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Regulation of Wnt Signaling by HOXA9 in Glioblastoma: Mechanistic, Prognostic and Therapeutic Insights

Tese de Mestrado
Mestrado em Ciências da Saúde

Trabalho efectuado sob a orientação do
Doutor Bruno Marques Costa

Maio de 2013

Agradecimentos

"Aqueles que passam por nós, não vão sós, não nos deixam sós.
Deixam um pouco de si, levam um pouco de nós." – Antoine Saint-Exupéry

Agradeço a honra de terem "passado" por mim, durante esta caminhada, pessoas que partilharam comigo o seu saber e a quem quero dar o meu mais sentido agradecimento.

Começo por endereçar os meus agradecimentos ao meu orientador, o Dr. Bruno Costa, pelas oportunidades: de trabalhar no seu grupo, de crescer profissionalmente e pessoalmente, de errar, de perguntar. Mas também, pela paciência, pelos ensinamentos e pela confiança que depositou em mim e no meu trabalho, do qual espero sinceramente que se possa orgulhar.

À Marta Pojo, por me ter convencido a ficar, por ter acreditado na minha ideia e se ter envolvido nela, ajudando-me a construí-la, e sem a qual parte deste trabalho não teria sido feito. Pelo olhar crítico, pelo incentivo e pelas conversas e discussões que ajudaram a tornar certos factos mais claros. À Tatiana, pela disponibilidade, pelo incentivo e apoio, pela palavra amiga. Mas sobretudo, pela paciência e compreensão! À Ana Oliveira quero agradecer pela ajuda, crucial na recta final deste trabalho, e por ter abdicado das suas tarefas em prol das minhas.

Às restantes meninas do grupo, Carina, Joana e Ana Xavier pela sempre disponibilidade, pelas conversas, mas também pelos momentos de descontracção. Estes agradecimentos alargam-se a todas as pessoas que, mesmo que brevemente, fizeram parte deste grupo. À Márcia, ao Filipe, à Marta Pereira, à Nelma, à Ângela e a todos os restantes elementos do laboratório, o meu muito obrigada pelos risos que geraram boa disposição.

A todos os meus professores do curso e do mestrado mas também a todos aqueles que pararam para me ensinar porque hoje sou o resultado de muito daquilo que vocês me ensinaram. Aos meus colegas/amigos, porque sem vós as coisas teriam sido bem mais difíceis.

Aos meus pais e às minhas irmãs que viveram comigo este passar das páginas dos artigos, livros e cadernos. A eles que aguentaram e compreenderam tudo, que conheceram os dias riscados, as dobras do tempo e me deram sempre coragem e alento para ir mais longe fossem quais fossem os obstáculos. Ao Sandro que, tal como eles, suportou tudo, me apoiou, me incentivou e me ajudou, ouvindo-me quantas vezes fossem necessárias para acalmar a minha alma angustiada e ansiosa pela responsabilidade deste trabalho. À minha família mais próxima e a todos aqueles que acreditaram em mim e que contribuíram para o meu sucesso e crescimento como pessoa. Espero não vos desiludir.

A todos, o meu sincero MUITO OBRIGADA.

Summary

Glioblastomas (GBMs) are the most common and malignant type of gliomas, a heterogeneous group of neoplasias that account for the majority of primary brain tumors. Clinically, GBMs present one of the highest cancer-related average years of life lost with a median survival of ≈15 months. Moreover, while the clinical outcome of GBM patients is particularly unpredictable, patients are equally treated with a standardized approach, consisting of surgical resection, radiotherapy and chemotherapy, mostly with temozolomide. To overcome this problem of a universal therapy to strikingly heterogeneous tumors, the identification of molecular prognostic factors that allow the stratification of subgroups of GBM patients is crucial. Nowadays, none of the suggested prognostic factors is sufficiently satisfactory to be used in clinics. The identification of new biomarkers, together with the understanding of their functional roles in GBM, may be a first step in the design of novel therapeutic approaches. Our group has recently shown that *HOXA9* overexpression is associated with poor prognosis in GBM patients. Functionally, overexpression of *HOXA9* in GBM *in vitro* models is associated with pro-proliferative and anti-apoptotic features. Since HOXA9 is a transcription factor, its targets can be the true biological effectors of its aggressiveness. Bioinformatics analysis of HOXA9 transcriptome using GSEA revealed that it is enriched to several components of the Wnt pathway. Aberrant activation of Wnt signaling has been reported in several tumors and associated with cancer stem cell-like features, including increased proliferation and therapy resistance. By chromatin immunoprecipitation, we found that HOXA9 binds to the promoter region of *WNT6*, a key gene of this pathway. In addition, we demonstrated that *WNT6* is also regulated by methylation in GBM, and that *WNT6* DNA methylation levels are associated with *HOXA9* expression in our GBM *in vitro* models. Additionally, we found that *WNT6* is overexpressed in GBMs, but not low-grade gliomas nor normal brain. Moreover, high *WNT6* expression was particularly frequent in the proneural GBM subtype. Importantly, we provide the first evidence of the clinical prognostic value of *WNT6* in GBM, implicating high levels of *WNT6* as a novel independent negative prognostic marker. Preliminary data of pharmacological inhibitions of Wnt signaling suggests that the Wnt pathway can be an attractive therapeutic target directed to HOXA9-positive GBMs. Together, our findings provide mechanistic, prognostic and therapeutic insights into the role of Wnt pathway in GBM.

Resumo

Os Glioblastomas (GBMs) são o glioma mais comum e maligno, sendo um grupo heterogéneo de neoplasias que conta com a maioria dos tumores primários do cérebro. Clinicamente, os GBMs apresentam uma das maiores médias de anos de vida perdidos devido ao cancro, tendo como média de sobrevivência ≈15 meses. Embora a resposta clínica destes pacientes seja particularmente imprevisível, todos são tratados com uma terapia padrão, que consiste na ressecção cirúrgica, radioterapia e quimioterapia, maioritariamente feita com temozolamida. Para ultrapassar este problema duma terapia universal para tumores altamente heterogéneos, é crucial a identificação de factores moleculares de prognóstico que permitam a estratificação dos pacientes em subgrupos. Actualmente, nenhum dos fatores de prognóstico sugeridos é suficientemente satisfatório para ser usado na clínica. A identificação de novos biomarcadores, juntamente com a compreensão dos seus papéis em GBMs, pode ser o primeiro passo no desenho de novas abordagens terapêuticas. Recentemente, o nosso grupo mostrou que a sobre-expressão do *HOXA9* está associada a um pior prognóstico destes pacientes. Funcionalmente, esta sobre-expressão, em modelos *in vitro* de GBM, está associada a propriedades pró-proliferativas e anti-apoptóticas. Uma vez que o HOXA9 é um fator de transcrição, os seus alvos podem ser os verdadeiros efetores biológicos da sua agressividade. Análises bioinformáticas do transcriptoma do HOXA9, usando o GSEA, revelaram que este está enriquecido para diversos componentes da via Wnt. A ativação aberrante desta via foi reportada em cancro e associada a um fenótipo semelhante aos das células estaminais, incluindo um aumento da proliferação e resistência à terapia. Por imunoprecipitação da cromatina, vimos que o HOXA9 se liga à região promotora do *WNT6*, um gene crucial na via Wnt. Mais ainda, demonstramos que o *WNT6* é também regulado por metilação do DNA em GBM. Além disso, nos nossos modelos *in vitro* de GBM, os seus níveis de metilação estão associados aos níveis de expressão do *HOXA9*. Adicionalmente, vimos que o *WNT6* está exclusivamente sobre-expresso em GBM. Além disso, altos níveis de expressão do *WNT6* são particularmente frequentes no subtipo proneural dos GBMs. Mais importante ainda, fornecemos a primeira evidência do valor clínico de prognóstico do *WNT6* em GBM, implicando os seus altos níveis como um novo marcador de prognóstico negativo independente. Resultados preliminares da inibição farmacológica da via de Wnt sugerem que esta via pode ser um alvo terapêutico atrativo direcionado a células positivas para o *HOXA9*. Em conjunto, os nossos resultados fornecem uma perspectiva mecanística, prognóstica e terapêutica sobre o papel da via Wnt em GBMs.

Contents

Abbreviations list

5-Aza	5-Aza-2'-deoxycytidine
AD	Anno Domini
Akt	v-Akt Murine Thymoma viral oncogene homolog
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
ASCL1	Achaete-Scute Complex homolog 1 (Drosophila)
ATCC	American Type Culture Collection
BBB	Brain Blood Barrier
BC	Before Christ
BCR	Biospecimen Core Resource
bp	Base Pair
BSA	Bovine Serum Albumin
CCND1	Cyclin D1
CCND2	Cyclin D2
CDKN2A	Cyclin-Dependent Kinase inhibitor 2A; Arf
cDNA	Complementary DNA
CHI3L1	Chitinase 3-like 1
ChIP	Chromatin Immunoprecipitation
CK4	Cytokeratin 4
CNS	Central Nervous System
CpG	Cytosine-phospho-Guanine
CSC	Cancer Stem-Cell
CTNNB1	β-catenin
DAPI	4',6-diamidino-2-phenylindole
DCX	Doublecortin
df	Dilution Factor
DLL3	Delta-like 3
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
Dvl	Dishevelled
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine Tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ES	Enrichment Score
EZH2	Enhancer of Zeste Homolog 2
FBS	Fetal Bovine Serum
FDR	False Discovery Rate

FOXA2	Forkhead box A2
Fwd	Forward
Fzd	Frizzled family receptor
GABRA1	Gamma-Aminobutyric Acid (GABA) A Receptor, Alpha 1
GATA6	GATA binding protein 6
GBM	Glioblastomas Multiforme
GSEA	Gene Set Enrichment Analysis
GSK3	Glycogen Synthase Kinase 3
H3	Histone 3
HCL	Hydrochloric acid
hGUS	Human Glucuronidase
HOX	Class I Homeobox
HOXA10	Homeobox A10
HOXA9	Homeobox A9
IC_{50}	Half Maximal Inhibitory Concentration
IDH1/2	Isocitrate Dehydrogenase 1 and 2
IgG	Immunoglobulin G
IWR-1	4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-Benzamide
KPS	Karnofsky Performance Status
KRAS	v-Ki-ras2 Kirsten Rat Sarcoma viral oncogene homolog
Lef	Lymphoid Enhancer-binding Factor
LGG	Low-Grade Glioma
LOH	Loss of Heterozygosity
MAPK	Mitogen-Activated Protein Kinase
MET	Met proto-oncogene (hepatocyte growth factor receptor)
MGMT	O-6 methylguanine-DNA methyltransferase
MIT	Massachusetts Institute of Technology
MMP7	Matrix Metallopeptidase 7
MSCV	Murine Stem Cell Virus
MSP	Methylation-Specific PCR
mTOR	Mechanistic Target Of Rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MYC	v-Myc Myelocytomatosis viral oncogene homolog
NaCl	Sodium Chloride
NCDs	Noncommunicable Diseases
NEFL	Neurofilament, light polypeptide
NF1	Neurofibromin 1
NKX2-2	NK2 homeobox 2
NP-40	Nonyl Phenoxypolyethoxylethanol
OLIG2	Oligodendrocyte Lineage Transcription Factor 2
OS	Overall Survival
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PDGFRA	Platelet-Derived Growth Factor Receptor, Alpha polypeptide
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, Catalytic subunit Alpha
PMS	Phenazine Methosulfate
PMSF	Phenylmethylsulfonyl Fluoride
PTEN	Phosphatase and Tensin homolog
qMSP	quantitative MSP
qPCR	quantitative PCR
qRT-PCR	quantitative RT-PCR
RB	Retinoblastoma
Rev	Reverse
RNA	Ribonucleic Acid
RTK	Tyrosine Kinase Receptor
RT-PCR	Reverse Transcription-PCR
SDS	Sodium Dodecyl Sulfate
SFRP	Secreted Fzd-Related Protein
shRNA	short-hairpin RNA
siRNA	small interfering RNA
SLC12A5	Solute Carrier family 12 (potassium/chloride transporter), member 5
SNP	Single-Nucleotide Polymorphism
SOX	SRY (sex determining region Y)-box
SP5	Sp5 transcription factor
STAT	Signal Transducer and Activator of Transcription
SYT1	Synaptotagmin I
TBP	TATA box Binding Protein
Tcf	Transcription Factor family
TCF4	Transcription Factor 7-like 2 (T-cell specific, TCF7L2)
TCGA	The Cancer Genome Atlas
TGF-β	Transforming Growth Factor, beta
TIMP-3	Tissue Inhibitor of Metalloproteinases-3
TMZ	Temozolomide
TP53	Tumor Protein p53
WHO	World Health Organization
WIF1	WNT Inhibitory Factor 1
Wnt	Wingless-type MMTV integration site family

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1. INTRODUCTION

1 | Introduction

Cancer is not a new disease. Indeed, there are some evidences of the existence of this disease in Egyptian mummies, and even in the skeleton of prehistoric animals, long before the presence of men on Earth [1]. The first description of cancer, specifically of breast cancer, was written in a papyrus around 3000 BC by Edwin Smith concluding that there was no cure for it [1]. Hippocrates, in the 4th century BC, compares cancer to a crab in its spread all over the body and in its persistence using the term carcinoma to describe tumors, from the Greek "Karkinos" that means crab [1]. After Edwin S. and Hippocrates, a history of approximately 3000 years was written by all pioneers in oncology, such as Aulus Celsus (25 BC-50 AD), Claudius Galen (130-200) and Theodoric (1205-1296), that notwithstanding their short intervention contributes somewhat to the detection and diagnosis of cancer [1]. Between 1500 and 1750, cancer was systematically and intensively studied leading to the description of the tumor pathology [2]. After that, and using the microscope, R. C. Virchow (1821-1902) recognized gliomas as infiltrating primary brain tumors [3], which in turn had been first described in 1614 [2]. At the same time, emerged the microscopic definition of benign and malignant cells, and the idea that epithelial tumors arise from pre-existing epithelial cells [3]. Subsequently, it was established that, depending on the organ affected, cancer is completely different, and presents distinct morphologies, clinical manifestations and, more importantly, different prognosis [3].

