Pathology & Oncology Research (2019) 25:1659–1663
https://doi.org/10.1007/s12253-018-00567-z

SHORT COMMUNICATION

Up-Regulation of the Alpha Prime Subunit of Protein Kinase CK2 as a Marker of Fast Proliferation in GL261 Cultured Cells

Lucía Villamañan[1] · Estefanía Alcaraz[1] · Lorenzo A. Pinna[2] · Maria Ruzzene[2] · Emilio Itarte[1,3] · Carles Arús[1,3,4] · Maria Plana[1,3] · Ana Paula Candiota[1,3,4]

Received: 9 October 2018 / Accepted: 17 December 2018 / Published online: 4 January 2019

Abstract

Glioblastoma (GB) is the most prevalent malignant primary brain tumor in adults. The preclinical glioblastoma model GL261 is widely used for investigating new therapeutic strategies. GL261 cultured cells are used for assessing preliminary in vitro data for this model although very little is known about the molecular characteristics of this cell line. Protein Kinase CK2 is a pleiotropic serine-threonine kinase and its inhibition may be a promising therapeutic strategy for GB treatment. In our group we follow treatment response with CK2 inhibitors in vivo using the GL261 murine model. For that, it is of our interest to assess the differential expression of α, α', β CK2 subunits as well as CK2 activity in the GL261 GB model. CK2α' expression changed along the growth curve of GL261 cells, undergoing downregulation in postconfluent phase cells, whereas CK2α and CK2β expression remained essentially unchanged. Furthermore, a marked decrease in CK2 activity in slowly proliferating postconfluent phase GL261 cells was observed. Finally, CK2α' expression in orthotopic GL261 tumors was intermediate between CK2α' expression found in cultured cells in exponentially growing or postconfluent phase, reflecting the heterogeneous nature of GL261 tumours growing in vivo. The results obtained suggest that, in the GL261 cell line, CK2α' could play a specific role in highly proliferative cells. Also, the decrease in CK2 activity in slowly proliferating GL261 cells could imply a differential susceptibility to subunit-specific CK2 inhibitors in this cell line, although further studies are needed to confirm this hypothesis.

Keywords Cell cycle · GL261 glioma · Cyclin D1 · Preclinical brain tumour model · CK2 alpha prime

Introduction

Glioblastoma (GB) is the most prevalent malignant primary brain tumour in adults with a survival of 14–15 months even after aggressive treatment, being urgent to seek for new therapeutic strategies [1]. Protein Kinase CK2 is a tetrameric, ubiquitously expressed serine threonine kinase, consisting of two catalytic subunits (2α/αα'/2α') and two regulatory subunits (2β). CK2 has been reported to specifically phosphorylate Akt1 on Serine 129 [2], allowing to indirectly evaluate CK2 activity in vivo by assessing the phosphorylation status of Akt1 in protein extracts. Moreover, CK2 has been described to play an important role in the regulation of the Brain Tumor Initiating Cells of GB [3]. Cyclin D1 expression is a good biomarker of cell cycle progression, being higher in proliferating cells and decreasing when cells enter in G0/G1 phase [4].

The GL261 glioma model is an orthotopic, immunocompetent preclinical model of GB generated by stereotactic injection of GL261 glioma cells in C57BL/6j mice. CK2α expression was found to be upregulated in GL261 GB tumours compared to wild type brain parenchyma [5]. However, not much is known about the molecular characteristics of the GL261 cell line. A better understanding of GL261 cells in culture could be of help in planning treatment strategies for GL261 as an in vivo GB model. The aim of this study was to analyze the relative protein content of α, α' and β CK2 subunits in exponentially growing (EP, exponential phase) and

✉ Ana Paula Candiota
AnaPaula.Candiota@uab.cat

[1] Departament de Bioquímica i Biologia Molecular, Unitat de Bioquímica de Biociències, Edifici Cs, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Spain

[2] Department of Biomedical Sciences, University of Padova, and CNR Institute of Neurosciences, via U. Bassi 58B, 35131 Padova, Italy

[3] Centro de Investigación Biomédica en Red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Cerdanyola del Vallès, Barcelona, Spain

[4] Institut de Biotecnologia i de Biomedicina (IBB), Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Spain

partially growth arrested (PCP, postconfluent phase) GL261 cells in culture and also in GL261 established tumours. In order to assess the proliferation status of cells in the different growth curve phases, cyclin D1 expression was also analyzed. Furthermore, overall CK2 activity was checked through the phosphorylation status of the CK2-specific site S129 of Akt 1 in GL261 cells and tumours.

