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Role of PLC γ 1 in the modulation of cell migration and cell invasion in glioblastoma

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Abstract

Phosphoinositide-specific phospholipases C (PLCs) are a class of enzymes involved in several cell activities, such as cell cycle regulation, proliferation, differentiation and cytoskeletal dynamics. Among these enzymes, PLC γ 1 is one of the most expressed PLCs in the brain, contributing to a complex network in the developing nervous system. Several studies have shown that PLC γ 1 signaling imbalance is linked to several brain disorders, including glioblastoma, the most aggressive brain tumor in adults. Indeed, it has been demonstrated a link between PLC γ 1 inhibition and the arrest of glioma cell motility of fetal rat brain aggregates and the impairment of cell invasion abilities following its down-regulation. This study aims to determine the pathological influence of PLC γ 1 in glioblastoma, through a translational study which combines *in silico* data, data from glioblastoma patients' samples and data on engineered cell lines. We found out that PLC γ 1 gene expression correlates with the pathological grade of gliomas, and it is higher in fifty patients' glioblastoma tissue samples compared to twenty healthy controls. Moreover, it was demonstrated that PLC γ 1 silencing in U87-MG leads to a reduction in cell migration and invasion abilities. The opposite trend was observed following PLC γ 1 overexpression, suggesting an interesting possible involvement of PLC γ 1 in gliomas' aggressiveness.

Keywords: Glioblastoma, PLC γ 1, Migration, Invasion, Silencing, Overexpression, Biomarker

Introduction

Glioblastomas are the most common intrinsic brain tumors originating from neuroglial stem or progenitor cells (Le Rhun et al., 2019). According to the World Health Organization (WHO) grading system 2016 (Louis et al., 2016) they are classified as high-grade gliomas (HGG) and are characterized by a high tendency to infiltration and diffusion (Ramirez et al., 2013). This is the main reason why current therapies, based on neurosurgical approaches, chemotherapy and radiotherapy, are not effective and do not lead to an increase in the median survival of patients. Indeed, only 3-5% of patients with a diagnosis of glioblastoma survive up to 3 years or more (Batash et al., 2017). Glioblastoma can develop both *de novo* or evolve from a previous astrocytoma (Stoyanov et al., 2018) and, anyway, almost every tumor recurs (Campos et al., 2016) due to the cellular migration and invasion properties that make impossible the tumor complete removal, even with multi-therapeutic approaches (Zepecki et al., 2019). For this reason, the comprehension of the molecular and pathological mechanisms behind tumor development represents a great challenge and the identification of new potential prognostic and diagnostic biomarkers could pave the way for new therapeutic perspectives.

The role of phosphoinositide 3-kinase (PI3K)-mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) signaling, which could be downregulated by several chemotherapeutic approaches (Abrams et al., 2018), has been evidenced in glioblastoma (Amin et al., 2021). In addition to this pathway, there are many other signaling pathways of pathophysiological interest for this tumor. Indeed, several studies have shown the key role of lipids (Martelli et al., 2004) and phosphoinositide-specific phospholipases C (PLCs), a class of membrane-associated enzymes whose role is extensively studied in cancer (Manzoli et al., 1995; Owusu Obeng et al.,

