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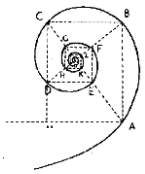

DOTTORATO IN MEDICINA MOLECOLARE E TRASLAZIONALE

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DESTABILIZE THE GLIOMA CELL NICHE THROUGH HSP90-GRP78 NETWORK TARGETING

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SOMMARIO

INTRODUZIONE: *I gliomi costituiscono i tumori maligni più comuni e letali del sistema nervoso centrale e rappresentano l'1,3% di tutti i tumori umani.*
I Glioblastoma (GBM, WHO IV grado) sono la neoplasia cerebrale di derivazione astrocitaria, più frequente negli adulti, in particolar modo nei pazienti anziani con età media di 55 anni. Essi sono caratterizzati da un comportamento altamente aggressivo e da prognosi infausta, con un'aspettativa di vita non superiore ai 14 mesi. Negli ultimi anni, l'assenza di opzioni terapeutiche adeguate ha spinto la comunità scientifica a ricercare differenti bersagli molecolari e bio-marcatori in grado di permettere una più adeguata stratificazione prognostica e terapeutica dei pazienti. Infatti, l'assenza di opzioni terapeutiche adeguate rendono i GBM una malattia mortale.
I gliomi sono caratterizzati dalla presenza di focolai necrotici circondati da cellule ipossiche. Questo microambiente sfavorevole suscita nelle cellule tumorali percorsi di compensazione come l'autofagia e la risposta a proteine con un errato ripiegamento spaziale (UPR). Essi sono due percorsi interconnessi e sfruttati dalle cellule tumorali per far fronte a condizioni di scarsità di nutrienti, ipossia o stress generato all'interno del microambiente neoplastico. Inoltre, è stato dimostrato come l'ipossia sia essenziale per la motilità cellulare e per la salvaguardia della cosiddetta nicchia di cellule staminali tumorali (GSC).
Le proteine dello shock termico (HSP) sono proteine altamente conservate, che vengono espresse in risposta a varie forme di stress e a cambiamenti ambientali, e in specifici stadi di sviluppo e differenziazione. Alcune di esse sono espresse anche in condizioni fisiologiche agendo da chaperon molecolari. Le HSP, quindi, compiono una varietà di funzioni biologiche, tra cui la regolazione della sintesi, piegatura, assemblaggio, e degradazione di differenti tipi di proteine. Inoltre, esse sono costitutivamente espresse in numerosi tipi di neoplasie ed in linee cellulari tumorali di diversa origine istogenetica. Lo chaperon GRP78, situato nel reticolo endoplasmatico, è anch'esso espresso in maniera aberrante in differenti tipi di tumori umani, e studi preliminari hanno dimostrato che la sua inibizione in modelli animali di glioma è in grado di ritardare la crescita neoplastica.
L'elevata espressione delle HSP nel glioma maligno potrebbe indicare un possibile coinvolgimento di queste proteine nella resistenza alla chemioterapia. Esse infatti, svolgono un ruolo di protezione all'interno delle cellule da agenti o condizioni di stress ambientale in grado di danneggiarle.
SCOPO DELLO STUDIO: *Valutare la relazione tra l'espressione dell'asse HSP90-GRP78, nelle aree peri-necrotiche tumorali e/o nelle cellule a pseudo-palizzata, contribuisce a mantenere un ambiente favorevole alla sopravvivenza e se essa è regolata da stimoli ipossici che svolgono un ruolo nel sostenere le cellule staminali di glioma e la motilità cellulare. Definire il contributo degli chaperon molecolari nella modulazione da parte di farmaci specifici sull'assetto HSP-GRP78 per fornire targets per nuove terapie utili per migliorare la prognosi dei pazienti affetti da tumori cerebrali.*
MATERIALI E METODI: *Casistica Clinica. E' stata raccolta una larga coorte di pazienti (n° 123), reclutati presso la Fondazione IRCCS Ca' Granda tra il 2013 e il 2016, con profili molecolari e caratteristiche clinico-patologiche associate. Da*

ciascun paziente è stato isolato l'RNA totale con Tizol® reagent (Thermo Fisher Scientific). Per la quantificazione dell'espressione genica sono stati utilizzati reagenti HumanTaqManAssays (Applied Biosystems). Linee cellulari di glioma. LN229, SW1088 e T98G provenienti dall'ATCC sono state mantenute come suggerito dal produttore. Le cellule U251-HRE sono state donate dalla Dott.ssa Ottobrini. Sono stati effettuati esperimenti di gene knock-out. Trasfezioni transienti di short interfering RNA (Sigma Aldrich) sono state effettuate utilizzando Lipofectamine3000 (Life Technologies). Colture tissutali organotipiche. Colture organotipiche di glioma sono state ottenute attraverso l'utilizzo del vibratome (VT1200 Leica Microsystems). Le fettine sono state coltivate su supporti dedicati (Millipore), in presenza o assenza di farmaci: Temozolomide e/o Gamitrinibs.
RISULTATI: *L'espressione di GRP78 e di TRAP1 appare aumentata nei tumori di alto grado rispetto a quelli di basso grado o al parenchima cerebrale normale. Elevati livelli di proteina correlano a prognosi infausta.*
Inoltre, GRP78 sembra essere arricchito nelle aree peri-necrotiche dei gliomi umani ad alto grado e la sua overespressione correla con HIF-1α. Modulando l'espressione di GRP78 in linee cellulari LN229, tramite siRNA o trattamento farmacologico con Gamitrinibs, si ottiene una diminuzione della popolazione side e della motilità delle cellule tumorali. Il regolatore di ipossia HIF1α diminuisce in cellule knock-out per TRAP1 rispetto al controllo, e un aumento dei geni dell'apoptosi si evidenzia dopo trattamento farmacologico. Infine il trattamento con Gamitrinibs, rispetto alla Temozolomide, influenza significativamente la vitalità tumorale in colture organotipiche di glioma.
CONCLUSIONI: *Questi dati suggeriscono che GRP78 rappresenta un marker prognostico negativo per i gliomi di alto grado, e che le HSPs sono arricchite nelle aree tumorali peri-necrotiche dove sostengono un ambiente pro-sopravvivenza. Inoltre, la sovra-espressione di GRP78 nei tumori cerebrali potrebbe preservare la popolazione di cellule staminali in condizioni di stress, come l'ipossia. Questo studio sembra confermare il potenziale ruolo di GRP78 come bersaglio molecolare nel trattamento del glioma maligno.*
PAROLE CHIAVE: *GBM, Ipossia, GSC, HSPs, GRP78, HSP90*

ABSTRACT

INTRODUCTION: *Gliomas are the most common and lethal malignant tumor of the central nervous system representing 1.3% of all human cancers. Glioblastoma (GBM, WHO grade IV) is the most common astrocytic-derived brain tumor in adults, more frequent in older patients (mean age 55 years). They are characterized by a highly aggressive behavior and poor prognosis. Survival rates for gliomas are poor with a median survival that is not exceeding 14 months.*
Over the last years, the absence of adequate therapeutic options has led the scientific community to seek different molecular targets and bio-markers able to allow a more adequate prognostic and therapeutic stratification of patients. In fact, the absence of adequate therapeutic options makes GBM a fatal disease.
A feature of gliomas is the presence of tumor necrotic foci surrounded by severely hypoxic pseudopalisading cells. This unfavorable microenvironment elicits in tumor cells compensatory pathways as autophagy and the unfolded-protein response (UPR). They are two interconnected pathways exploited by tumor cells to cope with low nutrient conditions, hypoxia or other microenvironment-generated stress. Moreover, evidences showed that tumor hypoxia fuel cell motility and preserve the so-called cancer stem cells niche (CSCs).
Heat shock proteins (HSPs) are highly conserved proteins which are expressed in response to various forms of stress, in response to environmental challenges, and in specific stages of development and differentiation. Some HSPs are also expressed under non stress conditions and act as molecular chaperones. Thus, HSPs fulfill a variety of biologic functions, including the regulation of synthesis, folding, assembly, and degradation of different proteins. HSPs are constitutively expressed in human carcinomas or cancer cell lines of various histogenetic origins. The endoplasmic reticulum chaperone GRP78 is aberrantly overexpressed by human cancers and preliminary reports showed that targeting of GRP78 in glioma animal model can delay cancer growth. The high expression of HSPs in malignant glioma indicates a possible role of these proteins in resistance to cancer chemotherapy. They in fact, protect cells from many cell-damaging agents or conditions of environmental stress.
AIM OF THE STUDY: *Assess whether the HSP90-GRP78 axe expression in peri-necrotic tumor areas and/or pseudo-palisading cells contributes in maintaining a pro-survival environment and whether it is regulated by hypoxic stimuli, plays roles in glioma cancer stem cell maintenance and in glioma cell motility.*
Define the contribution of molecular chaperones in glioma modulation of HSP-GRP78 axe by specific drugs to provide targets for novel therapies useful to improve prognosis of patients affected by brain tumors.
MATERIALS AND METHODS: *Clinical series. A series of patients' (n° 123) recruited at IRCCS Ca' Granda between 2013-2016, with correlation of molecular profiles to clinicopathological characteristic were collected. From each patient the total RNA was isolated with Tizol® reagent (Thermo Fisher Scientific). For gene expression quantification Human TaqManAssays (Applied Biosystems) and reagents were used. Glioma cell lines. LN229, SW1088 and T98G were from ATCC and maintained as suggested by manufacturer. U251-HRE cell were a generous gift from Dr. Ottobrini. Gene knock-out experiments were carried out as*

transient transfection of short interfering RNA (Sigma Aldrich) using Lipofectamine3000 as transfection reagent (LifeTechnologies). Organotypic tissue cultures. Organotypic glioma tissue were generated through vibratome (VT1200 Leica Microsystems) serial cutting of fresh tumors, and slices were cultured on dedicated supports from Millipore, were cultured with or without the drugs: Temozolomide and/or Gamitrinibs.

RESULTS: *The expression of GRP78 and TRAP1 are increased in high-grade compared to lower grade or normal brain parenchyma and, high protein levels correlated to poor prognosis. Moreover, GRP78 appears to be enriched in peri-necrotic areas of high-grade human gliomas where GRP78 overexpression correlates with HIF-1α expression. GRP78 targeting by siRNA or Gamitrinibs decreased the side population of LN229 cells as well as tumor cell motility. The hypoxia regulator HIF1α was decreased in TRAP1 knock-out cells respect to control. Lastly, an increase in apoptosis gene was evidenced after treatment with drugs. Finally, Gamitrinibs treatment of glioma organotypic cultures significantly affected tumor cell viability respect to Temozolomide.*

CONCLUSIONS: *These data suggest that GRP78 is a poor prognostic marker for high grades gliomas and HSPs are enriched in peri-necrotic tumor areas sustaining a pro-survival environment. Moreover, these data suggest that GRP78 over-expression in brain tumors could sustain the stem cell population in stress conditions such as hypoxia. This study confirms the potential role of GRP78 as a molecular target in the treatment of malignant glioma.*

KEY WORDS: *GBM, Hypoxia, GSCs, HSPs, GRP78, HSP90*

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SYMBOL LIST

ATF6	Activating Transcription Factor 6
ATRX	α-thalassemia/mental-retardation-syndrome-X-linked
CHT	Chemotherapy
CNS	Central Nervous System
CREB	cAMP Response-Element-Binding
CSC	Cancer Stem Cell
CT	Computed Tomography
EGFR	Epidermal Growth Factor Receptor
eIF2α	Eucaryotic Initiation Factor-2α
ER	Endoplasmic Reticulum
FGF2	Fibroblast Growth Factor-2
GA	Geldanamycin
GAM	Gamitrinibs
GBM	Glioblastoma
GSC	Glioma Stem Cell
HIF	Hypoxia-Inducible factors
HRE	Hypoxia-Responsive Elements
HSE	Heat Shock Element
HSF	Heat Shock Factor
HSP	Heat Shock Protein
IDH	Isocitrate Dehydrogenase
IRE 1	Inositol Requiring Protein 1

MGMT	O6-methylguanine methyltransferase
MRI	Resonance Magnetic Imaging
NF	Neurofibromatosis
NOD-SCID	Non Oobese Diabetic/Severe Combined Immunodeficiency
NOS	Not Otherwise Specified
NSC	Neural Stem Cell
Oct-4	Octamer-binding transcription factor 4
PDGFR	Platelet-derived Growth Factor Receptor
PEK	Pancreatic eIF-2alpha Kinase
PERK	PKR-like Endoplasmic Reticulum Kinase
PKR	Protein Kinase R
PTEN	Posphatase and Tensin Homolog
RB	Retinoblastoma
ROS	Reactive Oxygen Species
RT	Radiotherapy
TK	Tyrosine Kinase
TMZ	Temozolomide
UPR	Unfolded Protein Response
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	von Hippel-Lindau
WHO	World Health Organization

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INTRODUCTION

1. INTRODUCTION

1.1 Brain tumors

The 2016 World Health Organization (WHO) Classification of Tumors of Central Nervous System, for the first time uses molecular parameters in addition to histology to define many tumor entities, thus formulating a concept for how CNS tumor diagnoses should be structured in the molecular era. Most notably, in the past all astrocytic tumors had been grouped together. The 2007 WHO divides the diffuse gliomas into three main categories: astrocytomas (related to astrocytes), oligodendrogliomas (related to oligodendrocytes) and oligoastrocytomas (related to a mixture of these two cell types) [1]. Now all diffusely infiltrating gliomas (whether astrocytic or oligodendroglial) are grouped together: based not only on their growth pattern and behaviours, but also distinguishing on the entity of isocitrate dehydrogenase (IDH1 or IDH2) mutation and co-deletion of chromosomal arms *1p* and *19q* (Table 1) [2].

The use of “integrated” phenotypic and genotypic parameters for CNS tumor classification adds a level of objectivity that has been missing from some aspects of the diagnostic process in the past [3]. It is hoped that this additional objectivity will yield more biologically homogeneous and narrowly defined diagnostic entities than in prior classifications, which in turn should lead to greater diagnostic accuracy as well as improved patient management and more accurate determinations of prognosis and treatment response. Nevertheless, WHO grade determinations are still made on the basis of histologic criteria. The phenotype remains essential is that because there are individual tumors that do not meet the more narrowly defined phenotype and genotype criteria. Combining histopathological and molecular features with the grade is show in table 2.

The grade II diffuse astrocytomas, the grade III anaplastic astrocytomas and the grade IV glioblastomas are now each divided into IDH-mutant, IDH-wildtype and NOS (not otherwise specified) categories. The diagnosis of

oligodendroglioma and anaplastic oligodendroglioma requires the demonstration of both an IDH gene family mutation and combined whole-arm losses of *1p* and *19q* (*1p/19q* codeletion) (Figure 1).

Diffuse astrocytic and oligodendroglial tumours
Diffuse astrocytoma, IDH-mutant
  Gemistocytic astrocytoma, IDH-mutant
Diffuse astrocytoma, IDH-wildtype
Diffuse astrocytoma, NOS

Anaplastic astrocytoma, IDH-mutant
Anaplastic astrocytoma, IDH-wildtype
Anaplastic astrocytoma, NOS

Glioblastoma, IDH-wildtype
  Giant cell glioblastoma
  Gliosarcoma
  Epithelioid glioblastoma
Glioblastoma, IDH-mutant
Glioblastoma, NOS

Diffuse midline glioma, H3 K27M–mutant

Oligodendroglioma, IDH-mutant and
  1p/19q-codeleted
Oligodendroglioma, NOS

Anaplastic oligodendroglioma, IDH-mutant
  and 1p/19q-codeleted
Anaplastic oligodendroglioma, NOS

Oligoastrocytoma, NOS
Anaplastic oligoastrocytoma, NOS

Table 1. The 2016 World Health Organization Classification of Tumors of the Central Nervous System

WHO grades of select CNS tumours	
Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	II
Anaplastic astrocytoma, IDH-mutant	III
Glioblastoma, IDH-wildtype	IV
Glioblastoma, IDH-mutant	IV
Diffuse midline glioma, H3 K27M–mutant	IV
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	II
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	III

Table 2. Grading of selected CNS tumors according to the 2016 CNS WHO

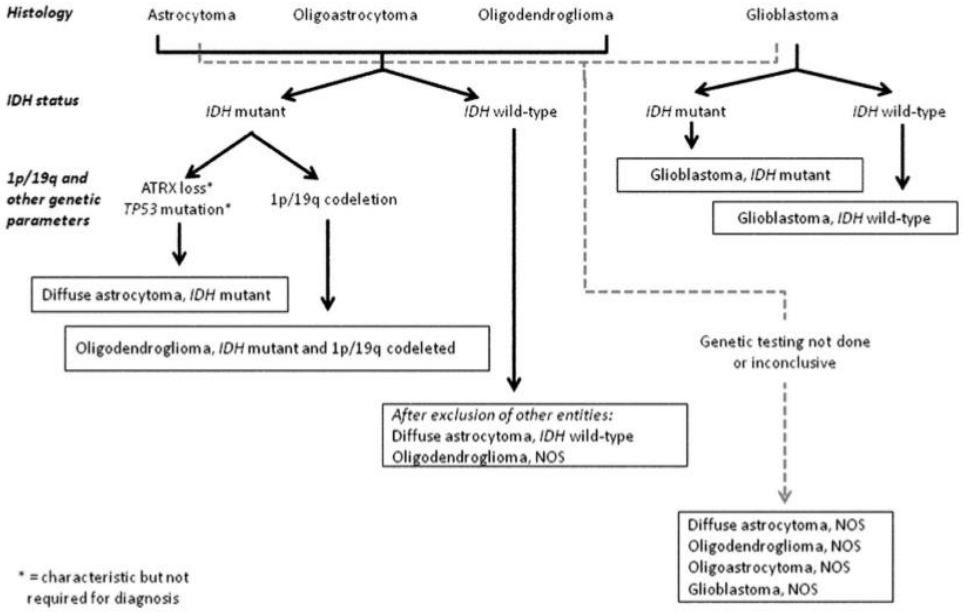


Figure 1. Algorithm for classification of the diffuse gliomas based on histological and genetic features

1.1.2 Epidemiology of gliomas

Malignant brain and CNS tumors are the 17th most common cancer and the 3rd most common cause of cancer death with more than 250.000 new cases diagnosed annually.

The worldwide incidence rate of primary malignant brain and CNS tumors in 2012, age-adjusted using the world standard population, was 3.4 per 100,000 [4].

Gliomas are the most common primary tumors of the central nervous system, they account for the 77% of primary malignant brain tumors [5].