Materials and Methods

Cell Culture and Animal Model

GL261 mouse glioma cells were cultured as stated in [5]. For the GL261 cells growth curve, 5×10^5 cells were seeded in 75 cm^2 flasks and counted with TC10 counter (Bio-Rad) from day 1 to 11 post-seeding ($n = 3$–6 for each day). For Western blot analysis, GL261 cells were seeded and collected after 6 days to obtain exponential cells and 9 days to get postconfluent cells. GL261 mice were generated as described in [5]. At day 17 p.i., when tumour volumes were 105.5 ± 12.8 mm^3, animals were euthanized and tumours resected.

Protein Extraction, Western Blot and Blot Quantification

Protein was extracted from cells and tumor samples as described in [6]. Blots were quantified with the ImageJ software and protein levels were normalized with the respective loading control (β-tubulin). To perform comparative analysis in the experiments shown in Figs. 1b, d, 2a, c and e (corresponding to different membranes), the highest average value found in quantitation was attributed the value "1", and the remaining values were proportionally

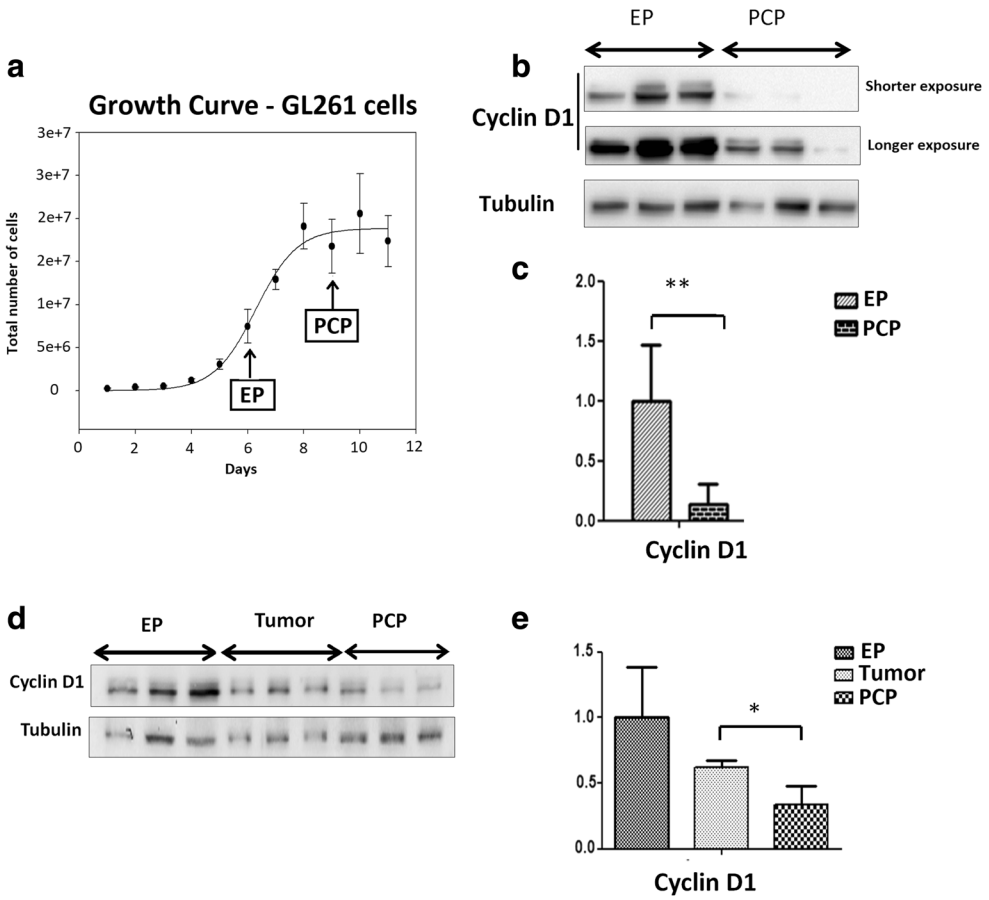

Fig. 1 GL261 cells growth curve and proliferative status along growth phases (**a**) Growth curve carried out with $n = 3$ to 6, depending on the time point evaluated, showing mean ± SD for each time point. (**b**) Western blot for EP and PCP GL261 protein extracts ($n = 3$ for each condition) in which Cyclin D1 and Tubulin expression were analyzed. Second image (below 1st row) corresponds to a blot at longer exposure time with the Chemidoc Image System. While not used for quantification, this second image allows to visually confirm Cyclin D1 expression in PCP cells. (**c**) Comparative quantification of Cyclin D1 normalized with tubulin expression ($n = 13$ for