2020) or in the regulation of several activities in the Central Nervous System (CNS)(García del Caño et al., 2015; Montaña et al., 2012; Ratti et al., 2019), such as neuronal positioning and synaptic transmission (Kim et al., 1997; Lo Vasco, 2012). Moreover, the signaling imbalance of PLC γ 1, one of the most expressed PLCs in the brain, is linked to several disorders, including epilepsy, behavior disorders, neurodegenerative diseases and also glioblastoma (Khoshyomn et al., 1999; Rusciano et al., 2021). In particular, it was evidenced a potential link between PLC γ 1 inhibition and the arrest of glioma cell motility and invasion of fetal rat brain aggregates (Khoshyomn et al., 1999). It is well known that in glioblastoma the upregulation of platelet-derived growth factor receptors (PDGF-R), nerve growth factor (NGF), insulin-like growth factor (IGF-1) and, most importantly, epidermal growth factor receptor (EGF-R) can mediate migration of glioblastoma cells into normal brain tissues. Interestingly, EGF and PDGF are upstream activators of PLC γ 1 and also major regulators of cell growth and proliferation (Engebraaten et al., 1993). Accordingly, PLC γ 1 inhibition leads to the arrest of glioblastoma cells invasion into normal brain tissues, careless of the combined signaling effects of PDGFR, NGF, IGF-1, and EGFR upregulation, suggesting possible anti-invasive therapeutic strategies for glioblastoma (Khoshyomn et al., 1999; Owusu Obeng et al., 2020; Penar et al., 1998). Moreover, it has been already evidenced PLC γ 1 involvement in tumor progression and metastasis in other types of cancer, including breast cancer (Sala et al., 2008). Sala et al. demonstrated that PLC γ 1 knockdown resulted in a complete impairment of cell invasion in breast cancer, using U87-MG glioblastoma cells as control, due to their migration mechanism known to be PLC-dependent. In this latter investigation it was shown a critical role of PLC γ 1 in the metastatic potential of cancer cells, indicating its inhibition as a potential therapeutic strategy for the treatment of metastasis dissemination (Sala et al., 2008). All in all, cell motility and invasion play an essential role in tumor development and the understanding of these mechanisms and of the relation between the inositide signaling and glioblastoma transformation could be critical for the total knowledge of this tumor. This article suggests PLC γ 1 as a putative biomarker and evaluates its potential future role for diagnostic and prognostic purposes in gliomas' molecular classification field, combining *in silico* data of glioblastoma patients, data on glioblastoma sample tissues and *in vitro* data carried out on engineered U87-MG cell line.

Materials and Methods

***In silico* data and collection of glioblastoma tumour samples**

PLC γ 1 distribution and patients' survival information were downloaded from the Chinese Glioma Genome Atlas (CGGA <http://www.cgga.org.cn/>) RNA sequencing (RNA-seq) dataset (mRNAseq_325) which collects the information relating to 325 glioma samples, including 103 WHO II gliomas, 79 WHO III gliomas, 139 WHO IV gliomas and 4 samples N/A of unknown nature. These patients were divided into high- PLC γ 1 and low-PLC γ 1 expression group according to the cut-off value of PLC γ 1 expression. Moreover, we collected fifty glioblastoma samples from the IRCCS Istituto delle Scienze Neurologiche di Bologna (Italy), diagnosed following the WHO 2016 and cIMPACT-NOW guidelines (Louis et al., 2020; Wesseling and Capper, 2018).

The study was approved by the AUSL Bologna Ethical Committee and informed consents were obtained from all participants.

Cell culture and lentiviral transduction

Human glioblastoma U87-MG cells (HTB-14 ATCC, Old Town Manassas, Virginia, US) were cultured in Minimum Essential Medium Eagle (MEM) (Corning, New York, US) with 10% FBS and 1% Penicillin/Streptomycin (Sigma-Aldrich) and Dulbecco's Modification of Eagle's Medium (DMEM) (Corning) with 5% FBS and 1% Penicillin/Streptomycin. Human embryonic kidney HEK 293 T cells (Genecopoeia Inc, US) were cultured in DMEM (Corning) with 10% FBS and 1% Penicillin/Streptomycin. Both cell lines were maintained in a humidified incubator at 37 °C with 5% CO₂.

HSH013238-LVRH1GH-a, HSH013238-LVRH1GH-c, HSH013238-LVRH1GH-d and CSHCTR001-LVRH1GH vectors (Genecopoeia) were used to construct lentiviruses to silence PLC γ 1 and express green fluorescent protein (GFP) as well as lentiviruses coding only for GFP, as control. EX-Z4417-Lv207 coding for Homo Sapiens PLC γ 1 and EX-NEG-Lv207 vectors (Genecopoeia) were used to construct lentiviruses used to overexpress both PLC γ 1 and GFP protein or only GFP, respectively.