Individuals of all ages can be afflicted but the incidence rates of glioma dramatically increases with advancing age with a peak around 50-55 years. Overall, 42.1% of all tumors diagnosed between 2008 and 2012 occurred in males and 57.9% in females but the malignant tumors occurred higher in males (55%) than in females (45%) (Figure 2) [6]. Furthermore, the incidence is 2-3 times higher in white than in black people [7]. Prognosis is poor and the median survival is 14.6 months; only few patients survive for three or more years. Infact, the survival after diagnosis with a primary malignant tumor in the US varies significantly by age, histology and behaviour Five-year relative survival rates following diagnosis of a primary malignant brain and CNS tumours 34.4% [8].

No underlying cause has been identified for the majority of malignant gliomas. The only established risk factor is exposure to ionizing radiation [9]. Approximately 5% of patients with malignant gliomas have a family history of gliomas. Some of these familial cases are associated with rare genetic syndromes, such as neurofibromatosis types 1 and 2, the Li−Fraumeni syndrome and Turcot's syndrome [10].

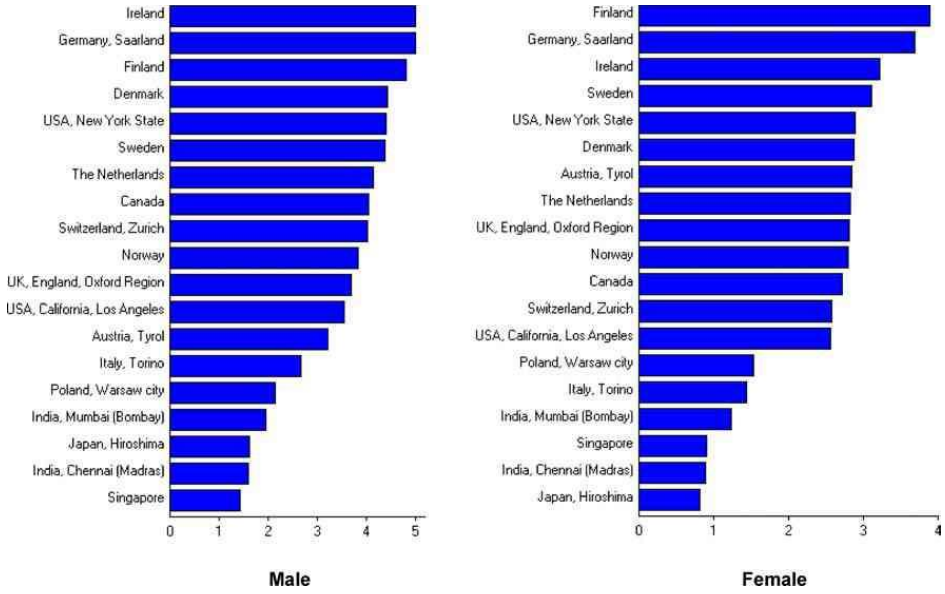


Figure 2. Epidemiology of gliomas in the world divided by the sexes

1.1.3 Glioma diagnosis

Patients with a malignant glioma may present either focal or generalized neurologic symptoms, including headache, confusion and loss of memory, neurological deficits and personality changes. Generalized symptoms reflect increased intracranial pressure and consist of headache, vomiting, and a cranial-nerve palsies. Focal symptoms and signs, such as hemiparesis and aphasia, reflect the intracranial location of the tumor. When a brain tumors is clinically suspected, the only test needed to diagnose a brain tumor is cranial resonance magnetic imaging (MRI), with or without gadolinium infusion. Computed Tomography (CT) is appropriate as a first-line procedure to obtain a quick assessment of the lesion, although is less sensitive than MRI. The diagnosis is must be confirmed by histological examination of tumour biopsy sample or by surgical resection. These imaging studies typically show a heterogeneously enhancing mass

with surrounding edema. Glioblastoma (GBM) frequently have central areas of necrosis and more extensive peritumoral edema [11–13].

1.1.4 Glioma prognosis

Despite decades of research and clinical trials, life expectancy for glioma patients has not improved considerably and is only about 2-3 years for anaplastic astrocytoma and 15 months for GBM [14,15].

There are several reasons because it has been so difficult to find new effective therapies against glioma. Drug delivery is limited by the blood-brain-barrier impediment and the distorted glioma vessels [16].

The invasive nature of gliomas makes the complete surgical resection of the tumor impossible. Tumor cells also have a strong intrinsic attitude for malignant progression and some cells, supposedly the cancer stem cells, are resistant to therapy. Lastly, over-expression of proteins involved in DNA repair machinery could dampen the effects of radio- and chemotherapy [17].

1.1.5 Glioma treatment

The standard treatment for gliomas is the surgical resection, radiotherapy and chemotherapy using alkylating agents. The size and localization of the tumor is important for the possibility to perform optimal surgery. Due to their invasive growth, gliomas indeed are impossible to completely resect. Surgery remains an important component in the treatment of GBM. It allows for a histologic confirmation of the diagnosis as well as cytoreduction. It may also serve a therapeutic role by reducing the intracranial pressure or to recovery of some neurological function. Advances in surgical imaging techniques, such as intraoperative magnetic resonance imaging (MRI), diffusion tensor imaging, awake craniotomy, cortical mapping, stereotactic guidance, and fluorescent-guided resection, have facilitated delineation of

tumor borders and have increased the extent of resection that can be achieved, improving the safety of surgery [18–21].

The combination of radiotherapy (RT) plus chemotherapy (CHT) is the most efficacious adjuvant therapy to prolong survival after primary resection. Temozolomide (TMZ), an oral alkylating chemotherapeutic agent, causes DNA damage and triggers a cascade of events leading to tumor cell apoptosis. The combination of RT and TMZ, as compared with radiotherapy alone, prolonged the median survival (14.6 months versus 12.1 months) [14].

TMZ adding a methyl group to purine bases of DNA, which leads to DNA damage and triggers a cascade of events that leads to tumor cell apoptosis [22,23]. The primary cytotoxic target of TMZ is O6-methylguanine. The methyl group added to O6-methylguanine can be removed by O6-methylguanine methyltransferase (MGMT), which is a DNA repair protein that functions to remove methyl groups from the O6 position of guanine. Removal of this methyl group confers resistance of tumor cells to TMZ and other alkylating chemotherapeutic agents by protecting cells from their DNA-damaging effects. In some patients, MGMT expression has been decreased or silenced by methylation of the promoter regions of the MGMT gene, preventing it from removing methyl groups from the O6 position of guanine [24]. The methylation status of the promoter region of the MGMT gene is one of the main mechanisms contributing to TMZ sensitivity (prolonged the survival to 2 years) or resistance in patients with GBM [25].

Genetically, GBM is a highly heterogeneous tumor harboring multiple recurrent and non-recurrent genetic alterations. Within the same tumor, cytogenetically related and unrelated clones coexist. These findings have inspired the investigation of molecularly targeted therapies designed to target tumor-specific recurrent genetic alterations as a novel approach to treating GBM. Particular interest has focused on inhibitors that target

receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR). Amplification of EGFR is one of the most common genetic alterations seen in GBM. Overexpression is the result of a mutant form of the receptor, EGFRvIII, which has a constitutively active kinase domain [26].

Gefinitib and Erlotinib are oral small molecule EGFR TKIs, which binding to intracellular kinase domain of EGFR, inhibit the activation of its downstream signaling pathways [27,28].

Overexpression of PDGFR signaling is another frequent alteration found in GBM. Imatinib is a small molecule multikinase inhibitor that binds PDGFR as well as several other RTKs. Imatinib blocks the PDGFR kinase domain to prevent activation of its downstream signaling pathways. Unfortunately, similar to EGFR inhibitors, clinical trials with Imatinib demonstrated a lack of therapeutic efficacy and minimal clinical benefit [29].

Malignant glioma are among the most vascular of human tumors characterized by extensive angiogenesis. Vascular endothelial growth factor (VEGF), a critical mediator of angiogenesis, is highly overexpressed in GBM. Bevacizumab is a humanized monoclonal antibody that binds and neutralizes the VEGF ligand, preventing activation of its receptors [30,31]. Bevacizumab is thought to improve outcomes by reducing angiogenesis, decreasing growth of tumor cells expressing VEGF, disrupting the microvasculature of the tumor leading to increased tumor hypoxia, and increasing tumor cell sensitivity to RT. In clinical trials of recurrent GBM, bevacizumab demonstrated clinical benefit and improved progression-free survival [32].

1.2 Glioblastoma

Glioblastoma (WHO grade IV), is the most common and lethal glial tumors, accounts for 75% of all adult glioma diagnoses (Figure 3). Incidence of glioblastoma increases with age, with rates highest in the 75 to 84 years, and it is 1.6 times more common in males [4].

Glioblastoma and other malignant gliomas are highly invasive, infiltrating surrounding brain parenchyma, yet they are typically confined to the central nervous system (CNS) and do not metastasize [33].

It is characterized histologically by considerable cellularity and mitotic activity, vascular proliferation, and necrosis with cells arranged around the edge of the necrotic tissue (pseudopalisading cells) (Figure 4) [34,35].

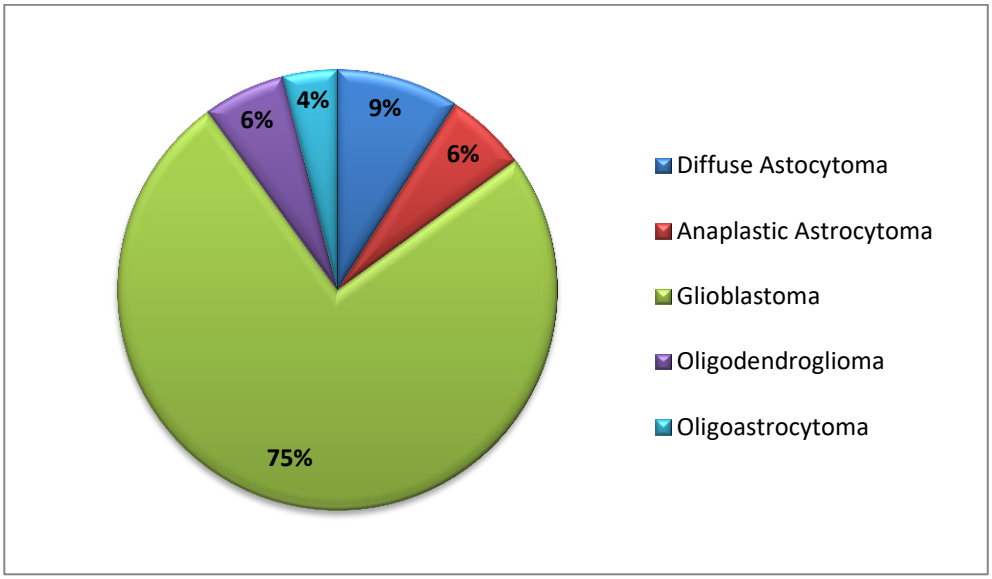


Figure 3. Distribution of diffuse astrocytic and oligodendroglial tumours

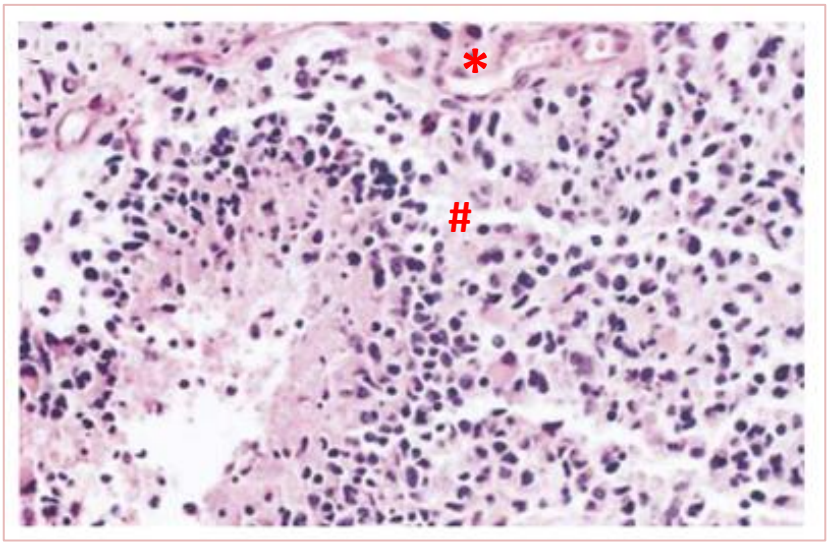


Figure 4. Hematoxylin and eosin of glioblastoma (# pseudopalisading cell; ***** vascular proliferation)

On the basis of their genetic features GBMs are divided into IDH-wildtype or IDH-mutant. IDH-wildtype corresponds most frequently with clinically defined primary glioblastoma which is characterized by short presenting history and it arises de-novo with no detectable pre-existing lower grade tumor. It is predominant in elderly patients (mean age = 55 years). IDH-mutant corresponds closely to so called secondary GBM with prolonged history and is typically preceded by a lower grade diffuse glioma. Preferentially arise in younger patients (mean age = 40 years) The majority of GBMs (~90%) are IDH-wildtype (Table 3) [2,36].

Whether a tumor is primary or secondary GBM cannot be predicted on basis of morphologic features. However the presence of large vessel thrombosis with large areas of infarction-like necrosis seems to be more prevalent in primary GBM. Prognosis and increased overall survival time seems to be better for the secondary tumors [37,38].

	IDH-wildtype glioblastoma	IDH-mutant glioblastoma
Synonym	Primary glioblastoma, IDH-wildtype	Secondary glioblastoma, IDH-mutant
Precursor lesion	Not identifiable; develops de novo	Diffuse astrocytoma Anaplastic astrocytoma
Proportion of glioblastomas	~90%	~10%
Median age at diagnosis	~62 years	~44 years
Male-to-female ratio	1.42:1	1.05:1
Mean length of clinical history	4 months	15 months
Median overall survival		
Surgery + radiotherapy	9.9 months	24 months
Surgery + radiotherapy + chemotherapy	15 months	31 months
Location	Supratentorial	Preferentially frontal
Necrosis	Extensive	Limited
TERT promoter mutations	72%	26%
TP53 mutations	27%	81%
ATRX mutations	Exceptional	71%
EGFR amplification	35%	Exceptional
PTEN mutations	24%	Exceptional

Table 3. Differences and characteristics between IDH-wildtype and IDH-mutant glioblastomas

Primary and secondary GBMs evolve from different genetic precursors and harbor distinct genetic alterations. Genetic alterations more typical for primary GBM are epidermal growth factor receptor (EGFR) overexpression, phosphatase and tensin homolog (PTEN) mutations, and loss of chromosome 10. Whereas genetic alterations more commonly seen in secondary GBM include isocitrate dehydrogenase-1 (IDH1) mutations, TP53 mutations, and 19q loss. Recent studies have also described mutations in the α-thalassemia/mental-retardation-syndrome-X-linked (ATRX) gene that are often copresent with IDH1/2 mutations and TP53 mutations in diffuse astrocytomas and secondary glioblastomas (Figure 5) [39,40]. Although different gene expression patterns in primary and secondary GBM were identified, characterization of the IDH1 mutation has allowed for reliable molecular differentiation of primary from secondary GBM. Additionally, IDH1 mutations appear to be associated with better prognosis and increase in overall survival. Interestingly, IDH1 mutations are also found in greater than 80% of diffuse astrocytoma and anaplastic astrocytomas, which are precursors of secondary GBM and less than 5% of primary GBM. Thus, IDH1 mutations are a reliable, objective molecular marker for secondary GBM over clinical and pathological criteria [41–43].

Using gene expression-based molecular classification of GBM that integrates multidimensional genomic data to establish patterns of aberrant gene expression and copy number alterations seen in GBM, four subtypes of GBM were identified. These GBM subtypes are classical, mesenchymal, proneural, and neural. Each subtype harbors distinct genetic alterations and expression profiles. The classical subtype is strongly enriched in the gene expression pattern observed in astrocytes. It is characterized by aberrant EGFR activity, leading to EGFR overexpression and loss of chromosome 10. Mutations in TP53, NF1, and IDH1 are uncommonly found in the classical subtype. The mesenchymal subtype is also enriched in the

gene expression pattern seen in astrocytes, but in addition, expresses mesenchymal markers as well as microglia markers. It is characterized by alterations in the gene for NF1 and PTEN deletions. EGFR overexpression is less commonly seen in the mesenchymal subtype compared with other GBM subtypes. The proneural subtype is enriched in proneural genes as well as the gene expression patterns seen in oligodendrocytes. It is characterized by alterations in TP53, PDGFR, and IDH1. Neural subtype gene expression pattern is the most similar to that of normal brain tissue. It is strongly enriched in the gene expression pattern seen in neurons, and expresses both astrocytic and oligodendrocytic markers [44–46].

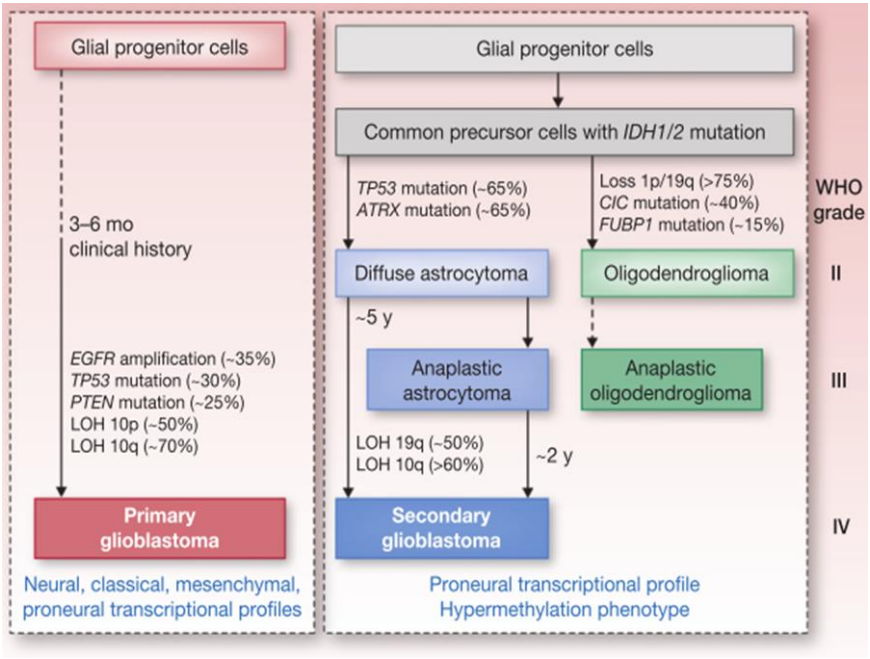


Figure 5. Genetic pathways to primary and secondary glioblastomas

1.3 Neural Stem Cells

Neural stem cells (NSCs) are multipotent cells within the brain capable of self-renewal and differentiation into all major cell types of the central nervous system (neurons, astrocytes and oligodendrocytes) [47].

The neural stem cell can be isolated from the walls of the ventricular system. The wall of the ventricular system is composed of a single layer of ependymal cells facing the lumen and a subventricular zone lying beneath the ependymal layer.