Nowadays, we know that not only cancer is completely different between organs, but its underlying causes also differ enormously between cancer types. A great percentage of cancers (90-95%) are sporadic being for most a disease of old age [4,5]. However, it was recognized that some inherited mutations can be passed from one generation to the next and can cause rare forms of cancer, such as retinoblastoma [6]. Rapid changes in our lifestyle (reduced intake of fruits and vegetables, increased consumption of calories and fat, genotoxic exposures, physical inactivity and the injurious excessive use of alcohol), together with changes in the environment and increases in the life expectancy, have outpaced the ability of our genome to adapt to these alterations, resulting in a genetically predisposed population to cancer [7,8]. In 2008, it was estimated that 63% of the 57 million global deaths, corresponding to 36 million of death, were due to noncommunicable diseases (NCDs), of which 21% (7.6 million) were due to cancer [7,8]. In Portugal, specifically, NCDs are estimated to account for 86% of all deaths, of which 26% are due to cancer [7,8]. Despite all efforts, namely in cancer treatment strategies (surgery, radiotherapy and chemotherapy), it is projected that the annual global cancer deaths will

increase to 13 million in 2030, while deaths associated to infectious diseases are projected to decrease drastically over the next 20 years as a consequence of better sanitation, antibiotics use and vaccination [7,8]. Therefore, a growing interest in cancer, the 2nd leading cause of death after cardiovascular diseases, was observed in the past century [7,8]. This ultimately leads to increasing efforts to produce a firm body of knowledge around this dismal disease.

Cancer is not a single and isolated disease. Quite differently, several distinct tumor cells that interact with one another form a complex tissue with abnormal growth and with the ability to recruit normal cells that actively participate in the tumorigenesis and contribute to the development of some critical characteristics of the tumor cells – the hallmarks of cancer [Figure 1.1]. Ten hallmarks were proposed by Hanahan and Weinberg in 2011 [9], and include the capability of the tumor cells to (i) evade growth suppressors, (ii) sustain proliferative signaling, (iii) resist to cell death, (iv) avoid the immune destruction, (v) enable the replicative immortality, (vi) induce angiogenesis, (vii) activate invasion and metastasis, and to promote (viii) the deregulation of the cellular energetics, (iv) tumor inflammation and (x) genome instability and mutation.

Simplifying, tumor cells arise from a healthy cell that acquires a first mutation, which predisposes this cell to proliferate excessively, and next this cell acquires a second, a third, and subsequent mutations that lead to uncontrolled growth. In this case, the severity of the cancer is based on the type of mutations acquired, and also on which gene the mutations occurrred. However, a broad vision of the basis of cancer contemplates not only DNA mutations, but also copy number aberrations, chromosomal rearrangements, and DNA and histone epigenetic modifications that lead ultimately to alterations in the gene expression profile [10]. These, in turn, are responsible for the imbalance in the homeostasis of the cells. Recent studies have suggested that global genome-wide hypomethylation occurs early in the tumorigenic process and leads to chromosomal instability and oncogene activation [11-13]. On the other hand, the hypermethylation of specific promoter regions, typically in tumor suppressor genes, occurs during tumor progression [12,13].

In the last decades, several molecular studies have identified critical genetic alterations that affect many key signaling pathways, such as KRAS, TGF-β, Sonic hedgehog, notch and wnt signaling, which are responsible for the regulation of typical cancer phenotypes, namely modifications in the cell cycle, migration, proliferation, survival, angiogenesis, invasion, and apoptosis [14-18]. A better understanding of cancer as a complex and dynamic tissue is crucial to concentrate our efforts in a more focused therapy.

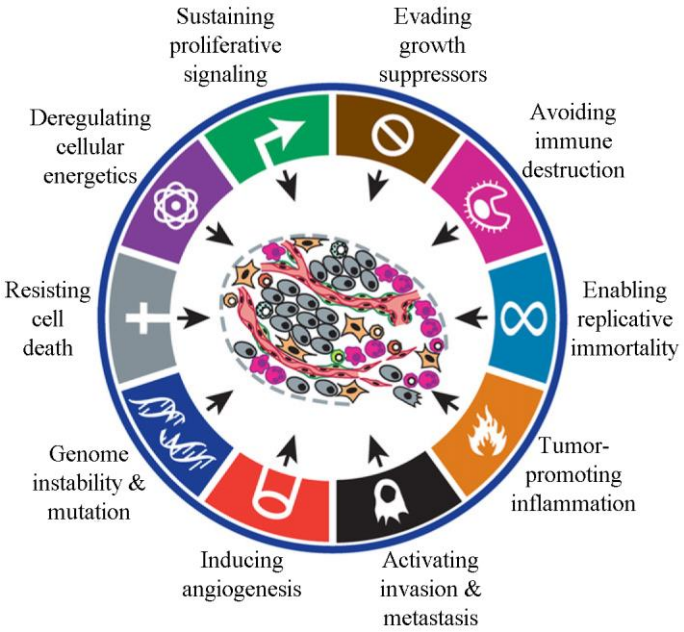

Figure 1.1| Hallmarks of cancer proposed by Hanahan and Weinberg.
Cancer cells are characterized by several capabilities such as sustained proliferation, immortality, invasion, angiogenesis, resistance to cell death and deregulation of the metabolism. (Adapted from Hanahan and Weinberg, 2011) [9]

1.1| Primary Brain Tumors

Primary brain tumors include all abnormal mass of cells that start in the brain, in opposition of metastatic tumors which spread into this region [19]. Among the extensive list of known tumor types, primary brain and central nervous system (CNS) tumors present a low incidence rate (only 2% of all primary tumors) [20], which does not stop them from having one of the highest mortality rate and rank first in average of years of life lost among all tumor types [21]. In 2008, the world estimated incidence of tumors from brain and nervous system for both sexes and all ages lies in approximately 238000 new cases [Figure 1.2]. In turn, approximately 175000 brain tumor-related deaths worldwide were estimated for the same year (for both sexes and all ages) [Figure 1.2]. However, the worldwide age-standardized incidence rate between sexes is different, being higher for males [22]. In fact, an estimated incidence of 3.9 new cases for males, against 3.2 in females, per 100000, was reported. Moreover, the estimated mortality for these tumors was 3 per 100000 for males, as opposed to 2.2 in females [22].

Geographically, the highest rates of new cases and deaths due to these tumors are observed in developed countries (Europe, North America, etc), suggesting that in developing countries the incidence is probably underestimated [23]. However, the relation between ethnicity

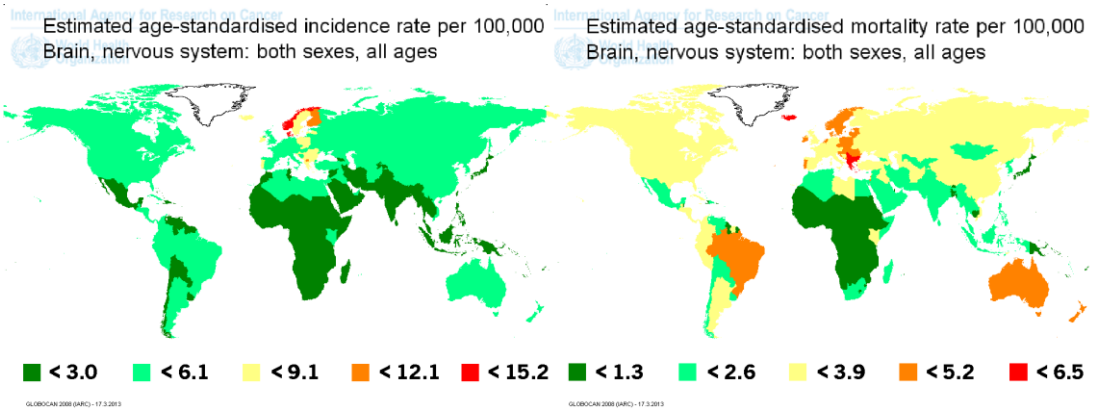

Figure 1.2| Primary brain and central nervous system tumors global estimated age-standardized incidence (left) and mortality (right) rates adjusted to the World Standard Population (all ages and per 100000 persons per year).
Highest rates are observed in developed countries. Portugal ranks among the 20 with worst rates worldwide. (Adapted from Globocan, 2008; http://globocan.iarc.fr) [22]

and brain tumor development susceptibility cannot be excluded. Such remark highlights the importance of the availability of easy and efficient diagnoses to prevent and restrain this deadly disease even in areas with lower medical support [22].

In Portugal, specifically, the estimated age-standardized incidence rate of brain and nervous system tumors, for both sexes and all ages, is 6.35 per 100000 and the estimated age-standardized mortality rate is 4.31 per 100000 [Figure 1.2]. These reported values place Portugal as one of the European countries with highest rates and among the 20 worst worldwide [22].

To date, several studies have tried to find an environmental epidemiology for primary brain tumors, but therapeutic X-irradiation is the only factor unequivocally linked to an increase of brain tumor risk. Nevertheless, other studies tried to correlate it with certain occupations, like physicians, firefighters, farmers, rubber manufacturers, among others, as well as socioeconomic status and education level, diet, smoking, electromagnetic fields and cell phones exposures, although none were conclusive and controversy remains [23].

1.2| Gliomas

After several decades of research, the cellular origin of brain and CNS tumors remains enigmatic [24]. The central nervous system is composed mainly by two types of cells: neurons and glial cells [Figure 1.3]. Based on their morphology, glial cells are classified as astrocytes, oligodendrocytes, ependymal cells, radial glial cells and microglial cells [25]. In opposition to most neurons that acquire their terminally differentiated status after fetal life, glial cells retain their proliferation capability during gestational and postnatal life [24,25]. Thus, is not irrational to

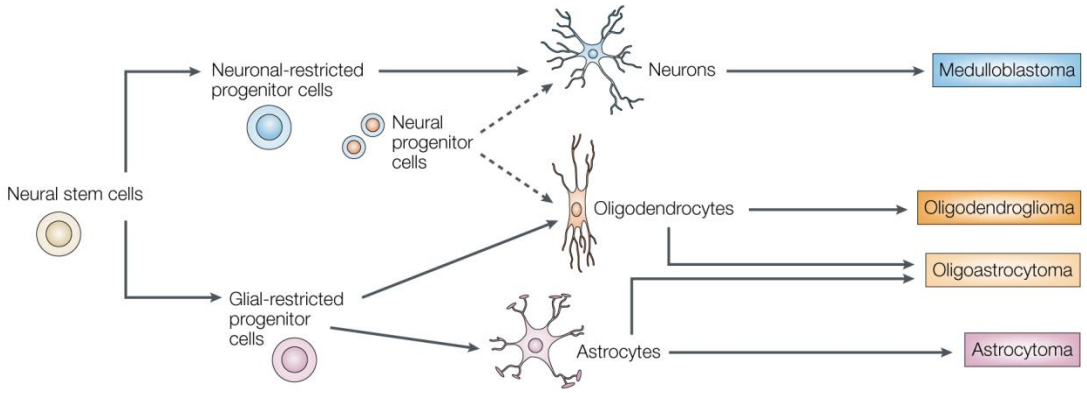

Figure 1.3| CNS tumor classification based on cell morphology.
CNS tumors are classified based on their morphological similarity to neurons (medulloblastoma), oligodendrocytes (oligodendrogliomas), astrocytes (astrocytoma) or to a mixture of astrocytes and oligodendrocytes (oligoastrocytoma). (Adapted from Zhu and Parada, 2002) [25]

raise the hypothesis that most brain and CNS tumors in adults may have their origin in glial cells. Taking this into account, and for simplicity, this heterogeneous group of tumors may be classified as gliomas or nongliomas [20,24]. Nongliomas comprise mainly benign tumors, such as meningiomas, the most common benign brain tumor, and pituitary adenomas, but also malignant tumors, such as medulloblastomas. On the other hand, gliomas are characterized by diffuse infiltration of white matter tracts, making these tumors highly malignant and hampering their surgical removal. Notably, even benign tumors can be fatal if excision and radiation therapy are not possible, due to the excessive cellular growth inside the closed skull space [20].