The Lenti-Pac HIV expression packaging kit (Genecopoeia) was used to transfect HEK293T cells according to manufacturer's protocol. The viruses contained in the supernatants were harvested 24-48 hours after transfection and filtered through a 0.45 μ m cellulose acetate filter. Virus supernatants and 8 μ g/ml of polybrene were added to the target cells and replaced with fresh media the next day. Cells were selected for 48 h in growth media supplemented with 750 μ g/ml of Hygromycin B (CSNpharm, Inc. US). PLC γ 1 expression analysis and migration or invasion tests were carried out 96 h after the transduction.

RNA extraction, reverse transcription, and real-time PCR

Total RNA from fresh-frozen tissues was extracted through RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Total RNA extracted from different brain lobes of healthy individuals (Biochain, Newark, CA: R1234062-P, R1234078-P, R1234051-P, R1234066-P) was used as control. A total of four different pools were used, each containing RNA from five different healthy donors (twenty individuals in total). RNeasy Mini Kit (Qiagen) was used to extract total RNA from U87-MG cells. Using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), 1 μ g of total RNA was reverse transcribed. Real-time PCR (10ng cDNA/reaction) was performed by QuantStudio1 Real-time PCR system (Thermo Fisher Scientific). GAPDH and 18S were used as housekeeping genes. Data were presented as fold changes relative to the expression levels of control samples in accordance with the $2^{-\Delta\Delta C_t}$ formula. Validated gene probes used are: 18S Hs99999901_s1, GAPDH Hs99999905_m1, PLC γ 1 Hs01008225_m1 (Thermo Fisher Scientific).

Transwell migration and invasion assays

U87-MG cells were trypsinized and suspended in serum-free medium 96 h after transduction. 100 μ l of the cell suspensions, containing 3×10^4 cells/ml for migration assay and 6×10^4 cells/ml for invasion assay, were seeded into the upper chamber of a 24-well transwell of 8 μ m pore size (Sarstedt, Nümbrecht, Germany). For invasion assay, a coating with Geltrex (Thermo Fisher Scientific) was carried out 2 hours before the seeding. Medium containing 10% FBS was added to the lower wells and plates were later incubated at 37°C for 18h. Transwell insert membranes were fixed and then stained with 0,2% Crystal Violet. The percentage of migrating and invading cells was calculated through manual counting in 4 random fields under an optical microscope (Magnification 20x). Each assay was performed in triplicate.

Statistical analysis

Statistical analysis was carried out using Graph Pad Prism 5.0 software (San Diego, CA, US). Using the Shapiro-Wilk Test for patient's RNA expression analysis, data resulted not normally distributed. The difference between Healthy Controls and Glioblastoma samples was evaluated using the Mann-Whitney test. One-way ANOVA test was used for cell migration and invasion analyses. The differences were considered statistically significant with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

1. PLC γ 1 gene expression correlates with gliomas' pathological grade

In order to evaluate the potential role of PLC γ 1 in the pathogenesis of high-grade gliomas, we started from the analysis of 325 samples collected in the CGGA online public database containing PLC γ 1 RNA-seq and survival data of patients with low- or high- grade of gliomas (from grade II to grade IV). From this analysis it was evidenced that PLC γ 1 gene expression positively correlates with the pathological grade of gliomas. Indeed, WHO IV gliomas showed a higher PLC γ 1 gene expression compared to WHO II and WHO III (Fig. 1a). Moreover, Kaplan-Meier curves, processed with the CGGA database, support the hypothesis that patients characterized by an overall higher PLC γ 1 gene expression have a lower survival probability, regardless of the grade of gliomas in both primary and recurrent ones (Fig. 1b).

