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Role of PLCγ1 in the modulation of cell migration and cell invasion in glioblastoma

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Declaration of competing interest:

All the authors declare no conflict of interest.

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, PLCγ1 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 PLCγ1 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 PLCγ1 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 PLCγ1 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 PLCγ1 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 PLCγ1 silencing in U87-MG leads to a reduction in cell migration and invasion abilities. The opposite trend was observed following PLCγ1 overexpression, suggesting an interesting possible involvement of PLCγ1 in gliomas' aggressiveness.

Keywords: Glioblastoma, PLCγ1, 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 PLCγ1, 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 PLCγ1 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 PLCγ1 and also major regulators of cell growth and proliferation (Engebraaten et al., 1993). Accordingly, PLCγ1 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 PLCγ1 involvement in tumor progression and metastasis in other types of cancer, including breast cancer (Sala et al., 2008). Sala et al. demonstrated that PLCγ1 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 PLCγ1 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 PLCγ1 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

PLCγ1 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- PLCγ1 and low-PLCγ1 expression group according to the cut-off value of PLCγ1 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 PLCγ1 and express green fluorescent protein (GFP) as well as lentiviruses coding only for GFP, as control. EX-Z4417-Lv207 coding for Homo Sapiens PLCγ1 and EX-NEG-Lv207 vectors (Genecopoeia) were used to construct lentiviruses used to overexpress both PLCγ1 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 µm cellulose acetate filter. Virus supernatants and 8 µg/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 µg/ml of Hygromycin B (CSNpharm, Inc. US). PLCγ1 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 µg of total RNA was reverse transcribed. Real-time PCR (10ng cDNA/reaction) was performed by QuantStudio1 Real-time PCR system (Thermo Fisher Scientifc). 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 $2^{-\Delta\Delta Ct}$ formula. Validated gene probes used are: 18S Hs99999901_s1, GAPDH Hs99999905_m1, PLCγ1 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 µl of the cell suspensions, containing $3x10^4$ cells/ml for migration assay and $6x10^4$ cells/ml for invasion assay, were seeded into the upper chamber of a 24-well transwell of 8 µm 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. PLCγ1 gene expression correlates with gliomas' pathological grade

In order to evaluate the potential role of PLCγ1 in the pathogenesis of high-grade gliomas, we started from the analysis of 325 samples collected in the CGGA online public database containing PLCγ1 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 PLCγ1 gene expression positively correlates with the pathological grade of gliomas. Indeed, WHO IV gliomas showed a higher PLCγ1 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 PLCγ1 gene expression have a lower survival probability, regardless of the grade of gliomas in both primary and recurrent ones (Fig. 1b).

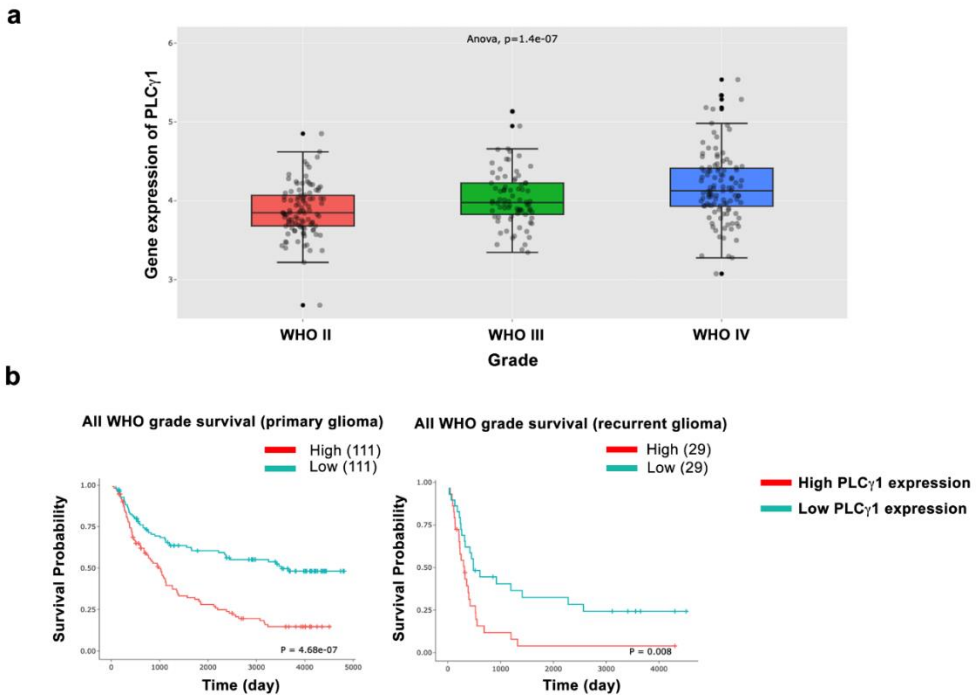

Figure 1

2. Glioblastoma patients are characterized by higher PLCγ1 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 PLCγ1 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 PLCγ1 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.

