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Tesis Doctoral:

Regulación epigenética, asociada a hipoxia, de ODZ1, un nuevo marcador de invasión en Glioblastoma

PhD Thesis:

Hypoxia-dependent epigenetic regulation of ODZ1: a novel invasion marker in Glioblastoma

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To my wife Rocío, whose sacrifice, support and unconditional love are reflected within these pages…

To my parents Rosa Lila & Alex, who gave me an education as the best heritage and taught me perseverance as an essential daily value…

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ABSTRACT

ODZ1 has been associated with cell invasion in Glioblastoma through upregulation of RhoA/ROCK signaling and its increased expression entails a worse overall prognosis. Notwithstanding, the role ODZ1 in the clinical practice is still unknown and the mechanisms triggering the ODZ1-pathway remain elusive. We aim to assess the association of ODZ1 expression and clinically relevant radiological tumor features. Besides, we hypothesize that hypoxia, a main biological glioblastoma hallmark, plays a role in regulating ODZ1-dependent invasion.

Pre- and postoperative radiological tumor features and ODZ1 tumor expression were assessed in patients with glioblastoma. The association between the pattern of tumor recurrence and ODZ1 expression was assessed. Similarly, the preoperative tumor geometrical features associated with ODZ1 were identified. Although the majority of the tumors with distant recurrences (>2cm from de primary tumor) had an increased ODZ1 expression when compared to focal recurrences, this difference didn´t reach statistical significance. Furthermore, among the tumor geometrical features analyzed, the tumor contrast-enhanced rim width was significantly associated with ODZ1 expression.

On the other hand, Pimonidazole (PIMO) was used to identify the hypoxic tumor cells in specimens from 17 IDHwt glioblastomas. The Illumina Infinium Human Methylation EPIC Array was used for ODZ1-promoter methylation profiling in FACS-sorted PIMO-positive and PIMO-negative cells and ODZ1 expression in hypoxic and normoxic regions was assessed by IHC. Glioblastoma cells were transfected with the ODZ1 promoter cloned into pGL2-luciferase reporter comparing wild type vs mutant and methylated vs unmethylated promoter and then cultured under hypoxic or normoxic conditions. ODZ1 mRNA expression and cell migration were determined with RT-PCR and a modified Boyden-chamber assay, respectively. Hypoxic tumor regions express higher levels of ODZ1, and hypoxia induces ODZ1 expression in glioblastoma cells by regulating the methylation status of the ODZ1 promoter. Hypoxia-induced upregulation of ODZ1 correlates with higher migration capacity, which is drastically reduced by knocking-down ODZ1. A functionally-active CpG-site is located at the 3'end of the promoter, which is hypermethylated in somatic neural cells and mainly hypomethylated in glioblastoma cells.

In vitro methylation of the promoter or mutagenesis of this CpG-site reduces the promoter activity in response to hypoxia.

The results presented here suggest that the tumor contrast-enhanced rim width, which has been associated with a poor outcome, might be related to ODZ1-dependent invasion. Furthermore, here we identify hypoxia as the first extracellular activator of ODZ1 expression and describe that hypoxia controls the levels of this migration-inducer, at least in part, by regulating the methylation status of the ODZ1-promoter.

RESUMEN

ODZ1 se ha asociado con la invasión celular en el glioblastoma a través de la regulación positiva de la señalización RhoA / ROCK y, en general, su mayor expresión conlleva un peor pronóstico. Sin embargo, aún se desconocen el valor de ODZ1 en la práctica clínica y los mecanismos que activan la vía de ODZ1. El objetivo del presente trabajo era evaluar la asociación entre la expresión de ODZ1 y las características radiológicas del tumor relevantes clínicamente. Además, planteamos la hipótesis de que la hipoxia, una característica biológica distintiva del glioblastoma, desempeña un papel significativo en la regulación de la invasión tumoral dependiente de ODZ1.

Se evaluaron las características tumorales radiológicas pre y posoperatorias y la expresión tumoral de ODZ1 en pacientes con glioblastoma. Específicamente, se evaluó la asociación entre el patrón de recurrencia tumoral y la expresión de ODZ1. Además, de forma similar, se identificaron las características radiológicas preoperatorias del tumor asociadas con ODZ1. Aunque la mayoría de los tumores con recurrencias distantes (> 2 cm del tumor primario) tenían una mayor expresión de ODZ1 en comparación con las recurrencias focales, esta diferencia no alcanzó significación estadística. Además, entre las características geométricas del tumor, el ancho del anillo tumoral captante de contraste se asoció significativamente con la expresión de ODZ1.

Por otro lado, se utilizó pimonidazol (PIMO) para identificar las células tumorales hipóxicas en muestras de 17 glioblastomas IDHwt. El Illumina Infinium HumanMethylationEPIC Array se usó para determinar el estado de metilación del promotor de ODZ1 en células PIMO-positivas y PIMO-negativas clasificadas por FACS. Además, la expresión de ODZ1 en regiones hipóxicas y normóxicas fue evaluada por IHQ. *In vitro*, las células de glioblastoma se transfectaron con el promotor ODZ1 clonado o metilado en el vector pGL2-luciferasa para comparar el promotor de tipo salvaje frente a promotor mutante y el metilado frente a no metilado, y luego se cultivaron en condiciones hipóxicas o normóxicas. La expresión de ARNm de ODZ1 y la migración celular se determinaron con RT-PCR y un ensayo de cámara de Boyden modificado, respectivamente.

Las regiones tumorales hipóxicas expresaron niveles más altos de ODZ1, y la hipoxia induce la expresión de ODZ1 en células de glioblastoma regulando el estado de metilación

del promotor ODZ1. La regulación positiva inducida por la hipoxia de ODZ1 se correlaciona con una mayor capacidad de migración, que se reduce drásticamente al bloquear ODZ1. Un sitio CpG funcionalmente activo se encuentra en el extremo 3´ del promotor, que está hipermetilado en las células neuronales somáticas y principalmente hipometilado en las células glioblastoma. La metilación in vitro del promotor o la mutagénesis de este sitio CpG reduce la actividad del promotor en respuesta a la hipoxia.

Los resultados presentados aquí sugieren que el ancho del anillo tumoral captante de contraste, que se ha asociado con un mal resultado, podría estar relacionado con la invasión dependiente de ODZ1. Además, aquí identificamos la hipoxia como el primer activador extracelular de la expresión de ODZ1 y describimos que la hipoxia controla los niveles de este inductor de migración, al menos en parte, regulando el estado de metilación del promotor ODZ1.

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ABBREVIATIONS

A

ADAM: A disintegrin and metalloproteinases
ADC: Apparent diffusion coefficient
APC: Adenomatous polyposis coli
ATRX: Alpha-thalassemia/mental retardation X-linked

B

BBB: Blood-brain barrier

C

CCND1: Cyclin D1
CDK: Cyclin Dependent Kinase
CDKN2A: Cyclin Dependent Kinase Inhibitor 2A
CIMP: CpG- island methylator phenotype
CNS: Central nervous system
CNV: Copy number variation
CSF-1: Colony stimulating factor-1
CSPGs: glycosylated chondroitin sulfate proteoglycans
CTV: Clinical target volumes
CX3CL1: Fractalkine.

D

DHH: Desert hedgehog
DICOM: Digital Imaging and Communication in Medicine
DTI: Difussion tensor imaginging

E

ECM: Extracellular matrix
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
EMT: Endothelial-mesenchymal transition
EMX2: Empty Spiracles Homeobox 2

F

FAK: Focal adhesion kinase
FGF8: Fibroblast growth factor 8
FLAIR: Fluid-attenuated inversion recovery

G

GAM: glioma-associated macrophages
gCIMP: CIMP glioma
GDNF: Glial cell–derived neurotrophic factor
GFAP: Glial fibrillary acidic protein
GPNMB: Glycoprotein non-metastatic b
GTR: Gross total resection
GWAS: genome wide association studies

H

HA: Hyaluronic acid
HGF: Hepatocyte growth factor
HIF1a: Hypoxia inducible factor alfa
HINT1: Histidine triad nucleotide binding protein 1

I

ICAM1: Intercellular adhesion molecule-1

icODZ1: ODZ1 intracellular domain

IDH: Isocitrate dehydrogenase

IHH: Indian hedgehog

ILK: Integrin-linked kinase

IMRT: intensity-modulated radiotherapy

ITG: Integrin

L

LIMK: LIM kinase

M

MAPK: Mitogen-activated protein kinase

MBD1: Methyl-CpG binding protein 1

MCP-1: Monocyte chemo-attractant protein-1

MDM2: Mouse double minute 2 homolog

MGMT: O6-methylguanine-DNA methyltransferase

MITF: Microphthalmia-associated transcription factor

MMP: Matrix metallopeptidases

MT1-MMP: Membrane type 1–matrix metalloproteinase

mTOR: Mammalian target of rapamycin

N

NCAM: neuronal cell adhesion molecule

NF1: Neurofibromin 1

NFIA: Nuclear factor I-A

NFκB: Nuclear factor kappa B

NSC: Neural stem cell

O

OS: Overall survival

P

PCP: Planar cell polarity

PD-1: Programmed cell death protein 1

PDGF: Platelet Derived Growth Factor

PDGFR: Platelet Derived Growth Factor Receptor

PET: positron emission tomography

PFS: Progression-free survival

PI3K: Phosphoinositide 3-kinase

PKB: Protein kinase B

PTCH1: PTCH1

PTEN: phosphatase and tensin homolog

R

RANKL: receptor activator of NF-κB ligand.

RB: Retinoblastoma

ROCK: RHO-associated kinase

S

RTK: Receptor tyrosine kinase

SHH: Sonic hedgehog

SMO: Smoothened

SVZ: Subventricular zone

T

TCAP: Teneurin C-terminal associated peptide

TCGA: The Cancer Genome Atlas

TERT: Telomerase reverse transcriptase

TET1: Ten-eleven translocation methylcytosine dioxygenase 1

TGFß: Transforming growth factor beta

TIMP-Tissue inhibitor of metalloproteinases

TMZ: Temozolomide

TNFα: Tumor Necrosis Factor-α

tPA: tissue plasminogen activator

TTFields: Tumor treating fields

U

uPA: urokinase-type plasminogen activator

V

VEGF: Vascular endothelial growth factor

Z

ZEB1/2: zinc-finger E-box-binding homeobox 1/2

LIST OF FIGURES

1. INTRODUCTION

1.1. Glioblastoma

1.1.1. Definition and epidemiology

In adults, glioblastoma is the most common malignant primary tumor in the central nervous system (CNS), with an incidence of 3.2 cases per 100,000 inhabitants, accounting for 47.7% of cases (Figure 1) (Ostrom et al. 2018). One of the key hallmarks of glioblastoma hindering effective therapy is the diffuse invasiveness of the tumor cells through the normal parenchyma, causing tumor recurrence in close proximity or distant from the original tumor site. This feature appears to be independent of tumor grade, as both higher and lower grade gliomas tend to recur as a result of invasion of tumor cells into surrounding brain tissue (Soffietti et al. 2010). Therefore, the median survival remains 14 months despite aggressive standard treatment protocols (Stupp et al. 2005).

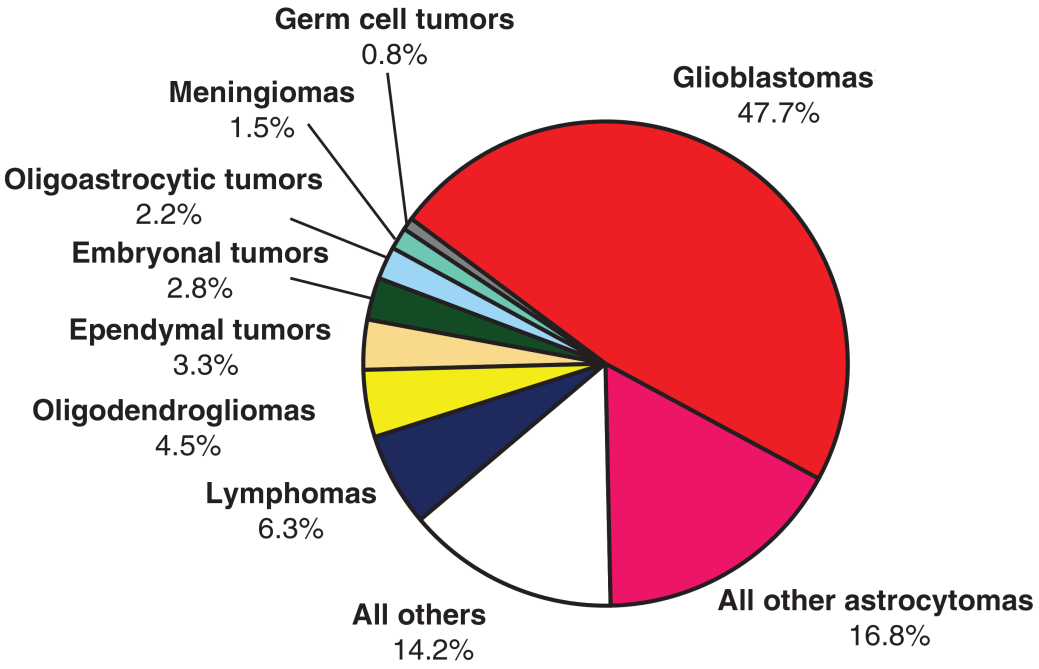

Figure 1 *– Distribution of malignant CNS tumors according to the Central Brain Tumor Registry of the United States (CBTRUS) statiscal report. Reproduced from* (Ostrom et al. 2018)

National registry-based studies in Korea, USA, United Kingdom and The Netherlands have reported and incidence of 0,59, 3,2, 4,64 y 5,9 cases/100,000 hab, respectively (Ostrom et al. 2015; Ho et al. 2014; Brodbelt et al. 2015; C.-H. Lee et al. 2010). Nevertheless, the lack of consistency in the diagnostic criteria and the differences in data collection processes add complexity to the comparison of the different incidences (Ostrom et al. 2014).

Regarding glioblastoma distribution by gender, it´s 1,6 times more frequent in males than in females. Besides, it´s more frequent in Caucasians that in blacks with a 2:1 proportion (Ostrom et al. 2015).

On the other hand, glioblastoma is uncommon in the pediatric population, with an incidence of 0.16 cases / 100,000 inhabitants below 20 years. This increases to 3.54 cases / 100,000 inhabitants in the age group of 45-54 years. After 55 years, its incidence undergoes a significant and progressive increase, reaching a maximum incidence of 15.24 cases / 100,000 inhabitants between 75 and 84 years. In the population older than 55 years it is the second most common primary CNS tumor and the first of the malignant tumors. (Ostrom et al. 2015)

A small proportion of glioblastoma cases, less than 1%, is associated with familial inherited syndromes such as Li-Fraumeni, Neurofibromatosis and Turcot. Most cases due to the malignization of gliomas of lesser histological grade (Ohgaki, Kim, and Steinbach 2010; Goodenberger and Jenkins 2012).

On the other hand, despite numerous studies trying to address the role of potential environmental risk factors, there is still no conclusive evidence for the majority of them. (Weller et al. 2015). Among the most studied ones, ionizing radiation has proven to be significantly associated with CNS tumors (Ostrom et al. 2014; Weller et al. 2015). It has been described that irradiation in childhood, as part of the treatment of tinea capitis with an average dose of 1.5Gy, is associated with a relative risk of 2.6 (95% CI = 0.8–8.6) of developing glial tumors and a relative excess risk of 1.98 / Gy (95% CI = 0.73–4.69) of developing malignant CNS tumors (Ron et al. 1988; Sadetzki et al. 2005).

Mobile phone radiation, classify as possibly carcinogenic to humans, has been also studied as a potential risk factor for glioma and glioblastoma. Nevertheless, the evidence remains inconclusive and population-based studies have failed to show a significant association between mobile phone use and the incidence of brain tumors (Karipidis et al. 2018; Little et al. 2012; Barchana, Margaliot, and Liphshitz 2012; Deltour et al. 2012). On the other hand, recent systematic reviews and meta-analysis have described a higher risk of glioma in long-term mobile phone users (Prasad et al. 2017; M. Yang et al. 2017).

Sugar intake has also been described as a potential risk factor (Nelson et al. 2012). Nevertheless, BMI, overweight and obesity were not associated with glioblastoma (Cote et al. 2018; Wiedmann et al. 2017) and long-term diabetes and increased HbA1c showed a decreased risk of glioma (Seliger et al. 2016; Kitahara et al. 2014).

Other environmental risk factors that have been associated to glioma include exposure to solvents as carbon tetrachloride (Nelson et al. 2012), alcohol intake (Baglietto et al. 2011) and higher socio-economic status (Porter, Lachance, and Johnson 2015). On the other hand, there is no evidence for traumatic brain injury as a risk factor for glioma (Munch et al. 2015) and whereas some other factors, as Cytomegalovirus infection, might be a protective factor in childhood and a risk factor later in life (Lehrer 2012).

1.1.2. Glioblastoma molecular and histological classifications

Several classification systems have been proposed according to the tumor molecular and histopathological features in Glioblastoma. Historically, from a histopathological perspective, multiple classification systems have been used, such as the Ringertz and St. Anne-Mayo CNS tumor classifications.

In the current World Health Organization Classification of CNS tumors, glioblastoma accounts as a Grade IV glioma based on three main features: 1) cellular atypia, 2) anaplasia and mitotic index and 3) microvascular proliferation and necrosis. This system also includes several somatic genomic alterations for the classification of diffuse gliomas. Among them isocitrate dehydrogenase (IDH) mutation status, Alpha-thalassemia/mental retardation X-linked (ATRX) loss, 1p / 19q codeletion are de most important ones. Thus, a glioblastoma harboring an IDH mutation is considered a secondary glioblastoma arising

from a prior lower grade diffuse glioma. Whereas, the IDH wild-type glioblastoma, accounting for 90% of cases, is thought to arise the novo and it also includes three histological variants: Giant cell glioblastoma, Gliosarcoma and Epithelioid glioblastoma (Louis et al. 2016).

Furthermore, Glioblastomas may be also classified depending on transcriptomic and DNA-methylome profiles and to specific somatic genomic alterations. According to the DNA-methylome, they can be divided according to whether or not they harbor a CpG-island methylator phenotype (CIMP). Consequently, this results in two different groups: CIMP gliomas (gCIMP) with and non-gCIMP. The CIMP in Glioblastoma has also prognostic value as the gCIMPs have better longer survival (E. Lee et al. 2018). Moreover, DNA methylation profiling is able to identify the different glioma subgroups by their specific DNA-methylation (Barritault, Meyronet, and Ducray 2018)

Transcriptomic profiles classify glioblastomas into four types: classical, neural, proneural and mesenchymal. Classical and mesenchymal Glioblastomas entail a more proliferative and invasive phenotypes, respectively. They are associated with worse prognosis, whereas, the proneural glioblastoma subtype is associated with better outcome. The neural group is associated with normal brain tissue in which tumor cells express neuronal markers. (Verhaak et al. 2010).

When non-GCIMP subgroups are analyzed regarding copy number, mRNA expression, somatic mutations and promoter methylation, proneural-glioblastoma´s are found to be phylogenetically distinct form other non-GCIMP subtypes (mesenchymal, neural and classical). (Ozawa et al. 2014)

Furthermore, somatic genomic alterations also include copy number variation (CNV), being the amplifications of chromosome 7, 12 and 4 and the deletion of chromosomes 10, 1 and 9 the most common ones. Interestingly, each transcriptomic-based subtype harbors a specific CNVs, accordingly epidermal growth factor receptor (EGFR) amplification and Cyclin Dependent Kinase Inhibitor 2A (CDKN2A) deletion are associated to the classical subtype, Neurofibromin 1 (NF1) loss with the Mesenchymal subtype, and Platelet Derived Growth Factor Receptor Alpha (PDGFRA) amplification with the proneural subtype (E. Lee et al. 2018).

Based on the integration of these classification systems, six different molecular subgroups of glioblastoma can be defined. Two pediatric subgroups, each of them harboring a specific histone mutation, as H3F3A K27 or H3F3A G34. The remaining four molecular subgroups present in adults and include: 1) IDH mutated glioblastoma with CIMP, which is characterized by a proneural transcriptomic signature; 2) Receptor tyrosine kinase (RTK) I, defined by PDGFRA gene amplification and similarly associated to the proneural signature; 3) RTK II, expressing the "classic" profile, and characterized by EGFR genomic alterations, either amplification or mutation; and 4) Mesenchymal glioblastoma, which harbors a distinct mRNA expression profile. The majority of the adult glioblastoma share other genomic alterations as chromosome 7 gain, chromosome 10 monosomy, phosphatase and tensin homolog (PTEN) loss, Telomerase reverse transcriptase (TERT) promoter mutation, aberrant activation of Mitogen-activated protein kinase (MAPK) and protein kinase B (PKB) signaling, and p53 and RB1 impaired activity. Furthermore, the IDH mutated and RTK I groups are more frequent in young adults, whereas the RTK II and Mesenchymal glioblastomas predominate in older adults (Weller et al. 2015; Masui, Mischel, and Reifenberger 2016).

1.1.3. Etiopathogenesis and gliomagenesis

1.1.3.1. Tumorigenesis

It has been described that the tumor arises from neural stem cells (NSC) located in the subventricular zone (SVZ). It was recently described that astrocyte-like NSCs, harboring driver mutations due to replicative errors, are capable of migrating away from the SVZ, acquiring other somatic alterations, as CNV and private mutations, and finally clonally evolving into high-grade gliomas in other brain regions. The genes with driver mutations that had proven to play a role in gliomagenesis arising from the SVZ include: TERT, EGFR, TP53, and PTEN among others (J. H. Lee et al. 2018). Furthermore, it has been proposed that gliomagenesis might follow a similar and parallel process to gliogenesis, involving glial transcription factors as Nuclear factor I-A (NFIA) and Olig2, among others (Laug, Glasgow, and Deneen 2018).

On the other hand, in a more detailed model recently proposed by Barthel et al., the tumorigenesis process in glial tumors is described in five phases: 1) Initial growth; 2) Oncogene-induced senescence; 3) Stressed growth; 4) Replicative senescence; and 5) immortal growth. The first phase, the initial growth, is thought to be precede by a glioma initiation event, most likely IDH1/IDH2 mutation and loss of chromosome 10 and gain of chromosome 7 in IDH-mutant and IDH-wildtype gliomas, respectively. These events result in oncogene activation and loss of tumor suppressor genes (PTEN and Ten-eleven translocation methylcytosine dioxygenase 1 (TET1), among others), leading to an increased proliferation. Then, as a reaction to this increased oncogenic signaling, the tumor cells enter a cell cycle arrest by activation of tumor suppressor genes as $p16^{INK4a}$/$p14^{ARF}$, RB and p53 (Phase II). Consequently, a subclone with an impairment of this tumor suppressive signaling, as loss or mutation of TP53 in IDH-mutant gliomas and CDKN2a/b in IDH-wildtype gliomas, will move to a second phase of cellular growth under stress conditions and characterized by incremental genomic instability. In turn, this results in a second period of growth arrest (replicative senescence) and, finally, a subpopulation of these cells manage to develop stem-like properties, leading to immortal growth (Barthel, Wesseling, and Verhaak 2018),

Interestingly, several of these genomic alterations highlights the relevance of RTKs pathways, including Phosphoinositide 3-kinase (PI3K) and MAPK signaling, in gliomagenesis. In fact, it has been described in vivo the collaborative role of this pathways in glioma tumorigenesis (Kondo 2017; Schmid et al. 2016; Vitucci et al. 2013). Another key alteration in gliomagenesis is the IDH mutation in the early phases of tumorigenesis, resulting in the accumulation of the oncometabolite 2-hydroxiglutarate, which inhibit several α-ketoglutarate-dependent pathways, causing at the end epigenetic alterations and Hypoxia inducible factor 1α (HIF1α)-dependent oncogene expression, among other pro-oncogenic changes (Ohba and Hirose 2016).