Traditionally, gliomas are histologically divided based on the microscopic similarities with glial cells [Figure 1.3] as astrocytomas (accounting for 75.1% of all gliomas), oligodendrogliomas (9.2%), oligoastrocytomas (also known as mixed gliomas, with 10.1%) and ependymomas (5.6%) [20,24-27]. Aside from being the most common glioma, astrocytomas include the most malignant type of brain tumors. Different grading systems have been proposed and used for this type of gliomas, resulting in a significant confusion [20]. The World Health Organization (WHO) grading classification has been, since 1993, the most reliable and meaningful indicator for the patient outcome. This system divides astrocytomas in 4 main grades [Table 1.1] according to their localization and histopathological features, such as increased cellularity, mitoses, endothelial proliferation and necrosis [25,27,28]. Grade I comprehends benign tumors, such as pilocytic astrocytomas, while grade II to IV include the malignant astrocytomas. Grade II, clinically named as diffuse astrocytoma, is characterized by diffuse infiltrating tumors and associated with 5 to 15 years of survival. Although their quite slow growth, they present a tendency to recur after resection and to progress to grade III or IV astrocytomas [25,27,28]. Grade III astrocytomas present a rapid cellular growth that, despite the usage of more aggressive adjuvant treatments,

Table 1.1| Astrocytomas WHO grading system.
Grade I are benign tumors while the grade II-IV are malignant tumors. The malignancy increases along the grades being glioblastoma one of the most malignant astrocytoma. [26]

Astrocytomas	I	II	III	IV
Subependymal giant cell astrocytoma	•			
Pilocytic astrocytoma	•			
Pilomyxoid astrocytoma		•		
Diffuse astrocytoma		•		
Pleomorphic xanthoastrocytoma		•		
Anaplastic astrocytoma			•	
Glioblastoma				•
Giant cell glioblastoma				•
Gliosarcoma				•

patients often exhibit a survival time less than 3 years [27-29]. Finally, grade IV includes the most malignant type of astrocytoma, namely glioblastoma multiforme (GBM; described in detail in section 1.3) [Table 1.1].

1.3| Glioblastoma Multiforme (GBM)

Glioblastoma multiforme, accounting for 50% of all gliomas, are the most common (25%) and most malignant type of all primary tumors of the CNS [27-29]. Although GBMs can occur in all age groups, they present an average age of diagnosis of 55 years [20]. They are classified with respect to their clinical history [30] as primary or secondary GBM [Figure 1.4]. Primary (or *de novo*) GBM are more frequent (95% of all GBM) and arise rapidly (<3 months) without a previous evidence of a histopathological or clinical history of a less malignant tumor. In opposition, secondary GBM (5%) progress slowly, generally over a period of 5 to 10 years, from a lower grade lesion [25,28,29,31,32]. Patients with GBM are frequently asymptomatic until the tumor reaches a massive size, and the onset of symptoms begins abruptly due to the mass effect (increased intracranial pressure) [20,33].

Microscopically, the histopathological features of GBM comprise increased cellularity, nuclear polymorphism and atypia, frequent mitoses and vascular thrombosis. Moreover, tumor necrosis is typically observed in the central portion of the tumor, while microvascular proliferation is observed in the margin [34]. Topographically, GBM, as other astrocytomas, are infiltrating and

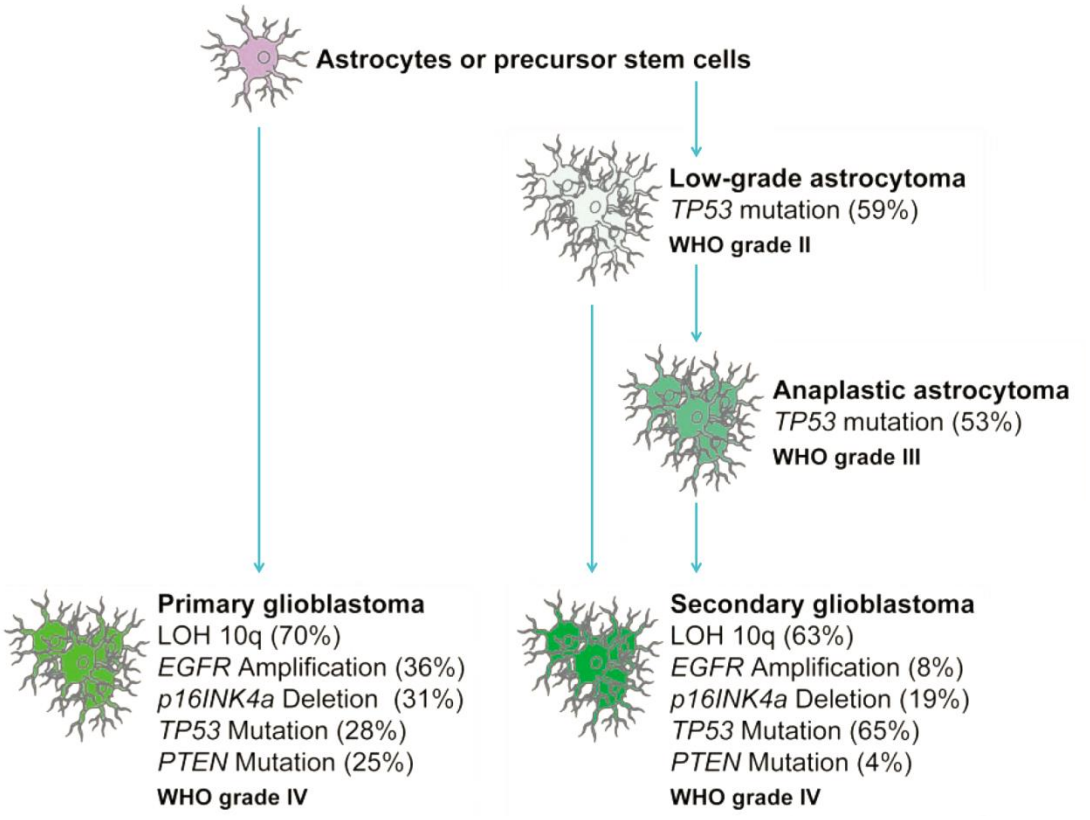

Figure 1.4| Genetic pathways in the evolution of primary (*de novo*) and secondary glioblastoma. Primary GBM arise *de novo* without previous evidence of a less malignant lesion, contrarily to secondary GBM that progress from a lower grade or anaplastic precursor lesion. LOH on chromosome 10 is the most frequent genetic alteration occurring in both primary and secondary GBMs. However, TP53 mutation is a feature more common of secondary GBMs and occurs early in the precursor lesions. LOH=Loss of heterozygosity. (Adapted from Ohgaki and Kleihues, 2007) [30]

diffuse, being a weakly delimited mass with no capsule. This characteristic makes complete surgical resection virtually impossible and relapses are nearly certain [20,26,29]. Additionally, other GBM hallmarks render the treatment of these tumors very difficult, including (i) GBM cells present a rapid growth and cell cycle, (ii) they are resistant to most common conventional therapies, (iii) brain damage may be definitive and significant due to its incapacity to self-repair, (iv) the drug penetration through the brain blood barrier (BBB) is difficult and for most chemotherapeutics is impossible without increasing its concentration and consequently leading to systemic side effects [33].

Since the phase III clinical trial of Walker *et al.* [35], radiotherapy was used as standard care for treatment of GBM after surgical resection. Although rarely effective in curing GBM, it was associated with an improvement in the previous median survival by twice its value. In the early 21th century, temozolomide (TMZ) was introduced in the standard of care of these patients after phase II/III clinical trials of Stupp *et al.* showing that radiotherapy, concomitant TMZ and 6 cycles of adjuvant TMZ improve the survival of GBM patients (≈15 months and 26% of 2-year survival)

comparatively to radiotherapy alone (≈12 months and 10% of 2-year survival) [36,37]. TMZ, as other methylating agents, presents the benefits of being well tolerated with low toxicity for normal cells, but most importantly TMZ has the ability to cross the BBB without the need for high doses [33,36].

Despite the recent evolution in the understanding of molecular and genetic tumorigenic mechanisms and improvements in neuroimaging technology, surgery and adjuvant treatments, patients with GBM exhibit a rapid progression of the disease and still present a median survival of approximately 15 months after diagnosis [20,24,27,36]. Moreover, the 5-year survival rate is less than 3% [23,37]. Among the multitude of clinical variables, an increased age, a low Karnofsky performance status (KPS), an increased severity of neurologic deficits at diagnosis, and the lower extent of tumor resection constitute poor prognostic variables for this deadly disease [20]. This unpredictable prognosis arise from the variability of response to the therapy due to the pathological, clinical and genetic heterogeneity of GBMs, thence the word "multiforme" [20].

1.4| Genetic alterations in GBMs

As referred above, cancer is a disease that contemplates several genetic and epigenetic alterations (DNA mutations, copy number aberrations, chromosomal rearrangements, DNA and histones epigenetic modifications) resulting in alterations in gene expression profiles and ultimately in signaling pathways [38]. GBM, particularly due to its high genetic heterogeneity, presents a huge number of critical alterations that affect many key pathways involved in the regulation of typical cancer hallmarks, such as cell cycle, migration, proliferation, survival, angiogenesis, invasion and apoptosis [38].

Nowadays, it was recognized that primary and secondary GBMs affect distinct age and gender groups and show distinct RNA and protein expression profiles, consequently leading to alterations in different molecular pathways and different responses to radiochemotherapy [30,32,39,40]. Loss of heterozygosity (LOH) on chromosome 10 is the most frequent genetic abnormality (80%) occurring in both primary and secondary GBMs [30,41]. However, while LOH on 10q occur in similar percentages in both types [Figure 1.4], LOH on 10p is practically exclusive of primary GBM (47% vs. 8%). Moreover, the loss of the entire chromosome 10 in primary GBM is a frequent event. In GBMs with partial loss, it was identified at least 3 commonly deleted loci, one of which affecting the tumor suppressor gene *PTEN*, which is also frequently mutated in primary GBMs [30,41,42]. Moreover, LOH on 22q was observed in 82% of secondary GBMs against 41% in primary GBMs [30]. One of the regions affected by this deletion contains

the tissue inhibitor of metalloproteinases-3 (*TIMP-3*) that, curiously, frequently presents high levels of DNA methylation at its promoter in secondary GBMs, leading to the loss of its expression. Additionally, LOH on 19q and 13q is more frequent in secondary GBMs (54% and 38% for secondary against 6% and 12% for primary GBMs, respectively) [30]. In opposition, LOH on 1p is equally frequent (≈15%) in both types of GBMs [30].

Mutations in genes implicated in cell cycle regulation, which allow cells to proliferate uncontrollably, have been frequently identified in GBM [43-45]. The retinoblastoma (RB) pathway [Figure 1.5], which is important in the G1/S transition, is aberrantly inactivated in GBM through

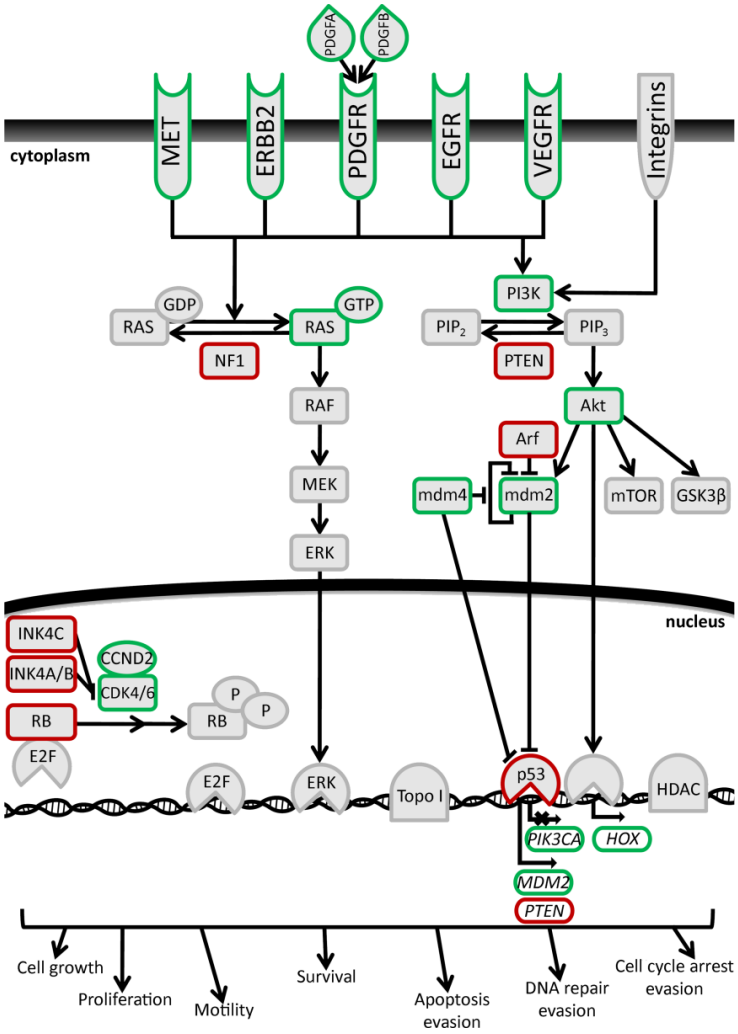

Figure 1.5| Common genetic alterations in GBM affect the RB, p53 and RTKs pathways.
The aberrant deregulation of these pathways in GBM leads to alterations in cell cycle, migration, proliferation, angiogenesis, and apoptosis. Known proto-oncogenes or growth-promoting genes (shown in green), such as *EGFR*, *PIK3CA* (*p110α*) and *AKT*, are activated by mutations, overexpression and amplification, while tumor suppressor genes (show in red), such as *PTEN*, *CDKN2A* (Arf) and *TP53*, are lost or inactivated by mutations, deletions, loss of heterozygosity and epigenetic changes. (Adapted from Gonçalves et al., 2013) [55]

the alteration of several genes and proteins [45]. In GBM, the *RB1* gene, the first described tumor suppressor gene, which codes for a negative regulator of the cell cycle, is frequently mutated [43,44]. The *TP53* gene is also frequently inactivated in GBM [Figure 1.5]. This gene encodes a protein (p53) that also controls the cell cycle by regulating target genes involved in cell cycle arrest, apoptosis and senescence [43]. p53 has been named as the "guardian of the genome" because it leads to the arrest of cells with DNA damage in G1 phase, in order to promote DNA repair processes [46]. However, if irreparable genetic injuries occur, p53 induces cell death by activating the apoptotic machinery [46]. In this context, p53 loss of function may lead, for example, to uncontrolled growth and increased genetic instability [44,47,48].