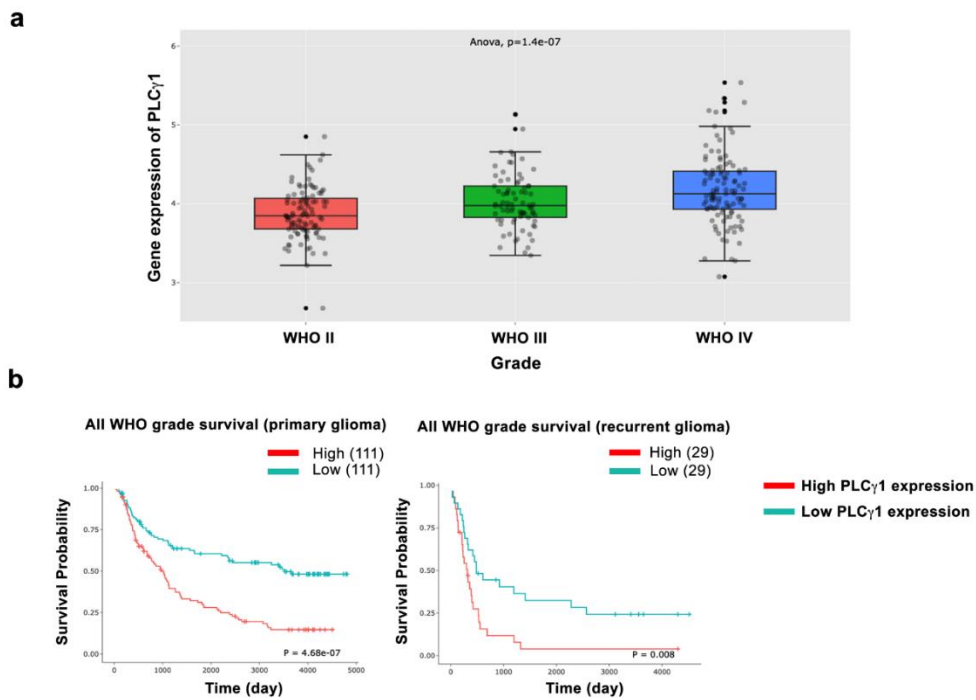


Figure 1

2. Glioblastoma patients are characterized by higher PLC γ 1 gene expression compared to healthy individuals

To deepen this study, fifty tissue samples from fresh-frozen retrospective glioblastoma patients collected in the last ten years were analyzed for their PLC γ 1 gene expression, comparing them with four healthy samples pools, each one containing total RNA derived from five different healthy individuals (twenty donors in total). All fifty patients' samples were characterized following the WHO 2016 and cIMPACT-NOW guidelines (Louis et al., 2016) before deepening the analysis. The expression analysis showed that glioblastoma patients were characterized by an overall higher PLC γ 1 gene expression compared to healthy controls (Fig. 2). This result led to the idea that this enzyme could play a key role in the pathogenesis of glioblastoma and that it could be considered as a signature gene for the molecular classification of high-grade tumors.