1.1.3.2. Genomic characterization and main molecular pathways

It has been well established that glioblastoma pathogenesis articulates around three main signaling pathways, RTKs, p53 and RB, that work together and interact between them to induce tumor growth and progression. The pilot project The Cancer Genome Atlas (TCGA) analysis showed that, in glioblastoma, up to 88%, 87% and 78% of samples

harbor somatic alterations affecting these pathways respectively (Figure 2). Interestingly, the genomic alterations within one pathway tended to be mutually exclusive, whereas in up to 74% of cases the three pathways were deregulated, suggesting a synergic mechanism between them necessary for glioblastoma pathogenesis (Crespo et al. 2015; McLendon et al. 2008; Parsons et al. 2008).

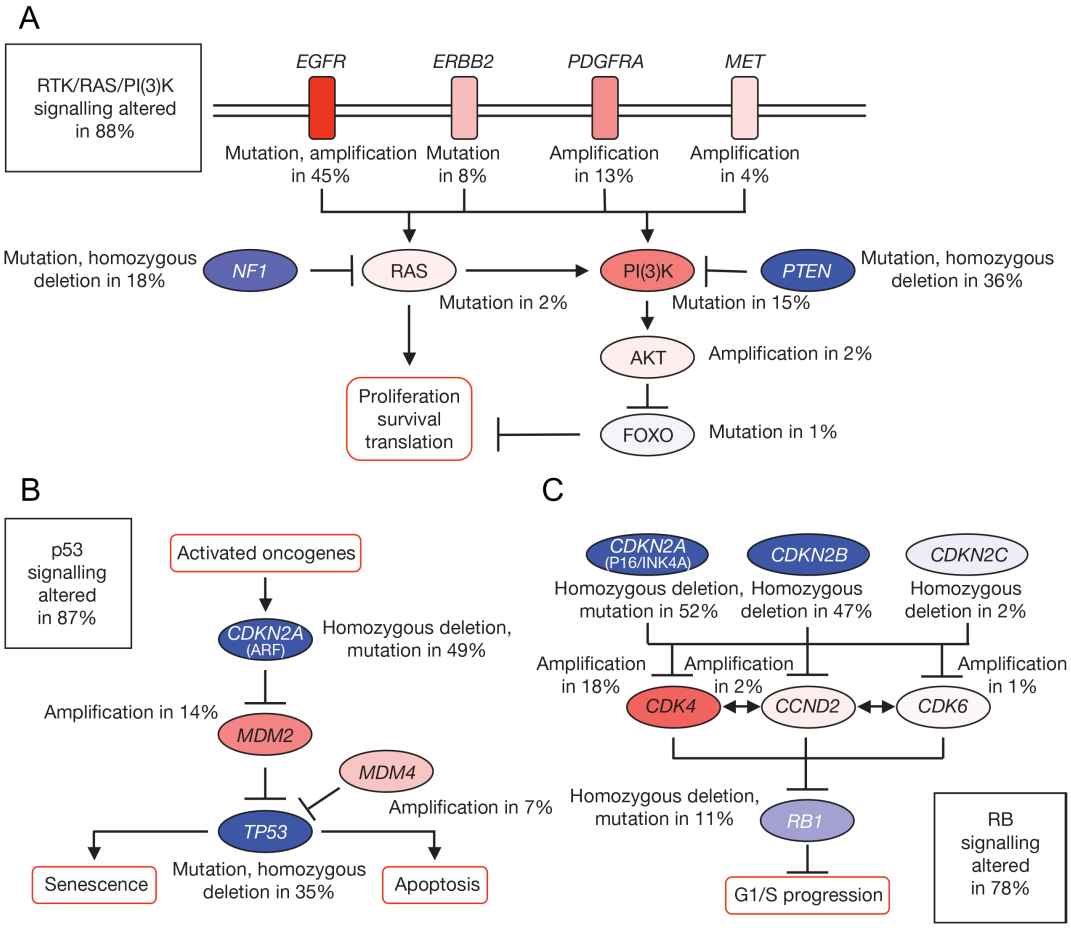

Figure 2 *– The Cancer Genome Atlas (TCGA) glioblastoma genomic characterization showing the main genetic alterations. The diagram illustrates the primary sequence alterations and copy number changes for components of the (A) RTK/RAS/PI(3)K, (B) p53 and (C) RB (c) signalling pathways. Red indicates activating alterations whereas blue inactivating alterations. Reproduced from* (McLendon et al. 2008)

Furthermore, previous genome wide association studies (GWAS) have identified several SNPs independently associated with glioma: TERT, EGFR, TP53 and CDKN2B are the most important ones among them (Ostrom et al. 2014).

1.1.3.2.1. RTK signaling

Genomic alterations affecting RTK signaling are present in up to 86% of cases. In the majority of glioblastoma cases there are genomic alteration in at least one of the RTKs. The most important RTKs in glioblastoma´s pathogenesis are the PDGRFA and the EGFR, both of them expressed in the tumoral cells, enabling autocrine and paracrine signaling. They are affected by CNV or mutations in 13% and 57,4% of cases, respectively. Genomic aberrations in other RTKs, as ERBB2 and MET, have also been described in a smaller proportion. Besides, more than 40% of the tumors have more than one RTK altered (McLendon et al. 2008; Brennan et al. 2013).

In gliomas, PDGFRA and EFGR activity might be deregulated due to gene amplification / mutation or by overexpression of the ligand and the receptor. Regarding EGFR, there are 7 different variants, EGFRvIII being the most frequent, present in 20-50% of glioblastomas with EGFR amplification. Downstream, RTKs activate a complex network of different pathways, the most relevant ones are: 1) Ras/Raf/MAPK, 2) PI3K/AKT, 3) PKC, and 4) STAT. Their activation results in cell proliferation, migration, invasion, resistance to apoptosis and neovascularization (Crespo et al. 2015).

Ras signaling is upregulated in the majority of glioblastoma cases, most likely due to RTK activation. Through this G-protein, RTKs activate several pathways leading to cell proliferation and survival, including the protein kinase cascade Raf/MEK/MAPK and also the PI3K/AKT/ Mammalian target of rapamycin (mTOR) pathway (Crespo et al. 2015). The latter has a central role in glioblastoma pathogenesis, and it is also regulated directly by upstream RTKs activity. RTK activation induce the releasing of the PI3K catalytic subunit, which in turn enables the phosphorylation of PIP2 into PIP3. Interestingly, this process can be reverted by PTEN. Then PIP3 induces Akt activation, which by upregulation of mTOR, Nuclear factor kappa B (NFκB)/ Multi-Drug Resistance Gene 1 (MDR1) and B-Catenin signaling among others, results in cell proliferation, cell growth, invasion, cell cycle regulation and drug resistance (X. Li et al. 2016).

1.1.3.2.2. p53 pathway

Similarly to other neoplasms, p53 plays a central role in glioblastoma pathogenesis, in which it is dysregulated in 85-87% of cases, according to the TCGA reports (Brennan et al. 2013; McLendon et al. 2008).

Functionally, the p53 pathway is crucial in regulating the cellular response to DNA damage. Consequently, it can be triggered by several cellular stress factors, including hypoxia, DNA damage, ribosome biogenesis, rNTP depletion, spindle damage, and heat or cold shock, among others. Besides, it also has a role in regulating other process as cell cycle, cell death, proliferation and differentiation of stem cells. Therefore, its final goal is to prevent perpetuation of genomic alterations (Harris and Levine 2005; Crespo et al. 2015).

The p53 pathway is regulated by a complex combination of negative and positive feedback mechanisms. Mouse double minute 2 homolog (MDM2), a TP53-induced factor, is one of the most important ones, as it blocks p53 activity, resulting in a negative feedback loop. Furthermore, CDKN2A-$p14^{Arf}$ also plays a role in this loop as it is downregulated by p53 and downregulates in turn MDM2 (Harris and Levine 2005). In glioblastoma, this network is dysregulated due to TP53 mutation or deletion in 27.9% of cases, MDM amplification in 15.1%, and/or CDKN2A deletion in 57.8%. Interestingly, TP53 alterations and MDM amplification and/or CDKN2A deletion are mutually exclusive (Brennan et al. 2013).

1.1.3.2.3. RB pathway

The RB pathway is widely known to be involved in cell proliferation and cell cycle regulation by inhibiting progression into the S phase, and it´s frequently altered in cancer. It has been described that Rb, in its hypophosphorylated and consequently active state, it's bound to the E2F-family of transcriptions factor, preventing progression from cell G1 phase into the S phase by hindering transcription of mitosis key genes. Contrarily, during cell proliferation, growth factors induce cyclin D1 and degradation of $p27^{Kip1}$, resulting in activation of cyclin complexes (Cyclin Dependent Kinase 2 (CDK2)/cyclin E). In turn, this complex, inactivates Rb, by inducing its phosphorylation into pRb, releasing E2F and resulting in transcriptional activation of proliferation genes, allowing de cell to progress from G1 phase into S phase (Du and Searle 2009; Crespo et al. 2015) .

During cell cycle progression, Rb is phosphorylated by the cyclin-dependent kinases CDK 4 and CDK6. In turn, these are inhibited by the cell cycle inhibitors, the Ink4 family of proteins, including CDKN2A-p16INK4a and CDKN2B-p15INK4b among others (Hamilton and Infante 2016).

In glioblastoma the RB pathway is dysregulated more commonly by deletion of CDKN2A/2B, in 61% of cases, and amplification of CDK4/6, in 15.5% of cases. Less frequently, the pathway can be affected by direct mutation or deletion of RB1 and deletion of CDKN2C. In 78.9% of cases, RB genomic alteration concur with a a dysregulated p53 pathway (Cohen and Colman 2015; Brennan et al. 2013).

1.1.3.3. Heterogeneity

In the last four decades, the tumor histological heterogeneity has been well recognized as a key biological feature in cancer pathogenesis. In glioblastoma, for instance, it is widely accepted that contiguous cells share a similar cytoarchitecture, whereas distant cells are more likely to be different, which suggest a clonal expansion. In general, intra-tumoral heterogeneity is associated to worse outcome (Eder and Kalman 2014; Reinartz et al. 2017)

Furthermore, inter- and intratumoral heterogeneity is one of the major biological hallmarks in glioblastoma and it is directly associated to treatment resistance (Qazi et al. 2017). Moreover, it has been proposed that recognizing the *ex vivo* single-cell derived subclone-specific drug resistance might help to overcome the glioblastoma heterogeneity-resistance (Reinartz et al. 2017).

Intratumoral heterogeneity results in tumor cell subpopulations with different molecular signatures resulting in different phenotypes. For instance, within the same tumor, specimens taken from different locations harbor different molecular signatures, falling in different categories of the Verhaak classification (Sottoriva et al. 2013; Verhaak et al. 2010).

Besides, single-cell RNA-seq analysis has also helped to demonstrate this intratumural heterogeneity in Glioblastoma, showing a significant cells-to-cell transcriptional variability and stemness status. Moreover, it also classify the tumor cells into the different Verhaak et al. molecular tumor subtypes (Patel et al. 2014).

Interestingly, glioblastoma intratumoral heterogeneity has also been identified at the epigenetic level, by analyzing the O6-methylguanine-DNA methyltransferase (MGMT)-promoter methylation status in in samples from different locations within the same tumor (Parker et al. 2016). Fuerthermore, multiple commonly dysregulated pathways can be differently affected in adjacent tumor cells (Sturm et al. 2014).

1.1.3.4. Hypoxia

Hypoxia is an important feature of glioblastoma and several previous *in vivo* and *in vitro* studies have implicated it in tumor proliferation and invasion (Monteiro et al. 2017). Furthermore, the clinical relevance of hypoxia in glioblastoma and its correlation with resistance to treatment has been previously established (Kawai et al. 2011; L. Yang et al. 2012).

Hypoxia promotes the expression of HIF-1a which, in turn, regulates angiogenesis, metabolic and transcriptional signaling pathways as EGFR, PI3K/Akt and MAPK/ERK. Besides, HIF-1a induces upregulation of the vascular endothelial growth factor (VEGF), involved in tumor neovascularization and invasion (Womeldorff, Gillespie, and Jensen 2014; Gang et al. 2017).

Besides, it is widely accepted that HIF-1a upregulation facilitates the adaptation of glioblastoma cells to their hypoxic environment, thus providing them an increased chance of survival (Colwell et al. 2017). In addition, it has been recognized that HIF-1a promotes tumor infiltration and invasion through several mechanisms (J.-W. E. Chen et al. 2018; Monteiro et al. 2017; Rosa et al. 2018).

1.1.3.5. Invasion

Glioblastoma invasion hinders the effect of treatment and lead to tumor recurrence. Besides, the molecular pathways involved in glioblastoma cell invasion regulation, play a major role in its pathogenicity (Lefranc et al. 2018). In fact, infiltrative glioblastoma stem-like cells have been described in the peritumoral parenchyma (Ruiz-Ontañon et al. 2013)

and their role in developing resistance to chemo- and radiotherapy is widely accepted (J. Chen et al. 2012; Bao et al. 2006).

The mechanism of glioma cell invasion involves both biochemical and biophysical processes that regulate cell shape and its movement across the intercellular space, concurrent with rearrangement of the extracellular matrix (ECM), these mechanisms are summarized in Figure 3 and described in the following sections.

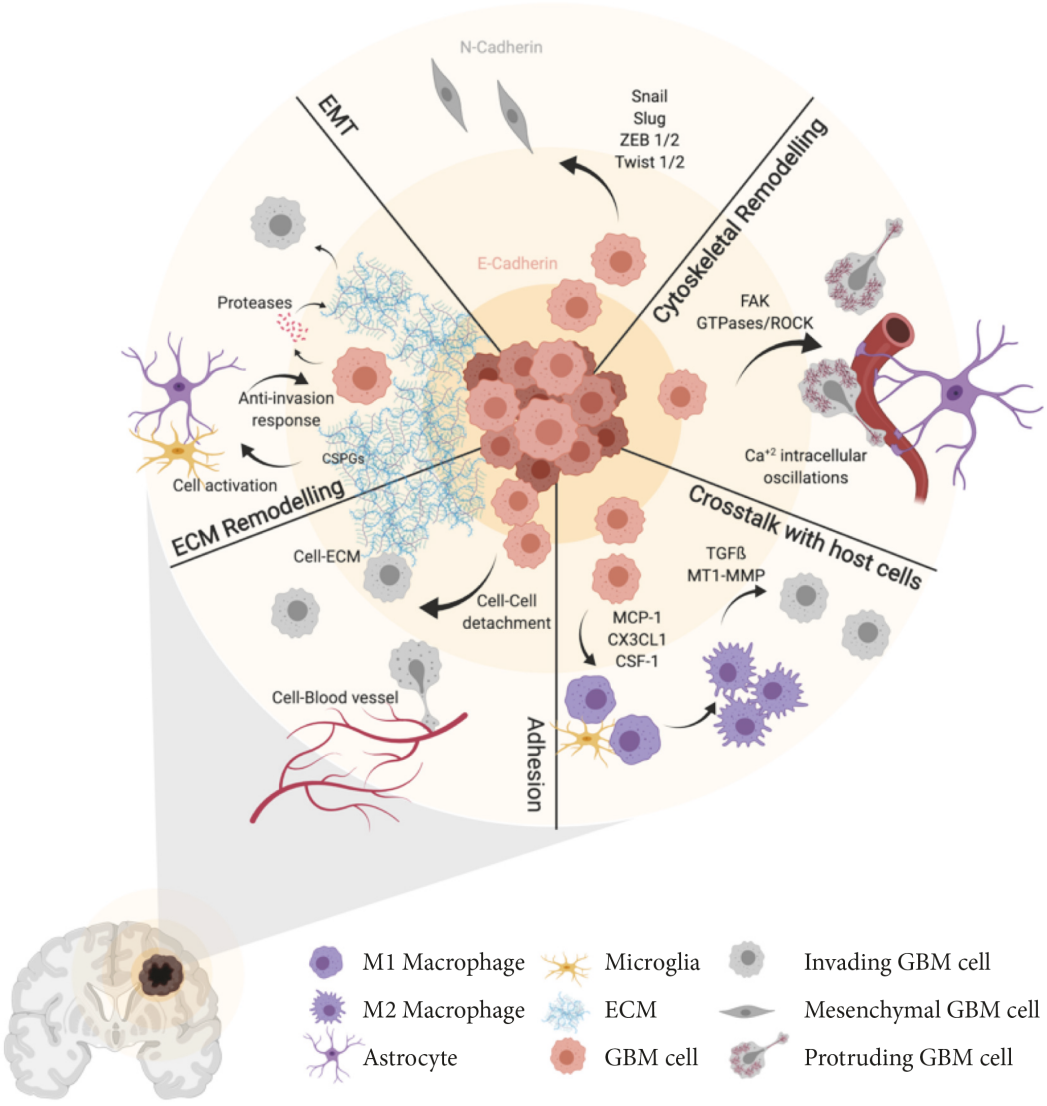

Figure 3 *- Cellular processes involved in glioblastoma cell invasion. Schematic summary of the processes involved in the invasive capacity of glioblastoma cells including cell-to-cell and cell-to-ECM adhesion, ECM remodeling, Endothelial-mesenchymal transition (EMT), cytoskeletal remodelling, and cross-talk with host cells. See text for details. Reproduced from* (Velásquez et al. 2019).

1.1.3.5.1. Adhesion molecules and ECM

Glioblastoma cell invasion begins with the cell detachment from the surrounding tumor tissue, a process that involves cell surface adhesion molecules such as neuronal cell adhesion molecule (NCAM) and cadherins. In fact, it has been demonstrated that cadherin instability leads to glioma cell migration (Asano et al. 2004) and NCAMs modify the ECM by down-regulating the expression of matrix metalloproteinases that degrade cadherins and, thereby, hinder tumor cell motility (Claes, Idema, and Wesseling 2007). Furthermore, the expression of NCAMs is inversely related to glioma grade, which is in agreement with data showing that loss of this molecule enhances tumor cell migration (Duenisch et al. 2011). Recent transcriptomic and proteomic analyses have reproduced these findings and have identified a new splice variant of NCAM1 with potential implications in cell signaling (Jayaram et al. 2018).

Intercellular adhesion molecule-1 (ICAM1), a member of the immunoglobulin family of genes, has also been shown to contribute to glioma cell invasion (Yu et al. 2012). Although ICAM1 is involved in several cellular processes, it has been described that, upon induction of inflammation, leukocytes interact with ICAM1 on the endothelial cells, which allows them to cross the barrier vessel wall (Frank and Lisanti 2008). Furthermore, in glioma, radiation increased ICAM1 expression resulting in migration and invasion of the tumor cells (Kesanakurti et al. 2013), enhancing the invasiveness of glioblastoma cells into the healthy brain tissue (Lin et al. 2019).

Integrins (ITGs), another key component of the interface between tumor and non-tumor cells, function as receptors that regulate cell adhesion to ECM proteins or cell surface proteins on other cells (Takada, Ye, and Simon 2007). Besides, ITGs also link extracellular contacts with the intracellular cytoskeleton through different signalling mechanisms. They are capable of clustering in the cell membrane and transducing intracellular signals upon extracellular ligands binding. The intracellular signals are transduced through their cytoplasmic domain (β subunit) by activation of kinases such as Focal Adhesion Kinase (FAK), Integrin-Linked Kinase (ILK) and Rho-GTPases. Through this mechanism, ITGs then activate pathways leading expression of genes that modulate cell proliferation, survival, differentiation and migration (outside-in signalling) (Takada, Ye, and Simon 2007). On the other hand, cytoplasmic proteins are capable of modulating the extracellular affinity of

ITGs for their ligands (inside-out signalling) and contribute to cell migration and invasion (Paolillo, Serra, and Schinelli 2016).

Besides, ITGs can regulate invasion and metastasis by providing the traction necessary for cell migration (Desgrosellier and Cheresh 2010). They also modulate the expression of proteases that play a role in modelling the ECM. In glioblastoma, they are involved in epithelial to mesenchymal transition (EMT), cell migration and invasion by improving adhesion to the ECM, and activation of intracellular signalling pathways such as FAK, Rho-GTPases, Shc/MAP-Kinases, and Src Family Kinases (Renner et al. 2016; Serres et al. 2014; Mikheev et al. 2015; Ding et al. 2002; Hehlgans, Haase, and Cordes 2007; Desgrosellier and Cheresh 2010; Lawson and Burridge 2014). αvβ3 also enhances glioblastoma invasion through the activation of matrix metalloprotease 2 (MMP2) at the plasma membrane (Desgrosellier and Cheresh 2010). Interestingly, ITG´s role in invasion has been validated in vitro and in vivo, as inhibition of αvβ3/αvβ5 in mouse models reduces glioblastoma cell migration and invasion (Scaringi et al. 2012), and αvβ3/αvβ5, α6β1 is associated with invasive phenotype in U87 glioblastoma cell line (Delamarre et al. 2009).

On the other hand, ECM composition also plays a critical role in the invasion process. This is reflected in the fact that the tumor-associated ECM is intrinsically different from the ECM within the normal parenchyma (Herrera-Perez, Voytik-Harbin, and Rickus 2015; de Gooijer et al. 2018). For instance, hyaluronic acid (HA) enrichment in tumor microenvironment promotes cell invasion through positive feedback regulation of NFkB (Yoo et al. 2018; Ferrandez et al. 2018; J.-W. E. Chen et al. 2018) and, on contrary, a glycosylated chondroitin sulfate proteoglycans (CSPGs)-enriched ECM is associated with non-invasive lesions. Besides, up-regulation of LAR-CSPG binding complexes results in strong binding of the tumor cells to the ECM, preventing cell invasion and high levels of CSPGs elicit an astrocyte/microglia-mediated anti-invasion response. Conversely, diffusely infiltrating tumor ECM lacks glycosylated CSPGs (Y. Kim et al. 2018).

Besides, in glioblastoma, matrix metalloproteinases (MMP), such as MMP-2 and MMP-9 degrade the ECM and therefore they are related to the tumor grade and the invasive capacity of glioma (M. Wang et al. 2003). Other molecules involved in the degradation of the ECM are cysteine proteases, A disintegrin and metalloproteinases (ADAMs), and

urokinase-type plasminogen activator (uPA). However, since low-grade gliomas with normal proteases levels are capable of invading the surrounding tissue, the role of proteases in the invasion of gliomas remains uncertain (Lakka et al. 2004). Notwithstanding, in vitro assays show that a high migration capacity is associated with expression of MMP-2, MMP-9, uPA, and tissue plasminogen activator (tPA) (Kaphle, Li, and Yao 2019).