each group). (**d**) Cyclin D1 expression in EP and PCP cells and in GL261 tumors. (**e**) Comparative quantification of Cyclin D1 normalized with tubulin expression (n = 3 for GL261 cultured cells and GL261 tumors). No significant differences were found in Cyclin expression between EP and Tumor. * = $p < 0.05$, ** = $p < 0.01$

calculated referencing to it. Graphs were performed with GraphPad Prism software.

Results

GL261 Growth Curve: CK2 Expression and Activity in EP and PCP GL261 Cells

The GL261 growth curve showed an exponential phase (EP) and a postconfluent phase (PCP) (Fig. 1a). Exponential phase is observed at days 4 to 7 and postconfluent phase at days 8 to 11 from the growth curve. CK2 expression and activity were assessed in EP and PCP GL261 cells at days 6 and 9 of the growth curve, respectively.

Cyclin D1 expression level was analyzed in GL261 cells (both EP and PCP) and GL261 tumours (Fig. 1b, c, Table 1) showing a significant ($p < 0.05$) decrease during the PCP phase compared to the EP. GL261 tumours presented an intermediate expression level between EP and PCP cells (Fig. 1d, e, Table 2).

The expression of different CK2 subunits is shown in Fig. 2a, b. CK2α' expression decreased during the postconfluent phase compared to the exponential phase ($p < 0.05$, Table 1). On the other hand, CK2α and β protein levels remained essentially unchanged (Table 1). In addition, CK2 activity, indirectly measured by phosphorylation of Akt S129, decreased during the postconfluent phase (3.8 fold decrease, $p < 0.05$) (Fig. 2c, d, Table 1), which would agree with CK2 activity diminishing in less proliferative cells.

Assessment of CK2 Expression in GL261 Tumours

No significant differences were found for CK2α or CK2β expression when comparing EP, PCP cells and GL261 tumors