Reynolds and Weiss [48] isolated cells from the adult mouse striatum, induced the proliferation of precursors within the cultures by administering epidermal growth factor, and found that the precursors differentiated into neurons and glia when the epidermal growth factor was withdrawn. Richards and colleagues, [49] showed that precursors could be induced to differentiate into neuronal cells when they were initially stimulated in vitro with fibroblast growth factor-2 (FGF2) and then with medium conditioned by an astrocyte cell line. The multi-potential nature of these cells has been shown by clonal analysis, with such clones being able to give rise to both neurons and glia. The role of such cells, in rodents at least, might be to migrate to the olfactory bulb and differentiate into interneurons [50,51].

The NSCs use several signalling pathways important for the proliferation. The Notch pathway is known to promote the survival and proliferation of neural stem cells and to inhibit their differentiation [52].

In addition, the Notch pathway is also associated with tumor development, potentially as an oncogene [53]. Notch also promotes the expression of Nestin, a neural stem cell marker, in glioma cells [54].

Nestin is most highly expressed in ependymal cells and at lower levels in the rapidly proliferating subventricular zone progenitor cells in adult mammals. Interestingly, in response to spinal cord injury, Nestin expression is increased in ependymal cells, and with time, increasing numbers of Nestin-positive cells can be seen outside the ependymal layer, suggesting

that new cells may be generated from these cells in response to injury [55]. In the adult human hippocampus were demonstrated the presence of neurogenesis. The NSCs persist in the adult brain into senescence. The potential to generate new neurons may potentially be utilized to develop therapies in neurodegenerative diseases [56].

1.3.1 Glioma Stem Cells

The tumors are composed of heterogeneous populations of cells. Malignant cells with functional properties, such as self-renewal, the capability to develop into multiple lineages, and the potential to proliferate extensively, that drive the formation and growth of tumours, have been called "cancer stem cells" [57,58].

In the light of the repute only a few atypical cells within the cancerous mass might be responsible for the growth and recurrence of some tumours. Two hypothetical models have been proposed to explain this event. The stochastic model predicts that all cells in a tumor have a similar tumorigenic potential, which is activated asynchronously and at a low frequency in certain cells. Conversely, the hierarchical model that holds only in rare subset of cells within the tumor have significant proliferation capacity and, particularly, the ability to generate new tumours, with the remainder of the tumor cells or terminally differentiated cells representing differentiating. The latter hypothesis fits with the cancer stem cell theory (Figure 6) [59].

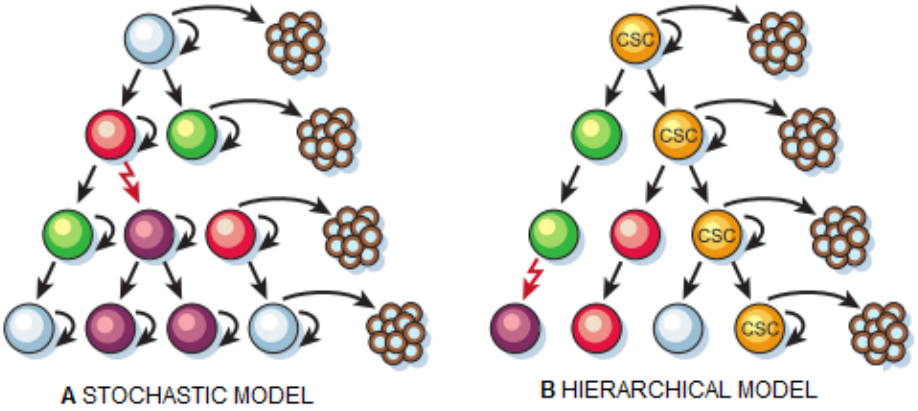


Figure 6. Stochastic and hierarchical model of tumor progression. **A**) Tumour cells are heterogeneous, but most cells can proliferate extensively and form new tumours; **B**) Tumour cells are heterogeneous and only the cancer stem cell subset (CSC, yellow) has the ability to proliferate extensively and form new tumours

The first evidence for the existence of CSCs came from acute myeloid leukemia, in which a rare subset of cells comprising 0.01-1% of the total population could induce leukemia when transplanted into immunodeficient mice [60,61]. These concepts and experimental approaches were then applied to other tumors such as breast [62], brain [63,64], colon [65], head and neck [66] and pancreas [67] cancers.

In the brain tumors, the glioma stem cells (GSCs) as the neural stem cells (NSCs), can implement a long-term proliferation, differentiate into neurons, astrocytes and oligodendrocytes, but there are also some differences: the proliferation in vitro and in vivo of GSCs is greater than that of NSCs, their growth can to be independent of exogenous mitogens, so aberrantly express surface markers, chromosomal abnormalities, and possess, once implanted in vivo, are able to form tumors the histologically identical to which they were derived [68]. According to the hypothesis of GSCs, the

their definition and identification provide a multimodal approach that includes: 1. the analysis of the self-renewal capacity; 2. the analysis of the differentiation capacity in all cell lineages of the CNS; 3. the search for common markers with adult stem cells (for example, Nestin, CD133 etc.);
4. verification of tumorigenicity when implanted into the brains of mice NOD-SCID [64,69].

The study of so-called "stem cell markers" led to the discovery of a subset of tumor cells that express a membrane protein called Prominina-1 (CD133). The CD133+ cells are more resistant to radiation therapy, compared to CD133- counterparty both in vitro and in vivo and have increased expression of anti-apoptotic genes. It is a possible correlation between greater expression of CD133 and worst outcome of patients has also been described [70,71].

Although CD133, appears to be a robust marker of brain tumor stem cells in initial studies, more recent studies have found that this marker does not distinguish tumorigenic from non-tumorigenic cells, as there are GSCs CD133- populations also tumorigenic and capable of self-renewal [72,73].

However, the most convincing demonstration of CSC identity comes from serial transplantation of a cellular population into the animal model, the universally recognized method for the functional evaluation of self-renewal and tumor propagation is precisely the ability to propagate the GSCs in a non-differentiated in vitro and to verify the tumorigenic capacity when injected into vivo [74].

1.3.2 Glioma Stem Cells Niche

The niche is a defined anatomical compartment that includes cellular and acellular components of residence, the stem cells - physiological and tumoral. The niche is constituted by a set of cells, blood vessels, glycoproteins and three-dimensional spaces which, together, form a highly specialized micro-environment in which the contact and communication between these components are critical for the maintenance of multipotency and the self-renewal of stem cells contained therein [75,76].

Oxygen plays a fundamental role in maintaining the integrity of these microenvironments (Figure 7). It has been shown how the oxygen is able to regulate the fate in differentiative or apoptotic effect of various types of neural precursors, since it represents a source of free radicals and that oxidative stress induces apoptosis of oligodendrocytes and other cells of the central nervous system. Hypoxia is also able to influence the survival and cell proliferation by inhibiting apoptosis. These observations have been crucial for the study of GSCs. Although initially the GSCs had been described mainly in niches situated around the tumor vessels, recent evidence suggests the presence of niches away from vases, precisely defined hypoxic niches, adjacent to the necrotic edge of the lesion and where the GSCs are modulated in their activity, from genes induced by hypoxia, such as HIF (HIF-1 and HIF-2), Oct-4, VEGF, Notch, and c-Myc [77–79].

The hypoxic microenvironment present in the tumor mass is a result of rapid cell proliferation in which the newly formed vasculature are unable to compensate. In this model, the presence of perivascular GSCs could represent the result of the so-called "angiogenic switch" whose first triggering event would be precisely the presence of hypoxia [80,81].

The cellular responses to hypoxia are regulated by a family of transcription factors Hypoxia Inducible Factors (HIFs). The HIFs factors are heterodimers consisting of an oxygen sensitive component, the α subunit

(HIFα), and by a component constitutively expressed, HIFβ. In normoxic conditions, HIFα is it is ubiquitinated by the product of the tumor suppressor gene von Hippel-Lindau (VHL) and directed to a proteasomal degradation; in hypoxia the interaction between HIFα and VHL protein is not leading to a stabilization of HIFα, which dimerizes with HIFβ and binds to hypoxia-responsive elements (Hypoxia-Responsive Elements, HRE) among the promoters of the genes regulated by low levels of oxygen. The genes transcribed later the effect of HIF are hundreds and modulate the survival, motility, metabolism and angiogenesis. There are two forms of the protein HIFα, HIF1α and HIF2α, highly homologous sequences and that bind HRE similar [82]. Regions with poor vascularity are characterized by severe hypoxia, low pH and lack of nutrients; these areas of necrosis are frequent in rapidly proliferating solid tumors and hypoxia has been associated with resistance to treatments, local invasion and poor prognosis. Glioma stem cells might be protected further from conventional therapies by factors within the vasculature niche. Treatments that disrupt aberrant vascular stem cell niches could therefore prove active against gliomas, because they might also function to disrupt stem cell maintenance [76,83].

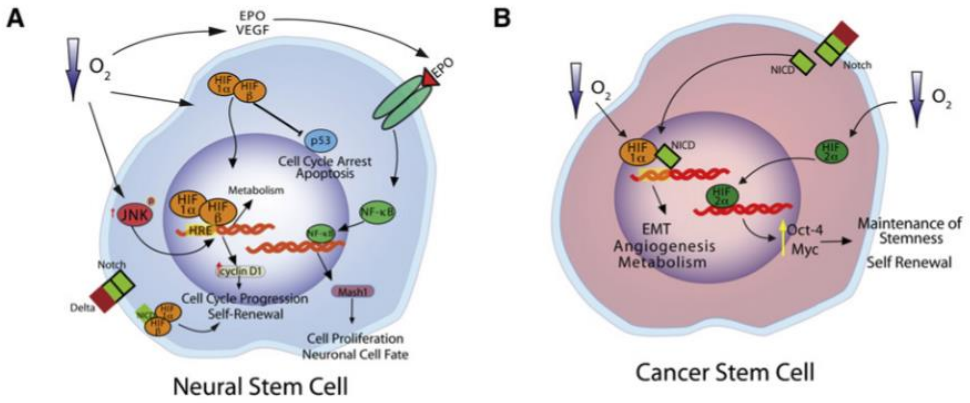


Figure 7. Role of oxygen in the maintenance of stemness in the Neural (**A**) and Cancer stem Cell (**B**)

Calabrese and colleagues demonstrated that stem cells from various brain tumors, including GBM, are maintained within vasculature niches that mimic the neural stem cell niche [81].

Notably, co-transplanting brain tumor stem cells and endothelial cells into immunocompromised mice, the initiation and growth of tumors in the brain were accelerated by the endothelial derived factors. Brain tumor stem cells seem to have potent angiogenic properties and can recruit vessels during tumorigenesis. It was shown that CD133-positive human GBM produced high level of VEGF and formed highly vascular and hemorrhagic tumors in the brains of immunocompromised mice.

In vivo, GSCs reside in a micro-niche composed of microvasculature readily accessible to the serum, and attachments to the stromal and more differentiated cells, and their biological behaviors are thus highly dependent on their microenvironment. Proliferating cells are found in the hypoxic center. In fact, it was observed that GBM stem cells directly differentiate into endothelial cells lining tumor vessels [84,85]. As well as regulating

stem cell proliferation and cell-fate decisions, niches also have a protective role defending stem cells from environmental insults [86].

1.4 Heat Shock Proteins

Heat Shock Proteins (HSPs) are a highly conserved proteins family at cellular level, expressed in response to different types of stressors, capable of causing structural and functional alterations of cellular proteins.

Among the physiological functions exerted by these chaperone there are: the movement of proteins within the cell compartments; folding of cytosolic proteins of the endoplasmic reticulum and mitochondrial; the contribution to the degradation of unstable proteins; the preservation by the formation of complexes and protein aggregates; and the action of re-folding of proteins partially denatured or incorrectly shaped [87–89].

The HSPs, in fact, have the task of ensuring the proper folding of proteins in their structure and facilitate transport within the cell compartments; they have also an important role in inducing thermo tolerance against strong thermal stress [90].

Heat Shock Proteins are so called because they produced following heat shock, but subsequently, were also observed as a consequence of pharmacological treatments or oxidative stress.

The discovery of HSPs is often attributed to Ritossa which, in 1962, observed in the salivary cells of the fruit fly, Drosophila, as a result of thermal shock [91]. Following this discovery many researchers were interested to HSPs, highlighting the expression of these proteins in other organisms such as chicken embryos, yeasts and plants.

It is believed that the expression of HSPs takes place in consequence of the binding of a transcription factor, HSF (Heat Shock Factor), for the HSP activated, with particular promoter regions called HSE (Heat Shock Element) located close to coding genes for the HSP [92,93].

Most of these genes do not contain introns, and the mRNA can be immediately translated into protein within a few minutes of exposure to the stressor. The stressor causes the transformation HSF from its inactive to active form shape, resulting in synthesis of HSP proteins. Although there are many hypotheses, about the mechanism through which the stress "active" the HSF, about the cell can “sense” the stress signal and translate that signal in response that lead to protein synthesis, it has not yet been clarified.

1.4.1 Classification of HSPs

Mammalian HSPs have been classified in two groups according to their size: high molecular weight HSPs and small molecular weight HSPs (Table 4). The first group includes three mayor families: HSP90 (Hsp90α, Hsp90β, TRAP1), HSP70 (HSP70, Hsc70, HSP73, GRP78), and HSP60.

Some of them are expressed constitutively; whereas, expression of others is induced only by stressful conditions and are confined to different cellular compartments such as cytosol, endoplasmic reticulum, mitochondria.

These proteins are molecular chaperones ATP-dependent and require the presence of cofactors called co-chaperones, such as Hsp40, Hop, Hip, Hip, bag1, to regulate their conformation and ATP-binding [94].

The HSP70 family constitutes the most conserved and best studied class of HSPs. It encompasses proteins ranging from 66 to 78 kDa (kiloDalton) localized mainly in cytoplasm (HSP70 or HSP72) or in mitochondria (mtHSP70) and one in the endoplasmic reticulum (GRP78/BiP). HSP70 proteins assist the folding of newly synthesized polypeptides, the assembly of multi-protein complexes, transport of proteins across cellular membranes and lastly, act as molecular chaperones [95].

Prominent members of the HSP90 family of proteins, that encompasses proteins ranging from 85 to 90 kDa, are Hsp90α, Hsp90β. These two

HSP90 isoforms are essential for the viability of eukaryotic cells, and are associates with a number of signalling proteins including ligand-dependent transcription factors, such as steroid receptor [96,97].

Mammalian HSP60, also called chaperonin, is mostly contained within the mitochondrial matrix although it can also be detected in cytosol. HSP60 is a constitutively expressed HSP, but its expression can increase after stress. HSP60 participates in the folding of mitochondrial proteins and facilitates the proteolytic degradation of misfolded or denatured proteins [98,99].

The second group includes small HSPs proteins that vary in size from 15 to 30 kDa.

HSP27, probably the most studied member of this family, is an ATP-independent chaperone whose main function is protection against protein aggregation. It is expressed in many cell types and tissues, at specific stages of development and differentiation. In cancer cells, the basal expression of HSP27 is abnormally high and associates with cell resistance to anticancer therapy [100].

1.4.2 HSP90/TRAP1

The main member of the Heat Shock Proteins family is HSP90, one of the most conserved molecular chaperones and present in many organisms from bacteria to humans. Participates to the regulation of many client proteins involved in signaling processes, cell proliferation and survival. The HSP90 family is distinguished compared to the other molecular chaperones because many of its substrates are known to be protein transduction signals, such as for example the receptors for steroid hormones and signals for the kinase [97,101].

HSP90 is one of the main species of molecular chaperones that need ATP to carry out their functions. They are among the most abundant proteins

present in the cells in fact represent 1-2% of total cellular protein, the value of up to 4-6% in the cellular stress conditions [102].

In particular, HSP90 appears to be very abundant in the cytosol, the endoplasmic reticulum, and in mitochondria, and their natural abundance can make difficult the identification of other classes of heat shock proteins whose synthesis can be induced exclusively by exposure to stress. The main task of HSP90 is to stabilize protein before occur their completed folding or their activation, but they also play a key role in the dense network of signal transduction, cell cycle control, in the degradation of proteins and in the movement protein [103].

The association of the HSP90 with many cellular proteins seems that induces or inhibits the normal function of these proteins, also they form stable complexes with transcription factors. To fulfill their role within the cells, HSP90 act as part of a multi chaperon machine in association with HSP70 and cooperate with a number of co-chaperones.

HSP90 is ubiquitously expressed in all normal cells and is over-expressed in tumor cells. HSP90 has a critical role for tumor cells, since it allows to maintain the functions of mutant proteins built up during the process of carcinogenesis and also seems to be actively involved in the protection of cells from apoptosis; so the over-expression of HSP90 corresponds in many types of cancers, a negative prognosis in terms of survival and response to therapy. The molecular mechanisms that involve this chaperone in resistance to anticancer therapies concern, in reality, different aspects: HSP90 efficiently ensures cytoprotection repairing the damage caused by the administration of cytotoxic drugs; protects cancer cells from apoptosis spontaneous, preserves the internal micro-circulation to the tumor and even improves the repair of DNA damage. In light of this, HSP90 emerges as a promising target in cancer treatment [104].

HSP90 exists in the form of a homodimer composed of four parts: N-terminal part contains the binding site for ATP; a linker between the N-terminal region and the intermediate region; intermediate domain; C-terminal domain where there is a second binding site for ATP.

They have been identified five isoforms of HSP90 which differ in the cellular localization. The two HSP90α and HSP90β, GRP94 that is in the ER, TRAP1 in the mitochondrial matrix [96].

TRAP1 was initially identified as a homologue of HSP90, since it has a high homology in the most conserved region, the N-terminal. The first function attributed to TRAP1 was the protection of mitochondria against oxidative stress induced by ROS (Reactive Oxygen Species). It can interact with the TNF receptor and the retinoblastoma protein (Rb). Hsp90 and TRAP1 are abundantly present in the mitochondrial compartment of cancer cells, while found at low levels in normal cells. TRAP1 represents a protection factor of cell apoptosis, inhibits with HSP90 the intrinsic pathway by blocking the apoptotic process in the early stages [105,106].

The use of inhibitory drugs, such as Geldanamycin (GA) and the Radicicolo (inhibitors of the ATPase pocket) leads to a block in the malignant cell proliferation. These molecules in fact, prevent HSP90 to fold and stabilize the client proteins, which are associated in a non-functional complex and therefore are degraded by the proteasome dependent ubiquitination and proteolysis [107,108].