PLC γ 1 silencing on U87-MG

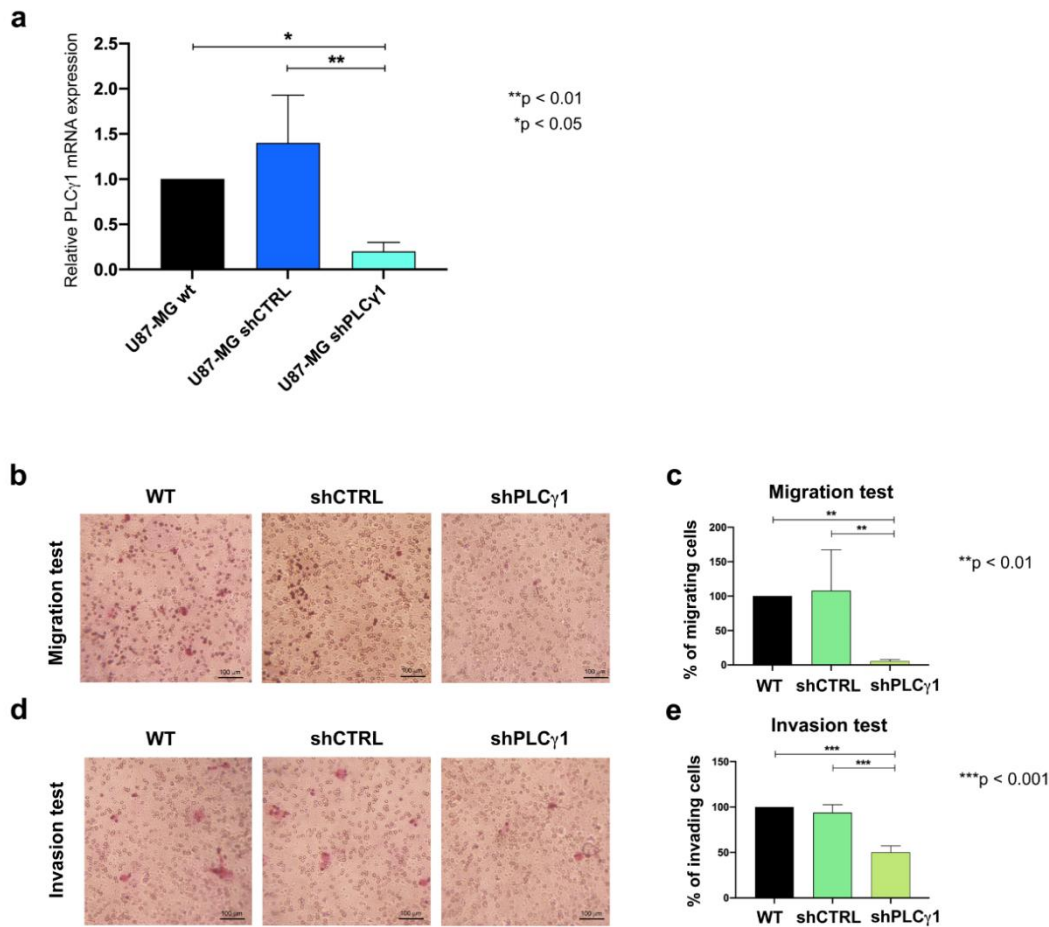


Figure 3

4. PLC γ 1 overexpression leads to increased cell migration and invasion in U87-MG

To reinforce all these data, we subsequently carried out a transient PLC γ 1 overexpression on the same cell model. Even under these circumstances, PLC γ 1 gene expression and the relative overexpression were first confirmed through a Real-Time PCR analysis (Fig. 4a), comparing U87-MG cells overexpressing PLC γ 1 (ovPLC γ 1) to the respective controls (WT and negCTRL). Therefore, transwell migration assay revealed that cells overexpressing PLC γ 1 had a higher ability to migrate than controls (Fig. 4b,c). The transwell invasion assay with the Geltrex coating was further carried out, confirming an opposite attitude to the one revealed following PLC γ 1 silencing. Indeed, following PLC γ 1 overexpression there was an increase in the ability of U87-MG cells to invade (Fig. 4d,e). Therefore, a positive correlation between PLC γ 1 expression level and the cellular migration and invasion abilities was confirmed in our cell model, strengthening what is already present in literature.