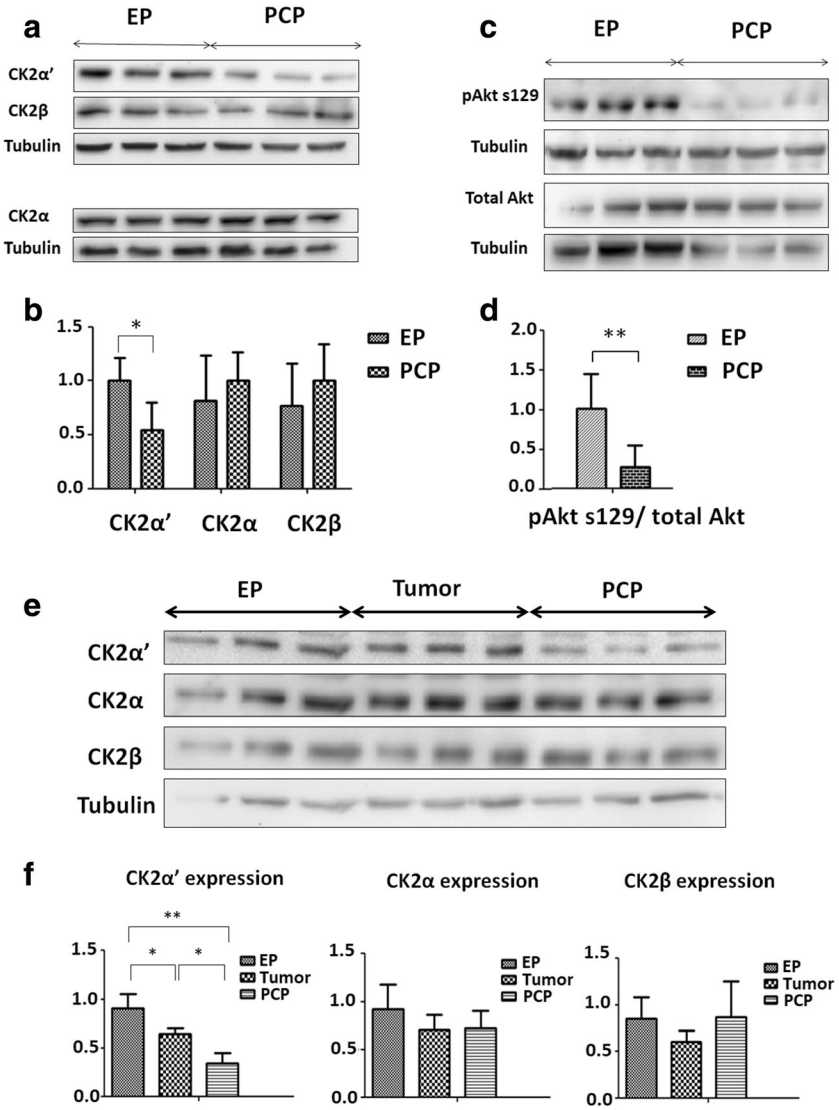

Fig. 2 CK2 expression and CK2 activity in EP and PCP GL261 cells and in GL261 tumours. (**a**) Western blot analysis of GL261 cell protein extracts. (**b**) Comparative quantification of CK2α', α and β expression normalized with Tubulin expression in EP and PCP cells ($n = 13$ for each group). The CK2α' levels were found 1.42 fold lower in PCP cells in comparison with EP ones (** = $p < 0.01$), whereas the CK2α and CK2β expressions remained constant in both phases. (**c**) Western blot for EP and PCP GL261 cell protein extracts in which total Akt, Tubulin expression and Akt s129 phosphorylation status were analyzed. (**d**) Comparative quantification of pAkt s129/total Akt ratio normalized with tubulin expression in which a 3.8 fold decrease is seen in PCP cells (n = 13 for each group). ** = $p < 0.01$. (**e**) Western blot for EP and PCP GL261 cells and GL261 tumor protein extracts in which CK2α, α' and β expression was analyzed. (**f**) Comparative quantification of CK2α, α' and β expression, normalized with tubulin expression ($n = 3$ for each group). * = $p < 0.05$, ** = $p < 0.01$

Table 1 Comparative analysis of CK2 expression and activity, and Cyclin D1 expression, in GL261 cells

	EP	PCP	p- value
CK2α'	1.00 ± 0.21	0.54 ± 0.26	3.75E-05**
CK2α	0.81 ± 0.42	1.00 ± 0.26	0.091
CK2β	0.77 ± 0.39	1.00 ± 0.34	0.233
Cyclin D1	1.00 ± 0.46	0.14 ± 0.16	2.31E-06**
pAkt s129/total Akt	1.00 ± 0.42	0.26 ± 0.27	1.87E-05**
CK2α'/ CK2α	1.56 ± 0.76	0.6 ± 0.29	0.0006**
CK2α'/CK2β	1.48 ± 0.72	0.67 ± 0.44	0.004**
CK2α/CK2β	1.16 ± 0.64	1.15 ± 0.59	0.97

Analysis of CK2 α', α and β and Cyclin D1 expression and pAkt S129 phosphorylation/ total Akt ratio in different growth phases of cultured GL261 cells, as well as ratios of different subunits. EP = exponential phase, PCP = postconfluent phase. Mean ± SD is shown, and experiments were performed with $n = 13$. Please refer to "Protein Extraction, Western Blot and Blot Quantification" section for details about quantitation.

** = $p < 0.01$

(Fig.2). Regarding CK2α' expression, tumours presented an intermediate value between EP cells and PCP cells ($p < 0.05$ for both comparisons).

Unbalanced CK2 Expression

CK2α'/α and CK2α'/β ratios were found 2.6 and 2.2 fold lower, respectively, in PCP cells in comparison with EP cells, while no significant differences were found for CK2α/β (Table 1). This decrease in CK2α', which is not fully compensated by the increase in CK2α, changes the proportion between catalytic and regulatory subunits. In tumors, the ratios calculated suggested higher similarity with EP cells (Table 2). CK2α'/α and CK2α'/β ratios were significantly higher in GL261 tumors compared to PCP cells, underlining the CK2α' downregulation in PCP cells.