GBM cells also commonly present a constitutive activation of cell growth signaling pathways [Figure 1.5] by the overexpression of several mitogens and their specific membrane receptors [38,44,49,50]. GBM cells can also acquire mutations in the membrane receptors becoming independent of exogenous growth stimulation, increasing survival and motility [38,44,49-51]. The deregulation of growth factor signaling occurs frequently by the amplification and/or activating mutations of tyrosine kinase receptors (RTKs) [38,44]. These play critical roles in several cellular processes, including cell growth, motility, survival and proliferation, and are tightly controlled by various physiological mechanisms [44]. One of the most described RTK alterations in GBM is the deletion of exons 2-7 of epidermal growth factor receptor (*EGFR*) gene resulting in the loss of the extracellular domain (EGFR-vIII mutant) [44]. RTKs mediate its functions by downstream effectors, namely phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) signaling cascades [51]. Among them, the PI3K pathway is the most described in GBM and is involved in cell growth, proliferation, differentiation, motility and survival [44,51]. The most frequent alterations in this pathways include inactivating mutations and homozygous deletions of the tumor suppressor *PTEN* [44]. In addition to its critical effects in cell growth, motility, and survival, the PI3K pathway seems to be also important in the activation of *HOX* genes, which were recently described to be important for the aggressive phenotype of some GBMs [52-54].

In addition to the most common genetic alterations found in GBM, several other aberrations have been described [55]. For example, recent reports show that the Wnt pathway is aberrantly activated in glioma, including in glioblastoma cells [56-60]. This pathway is particularly relevant due to its association with cancer stem-cells (CSC) and resistance to radiochemotherapy [17,50,56,59].

The development of new and more sophisticated platforms of genome-wide screenings has allowed a more robust identification of the accumulation of genetic and epigenetic alterations. The Cancer Genome Atlas (TCGA) project, for example, was established with the aim of using genome-scale analysis, which include DNA copy number, gene expression, DNA methylation and nucleotide sequencing, to understand the molecular basis of cancer [44]. With this multiplatform profiling and using an integrative analysis, they identified a highly interconnected network of molecular aberrations in GBM that include the three pathways described above: RB, p53 and RTKs (e.g. PI3K pathway) [44]. Interestingly, this integrative analysis showed a statistical tendency to mutual exclusivity for the specific alterations of components within each pathway [Figure 1.5]. Nonetheless, a great percentage of samples harbored aberrations in all signaling pathways [44], which is in agreement with the hypothesis that these pathways are a core prerequisite for GBM disease.

1.5| Molecular prognostic factors in GBMs

As stated above, GBMs are extremely heterogeneous tumors and, although we continue to learn a lot about some of the most common genetic alterations of this tumor, a critical question remains unanswered: how can the treatment of GBM be optimized for the benefit of individual patients?

A great majority of GBM patients present resistant tumors to the current standard care; thus, it is important to identify these patients to preserve them from deleterious, expensive and ineffective therapy, and allow them to be treated with alternative or additional therapies with which they may have a better chance of a stronger and robust response [61]. In the last decade, there was an increasing effort in the identification and use of molecular markers for the evaluation and management of GBMs. The main goal of these molecular markers is to distinguish patients who will probably present a good response to the standard therapy, from those will not. In addition, it is critical to identify biomarkers of drug resistance and their underlying mechanisms to propose new therapeutic targets and consequently new alternatives to the current therapy [61]. Several molecular markers have been described to have clinical potential for the management of GBM, such as the promoter methylation of the gene O-6-methylguanine-DNA methyltransferase (*MGMT*) [62], the mutation of the isocitrate dehydrogenase 1 and 2 [63,64], or the expression of class I homeobox (*HOX*) genes [54]. However, none of these has been widely included in the clinical setting.

The promoter methylation of the *MGMT* gene is so far the most promising biomarker [62,65]. Alkylating agents, like TMZ, methylate the O^6 position of guanine, which triggers apoptosis of the tumor cells. *MGMT* encodes a protein with the capacity to irreversibly transfer this methyl group to its cysteine residue, representing a potential mechanism of resistance to TMZ treatment [61]. Nevertheless, about 50% of GBMs present decreased expression of *MGMT*, rendering these tumors more vulnerable to TMZ. The most frequent mechanism of down-regulation of *MGMT* in GBMs seems to be *MGMT* promoter methylation, which confers therapeutic advantages [66]. Hegi and colleagues observed that 46% of GBM patients with *MGMT*-methylated tumors were alive at 2 years (with an increase of the median overall survival, OS, to 21.7 months) against 23% of patients with *MGMT*-unmethylated tumors (median OS 12.7 months) [62,65]. However, in the same study they saw that the group with unmethylated *MGMT* also presents prognostic benefits with TMZ therapy, although inferior to what observed in *MGMT*-methylated tumors, and explanations for this remain unclear [62]. This beneficial response of *MGMT*-unmethylated tumors suggests that treatment cannot be detained based on the methylation status of *MGMT*-promoter, even more given the well tolerated nature of TMZ.

A few years ago, a genome-scale study identified *IDH1* mutation as highly frequent in younger patients with secondary GBM (median age of approximately 45 years against 60 years for primary GBMs) [63]. Even after adjustment for patient age, this somatic mutation seems to confer a prognostic benefit [63]. In a subsequent study, it was observed a longer prognosis not only for *IDH1* mutations, but also for *IDH2*, with a median OS of 31 months in contrast to 15 months for those without these mutations [64]. Although the association between IDH1/2 mutations and oncogenesis remains unknown, some reports show that these mutations lead to a new pro-oncogenic activity of *IDH1/2* with the production of R(-)-2-hydroxyglutarate, an onco-metabolite [67,68]. In this context, the role of *IDH1/2* mutations as prognostic biomarker is still being defined. The presence of IDH1/2 mutants has potential to help in the diagnosis, since these mutations occur at higher frequencies in secondary GBM (84.6%) and low-grade gliomas (LGGs; 69.2 to 100%) and rarely in primary GBMs (0.05%).

Using an unsupervised hierarchical clustering analysis, Verhaak *et al*. [69] used TCGA data to successfully classify GBM into four subtypes – classical, mesenchymal, proneural and neural – improving and validating previous classifications of GBM [54,70-76]. The classical subtype combine the most common alterations observed in GBM, with 100% of the samples harboring chromosome 7 amplification paired with chromosome 10 loss, 97% presenting high

levels of *EGFR* amplification and 95% showing homozygous deletion targeting *CDKN2A* locus. Moreover, this subtype presents *TP53*, *NF1*, *PDGFRA*, *IDH1*, *RB1*, *CK4* and *CCND2* abnormalities, the last 3 from the RB pathway. However, *NF1* hemizygous deletions, which translates into lower *NF1* expression levels, was the predominant feature of the mesenchymal subtype. Moreover, 87% of mesenchymal samples present co-mutations of *NF1* and *PTEN*. Another characteristic of this class is the expression of mesenchymal markers, such as *CHI3L1* and *MET*. The neural subtype is recognized as the most similar to normal brain tissue and express neuron markers such as *NEFL*, *GABRA1*, *SYT1* and *SLC12A5*. However, samples of the neural subtype were undeniably GBMs regarding their morphology by light microscopy. The proneural subtype was linked to younger age and longer survival. Furthermore, the most common features are *PDGFRA* abnormalities and *IDH1* mutations. Besides this, *TP53* mutations and LOH were more frequent in this subtype. Although less prevalent than in the classical subtype, 54% of this samples also presented amplification of the chromosome 7, paired with chromosome 10 loss [69].

However, the actual clinical relevance of these subtypes, or even of *MGMT* promoter methylation, *IDH1/2* mutations or others prognostic markers, is still controversial, and there is no solid evidence supporting their routine use. Indeed, some studies report no statistical significance between these biomarkers and the prognosis of patients with GBM [53,77].

Recently, *HOX* genes were described as aberrantly expressed in several tumors, including GBMs. *HOX* genes are part of the homeobox genes family, which encodes transcription factors crucial in the development of the embryo [78,79] but also in the postdevelopment regulation [80-83]. In humans, 39 *HOX* genes were identified and divided into 4 clusters (A – D) according to their localization (7p15, 17p21, 12q13 and 2q31 for HOXA to HOXD, respectively) [78]. During the embryonic development, *HOX* genes are expressed in a tightly-regulated temporo-spatial pattern (from 3' to 5') along the anterior-posterior axis according to the strict collinearity principle (spatial collinearity, posterior prevalence and temporal collinearity) [78,84]. Alterations interfering with these mechanisms, required to preserve the embryonic determination, may lead to tumorigenesis in adults. Deregulation of *HOX* genes occurs following different patterns, leading to the aberrant silencing or overexpression of different *HOX's* with tumor suppressive or oncogenic properties, respectively [78,84]. Three main mechanisms that drive to these deregulations have been proposed by Abate-Shen: temporospatial deregulation, gene dominance and epigenetic deregulation [85]. Not only due to their importance at the embryonic

development, but also due to their contribution in the control of cell identity, growth, angiogenesis, invasion, apoptosis, differentiation and cell-cell or cell-extracellular matrix interactions, the deregulation of *HOX* genes are ultimately correlated with the failure of the treatment and patients' poor prognosis [52,86,87]. Thus, until now, several primary tumors were reported to have deregulated expression of *HOX* genes, such as breast [88-90], brain [52-54,86,91,92], lung [93,94], colon [95], cervix [96], bladder [97,98], kidney [99] and leukemia [87,100-102].

Recently, Abdel-Fattah *et al.* reported the aberrant expression of several *HOX* genes in malignant astrocytomas compared to normal astrocytes [86]. Posteriorly, Murat *et al.* suggested a *HOX*-dominated gene cluster as an independent predictive factor of therapy resistance in GBMs [54]. Interestingly, they described an enrichment between the same *HOX* gene cluster and a self-renewal signature being the first evidence of a glioma stem cell-like phenotype, associated with resistance to therapy. More recently, Costa *et al.* described the aberrant high expression of *HOXA* genes in a subset of GBM, as compared to LGGs and normal brain tissue, suggesting a role for these genes in glioma malignancy [52]. Moreover, by pharmacological manipulation of a GBM cell line and two sublines of a primary GBM grown as neurospheres, they identified the PI3K pathway as the molecular mechanism by which *HOXA* genes are regulated. Additionally, they observed that this regulation by the PI3K pathway was through reversible regulation of histone modifications [52]. Of all *HOXA* genes, they focused on *HOXA9,* whose expression was associated with poor prognosis in GBM, independently of other prognostic factors, such as *MGMT* promoter methylation. Importantly, they observed that in all patients with *MGMT* promoter methylation, *HOXA9* expression was sufficient to identify a subset of patients with poor prognosis. Functionally, they showed that *HOXA9* retroviral infection in immortalized astrocytes and GBM cell lines leads to increased proliferation of the cells and anti-apoptotic features. More recently, Gaspar *et al.* identified a pediatric GBM cell line that, although lacking of *MGMT* expression, was resistant to TMZ, independently of the mismatch repair and base excision repair system [53]. Interestingly, the resistance was associated to an enrichment in HOX/stem cell gene expression signature, namely to *HOXA9* and *HOXA10* that were associated to a shorter survival of pediatric high-grade glioma patients [53]. Similarly to what Costa *et al.* had observed, they suggested that the PI3K pathway is responsible for the aberrant expression of these genes, and interestingly, they report an evidence of a synergistic interaction of TMZ and PI3K inhibitors in GBM models, both *in vitro* and *in vivo* [53].

As a whole, these reports, and those linking *HOXA9* expression with poor survival in other types of tumors [87,90,94,98,100,102,103], emerge as base to affirm the importance of this gene in tumorigenesis. Although the mechanisms behind HOXA9-associated aggressiveness remain to unveil, it is rational to hypothesize that HOXA9-transcriptional target genes can be the true biological effectors of its aggressiveness. In this respect, our group has recently performed transcriptomic analysis by expression microarrays in U87MG GBM cells, retrovirally infected to overexpress *HOXA9*. After microarrays validation, we identified a set of genes belonging to the Wnt pathway whose expression is altered after *HOXA9* overexpression (e.g.: *WNT6*, *CCND1*, *TCF7*, *FZD5*, *FZD8* and *MMP7*) [Figure 1.6]. Interestingly, *WNT6* encodes to the WNT6 protein that plays a crucial role in the activation of the Wnt pathway. Its critical function in the development of the embryo predisposes this gene to have a role in the tumorigenic and metastatic process when inappropriately expressed in adult. Indeed, it raises the assumption that genes expressed early in life but not in specific adult tissues might be re-expressed in these tissues during the neoplastic and malignant transformation [78].