1.1.3.5.2. Epithelial to mesenchymal transition

Epithelial to mesenchymal transition (EMT) is the process through which the cell cytoskeleton is remodelled to a non-polarized mesenchymal phenotype with an enhanced invasion capacity. Specific transcription factors such as Snail and Slug, the zinc-finger E-box-binding homeobox (ZEB)1/2, and Twist1/2 are considered the main regulators of the EMT process and in glioblastoma they lead to invasion, migration and proliferation (Myung et al. 2014; Siebzehnrubl et al. 2013; Mikheeva et al. 2010). These factors are implicated in regulating the transcription of genes, including N-cadherin, vimentin, and fibronectin that are typically expressed in mesenchymal cells (Kahlert, Joseph, and Kruyt 2017) and at the same time, they suppress the expression of epithelial markers such as E-cadherin, claudins, occludins, and cytokeratins. Loss of E-cadherin, in turn, results in Wnt signalling and accumulation of β-catenin, which leads to increased transcription of genes that promote cell proliferation and invasion (McCrea and Gottardi 2016). Besides, it was shown that expression of N-cadherin negatively correlated with glioblastoma tumor cell invasiveness, and its overexpression *in vitro* reduced cell migration and restored cell polarity (Asano et al. 2004; Camand et al. 2012). The Tumor Necrosis Factor-α (TNFα) ans IL6 play also an essential role for EMT induction. Other pathways involved in EMT regulation include the Hepatocyte growth factor (HGF), the Epidermal growth factor (EGF), and the Platelet derived growth factor (PDGF) (J. Kim et al. 2016; F. Liu et al. 2017; Wu et al. 2013; Storci et al. 2010).

Interestingly, clinical factors like the radiation treatment or the anti-angiogenic therapy for primary glioblastomas resulted in transition to a mesenchymal phenotype in the recurrent tumors. Moreover, genes involved in EMT signaling are upregulated in radio-resistant glioma cells and *in vivo* studies in xenograft glioblastoma mouse models have demonstrated a gene expression profile shift from proneural a mesenchymal signatures upon radiation treatment (Mahabir et al. 2014; Y.-H. Kim et al. 2014; Halliday et al. 2014).

1.1.3.5.3. Cytoskeletal remodelling and cell motility

Glioma cells, which show a mesenchymal pattern of migration, are capable of passing through extracellular spaces smaller than their own nuclei. This capacity is possible thanks to cytoskeletal remodeling, which leads to the outward extension of the cell membrane at the leading edge (pseudopod). Then, the pseudopod is in contact with the ECM through ITGs localized on the cell membrane, and they, in turn, activate phosphorylation and de-phosphorylation intracellular signals via FAK(Hynes 2002; Stylli, Kaye, and Lock 2008). As a consequence, membrane-type MMPs are recruited at the focal contacts to degrade and restructure the ECM via the production of soluble matrix metalloproteases, including MMP-2 and MMP-9. Finally, the cells contract by the acto-myosin complex engagement, resulting in focal contact disassembly, integrin recycling, detachment of the trailing edge, and ultimately, cell invasion (Tam et al. 2002; Wear, Schafer, and Cooper n.d.).

RHO GTPases also play an important role in regulating acto-myosin complex engagement during EMT. Among them, RHOA promotes formation of actin stress fibres and, RAC1 and CDC42, on the other hand, regulate the formation of lamellipodia and filopodia. Once activated, GTPases upregulate and promote the RHO-associated kinase (ROCK)-dependent actin polymerization and also induce the phosphorylation of myosin light chain to promote acto-myosin contraction and activation of LIM kinase (LIMK) (Narumiya, Tanji, and Ishizaki 2009). In glioma cells, RHO GTPases including RHOA and RAC regulate cytoskeletal rearrangements resulting in ameboid and mesenchymal cell motility and have been shown to promote migration and growth of glioma cells *in vitro* and *ex vivo* (H. Wang et al. 2014). Furthermore, it has been described that trans-membrane ion co-transporters induce cell migration and EMT through downstream activation of RHOA and RAC pathways (Ma et al. 2019). Besides, several pathways including Wnt and PI3K/Akt have been shown to be associated with RhoA to regulate cytoskeletal changes that allows migration(G. Liu et al. 2018; Drappatz, Norden, and Wen 2009).

Glioma cell motility is also regulated by biophysical properties such as cell density and the rigidity and geometry of the ECM (Discher, Janmey, and Wang 2005). In example, the increased rigidity of the ECM in gliomas results in formation of stress fibres and focal adhesions that enable more rapid migration of the cells(Ulrich, de Juan Pardo, and Kumar 2009).

Glioma cells interaction with the vascular walls is another described invasion mechanism. It has been demonstrated that glioma cells associate with the vascular walls and migrate along the vessels. Furthemrore, bradykinin, secreted by the endothelial cells, functions as a chemotactic signal for glioma cells through binding to its receptor (BR-2) on the glioma cell surface resulting in subsequent intracellular Ca2+ oscillations. Changes in Ca2+ levels in turn, regulate cell motility through acto-myosin-mediated contraction, regulation of tubulin dynamics, and controlling the activation of focal adhesion kinases that mediate cell adhesion to substrates in the ECM (Martini and Valdeolmillos 2010; Montana and Sontheimer 2011). Besides, movement of glioma cells along the vascular walls in turn disrupts the astrocyte-endothelial cell interaction resulting in disruption and breakdown of the blood-brain barrier (BBB) and alterations in blood vessel diameter (Watkins et al. 2014). This enables glioma cells to gain access to oxygen and nutrients from the bloodstream. In addition to the cytoskeletal rearrangement, regulation of cell volume by voltage-gated chloride and potassium channels is another mechanism that regulates glioma cell migration (Cuddapah et al. 2014).

1.1.3.6. Immune modulation

Several types of supportive stromal cells are active components of the tumor microenvironment. In fact, they are capable of secreting growth factors and molecules with a significant impact in tumor cell invasion and proliferation. In fact, the microenvironment has been demonstrated to play key regulatory roles in response to therapy and tumor progression (Hirata and Sahai 2017). It has recently been shown that astrocytic and oligodendrogliotic gliomas share similar glial lineages and that differences in bulk expression profiles between these glial tumors is primarily driven by composition of the tumor microenvironment (Venteicher et al. 2017).

Furthermore, it is widely accepted that alterations in local immune and vascular networks facilitate tumor growth in glioblastoma. In fact, nearly a third of glioblastoma mass is composed of glioma-associated macrophages (GAMs), which are derived primarily from bone-marrow derived cells and resident inflammatory cells (De Palma 2016). GAMs have been shown to play an important role in tumor invasion and in the ability of tumor cells to invade the brain parenchyma (Biswas and Mantovani 2010; F. Hu et al. 2015; Coniglio

and Segall 2013). They are recruited by glioma cell chemo-attractans, such as monocyte chemo-attractant protein-1 (MCP-1), fractalkine (CX3CL1), glial cell–derived neurotrophic factor (GDNF), and colony stimulating factor-1 (CSF)-1) (Hambardzumyan, Gutmann, and Kettenmann 2016). Then, GAMs either adopt a pro-inflammatory M1 phenotype or anti-inflammatory M2 phenotype. For instance, CSF-1 also promotes recruited macrophages to adopt M2 phenotype that contributes to tumor invasion. In fact, immunomodulation of CSF-1 signalling using a CSF-1R inhibitor has demonstrated to shift macrophages back to M1 phenotype (Pyonteck et al. 2013).

On the other hand, GAMs secrete several factors with primary effects on tumor cells. In example, when exposed to glioma cells, GAMs upregulate expression of membrane type 1–matrix metalloproteinase (MT1-MMP) that cleaves pro-MMP2 to facilitate degradation of the extracellular matrix and glioblastoma invasion. Moreover, GAMs secrete several oncogenic factors such as transforming growth factor beta (TGFß), which enhances glioma cell migration by up-regulating integrin expression, and contributes to the degradation of extra-cellular matrix components by inducing MMP2 expression and suppressing the expression of tissue inhibitor of metalloproteinases (TIMP)-2 (Markovic et al. 2009; Wesolowska et al. 2008).

1.1.3.6.1. Molecular pathways in glioblastoma invasion

Several signalling networks are employed by glioblastoma cells to promote tumor growth and invasion. The most important pathways involved in glioblastoma invasion include RTK signaling, which have been extensibly described before, the Wnt/ß-catenin pathway and Hedegehog signaling (Singh et al. 2004; Hanahan and Weinberg 2011).

RTK signaling, including Ras/Raf/MAPK, Raf/JNK, Rho/Rac/PAK and PI3K/Akt/mTOR activation, has a significant role in glioblastoma invasiveness and aggressiveness. Furthermore, all these pathways might be activated by several growth factors including VEGF, EGF, PDGF and TGF-β, commonly found in the ECM, resulting in tumor cell invasion (Plotnikov et al. 2012; Streuli and Akhtar 2009).

The Phosphoinositide-3-kinase (PI3K) signalling cascade, activated by interaction of EGF and TGFß with their respective RTKs, regulates a series of biological processes such as

cellular metabolism, growth, survival and invasion (C. Liu et al. 2017). Constitutional activation of the PI3K-Akt pathway results in increased expression of matrix metalloproteases including MMP-2 and MMP9 that facilitate degradation of ECM and lead to tumor invasiveness (Lal et al. 2002; McNeill et al. 2018).

Other RTKs haven been also implicated in glioblastoma invasion. For instance, the RTK c-Met and its ligand HGF/Scatter factor are overexpressed in gliomas and they have been shown to play a role in cell proliferation, invasion, angiogenesis and survival in several cancers(Gentile, Trusolino, and Comoglio 2008). Interestengly, co-expression of EGFR and c-Met in glioblastoma leads to deregulated EGFR signalling and increased HGF binding to c-Met, which in turn, promotes cell invasion (Velpula et al. 2012). The RTK Mer (MerTK) is also overexpressed in glioblastoma and *in vitro* depletion of MerTK disrupts the round morphology of glioma cells and decreases their invasiveness Besides, the expression and phosphorylation of myosin light chain correlated with activation of MerTK, suggesting that the effect of MerTK on glioma cell invasion is mediated by the ability of acto-myosin to contract (Y. Wang et al. 2013). Interestingly, ECM dysfunction may lead to aberrant activation of RTK signaling pathways, and therefore it might regulate tumor growth, angiogenesis, and invasion (Manini et al. 2018).

Wnt signaling. WNT signaling, involved in cell self-renewal, differentiation and migration during CNS embryogenesis, is implicated in several types of cancer when pathologically activated (Dijksterhuis, Petersen, and Schulte 2014; Kahlert et al. 2012; Cui et al. 2018; Y. Lee et al. 2016). In glioblastoma, WNT signaling is involved in invasion and EMT. In brief, the WNT ligands bind to the transmembrane Frizzled receptors activating two intracellular molecular cascades: the canonical ß-catenin-dependent and ß-catenin-independent pathways (Dijksterhuis, Petersen, and Schulte 2014). The first leads to disassembly of the complex consisting of AXIN, adenomatous polyposis coli (APC), and GSK3β, thereby stabilizing β-catenin (Dijksterhuis, Petersen, and Schulte 2014). Then, β-catenin translocates into the nucleus where it regulates transcription of EMT and cell invasion related target genes (Kamino et al. 2011; Kahlert et al. 2012). Overexpression of positive WNT signaling is associated with upregulation of cyclin D1 (CCND1), c-myc, COX2, ZEB1, SNAIL, TWIST, SLUG, N-cadherin, and SOX2, among others (Y. Lee et al. 2016). On the other hand, the ß-catenin-independent pathway is activated through WNT2, WNT4, WNT5A, WNT6 and WNT11 factors and leads to up-regulation of the planar cell

polarity (PCP) and calcium pathways (Dijksterhuis, Petersen, and Schulte 2014). It has been described that the canonical pathway is important for glioma stem cell maintenance and the ß-catenin independent pathway mainly regulates cell motility and polarity (Bhuvanalakshmi et al. 2018; G. Wang et al. 2017).

It has been demonstrated that, in glioblastoma, activation of ß-catenin leads to increased tumor invasion, while inhibition of ß-catenin suppressed cell proliferation and invasion. Furthermore, WNT signaling promotes tumor cell migration *in vitro* by inducing the expression of Zeb1, Twist1 and Slug. Interestingly, Wnt/β-catenin pathway is mainly activated within cells located at the invasive edge of the mesenchymal patient tumors. (Kahlert et al. 2012). On the other hand, knockdown of WNT5A downregulated expression of MMP, and suppressed glioma cell migration and invasion (Kamino et al. 2011; Kahlert et al. 2012)

Hedegehog-GLI1. The Hedgehog pathway also plays a role in CNS development. In glioblastoma, Hedgehog signaling is upregulated and it is involved in tumorigenesis and tumor progression. In brief, Hedgehog signaling can be activated by three ligands: Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH). They bind to the transmembrane protein Patched 1 (PTCH1), which allows Smoothened (SMO) to prevent the cleavage of GLI1. Then, GLI1 translocates to the nucleus and act as a transcription factor (Paw et al. 2015). Hedgehog signaling activation leads to up-regulation of GLI1, PTCH1, CCND2, B-cell lymphoma 2 (Bcl-2) and VEGF. In addition, the Hedgehog pathway modulates the expression of stemness genes, such as NANOG, OCT4, and SOX (Carpenter and Lo 2012). Interestingly, a novel alternatively spliced isoform, tGLI1, is undetectable in normal cells but expressed in glioblastoma, has been linked to increased cell motility and tumor invasion (Rimkus et al. 2018; Sirkisoon et al. 2018). Furthermore, tGLI1 up-regulates heparanase expression, which remodels the ECM and releases angiogenic factors (Carpenter et al. 2015). The inhibition of hedgehog pathway with cyclopamine and RNA interference techniques inhibited glioma cell migration and tumor invasion (K. Wang et al. 2010; Uchida et al. 2011).

Nuclear factor-κB. NF-κB, a family of highly regulated dimer transcription factors, is usually overexpressed in glioblastoma and contributes to the survival of migratory tumor cells (Smith et al. 2008). Signalling pathways triggered by growth factor receptors, including

EGFR and PDGFR, contribute to tumor development in glioblastoma and NF-κB plays key roles in these pathways (Shih and Holland 2006; Bonavia et al. 2012). Among glioblastoma subtypes, the mesenchymal phenotype is the most aggressive because it is highly invasive and radio-resistant (Carro et al. 2010) and associates with poor patient outcome. A transition of glioblastoma cells from less aggressive phenotypes (i.e., proneural) to cells with mesenchymal features can be promoted by activation of NFkB signaling (Bhat et al. 2013). Moreover, NFkB activation in mesenchymal glioblastoma cells mediates cell migration and tumor invasion through up-regulation of NFkB target genes, including cell chemo-attractants (IL-8, MCP-1) and matrix metalloproteinases (MMP-9) (Tchoghandjian et al. 2013). This signalling pathway can be activated by a number of stimuli, including ECM components such as hyaluronic acid, through binding to TLR4, differentiation of glioblastoma stem-like cells (Ferrandez et al. 2018; Nogueira et al. 2011) and cytokines, that may be released by infiltrating monocytes/macrophages or surrounding parenchymal cells. To this end, when the receptor activator of NF-κB ligand (RANKL), a member of the TNF family, is upregulated in glioblastoma cells, it activates neighbouring astrocytes through NFkB signalling which leads to secretion of cytokines, such as TGFß, and promotes glioblastoma cell invasion (J.-K. Kim et al. 2014). Thus, NFkB-mediated invasiveness may occur when this signalling pathway is activated either in glioblastoma cells or cells in the tumor microenvironment.

1.1.4. Prognostic Markers

In gliomas in general, several molecular markers have been identified to have prognostic value. The most important ones are the IDH mutation status, the 1p/19q codeletion, the MGMT promoter methylation, the TERT promoter mutations and EGFR alterations, among others. Some of them, as IDH mutation, 1p/19q codeletion and MGMT promoter methylation are assessed routinely performed in the clinical setting (Aquilanti et al. 2018).

IDH mutation. IDH mutation status is the most important predictor factor in glioblastoma. IDH mutation entails a favorable prognosis when compared to IDHwt gliomas. Interestingly, IDH mutation is the strongest predictor of overall survival (OS) in gliomas, over other important factors as age and tumor grade (Hartmann et al. 2010). Immunohistochemistry for IDH-R132H is usually performed to assess IDH mutation

status. This is usually enough to identify IDH wild type from IDH mutant glioblastomas in patients older than 55 years of age (Chen et al., 2014). Nevertheless, in younger patients and/or patients suspected to have secondary glioblastomas, if IDH-R132H immunostaining is negative, IDH sequencing might be necessary to rule out false negatives (Masui, Mischel, and Reifenberger 2016).

MGMT promoter methylation. Methylated MGMT promoter glioblastomas show a favorable response to treatment with temozolomide when compared with the unmethylated ones, with a significant increase in OS. MGMT has a critical role in DNA repair by removing specific DNA alkyl groups, protecting then the tumor cell from the action of alkylating drugs (Hegi et al. 2005). Besides, it also has a prognostic value in regardless of the treatment received, as MGMT methylation is associated with a longer OS (Aquilanti et al. 2018). Therefore, although MGMT testing value in the clinical setting remains controversial, in clinical trials it is important to adequately stratify patients (Wick et al. 2014).

1.1.5. Current treatment protocols

The current standard of care for patients with glioblastoma is the protocol described by Stupp et al. in 2005. It consists in surgical resection and adjuvant chemo-radiation with temozolomide, an oral alkylating drug, followed by six cycles of adjuvant temozolomide. It has been proven that adding temozolomide to a total dose of 60Gy of radiation results in a significant increase in OS when compared to radiotherapy alone (Stupp et al. 2005). Since the introduction of the Stupp protocol, there hasn´t been another significative therapeutic progress for glioblastoma (Kawano et al. 2015). In fact, promising agents as Bevacizumab, an anti-VEGF-A molecule, hasn´t proven to improve OS in glioblastoma patients (Chinot et al. 2014).

Nevertheless, the Stupp protocol was based in a trial that included a cohort of patients of 70-year-old or younger. Therefore, the question of whether this protocol was beneficial for elderly patients had remained unanswered. More recently, Perry et al. analyzed the addition of temozolomide to a shorter course of radiation, proving that this combination was associated with a longer OS than radiotherapy alone (Perry et al. 2017).

On the other hand, it´s widely accepted that the infiltrated parenchyma is associated with recurrence and resistance to treatment, thereby playing a central role in each step of the treatment (Lefranc et al. 2018).

1.1.5.1. Surgical resection of the infiltrative tumor

In glioblastoma, tumor cell invasiveness can lead to the infiltration or destruction of surrounding parenchyma resulting in neurological deficits (Mandonnet, Capelle, and Duffau 2006; Cuddapah et al. 2014). It has been proven that gross total resection of the contrast-enhanced tumor improves overall outcome (Chaichana et al. 2014; D. S. Xu et al. 2018). However, this approach might disregard the tumor burden invading the surrounding parenchyma, which could be potentially resected if eloquent areas are not compromised(Y. M. Li et al. 2016).

Thus far, several studies have shown that resection of the infiltrative portion of the tumor, based on DTI, ADC or T2/FLAIR abnormalities, is associated with longer progression-free survival (PFS) and OS (Yan et al. 2017; Y. M. Li et al. 2016; Pessina et al. 2017; Elson et al. 2015; Grossman et al. 2017). However, a recent analysis of 245 primary glioblastomas did not find a significant difference in recurrence and survival associated with the postoperative FLAIR volume(Mampre et al. 2018).

Although there is evidence supporting that resection of the infiltrative tumor can result in better outcomes, opposite results highlight the need for further research, as it remains unclear the more appropriate method to identify the areas of the surrounding parenchyma with greater tumor cell density and to distinguish them from the oedematous brain(P. D. Chang et al. 2017).

1.1.5.2. Radiation therapy targeting glioblastoma invasiveness

Typically, since the Stupp et al protocol, a 60Gy total dose is given to patients with glioblastoma (Stupp et al. 2005). A more recent approach for elderly patients consists in a total dose of 40Gy with concomitant temozolamide (Perry et al. 2017).

Accurate tumor volume definition is critical in conformal or intensity-modulated radiotherapy (IMRT) planning. Analogously to surgical approaches, a sub-therapeutic radiation dose within the tumor may result in treatment failure and recurrence, whereas whole-brain dose increments may lead to radiation-induced toxicity (Harat et al. 2017). Moreover, a sub-lethal irradiation dose may enhance invasion in glioblastoma (Pei et al. 2015; Zaboronok et al. 2014). Another suggested mechanism of tumor recurrence is the pro-invasive ECM remodelling in the tumor microenvironment in response to ionizing radiation (Yoo et al. 2018).

Despite the infiltrative nature of glioblastoma, radiation planning protocols have evolved from whole brain radiotherapy towards more tailored tumor volume targets, partially based on that the great majority of recurrences arise within 2 cm from the primary site(Minniti et al. 2010; E. L. Chang et al. 2007). In this context, it remains unclear if targeting the MRI-defined infiltrative tumor results in better PFS and OS. Moreover, in clinical practice there is a considerable variation in target volume definition without significant differences in outcome, from using a 2-3 cm margin on the T1 contrast-enhanced tumor to a 2 cm margin on the T2/FLAIR hyper-intensity, as recommended by the European Organization for Research and Treatment of Cancer or the Radiation Therapy Oncology Group, respectively(Cabrera et al. 2016; Wernicke et al. 2016). In fact, by targeting the tumor area with a margin of 2 cm and without using the peri-tumoral oedema as tumor volume, Chang et al. achieved similar recurrence pattern results(E. L. Chang et al. 2007). Further research is needed to assess whether this is a result of the overall lack of benefit from radiation therapy or if targeting the infiltrative tumor burden with radiation does not significantly impact the outcome(Wernicke et al. 2016).

On the other hand, the use of DTI-based clinical target volumes (CTV) has been proposed, as they are smaller than the ones based on the T2-hyperintensity, sparing the peri-tumoral oedema. Besides, this reduction in the CTVs could allow dose escalation (Berberat et al. 2014; Jena et al. 2005). Furthermore, approaches taking into account tumor growth dynamics have been developed, by defining the CTVs based on DTI-derived mathematical growth models. Although this approach could be more effective at targeting cancer cells and preserving healthy tissue, further research is warranted to assess its outcome and tumor recurrence (Jensen et al. 2017; Konukoglu et al. 2010)

Other approaches for CTV definition are based on PET findings. For instance, a higher dose coverage of 18F-FET-PET tumor regions is positively correlated with time to progression and PET-based CTVs better-predicted failure sites when compared to MRI-based CTVs (Harat, Małkowski, and Makarewicz 2016; Harat et al. 2017). Although current ongoing protocols are trying to better define the impact of PET-based tumor delineation in outcome (Oehlke et al. 2016).

1.1.5.3. Therapeutic targets in glioblastoma invasion

Overall, current commonly used therapies for glioblastoma, including alkylating agents as Temozolomide (TMZ) and the anti-VEGF compound Bevacizumab, failed in targeting glioma cell invasion. Despite that TMZ can potentially inhibit invasion in vitro(Wick et al. 2002), this effect is not significant in the clinical practice and several resistance mechanisms to alkylating agents have been proposed (Hombach-Klonisch et al. 2018). Among them, the lack of blood-brain permeability in T2/FLAIR hyperintensity areas(Sarkaria et al. 2018; Goldwirt et al. 2015) and the resistance mechanisms intrinsic to GSC in the infiltrative tumor are intimately associated with the glioblastoma invasive capacity (Safari and Khoshnevisan 2015; Ruiz-Ontañon et al. 2013). On the other hand, Bevacizumab could lead to a hypoxic environment resulting in enhanced glioma cell invasion of the normal parenchyma(Keunen et al. 2011; Rahman et al. 2013).