Discussion

Results reported here show, for the first time and to the authors' knowledge, that CK2α' is specifically downregulated in postconfluent, slowly proliferating GL261 cells. This agrees with Pinna's and Oliviero's work [7], in which an induction of CK2α' expression was observed during G0/G1 transition in fibroblasts, suggesting a differential expression in senescent and proliferating fibroblasts. Taken together, these findings suggest that CK2α' expression could be proposed as a proliferation marker for GL261 cells. However, it is still unclear whether CK2α' itself plays specific roles in quiescent GL261 cells, or whether there is a direct relationship between CK2 activity decrease and CK2α' expression decrease in PCP cells of this murine cell line. One hypothesis could be that the holoenzyme containing CK2α' would be able to phosphorylate Akt1 in S129 better than the one containing CK2α. We have observed also that CK2α' expression in GL261 tumors reached an intermediate value between EP and PCP GL261 cells, probably due to the heterogeneous nature of GL261 tumors, composed by both highly proliferating and slowly proliferating cells [8] (similar to EP and PCP, respectively). The environment and growth regulatory conditions in each growth phase could present similarities with a different tumor region. The EP cells would be similar to the peripheral tumor cells: ample access to nutrients and oxygen and, accordingly, a very high proliferative rate. On the other hand, the PCP cells would resemble more cells in the inner part of the tumor, where growth factors, nutrients and oxygen may become limiting, decreasing the proliferation rate and where cell death-related phenomena may be also taking place. This is in agreement with results reported by [8], in which the same GL261 tumor can present regions with different Ki67% values ranging from 40 to 80%, with higher values on the highly proliferative periphery, reflecting the mixed contribution that may be taking place also in the investigated GL261 tumors in this work.

Table 2 Comparative analysis of CK2α, α' and β and Cyclin D1 for GL261 tumors

				p value		
	EP	Tumor	PCP	EP-Tumor	EP-PCP	Tumor-PCP
CK2α'	0.90 ± 0.15	0.65 ± 0.06	0.35 ± 0.1	0.048*	0.006**	0.011*
CK2α	0.92 ± 0.26	0.70 ± 0.16	0.72 ± 0.18	0.274	0.327	0.910
CK2β	0.85 ± 0.23	0.61 ± 0.12	0.87 ± 0.38	0.200	0.952	0.400
Cyclin D1	1 ± 0.39	0.62 ± 0.05	0.33 ± 0.14	0.23	0.049*	0.029*
CK2α'/ CK2α	1.01 ± 0.14	0.95 ± 0.21	0.48 ± 0.09	0.726	0.006**	0.006**
CK2α'/CK2β	1.08 ± 0.14	1.08 ± 0.11	0.43 ± 0.17	1.000	0.007**	0.007**
CK2α/CK2β	1.08 ± 0.08	1.16 ± 0.02	0.88 ± 0.2	0.544	0.174	0.174

Analysis of CK2α', α and β and Cyclin D1 expression, as well as the ratios of different subunits for GL261 tumors. Ratios were obtained for each individual sample (not from division of average values). EP = exponential phase, PCP = postconfluent phase. Mean ± SD is shown, experiments were performed with $n = 3$. Please refer to "Protein Extraction, Western Blot and Blot Quantification" section for details about quantitation.

* = $p < 0.05$, ** = $p < 0.01$.

If we accept that GL261 tumors are composed by cells with mixed proliferative characteristics, these results point that cells with high CK2α' levels could benefit from a protective effect against apoptotic death triggered by treatment, and this could lead to resistance to such treatment. Moreover, differences between the expression and activity of CK2 catalytic subunits may become relevant to improve therapeutic success, since CK2α' has been described to be more sensitive to some CK2 inhibitors than CK2α [9] and efforts are being made to develop CK2α and CK2α' specific inhibitors [10]. Still, further work will be needed to clarify whether the susceptibility to CK2 inhibitors is actually different according to GL261 cell proliferative status, but data presented here suggest that whenever CK2α' selective inhibitors were available, it may be relevant to evaluate them in GL261 mice. In this respect, a word of caution should be raised if such a treatment becomes available: immune system participation in response to therapy, which cannot be simulated with in vitro assays, could be affected if an inappropriate schedule of administration is applied [6], reverting the expected beneficial effect.

Acknowledgements Time allocation in the joint NMR facility of UAB and CIBER-BBN, Unit 25 of NANBIOSIS, for MRI follow-up of GL261 murine tumour evolution, is gratefully acknowledged.

Author's Contributions Conception and design, A.C., C.A., E.A., E.I. and M.P.; analysis and interpretation, L.V.; Writing-Drafting, L.V., A.C: and C.A.; Revising, L.P and M.R..; Supervision, A.C. and C.A.; Guarantor: A.C.