When Wnt ligands interact with their specific receptor, the formation of the complex Dvl-Fzd promotes the relocation of Axin to the membrane and the inactivation of the β-catenin destruction complex, to which Axin belongs to. In this way, β-catenin may enter and accumulate in the nucleus [Figure 1.6]. Here, a complex is formed between β-catenin and Tcf/Lef family proteins, leading to the transcriptional activation of several target genes that promote cell proliferation, migration and invasion (such as *cyclin D1* and *MMP7,* which interestingly were found to be up-regulated in our microarrays). On the other hand, when no ligands bind to its receptor, the destruction complex formed by Axin, APC and GSK3 sequesters β-catenin and promotes its phosphorylation and consequent destruction. The first association of this pathway to cancer was when APC mutations were involved with familial adenomatous polyposis, an autosomal, dominantly inherited disease that usually leads to a spontaneous and invasive form of colon carcinoma [104-106]. Indeed, approximately 90% of colon cancer patients present aberrant Wnt pathway activity, as result of *APC* mutation (in 80% of patients) [106-109] or due to β-catenin (*CTNNB1*) [110,111] or *AXIN2* mutations [112,113]. While the mutations in *APC* and *AXIN2* compromise their function on the β-catenin destruction complex, β-catenin mutations protect this protein against phosphorylation, ultimately blocking its degradation. However, epigenetic silencing of Wnt antagonist, such as members of the secreted Fzd-related protein (*SFRP*) [114,115] or Wnt inhibitory factor (*WIF1*) [116], were also described in colon cancer.

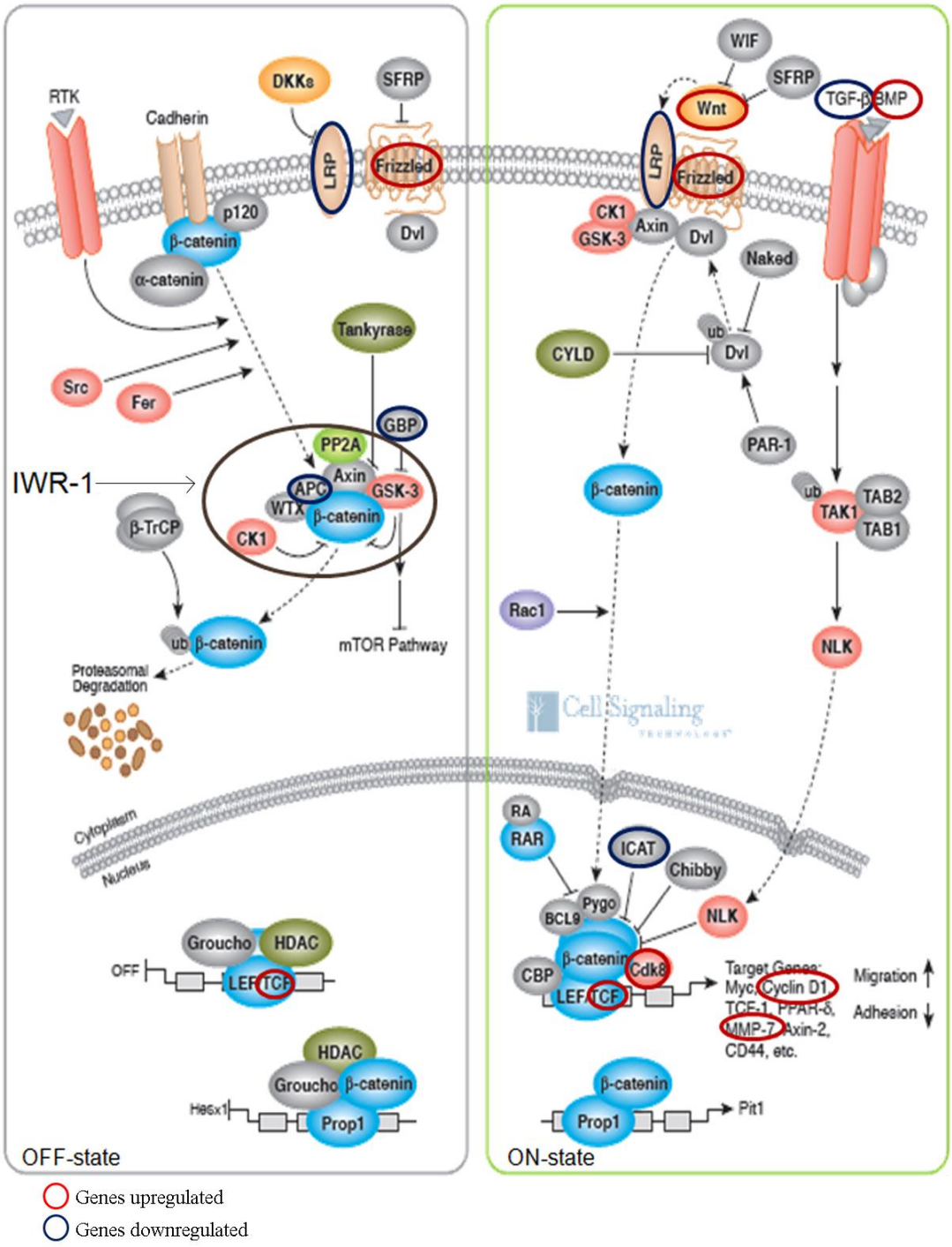

Figure 1.6| Diagram representing the Wnt pathway.

Blue and red circles represent down-regulation and up-regulation, after HOXA9 overexpression in U87MG GBM cell line, respectively.

On state: the interaction of Wnt ligand with its specific receptor leads to the formation of Dvl-Fzd complex promoting the relocation of Axin to the plasma membrane and the inactivation of β-catenin destruction complex. Thereby β-catenin may enter the nucleus and interact with members of Tcf/Lef family activating several target genes that promote cell proliferation, migration and invasion, such as *cyclin D1* and *MMP7*.

Off-state: In cells without Wnt activation, the complex β-catenin, Axin, APC and GSK3 causes β-catenin phosphorylation and its consequent destruction. IWR-1, an inhibitor of this pathway, was reported to be associated with the stabilization of this complex and consequently destruction of β-catenin and inhibition of Wnt pathway. (Adapted from Cell Signaling Technology).

Interestingly, in colon tumor, the inhibition of Wnt pathway activity by overexpression of dominant-negative Tcf proteins, by depletion of *β-catenin* by short-hairpin RNA (shRNA) or by the re-expression of *SFRP*, efficiently represses the tumor growth [115,117-119]. In fact, the importance of the Wnt signaling seems to be well known and studied in this cancer comparatively to other human cancers. Notwithstanding, similarly to colon cancer, *AXIN* and/or β-catenin mutations were also reported in several other tumors, such as breast [120], prostate [121], skin [122], endometrial [123], medulloblastoma [124,125], hepatocellular [126,127], uterine endometrium [128] and esophageal squamous cell carcinomas [129]. Even though most non-colon cancers do not present the classical mutation in *APC*, aberrant Wnt signaling activity was observed in a range of other human cancers [121,124,130].

In normal brain, the Wnt pathway presents clearly defined roles in the regulation of brain development and in the proliferation and self-renewal of neural stem-cells and neural progenitor cells from the hippocampus, the postnatal subventricular zone and the fetal ventricular zone [17]. Recently, some reports have described a role for this pathway in brain cancer, namely astrocytomas [130,131], and its association with glioblastoma stem-cell maintenance and, consequently, resistance to radiochemotherapy [17,56,59,60]. Contrarily to other tumors, in GBM, while no mutations were observed in this gene, some reports showed *CTNNB1* overexpression and consequent accumulation in the nucleus [132-135]. In this context, it was described that *CTNNB1* overexpression can be achieved by epigenetic silencing of Wnt inhibitors (such as *SFRP* and *WIF1*) or increased expression of Wnt ligands and/or other components of this pathway (such as Fzd and Dvl family members) [58,136-140]. Interestingly, depletion of β-catenin by shRNA or ectopic expression of *WIF1* was sufficient to repress cellular growth, invasion and migration of GBM cells [135,136,141].

Clearly, strong evidences for aberrant activation of the Wnt pathway being a driving force in several tumors, including GBMs, highlight the need for more studies, which may allow a better understanding of the molecular mechanisms underlying its over-activation.

1.6| Objective

GBMs are the most common and lethal tumors of the CNS. Although their clinical response is poor and unpredictable, patients with GBM are equally treated with a standardized approach that includes, after surgical resection, radio- and chemo-therapy, mostly with TMZ. Thus, the identification of prognostic biomarkers is crucial to allow the stratification of these patients in molecular subgroups. Our group has recently showed that *HOXA9* overexpression in GBM is associated with poor prognosis and pro-proliferative properties [52]. Furthermore, we found some clues suggesting that the Wnt pathway is altered in HOXA9-positive cells. Since aberrant activation of the Wnt pathway is reported in several tumors and associated with increased proliferation and therapy resistance, we hypothesized that the over-activation of this pathway by HOXA9 may partially contribute to the resistance of these cells to temozolomide therapy. In order to clarify this hypothesis, and considering the lack of studies regarding Wnt pathway, mainly the role of *WNT6*, in the context of brain tumors, we intend to:

1) Characterize the molecular alterations of *WNT6* in GBM clinical samples and cell lines;
2) Evaluate the influence of the Wnt pathway in cell viability;
3) Investigate the efficacy of a combinatorial treatment of TMZ with an inhibitor of the Wnt pathway to more efficiently target HOXA9-positive cells;
4) Assess the clinical significance of *WNT6* in GBM.

2. MATERIALS AND METHODS

2| Materials and Methods

2.1| Cell lines and culture conditions

The human glioblastoma cell lines, U87MG and A172 from American Type Culture Collection (ATCC), were used in this study. The U87MG cell line was previously [52] genetically retrovirally infected with murine stem cell virus (MSCV) containing the *HOXA9* coding region to overexpress this gene (U87MG-HOXA9) or with an empty vector (U87MG-MSCV, control). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco®) supplemented with 10% Fetal Bovine Serum (FBS; Biochrom) and 1% Penicillin-Streptomycin (Invitrogen). As the MSCV expression vector carries the bacterial neomycin gene that confers resistance to G418, U87MG-MSCV and U87MG-HOXA9 cells medium also contained 500ng/µl of G418 (Sigma-Aldrich®), in order to continuously select the retrovirally infected cells. Cells were maintained in a humidified atmosphere at 37°C and 5% (v/v) CO_2.

2.2| Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA; www.broad.mit.edu/gsea/) was performed as previously described [142] using the microarray expression data from U87MG-MSCV and U87MG-HOXA9 GBM cells, in order to understand if the HOXA9-transcriptome coordinately enriches for particular classes of genes. Gene sets databases from MSigDB C2 collection version 3 were used (available online). The permutation type used was “gene sets”, while the default option was used for all other parameters. Only results with a false discovery rate (FDR) <0.25 were considered significant.

2.3| RT-PCR and qRT-PCR

The TRIzol method (Invitrogen) was used to extract the total cellular RNA and DNA from 11 glioma-patients tissues (from Hospital of Braga) and cell lines U87MG-MSCV, U87MG-HOXA9, RES186, RES259, UW479, KNS42, SF188, U87, U251, U373 and SNB19. After quantification by spectrophotometry, cDNA was synthesized from up to 1µg of the total RNA (RT-Phusion Kit, Thermo Scientific). We assessed by a standard reverse transcription-polymerase chain reaction (RT-PCR) or quantitative RT-PCR (qRT-PCR), the levels of *WNT6, HOXA9, AXIN2* and housekeeping genes *hGUS* or *TBP*, used for PCR or qPCR, respectively, with the following sets of primers: *WNT6* Fwd 5’-GACGAGAAGTCGAGGCTCTTT-3’ and Rev 5’-CGAAATGGAGGCAGCTTCT-3’; *HOXA9* Fwd 5’-GCCCGTGCAGCTTCCAGTCC-3’ and Rev 5’-GAGCGCGCATGAAGCCAGTTG-3’;

AXIN2 Fwd 5'- TCAGCAGAGGGACAGGAATC-3' and Rev 5'- AGCTCTGAGCCTTCAGCATC-3'; *hGUS* Fwd 5'-CCTGTGACCTTTGTGAGCAA-3' and Rev 5'-GTGCCCGTAGTCGTGATACC-3'; *TBP* Fwd 5'-GAGCTGTGATGTGAAGTTTCC-3' and Rev 5'-TCTGGGTTTGATCATTCTGTAG-3'. The annealing temperature was 60°C for *WNT6* and *TBP* and 57°C for *hGUS*. A touchdown PCR was performed for *AXIN2*, the annealing temperature starts at 63°C (decrement of 1°C per cycle for 10 cycles) and end at 56°C. For the Chromatin Immunoprecipitation assay (ChIP), primers were designed to detect 1000bp upstream the transcription start region: Fwd 5'-CAGGGGCATCAAAGACATTT-3' and Rev 5'-TCAAGAGATCGAGGGGTCAG-3'. The annealing temperature was 60°C. Finally, the running was accomplished using a 2% agarose gel for all reactions. For the qPCR, levels were determined based on the ΔΔCt method as described previously [143].