Considering the lack of an effective therapeutic approach against glioblastoma invasiveness, further research is warranted to better understand the invasion pathways contributing to glioma cell infiltration and consequently, to develop new therapeutic agents. An effective therapeutic strategy should target both infiltrative glioblastoma cells and the tumor cell-stroma interaction (Xie, Mittal, and Berens 2014).

Up to now, no clinically transferable results have been achieved after trying to target some of the mechanisms involved in glioblastoma invasion, including cytoskeleton reorganization and cell motility, cell adhesion, and degradation of ECM (Drappatz, Norden, and Wen 2009; Lefranc et al. 2018)

Current areas of research include several potential targets in glioma cell invasion pathways. Glutamate-mediated infiltration inhibition has been assessed in several Phase I-II trials

with promising results. Besides, the role of different tumor cell ion channels and transporters, microtubule-based tumor cell network, microRNA-related invasion and the mechanisms involved in the interaction between the tumor and host open potential opportunities for targeted therapy approaches (Vehlow and Cordes 2013; Xie, Mittal, and Berens 2014; Lefranc et al. 2018)

1.1.5.4. Immunotherapy

Current efforts for analyzing the effect of immunotherapy in glioblastoma include oncolytic viral therapy as herpes simplex virus or poliovirus, vaccination therapy, immune check-point blockade as the anti-PD-1 nivolumab, and CAR-T cell therapy against EGFRvIII. Nevertheless, some disappointing results suggest that glioblastoma might be considered as a "cold tumor", probably in relation with a strong immunosuppression in the tumor microenvironment (Lim et al. 2018).

1.1.5.5. Tumor treating fields (TTFields)

TTFields are low intensity alternating electric fields apply the whole brain, that interfere and prolongs cell division, making the tumor cells, with a high mitotic activity, the most susceptible ones. It has proven efficacy in increasing OS and PFS in an interim analysis of a prospective randomized phase-3 clinical trial (Stupp et al. 2015; Burri et al. 2018).

1.1.6. Prognosis: Overall and Progression Free Survival

Despite the different therapeutic approaches that have been described for glioblastoma, the prognosis in glioblastoma remains dismal. The median OS is 15 months despite surgical resection plus chemoradiation and the 5-year survival rate is only 5.6%. Moreover, no advances have been made over the past 15 years to have a significant impact on OS (Fine 2015; Kawano et al. 2015; Ostrom et al. 2018). Nevertheless, some retrospective studies, have described an increase in OS in a 4-year timeframe (2006-2008 to 2010-2012) by using population-based databases. Interestingly, this increased in OS coincided with the introduction of Bevacizumab for the treatment of patients with glioblastoma (2009) (Johnson et al. 2018). However, later randomized trials failed to prove a benefit in OS

from Bevacizumab (Chinot et al. 2014; Kaka et al. 2019) and, consequently, other factors might be having an impact on OS.

In the Stupp et al. trial the median PFS was 6.9 months with the adjuvant chemoradiation (Stupp et al. 2005). Since then, despite several different combination of chemotherapy protocols, no significant improvements in PFS have been achieved (Weller et al. 2013). However, more recently bevacizumab and TTFields have proven to have an impact in delaying recurrence (Chinot et al. 2014; Stupp et al. 2015).

After surgical resection and adjuvant chemoradiation, tumor recurrence occurs virtually in all cases. The RANO (MacDonald) criteria are recommended to assess tumor recurrence or progression in high-grade gliomas (Chukwueke and Wen 2019). The tumor recurrence may have different patterns according to the distance to the primary surgical site and morphology. Arbitrarily, a local recurrence has been define as the one located within 20 mm from the resection cavity margin, whereas a distance recurrence is located more than 20 mm away from the margin (Konishi et al. 2012). Besides, some cases might present an increase in the contrast-enhancement in the post-treatment MRI without a true progression. This phenomenon is called pseudoprogression and improves over time, contrary to true progression (Chukwueke and Wen 2019).

1.1.7. Mathematical Models in Glioblastoma

Mathematical models have been applied to oncology with different purposes, including providing quantitative predictions of the natural history of the disease and the tumor biological processes, allowing further clinical and experimental validation (Altrock, Liu, and Michor 2015).

In glioma, Swanson et al. have described a mathematical model based on biological parameters intrinsic to the tumor, such as invasion, proliferation and heterogeneity (Swanson, Alvord, and Murray 2000; L. S. Hu et al. 2017; Molina, Pérez-Beteta, Luque, et al. 2016)

From the clinical perspective, these models have been then applied in low grade gliomas to predict the speed of growth (Mandonnet et al. 2003) and prognostic value (Pallud et al. 2006). Some of these models have been successful in predicting survival and response to treatment. (Swanson, Rostomily, and Alvord 2008; Rockne et al. 2010; Molina, Pérez-Beteta, Luque, et al. 2016; Pérez-Beteta et al. 2018, 2019)

1.2. Teneurins

1.2.1. Definition and structure

Teneurins constitute a family of transmembrane glycoproteins which play an important role in nervous system morphogenesis, where they are related to cell and axonal migration (Young and Leamey 2009). More recently, their role in cancer has been increasingly recognize (Rebolledo-Jaramillo and Ziegler 2018). For the sake of simplicity we refer to Teneurin1 as ODZ1 throughout the text.

Teneurins were first described in *Drosophila melanogaster* in two independent studies by Baumgartner et al. and Levine et al. in 1993 and 1994, respectively (Levine et al. 1994; S Baumgartner et al. 1994; Stefan Baumgartner and Chiquet-Ehrismann 1993). Initially, teneurins were discover while looking for the tenascin-C homologues in *Drosophila*, based on their epidermal growth factor (EGF)-like repeats, and consequently they were called Ten-a, from "tenascin accessory" and Ten-m, from "tenascin major", according to the protein size. They were thought to be secreted proteins and part of the ECM. Independently and by using a different approach, Ten-m was discovered and described as a pair-rule gen by Levine et al. At that moment, it was considered a Type I transmembrane protein and received the name ODZ, from odd Oz, reflecting the "oddless" of its pair-rule nature and its presumed role in the heart and in the brain, in reference to the Wizard of Oz. Later on, it was recognized that ODZ was in fact a type II transmembrane protein with no association with the pair-rule phenotype described in the first place. Henceforth, four teneurins paralogs (Tenm1-Tenm4) have been described and validated in several vertebrate species including human, mouse, chicken and zebrafish (Stefan Baumgartner and Wides 2019) and its current name was introduced in 1999, reflecting its invertebrates homologues and its most important site of expression (Minet et al. 1999)

Several key structural features of the teneurins family of proteins are phylogenetically maintained. Interestingly, teunerins were found in the choanoflagellate *Monosiga brevicollis*, which has been considered the closest living relatives of animals. It has been proposed that teneurin genes evolved in choanoflagellates after a horizontal gene transfer process from diatoms and bacteria (Richard P. Tucker et al. 2012; Richard P. Tucker 2018). Then, in arthropods, the teneurin gene experimented a duplication leading to the two paralogs in Drosophila. Similarly, the four paralogs in vertebrates arose from a two-step quadruplication (Figure 4) (Wides 2019).

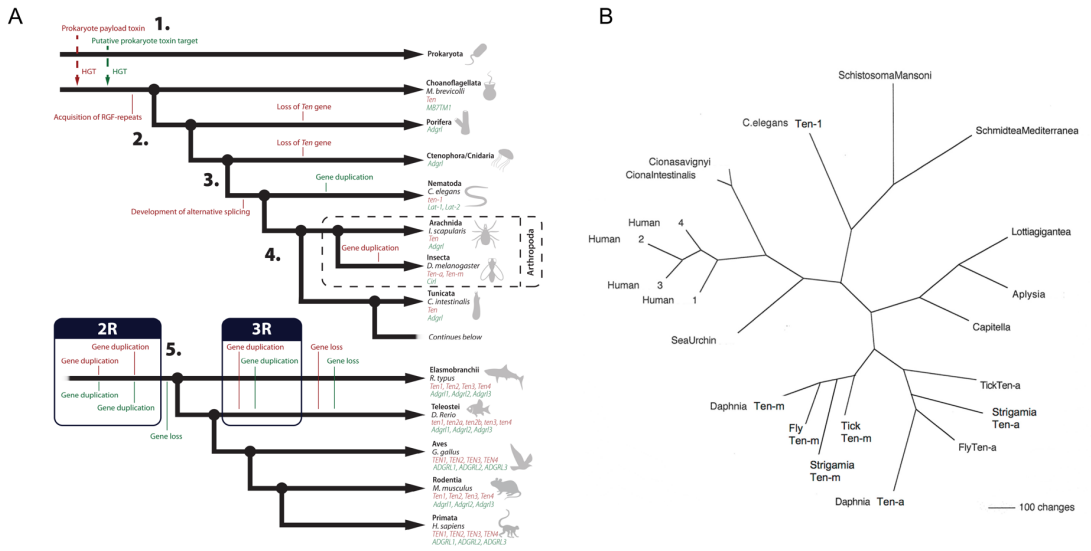

Figure 4 *– Teneurins phylogenetic evoluction. (A) Phylogenetic history of teneurins and (B) teneurins from selected representatives of metazoans in an unrooted phylogram of Teneurin protein sequences showing the relatedness of examples from several phyla. Reproduced from (Sita et al. 2019) and (Wides 2019) respectively.*

Teneurins are transmembrane type II proteins with an approximate molecular weight of 300kDa and approximately 2800 aa. Their basic structure is characterized by a large extracellular C-terminal domain (2400 aa) and a smaller intracellular N-terminal (400 aa) (Figure 5) (Richard P. Tucker et al. 2007).

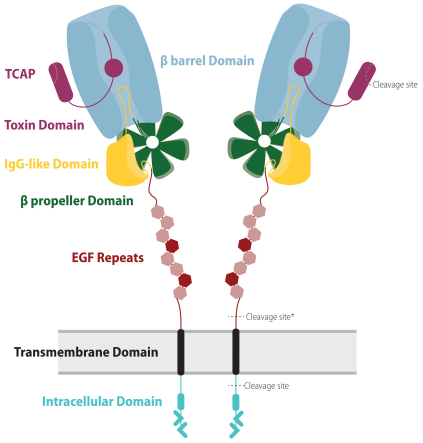

Figure 5 *– Schematic diagram of the overall molecular structure of a teneurin Reproduced from (Sita et al. 2019).*

Most of teneurins detailed structure has been described after recent electron-microscopic analysis of Ten2 and Ten3 (V. A. Jackson et al. 2018; J. Li et al. 2018). Their structure can be further divided into seven well identified domains, one intracellular, one transmembrane and five extracellular domains. The extracellular domains, from the membrane to the C-terminal, are: 1) EGF-like repeats, 2) Ig-like domain, 3) B-propeller domain, 4) B-Barrel domain and 5) Toxic domain (Figure 5) (Sita et al. 2019; Araç and Li 2019).

Overall, the extracellular domains are disposed in a way that the cylindrical β-barrel, sealed at the bottom by the Ig-like and the β-propeller domains, has the toxic domain partially protruding at the top. Furthermore, this teneurin B-barrel configuration is remarkably similar to bacterial secreted Tc-toxins (Araç and Li 2019; Richard P. Tucker 2018).

The transmembrane region is composed of approximately 30 hydrophobic residues (Young and Leamey 2009). A linker region of 200 aa precedes eight tenascin-type EGF-like repeats, each one of them formed by six cysteines. The EFG-like repeats allow dimerization of the protein through the junction of the second and fifth, which are modified and formed by only five cysteine residues, allowing them to form disulfide covalent bonds with the adjacent teneurin, resulting in teneurin cis-dimers (Richard P. Tucker 2018). The EGF-like repeats are followed by an Ig-like and a 6-bladed B-propeller, which is formed by a series of NCL-1/HT2A/Lin-41 (NHL) repeats. Besides, it has been recently proposed that alternative splicing of a specific region within the B-propeller plays

a role in the regulation of teneurin adhesion to other proteins as latrophilins. The B-barrel domain is formed by several YD repeats, which conform a hollow shell, comprising a rearrangement hot spot (Rhs)-associated core domain (V. A. Jackson et al. 2018; J. Li et al. 2018).

The toxin-like domain, located C-terminal to the Rhs-associated core domain, has an amino acid sequence that greatly resembles to bacterial GHH toxins ones. In teneurins, the C-terminal end of the GHH toxin-like domain exits the B-barrel and is tethered to the outer surface. Finally, in teneurins, the 40-41 C-terminal residues, overlapping with the toxin-like domain, has been called the teneurin C-terminal associated peptide (TCAP). This peptide, depicting a great similarity to corticotrophin, might represent de modern homolog of the original bacterial toxin and it may be synthetized independent of the teneurin (Sita et al. 2019).

The intracellular domain the most variable part of the protein and it can be cleaved and translocated to the nucleus. It features proline-rich sites or SH3-binding domains and EF-hand-like Ca2+ bindings motifs (Richard P. Tucker et al. 2007). The former ones are able to bind CAP/Ponsin, also known as sorbin, an adapter protein involved in cytoskeletal remmodelation and intracellular signaling. This binding results in nuclear translocation of both, the teneurin intracellular domain and CAP/ponsin. Besides, it has been described that the intracellular domain is also capable of binding other proteins as the methyl-CpG binding protein 1 (MBD1) a transcriptional repressor (Nunes et al. 2005). Furthermore, it has also been described that the intracellular domain of teneurin-2 has a repressive activity on zic-1-mediated transcription (Bagutti et al. 2003).

1.2.2. Expression and function

Overall, teneurins are usually expressed in the nervous system, especially during development as they have a significant role in neural network formation. Alterations leading to teneurin aberrant or impaired expression result in abnormal neural connections and abnormal nervous system development. Nevertheless, there are also several examples of teneurin expression in non-neural tissue during development (Richard P. Tucker 2018; Sita et al. 2019).

In invertebrates, teneurin expression has been widely described in Drosophila melanogaster and Caenorhabditis elegans. In the first, two paralogs have been reported, Ten-a and Ten-m, whereas in the latter, one single gene with two different transcripts have been found, Ten-1L and Ten-1S. All of them are expressed predominantly in the nervous system in different stages of development. In vertebrates, four paralogs have been identified (Ten1-Ten4) in during neural development with different but overlapping expression patterns. In the chicken and mouse embryos, Teneurins in general, and Ten1 among them, have been found in the diencephalon, in the optic tectum and in specific thalamic nuclei. This is more relevant in the optic pathway, where may have an important role in the correct development of the optic decussation circuits and teneurin knockout mice result in abnormal ipsilateral projections to the geniculate ganglion and from there to the visual cortex. Interestingly, Teneurins are expresses in the mouse hippocampus. Each one of them is expressed predominately in one or two hippocampal regions. Specifically, Ten1 is found in the CA3 region, Ten2 in the CA1 and CA3 regions Ten3 in the stratum lacunosum moleculare and Ten4 in the molecular layer of the dentate gyrus, in the stratum lacunosum moleculare and in the CA3 region (Richard P. Tucker 2018; Leamey and Sawatari 2014).

Moreover, it has been recently proposed that ten3 is expressed in several hippocampal regions and that plays a central role in the proper targeting of the axons of the CA3 and subicular neurons (Berns et al. 2018). On the other hand, the ten-1 B-propeller domain seems to be critical for the development of the olfactory circuit, as its mutation results in congenital anosmia in humans (Alkelai et al. 2016).

Furthermore, it is widely accepted that teneurins have a central role in guiding the neural projection and help to establish the neural network connectivity within the nervous system (Young and Leamey 2009; Leamey and Sawatari 2014). Nevertheless, the molecular mechanisms underneath remains elusive. In relation to this, it has been shown that the intracellular domain has a nuclear localization signal that is necessary for translocation to the nucleus (Kenzelmann et al. 2008). In fact, there´s evidence of the nuclear translocation of the intracellular domain in chicken embryo fibroblasts (Nunes et al. 2005). Besides, it has been suggested that the mechanism of the intracellular domain release is similar to other transmembrane proteins such as Notch1 and APP, in a process known as Regulated Intramembrane Proteolysis (RIP) (Young and Leamey 2009).

In the nucleus, the cleaved intracellular domain can interact with several proteins, including MBD1, the transcription factor zic and, more recently described, the transcription factor Myc. Similarly, the extracellular domain can be also cleaved and released, as TCAP, which has been proposed to have signaling properties (R.P. Tucker and Chiquet-Ehrismann 2006; Talamillo et al. 2017; Sita et al. 2019; Young and Leamey 2009).

1.2.3. Transcriptional and epigenetic regulation

The human teneurin-1 gene is located on the X chromosome at position Xq25 and despite significant advances on teneurins over the last 25 years, the mechanisms that regulates its expression remain elusive and poorly understood. In an interesting report, Beckmann et al. identified three additional exons located 80kb to 200kb upstream the first coding exon. The first of these additional exons, a highly phylogenetically conserved region with surrounding CpG islands, was identified as a second alternate promoter. Furthermore, this alternate promoter, but not the first one, has a conserved site for Empty Spiracles Homeobox 2 (EMX2), a homeobox transcription factor involve in brain development and commonly co-express with Teneurin-1 in similar brain regions. In fact, EMX2 specifically induces the transcription of the alternate promoter by binding a homeobox binding motif within the promoter sequence (Beckmann et al. 2011). Besides, other studies have proposed that EMX2 is involved in the regulation of the remaining teneurins as well (H. Li, Bishop, and O'Leary 2006).

Similarly, Tucker et al. reported that Teneurin-2 is induced by exogenous Fibroblast growth factor 8 (FGF8) in vertebrates' limbs. Besides, they proved that Teneurin-2 and FGF8 coexpressed in similar regions during limb development. Notwithstanding, the precise mechanism by which FGF8 regulates Teneurin-2 remains unknown (Richard P. Tucker et al. 2001).

In addition to transcriptional factors, epigenetic mechanisms might have a significant role in regulating teneurins expression, although up to know these mechanisms have been described only in tumor cells and not during developmental processes. The CpG islands found in the teneurins genes support this mechanism. However, epigenetic regulation has

been demonstrated only for ODZ1 and not for other teneurins. This might be explained by the singular epigenetic control of X-chromosome. (Rebolledo-Jaramillo and Ziegler 2018; Ziegler et al. 2012; Talamillo et al. 2016; Beckmann et al. 2011)

1.2.4. Teneurins and cancer

The evidence supporting teneurins role in tumorigenesis has significantly growth in the last five years. As occurs with oncogenes and tumor suppressor genes in general, teneurins might be affected by widely known carcinogenic mechanisms, including genomic alterations and abnormal expression, leading to pathological teneurin up- or downregulation within the tumor, and resulting in carcinogenesis and tumor progression (Rebolledo-Jaramillo and Ziegler 2018).

Teneurins abnormal expression has been described in several human cancers. For instance, Teneurin-2 expression is decreased in breast hyperplasia, hepatocellular carcinoma and esophageal squamous cell carcinoma whereas it is increased in lymphomas, cervical cancer, malignant pleural mesothelioma and ovarian carcinoma. Similarly, Teneurin-4 expression is decreased in renal carcinoma and augmented in breast carcinoma and brain tumors (Ziegler et al. 2012).

Regarding ODZ1, altered expression in cancer has only been described in prolactinoma metastases (Zhang et al. 2014), in papillary thyroid carcinoma (Cheng et al. 2017) and glioblastoma (Talamillo et al. 2016). Nonetheless, in a recent analysis of public cancer databases, a better survival outcome was associated to ODZ1 high expression in different cancer types as breast, head and neck, lung, pancreatic and thyroid cancer, and to a low ODZ1 expression in endometrial, renal, gastric cancer and in gliomas (Rebolledo-Jaramillo and Ziegler 2018)

Teneurin genomic alterations include chromosome rearrangements. After an elaborate analysis of Teneurin´s chromosome rearrangements, Rebolledo-Jaramillo et al. have proposed that teneurins should be considered as transcribed units prone to genetic instability. Teneurin chromosome rearrangements include translocations, CNVs, chromothripsis, and viral genome integration and they might result in either oncogenes or

tumor suppressor genes. Moreover, Ten4 chromosomal rearrangements have been the more frequently described, especially in breast adenocarcinoma, microcytic and non-microcytic lung carcinoma. Nonetheless, chromosomal alteration affecting teneurins Ten1 and Ten2 have also been identified in several tumors. Ten1 rearrangements have been described in prostate cancer, kidney adenocarcinoma and acute myeloid and myeloblastic leukemia but not in glioma. Up to now, there are no reported translocations for Ten-3. (Rebolledo-Jaramillo and Ziegler 2018)

On the other hand, mis-regulation of teneurin expression might have also a role in tumorigenesis and tumor progression. Interestingly, EMX2, one of the transcription factors regulating teneurins, might have a role in cancer. (Ziegler et al. 2012). Besides, Teneurin pathways have been related with WNT signaling and they might have a role as WNT regulators. Similarly, teneurin-4 and teneurin 1, have been linked to widely known cancer signaling pathways as PI3K/AKT and MEK/ERK, through Neuroregulin-1 and MITF, respectively (Ziegler et al. 2012; Rebolledo-Jaramillo and Ziegler 2018).

Finally, teneurin epigenetic regulation in cancer has been considered as a probable mechanism contributing to carcinogenesis. Talamillo et al. and Rebolledo-Jaramillo et al. have proposed that Ten1 might subjected to epigenetic regulation, as demonstrated by increase Ten expression after treating glioblastoma cells with the demethylating agent 5-aza (Rebolledo-Jaramillo and Ziegler 2018; Talamillo et al. 2016). Interestingly, Ten1 promoter is hypomethylated in brain metastases when compared to their primary tumor, suggesting that hypomethylation may have a role in the mestastatic process (Vizoso et al. 2015). Nonetheless, Ten-2 and -4 DNA demethylation with 5-aza didn´t result in an increase expression in tumor cells in ovarian cancer (Graumann et al. 2017).

1.2.5. Teneurins and glioblastoma

Teneurin-1 expression in glioma cells has been described by several authors. Moreover, results obtained from population databases of clinical and genetic variables of patients with glioblastoma show a decrease in survival in cases with a greater expression of Teneurin-1 and that the expression of Teneurin-1 increases in high-grade gliomas (Rebolledo-Jaramillo and Ziegler 2018; Talamillo et al. 2016; Schöler et al. 2015).

Furthermore, transcriptional regulation by teneurin-1 has been previously demonstrated in glioblastoma. Schöler et al. recently reported that Teneurin-1 binds the histidine triad nucleotide binding protein 1 (HINT1), a known transcription repressor, resulting in activation of the microphthalmia-associated transcription factor (MITF) and subsequent upregulation of its target gene the glycoprotein non-metastatic b (GPNMB). The precise mechanism by which Teneurin-1 activates MIFT-dependent transcription remains unknown, whether it releases MIFT by binding HINT or it competes for binding to MITF. In any manner, the resulting upregulation in of GPNMB glioblastoma leads to an increase invasiveness of tumor cells (Schöler et al. 2015).