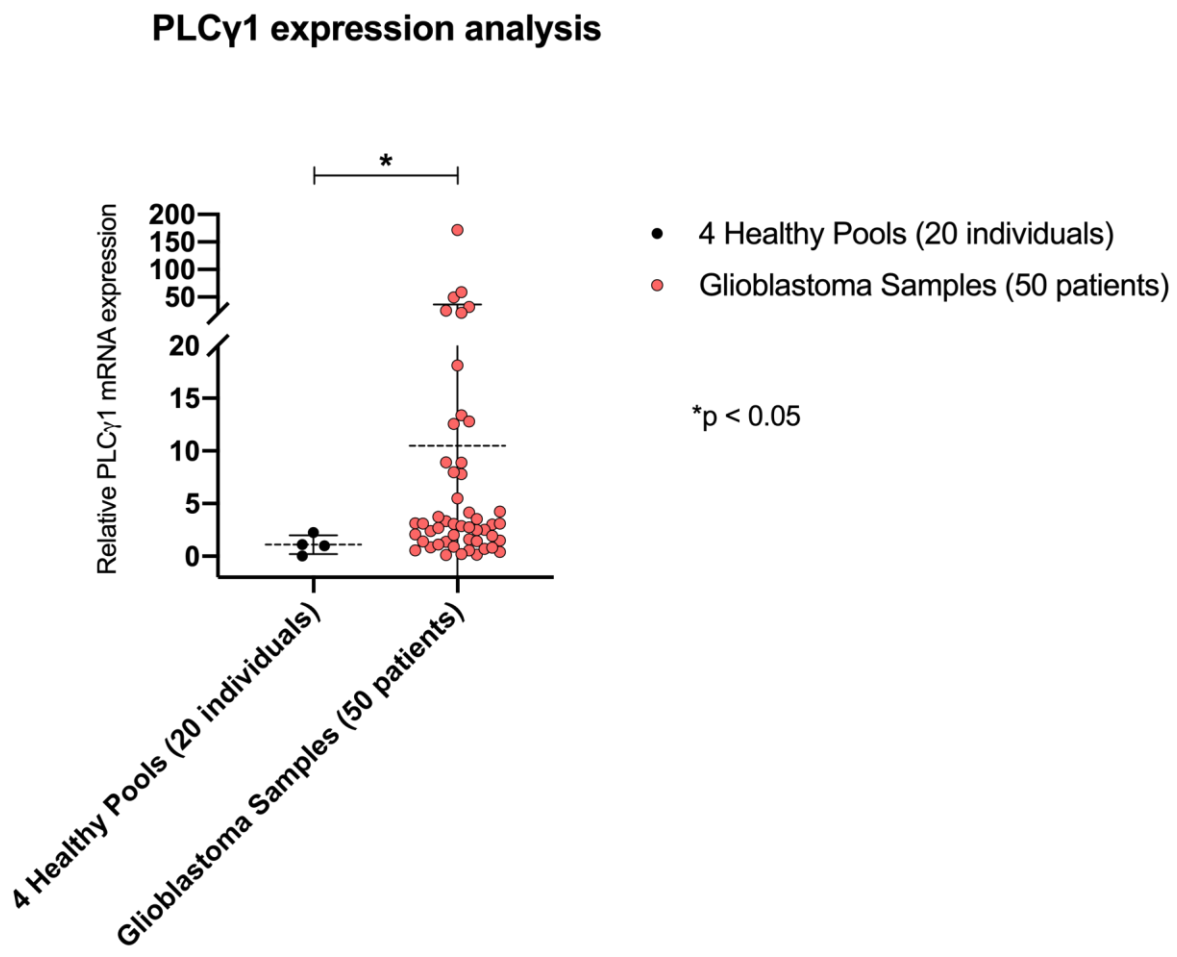

Figure 2

3. PLCγ1 silencing leads to decreased cell migration and invasion in U87-MG

In order to understand the role of PLCγ1 in glioblastoma, we decided to create an *in vitro* model based on U87-MG glioblastoma cell line. We transiently silenced PLCγ1 in this cell model to analyze the effect on the main aggressiveness mechanisms related to tumor transformation, such as migration and invasion. Firstly, U87-MG silenced cells (shPLCγ1) were tested for PLCγ1 gene expression and compared to wild type (WT) cells and to cells transduced with empty vectors coding only for GFP (shCTRL) (Fig. 3a). Next, we examined the migration potential of PLCγ1-silenced cells through a transwell method. It was evidenced that, following PLCγ1 silencing, U87-MG cells exhibited significantly reduced ability of migration compared to control cells (Fig.3b, c). Later, the same transwell assay was performed with the addition of a Geltrex coating in order to assess the consequences of PLCγ1 silencing on the invasion ability of the cells. This test displayed that PLCγ1-silenced U87-MG had significantly lower invasion ability than the respective controls (Fig. 3d,e).