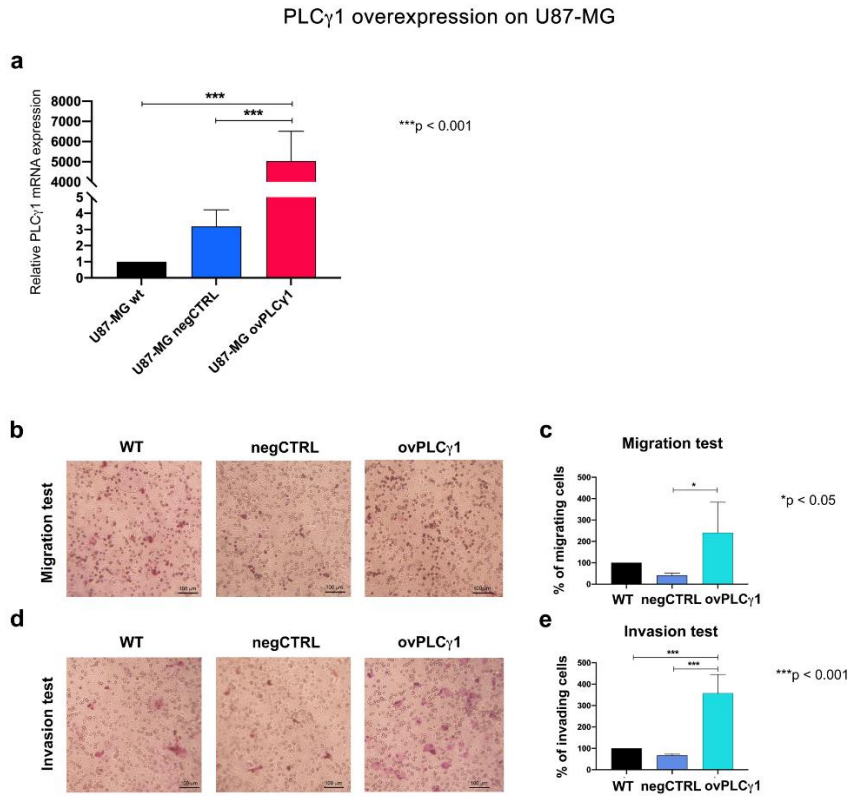


Figure 4

Discussion

Phosphoinositides (PI) and Phospholipases represent key elements in the overall regulation of numerous cellular processes (Faenza et al., 2003; Lo Vasco et al., 2004; Xian et al., 2020) including cellular growth, differentiation, proliferation and many other cellular homeostasis pathways (Cocco et al., 2015; Manzoli et al., 2005) which are interestingly altered in glioblastoma (Ratti et al., 2019), the most common and lethal brain tumor in adults. Despite the efforts to develop new therapies, glioblastomas often develop resistance to treatments and recur quickly, due to the great tumor complexity and heterogeneity (Liu et al., 2018). For this reason, the management of glioblastoma represents today a great challenge and the understanding of the molecular pathways that drive malignancy in glioblastoma could help to find a successful targeted therapeutic strategy and the development of new ideal biomarkers. In this paper it is highlighted how PLC γ 1, one of the most PLCs expressed in the brain (Rusciano et al., 2021), could represent a key gene in the molecular classification of high-grade gliomas. It has been already evidenced in literature the possible involvement of PLC γ 1 in glioblastoma progression and aggressiveness, also considering its upstream activators: PDGF and EGF, which are dominant mediators of cell growth and proliferation (Engebraaten et al., 1993). Moreover, PLC γ 1 mutation and involvement in the processes of tumorigenesis, including proliferation, migration, and invasion in different types of cancer are frequently reported (Shin et al., 2021). For example, PLC γ 1 activation is associated with angiosarcoma progression, and its upregulation and mutation have been consistently linked

to poor patient survival (Behjati et al., 2014). PLC γ 1 role is associated also to squamous cell carcinoma (Xie et al., 2010), colorectal and gastric cancers (Li et al., 2005). PLC γ 1 centrality in the progression of various types of cancer is probably linked to PLC γ 1 strategic position at a convergence point of various signaling pathways, including growth factor receptor signaling and adhesion receptor signaling for cell spreading, invasion, and migration. This work suggests PLC γ 1 as an ideal future biomarker with the potential to assess cancer risk and to promote early diagnosis, due to the positive correlation between its gene expression and the pathological grade of gliomas. Indeed, this translational study, which correlates data extracted from *online* database and retrospective analyses on fresh/frozen glioblastoma tissue samples, revealed that PLC γ 1 mRNA expression increased in high-grade gliomas compared to low-grade ones and also compared to healthy individuals.