Similarly, Talamillo et al. have recently shown that teneurin-1 is activated during the GSC differentiation process, promoting morphological changes of the tumor cell phenotype resulting in cytoskeletal remodeling, invadopodia and, subsequently, an enhanced invasive capacity of tumor cells. During the differentiation process, ODZ1 intracellular domain (icODZ1) is cleaved by SPPL2a, a membrane protease, and released to the cytoplasm. Then, the icODZ1 is translocated into the nucleus where, by binding the transcriptional factor Myc, it acts as a transcription factor in the E-box E6 of the RhoA promoter. Therefore, icODZ1-dependent transcription promotes the invasion of the extra-cellular matrix through a Myc-dependent post-transcriptional cascade that promotes RhoA expression through activation the RhoA-ROCK1 pathway, involved in the remodeling of the cytoskeleton. (Talamillo et al. 2016).

Interestingly, ODZ1 expression in tumor cells is higher within the tumor boundaries, along the brain-tumor interface. Besides, the percentage Ten1-positive tumor cells correlated with poor outcome and a decrease in overall survival and disease-free survival in a group of 152 patients with glioblastoma (Talamillo et al. 2016).

Despite the advances on the mechanistics of the ODZ1 transcriptional regulation, the extracellular trigger stimuli capable to upregulate Ten1 and activate its pathway have not yet been described. Therefore, Ten1 is a potential prognostic marker and a possible therapeutic target in glioblastoma. However, studies focusing on their prognostic value are still needed using homogeneous groups of patients and trials that allow determining the triggers of the activation of the ODZ1 pathway.

1.3. Problem statement and objectives

1.3.1. Problem statement

At present, glioblastoma remains to be one of the most lethal types of cancer. The recent discovery of ODZ1 as a factor involved in the invasive capacity of tumor cells raises new insights on the invasion pathogenesis and it could be potentially used as a prognostic marker and therapeutic target.

In this sense, it is essential to determine its prognostic value in a clinical context and its association with non-invasive tumor geometrical features. Furthermore, the regulation mechanisms controlling ODZ1 signaling and ODZ1-dependent tumor cell invasion are still unknown. The description of such mechanisms could improve our understanding on ODZ1 pathogenesis in glioblastoma and, consecuently, lead to the identification of potential therapeutic targets within the pathway.

1.3.2. Aim and Objectives

1.3.2.1. Aims

To describe the association of tumor ODZ1 expression and putative radiological markers of invasion and hypoxia in glioblastoma and to analyse the role of the tumor hypoxia in the activation and regulation of ODZ1-dependent cell invasion in Glioblastoma.

1.3.2.2. Specific objectives

- To determine the expression of ODZ1 in the tumor specimens of patients with glioblastoma and its association with the tumor geometrical features and the radiological pattern of tumor recurrence.

- To determine the ODZ1 expression in the tumor hypoxic microenvironment in surgical tumor specimens from patients with glioblastoma.

- To describe the mechanism of the hypoxia-dependent regulation on ODZ1 transcription.

- To assess the role of the tumor hypoxic microenvironment on the methylation status of the ODZ1 promoter and ODZ1-mediated invasion.

2. METHODS

2.1. Patients

Three different and overlapping cohorts of patients have been included for the different analysis from two different institutions: Hospital Universitario Marqués de Valdecilla (HUMV; Santander, Spain) and Toronto Western Hospital (TWH; Toronto, Canada). All the patients included in the three different cohorts met the following inclusion criteria: 1) age>18 years and 2) Histological confirmation of IDH wild-type glioblastoma, and 3) Primary tumor with no prior treatment. Besides, retrospective clinical and demographic variables were collected from all patients.

The first cohort of patients was used to analyse the radiological pattern of tumor recurrence and a total of 22 consecutive patients with glioblastoma where included, all of them were treated at the HUMV in the period from 2007 to 2016. In order to reduce the possible cofounding factors, the aim was to gather a homogenous group of patients regarding the treatment received. Therefore, all of the patients included in this cohort met the following additional inclusion criteria: 1) Surgically treated with gross total resection (GTR), 2) Adjuvant chemoradiation according to Stupp protocol (Stupp et al. 2005), 3) No treatment with carmustine, bevacizumab or enrolled in clinical trials prior the first radiological recurrence, and 4) Clinical follow-up until the first radiological evidence of tumor recurrence.

In addition, a second group including 54 patients treated at HUMV, was used to determine the ODZ1 tumor expression and its relationship with the pretreatment tumor radiological geometrical features from the preoperative MRI.

Finally, a third cohort of patients was used to determine the relation between the tumor hypoxic microenvironment and the regulation and activation of ODZ1. This group included 17 patients treated at the TWH. Besides, a subset of these patients was used to assess ODZ1 gene methylation statuts. The patients in this cohort received preoperatively

Pimonidazole hydrochloride (PIMO) (Hpoxyprobe-1; Natural Pharmacia International Inc, Burlington, MA), an exogenous hypoxia marker with an IND (Investigational New Drug) status for use in the clinical evaluation of hypoxia. PIMO was administered to all patients 16-20h prior surgical resection. Tumor specimens were obtained at the time of surgery and processed for further analysis (Figure 6).

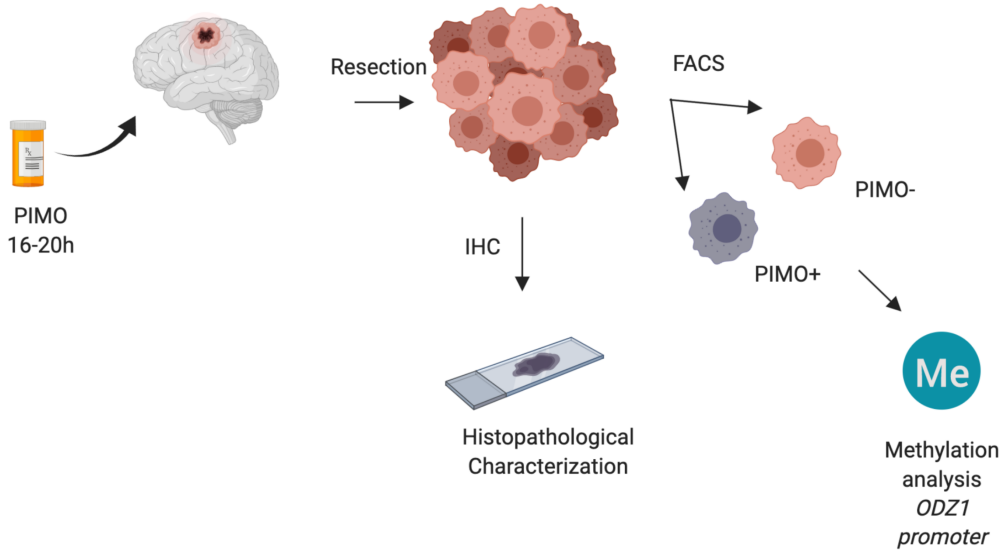

Figure 6 *- Study pipeline. Diagram illustrating the collection and processing of the glioblastoma specimens.*

Approval of Research Ethics Board from University Health Network (Toronto, Canada) or Hospital Universitario Marques de Valdecilla (Santander, Spain) was obtained for each patient included in the study in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.2. Radiological imaging analysis

2.2.1. Radiological recurrence pattern

Complete tumor resection was defined as the absence of contrast enhancing lesions in the immediate postoperative cranial CT scan. In addition, tumor recurrence was defined as the appearance of new contrast enhancing lesions in serial follow-up neuroimaging studies.

The pattern of recurrence was classified, according to previous reports, as: 1) Focal: in lesions located within 2cm from the edge of the tumor resection cavity and, 2) Distance: lesions located more than 2cm from the edge of the tumor resection cavity.

2.2.2. Tumor geometrical features analysis

All the analysis of the tumor geometrical features was carried out at the Mathematical Oncology Laboratory at the Universidad de Castilla – La Mancha (Ciudad Real, Spain).

2.2.2.1. Acquisition of images

The tumor geometrical features were determined in the preoperative gadolinium-enhanced T1-weighted and FLAIR MRI sequences following the a processing pipeline widely described previously (Pérez-Beteta et al. 2019; Molina, Pérez-Beteta, Martínez-González, et al. 2016; Pérez-Beteta et al. 2018). Briefly, high spatial resolution MRIs were obtained in 1.5 or 3 Tesla strength fields. The MRI Digital Imaging and Communication in Medicine (DICOM) files were imported into the scientific software package Matlab (R2015b, The MathWorks, Inc., Natick, MA, USA). Then, the images were semi-automatically segmented considering two possible regions: 1) the contrast enhancing tumor and 2) the hypointense necrotic tumor core. Segmentation accuracy was checked and manually corrected (Figure 7).

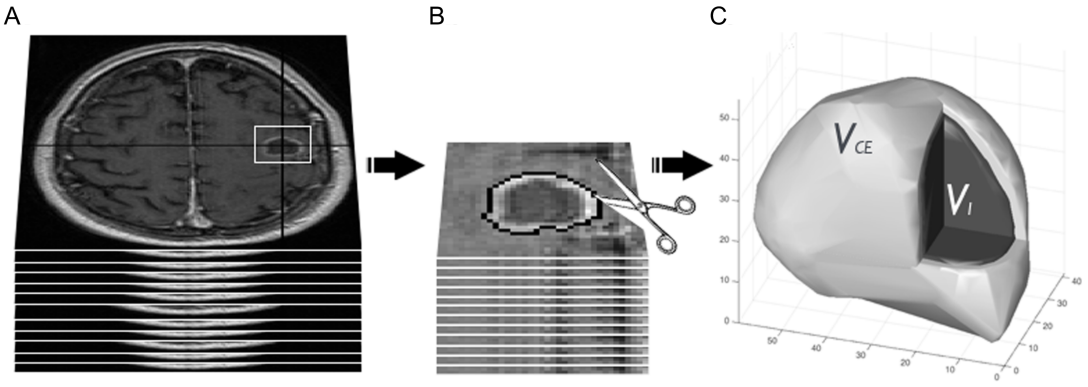

Figure 7 *– Diagram summarizing the MRI segmentation process. (A) Semi-automatic tumor segmentation and manual correction by an image expert, (B) Resulting segmented slices and (C) 3D reconstruction of the tumor. Reproduced from* (Pérez-Beteta et al. 2017)

2.2.2.2. Tumor geometric variables

Once the tumor was segmented, the geometrical 3D measures were automatically computed. They included the following volumetric and tumor invasion geometrical measures:

- Contrast enhancing T1 volume (CET1V): contrast enhanced tumor volume, excluding the hypointense necrotic tumor core.
- Infiltration volume (IV): FLAIR hyperintense signal volume.
- Total tumor volume (TTV): the T1 tumor volume plus the Infiltration volume.
- Volume infiltration ratio (VIR): total tumor volume and T1 tumor volume ratio (TTV/T1TV).
- Maximun tumor diameter (TDmax): the maximum tridimensional tumor diameter considering the contrast enhancing T1 volume and the infiltration volume.
- Infiltration diameter (ID): FLAIR/T2-hyperintense signal average width.
- Tumor surface (TS): tumor surface area.
- Tumor surface regularity (TSR): it describes the regularity of the tumor surface. It would be worth 1 when the tumor was a perfect sphere and the value goes down as the tumor becomes irregular and infiltrative resembling a fractal shape.
- Diameter infiltration ratio (DIR): is the ratio of the maximum tumor diameter and the maximum diameter of the T1 contrast enhancing tumor.
- Contrast-enhancing rim width (CE rim width): average size for the CE rim obtained from a spherical approximation from the TTV and and the CET1V.

2.3. Immunostaining

2.3.1. Immunohistochemical staining and analysis

In all cases, the determination of ODZ1 expression in histological samples of tumor tissue was determined by immunohistochemistry. After reviewing the H&E slides and best blocks, with highest tumor cellularity, were selected aiming at 70% tumor cellularity.

Immunohistochemical staining was performed using an in-house immunoabsorbed anti-icODZ1 antibody against the N-terminal region of ODZ1 (Talamillo et al. 2017).

Briefly, after high pH deparaffinization, rehydration, epitope retrieval and peroxidase and protein blocking, slides where incubated overnight with the primary antibody (AntiODZ1, 1/100) at 4°C in a humid environment. Then, the slides were incubated with a rabbit secondary during 45´. Finally, DAB Chromogen and hematoxylin counterstain were used to analyze the FFPE sections. In all cases, ODZ1 positivity and pattern of expression were assessed. For the tumor recurrence and radiomics analysis, ODZ1 positivity was determined by a qualitative scale ranging from 0, no expression, to 4, intense expression. Nuclear staining and cytoplasmatic ODZ1 positivity were assessed separately following the same scale. Besides, the pattern of expression was also noted and classified in to three different types: nuclear, cytoplasmatic or mixed.

Similarly, Hypoxyprobe, a peroxidase-based immunostaining kit containing an anti-PIMO monoclonal antibody (NPI Inc, Burlington, MA). PIMO is a 2-nitroimidazole is selectively reduced and covalently bound to intracellular macromolecules within severe hypoxic environments (with specific tissue oxygen tension, p02, of < 10mm Hg) (Walsh et al. 2014). The staining process was similar to the one described for ODZ1 staining.

Finally, in order to assess ODZ1 expression in the severe hypoxic regions, consecutive FFPE tumor sections were stained and reviewed with each antibody. For this group pf patients, PIMO uptake and ODZ1 nuclear and cytoplasmic expression were quantified using a pixel-based image analysis software (Aperio ImageScope). PIMO negative and positive regions were delineated and annotated. Then, ODZ1 expression was determined in each annotated PIMO positive and PIMO negative region. PIMO and ODZ1 positivity cut-offs were defined as 15% and 5% in the tumoral areas, respectively.

2.3.2. ODZ1 and PIMO double immunofluorescence staining

Immunofluorescence (IF) staining and analysis was completelyperformed at the Macfeeters-Hamilton Neuro-oncology Centre at the Princess Margaret Cancer Centre (Toronto, Canada). Fragments from the tumor specimens collected from patients who had

received PIMO prior surgical resection, were embedded in OCT and frozen by immersion in liquid nitrogen, then stored at -20ºC until further processing. Then, 8µm frozen section were obtained from the OCT block in the cryostat.

Frozen sections were allowed to dry at RT for 5min and then fixed with pre-cooled acetone at -20ºC for 10 min. Then, non-specific binding sites were block by incubating the sections with 3% bovine serum albumin (BSA) and 0.1% Triton for cell permeabilization for 60 minutes. After a 5 minutes PBS wash, the sections were incubated with the first primary antibody, anti-PIMO monoclonal antibody (NPI Inc, Burlington, MA), for 4 hours at a 1/800 dilution. Afterwards, Sections were washed with PBS and incubated overnight with the second primary antibody, AntiODZ1, at a 1/200 dilution. Sections were PBS wash again and incubated with the secondary antibody for 460 minutes at a 1: 150 dilution in PBS, in a humid and dark chamber. The secondary antibodies used are conjugated with Fluorescein (FITC) for or TexasRed (TxR) (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) for anti-PIMO and anti-ODZ1, respectively. DAPI was used for counterstaining.

2.4. Primary cells cultures

2.4.1. Glioblastoma specimens processing

Primary glioblastoma cells used in this study were previously established and described by our group (Nogueira et al. 2011; Talamillo et al. 2017). Tumor specimens for primary cells cultures were obtained from patients with glioblastoma diagnosed according WHO criteria and undergoing surgical tumor removal as part of the standard treatment protocol of the Neurosurgery Department at HUMV. The fresh tumor specimens were obtained at the operating room during surgical removal and they were kept at 4°C in Dulbecco´s modified Eagle medium medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA) until further processing in the laboratory.

Sample collection and handling of the specimens were performed under sterile conditions sterile during the whole process. First, specimens were washed in sterile phosphate buffered saline (PBS) (Gibco, Invitrogen) necrotic areas and vessels were manually

removed. Then, after mechanical fragmentation, specimens underwent enzymatic digestion with collagenase type Ia (Sigma-Aldrich, St. Louis, MO, USA), at a concentration of 12.5mg/mL in DMEM, for 60-90 minutes, at 37°C and under constant stirring. Finally, after cold precipitation of the undissociated tissue fragments during 15 min, the cell suspension in the supernatant was centrifuged at 1000 rpm for 5 minutes and resuspended in a complete medium (CM), a glioblastoma-initiating-cell (GIC)-specific culture medium.

2.4.2. Cell culture establishment and maintenance

After two to six days of culture in the presence of complete medium, the tumor cells were able to form neurospheres, floating clusters of glioblastoma stem cells. These cells forming neurospheres were gradually separated from the adherente cells during the successive passes. Cellular debris and red blood cells were also successively discarded by low speed centrifuges. All cultures were incubated at 37°C with atmosphere of 5% of CO2 and humidity of 95-99%.

The tumor cells were maintained as neurospheres in serum-free DMEM/F12 medium (Invitrogen, Carlsbad, CA) and plated at a density of 3×10^6 live cells/60-mm plate, adapting these parameters whenever needed to achieve the optimal conditions of each cell line. Cells were used between passages 10 and 20. The culture medium was renewed every 2 or 3 days and the neurospheres were dissociated every 4–5 days to facilitate cell growth. Dissociation was performed by incubating the neurospheres with Accutase proteolytic enzymes (Sigma-Aldrich) for 5-7 minutes at 37°C under gentle agitation, until individual cells in suspension were obtained. The cell suspension obtained will be subsequently cultured in fresh complete medium.

2.4.3. Cell culture under hypoxia

The cell cultures under hypoxia were performed by using a Hypoxia Incubator Chamber (StemCell Technologies, Vancouver, BC, Canada) and following the same protocols for cell culturing described before. Glioblastoma cells were incubated under hypoxia (1% O_2) and harvested after 24, 48 or 72 hours for further analysis

In order to confirm an effective cellular response to hypoxia, cells were treated with Hypoxiprobe-1 and immunolabeled with anti-PIMO. Glioblastoma cells incubated in normoxia (21% O_2) as described before, were used as controls for all experiments.

2.4.4. Cell lines characterization

Three different primary cell lines established from tumor specimens of patients with glioblastoma were used (G196, G52 and G63). G196 and G52 did not express IDH1 mutant protein, overexpressed EGFR and were GFAP (+) while G63 expressed mutant p53 protein.

2.5. Expression analyses

2.5.1. Total RNA extraction.

Phenolic Total RNA extraction was carried out using the Trizol reagent (Invitrogen, Carlsbad, CA). The collected cells were lysed in 1 ml of Trizol and were incubated 5 minutes at room temperature. Then, samples were centrifuged with 200 μL of chloroform-isoamyl alcohol (24:1) for 15 minutes at 13,000 rpm at 4°C. After centrifugation, the supernatant containing the RNA was recovered and transferred to a new tube for precipitation by using 500 μL of isopropanol. The precipitated RNA was washed in 1ml of Ethanol 80% in H_2O DEPC, H_2O with 0.1% diethylpyrocarbonate, a RNAse inhibitor (Sigma). Finally, after allowing the samples to dry, they were resuspended in a variable volume of H_2O DEPC. The Total RNA concentrations were subsequently quantified by absorption spectrophotometric (Nanodrop TMND-1000). The absorbance ratios 260/280 nm and 230/260 nm were used as criteria for sample quality. All samples obtained from RNA were stored at -80 until its use to minimize its degradation.

2.5.2. RT-PCR.

Complementary DNA (cDNA) was obtained, from the extracted mRNA, by reverse transcription (RT). Briefly, 1-2 μg of total RNA of each sample was diluted in a total volume of 10 μL of H_20 DEPC. In order to achieve mRNA denaturation, the samples were heated for 10 minutes at 65°C and then immediately cooled to 4°C. Subsequently, 500 pmol of random sequence oligonucleotides (Promega), 0.2 mM of dNTPs (Amersham Pharmacia), 1mM of dithiothreitol (DTT), 5U of ribonucleases inhibitor (Invitrogen) in a 1x cDNA buffer (50 mM Tris-HCl pH 8.3, 40 mM KCl, 6mM MgCl2) were added to each reaction. Additionally, H_2O DEPC was added up to a final volume of 20 μL per reaction.

The samples were incubated for 5 minutes at 25°C, 60 minutes at 42 ° C and finally 5 minutes at 95°C for enzyme inactivation. All synthesized cDNAs were stored at -20 ° C until use and amplification. PCR amplification was performed from 1 μL cDNA by using a 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2), 1.5 mM MgCl2, 0.2 mM dNTPs, 25 pmoles of each primer, 2.5U of Taq Polymerase (Bioline Ltd. London, United Kingdom Bound) and distilled H_2O to a final volume of 50 μL per reaction. The reaction conditions were as follows: denaturation phase (1 cycle) a 95°C for 5 minutes, followed by an amplification phase (18-30 cycles) with a denaturation step at 95°C for 30 seconds; a pairing phase at 55-65ºC for 30-45 seconds (variable according to the size of the amplicon and the Tm of the primers) and a extension phase at 72 ° C for 30 seconds. To detect and quantify the amplified cDNA products, they underwent electrophoresis (100mv) in 2% agarose gel to separate them based on their size. Finally, the gels were stained with bromide of 2% ethidium in 1x TAE buffer (Roche) and visualized under ultraviolet light in a transilluminator (Bio-Rad) and images were acquired using the system camera using the Quantity One (Bio-Rad) program.

2.5.3. Quantitative RT-PCR.

The expression of individual genes was evaluated by quantitative RT-PCR on total cellular RNA as previously described (Talamillo et al. 2017). In brief, to each 96-well plate reaction were added: 100 ng of each cDNA, 0.4 μM of each primer, 12.5 μL of commercial reagent 0.5x from Sybr Green PCR Master Mix (Applied Byosystems) necessary for amplification

and H_2O up to a final volume of 25 μL. The Sybr Green PCR Master Mix contains a DNA-binding fluorophore, dNTPs, a reaction buffer and the DNA polymerase enzyme AmpliTaqGold, which remains inactive until it is subjected to high temperatures, 10 minutes at 95 ° C, thereby avoiding nonspecific hybridization of the primers.

The PCR reaction was performed in a 7000-sequence detection system (Life Technologies, Carlsbad, CA), according to the following protocol: first a 2-minutes step at 60°C, followed by 10 minutes at 95°C for enzyme activation, and then 40 cycles of amplification with a denaturation step of 15 seconds at 95°C and another pairing and extension step of 1 minute at 60°C in each cycle.

The results were analyzed by using the ABI system PRISM 7000 software capable of processing the emitted fluorescence and therefore obtaining a Ct value (threshold cycle) for each sample. Dissociation curves confirmed the amplification of a specific product was checked. All samples were analyzed in triplicate and B-actin was used as an internal control and expression levels were calculated accordingly.

2.5.4. Primers used.

The design of specific primers for both, PCR and q-PCR, was done in Primer Express software (Applied Biosystems). The following primers were used: *β-Actin* (5'GCGGGAAATCGTGCGTGACATT3' and 5'GATGGAGTTGAAGGTAGTTTCGTG3') and *ODZ1* (5'ACTCAAGAGATGGAATTCTGTG3' and 5'CTTAGTGCATGGTCAGGTG 3').

2.6. Promoter methylation status and bioinformatics analysis

The methylation profiling and bioinformatics analysis was completely carried out at the Macfeeters-Hamilton Neuro-oncology Centre at the Princess Margaret Cancer Centre (Toronto, Canada).