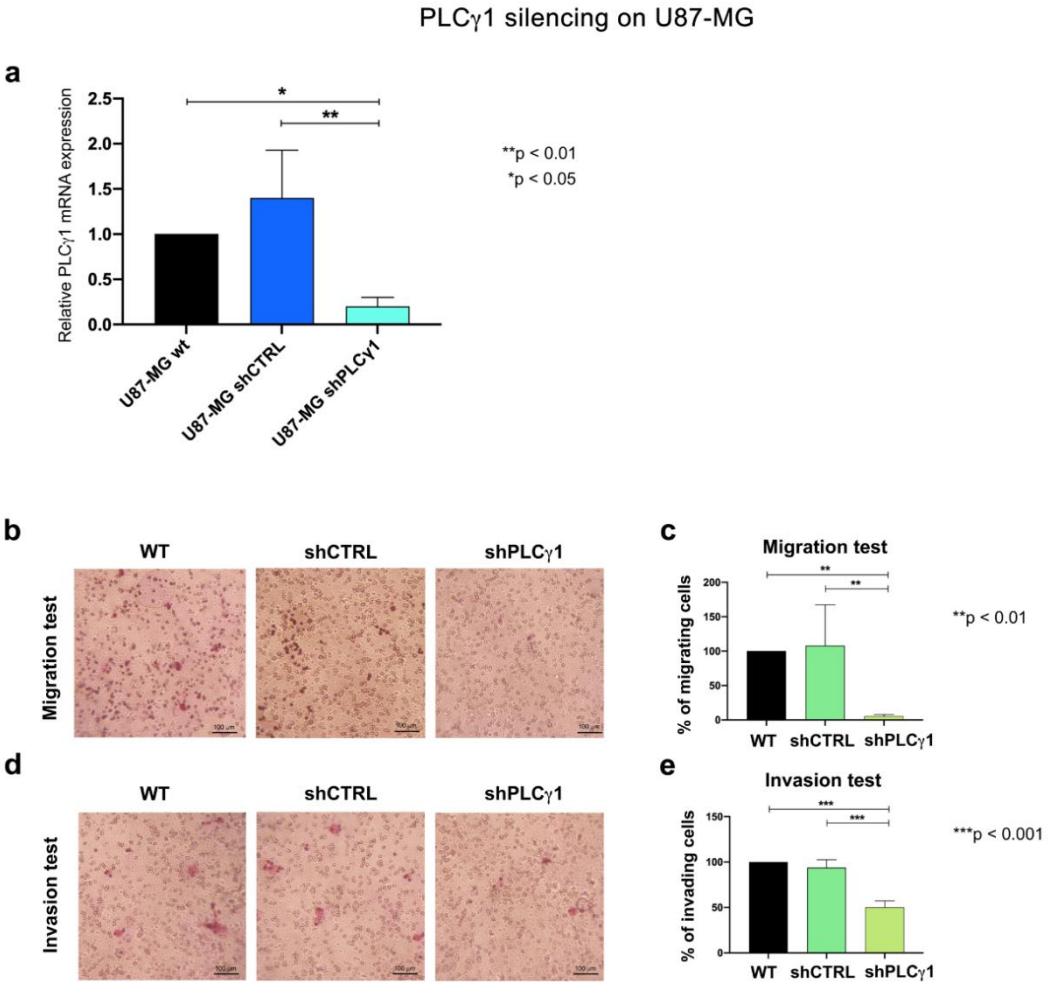

Figure 3

4. PLCγ1 overexpression leads to increased cell migration and invasion in U87-MG

To reinforce all these data, we subsequently carried out a transient PLCγ1 overexpression on the same cell model. Even under these circumstances, PLCγ1 gene expression and the relative overexpression were first confirmed through a Real-Time PCR analysis (Fig. 4a), comparing U87-MG cells overexpressing PLCγ1 (ovPLCγ1) to the respective controls (WT and negCTRL). Therefore, transwell migration assay revealed that cells overexpressing PLCγ1 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 PLCγ1 silencing. Indeed, following PLCγ1 overexpression there was an increase in the ability of U87-MG cells to invade (Fig. 4d,e). Therefore, a positive correlation between PLCγ1 expression level and the cellular migration and invasion abilities was confirmed in our cell model, strengthening what is already present in literature.

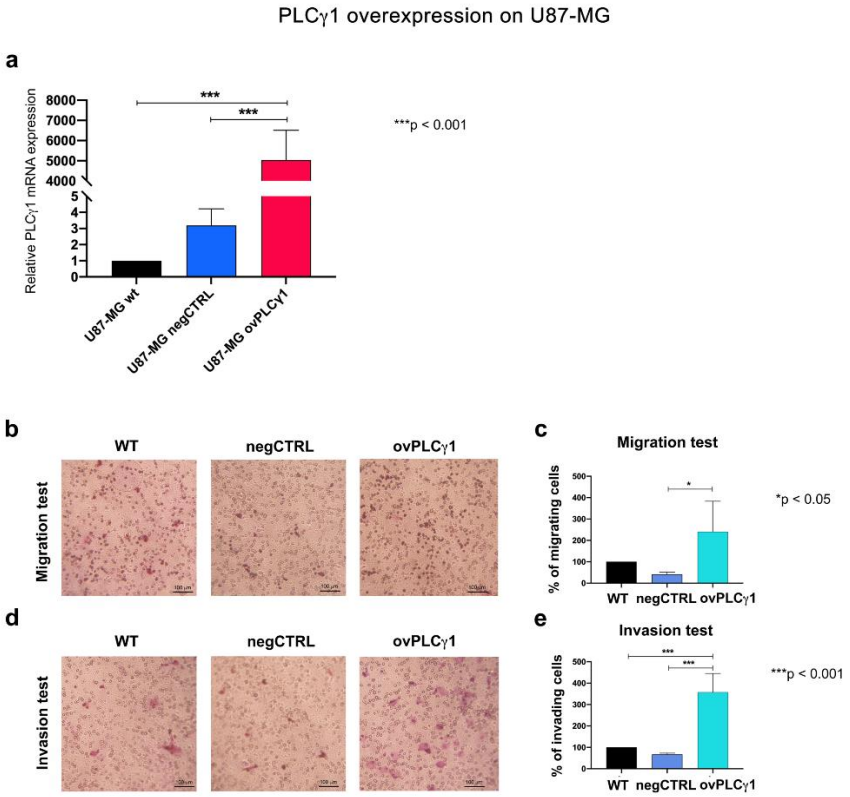

Figure 4

Discussion

Phoshoinositides (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 ideals biomarkers. In this paper it is highlighted how PLCγ1, 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 PLCγ1 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, PLCγ1 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, PLCγ1 activation is associated with angiosarcoma progression, and its upregulation and mutation have been consistently linked

to poor patient survival (Behjati et al., 2014). PLCγ1 role is associated also to squamous cell carcinoma (Xie et al., 2010), colorectal and gastric cancers (Li et al., 2005). PLCγ1 centrality in the progression of various types of cancer is probably linked to PLCγ1 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 PLCγ1 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 PLCγ1 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 PLCγ1 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, PLCγ1-silenced cells showed less ability to migrate and invade compared to the respective controls. On the contrary, cells overexpressing PLCγ1 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 PLCγ1 in gliomas' aggressiveness. However, the mechanisms by which PLCγ1 is upregulated in high-grade tumors are not clear yet. Further studies are certainly needed. In particular, it will be necessary to investigate the PLCγ1 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.