In order to highlight the importance that this enzyme could have in the pathogenesis of this complex tumor, it was carried out an *in vitro* study based on engineered U87-MG glioblastoma cell line, already cited in literature as a cell model whose migration is PLC-dependent (Sala et al., 2008). These cells were in parallel subjected to PLC γ 1 silencing and overexpression, in order to evaluate the effects of these modulations on cell migration and invasion, which represent the main mechanisms that govern tumor aggressiveness (Liu et al., 2018). As expected, PLC γ 1-silenced cells showed less ability to migrate and invade compared to the respective controls. On the contrary, cells overexpressing PLC γ 1 acquired greater aggressiveness linked to an increased ability of migration and invasion (Fig. 5). All in all, *in silico* data from CGGA database, clinical data collected on patient fresh-frozen retrospective samples, together with *in vitro* data on engineered cell line, suggest a prospective involvement of PLC γ 1 in gliomas' aggressiveness. However, the mechanisms by which PLC γ 1 is upregulated in high-grade tumors are not clear yet. Further studies are certainly needed. In particular, it will be necessary to investigate the PLC γ 1 downstream pathways and the key molecules involved in the regulation of migration and invasion, which represent necessary mechanisms for the acquisition of resistance to common therapies. The complete understanding of these events could pave the way to the identification of new diagnostic and prognostic bio-markers in gliomas.

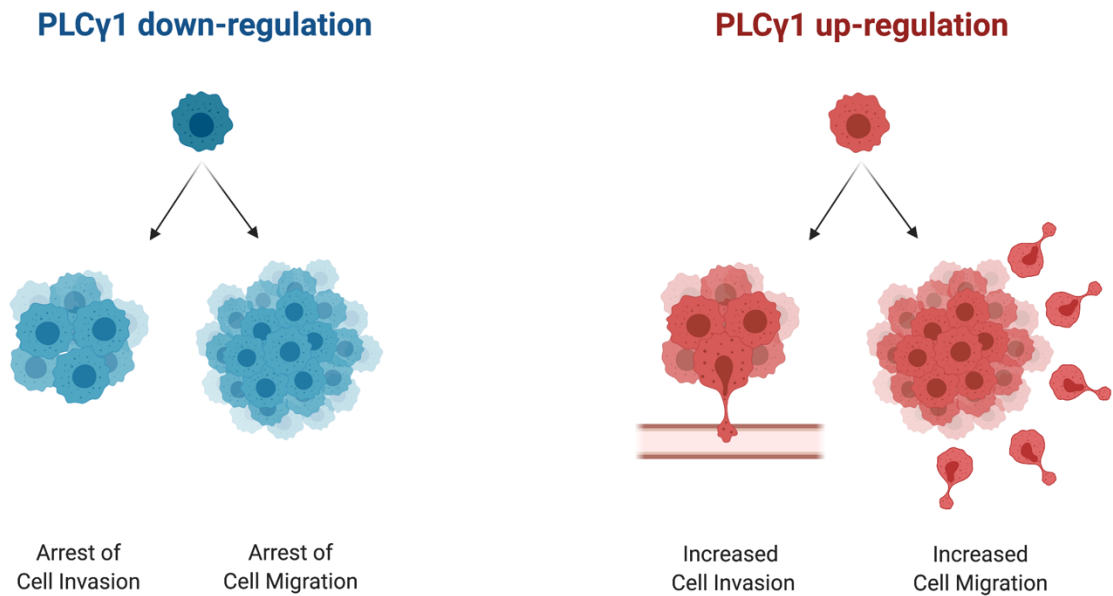


Figure 5

Author Contributions: S.R. and M.V.M designed the experiments; M.V.M. and L.Mor. conducted the experiments; L.M., L.C., P.G.S, J.A.M and S.R supervised the experiments; S.A., M.Z. and D.M. enrolled the patients; S.M., G.R., V.P. and A.M.B. analysed data; S.R. and M.V.M wrote the paper; S.R., G.R., M.Y.F., L.M. and L.C. funding acquisition.

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Footnote:

Given his role as Editor in Chief, Dr. Lucio Cocco had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Daniel Raben.

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Figure Legends

Fig. 1: PLC γ 1 gene expression correlates with gliomas' pathological grade

Panel a: Distribution of PLC γ 1 expression in gliomas according to WHO classification of patients collected in the CGGA database. WHO II, n=103; WHO III, n=79; WHO IV, n=139. PLC γ 1 mRNA expression positively correlates with gliomas' pathological grade ($p=1.4e-07$). **Panel b:** Kaplan-Meier survival curves, extracted from the CGGA dataset, of PLC γ 1 high or low expression patient groups. Patients were divided according to the median level of PLC γ 1 mRNA expression ($p < 4.68e-07$ and $p = 0.008$).