2.6.1. Specimen processing

In order to assess the methylation status of *ODZ1* promoter and body within hypoxic tumor cells, fresh glioblastoma specimens from 10 patients were dissociated and analysed. All of these patients received a preoperative dose of PIMO, as described before. After tumor dissociation, tumor cells were labelled with a FITC-conjugated PIMO-specific antibody and sorted by FACS. Finally, DNA was isolated from FACS-sorted PIMO positive and negative cells and processed for methylation profiling by bisulfite conversion with the EZ DNA Methylation kit (Zymo Research, Irvine, CA, US).

2.6.2. Methylation profiling

Methylation profiling was performed using the Illumina Infinium HumanMethylationEPIC Array (Illumina Inc., San Diego, CA). Raw data files (*.idat) were imported preprocessed and normalized with the ssNoob method using the minfi package (version 1.28.3) (Aryee et al. 2014) from the Bioconductor package (version 3.8) (Fortin, Triche, and Hansen 2017) together with appropriate quality control (detection P-value < 0.05) and analysis procedure. Methylation values (beta-values) of all CpG sites were obtained which range from 0 for unmethylated to 1 for fully methylated.

Additionally, *ODZ1* methylation status was determined in 155 glioblastoma samples obtained from The Cancer Genome Atlas (TCGA) following a protocol previously described (Mamatjan et al. 2017). In brief, the TCGA 450k methylation data set (level 3) and clinical information were downloaded from the National Cancer Institute Genomic Data Commons. Four CpGs sites located within the *ODZ1* gene were included in the analysis: cg08750326, cg24761295, cg01792733 and cg19331065.

Finally, overall methylation in glioblastoma cells cultured under hypoxia was assessed by using the colorimetric MethylFlash Global DNA Methylation ELISA kit (Epigentek, Farmingdale, NY) following the manufacturer protocol.

2.7. ODZ1-promoter enzymatic methylation

A CpG methyl-transferase (M.SssI) was used to methylate all CpG sites in the *ODZ1* promoter according to manufacturer instructions (New England Biolabs, Ipswich, MA). Then, the methylated promoter was cloned into KpnI and HindIII sites of an unmethylated pGL2-luciferase reporter plasmid (Promega, Madison, WI). DNA was isolated from transduced bacterial colonies and those maintaining the methylated *ODZ1* promoter were identified by digestion with the methylation-sensitive HpaII restriction enzyme (New England Biolabs) followed by PCR-amplification with primers flanking the CCGG site of interest (cg24761295) (5´TGCTGCAACCTCCAGCTTAAT3´ and 5´TGTGAGGAAATGCATCTGGCA3´). As a control, a fragment of the *ODZ1* promoter without HpaII sites was amplified with primers (5´TGCAACAGTGGACTGAAATGG3´ and 5´TCTTAGGGCCAGTAGAGGCAT3´).

2.8. ODZ1-promoter mutagenesis

Site-directed mutagenesis of cg24761295 in the ODZ1 promoter was performed by using the QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Cruz, CA) following the manufacturer's instructions, with primers: 5´CAGAGTGTTATTATTGCCTCAGGCTAGCTTCATGTCATCTAG3´ and 5´CTAGATGACATGAAGCTAGCCTGAGGCAATAATAACACTCTG3´. The modified promoter was sequenced to verify the mutation.

When indicated, glioblastoma cells were transfected with *ODZ1*-specific shRNAs (Thermo Fisher Scientific, Waltham, MA) by using nucleofection, as previously described (Talamillo et al. 2016).

2.9. Transfections and gene reporter assays

Both expression vectors, containing the methylated or mutated promoter, were amplified and purified. In brief, 20 μL of the vector was added to a vial of competent *E. coli* DH5alpha (Eppendorf North America, New York, NY) and follow incubation for 30

minutes at 4°C. Subsequently, in order to favor the entry of DNA through pores in the membrane of the bacterium, they were incubated 30 seconds in a bath at 42°C and followed by a 2 minutes incubation at 4°C. Next, 250 μL of LB medium was added and incubated for 1 hour at 37°C under stirring. Afterwards, 50 μL of the mixture was seeded in plates of LB-agar medium to select only the bacteria that incorporate the vectors. The next day, some of the colonies were selected and grown overnight at 37°C in an 250ml Erlenmeyer with 200 mL of liquid LB medium supplemented with antibiotics. The next day, plasmids were extracted and were purified with the EndoFree Plasmid Maxi Kit (Quiagen, Hilden, Germany) following the manufacturer's instructions. Finally, the DNA obtained was quantified with the Nanodrop system.

Glioblastoma cells were cotransfected with 2 μg wild type and mutant promoter or methylated and unmethylated promoter cloned into pGL2-luciferase reporter, and 0.2 μg pRSV-ß-gal by using nucleofection. Transfected cells were cultured under hypoxic or normoxic conditions for 48 h and cell extracts were prepared and analysed for the relative luciferase activity by a dual-light reporter gene assays (Applied Biosystems, Foster City, CA). Results were normalised for transfection efficiency with values obtained with pRSV-β-gal.

2.10. Migration assay

The migration capacity of glioblastoma cells was analysed by using a modified Boyden chamber assay in 24-well plates (Transwell, Corning Incorporated, NY). Briefly, a cell suspension containing 0.5-1.0 x 106 cells/mL was prepared. Then, 300 μL of the cell suspension was added into each insert and 500 μL of serum free media was added into the lower chamber. Cells were incubated for 24 or 48h under hypoxic and normoxic conditions. Afterwards, cells and media were carefully removed from the top side of the insert and migratory cells in the lower face of the membrane were fixed and stained. The stain from the underside of the insert was extracted and added to a 96-well microtiter plate suitable for optical density measurement at 560 nm.

2.11.Statistical analysis

All statistics were calculated with the SPSS statistical package (version 13.0). Data are presented as mean ± SD of three independent experiments. Differences between groups were tested for statistical significance using the unpaired 2-tailed Student's t-test or Chi Square accordingly. The significance level was set at $p< 0.05$.

3. RESULTS

3.1. ODZ1 expression and pre- and postreatment radiological features

3.1.1. Imaging geometrical features and ODZ1 expression

ODZ1 expression in glioblastoma specimens was determined by immunohistochemistry (IHC). In positive cases, a nuclear and a cytoplasmatic staining was noted (Figure 8). Positivity was determined by the number of positive nuclei and the intensity of the staining. Finally, the cases were divided in two different groups for the analysis: ODZ1 negative cases, without any nuclear positivity, and ODZ1 positive cases, depicting positive nuclei.

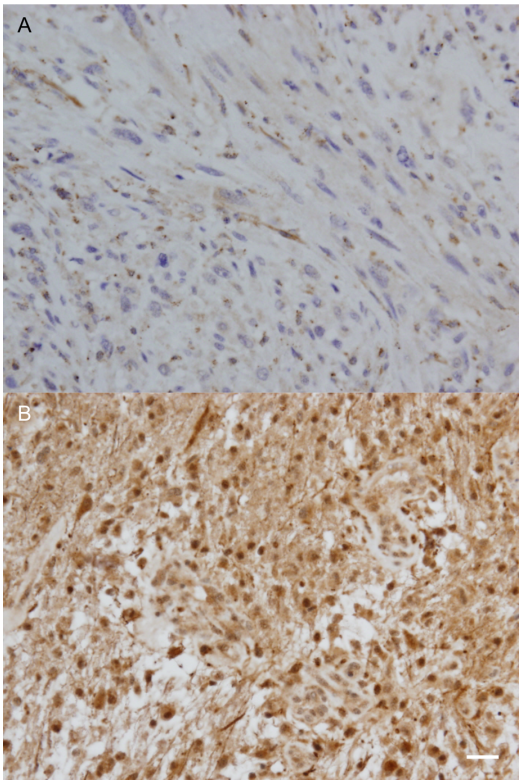

Figure 8 *- ODZ1 expression in glioblastoma. ODZ1 IHC showing expression in negative (A) and positive (B) cases. Scale bar: 20 µm.*

First, we assessed the association of each of the 10 tumor geometric features analyzed in the preoperative MRI and the ODZ1 expression. Spearman correlation analysis showed a strong association among the imaging geometric features. Nevertheless, no significant correlation was obtained between the image variables and those of ODZ1 expression (Figure 9).

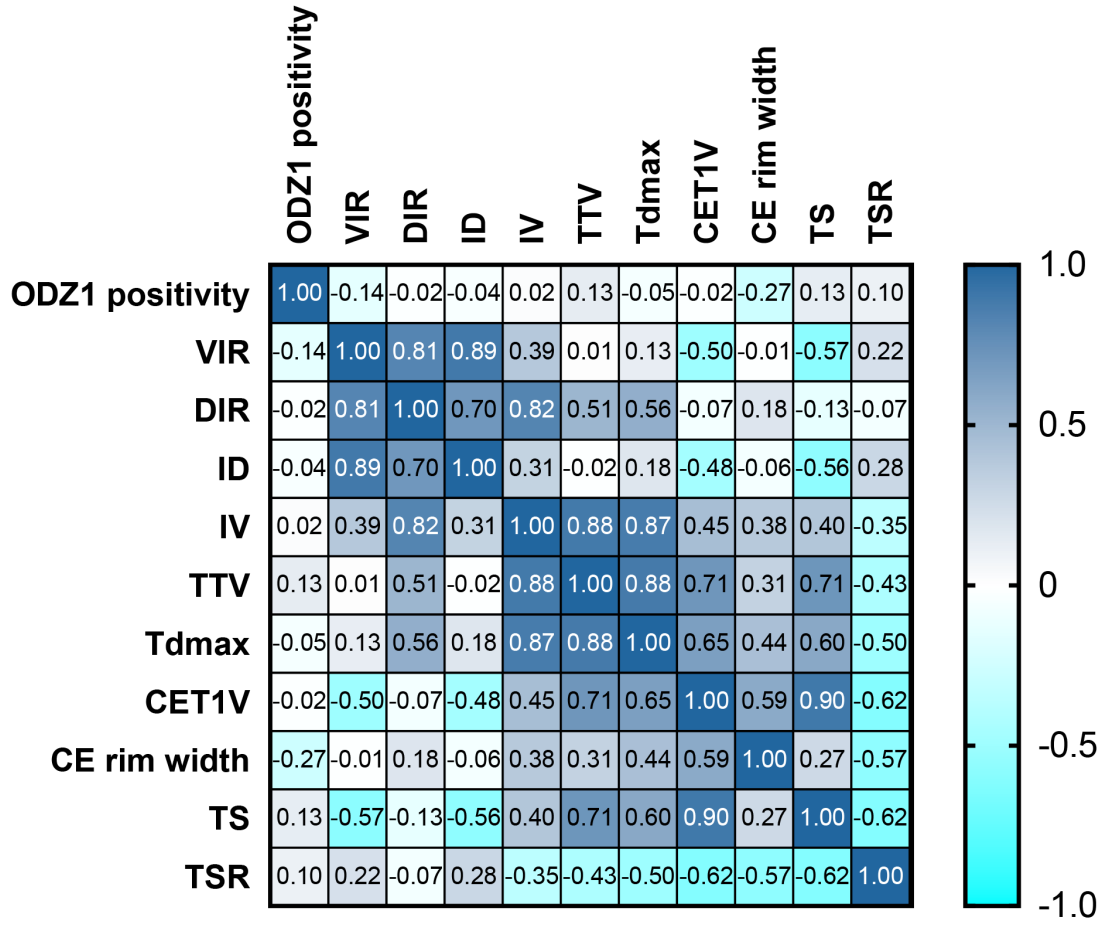

Figure 9 *- ODZ1 expression and imaging features. Heatmap of spearman correlations between ODZ1 expression and radiomics features. VIR: Volume infiltration ratio; DIR: Diameter infiltration ratio; ID: Infiltration diameter; IV: Infiltration volume; TTV: Total tumor volume; Tdmax: Maximun tumor diameter; CET1V: Contrast enhancing T1 volume; CE rim width: contrast-enhanced rim width; TS: Tumor surface; TSR: Tumor surface regularity.*

On the other hand, by using the Mann Whitney Test, the Contrast-enhancing rim width significantly associated with the intensity of nuclear and cytoplasmatic ODZ1 expression ($p = 0.033$ and $p=0.040$, respectively). Furthermore, when we considered the ODZ1 nuclear expression as dichotomous variable, by grouping on one side the patients without any ODZ1 expression (9 patients) and on the other the patients with poor, intermediate

and positive expression (29 patients). Then, using a Kruskal-Wallis test, the Contrast-enhancing rim was again the only geometrical tumor features that significantly associated with ODZ1 expression. In the group with no ODZ1 expression the contrast-enhancing rim width was smaller than in the tumors expressing ODZ1 (Figure 10).

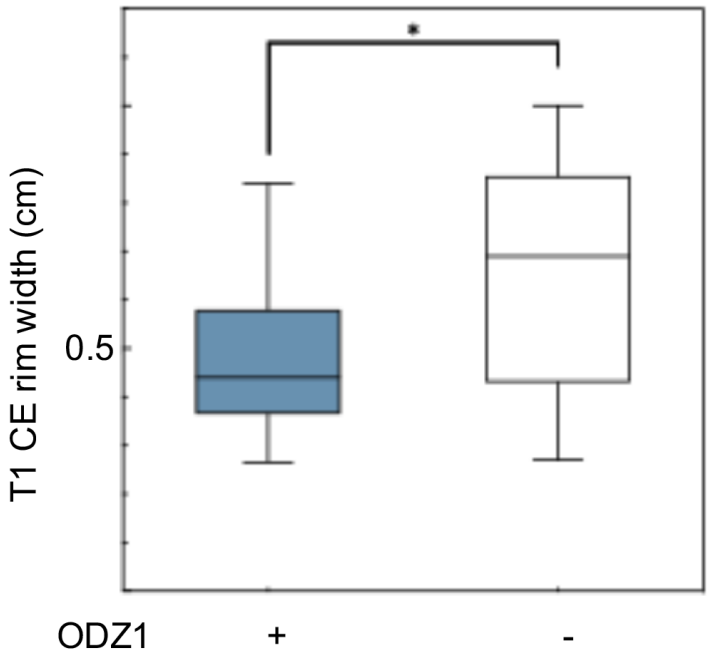

Figure 10 *- CE rim width and ODZ1 expression. ODZ1 expression is associated with an increased a tumor CE rim width (Chi-Square, *p < 0.05)*

3.1.2. Glioblastoma recurrence patterns and ODZ1 expression

In the 20 patients treated with complete tumor resection and chemoradiation according to the Stupp protocol, only 5 (25%) patients presented distance tumor recurrences whereas the remaining ones recurred focally within the surgical cavity margins (Figure 11). No differences were noted between the patients expressing high or low levels of ODZ1.

Among the five cases with distance tumor recurrences, 3 of them (60%) presented a high ODZ1 expression, whereas among the 15 cases presenting focal recurrences, only 6 cases presented a significant ODZ1 expression. Nevertheless, this difference was not statiscally significative (Figure 12).

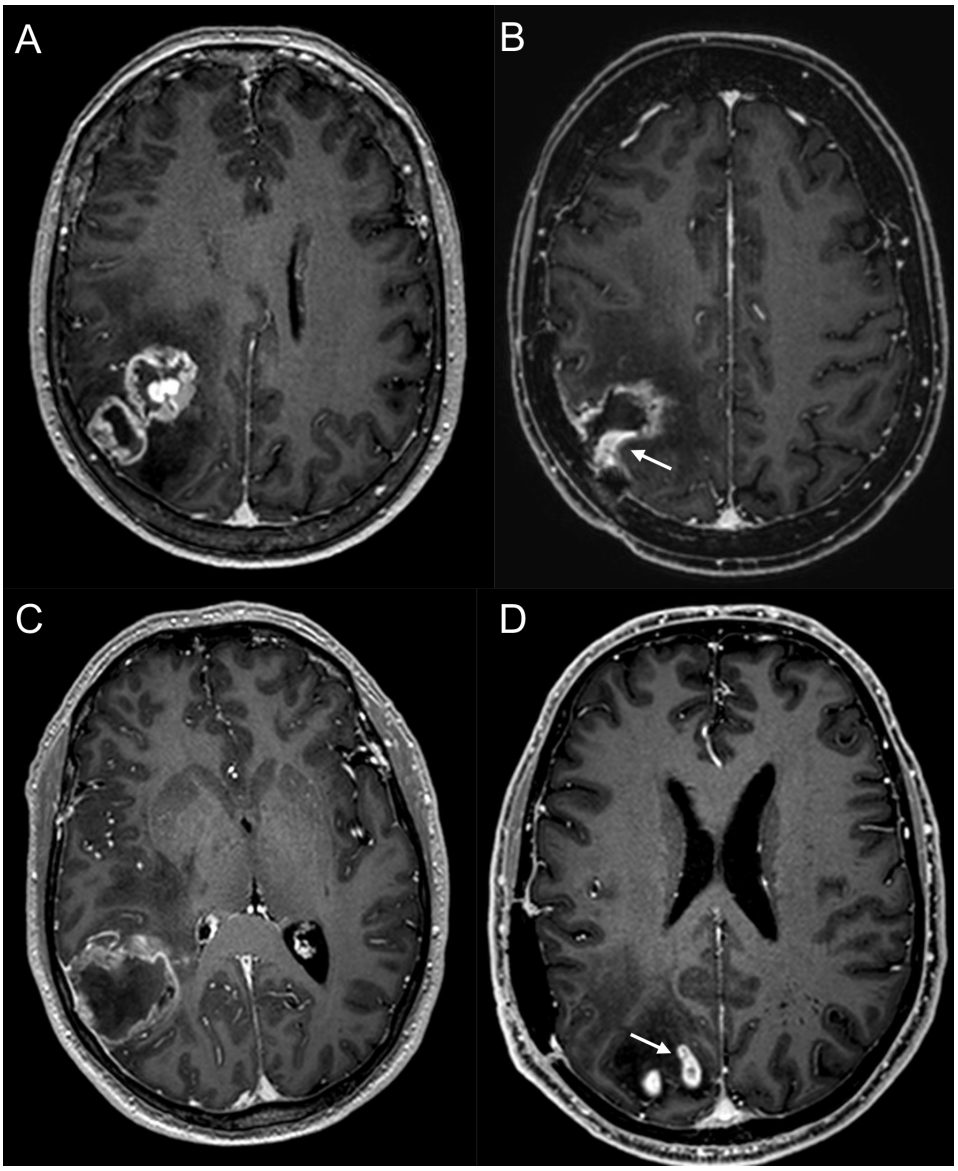

Figure 11 *– Illustrative cases of tumor recurrence in glioblastoma. Focal tumor recurrence: Axial gadolinium enhanced T1-weighted MRI images showing a right-sided parietal primary tumor (A) and the focal tumor recurrence (B, arrow). Distance tumor recurrence: Axial gadolinium enhanced T1-weighted MRI images showing a right-sided parietal primary tumor (C) and the distance tumor recurrence (D, arrow).*

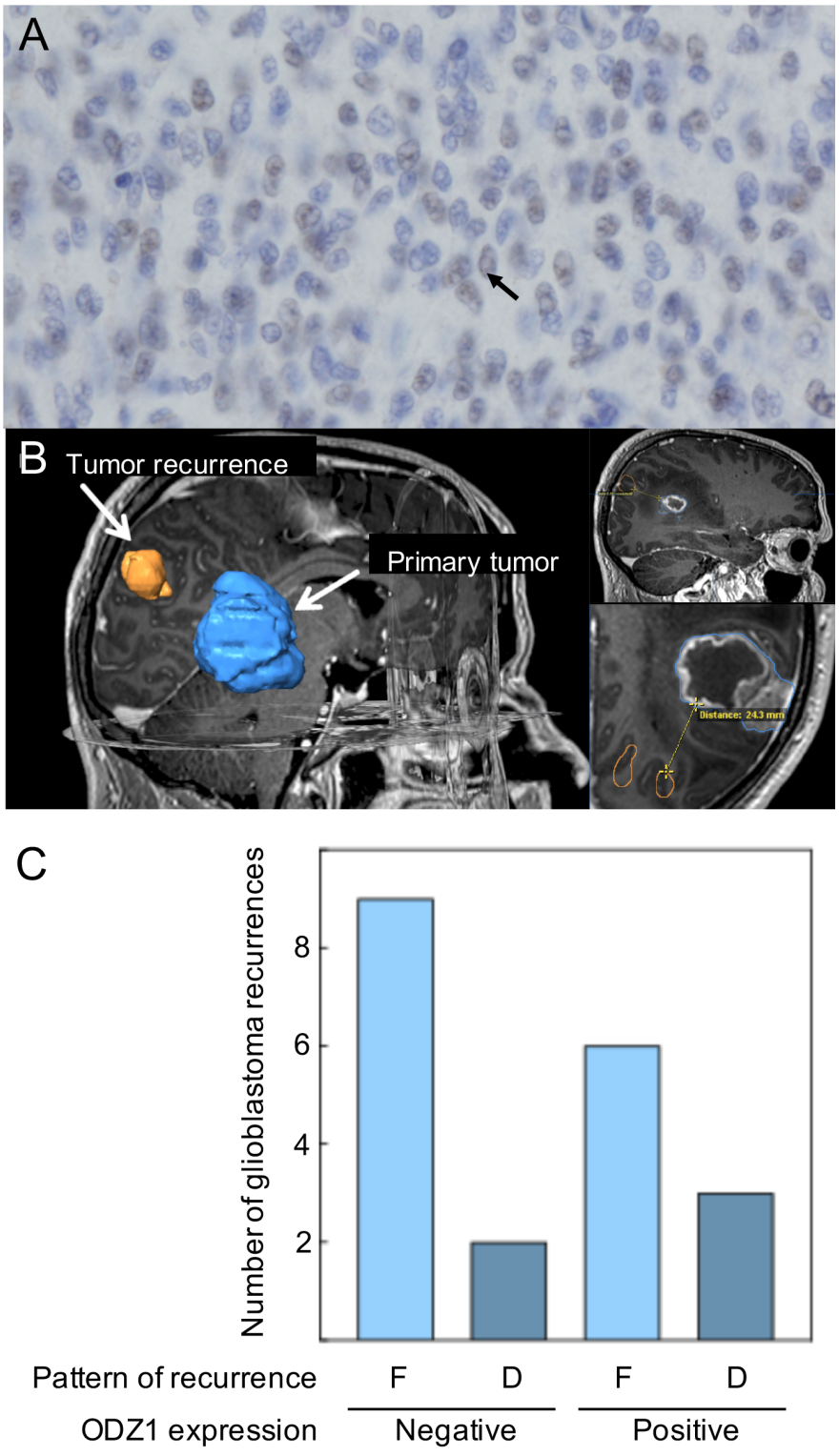

Figure 12 *- ODZ1 expression in glioblastoma with focal and distance recurrence. Illustrative case of glioblastoma with (A) ODZ1 IHC showing nuclear positivity and (B) Contrast-enhanced MRI scan showing reconstruction of primary tumor (blue) and distant recurrence (orange).*
. (B) Bar graph showing the distribution of focal and distant tumor recurrences in ODZ1 negative and positive glioblastoma (Chi-Square, $p > 0.05$).

3.2. Hypoxia-dependent ODZ1 regulation

3.2.1. Hypoxia upregulates ODZ1 in glioblastoma tumor specimens

Tumor cell migration is known to be triggered by microenvironmental stress including hypoxia, which is one of the hallmarks of glioblastoma. ODZ1 has been recently described as a novel inducer of migration/invasion in glioblastoma (Talamillo et al. 2017), however the external stimuli that promote the expression of *ODZ1* remain unknown.