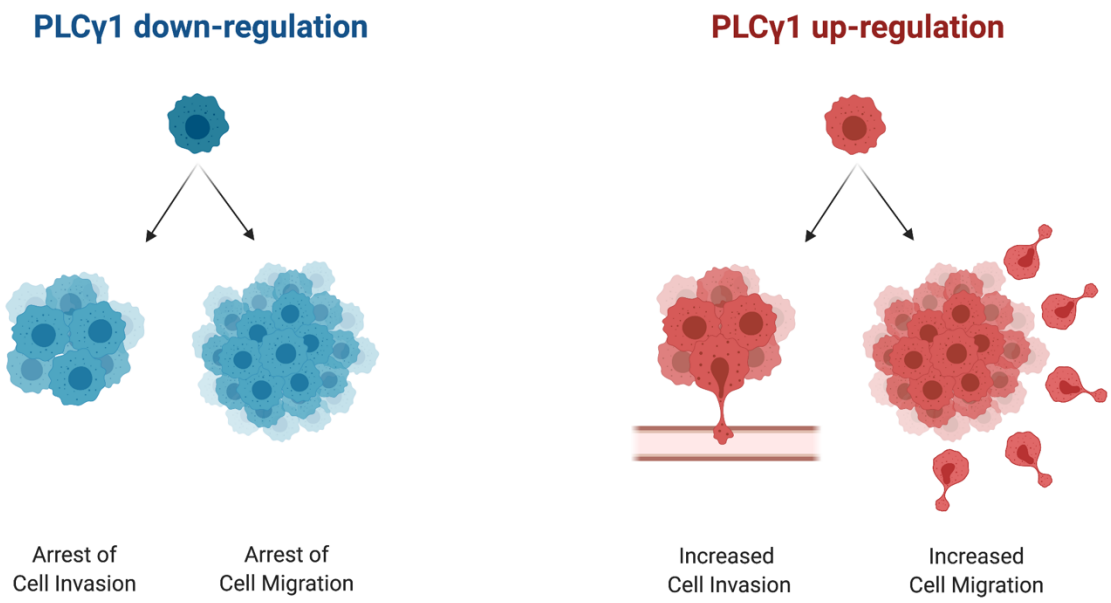

Figure 5

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: PLCγ1 gene expression correlates with gliomas' pathological grade

Panel a: Distribution of PLCγ1 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. PLCγ1 mRNA expression positively correlates with gliomas' pathological grade (p=1.4e−07). **Panel b:** Kaplan-Meier survival curves, extracted from the CGGA dataset, of PLCγ1 high or low expression patient groups. Patients were divided according to the median level of PLCγ1 mRNA expression ($p < 4.68e-07$ and $p = 0.008$).

Fig. 2: Glioblastoma patients are characterized by higher PLCγ1 gene expression compared to healthy individuals. PLCγ1 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 PLCγ1 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 $2^{-\Delta\Delta Ct}$ formula. Asterisks indicate statistically significant differences between the groups, with $*p < 0.05$.

Fig. 3: PLCγ1 silencing on U87-MG cell line and relative consequences analyses on migration and invasion abilities.

Panel a: PLCγ1 mRNA expression on U87-MG cell line after PLCγ1 silencing. PLCγ1-silenced cells (shPLCγ1) 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**). PLCγ1-silenced cells (shPLCγ1) were compared to wild type (WT) and mock-transduced (shCTRL) cells. Magnification 20x (bar: 100 μm). Columns show the mean ± 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**). PLCγ1-silenced cells (shPLCγ1) were compared to wild type (WT) and mock-transduced (shCTRL) cells. Magnification 20x (bar: 100 μm). Columns show the mean ± SD of three independent experiments with $***p < 0.001$.

Fig.4: PLCγ1 overexpression on U87-MG cell line and relative consequences analyses on migration and invasion cell abilities.

Panel a: PLCγ1 mRNA expression on U87-MG cell line after PLCγ1 overexpression. Cells overexpressing PLCγ1 (ovPLCγ1) 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 PLCγ1 (ovPLCγ1) were compared to wild type (WT) and mock-transduced (negCTRL) cells. Magnification 20x (bar: 100 μm). Columns show the mean ± 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 PLCγ1 (ovPLCγ1) were compared to wild type (WT) and mock-transduced (negCTRL) cells. Magnification 20x (bar: 100 μm). Columns show the mean ± 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 PLCγ1 expression level and the acquisition of a more aggressive cellular phenotype. Indeed, following PLCγ1 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 PLCγ1.