Fig. 2: Glioblastoma patients are characterized by higher PLC γ 1 gene expression compared to healthy individuals. PLC γ 1 gene expression in 50 glioblastoma patients' samples and 4 healthy pools of 5 donors each, used as controls (20 individuals in total). The distribution of PLC γ 1 gene expression in glioblastoma tissue samples compared to controls was displayed through scatter plots. 18S was used as housekeeping gene and data were presented as fold changes relative to the expression levels of control samples in accordance with the $2^{-\Delta\Delta C_t}$ formula. Asterisks indicate statistically significant differences between the groups, with $*p < 0.05$.

Fig. 3: PLC γ 1 silencing on U87-MG cell line and relative consequences analyses on migration and invasion abilities.

Panel a: PLC γ 1 mRNA expression on U87-MG cell line after PLC γ 1 silencing. PLC γ 1-silenced cells (shPLC γ 1) were compared to wild type (WT) and mock-transduced cells (shCTRL). U87-MG were analyzed after 48 h of hygromycin selection, i.e. 96 h after transduction overall. GAPDH was used as housekeeping gene and three independent experiments were carried out for the analysis, with $***p < 0.01$, $*p < 0.05$. **Panels b and c:** Representative images of transwell migration assay in U87-MG cell line (**b**), with the relative graphical representation (**c**). PLC γ 1-silenced cells (shPLC γ 1) were compared to wild type (WT) and mock-transduced (shCTRL) cells. Magnification 20x (bar: 100 μ m). Columns show the mean \pm SD of three independent experiments with $***p < 0.01$. **Panels d and e:** Representative images of transwell invasion assay, with a Geltrex coating, in U87-MG (**d**), with the relative graphical representation (**e**). PLC γ 1-silenced cells (shPLC γ 1) were compared to wild type (WT) and mock-transduced (shCTRL) cells. Magnification 20x (bar: 100 μ m). Columns show the mean \pm SD of three independent experiments with $***p < 0.001$.

Fig.4: PLC γ 1 overexpression on U87-MG cell line and relative consequences analyses on migration and invasion cell abilities.

Panel a: PLC γ 1 mRNA expression on U87-MG cell line after PLC γ 1 overexpression. Cells overexpressing PLC γ 1 (ovPLC γ 1) were compared to wild type (WT) and mock-transduced cells (negCTRL). U87-MG were

analyzed after 48 h of hygromycin selection, i.e. 96 h after transduction overall. GAPDH was used as housekeeping gene and three independent experiments were carried out for the analysis, with *** $p < 0.001$. **Panels b and c:** Representative images of transwell migration assay in U87-MG cell line (**b**), with the relative graphical representation (**c**). Cells overexpressing PLC γ 1 (ovPLC γ 1) were compared to wild type (WT) and mock-transduced (negCTRL) cells. Magnification 20x (bar: 100 μ m). Columns show the mean \pm SD of three independent experiments with * $p < 0.05$. **Panels d and e:** Representative images of transwell invasion assay, with a Geltrex coating, in U87-MG (**d**), with the relative graphical representation (**e**). Cells overexpressing PLC γ 1 (ovPLC γ 1) were compared to wild type (WT) and mock-transduced (negCTRL) cells. Magnification 20x (bar: 100 μ m). Columns show the mean \pm SD of three independent experiments with *** $p < 0.001$.

Fig. 5: Summary Figure of the demonstrated mechanisms. As already demonstrated in literature on various types of cancer and on fetal rat brain aggregates in glioblastoma (Khoshyomn et al., 1999), this study shows a strong correlation between PLC γ 1 expression level and the acquisition of a more aggressive cellular phenotype. Indeed, following PLC γ 1 down-regulation, U87-MG cells decreased their migration and invasion abilities compared to controls. On the contrary, the opposite trend, characterized by the acquisition of a greater ability to migrate and invade, was observed in cells overexpressing PLC γ 1.