Funding Lucia Villamañan held a PIF predoctoral fellowship from *Universitat Autònoma de Barcelona*. This work was funded by the *Ministerio de Economía y Competitividad* (MINECO) grant MOLIMAGLIO (SAF2014–52332-R). Also funded by *Centro de Investigación Biomédica en Red- Bioingeniería, Biomateriales y Nanomedicina* (CIBER-BBN, (http://www.ciber-bbn.es/en)), an initiative of the *Instituto de Salud Carlos III* (Spain) co-funded by EU *Fondo Europeo de Desarrollo Regional* (FEDER). Also funded by AIRC IG 18756 to LAP.

Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethical Approval All applicable regional and national guidelines for the care and use of animals were followed. The studies described in this paper were approved by the local ethics committee *Comissió d'Ètica en Experimentació Animal i Humana* (CEEAH) (http://www.uab.cat/etica-recerca/) (protocol CEEAH-3665).

Consent for Publication Not applicable.

Abbreviations *CK2,* Casein Kinase 2; *DTT,* Dithiothreitol; *EDTA,* Ethylenediaminetetraacetic acid; *EP,* Exponential phase; *GB,* Glioblastoma; *PCP,* Postconfluent phase; *PBS,* Phosphate buffered saline; *PMSF,* phenylmethylsulfonyl fluoride; *PVDF,* Polyvinylidene Difluoride; *SDS-PAGE,* sodium dodecyl sulfate polyacrylamide gel electrophoresis; *TBS,* Tris-buffered saline

References

1. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Caimcross JG, Eisenhauer E, Mirimanoff RO (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352(10):987–996. https://doi.org/10.1056/NEJMoa043330
2. Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, Brustolon F, Pinna LA, Ruzzene M (2005) Protein kinase CK2 phosphorylates and upregulates Akt/PKB. Cell Death Differ 12(6):668–677. https://doi.org/10.1038/sj.cdd.4401604
3. Rowse AL, Gibson SA, Meares GP, Rajbhandari R, Nozell SE, Dees KJ, Hjelmeland AB, McFarland BC, Benveniste EN (2017) Protein kinase CK2 is important for the function of glioblastoma brain tumor initiating cells. J Neuro-Oncol 132(2):219–229. https://doi.org/10.1007/s11060-017-2378-z
4. Stacey DW (2003) Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Current opinion in cell biology 15 (2): 158-163. https://doi.org/10.1016/S0955-0674(03)00008-5
5. Ferrer-Font L, Alcaraz E, Plana M, Candiota AP, Itarte E, Arus C (2016) Protein kinase CK2 content in GL261 mouse glioblastoma. Pathol Oncol Res 22(3):633–637. https://doi.org/10.1007/s12253-015-9987-7
6. Ferrer-Font L, Villamanan L, Arias-Ramos N, Vilardell J, Plana M, Ruzzene M, Pinna LA, Itarte E, Arus C, Candiota AP (2017) Targeting protein kinase CK2: evaluating CX-4945 potential for GL261 glioblastoma therapy in immunocompetent mice. Pharmaceuticals 10(1). https://doi.org/10.3390/ph10010024
7. Orlandini M, Semplici F, Ferruzzi R, Meggio F, Pinna LA, Oliviero S (1998) Protein kinase CK2alpha' is induced by serum as a delayed early gene and cooperates with Ha-ras in fibroblast transformation. J Biol Chem 273 (33):21291–21297. https://doi.org/10.1074/jbc.273.33.21291
8. Ortega-Martorell S, Lisboa PJG, Vellido A, Simoes RV, Pumarola M, Julià-Sapé M, Arús C (2012) Convex Non-Negative Matrix Factorization for Brain Tumor Delimitation from MRSI Data. PLoS One 7 (10):e47824. https://doi.org/10.1371/journal.pone.0047824
9. Janeczko M, Orzeszko A, Kazimierczuk Z, Szyszka R, Baier A (2012) CK2alpha and CK2alpha' subunits differ in their sensitivity to 4,5,6,7-tetrabromo- and 4,5,6,7-tetraiodo-1H-benzimidazole derivatives. Eur J Med Chem 47(1):345–350. https://doi.org/10.1016/j.ejmech.2011.11.002
10. Bollacke A, Nienberg C, Borgne ML, Jose J (2016) Toward selective CK2alpha and CK2alpha' inhibitors: development of a novel whole-cell kinase assay by autodisplay of catalytic CK2alpha'. J Pharm Biomed Anal 121:253–260. https://doi.org/10.1016/j.jpba.2016.01.011