The drugs used conventionally as HSP90 inhibitors can be effective also in inhibiting the function as the at the mitochondrial level. The first molecules synthesized to act on mitochondrial targets have been defined as Gamitrinibs (GA mitochondrial matrix inhibitors). These inhibit the ATPase activity of TRAP1 directly inducing the increase of mitochondrial permeability thus leading to the loss of membrane potential and the release of cytochrome *c* into the cytosol. The GAM derived from a combination of

three parts: the GA (Geldanamycin), or rather its derivative, 17-AAG; a repeated structure of guanidinium salts; a linker that links the two portions. The accumulation in the mitochondria due to a rapid loss of potential of the inner membrane and, only in the cytosol of the tumor cell, restores the release of cytochrome *c*. The GAM shows, in fact, a modest or no cytotoxicity on normal cells at doses sufficient to cause the death of cancer cells [109].

FAMILY	NAME	Other NAME	LOCALIZATION
HSP 100	HSP 110 HSP 104 GRP 100		NUCLEUS CYTOSOL ER/GOLGI
HSP 90	HSP 90 GRP 94	HSP 82 ERP 90	NUCLEUS/CYTOSOL ER
HSP 70	HSP 70 GRP 78 MTP 70	HSP 72, DnaK BIP GRP75	NUCLEUS/CYTOSOL ER MITOCHONDRIA
HSP 60	HSP 60 HSP 58	GroEL	CYTOSOL MITOCHONDRIA
HSP 40	HSP 40	DnaJ	NUCLEUS/CYTOSOL
HSP 30	HSP 32 HSP 35	 G3PDH	CYTOSOL CYTOSOL
SMALL HSP	HSP 27 HSP 10	 GroES	NUCLEUS/CYTOSOL MITOCHONDRIA

Table 4. Classification of mean heat shock proteins and subcellular localization

1.4.3 HSP70

It is the most conserved family, presents a high levels of sequence conservation in all species where it has been identified [110,111].

HSP70 are constituted by two major functional domains, the first portion is present in the N-terminal of the protein of about 40 kDa with ATPase function, and the second is present in the C-terminal portion of the protein of about 25 kDa peptide-binding. The two domains are separated by a region susceptible to the cut of the protease. Since the HSP70 are localized in the cytosol, the endoplasmic reticulum (ER), in the mitochondria, in the precursors of the members intended to be transported within these organelles, it is present, at the N-terminus, a transit peptide with a sequence variable. For many HSP70, the presence of a sub-domain to the C-terminus of about 5 kDa, it is essential to allow interaction with their co-chaperones. The wide spectrum of functions of HSP70 is due to the intervention of co-chaperon that perform specific cellular functions in cooperation with the HSP70 [112].

They are activated as a result of stress. The induction heat of Hsp70 is mediated by heat shock transcription factor (HSF) that correspond to the heat shock elements (HSE) in the promoters. The active trimeric HSF bind the HSE present in the promoters of many genes inducible by heat, including the HSP70. In conditions where there is no thermal shock, either HSP70 binds to inactive HSF or remains as free HSP70. During the heat shock, the pool of free HSP70 is reduced by binding denatured proteins by heat, that releases the HSF from HSP70. The presence trimeric HSF activated in the nucleus promotes transcription of HSP70 thereby increasing the amount. The HSP70 excess capture the HSF newly synthesized before they are brought to the nucleus and form a trimeric structure, so that from time to time attenuates the expression of HSP70-induced heat and others HSPs. The cytosolic HSP70 are involved in cellular processes such as protein folding, refolding of the denatured

proteins, prevention of protein aggregation and maintain the protein in a state of competent amount in eukaryotes [113,114].

It was therefore suggested that the cytoplasmic HSP70 are involved in the translocation of the ER protein precursors. The lumen of the ER, where the pathways of secretion of the precursor proteins are generally co-translocated, belongs a member of the HSP70 family, GRP78/BiP. It is considered one of several chaperones to be involved in the translocation of precursor proteins across the ER membrane, the protein folding, assembly and binding to proteins with incorrect folding into the lumen of the ER. The GRP78/BiP expression is induced by stress conditions which lead to proteins having an incorrect folding. Many studies have shown that overexpression of BiP relieves stress to the ER by restoring the processing of secreted proteins and the folding [115–117].

1.4.4 ER Stress

In eukaryotic cells the endoplasmic reticulum (ER) is the first compartment in the secretory pathway. Inside this organelle proteins are synthesized, correctly folded to get the right shape and modified to make them finally functional. The properly folded and modified proteins are then translocated to the Golgi complex, and are retained in the reticulum the not folded properly or proteins that must be degraded. The alteration of any of these processes cause ER stress. The ability of the cell to respond and solve these alterations is essential to maintain its normal homeostasis [118,119]. When the cell are exposed to any stress, one of the main consequences is the accumulation in the lumen reticulum of protein not folded correctly. To maintain their homeostasis, cells active two different answers; first is the "Unfolded Protein Response" (UPR), which leads to activation of transcriptional genes coding for molecules resident in the endoplasmic reticulum and facilitate proper protein folding. These molecules, called

"chaperones", such as GRP78/BiP and GRP94, promote the right setting protein folding trying to limit the increase of unfolded proteins.

The second answer is instead, a radical and rapid suppression of protein synthesis to prevent other proteins are synthesized. The repression of protein synthesis occurs in the stress response in mammalian cells through the increase of eIF2α phosphorylation. eIF2α phosphorylated interferes with the formation of translation initiation complex by blocking the translation itself. In ER stress eIF2α is phosphorylated by PKR and PEK/PERK, repressing the synthesis. Phosphorylation of eIF2α is the only way known that regulates the suppression of protein synthesis in response to the reticulum stress [120].

The UPR, on the other hand, begins as soon as the balance within the endoplasmic reticulum is compromised. Three different transmembrane proteins resident in the reticulum have been identified as "sensors" of the presence of ER stress: IRE1 (α and β), a kinase and endoribonuclease, the kinase PERK and the transcription factor ATF6 (α and β) (Figure 8).

IRE1 and PERK, activated after stress, homodimerizes, autophosphorylates and then are activated, while ATF6 translocates to the Golgi. Into the Golgi it is cut by the two proteases, S1P and S2P, generating a transcription factor activated.

The combined effect of the activation of all three of these molecules involves a positive regulation of genes encoding chaperones and for proteins involved in the degradation and a negative regulation of protein synthesis and the consequent inflow of nascent proteins within the reticulum.

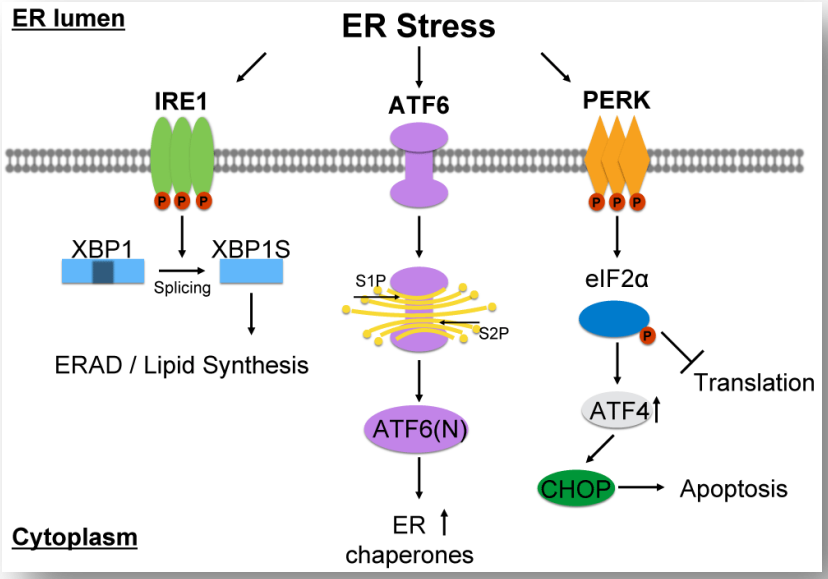


Figure 8. Upon accumulation of unfolded protein in the ER lumen, IRE1, ATF6, PERK are active UPR and/or suppression of synthesis

1.4.4.1 GRP78/BiP and Endoplasmic Reticulum Stress

The activation of all three UPR components depends on dissociation from these of a fourth molecule, enormously abundant in the lumen of the reticulum, GRP78/BiP.

BiP is an ATPase, member of the "heat shock protein 70 family". In unstressed cells IRE1, ATF6 and PERK are retained in the lumen of the reticulum by BiP, which is associated to their luminal domain. BiP on the other hand, is also the main chaperone of the cell, is associated so even with the newly synthesized proteins to allow the correct folding [121].

In a cell not stressed, BiP is in continuous balance between binding to proteins to fold and binding to the stress sensors. If, however, any stress leads to increased protein not folded properly, the balance shifts; most of BiP is bound to proteins which have to be folded and the sensors, IRE1,

ATF6 and PERK are free and can be activated. As previously described IRE1 and PERK are free to form dimers and phosphorylate themselves, while ATF6 is free to transit the Golgi complex, and there be cut from its two proteases. Though IRE1, PERK and ATF6 are all activated simultaneously by the dissociation of BiP, each of the pathways activated by these is temporally distinct. This allows that different parts of the same UPR are adjusted each from different molecules [119].

After exposure to stress the way as quickly activated is the repression of protein synthesis mediated by PERK. Given that eIF2α is directed substrate of kinase, phosphorylation occurs immediately and blocks very quickly cellular translation [122].

Also the proteolytic cleavage of ATF6, and then its activation occur quickly after the stress occurred, but the expression of genes under its control, however, requires the nuclear translocation of its cytoplasmic domain activated, the induction of the transcription and synthesis, then longer times. Since the activation of ATF6 leads to the induction of many genes for chaperones, such as for example the same BiP and GRP94, this increases the ability of the protein folding pattern, and then the restoration of homeostasis. Following the synthesis block operated by eIF2α this can be seen in a certain way as the second stage of the UPR response [123].

During UPR, the transcription it is also positively regulated by ATF4, a transcription factor member of cAMP Response-Element-Binding (CREB) family. ATF4 requires for his translation the eIF2α phosphorylation. This transcriptional factor in fact, contains ORF at its 5' non-translated which in normal conditions not allow the translation, but in the presence of phosphorylated eIF2α allow the production of ATF4. The activation of IRE1 leads to transcriptional induction, but the activation of this pathway occurs after the activation of ATF4 and ATF6 [124,125].

AIM OF THE STUDY

2. AIM OF THE STUDY

The aim of this study was to assess whether the HSP90-GRP78 axe expression in peri-necrotic tumor areas or pseudo-palisading cells contributes in maintaining a pro-survival environment and whether it is regulated by hypoxic stimuli. Identify a subset of human gliomas in which the cancer-stem cell niche is sustained by high GRP78 expression and a hypoxic environment and determine whether GRP78 also promotes tumor cell migration, thus further plays roles in glioma cancer stem cell maintenance and in glioma cell motility and contributing in brain tumor relapse. At last, to define the contribution of molecular chaperones in glioma modulation of HSP-GRP78 axe by specific drugs in ex vivo glioma tissue cultures, to provide targets for novel therapies useful to improve prognosis of patients affected by brain tumors.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Human samples

An Ethical Committee approved the study and informed consent was signed by all enrolled patients. A series of patients' recruited at IRCCS Ca' Granda between 2013-2016, with correlation of molecular profiles to clinico-pathological characteristic were enrolled (Table 5). We collected 123 fresh human samples of brain cancers (89 grade IV, 16 grade III, 16 grade II, 2 grade I).

3.2 RNA purification and qRT-PCR

Samples were homogenized in 1 ml of Tizol® reagent (Ambion Thermo Fisher Scientific) using a tissue lyser (Qiagen). Tissue lysates were incubated for 5 min at room temperature. 200 µl of chloroform were added. Samples were centrifuged for 15 min at 13000 rpm and 4 °C and the upper aqueous phases were recovered. 500 µl of 100% isospropanol were added and samples were incubated at -20 °C over night. Samples were centrifuged for 15 min at 13000 rpm and 4 °C. Pellets were washed with 75% cold ethanol and dissolved in H_2O. The total RNA was quantified by spectrophotometer Nanodrop1000 (Thermo Fisher Scientific).

We performed retro-transcription with 300 ng (gliomas samples) of RNA using High Capacity cDNA Reverse Trascription Archive Kit (Thermo Fisher Scientific), according to the manufacturer's.

For gene expression quantification Human TaqMan Assays (Applied Biosystems) and reagents were used following the manufacturer's specification. Relative gene expression levels were normalized to ribosomal RNA 18s levels (housekeeping gene) and presented as Log $2^{-\Delta Ct}$. The instrument ABI PRISM® 7900HT (Applied Biosystems) was used for the analysis.

3.3 Tissue Micro Array (TMAs)

Tissue micro-arrays (TMAs) with representative cores of tumor (four per case) or normal (one per case) parenchyma was generated from 187 patients affected by brain cancers and by 100 corresponding normal brain counterparts (Table 6). Tissue cores were arranged in seven TMA blocks. Tumor histotypes included astrocytomas WHO Grade II or III (anaplastic), oligodendroglomas WHO Grade II or III (anaplastic), and glioblastomas WHO Grade IV. Hematoxylin and eosin (H&E) staining was used to control suitability of TMAs prior to immunohistochemistry.

3.4 Immunohistochemistry (IHC)

For each TMA block, 4 µm-thick sections were stained with GRP78 (1:1000, C50B12, CellSignaling), TRAP1 (1:200 HPA044227, Sigma Aldrich), Bcl2 (prediluited, 124, RocheVentana), and HIF-1α (1:200, 54/HIF1α, BDBioScience) using citrate buffer in antigen retrieval step. Immunohistochemistry was performed using Benchmark Ultra Roche Ventana immunostainer. Endogenous peroxidase activity was blocked by hydrogen peroxide (0,3%) for 10 minutes. The chromogen diaminobenzidine (DAB) was incubated for 8 min at room temperature. All slides were counterstained with haematoxylin.

A pathologist blinded to clinical data evaluated immunohistochemical results.

3.5 Cell lines

Human Glioma LN229 (Glioblastoma), SW1088 (Astrocytoma) and T98G (Glioblastoma) cells were purchased from the American Type Culture Collection (ATCC). Glioma cell lines were cultured in RPMI (Gibco-Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco-Thermo Fisher Scientific) and 1% of Penicillin and Streptomycin (Pen-

Strep, Gibco-Thermo Fisher Scientific) and were maintained in 5% CO2 humidified incubator at 37°C, as recommended by the manufacturer.
Human glioblastoma U251-HRE cell were a generous gift from Dr. Ottobrini (University of Milan - Centro di Imaging Molecolare e Cellulare IMAGO). Cells were cultured in RPMI supplemented with 10% FBS and 1% Pen/Strep and maintained in a humidified atmosphere of 5% of CO2 at 37°C. U251 cell line expressing the Luciferase a reporter gene under the control of a hypoxia responsive element (HRE) a inducible promoter, were used to assess the modulation of HIF-1α activity as consequences of a reduced O_2. In this way, the activity of HIF-1α can be visualized by non-invasive techniques, using luciferase activity as a hypoxia biosensor.

3.6 Cell lines transfection

LN229, SW1088, T98G, and U251 cells were seeded at $1x10^5$ cells/well in 6-wells plates and cultured in 2 ml of complete media for 24 h. Cells were, then transfected with 100 pM of MissionEsiRNA GRP78 and siRNA TRAP1 (Sigma-Aldrich) or negative controls (Sigma-Aldrich). Were utilized 5 µl Lipofectamine 3000 (Thermo Fisher Scientific) as transfection reagent in a final volume of 500 µl of OptiMem medium (Gibco-Thermo Fisher Scientific). After 5 h the transfection medium was replaced with 2 ml of RPMI (Gibco-Thermo Fisher Scientific) complete medium and cells were maintained in a 5% CO_2 humidified incubator at 37°C for 48 h and processed for individual experiments.

3.7 Apoptosis and cell cycle analysis

After 48 h from transfection, LN229, T98G or SW1088 cells were trypsinized and cells suspension was centrifuged at 1000 rpm for 5 min. Each pellet was washed with HBSS (Gibco-Thermo Fisher Scientific) and re-suspended in 600 µl of 1% FBS in PBS (Gibco-Thermo Fisher Scientific).

Then, 1.4 ml of cold absolute ethanol were added drop to drop and cells were incubated at -20°C over night. Fixed cells were centrifuged at 1000 rpm for 3 min, the pellet was washed with PBS and re-centrifuged at 1000 rpm for 3 min. The pellet was re-suspended in 500 µl of propidium iodide (PI)RNAase Stainig Buffer (BD Pharrmigen), to analyze cell cycle transition by FACS Canto I Flow Cytometer (BD Biosciences).
Alternatively, apoptosis cells were stained with Annexin V/PI using the FITC Annexin V Apoptosis Detection Kit (BD Bioscience, San Diego, CA, USA), according to the manufacturer's.
Evaluated by multiparametric flow cytometry and analyzed using FlowJo software.

3.8 Side population analysis

Transfected LN229 cells were resuspended at $1x10^6$ cells/ml in pre-warmed DMEM supplemented with 2% FBS and 10mM HEPES (Thermo Fisher Scientific). Hoechst 33342 (Cell Signaling Technology Inc) dye was added at final concentration of 5 µg/ml in presence or absence of 50 µM Verapamil (Sigma-Aldrich) and cells were incubated at 37°C for 2 h with intermittent shaking. At the end of the incubation, cells were washed by centrifugation at 4°C with ice-cold HBSS (Gibco-Thermo Fisher Scientific) and resuspended at the final concentration of $2x10^7$ cells/ml with cold HBSS supplemented with 2% FBS and 10 mM HEPES. In order to exclude dead cells, propidium iodide (Sigma-Aldrich) was added at the final concentration of 5 µg/ml. SP analysis and cell sorting were conducted on FACS Aria III (Bencton Dickinson, San Josè, CA, USA) equipped with a FACS Diva software (version 7.0).

3.9 Cell invasion analysis

After 48 h from transfection, the pellet of cells were counted using trypan blue (Cambrex Bio Sciences) and diluted at the concentrations of $1.25x10^5$ cells/ml using a medium devoid of FBS. Rehydrated Matrigel insert (BD BioCoat Matrigel Invasion Chambers BD Biosciences) were placed in a 24-wells plate and immersed in a chemotactic medium containing 10% FBS. Then, 500 µl ($2.5x10^4$ cells) of cells suspension were seeded into Matrigel-inserts and incubated in a 5% CO_2 humidified incubator at 37°C for 24 h. Cells on the lower surface of the membrane can be fixed, stained with Toluidine Blue and counted at the microscope.

3.10 Cell migration analysis

After 48 h from transfection, a wound was created in the monolayer of LN229 and SW1088 cells using a P200 micropipette tip. Then the cells were washed in PBS and incubated in complete medium for additional 24 h before analysis. For the analysis, random pictures were taken at 5x magnification when the scratch was performed (T0) and after 24 h (T24). The migration (units) was determined as reduction in the wound's gap using NIH Image-J software.

