subject raised by the observation that mCHI3L1 was detected in mice bearing WT tumors (Fig. 4.17B, left) – suggests that this increase that can be associated with the response of the tumor microenvironment i.e., astrocytes, pericytes, endothelium, accumulated macrophages coming from the host.

Thus, growing glioma tumors stimulate the TME to release CHI3L1 with no evidence for autocrine signaling via CHI3L1 in glioma cells. The question arises: does the CH3L1 need cell-to-cell contact?

The vessel normalization effect of CH3L1 KO which is presented as highly desirable for effective anti-tumor activity looks very promising if CHI3L1 is confirmed as a good target. Since the model proposed in Fig 4.28 is too basic, it could it be suggested a place for CHI3L1 in the tumor angiogenesis molecular cascade comparatively to the Gal1-VEGFrec-VEGF cascade in the same process.

In conclusion:

This thesis is a very valuable manuscript that reflects innovative and highly significant scientific strategy and data. The candidate has participated to 12 publications according to WOS and is the first author in one paper published in Neuro-oncology in 2022, and a review in annals in Expt. Med. and Biol., second author in Annals in Oncology in 2019.

In summary, I declare that the data obtained represent a high scientific value and the author shows that he can approach and also explain and discuss the value of his experiments and data.

Mgr **Salwador Cyranowski** showed that he knows and manages with a large number of sophisticated techniques and that he can use them adequately, present, criticize and interpret them.

Z pełnym przekonaniem stwierdzam ze rozprawa doktorska mgr Salwador Cyranowski pt. :
„The role of chitinase-3-like protein 1 in the pathobiology of gliomas**", ma oryginalny charakter, swiaczy umiejetnocsi samodzielnego wprowadzenia badan naukowych i zasługuje na bardzo wysoko ocene.**
Rozprawa doktorska spełnia warunki okreslone w art.187 ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyszym i nauce (Dz. U.2018 poz. 1668),. W zwiazku z powyszym, zwracam sie do Wysokiej Rady Dyscypliny Nauk Medycznych Warszawskiego Unywersytet Medycznego o dopuszczenie Magister Salwador Cyranowski do dalszych etapow przewodu doktorskiego.
***Jednoczesnie wnioskuje o odpowiednie wyroznienie rozprawy doktorskiej* Magister Salwador Cyranowski.**

Profesor Dr Hab Claudine Kieda (PhD, DSc)
WIM PIB