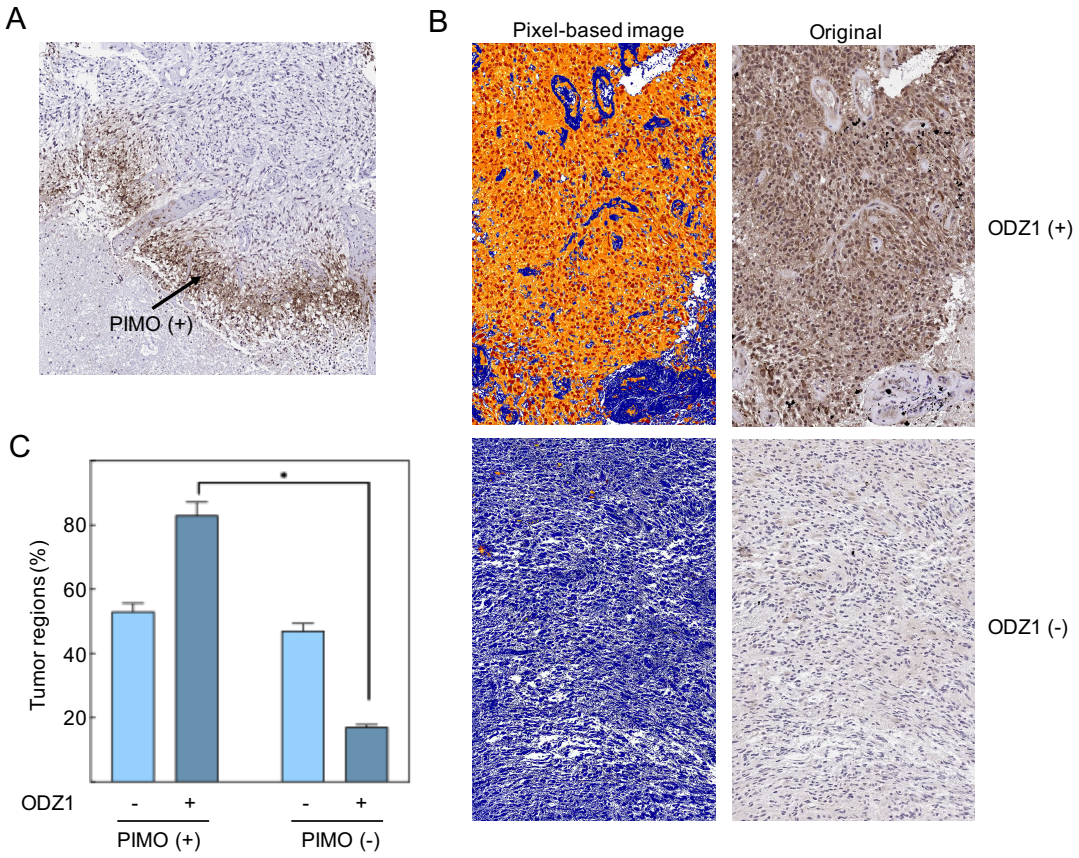

Figure 13 *- In vivo ODZ1 expression in hypoxic and normoxic tumor regions. (A) Immunohistochemistry of glioblastoma tumor tissue with anti-PIMO antibody showing the presence of PIMO positive tumor regions. (B) Glioblastoma tissue specimens were immunostained with anti-ODZ1 antibody. Original and pixel-based image analysis of ODZ1 nuclear and cytoplasmic expression in positive and negative representative cases, are presented. (C) PIMO and ODZ1 immunostaining was determined in consecutive histological sections in 54 tumor regions. (Chi-Square, *p < 0.05).*

In order to determine whether *ODZ1* expression was linked to hypoxia in glioblastoma, we first examined the cellular uptake of the exogenous hypoxia marker pimonidazole (PIMO) administered to the patients prior to surgery to delineate the severely hypoxic

tumor regions and distinguish them from the non-hypoxic areas (Figure 13A). The PIMO uptake allowed us to clearly annotate the hypoxic and normoxic tumor regions. Consequently, we used these annotated regions to analyse the expression of ODZ1 protein in hypoxic and normoxic regions (Figure 13B).

In the 54 tumor regions analysed, ODZ1 nuclear and cytoplasmic expression increased in the severe hypoxic regions when compared with the normoxic tumor regions in glioblastoma. Up to 80% of regions that stained positive for the hypoxia marker PIMO were ODZ1-positive, whereas among the PIMO-negative regions less than 20% were ODZ1-positive ($p<0.05$) (Figure 13C).

Furthermore, ODZ1 and PIMO IF double staining of frozen tumor sections from a patient with glioblastoma, showed colocalization of both antibodies, suggesting that ODZ1 might be overexpressed in hypoxic tumor cells exhibiting PIMO uptake.

3.2.2. Hypoxia upregulates ODZ1 transcription and promotes migration of glioblastoma cells

Once it was proven that ODZ1 is overexpressed in the hypoxic regions of the tumor, the next step was to determine whether the tumor cell hypoxic microenvironment could lead to increased expression of *ODZ1 in vitro*.

Therefore, we first cultured primary glioblastoma cells under hypoxic conditions (1% O_2) in order to assess cell viability and to establish the optimal conditions for the experiment. We found that the majority of the cells remained viable (over 85% by Trypan blue assay). Besides, we treated cells with PIMO before harvesting and performed immunofluorescence analysis to confirm that cells cultured in hypoxic conditions were able to uptake PIMO from culture media, while those grown in normoxia did not (Figure 14). Then, we determined ODZ1 expression in glioblastoma cells cultured under hypoxic conditions. Consistent with our *ex vivo* previous findings in glioblastoma tumor sections from patients, *in vitro* analysis of *ODZ1* expression in glioblastoma cells cultured in hypoxia during 24h indicated a 4-fold increase in *ODZ1* mRNA expression ($p<0.05$). These findings were similar in three different primary glioblastoma cell lines (G196, G52 and

G63). Interestingly, the effect of hypoxia in ODZ1 expression could be significantly reduced by transfecting the glioblastoma cells with ODZ1-specific shRNAs (Figure 15). Additionally, we cloned the *ODZ1* promoter into a luciferase reporter-containing plasmid. Glioblastoma cells transfected with this construct increased the level of luciferase activity when cultured in low oxygen (2-fold increase, $p<0.05$) further demonstrating that hypoxia was relevant for the expression of *ODZ1* and that the promoter cloned contained hypoxia-responsive elements (Figure 15).

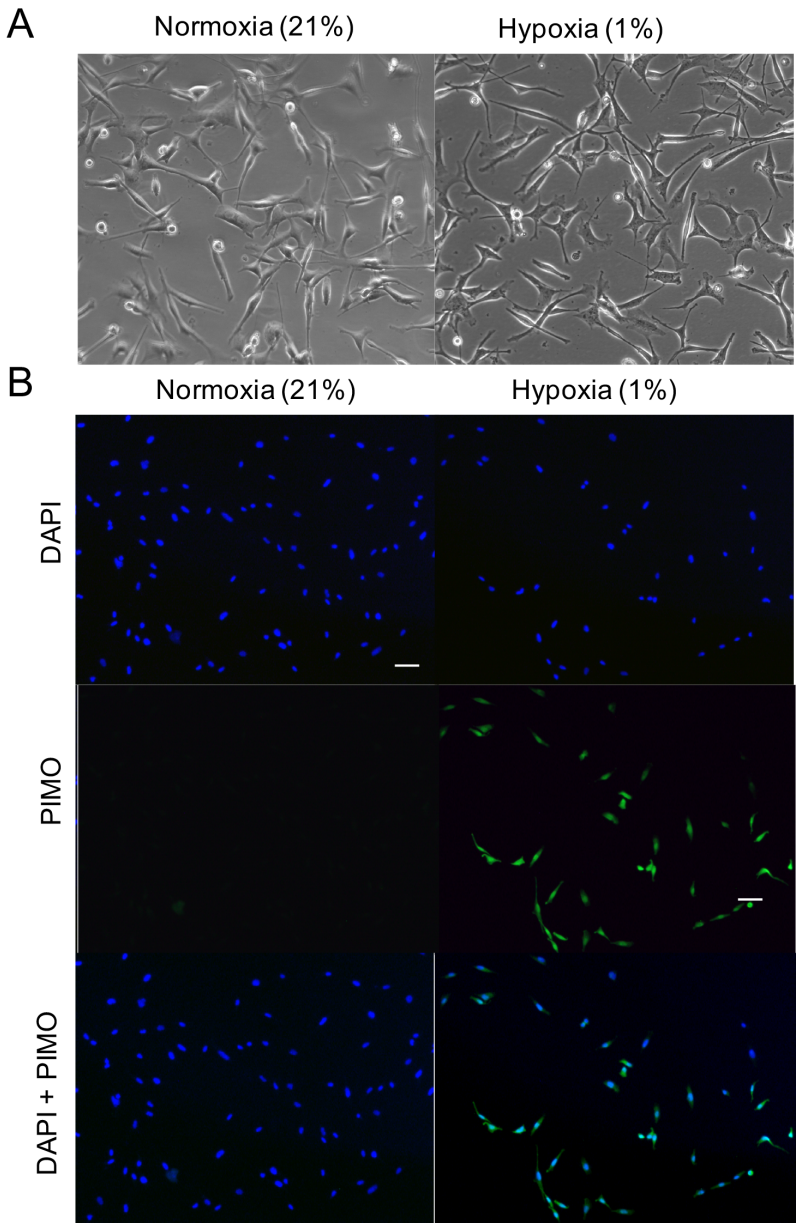

Figure 14 *- Primary glioblastoma cells cultured under hypoxia. (A) After 48h under hypoxia glioblastoma cells maintain their phenotype with a viability higher than 80%. (B) Cells cultured in 1% O2 for 48 h stained positive for the hypoxia marker PIMO as confirmed by immunofluorescence with anti-PIMO antibody (more than 95% of PIMO positive cells). Scale bar: 10 µm.*

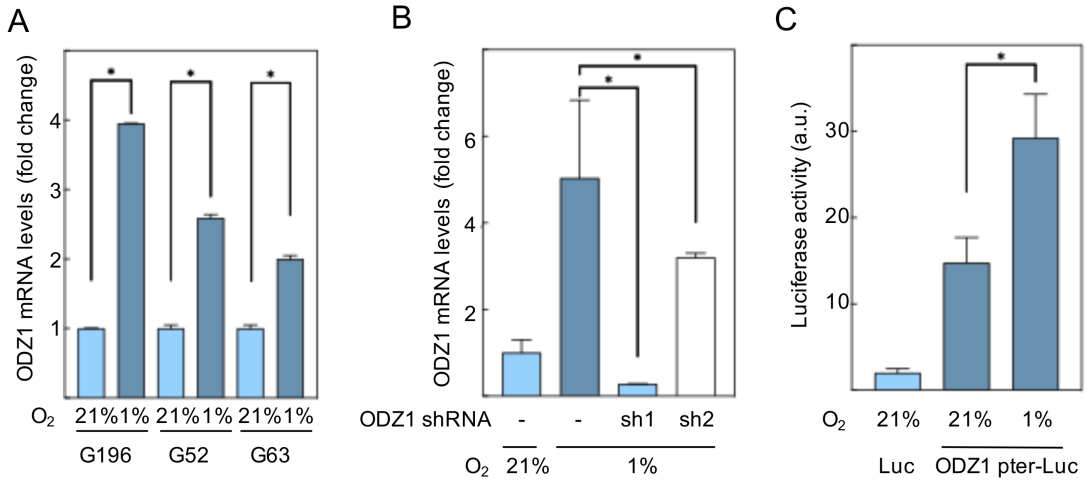

Figure 15 *- Hypoxia upregulates ODZ1 expression. (A) ODZ1 mRNA levels in glioblastoma cells under hypoxia (1% O2) and normoxia (21% O2) for 24 h were analyzed by qPCR in three different glioblastoma primary cell lines (G196, G52, and G63). (B) Downregulation of hypoxia-induced ODZ1 mRNA levels in G196 cells transfected with two ODZ1-specific shRNAs and cultured under hypoxia for 48 h. (C) Cells transfected with ODZ1 promoter cloned into a luciferase reporter plasmid were analyzed for luciferase activity under hypoxia (48 h). All histograms show the mean ± SD of three independent experiments. Student t-test *p < 0.05.*

Afterwards, in order to determine if the hypoxia-induced ODZ1 upregulation was meaningfully affecting the glioblastoma cell migratory capacity, we performed migration assay under hypoxic and normoxic conditions.

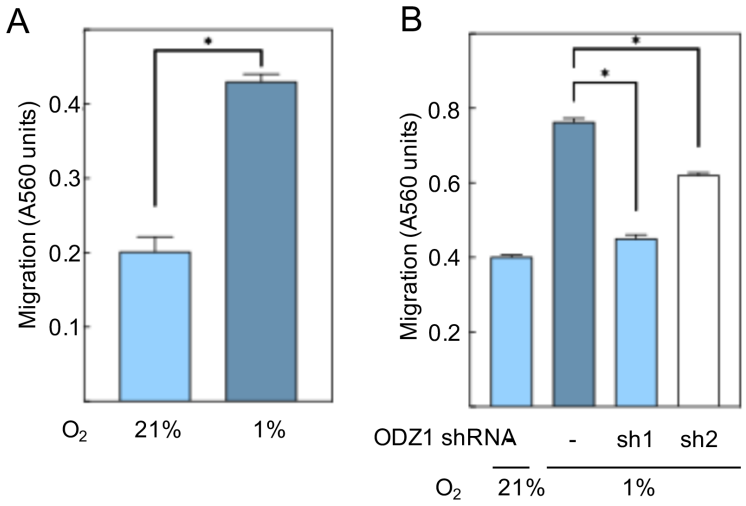

Figure 16 *– Hypoxia promotes ODZ1-dependent migration of glioblastoma cells. (A) A modified Boyden Chamber assay was used to assess cell migration under hypoxia and normoxia for 48 h. (B) Cell migration capacity under hypoxia (48 h) in the presence of ODZ1 shRNAs. All histograms show the mean ± SD of three independent experiments. Student t-test *p < 0.05.*

Interestingly, as shown in Figure 16, hypoxia promoted migration of glioblastoma cells with more than a 2-fold increase ($p<0.05$). Next, ODZ1 contribution to the hypoxia-induced migration was determined by knocking down ODZ1. Transfection of glioblastoma cells with ODZ1-specific shRNAs showed that efficient downregulation of *ODZ1* mRNA levels correlated with reduced migration of glioblastoma cells under hypoxia ($p<0.05$) (Figure 16).

3.2.3. ODZ1-promoter methylation status is modified by hypoxia

As soon as the hypoxia-dependent and pathophysiologically meaningful ODZ1 upregulation was demonstrated *in vivo* and *in vitro*, several questions arose, regarding the mechanisms by which the hypoxic microenvironment is able to regulate ODZ1 expression. Based on previously described observations on the role of hypoxia in epigenetic regulation in cancer and that ODZ1 was upregulated after demethylation, we assessed the role of hypoxia-dependent ODZ1 epigenetic regulation.

First, we assessed the effect of hypoxia on overall DNA methylation status. Incubation of glioblastoma cells under hypoxic conditions resulted in overall DNA methylation when compared with the cells cultured in normoxia (Figure 17A).

Then, in order to assess whether the tumor hypoxic microenvironment could regulate the methylation status of the ODZ1 gene promoter and gene, we determined the ODZ1 methylation status in tumor hypoxic and non-hypoxic cells. Therefore, we isolated PIMO positive and PIMO negative tumor cells from surgical specimens of 10 patients with glioblastoma by using a cell sorter. For the analysis of the methylation status of the *ODZ1* promoter, we focused on a fragment of 1.4 kb upstream of the transcription start site that contained no canonical CpG island but 18 CpG sites. Overall, we identified four CpG sites in the ODZ1 gene, two of them were located within the promoter (cg08750326 and cg24761295) and the other two in the body of the gene (cg01792733 and cg19331065). Among these four probes, only one CpG site, cg24761295, was found differentially methylated in sorted PIMO positive and PIMO negative tumor cells. The cg24761295 site was hypomethylated in PIMO positive cells and this difference in the methylation status was significant, which is consistent with a higher expression of ODZ1 under hypoxia. The

methylation status of the other CpG sites in the *ODZ1* promoter (cg24761295) and gene body (cg01792733 and cg19331065) showed no differences between the hypoxic and normoxic tumor cells (Figure 17B).

Interestingly, an analysis of 155 glioblastoma cases from TCGA showed that the two CpG sites located within the promoter (cg08750326 and cg24761295) were hypomethylated in tumor specimens when compared to normal brain tissue, while the two CpG sites located within the body of the gene (cg01792733 and cg19331065), far from the transcription start site, had similar methylation status both in normal brain and glioblastoma (Figure 17C).

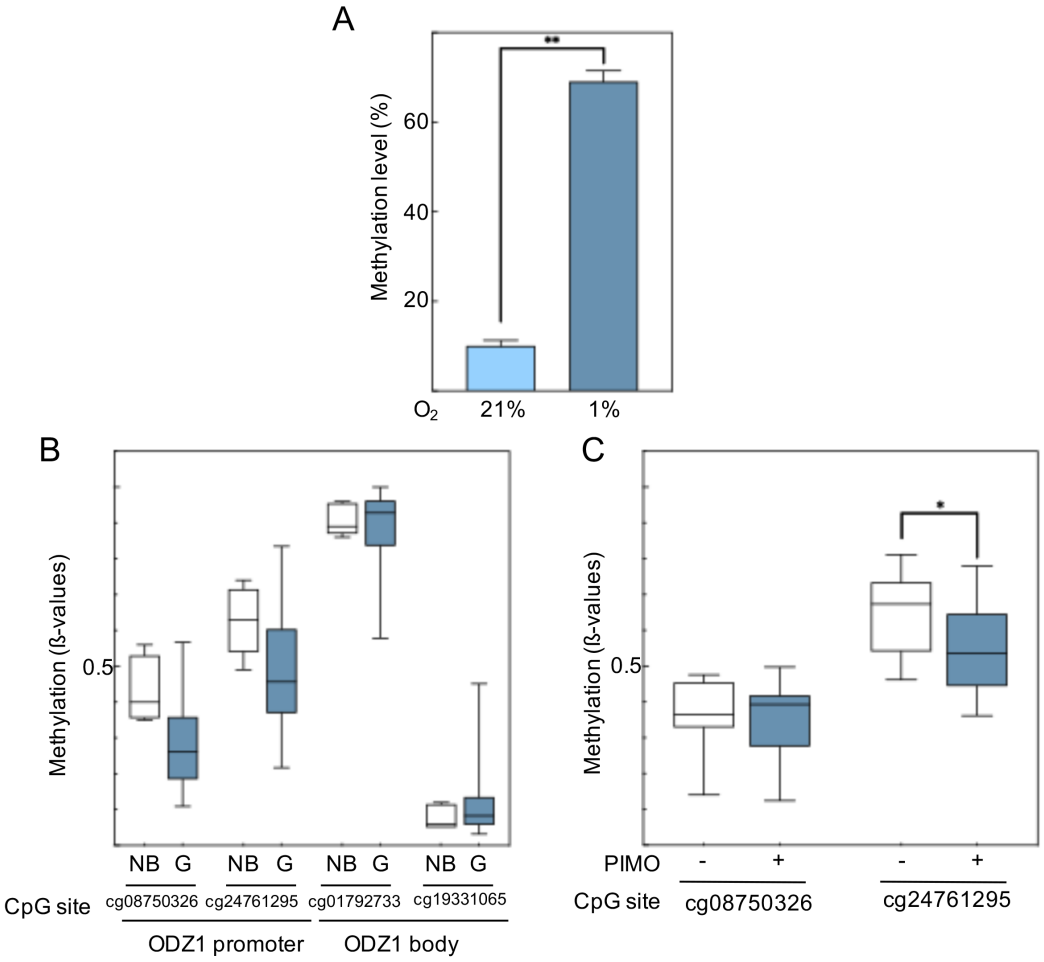

Figure 17 *- Hypoxia-dependent regulation of ODZ1 promoter methylation. (A) Overall methylation level of DNA from glioblastoma cells cultured under hypoxic and normoxic conditions (48 h). (B) Methylation status of CpG sites in the ODZ1 gene in glioblastoma tissue (n = 155) and normal brain (n = 5). Data obtained from TCGA. (C) Methylation of CpG sites located at the ODZ1 promoter in PIMO negative and PIMO positive cells in tumor specimens from 10 patients. Histograms show the mean ± SD of three independent experiments. Boxes show the median with whiskers extended to minimum and maximum values. Student t-test *p < 0.05, **p < 0.01.*

3.2.4. Methylation or Mutagenesis of cg24761295 in the ODZ1-promoter reduces gene expression

We aimed to determine if the differentially methylated CpG sites found *ex vivo* in the ODZ1 promoter significantly affected gene transcription by cloning a methylated promoter into a luciferase reporter and transfecting the plasmid into glioblastoma cells.

First, the CpG sites in the *ODZ1* promoter were enzymatically methylated by M. SssI methyltransferase. Enzymatically digestion with HpaII, which cuts the unmethylated CCGG sites, was used in order to confirm effective methylation of the promoter. Then, the methylated ODZ1 promoter was cloned into KpnI and HindIII sites of an unmethylated pGL2 luciferase reporter plasmid.

We confirmed the presence of a methylated promoter by digesting the plasmid with HpaII, which cuts the unmethylated CCGG sites of pGL2-luciferase that flanked the promoter and leaves the methylated CCGG sites within the promoter uncut In this way, we were able to assess only the role of the promoter methylation in gene expression without hindering the plasmid functionality. Then, the plasmid was transfected and amplified in E. coli. The E. coli colonies expressing the plasmid containing the methylated promoter were selected by HpaII digestion and PCR amplification with primers flanking the cg24761295 site to assure the methylation status of this site. Only the plasmid with methylated (uncut) promoter gave an amplification signal (Figures 18 and 19).

Finally, the reporter plasmid containing an *ODZ1* methylated promoter was transfected into glioblastoma cells and these tumor cells were cultured under hypoxia. Hypoxia induced luciferase activity through the unmethylated *ODZ1* promoter. However, this activity was abolished when CpG sites within the promoter were methylated (Figure 19).