3.11 Immunoblotting

Aliquots of U251 cells were harvested, 48 h post-transfection, and solubilized in 100 µl of RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1%
SDS, 0.5% sodium deoxycholate, 1% TritonX) supplemented with 1X complete protease inhibitor cocktail (Roche). Cell lysates (50 µg) were separated by electrophoresis on 8% SDS-polyacrilamide gels, transferred to PVDF membranes (Millipore), probed with primary antibodies against GRP78 (Cell Signaling), TRAP1 (Sigma-Aldrich), HIF1α (BD Biosciences), or β-Tubulin (Sigma-Aldrich). Primary antibodies were probed with anti-

mouse or rabbit secondary antibodies (Bio-Rad) and reactive bands were visualized in chemiluminescence with ECL Plus reagents (GE Health Care).

3.12 Hypoxia by Petaka system

Post-transfection, aliquots of U251 cells were harvested ($6x10^5$ cells). The pellet was added with 15 ml of RPMI supplemented with 10% FBS and 1% Pen/Strep. Through a 18-gauge needle, the cells were injected into PetakaG3™ Low Oxygen Transfer (Celartia) and maintained in a humidified atmosphere of 5% of CO2 at 37°C, for 24 h and processed for individual experiments.

3.13 Luciferase reporter assay

U251 cells, 48 h post-transfection, were rinsed with 1X PBS. Then, removed all rinse solution were dispensed 500 µl of 1X PLB for 15 min at room temperature, to perform lysis. Into a luminometer tube pre-dispensed 100 µl of LAR II (Dual-Luciferase Reporter assay, Promega) and were added 20 µl of PLB lysate. Briefly, lysates were analyzed by a luminometer GloMax20/20 - Detection System, Promega) data were normalized to the amount of proteins (Bradford assay) and expressed as relative luminescence/fluorescence units (RLU/FLU = counts/mg proteins).

3.14 Organotypic tissue culture

Organotypic tissue slices were generated through vibratome (VT1200 Leica Microsystems) serial cutting of fresh tumors (300 µm), and slices were cultured on dedicated supports from Millipore. Ex vivo organotypic glioma tissue or glioma cell lines were cultured up to 72 hours in presence or absence of 100 µM or 200 µM Temozolomide, Gamitrinibs (to inhibit HSP signaling). As control sample we used an untreated tissue slice cultivated in complete media supplemented with vehicle (1 µl of DMSO in 1 ml of

media). Tissue cultures were formalin-fixed and paraffin-embedded (FFPE) for molecular or morphological (hematoxylin and eosin staining) and immunohistochemistry analyses. For each glioma culture tissue proliferation (Ki67 labeling), apoptosis (cleaved Caspase-3), and PI3 Kinase/AKT signaling, protein presence and expression in cultivated tissue slices were evaluated by immunohistochemistry (IHC) and scored as percentage of positive cells out of the total. IHC scores in cultivated tissue slices are expressed relative to baseline uncultured sample (T0).

3.15 Statistical analyses

Groups' comparisons were performed by Student *t* Test with Welch correction if appropriate. Survival curves were made according to Kaplan-Meier method and analysis were performed using log Rank test.

P values ≤0.05 were considered statistically significant.

For human samples (n=123 patients), data were analyzed using a GraphPad Prism©5 (GraphPad Software Inc., San Diego, CA, USA) software package for Windows.

GRADE	GRADE IV PRIM 82	GRADE IV SEC 7	GRADE III 16	GRADE II 18
GENDER (F/M)	F 34 / M 48	F 0 / M 7	F 6 / M 10	F 7 / M 11
AGE (years*)	64	43	47	46
KI67 (%*)	38	61	18	6
IDH WT (n)	82	0	4	4
IDH M (n)	0	7	6	11

Table 5. Characteristics clinico-pathological of 123 patients' enrolled in the study (* average)

Grade	Tumor type (abbreviation)	n	Age (years)	Gender (M/F)	Ki67 (%)	MGMT methylated cases (n, %)	$IDH1^{R132H}$ (n, %)	1p/19q LOH (n, %)
Grade II (n=60)	Astrocytoma (Astr)	13	41 (19-73)	9M/4F	-	7 (53.8%)	9 (69%)	-
	Oligodendroglioma (OD)	47	45 (25-72)	28M/19F	-	39 (83%)	31 (66%)	-
Grade III (n=29)	Anaplastic Astrocytoma (AA)	19	46 (22-78)	11M/8F	7 (1-50)	10 (52.6%)	8 (42%)	10 (59%)
	Anaplastic Oligodendroglioma (AO)	10	52 (34-72)	4M/6F	8.7 (1-30)	9 (90%)	8 (80%)	8 (100%)
Grade IV	Glioblastoma (GBM)	98	55 (24-78)	59M/39F	10 (2-75)	48 (48.9%)	11 (11%)	-

Table 6. Characteristics clinico-pathological of 187 patients' arranged in tissue microarray

RESULTS

4. RESULTS

4.1 GRP78 is up-regulated in human gliomas

The presence of GRP78 was confirmed at gene expression level in a patient's clinical series of fresh samples composed by 82 GBM IDH1 wild type, 7 GBM IDH1-mutated, 16 grade III and 18 low grade and correlated to their clinical features and/or prognosis. The GPR78 expression was significantly enriched in high grade gliomas respect to lower grades (**, $P<0.001$; Figure 9).

Therefore, were analyzed GRP78 and TRAP1 expression in a second larger set of gliomas arranged in tissue microarrays (TMAs) derived from 187 patients and in 85 normal brain counterparts.

Immunohistochemical analysis showed that GRP78 (***, $P<0.001$; Figure 10), and TRAP1 (*, $P<0.01$; Figure 11) are overexpressed by brain tumors compared to normal counterparts independent of tumor grade, and by high-grade gliomas compared to lower grade tumors. Investigating expression pro-survival and hypoxia markers, such as Bcl2 and HIF-1α respectively, we could detect an association between high GRP78 expression in peri-necrotic tumor areas (asterisk in Figure 12) and increased expression of Bcl2 and HIF-1α.

Furthermore, in regards to prognosis, higher protein levels of GRP78 were correlated to poor prognosis in the series of glioma patients as showed by survival curves ($P<0.0001$; Figure 13).

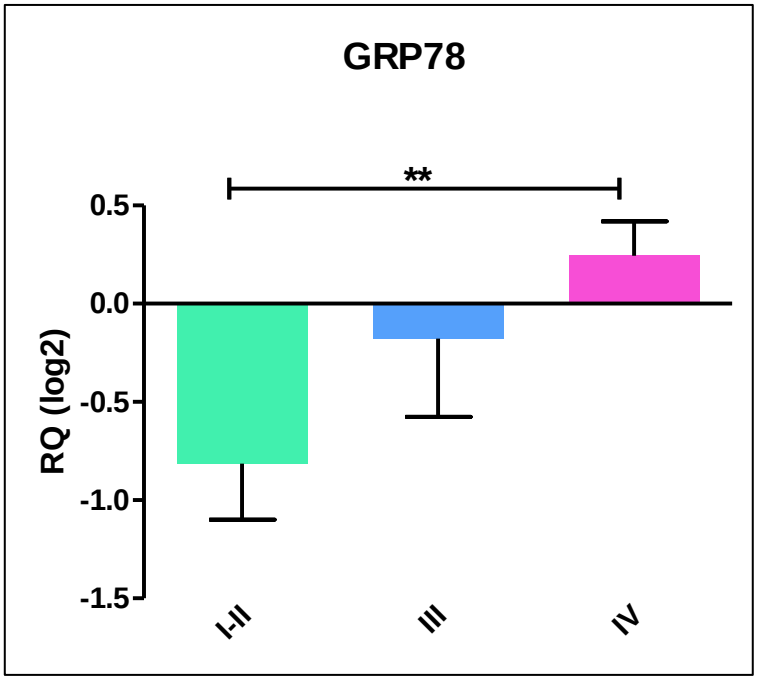


Figure 9. Real Time q-PCR analysis of GRP78 expression in human glioma with different grade (**, P<0.001)

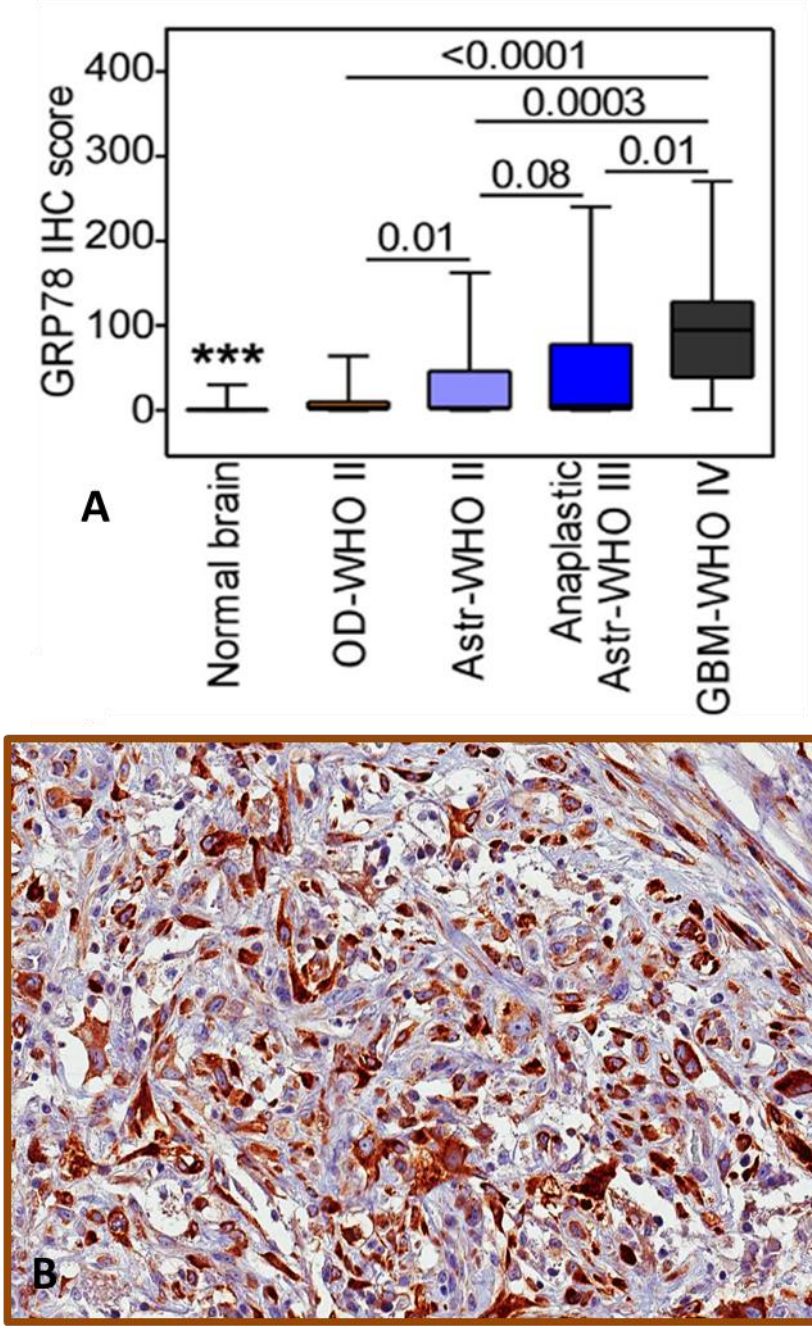


Figure 10. **A)** Quantification of GRP78 in human gliomas with different grades and in normal brain parenchyma. **B**) Representative picture of human gbm obtained by GRP78 staining (20x magnification)

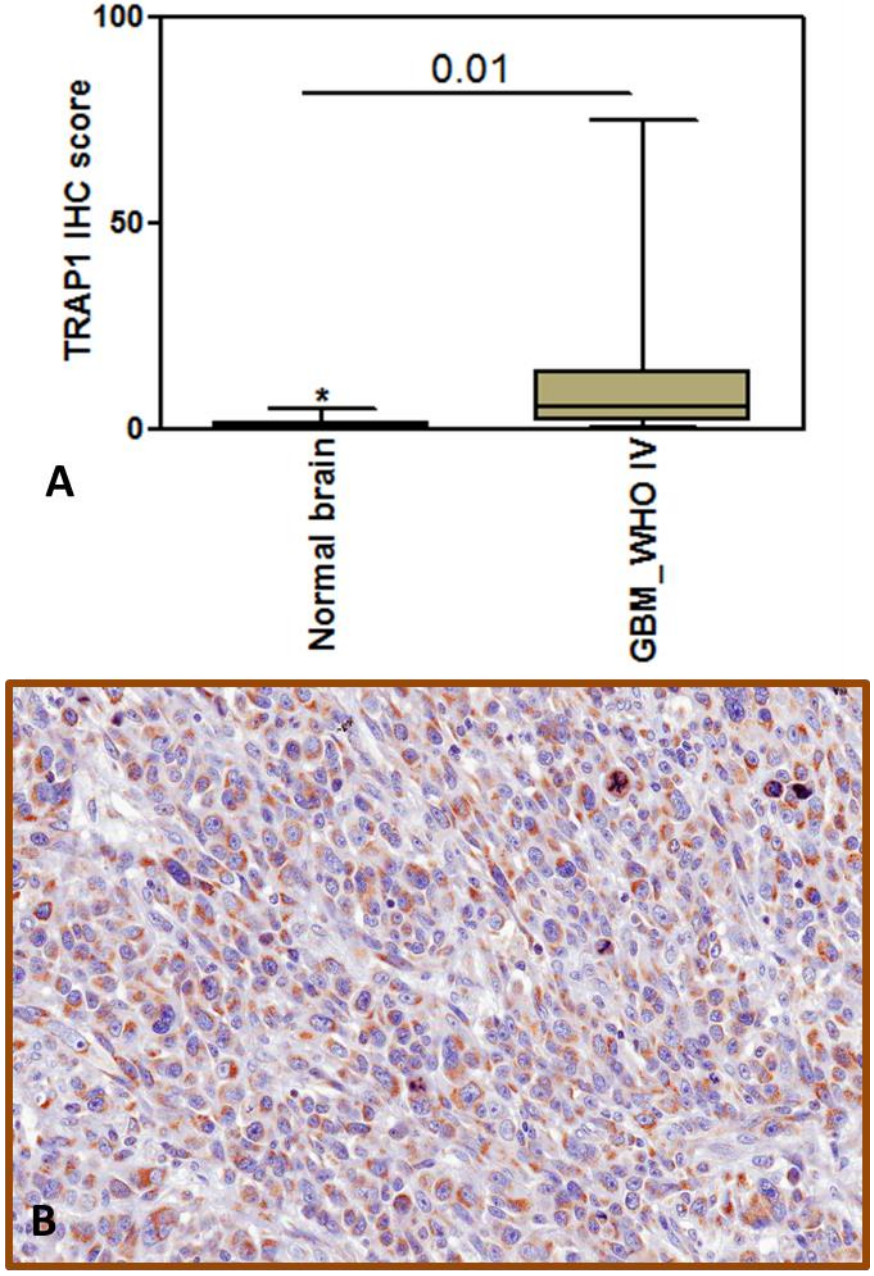


Figure 11. **A)** Quantification of TRAP1 in human glioblastomas and in normal brain parenchyma. **B**) Representative picture of human gbm obtained by TRAP1 staining (20x magnification)

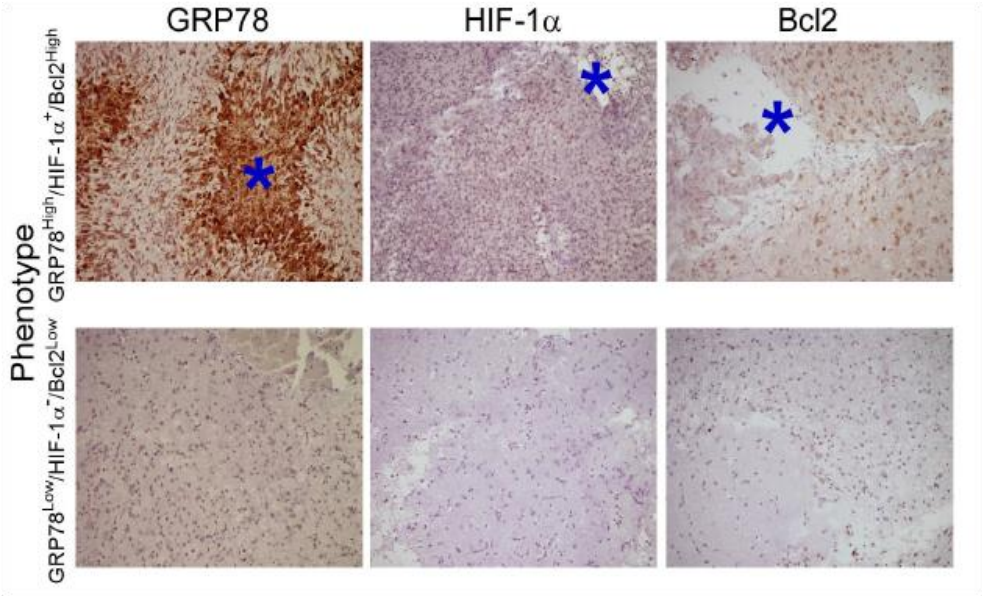


Figure 12. GRP78 is enriched in glioma areas characterized by elevated HIF1α and Bcl2 expression (*necrotic area)

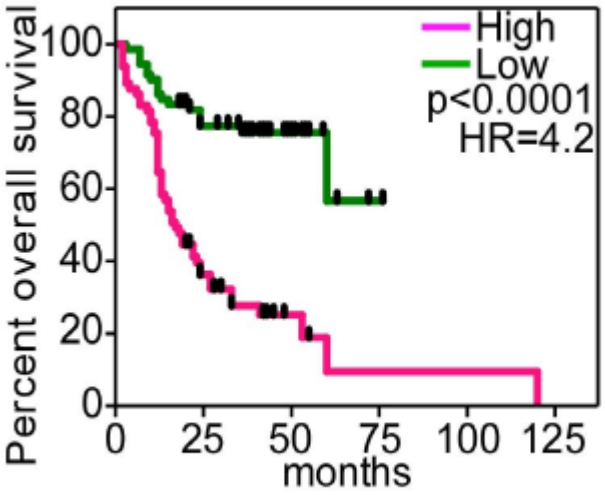


Figure 13. Glioma patients' overall survival according to GRP78 expression

4.2 GRP78 inhibition decrease viability and motility of GBM cell lines

Functional experiments were performed on a panel of glioma cells lines such as LN229 (GBM), T98G (GBM), SW1088 (Astrocytoma) and U251-HRE (GBM) to understand whether knock-down of HSPs influenced the vitality of tumor cells. The transient silencing of cell lines were modulated for GRP78 and TRAP1 expression by siRNA or esiRNA.