2.4| Western blot

Cells were trypsinized, washed with PBS and lysed for 1 hour at 4°C using a lysis buffer containing Tris-HCL 50 mM, EDTA 5 mM, NaCl 150 mM, 1% NP-40, PMSF 1 mM and inhibitors of proteases 25x. Then, the protein concentration was determined using the Bradford method [144]. Using a 10% SDS-polyacrylamide gel, the total protein extracts (50 ug/lane) were separated by electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Life Sciences). The immunodetection was achieved using antibodies against WNT6 (1:1000, Abcam) and α-tubulin (1:100; Santa Cruz Biotechnologies). Blots were revealed with peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (Santa Cruz) followed by enhanced chemiluminescence (ECL) solution (SuperSignal West Femto Chemiluminescent Substrate; Thermo Scientific).

2.5| Immunofluorescence

U87MG-MSCV and U87MG-HOXA9 cells were fixed with Paraformaldehyde (PFA) 4% and then incubated in 1% BSA in 0.1 PBS-Tween for 1h. Next, the cells were incubated with the primary antibody against WNT6 (ab50030, abcam; 1µg/ml) overnight at 4°C. The secondary antibody (red) was Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L; Alfagene) used at a dilution of 1:1000 for 1h in the dark. DAPI (VECTASHIELD® Mounting Medium with DAPI, Vector Laboratories) was used to stain the cell nucleus (blue) at a concentration of 1.5µg/ml.

2.6| TCGA data meta-analysis in glioma patients

The Cancer Genome Atlas (TCGA) was used to obtain the information about gene expression, copy number alterations, mutations and DNA methylation status, as well as clinical information, when available, from low-grade glioma (LGG), GBM and normal patients [145].

2.6.1| Gene expression data

All the gene expression data from samples hybridized by the University of North Carolina, Lineberger Comprehensive Cancer Center, using Agilent G4502A 244K, were downloaded from TCGA [145] data matrix (http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp). This dataset included 572 glioblastomas, 27 low-grade gliomas, and 10 unmatched normal patient samples. To prevent duplicates, when there is more than one portion per patient, the median was used to the study. Three probe sets hit *WNT6* gene (A_23_P119916, A_32_P159877 and A_24_P208513). The provided value was pre-processed and normalized according to "level 3" specifications of TCGA (see *http://cancergenome.nih.gov/dataportal/* for details). Overexpressed genes are the ones with a "level 3" value ≥0.41.

2.6.2| Gene copy number data

Gene copy number data from 372 glioblastoma samples was assessed by the Eli and Edythe L. Broad Institute of the Massachusetts Institute of Technology (MIT) and Harvard University, using Affymetrix Genome-Wide Human SNP Array 6.0, and were exported from the TCGA [145] data portal (*http://tcga-portal.nci.nih.gov/tcga-portal/AnomalySearch.jsp*).

Gene amplifications or deletions were considered for Log_2 copy number tumor/normal ≥0.5 (gene copy number ≥3) or ≤-0.5 (gene copy number ≤1), respectively.

2.6.3| DNA methylation data

DNA methylation profiles were downloaded from the TCGA data matrix (*http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp*). The data was evaluated by the John Hopkins University and the University of Southern California joint group using Illumina Infinium Human DNA Methylation 450 array, and include the methylation status of 74 glioblastoma samples. For this study, 29 probes that span from 5000bp upstream to 5000bp downstream of *WNT6* gene were selected. This region of approximately 24000bp includes 3 CpG islands. The methylation level was determined at each locus by the intensity of the two fluorescent signals, from the T (unmethylated) and C (methylated) alleles. The relative methylation level (β-value) was calculated

as the ratio of methylated-probe signal to total locus signal intensity. High methylation was considered for β-values higher than 0.5.

2.6.4| Clinical data

Clinical data of each patient was provided by the Biospecimen Core Resources (BCRs) and include information about age at diagnosis, gender, Karnofsky performance status (KPS) and days to death and to last follow-up. This data is available for download through TCGA data matrix (*http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm*).

2.7| 5-Aza-2′-deoxycytidine (5-Aza) treatment

To study the effect of *WNT6* DNA methylation in HOXA9-negative and -positive cells, U87MG-MSCV and U87MG-HOXA9 cells were plated in T25-flasks at an initial concentration of 75000 cells per T25. DMEM (Gibco®) supplemented with 10% FBS (Biochrom) was used. Treatment with 5μM of 5-Aza-2'-deoxycytidine (5-Aza; Sigma-Aldrich®), a specific inhibitor of DNA methylation, or vehicle (DMSO) started 24 hours (day 0) after platting. 5-Aza is a pro-drug that upon transport into cells is phosphorylated to become active. Next, it acts by incorporating into DNA leading to the disruption of the interaction between the DNA and DNMTs (DNA Methyltransferases) [146]. Since the S-phase is required for the effective incorporation into the DNA [146], media with drug (5-Aza) or vehicle (DMSO) was renewed every 24 hours for 2 days. After this, cells were washed, harvested by trypsinization and centrifuged (1200 x g). The collected pellets were used for DNA and RNA extraction.

2.8| Methylation-Specific PCR (MSP)

DNA methylation of *WNT6* was evaluated by MSP after sodim bisulfite treatment. After DNA extraction (according to the TRIzol method) and quantification, it was submitted to a chemical reaction leading to the conversion of unmethylated cytosines to uracil residues, while 5-methylcytosines remains as cytosines – bisulfite modification (according to EZ DNA Methylation-Gold™ Kit; Zymo Research, manufacturer's instructions). The subsequent described procedure was based on this selective modification. Methylation was assessed by MSP using the following sets of primers: Unmethylated set, Fwd 5'-TTTTGTGTTCGGCGTACGT-3' and Rev 5'-AATCTATCCTAAATCCCGAA-3'; Methylated set, Fwd 5'-TGTTGTTGTTTTTGTGTTTGGTGTAT-3' and Rev 5'-CCCCAATCTATCCTAAATCCCA-3'. A touchdown MSP was performed. For the unmethylated or methylated set, the annealing temperature starts at 62°C or 60°C (decrement of

1°C per cycle for 10 cycles) and end at 52°C or 50°C for 28 cycles, respectively. All MSP products were loaded onto a 3.5% agarose gel for all reactions.

2.9| Cell Viability

To access the effect of Wnt pathway inhibition in the viability of HOXA9-negative and HOXA9-positive cells, U87MG-MSCV and U87MG-HOXA9 cell lines were seeded, in duplicate, at an initial density of 20000 cells per well in 6-well plates, and 5000 cells per well in 12-well plates, for Trypan Blue and MTT assays, respectively. The media used was DMEM (Gibco®) supplemented with 10% FBS (Biochrom). Treatment with IWR-1 (Sigma-Aldrich®), an inhibitor of the Wnt pathway recently described [147], Temozolomide (TMZ; Sigma-Aldrich®) or vehicle (DMSO) started 24 hours (day 0) after platting. Media with compound (IWR-1, TMZ or the combination) or vehicle (DMSO) was renewed every 24 hours for 2 days. In our assay, IWR-1 was used at several concentrations ranging from 1μM to 100μM for IC_{50} determination, or at the fixed concentration of 1μM in comparison to the reported in [147]. On the other hand, TMZ was used at 300μM or 600μM (IC_{50} value).

2.9.1| Trypan Blue Assay

At day 3, cells were recovered by trispinization. The suspension was mixed, at the ratio 1:1, with trypan blue dye (Gilco®), which provides information regarding the cellular membrane integrity. This allows us to identify the viable cells since these cells don't let the dye to pass through the membrane, as opposed to dead cells, which have compromised membrane integrity. Counting of viable cells was performed with the help of the Neubauer chamber under the microscope (using the 20x objective) in two independent samples of each well. The total number of cells in each well was calculated using the following formula:

$$Total\ cells = average \times df \times \frac{1}{10^{-4}}\ {}^{cells}/_{ml}$$

Formula 1| Formula to obtain the total number of cells counted by Trypan Blue using the Neubauer chamber.
average = average of viable cells counted in all quadrants; df= dilution factor; 10^{-4} ml – volume of the counting square region.

The remaining cell suspension was stored to further gene expression analysis. This technique was also used to obtain the desired concentration of cells to plate the viable cells at exact initial densities.

2.9.2| MTT Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is reduced in viable cells to purple formazan crystals by a mitochondrial reductase in the presence of PMS (phenazine methosulfate), providing information about the metabolic viability of the cells [148]. At day 3, cells medium was replaced with PBS 1x containing 0.5mg/ml of the water-soluble tetrazolium dye MTT (Invitrogen) and incubated, in the dark, during 2 hours, in a humidified atmosphere (37°C and 5% CO_2). After incubation, the solution with MTT was removed and 500µl of acid-isopropanol (0.4M of HCl in isopropanol) was added to each well to dissolve the crystals (which are insoluble in aqueous solution). The content of each well from the 12-well plate (100 to 150µl) was next transferred in triplicates to a 96-well plate, and the absorbance was measured at 570nm.

2.10| Statistical analyses

The Chi-square test was used to assess the differences between the distributions of tumors with high and low *WNT6* expression, stratified for LGGs and GBMs.

The correlation between methylation indexes of each probe and *WNT6* expression was measured by the Pearson correlation coefficient (r) calculated using GraphPad Prism 5.01 (GraphPad software, Inc.). Moderated and high correlations were considered when $r>0.3$ and $r>0.7$, respectively.

To evaluate the prognostic value of *WNT6* expression, multivariate analysis of survival was performed using the Cox proportional hazard model, where the potential confounding effect of some variables is considered. These analyses were made with SPSS 19.0 software (SPSS, Inc.).

One-way ANOVA (post hoc Tukey) was used to assess the differences between each condition of the qRT-PCR, Trypan Blue and MTT assays.

For all statistical tests, significance was considered when $p<0.05$.

3. RESULTS

3| Results

3.1| HOXA9-associated transcriptome is enriched to Wnt pathway related gene sets

Querying our HOXA9-associated transcriptomic signatures through gene set enrichment analysis (GSEA) [27] revealed that HOXA9-upregulated genes in U87MG cells are significantly associated with genes involved in the Wnt pathway (Enrichment Score, ES = 0.454323 and False

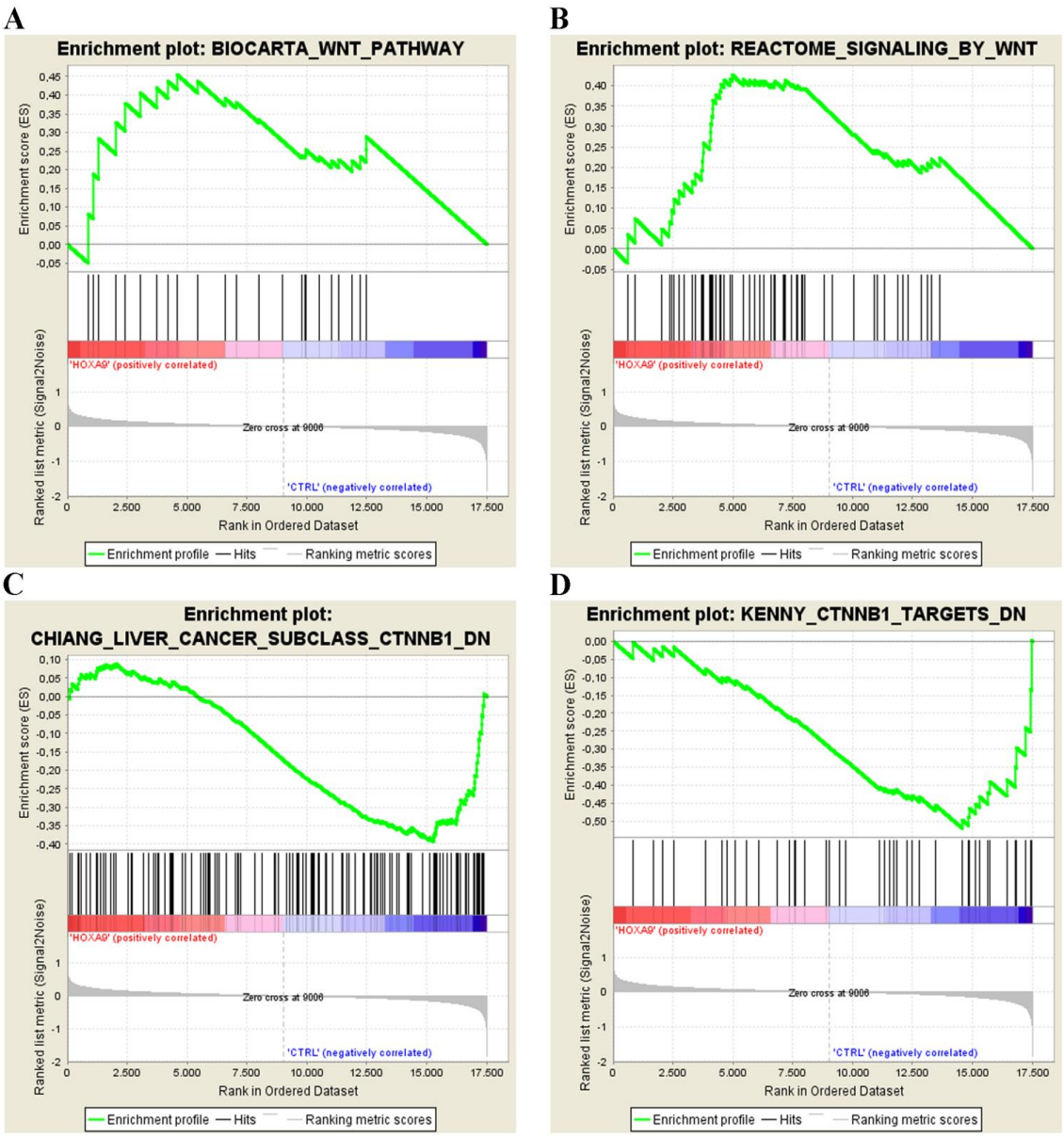

Figure 3.1| Enrichment of HOXA9-transcriptome to Wnt pathway.
(A and B) HOXA9-upregulated genes in U87MG cells are enriched to gene sets related to the Wnt pathway (ES = 0.454323 and FDR = 0.174527; A and ES = 0.426325 and FDR = 0.074905; B). **(C and D)** HOXA9-downregulated genes in U87MG GBM cell line are enriched to gene sets which reflects the downregulated genes by an constitutively active β-catenin (CTNNB1) both in a hepatocellular carcinoma (ES = -0.3926 and FDR = 0.124273; C) and in HC11 mammary epithelium cells (ES = -0.52033 and FDR = 0.061827257; D).