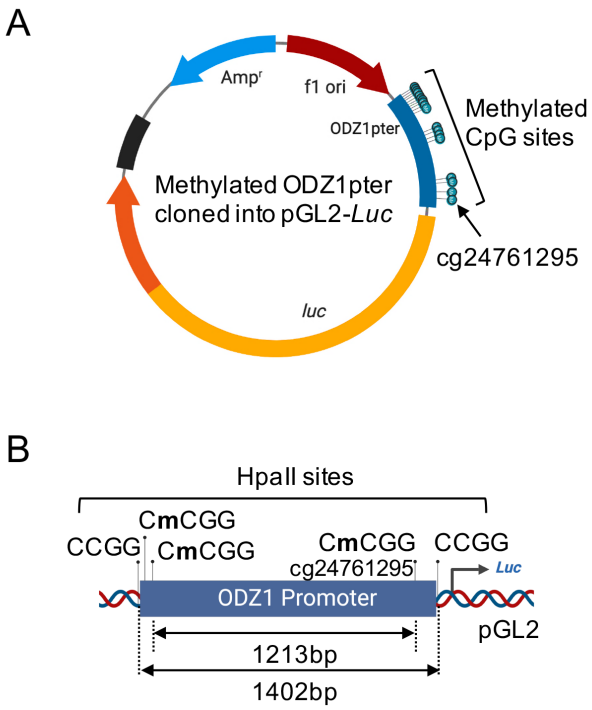

Figure 18 *- Schematic representation of (A and B) the methylated ODZ1 promoter cloned into a pGL2-Luciferase reporter vector highlighting the methylated CpG sites, including cg24761295 (Created with BioRender.com).*

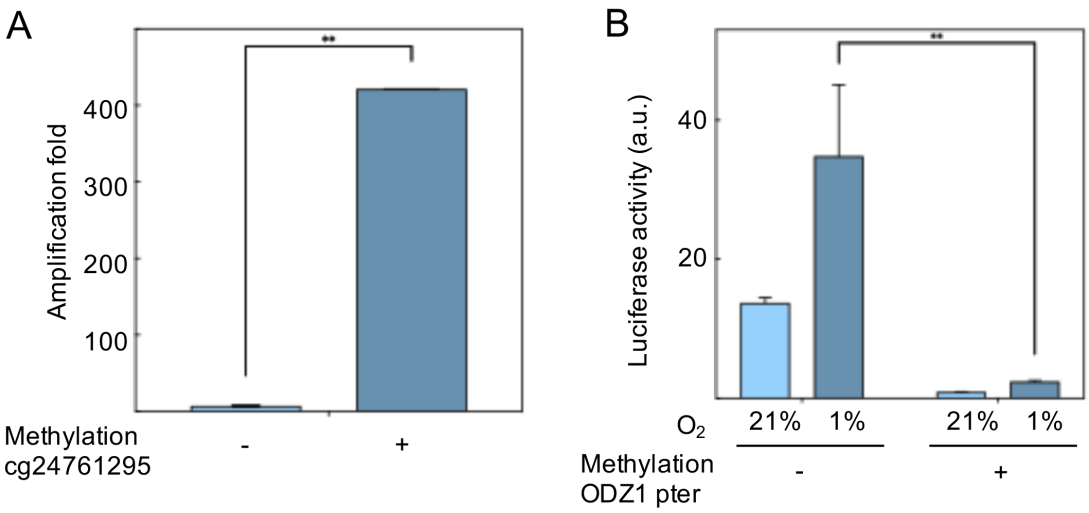

Figure 19 *- ODZ1 promoter methylation blocks gene transcription. (A) Enzymatically methylated ODZ1 promoter was amplified with primers flanking cg24761295 after digestion with methylation sensitive HpaII. Amplification signal (uncut fragment) confirms the methylation of this site. (B) Luciferase activity in cells transfected with the reporter plasmid containing a methylated ODZ1 promoter and maintained under normoxia or hypoxia (48 h). Histograms show the mean ± SD of three independent experiments. Student t-test **$p < 0.01$.*

Furthermore, to assess the specific relevance of cg24761295, a C to A change was introduced by site-directed mutagenesis and confirmed by HpaII digestion. Consistent with our previous results, glioblastoma cells transfected with the wild type promoter-reporter plasmid increased luciferase activity in response to hypoxia (p=0.035), whereas cells containing the mutant promoter did not (Figure 20).

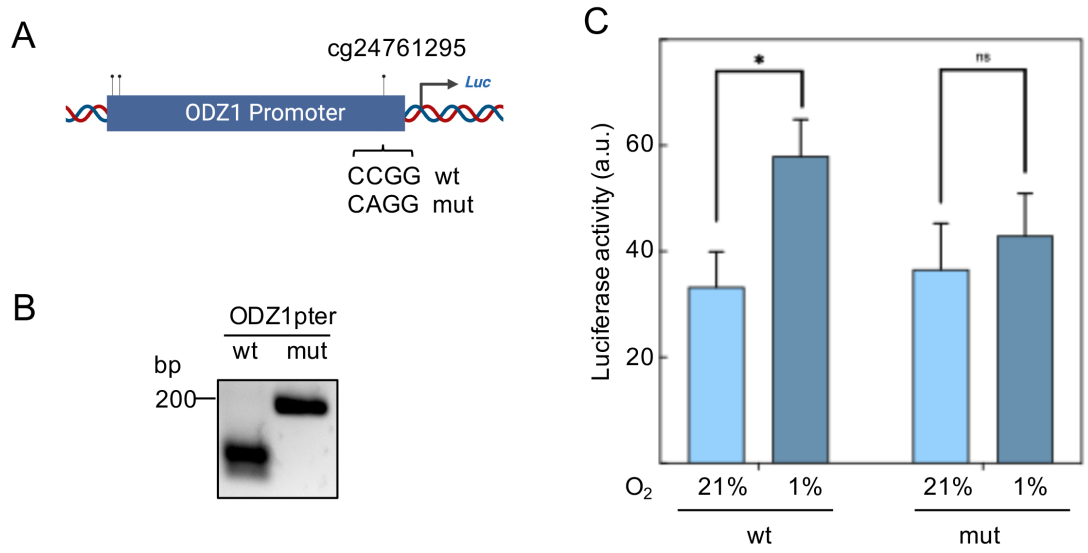

Figure 20 *- Mutation of cg24761295 reduces the transactivation capacity of the ODZ1 promoter. (A) Schematic representation of the mutated ODZ1 promoter cloned into a pGL2-Luciferase reporter vector highlighting the methylated CpG sites, including cg24761295 (Created with BioRender.com). (B) A fragment of 200 bp containing cg24761295 was amplified from wild type and mutant promoter and digested with HpaII to confirm mutation. (C) Glioblastoma cells were transfected with wild type and mutant promoter-containing plasmids and luciferase activity was determined under hypoxia and normoxia (48 h). Histograms show the mean ± SD of three independent experiments. Student t-test *p < 0.05. ns, not significant.*

4. DISCUSSION

Despite the current standard of care, glioblastoma still implies a dismal prognosis and represents a major challenge for the clinician (Stupp et al. 2005). The invasion capacity of glioma cells is one of the main pathogenic mechanisms involved in recurrence and treatment resistance (de Gooijer et al. 2018). In fact, multiple molecular pathways have been described to be implicated in glioma cell invasion, nonetheless, they haven´t been capable of fully elucidate the mechanisms underlying the tumor cell migratory and invasion capacities (Paw et al. 2015). Moreover, the cell invasion process in glioblastoma is intricately interrelated with other pathogenic tumor features, as hypoxia and heterogeneity.

Within this context, ODZ1, a member of the phylogenetically conserved teneurin family of proteins, has been recently associated with glioblastoma cell invasion. It has been recently proposed by our group that ODZ1 upregulates a RhoA/ROCK cascade resulting in cytoskeletal remodeling and cell migration, leading to poor rates of OS and PFS (Talamillo et al. 2016). Furthermore, teneurins´ role in tumorigenesis and tumor progression has been increasingly recognized in the last five years in a wide spectrum of malignancies (Rebolledo-Jaramillo and Ziegler 2018).

Notwithstanding, despite the recently described insights on ODZ1 signaling in glioblastoma, it remains unclear the ODZ1 clinical value. For instance, if whether ODZ1 expression correlates with clinical and radiological features in glioblastoma patients. Besides, the factors capable of triggering or activating the ODZ1 pathway haven´t been described. Consequently, the aim of this work is to describe the relation of ODZ1 expression with relevant tumor radiological variables, as the pretreatment geometrical tumor features and the radiological pattern of tumor recurrence. In addition, it explores the association of the hypoxic tumor microenvironment and ODZ1 expression and how the ODZ1 pathway is activated by hypoxia-dependent epigenetic regulation.

4.1. ODZ1 expression and tumor geometrical features

Recently, tumor geometrical features are emerging as surrogates of molecular tumor profiles, improving preoperative diagnosis and survival prediction, by identifying radiological prognostic biomarkers. Furthermore, the tumor geometrical features can be integrated with clinical and biological variables to build stronger prediction models (Chaddad et al. 2019).

In this context and taking into account that ODZ1 expression is significantly associated with a worse outcome in glioblastoma patients (Talamillo et al. 2016), the aim of this work was to analyze the tumor geometrical features that could be associated with increased ODZ1 expression, which then could be used as prognostic marker in patient with glioblastoma.

Among the tumor geometrical features analyzed here, only the CE rim width was associated with an increased ODZ1 expression in glioblastoma, showing a directly proportional relationship. Interestingly, the tumor CE rim width is an independent predictor of survival in glioblastoma. Moreover, it has been consistently demonstrated that an increase in tumor CE rim width correlates with poor outcome (Molina, Pérez-Beteta, Martínez-González, et al. 2016; Pérez-Beteta et al. 2019, 2017).

To the best of our knowledge, no other molecular tumor feature had been previously associated with the tumor CE rim width in glioblastoma. This fact may have several implications. First, it helps to better understand the biology underlying the CE rim width. It has been previously proposed by Pérez-Beteta et al. that in glioblastoma, the CE rim width could represent tumor invasion and proliferation. Moreover, this CE rim might represent the active border of tumor cells implicated in these processes, resulting in a higher tumor growth rate (Pérez-Beteta et al. 2019).

The association with ODZ1, which is directly involved in tumor invasion and proliferation, would support this hypothesis. Nevertheless, ODZ1 expression was not significantly associated with traditional radiomics invasion markers as the ones based on T2 hyperintensity peritumoral volume (P. R. Jackson et al. 2015). Although further studies will

be required to validate these findings, this might suggest that both, the CE rim width and ODZ1 tumor expression, are involved in the tumor proliferation-invasion process.

Secondly, the integration of other geometrical tumor features from the preoperative MRI might help to build models capable of predicting the ODZ1 tumor expression and, in combination with other markers, can help to improve survival prediction (Park et al. 2020; Choi et al. 2020; Y. Li et al. 2018).

4.2. ODZ1 and tumor recurrence

The glioblastoma recurrence pattern has been classified according different systems. In general, in a series of 57 patients, after the adjuvant treatment with temozolomide and radiotherapy, 80% of the tumor recurrences were local, 7.5% distant, 6.25% multifocal and 6.25% diffuse (Chamberlain 2011). The pattern of tumor recurrence classification can be further simplify by defining two arbitrary groups: focal recurrences, within 2 cm surrounding the surgical cavity and distant, located beyond the 2 cm margin. (Konishi et al. 2012).

Although the results presented here are not statistically significant, they show a trend that tumors with distant recurrences may have higher ODZ1 expression levels. Nevertheless, it´s important to take into account, that the pattern of recurrence is influenced by numerous biological and clinical factors, resulting in several potential confounding factors (Konishi et al. 2012). In order to try to overcome this issue, a homogenous patient sample was included in the analysis. However, other potential confounding variables were not taken into account.

For instance, the CTV in the radiation therapy planning, which has been associated with the tumor recurrence pattern, was not included in the analysis presented here. Furthermore, within this group of patients, the CTV was defined based on CT or MRI, which might yield worst local tumor control when compared to PET-based CTV delineation (Harat, Małkowski, and Makarewicz 2016). Among other variables to consider in further analysis with larger cohorts are the preoperative FLAIR/T2 hyperintensity extension and tumor size.

4.3. Hypoxia-induced ODZ1-mediated invasion

It is widely accepted that cell invasion and tumor hypoxia are two key biological hallmarks associated with glioblastoma pathogenesis and are both associated with poor prognosis (Cavazos and Brenner 2016; Lefranc et al. 2018). Besides, hypoxia plays a significant role in regulating tumor invasion through numerous molecular pathways (Monteiro et al. 2017). Nevertheless, the contribution of *ODZ1*, to the hypoxia-induced glioblastoma cell migration was still unknown.

There are reasons to think that the hypoxia-dependent invasion could be mediated at least in part through ODZ1 signaling. Among others, it has been described that the tumor hypoxic microenvironment activates a number of cellular processes directed to favor an invasive glioblastoma cell phenotype that allows cell migration. In fact, hypoxia-dependent upregulation of invasion pathways results in ECM remodelling, EMT and cytoskeletal dynamic changes (J.-W. E. Chen et al. 2018; Monteiro et al. 2017). Interestingly, as described before, ODZ1 upregulation also results in cytoskeletal remodeling by upregulating the RhoA/ROCK cascade (Talamillo et al. 2017).

In this study, the association between ODZ1 and hypoxia was examined, with emphasis in its impact on the regulation of tumor cell invasion. Interestingly, ODZ1 was mainly expressed in severely hypoxic tumor regions of surgical specimens from patients with glioblastoma. Moreover, this finding was validated by in vitro studies demonstrating that glioblastoma cells upregulate *ODZ1* mRNA and increase cell migration in response to hypoxia. These findings are consistent with the widely accepted fact that the hypoxic tumor microenvironment induces a tumor cell phenotype reacting to the hypoxia, especially in GSC (Colwell et al. 2017).

Besides, blockade of ODZ1 expression by interfering RNA reduced hypoxia-induced cell migration. This finding identifies the first extracellular stimulus capable of triggering the ODZ1-mediated tumor cell migration. Biologically, hypoxia-induced invasion mediated by ODZ1 could be one of the mechanisms that allows the tumor cell to escape away from a hostile microenvironment to a more hospitable niche. ODZ1 upregulation could act in harmony with other pathways involved in the HIF1a/RhoA/ROCK-associated cell invasion response to hipoxia (Gilkes et al. 2014). For instance, it has been recently

described that in hepatocellular cancer, hypoxia-induced upregulation of HIF1a/RhoA/ROCK induce cytoskeletal remodelling by regulating the binding CAPZA1 to F-actin via PIP2 (D. Huang et al. 2019). Similarly, hypoxia-induced galectin-3 promotes RhoA-dependent motility in non-small cell lung cancer (Kataoka et al. 2019). On the other hand, hypoxia can also induce the PI3K/Akt/mTOR/HIF-1α pathway which results in tumor cell migration and invasion in glioblastoma (W. Huang et al. 2018). Nevertheless, it remains unclear how hypoxia induces up-regulation of these pathways and how ODZ1 articulates with them to enhance tumor cell migration.

4.4. ODZ1 hypoxia-related epigenetic regulation

It was recently proposed that teneurins, and specifically ODZ1, might be part of an up- and downstream epigenetic regulation (Rebolledo-Jaramillo and Ziegler 2018). Moreover, it has been also described that the hypoxic microenvironment may influence the tumor DNA methylation status. Consistent with these observations, the results presented here show an increased overall methylation in glioblastoma cells under hypoxic conditions. However, when analysed specifically, the CpG sites within the *ODZ1* promoter were hypomethylated under hypoxia. In support of our findings, this mechanism has been already described. Thienpont et al. have shown that hypoxia-induced loss of TET activity increases hypermethylation at gene promoters in glioblastoma (Thienpont et al. 2016) and it has also been described that hypoxia increases the expression of a gene involved in the control of cell migration through promoter hypomethylation (Shi et al. 2015).

Ziegler et al. also proposed that teneurin epigenetic regulation may have a role in teneurin expression in cancer. Although the *ODZ1* promoter lacks a canonical CpG island (Esteller 2002; Unruh et al. 2019; Ando et al. 2019), it does contain an aggregation of CpG sites. The results presented here shows that this CpG site (cg24761295) is differentially methylated in hypoxic and normoxic glioblastoma cells, being hypomethylated in hypoxic microenvironments. In line with this, in the TCGA database this site is commonly hypomethylated in glioblastoma. Promoter methylation status controls gene expression through different mechanisms including histone binding or recruiting 5mC-dependent blocking proteins (S. B. Baylin et al. 2001; Stephen B Baylin 2005; Herman and Baylin 2003). Importantly, hypermethylation of *ODZ1* promoter or mutation of cg24761295

blocked the activating effect of hypoxia on ODZ1 expression, further confirming the role of this CpG site in regulating *ODZ1* promoter activity.

Cumulatively, these results suggest that *ODZ1* promoter methylation has a significant role in controlling the levels of this tumor invasion-associated gene in glioblastoma cells under hypoxia. Consistently, the 1.4 kb promoter fragment upstream of the transcription start site cloned in a reporter plasmid contained hypoxia-responsive elements, including cg24761295 at the 3' end of the promoter. Of note, cg24761295 has been shown to be hypomethylated in metastatic melanoma and colon cancer cells, compared with the primary counterparts (Vizoso et al. 2015) supporting the association of this CpG site with invasiveness. In line with this, methylation of CpG sites reduced the activity of the *ODZ1* promoter, being cg24761295 of major functional relevance. Although these findings support the role of hypoxia-induced epigenetic regulation of *ODZ1*, the contribution of other transcriptional pathways associated with the cellular response to hypoxia should be addressed in future studies. Taking into account the major role of HIF1a in the tumor cell response to hypoxia (Monteiro et al. 2017), it is conceivable that ODZ1 pathway may be regulated at least in part by HIF1a. Furthermore, other pathways may also be involved. For instance, it has been described that hypoxia-induced IL6 induce STAT3 phosphorylation (Xue et al. 2016). Moreover, STAT3 activation by hypoxia has been reported in non-tumor cells (C. Xu et al. 2017). Interestingly, by using in-silico tools, we have identified two potential HIF-binding sequences (GCGTG and CCGTG) and a STAT consensus site (TTCTTTGAA) within 1.5 kb from the transcription start site of the ODZ1 promoter.

It has also been proposed that hypoxia-dependent pathways and epigenetic alterations may contribute together to the regulation of the epithelial-to-mesenchymal transition giving rise to cells with migratory capacity (Yeo et al. 2017).

The results presented here suggest that the tumor hypoxic microenvironment plays a significant role in activating the ODZ1-mediated migration of glioblastoma cells. This activation pathway is controlled, at least in part, by the methylation status of the ODZ1 promoter being of special relevance a CpG site (cg24761295) proximal to the transcription start site. Thus, ODZ1 expression within the hypoxic tumor microenvironment may serve

as a prognostic marker and therapeutic target for the clinical management of glioblastoma patients.

4.5. Limitations

Two main limitations have been identified within the present project. First, the small number of patients enrolled in the study was probably a determinant factor leading to negative results. In the context of the intra- and intertumoral high heterogeneity in glioblastoma and the several clinical variables acting as potential confounding factors, a sample would reduce the power of the analysis leading to type II error.

On the other hand, the conclusions on ODZ1 epigenetic regulation are mainly based on results from *in vitro* experiments in primary glioblastoma cell lines. This have to be taken into account when extrapolating these results and further *in vivo* validation might be one of the next steps.

4.6. Future directions

Taking into account that the first description of teneurins is relatively recent (S Baumgartner et al. 1994; Levine et al. 1994), there are still several gaps in our knowledge on their role and regulation in cancer, and specifically in glioblastoma.

First, the results presented here raised several unanswered questions regarding the regulation of ODZ1 signaling. It has been described here the hypoxia-dependent epigenetic regulation of ODZ1 expression. However, it remains unknown the contribution of others regulating pathways to ODZ1 overall expression in glioblastoma cells. Considering the major role of HIF1a in the tumor cell response to hypoxia, it is conceivable that HIF1a signaling could also play an important role on ODZ1 transcriptional regulation.

On the other hand, further validation of the ODZ1 clinical prognostic value is still necessary. Hitherto, ODZ1 has been associated with reduced overall and progression free

survivals (Talamillo et al. 2016), however it remains unclear if ODZ1 expression entails a poor prognosis regardless of other tumor molecular features. Besides, the role of hypoxia-dependent methylation of the ODZ1-promoter needs to be assessed *in vivo* and, ultimately, in the clinical setting.

Finally, the role of other members within the teneurin family in glioblastoma pathogenesis remains to be elucidated. Although they have been involved in tumorigenesis in other types of cancer, their involvement in glioblastoma remains unknown. Furthermore, ODZ1 epigenetic regulation and its association with the hypoxic tumor microenvironment haven´t been described in other types of cancer. For instance, in metastatic cancer to the brain, where ODZ1 differentially methylation might play a significant role, the presence of these pathogenic mechanisms could help to better understand the metastasis process.

5. CONCLUSIONS

ODZ1 expression is associated with the CE rim width in a directly proportional relationship. Although the biological processes underlying the CE rim width remain unclear, this marker might be related with tumor prognosis and invasion, as previously described.

The results presented here suggest that ODZ1 increased expression is more frequent in glioblastomas with distant tumor recurrences, nevertheless, this differences didn´t reach statistical significance.

ODZ1 expression is increased in glioblastoma cells within the PIMO positive regions, representing the tumor hypoxic microenvironment. Furthermore, these findings were confirmed *in vitro*, as glioblastoma cells cultured under hypoxic conditions showed an increased ODZ1 expression. To the best of our knowledge, hypoxia is the first extracellular stimulus capable of activating ODZ1 signaling.

Hypoxia-induced invasion is mediated, at least in part, by upregulation of the ODZ1 pathway. Therefore, ODZ1 may contribute to tumor cell migration from the hostile hypoxic microenvironment to more hospitable niches.

ODZ1-related invasion is epigenetically regulated by hypoxia-dependent hypomethylation of the ODZ1 promoter. This is the first description of epigenetic regulation in the teneurin family.

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APPENDIX

Appendix A. Conference contributions and publications

Peer-reviewed publications

Velásquez C, Mansouri S, Gutiérrez O, Mamatjan Y, Mollinedo P, Karimi S, Singh O, Terán N, Martino J, Zadeh G, Fernández-Luna JL. **Hypoxia Can Induce Migration of Glioblastoma Cells Through a Methylation-Dependent Control of ODZ1 Gene Expression.** Front Oncol. 2019 Oct 10;9:1036. doi: 10.3389/fonc.2019.01036. eCollection 2019.

Velásquez C, Mansouri S, Mora C, Nassiri F, Suppiah S, Martino J, Zadeh G, Fernández-Luna JL. **Molecular and Clinical Insights into the Invasive Capacity of Glioblastoma Cells.** J Oncol. 2019 Jul 29;2019:1740763. doi: 10.1155/2019/1740763. eCollection 2019.

Conferences contributions

Hypoxia-induced ODZ1-promoter hypomethylation can regulate Glioblastoma cell invasion
Velásquez C, Mansouri S, Gutiérrez O, Mamatjan Y, Mollinedo P, Karimi S, Singh O, Terán N, Martino J, Zadeh G, Fernández-Luna JL.
Poster presentation at **XI Simposio GEINO.**
Madrid, Spain. 2019

GENE-05. Upregulation of ODZ1-mediated invasion in the hypoxic tumor microenvironment in glioblastoma
Velasquez C, Mollinedo P, Mansouri S, Martino J, Mamatjan Y, Talamillo A, Karimi S, Terán N, Aldape K, Zadeh G, Fernández-Luna JL
Poster presentation at **23rd Annual Scientific Meeting and Education Day of the Society for Neuro-Oncology (SNO)**
New Orleans, USA. 2018

Association of hypoxia with ODZ1, a novel invasion marker in glioblastoma
Velasquez C, Mollinedo P, Mansouri S, Martino J, Talamillo A, Karimi S, Mamatjan Y, Terán N, Zadeh G, Fernández-Luna JL
Poster presentation at **EANS2018, Congress of the European Association of Neurosurgical Societies (EANS)**
Brussels, Belgium. 2018