Effectively, a decreased of mRNA levels (***, $P<0.001$; Figure 14) and of proteins (Figure 15) in knock-down of GRP78 and TRAP1, respect to control were been demonstrated.

Moreover, to assess whether the GRP78 inhibition induce cell death we set up the Annexin-V assay. It was highlighted an increase in apoptotic cell death in GRP78-silenced cells respect to control transfected ones (Figure 16).

Depletion of GRP78 affected also cell motility, in fact cell invasion was decreased with a reduction of invaded cells of about 70% in LN229, and cell migration was decreased also in cell line low grade such as SW1088 respect to control (Figure 17A, 17B).

Globally, GRP78 silencing decreased cell migration and partially affected cell viability.

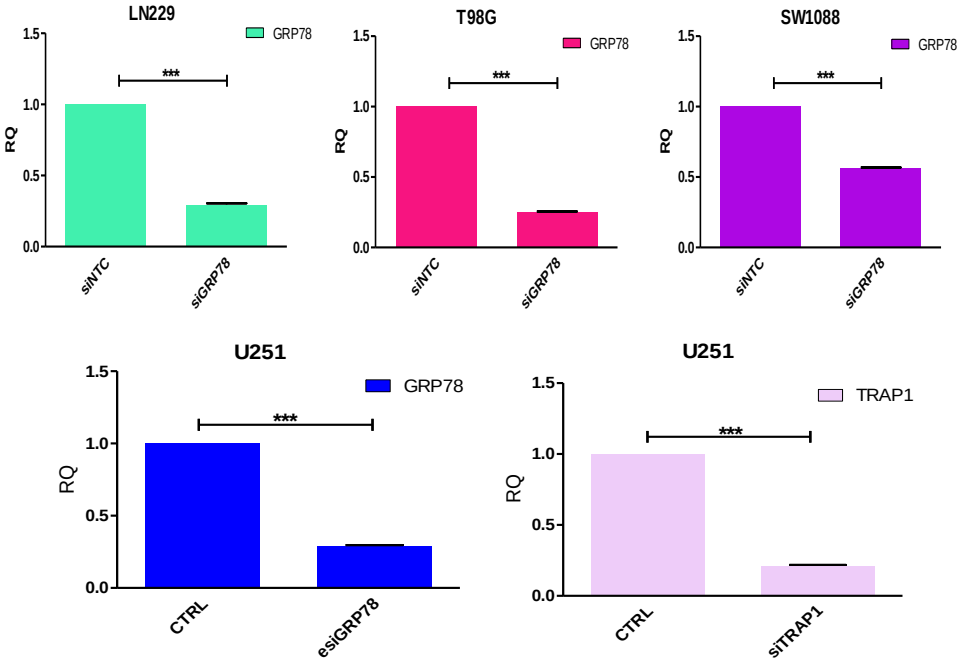


Figure 14. Real Time q-PCR analysis of GRP78 and TRAP1 expression in glioma cell line, LN229, T98G, SW1088, and U251 after gene silencing of GRP78 and TRAP1 (***, P<0.001)

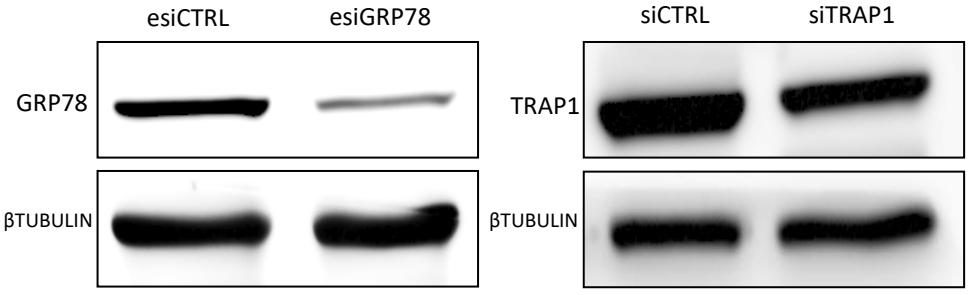


Figure 15. Immunoblot for GRP78, TRAP1 and βTUBULIN expression in U251 modulated cells

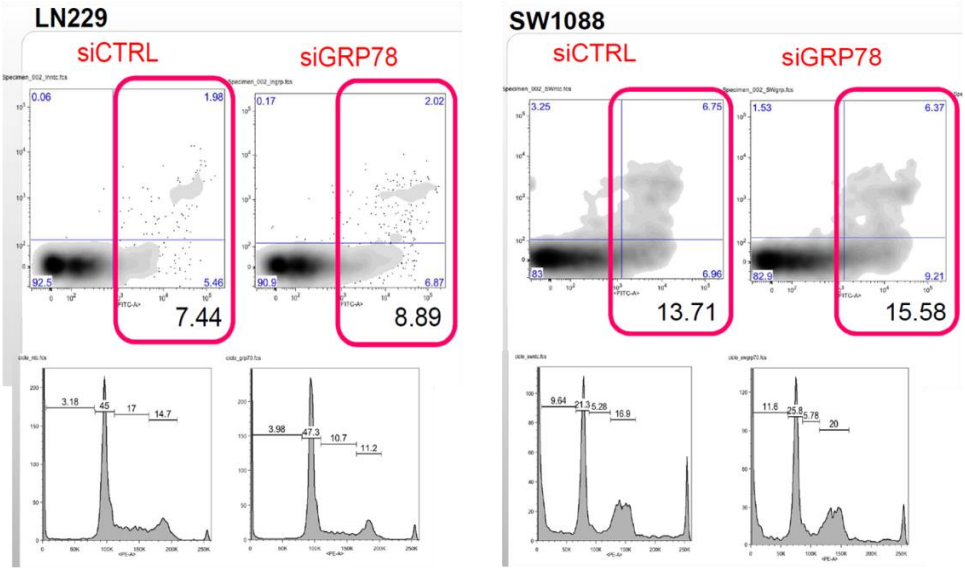


Figure 16. Annexin V assay. Increases of apoptosis in glioma cell lines (LN229 and SW1088) after siRNA silencing compared to control

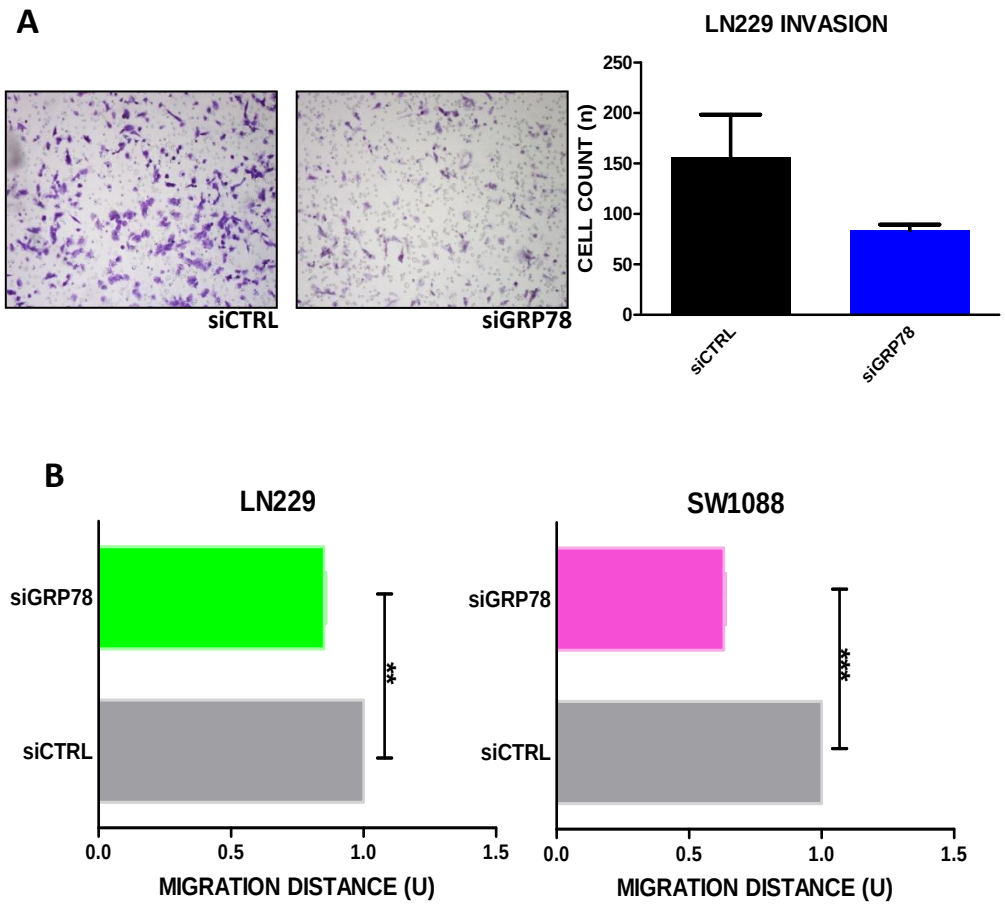


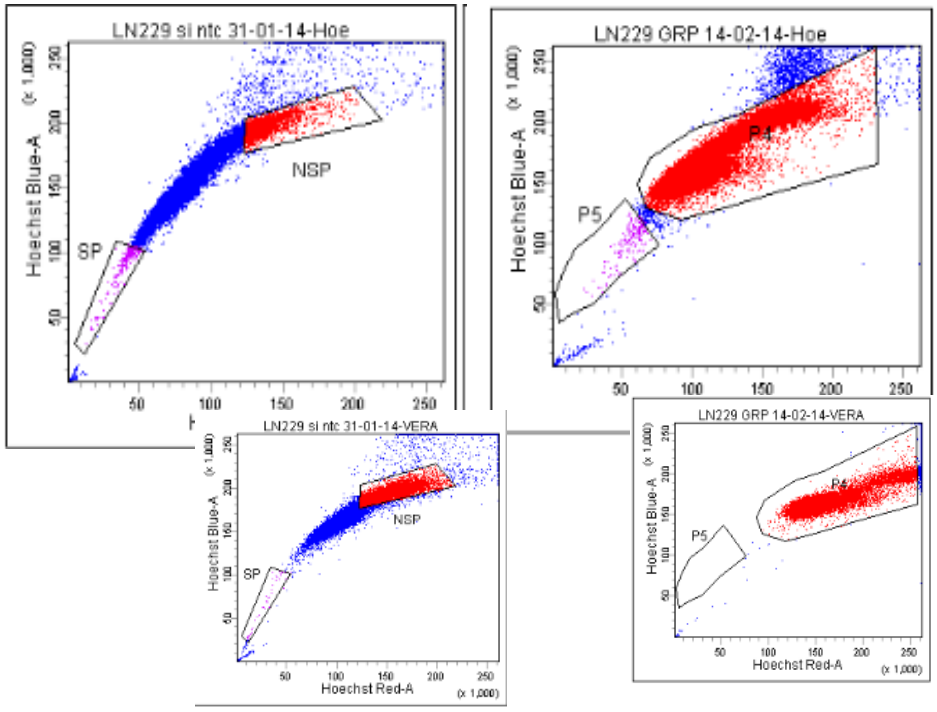
Figure 17. Motility in glioma cell lines. Decrease the ability of invasion (**A**) and migration (**B**) in glioma cell line, LN229 and SW1088, after gene silencing of GRP78. (**/***, P<0.005)

4.3 GRP78 is present in stem cell niche of GBM

Flow cytometry analysis for ABCG2 function usually identifies the side population as a very small fraction of cells in tumors. Consistent with this, we found a small percentage of tumor cells expressing ABCG2.

The presence of the side (SP) or the non-side (NSP) populations of glioma cell lines such as LN229 (GBM), T98G (GBM) were isolated through FACS-sorting procedures after GRP78 knockout through siRNA or control transfected cells: percentage of SP decreased in modulated cells respect to control (***, $P<0.001$; Figure 18). To characterize the cells that actively contribute to the SP phenotype, we performed a Hoechst dye exclusion assay on GBM cell lines. As most cells accumulate Hoechst 33342, SP cells can be isolated by dual-wavelenght flow cytometry based on their ability to efflux this dye, a process that requires the action of the ABC transporters. We identified a side population among cells isolated from tumor samples. This SP fraction was generally small, ranging from 0.5% to 1% of total cells.

Tumor samples exhibited some regions of cells positive for some typical stem cell marker. The expression of stem cell markers (ABCG2, NANOG), the hypoxia transcription factor (HIF1α), and the expression of GRP78 were analyzed by qRT-PCR in SP or NSP cells populations (Figure 19). Globally, gene expression levels of all analyzed markers were higher in the side-population (SP) respect to non-side population (NSP). This data is in line with the expectation of the presence of stem niche in glioma cancer.



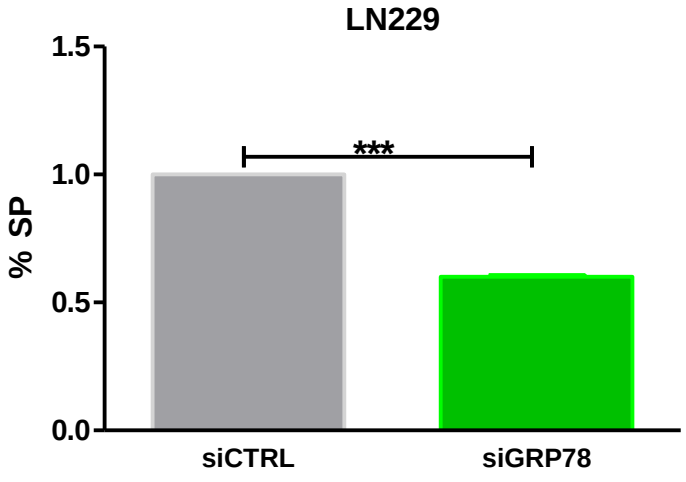


Figure 18. Flow cytometry analysis in glioma cell line (LN229), silenced for GRP78 to identified SP and NSP (***, P<0.001)

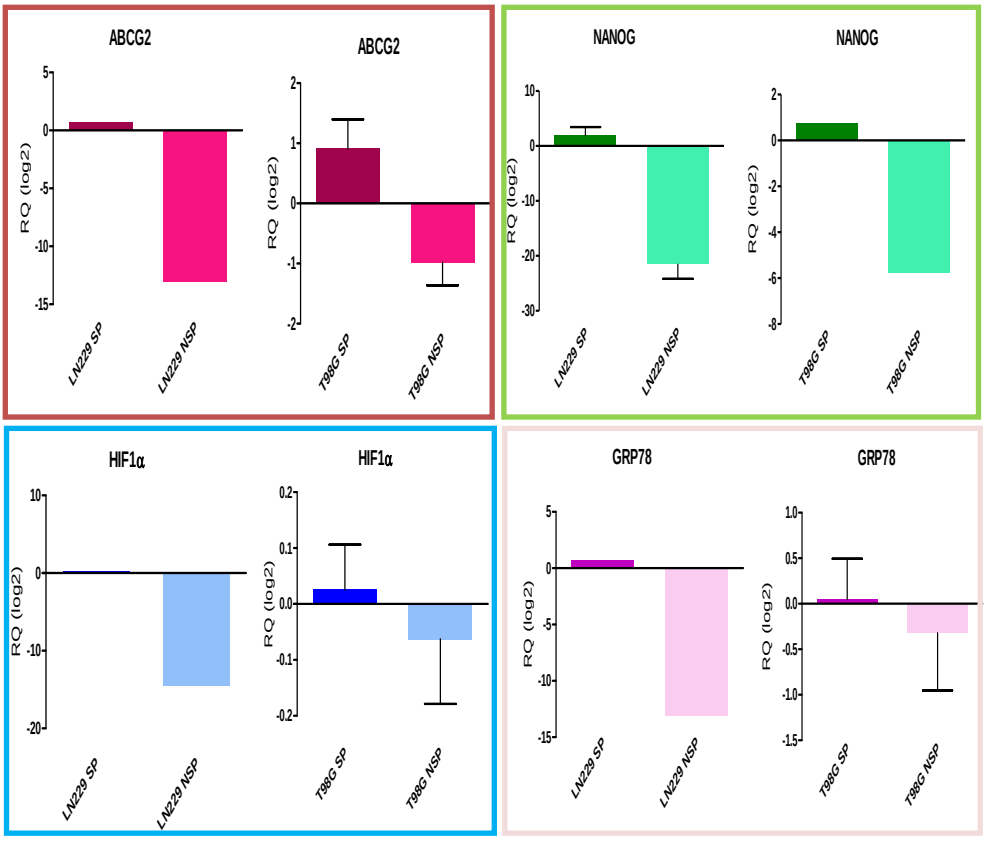


Figure 19. Real Time q-PCR analysis of ABCG2, NANOG, HIF1α and GRP78 expression in glioma cell line (LN229, T98G), SP and NSP. An increase of all marker in SP respect to NSP is shown

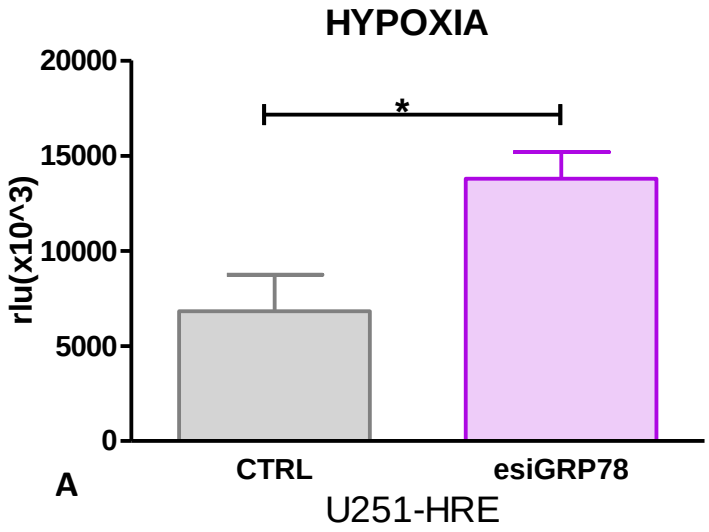
4.4 HSPs and hypoxia

To assess the role of HSP90-GRP78-HIF1α axe expression in hypoxia we used in vitro characterization of Luciferase Activity after HIF-1α modulation. To modulated hypoxia we used PetakaG3™LOT (Low Oxygen Concentration) system, which is an innovative bioreactor able to decrease oxygen concentration within the limits of the oxygen tensions in physiologic tissues (from 75 mmHg down to 15 mmHg). U251-HRE cells expressed the luciferase reporter gene in relation to HIF-1α nuclear accumulation.

Luciferase activity was seen to increase after esiGRP78 in U251-HRE cells in hypoxic conditions, respect to control (Figure 20A). Conversely, luciferase activity decreased in U251-HRE cells TRAP1-silenced after induction of hypoxia by LOT system, compared to control (Figure 20B).