Discovery Rate, FDR = 0.174527 [Figure 3.1A]; and ES = 0.426325 and FDR = 0.074905 [Figure 3.1B]). HOXA9-downregulated genes in U87MG cells were significantly associated with downregulated genes characterized by activated β-catenin both in hepatocellular carcinoma (ES = -0.3926 and FDR = 0.124273 [Figure 3.1C]) and in HC11 mammary epithelium cells (ES = -0.52033 and FDR = 0.061827257 [Figure 3.1D]). Such results are in accordance to the hypothesis raised that this pathway is over-activated in our HOXA9-positive cells, as suggested by the set of genes belonging to the Wnt pathway whose expression is altered in our microarrays [Figure 1.6]. Together, these results propose the enrichment of the HOXA9-transcriptome in the Wnt pathway, suggesting that this pathway may be relevant in mediating some of the hallmarks of HOXA9-positive GBM cells, including proliferation and migration.

3.2| HOXA9 directly binds to *WNT6* promoter region

The ligands of the Wnt signaling pathway are secreted glycoproteins of 365 amino acids that contain an N-terminal signal peptide. These proteins – Wnt proteins – were early associated with tumorigenic processes, such as WNT1, WNT2, WNT4 and WNT7 in breast cancer [149,150]. Analyzing our expression microarray, we found that one of the genes of the Wnt pathway whose expression was increased by *HOXA9* overexpression was *WNT6*. To confirm this

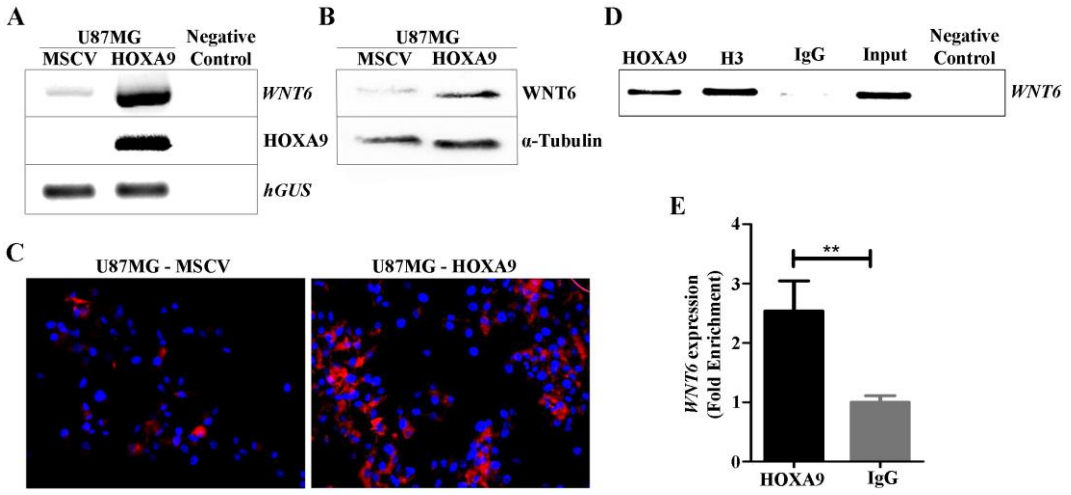

Figure 3.2| *WNT6* is overexpressed in HOXA9-positive GBM cells.
(A) *WNT6* expression was evaluated by RT-PCR in U87MG GBM cells validating the overexpression of this gene after *HOXA9* retroviral overexpression. **(B)** Western-Blot and **(C)** immunofluorescence showing higher WNT6 protein levels in HOXA9-positive cells. **(D and E)** Chromatin Immunoprecipitation (ChIP) was performed to access the putative binding of HOXA9 to the promoter region of *WNT6*, followed by PCR **(D)** or qPCR **(E)**. Antibodies anti-Histone 3 (H3) and IgG were used as positive and negative controls for the ChIP, respectively. The input reflects DNA not exposed to immunoprecipitation (PCR positive control). Chromatin immunoprecipitates with anti-HOXA9 antibody shows that HOXA9 binds to the promoter region of *WNT6*. In (E), the fold enrichment is normalized to input and to the IgG background signal. Three independent experiments (mean and standard deviation) are represented. ** p=0.0085

overexpression at the gene and protein levels, RT-PCR [Figure 3.2A], Western Blot [Figure 3.2B] and Immunofluorescence [Figure 3.2C] were performed in U87MG-MSCV (control) and U87MG-HOXA9 GBM cell lines. As expected, HOXA9-positive cells had an increased expression of *WNT6* mRNA [Figure 3.2A] and protein [Figure 3.2B and C]. To validate *WNT6* as a direct target of HOXA9, ChIP (Chromatin Immunoprecipitation) was performed on A172, a GBM cell line which endogenously express *HOXA9*, using an antibody against HOXA9 and a set of primers designed to amplify a portion of *WNT6* promoter region. As shown in Figure 3.2D-E, HOXA9-bound chromatin immunoprecipitates, but not precipitates from control immunoglobulin, contained a portion of the promoter region of *WNT6* (p=0.0085). Together, these results point out HOXA9 as a direct transcriptional activator of *WNT6* in GBM.

3.3| *WNT6* is overexpressed in GBM cell lines and primary tissues

While high *WNT6* expression levels were observed in different human cancer cell lines, such as colorectal, cervical [151], gastric cancer [152], and in GBM [60], until now little is known about the specific role of *WNT6* in tumors, mainly in GBM. In order to investigate the importance of *WNT6* in the aggressiveness of GBM, we analyzed the expression of this gene in a panel of glioma patients' samples (from the Hospital of Braga), glioma pediatric and adult cell lines. As we can observe [Figure 3.3], *WNT6* expression varies within grades and patients' ages. However, higher levels of expression were observed in a subset of grade IV adult glioma cell lines and primary samples. To validate this result in a larger dataset, we analyzed gene expression array

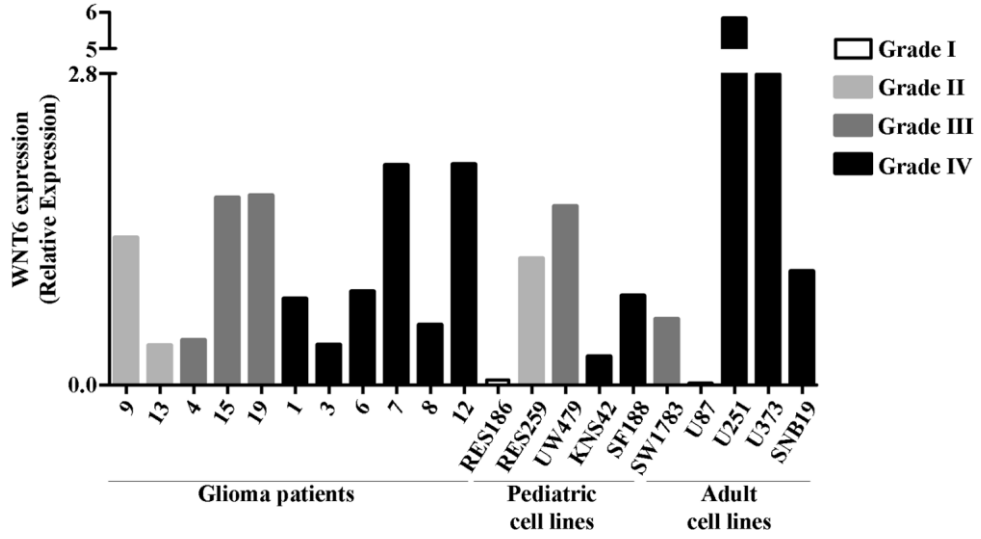

Figure 3.3| *WNT6* is overexpressed in a subset of grade IV adult glioma cell lines and primary tumors.
WNT6 expression was evaluated, by qRT-PCR, in glioma patients' tumors, pediatric cell lines and adult cell lines, identifying a subset of grade IV adult glioma cell lines and primary tissues with overexpression of *WNT6*.

data from low-grade gliomas (LGG) and GBM patients deposited in TCGA [145]. Strikingly, *WNT6* was not overexpressed in any of LGG patients (0/27), while 15.6% of GBM patients (89/572) presented high *WNT6* levels, when comparing to controls [Table 3.1 and Figure 3.4A]. Moreover, this difference between *WNT6* expression in LGG and GBM was statistically significant (p=0.026). These results suggest that *WNT6* is important in glial tumor malignancy.

Table 3.1| *WNT6* is overexpressed in GBM patients.
Chi-squared test shows that the pattern of *WNT6* expression is significantly different between LGG and GBM patients. *WNT6* expression in LGG is low; however, 15.6% of GBM patients have high expression of *WNT6*.

		***WNT6* expression**		***p*-value**
		Low	**High**	
GBM	n	483	89	0.026
	%	94.7	100	
LGG	n	27	0	
	%	5.3	0	

n=number of patients

3.4| *WNT6* high expression is more frequent in the proneural subtype

Several efforts have been performed to stratify GBM patients in molecular subgroups [54,70-76]. Recently, Verhaak *et al.* proposed the division of GBM patients in 4 main subgroups – classical, mesenchymal, neural and proneural [69]. Using this division, we evaluated the levels of *WNT6* expression among GBM subtype (in a total 201 patients) and found a different pattern of expression among them (p=0.042) [Table 3.2]. Interestingly, 48.5% of GBM patients with high *WNT6* expression belong to the proneural subtype, while low expressing patients are well dispersed among the subtypes [Table 3.2].

Interestingly, one of the characteristics of the proneural subtype is the high expression of *TCF4*, the partner of β-catenin in the transduction of the Wnt pathway [69]. In this context, at least two genes (*WNT6* and *TCF4*) important in the Wnt pathway are highly expressed in the proneural subtype. This suggests that the Wnt pathway may be over-activated in these clinical samples, validating what we detect in our GBM *in vitro* models and confirming the importance of the Wnt pathway in GBM.

Table 3.2| High expression of *WNT6* is more frequent in the proneural subtype.
The Chi-squared test shows that *WNT6* expression is significantly different between all GBM subtypes. The proneural subtype contains almost half of *WNT6* high expressing GBM patients.

		***WNT6* expression**		***p*-value**
		Low	**High**	
Classical	n	48	6	0.042
	%	28.6	18.2	
Mesenchymal	n	50	8	
	%	29.8	24.2	
Neural	n	29	3	
	%	17.3	9.1	
Proneural	n	41	16	
	%	24.4	48.5	

n=number of patients

3.5| *WNT6* expression is regulated by promoter DNA methylation

While *WNT6* was found to be a direct-target of HOXA9, we wanted to understand if other molecular mechanisms may be responsible for *WNT6* overexpression in GBM. Thus, we next evaluated *WNT6* gene copy number aberrations [Figure 3.4B] and alterations in DNA methylation levels in *WNT6* locus [Figure 3.4C].

Concerning gene copy number, in 372 GBMs analyzed, *WNT6* amplification was found in 2 samples (0.54%), and no deletions were found [Figure 3.4B]. While these 2 cases with *WNT6* amplifications also presented gene overexpression, considering the rareness of these amplifications in GBM, this is not likely the mechanism responsible for *WNT6* overexpression (observed in 0.54% of patients).

DNA methylation is an epigenetic feature that plays an important role in regulating gene expression. In fact, aberrant DNA methylation has been observed in many tumor types [10]. In this context, we next evaluated the levels of DNA methylation in the *WNT6* locus, spanning from 5000bp upstream to 5000bp downstream of *WNT6* coding sequence, in 74 GBM samples from TCGA. Evaluating a total of 29 methylation probes within this ≈24000bp region [Figure 3.4C], we found some consistent patterns of methylation across all samples (e.g., several probes in the first CpG island showed low methylation levels). However, the expression of *WNT6* varies greatly across these same samples, suggesting that these regions are not associated with *WNT6* expression [Figure 3.4C].