These data were confirmed by protein levels (Figure 21A, 21B), in fact the protein HIF1α results augmented in hypoxia conditions after GRP78 silencing in U251-HRE cells, while, the levels of Hypoxia Inducible Factors, decreased in TRAP1-silenced cells.

Therefore, mRNA levels by Real Time q-PCR show an increase of TRAP1 expression level in esiGRP78 cells. On the other hand, in siTRAP1 modulated cells the gene expression levels of GRP78 results lower than control (Figure 22A, 22B).



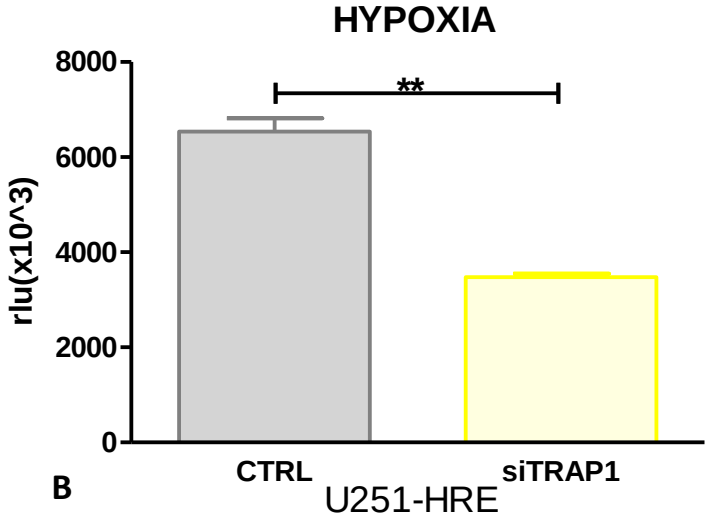


Figure 20. Lucifearse assay. Expression levels of luciferase in hypoxia conditions after esiGRP78 (**A**) and siTRAP1 (**B**) in U251-HRE cell line, respect to control (*/**, P<0.005)

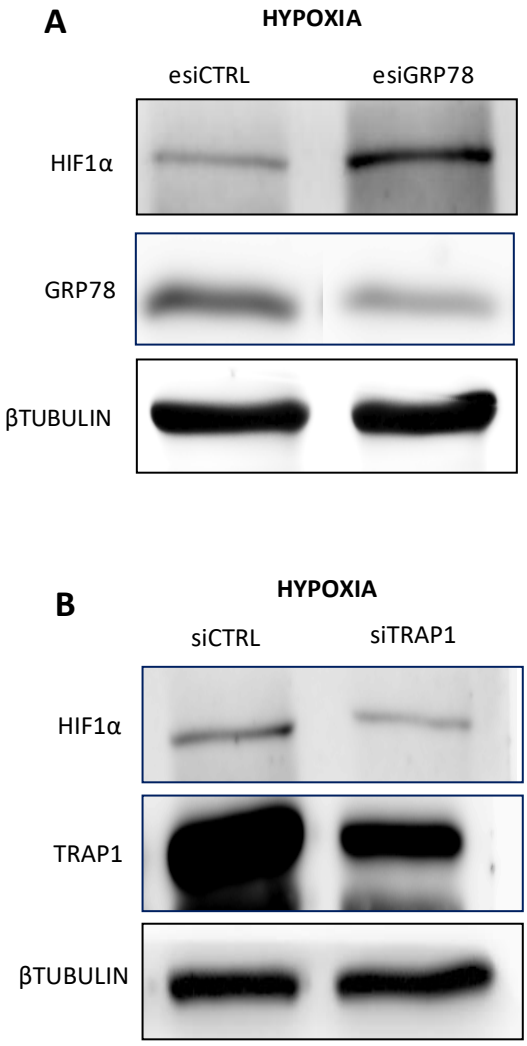


Figure 21. Immunoblot for HIF1α, GRP78 (**A**) and TRAP1 (**B**) and βTUBULIN expression in U251 modulated cells in hypoxia conditions

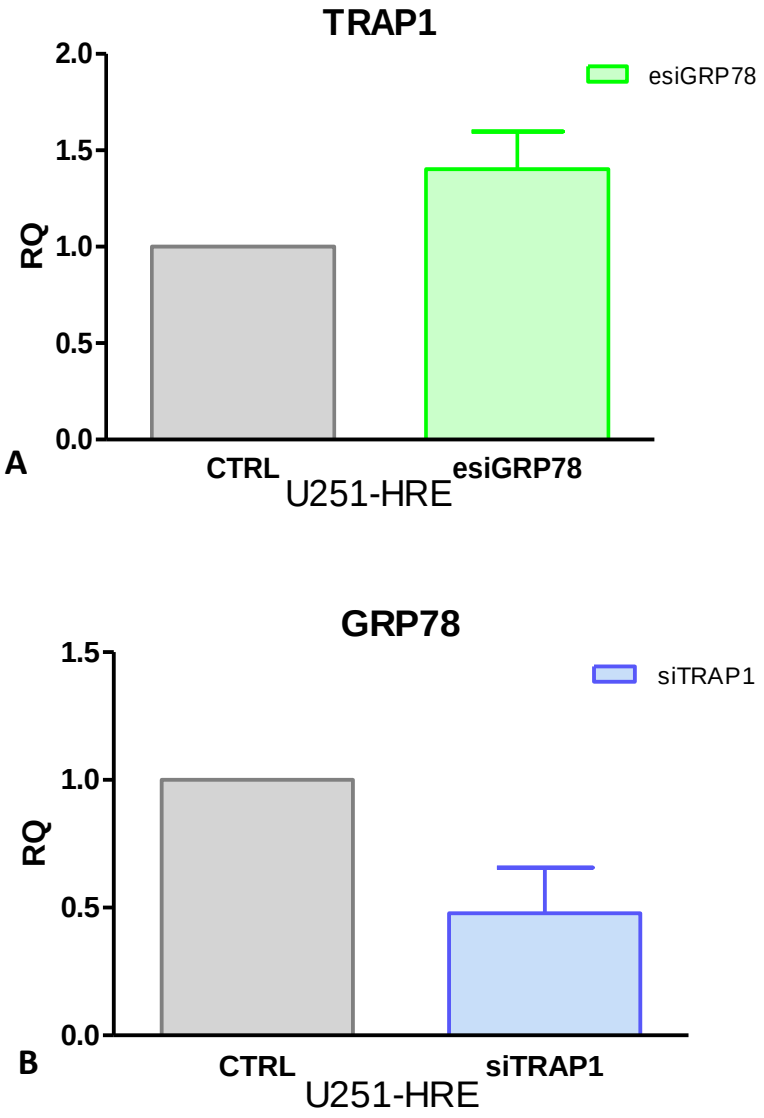


Figure 22. Real Time q-PCR analysis of TRAP1 (**A**) and GRP78 (**B**) expression in glioma cell line (U251-HRE) silenced for GRP78 and TRAP1

4.5 Treatment of GBM organotypic cultures with drugs induces GRP78 depletion expression

For ex vivo experiments we used patient-derived from organotypic glioma cultures which preserve the tri-dimensional tissue architecture and cellular heterogeneity of the original tumor. They were cultured through two pharmacological approaches. Temozolomide (TMZ) is the current gold standard in chemotherapy of GBM. It is an alkylating agent that, in an aqueous solution at physiological pH, dissolves into its bioactive form MTIC (5-(3-methyl-1-triazeno)imidazole-4-carboxamide), which is capable of penetrating the blood-brain barrier. Gamitrinibs (GA mitochondrial matrix inhibitors), are small molecules designed to disrupt the HSP90 network compartmentalized in tumor mitochondria. Gamitrinibs consist of 3 main parts, including a benzoquinone ansamycin backbone of 17-AAG, a linker region, and 1–4 tandem repeats of cyclic guanidinium (Gamitrinib-G1–G4) or triphenylphosphonium (Gamitrinib-TPP). Gamitrinibs are expected to interact with the HSP90 ATPase pocket via the 17-AAG component, whereas the guanidinium and triphenylphosphonium regions are responsible for mitochondrial penetration.

Globally, the expression of GRP78 decreased after treatment with high concentration of TMZ (100 µM, 200 µM), respect to control (Figure 23A).

We also evaluated the expression of hypoxia transcription factor (HIF1α), which results in a significantly decreased in treated sample respect to control (Figure 23B).

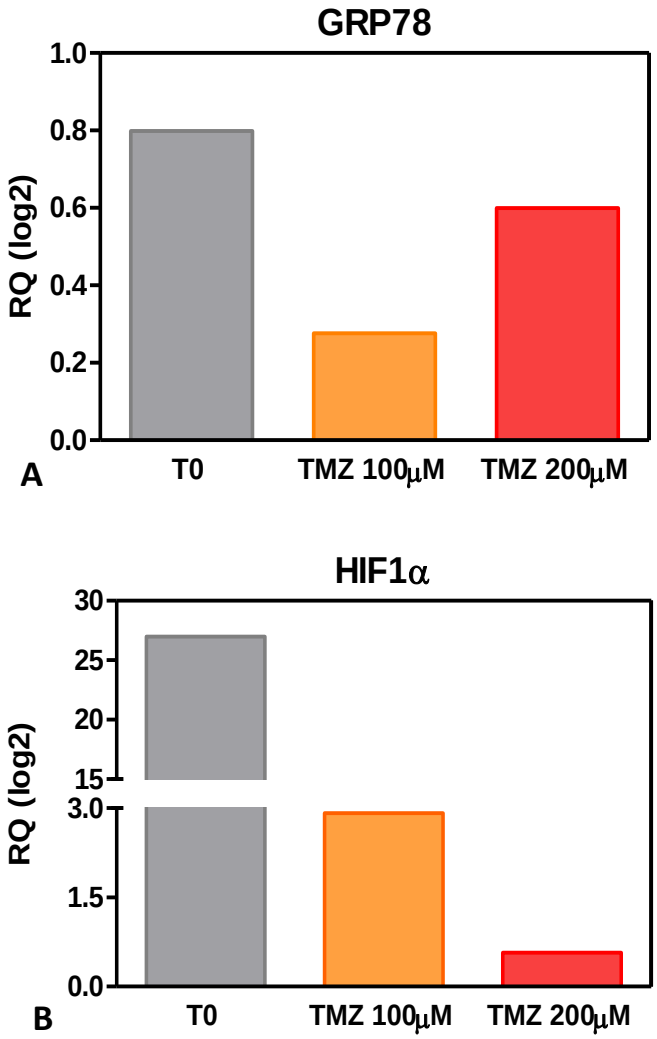


Figure 23. **A**) Real Time q-PCR analysis of GRP78 in human gliomas samples after treatment with Temozolomide. **B**) Real Time q-PCR analysis of HIF1α in human gliomas samples after treatment with Temozolomide

As regards the treatment with TMZ data were confirmed even in immunohistochemistry as shown in the figure 24. Moreover, the expression of GRP78 and proliferation index were evaluated also by immunohistochemistry after treatments with GAM. As shown in figure 25, the expression levels of GRP78 appear increased respect to control in human glioma samples.

The proliferation index (Ki67) was evaluated after treatments by two drugs, TMZ (100 µM, 200 µM), and GAM (10 µM, 25 µM). Globally, all treatment regimens caused a decreased in cell proliferation. To determined induction of programmed cell death we used cleaved caspase 3 (Cl-casp3). As shown in figure 26, apoptotic cells increased after treatment with low and high dosage respectively, of TMZ and GAM respect to controls (Figure 26A, 26B). Moreover, a limited modulation of pAKT and mTOR suggests the absence of autophagy (Figure 27).

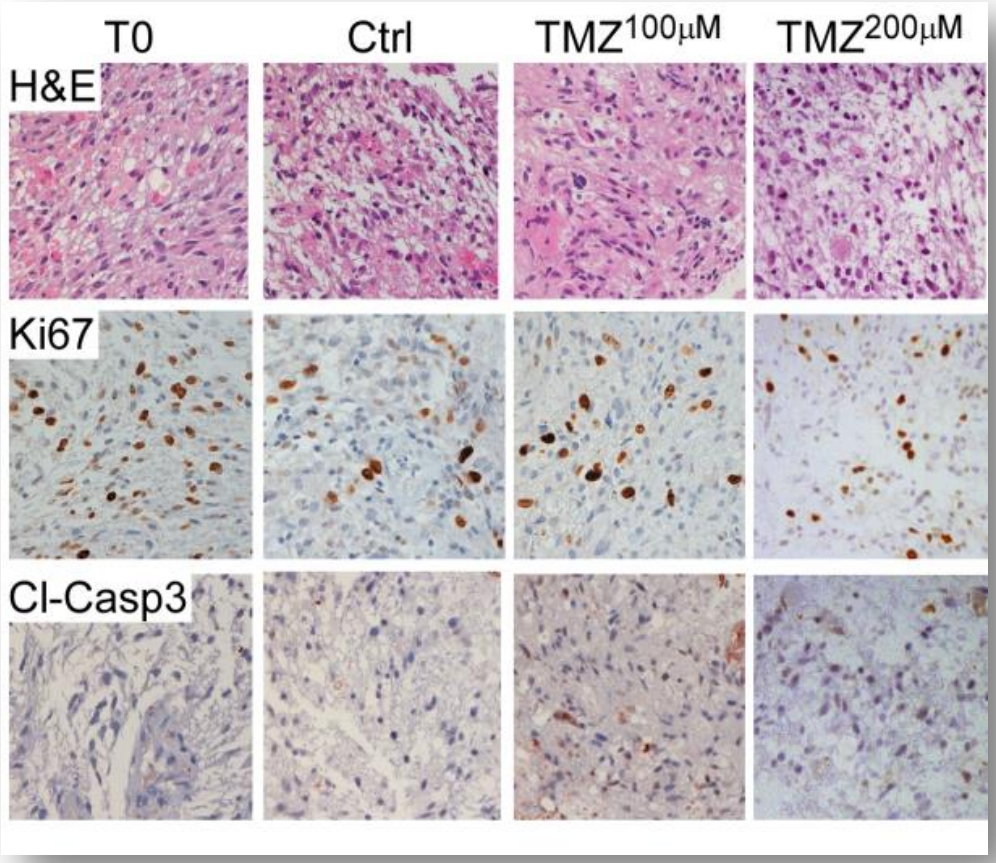


Figure 24. Immunohistochemistry analysis of Ki67 and Cleaved-Casp3 in human gliomas samples after treatment with TMZ

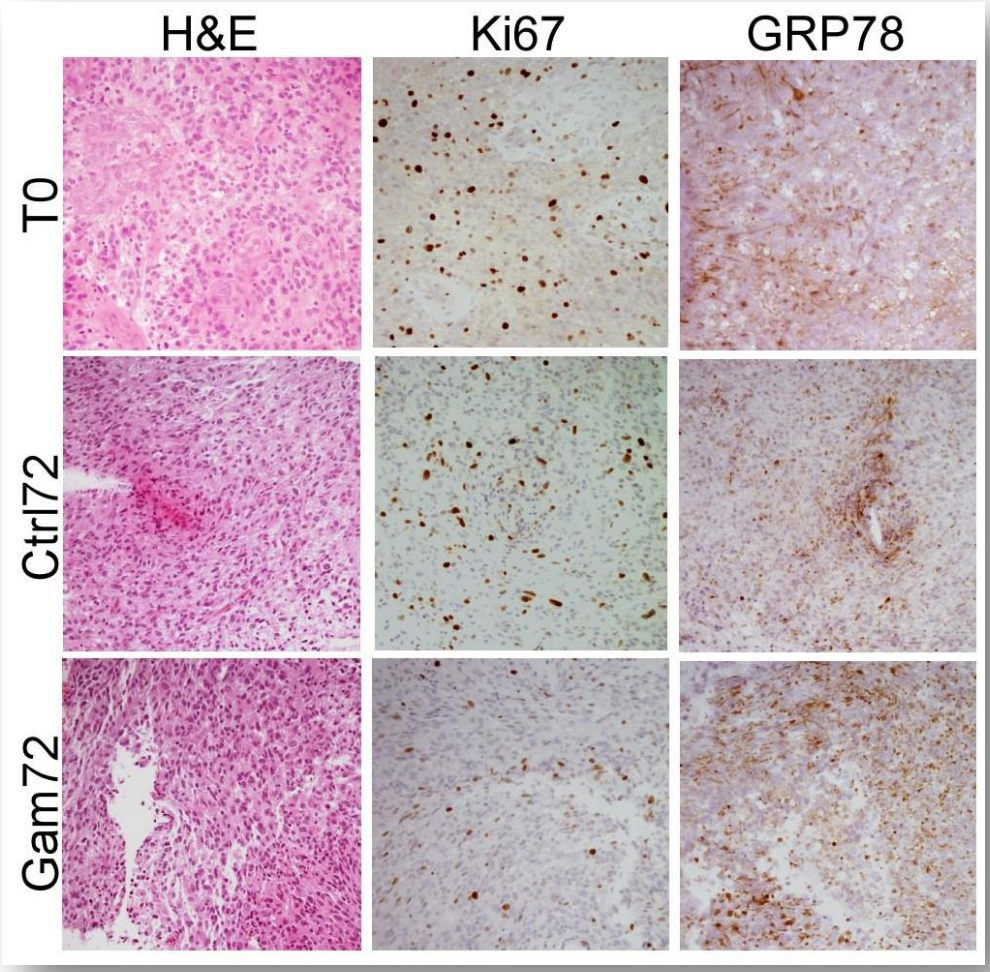
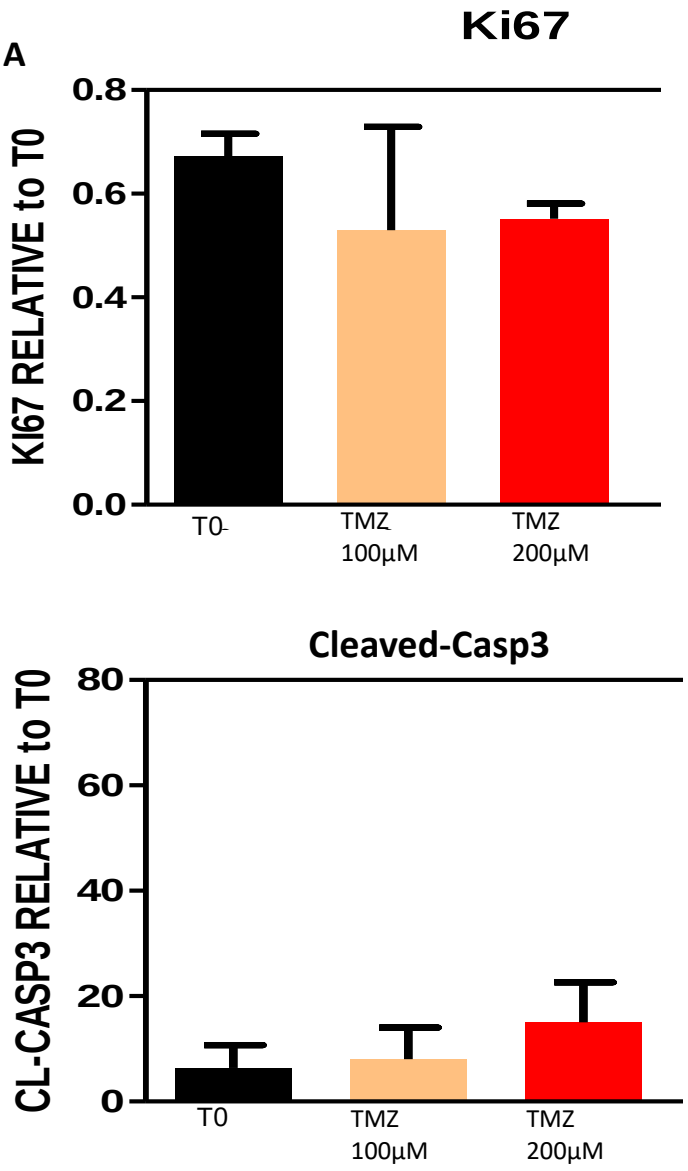


Figure 25. Immunohistochemistry analysis of Ki67 and GRP78 in human gliomas samples after treatment with GAM


A
Ki67
KI67 RELATIVE to T0
0.8
0.6
0.4
0.2
0.0
T0
TMZ 100µM
TMZ 200µM
Cleaved-Casp3
CL-CASP3 RELATIVE to T0
80
60
40
20
0
T0
TMZ 100µM
TMZ 200µM

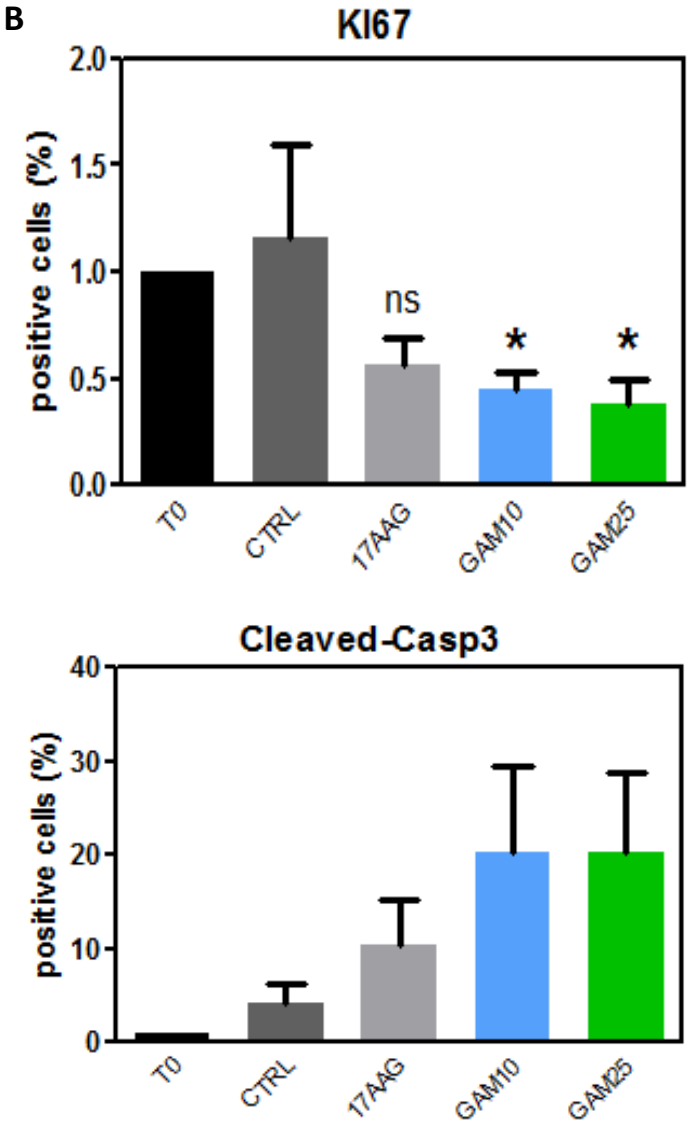


Figure 26. **A**) Percentage of positive cells analyzed for Ki67 and Cleaved-Casp3 in human gliomas samples after treatment with Temozolomide. **B**) Percentage of positive cells analyzed for Ki67 and Cleaved-Casp3 in human gliomas samples after treatment with Gamitrinibs

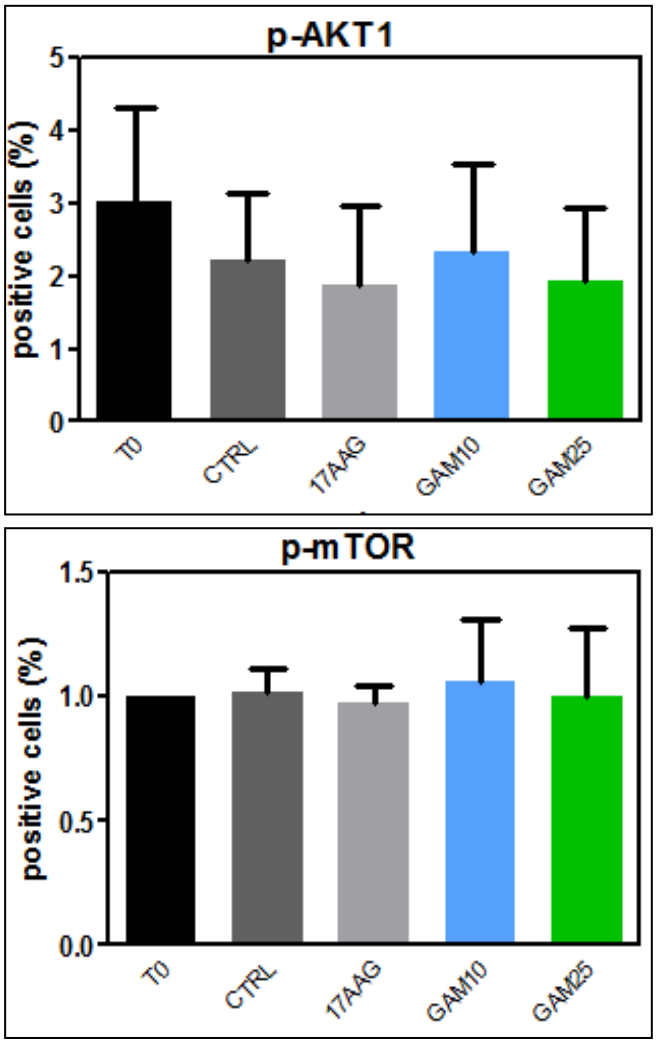


Figure 27. Percentage of positive cells analyzed for pAKT1 and p-mTOR in human gliomas samples after treatment with Gamitrinibs

DISCUSSION

5. DISCUSSION

Gliomas are the most common and lethal malignant tumor of the central nervous system representing 1.3% of all cancers. Survival rates for gliomas are poor, at around 12–13% for males, and 15% for females, with a median survival time of 14.6 months.

Survival rates differ depending on the grade of gliomas, with low grade glioma having the highest survival rate [126].

While diffused astrocytomas are the most common primary brain tumours, glioblastoma (GBM) is the most malignant.

Glioblastoma (GBM, WHO grade IV) is the most common astrocytic-derived brain tumor in adults, more frequent in older patients (mean age 55 years). It is characterized by an intrinsic heterogeneity among cell types comprising the tumor mass. It is characterized by a highly aggressive behavior and poor prognosis. Despite aggressive treatments including surgical resection, chemo- and radiotherapy, GBM remains an incurable disease that almost invariably leads to neurological demise and death. Due to its high degree of invasiveness, radical resection of the primary tumor mass is not curative. Infiltrating tumor cells invariably remain within the surrounding brain, leading to disease progression or recurrence, either locally or distant from primary tumor [37].

Due to its aggressiveness, together with inadequate therapeutic options, several molecular targets are being studied to find more efficient treatments or as prognostic biomarkers useful for patients' management.

A hallmark of GBM is the presence of tumor necrotic foci surrounded by severely hypoxic pseudopalisading cells [127].

Hypoxia has been show to fuel tumor cell migration [35], and to sustain the cancer-initiating cells niche [77].

Autophagy and the Unfolded Protein Response (UPR) are two interconnected pathways exploited by tumor cells to cope with low nutrient

conditions, hypoxia or other microenvironment-generated stress. Moreover, deregulation of these pathways contributes to neurodegenerative disorders
Besides their recognized role of master regulators of cell homeostasis, subcellular compartmentalized chaperones recently emerged as novel promising targets for anti-cancer therapy [128], also in animal models of gliomas [129].
Indeed, specific targeting of mitochondrial HSP90 induced selective cell killing of cancer cells [130], prevented primary and metastatic prostate cancer growth in vivo [104] and depletion of the endoplasmic reticulum chaperone GRP78 delayed glioma cell growth in vivo. GRP78 networks have also been uncovered, adding complexity to the molecular network that underpins stress responses in human cancer.
Heat shock proteins (HSPs) are highly conserved proteins that are expressed in response to various forms of stress, in response to environmental challenges, and in specific stages of development transitions and differentiation. Some HSPs are also expressed under non-stress conditions and act as molecular chaperones. Thus, HSPs fulfill a variety of biologic functions, including the regulation of synthesis, folding, assembly, and degradation of different proteins. HSPs are constitutively expressed in human carcinomas or cancer cell lines of various histogenetic origins. Malignant gliomas are highly aggressive neoplasms that are rather resistant to radiotherapy and chemotherapy. Preliminary observations indicated HSPs expression in glioma cell lines, but have also been reported to express several HSPs in vivo, including members of the small HSPs family, as well as HSP90. The high expression of HSPs in malignant glioma indicates a possible role of these proteins in resistance to cancer chemotherapy. This is because HSPs protect cells from many cell-damaging agents or conditions of environmental stress [131]. Several studies have reported a positive correlation of HSP expression with

histologic tumor grade, as shown for small HSP in astrocytic tumors [132] and in ovarian cancer and for HSP70 in colorectal cancer and endometrial carcinomas [133]. Because HSP are potential factors of cancer cell resistance to cytoablative therapy, we studied the expression and functional activity of HSP in human malignant gliomas in vitro and in vivo.

GRP78 is best known for its roles in the ER where it promotes the folding, maturation and assembly of nascent proteins and also coordinates the unfolded protein response (UPR) that alleviates ER stress [134].

Although it is constitutively expressed, GRP78 levels increase greatly in response to demanding conditions such as hypoxia and nutrient deprivation and it has been heavily implicated in cytoprotection and chemoresistance in the tumor microenvironment where these conditions prevail [116].

The first direct evidence for the involvement of GRP78 in cancer progression came from the demonstration that GRP78 knockdown in fibrosarcoma cells using antisense prevented tumor formation in nude mice [135]. It was further shown that a suicide transgene driven by the GRP78 promoter in breast cancer cells completely blocked tumor growth in mice. More recently, it was demonstrated that GRP78 heterozygous mice develop normally but are resistant to transgene-induced mammary tumor growth. In this study, it was demonstrated that reduced GRP78 levels in heterozygous mice resulted in decreased tumor cell proliferation, increased tumor cell apoptosis and reduced tumor angiogenesis [136,137].

Recently, the group of Chae YC et al., demonstrated that suboptimal mitochondrial HSP90 targeting by Gamitrinibs (small molecules that inhibit ATPase activity of mitochondrial HSP90-like chaperones) impairs tumor bioenergetics, activates pro-survival autophagy and stimulates endoplasmic reticulum UPR as evidenced by GRP78 over-expression.

Preliminary data show that GRP78 participates in cripto-mediated cell migration [138] and in embryonic or adult hematopoietic stem cell

maintenance [139]. Nevertheless, precise mechanisms that relate morphological features to specific molecular alterations have not been reported. Many recent studies converge to ask which roles, if any, cancer stem cells (CSCs) play in cancer pathogenesis. CSCs appear to be resistant to chemotherapy, suggesting that these cells persist after treatment and drive recurrence [59]. CSCs are defined as cells within a tumor that possess the capacity to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor [140].

They share many of the characteristics of normal tissue stem cells, which could help to explain certain clinical features such as tumor hierarchy, recurrence, metastasis and therapy resistance.

Our data are in line with the literature about GRP78 over-expression in brain tumors and as an indicator of poor prognosis and shorter overall survival. Performing immunohistochemistry for GRP78 on a glioma patients' tissue microarray we could evidence a significant overexpression of the HSP in higher grades gliomas compared to grade II tumors or normal brain parenchyma. Moreover GRP78 and TRAP1 appears to be enriched in peri-necrotic areas of high-grade human gliomas where GRP78 overexpression correlates with HIF-1α and Bcl2.

Up-regulation of GRP78 seems to happen at gene expression level, since its mRNA was increased in an independent set of gliomas. Finally, our data shows that GRP78 is a poor prognostic marker for high grades gliomas.

With advances in siRNA (small interference RNA), it is now possible to target important components involved in the development and progression of tumours.

Invasion, and migration are key processes of tumor progression and are tightly linked to tumor recurrence and therapeutic resistance in different tumor types: siRNA silencing of GRP78 induced loss of viability of H1299 and A549 lung cancer cells.

After silencing of GRP78 we set up functional assays to test whether the ability of invasion and migration of brain cancer cells suffer a change. For this purpose we used three tumor cell lines, two high-grade and one low-grade cell type. In agreement with the literature decreased cell viability and motility could be detected in GRP78-silenced cells, whereas a non-targeting silencing had no effect.

Cells with tumorigenic potential, and which have a self-renewing stem cell-like phenotype are defined cancer stem cells (CSCs). They have been identified in GBM, and many other tumors, are considered able to initiate tumor formation with fewer cells and specially, are responsible to chemotherapeutic agents resistant to therapy.

To isolated the side population (SP) we used the flow cytometry FACS-sorting which identified a subset of cells with differential efflux activity of the fluorescent DNA binding dye Hoechst 33342. Our data confirms the presence of glioma stem cell niche, in fact, stem cell markers and GRP78 results more express in SP than NSP. In GRP78 knockout percentage share of SP cells was decreased compared to the control non-transfected.

As well know heat shock proteins (HSPs) are involved in protein folding, aggregation, transport and/or stabilization by acting as a molecular chaperone, leading to inhibition of apoptosis by both caspase dependent and/or independent pathways. HSP70 and HSP90 are known to suppress apoptosis by directly associating with Apaf-1 and blocking the assembly of pro-caspase 9. In line with the literature our studies on apoptosis showed that GRP78 knockout was associated to an increase in the apoptotic fraction of glioma cells compared to control transfected cultures.

Hypoxia is considered to play a crucial role in tumor development and progression and to be a main factor in GBM, promoting angiogenesis, tumor invasiveness, loss of apoptotic potential, genomic instability, and chemotherapy and radiation resistance. For these reasons, hypoxia is

considered a promising target for the treatment of many different human cancers usually characterized by the presence of extensive hypoxic and necrotic areas, GBM in particular. In this context, to target HSP90-GRP78-HIF1α axe, we have induced a condition with low level of oxygen and we evaluated protein expression level in cell lines silenced for GRP78 and TRAP1. The results indicated a relationship between GRP78-TRAP1-HIF1α. In fact, in TRAP1-silenced cells, the expression level of GRP78/HIF1α appear decreased. Oppositely, the depletion of GRP78 appears to activate a compensatory pathway inducing the expression of TRAP1/HIF1α. Hypoxia and especially the oxygenation-dependent transcription factor, HIF-1, could be valid targets for the development of new cancer therapies.

Ex vivo culture of human gliomas tissues were provided experimental evidence for novel drugs combination strategies to inhibit glioma growth and dissemination.

Cancer cell ability to survive and invade extracellular matrix were provided preliminary evidence of therapeutic efficacy in preventing tumor ability to spread and recur. Indeed, the limitations imposed by the blood-brain barrier to obtain reliable serological markers from glioma patients is overcome in this study by the use of ex vivo tissue cultures of human glioma tumors.

In ex vivo, the organotypic glioma tissue were treated with two different pharmacological approaches. Globally, the expression of GRP78 decreased after treatment with TMZ respect to control. Also the expression of hypoxia transcription factor, results in a significantly decreased in treated sample respect to control. Therefore, all treatment regimens caused a decreased in cell density and an induction of programmed cell death. Treatments with GAM, oppositely induced pro-survival autophagy with over expression of GRP78.

CONCLUSIONS

6. CONCLUSIONS

GBM is the most common and lethal primary malignancy of the CNS. Even with surgical resection and aggressive treatment with chemo- and radiotherapy, the prognosis remains very poor. Due to continued advancements, the understanding of the complex biology of GBM and its pathogenesis, a wide variety of novel therapeutic approaches have been developed and are currently under study as potential treatments for GBM. Despite promising results in preclinical trials, many of these therapies have provided limited or no therapeutic efficacy in human clinical trials. Thus, although survival of patients with GBM continues to slowly improve, treatment of GBM remains extremely challenging. Continued research and development of new molecular targeted and immunotherapeutic approaches, based on a detailed understanding of molecular pathogenesis, can reasonably be expected to lead to increased survival and more favorable prognosis of patients with GBM.

HSPs are highly conserved proteins that have an important cellular function as molecular chaperones, protecting cells from various forms of stress. Further, the HSP family has been classified as a potential class of antiapoptotic genes that can down-regulate caspase activity, thus being important mediators of resistance to diverse apoptotic stimuli.

In the light of these evidences, the aim of this project were investigate GRP78-HSP90 axe expression in glioma, to understand which is their role in malignant brain cancer.

Different experimental approaches used in our research, highlighted that, high levels of GRP78 are characteristic of GBM and could influence the chemoresistance typical of high grades gliomas, indeed patients with a low GRP78 expression had a longer OS.

This results emphasizes from the molecular point of view the importance of the GRP78 as a poor prognostic marker for high grades gliomas and also, as HSPs are enriched in peri-necrotic tumor areas sustaining a pro-survival

environment. Moreover, these data suggest that GRP78 over-expression in brain tumors could sustain the stem cell population in stress conditions such as hypoxia.

Despite increased understanding of the molecular alterations that characterize different subsets of high-grade glioma, this tumor remain a fatal disease with inadequate treatment options. Therefore, there is a crucial need to identify proteins that could be effectively targeted the cancer cells. We have demonstrated that GAM could be an innovative and promising drug. This approach could provide fundamental evidences of a novel therapeutic strategy for GBM, to hamper disease recurrence, and potential markers to predict therapeutic efficacy, thereby improving prognosis of glioma patients.

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7. BIBLIOGRAPHY

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SCIENTIFIC PRODUCTS

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