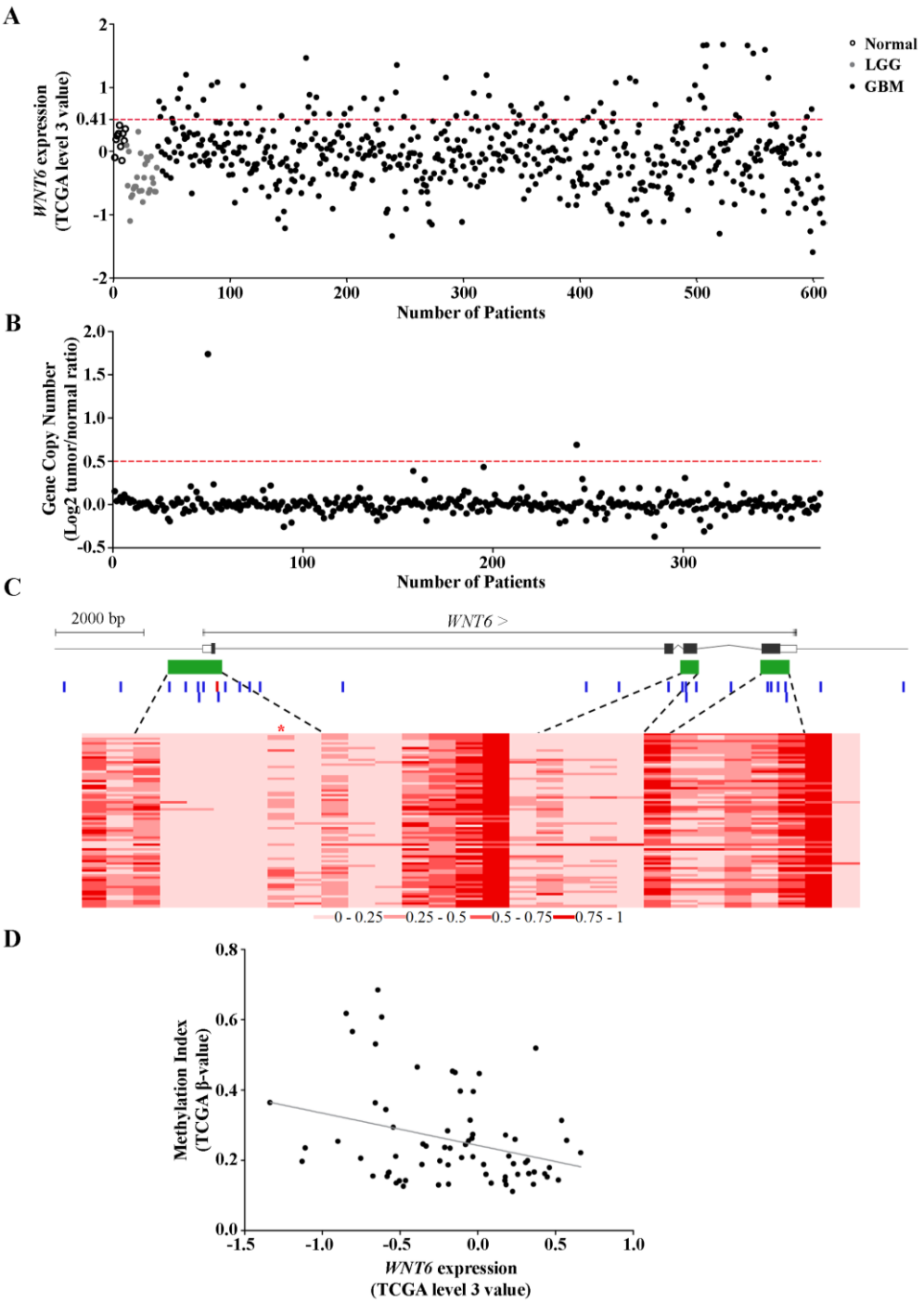

Figure 3.4| Molecular alterations of *WNT6* in gliomas.
(A) Expression levels of *WNT6* in 27 low-grade gliomas (LGG; grey dots), 572 glioblastomas (GBM; black dots) and 10 unmatched normal controls (black unfilled dots) from TCGA. *WNT6* is overexpressed (TCGA data level 3 values ≥0.41; above red dashed line) in 0.0% of LGG and in 15.6% (n=89) of GBM samples. **(B)** *WNT6* gene copy number status in 372 GBM specimens from TCGA. *WNT6* is amplified (Log2 Copy Number Tumor/Normal ≥0.5) in 0.54% (n=2) of GBM samples. The normal copy number interval is between the red dashed line and x axis. **(C)** Heatmap representations of DNA methylation levels (TCGA β-values) of the chromosomal region encompassing *WNT6* in 74 GBM samples from TCGA. A total of 29 methylation probes (vertical blue and red bars) were assessed. CpG islands >300bp are represented in green. The color code (grades of red color corresponding to different methylation indexes) is shown below the heatmap. Each column corresponds to a probe and each line to a patient. The lines within *WNT6* correspond to introns. **(D)** Correlation graph between *WNT6* expression (TCGA level 3 value) of 70 GBM and DNA methylation indexes (TCGA β-values) of one probe that hit the first CpG island (red probe*; C). The graph shows an inverse correlation between *WNT6* expression and methylation levels of this probe.

To further clarify this, we evaluated the correlation between *WNT6* expression and DNA methylation levels of each methylation probe in 70 GBMs for which both expression and methylation data were available. We found 1 probe (cg11175192) in the first CpG island moderately inversely correlated (r=0.308) with expression [Figure 3.4D]: higher values of methylation were correlated with lower values of *WNT6* expression. Taken together, our data suggest that this epigenetic mechanism, together with HOXA9 activation, may promote the aberrant *WNT6* overexpression observed in a subset of GBMs.

Having observed the correlation between *WNT6* methylation and expression in GBM clinical samples [Figure 3.4D], we attempted to elucidate the role of HOXA9 in the methylation of *WNT6* in our GBM cell models. First, U87MG-MSCV and HOXA9 cells were treated with 5-Aza-2'-deoxycytidine (5-Aza), a DNA demethylating agent, and the expression of *WNT6* and *HOXA9* was reevaluated by RT-PCR [Figure 3.5A]. As expected, U87MG-HOXA9 cells present higher *WNT6* mRNA levels than U87MG-MSCV cells [Figure 3.5A]. Moreover, after 5-Aza treatment the expression of *WNT6* increases drastically in both cell lines [Figure 3.5A]. Interestingly, *HOXA9* expression was detected in U87MG-MSCV cells after 5-Aza treatment, which in basal condition presents undetectable *HOXA9* expression [Figure 3.5A], suggesting that the genome-wide hypomethylation is sufficient to activate *HOXA9* expression in these cells. Next, primers for

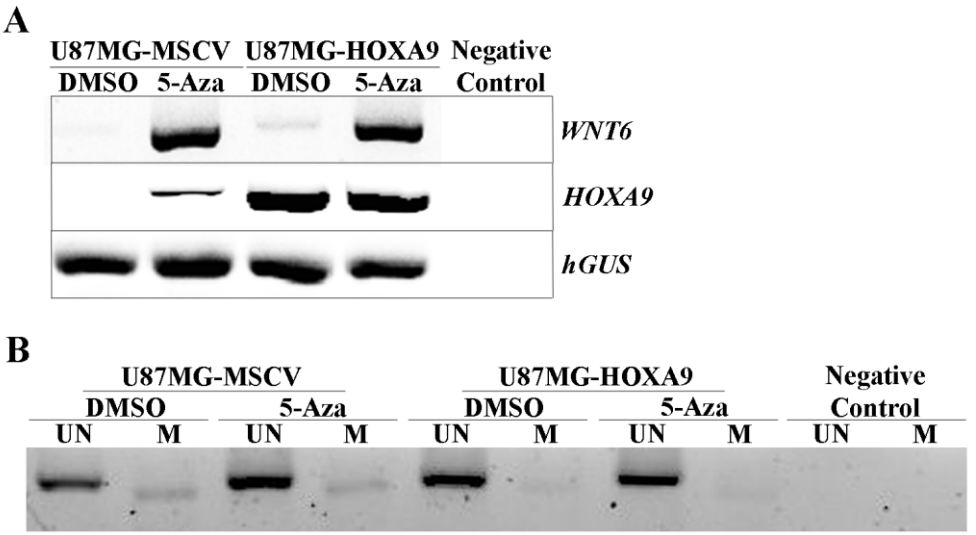

Figure 3.5| *WNT6* is partially repressed by methylation in U87MG-MSCV and U87MG-HOXA9 cells.
(A) *WNT6, HOXA9* and *hGUS* expressions were evaluated by RT-PCR in U87MG GBM cells before and after treatment with 5-Aza, a demethylating agent, showing that *HOXA9* and *WNT6* expressions increase after treatment in HOXA9-negative or both cells, respectively. **(B)** Methylation-specific PCR (MSP) was performed to evaluate the methylation levels of *WNT6* near the cg11175192 probe, showing lower levels of *WNT6* methylation in HOXA9-positive cells at basal conditions. As expected, the methylation decreases in U87MG-MSCV and U87MG-HOXA9 cells after 5-Aza treatment

Methylation-Specific PCR (MSP) were designed to detect the CpG's identified by the probe cg11175192. As expected, we observe lower levels of methylation after 5-Aza treatment in both cell lines, although for the U87MG-HOXA9 cells the difference was less pronounced. Interestingly, in basal conditions, HOXA9-positive cells present lower levels of *WNT6* promoter DNA methylation than their negative counterparts [Figure 3.5B], which can explain the higher levels of *WNT6* expression in HOXA9-positive cells. Together, these results suggest that although *WNT6* overexpression after 5-Aza treatment in U87MG-MSCV cells may be related to the activation of *HOXA9* expression, the methylation level of this region may also partly contribute to *WNT6* high expression after 5-Aza treatment.

3.6| High levels of *WNT6* expression associate with shorter survival in GBM patients

Since *HOXA9* overexpression was shown to correlate with poor survival in GBM patients [52], and considering *WNT6* is a putative HOXA9 effector molecule, we investigated the clinical significance of *WNT6* expression, in 407 GBM patients with available survival data from TCGA [Table 3.3]. To this end, a Cox model was used, adjusted to take into account the potential confounding effect of other putative prognostic factors, namely patient age, Karnosfsky Performance Status (KPS), gender and therapy, and allowing to use *WNT6* expression as a continuous variable.

Table 3.3| High levels of *WNT6* expression are significantly associated with shorter survival of GBM patients.
Cox multivariate survival analysis indicate that higher values of *WNT6* expression are associated with a statistically significant shorter overall survival of GBM patients (p=0.030), independently of other known prognostic factors (patient age, p<0.0001; KPS, p=0.001; gender, p=0.037; treatment with chemo- or radiotherapy, p<0.0001). Exp(B)≥1 means that higher values of the variable are associated with shorter survival; Exp(B)<1 means that higher values of the variable are associated with longer survival.

	Overall survival	
	***p*-value**	**Exp(B)**
***WNT6* expression**	0.030	1.288
Age	<0.0001	1.029
KPS	0.001	0.984
Gender[1,a]	0.037	0.771
Chemo- or Radiotherapy[1,b]	<0.001	0.192

[1]Gender and Chemo- or Radiotherapy were used as categorical variables.
[a]Comparison Female (n=154) vs Male (n=253).
[b]Comparison treatment (n=383) vs no treatment (n=24).

In this analysis, we observed a statistically significant association between higher values of *WNT6* expression and shorter survival of GBM patients (p=0.030; [Table 3.3]), independently of patient age (p<0.0001), KPS (p=0.001), gender (p=0.037) and the treatment with chemo- or radiotherapy (p<0.0001).

Such result was validated using an independent dataset from Rembrandt constituted by a low number of GBM patients (n=181), for which a significantly poorer OS was observed in 5 patients with higher *WNT6* expression than for those that present lower expression levels (p=0.046 by Log-rank test, data not shown). These results suggest that *WNT6* expression may be a prognostic factor in GBM patients.

3.7| Inhibition of Wnt signaling affects cell viability of HOXA9-positive GBM cells

Wnt signaling has been reported to be associated with the tumor progression and cancer stem-cells, mostly in the context of colorectal cancer [153,154]. Moreover, this pathway has been suggested as a potential therapeutic-target, not only in colorectal cancer but also in GBMs [17,50,155]. Since this pathway seems to be over-activated by HOXA9, whose overexpression is associated with a more aggressive phenotype, we investigated the effect of IWR-1, an inhibitor of this pathway, in the viability of HOXA9-positive and HOXA9-negative cells. With the aim of determining the IC_{50} concentration, U87MG-MSCV and U87MG-HOXA9 cells were treated with increasing concentrations of IWR-1 (10, 20, 30, 40, 70 and 100μM), and its effects on cell viability was assessed by MTT [Figure 3.6A]. The cells' response to Wnt inhibition was dose-independent, as increasing concentrations of IWR-1 did not increasingly affected cell viability [Figure 3.6A]. For the concentrations of 10, 70 and 100μM, statistical significant differences were observed between control and treatment conditions for the HOXA9-positive cells. The two highest concentrations also show significant differences for the HOXA9-negative cells when compared to control. Moreover, at 70μM, the viability of U87MG-HOXA9 cells was significantly lower than that of U87MG-MSCV cells. This experiment was repeated for other ranges of concentrations and the same tendency was obtained (data not shown), strongly suggesting the presence of a compensatory feedback in the cells.

Thus, based on the reported by Chen *et al.*, we decided to treat our cells with the minimal dose (1μM) capable of inhibiting the Wnt pathway in mouse fibroblast cells [147]. To evaluate cell viability at this concentration, Trypan Blue [Figure 3.6B] and MTT assays [Figure 3.6C] were performed. By Trypan Blue, we observed that the viability of HOXA9-positive cells was

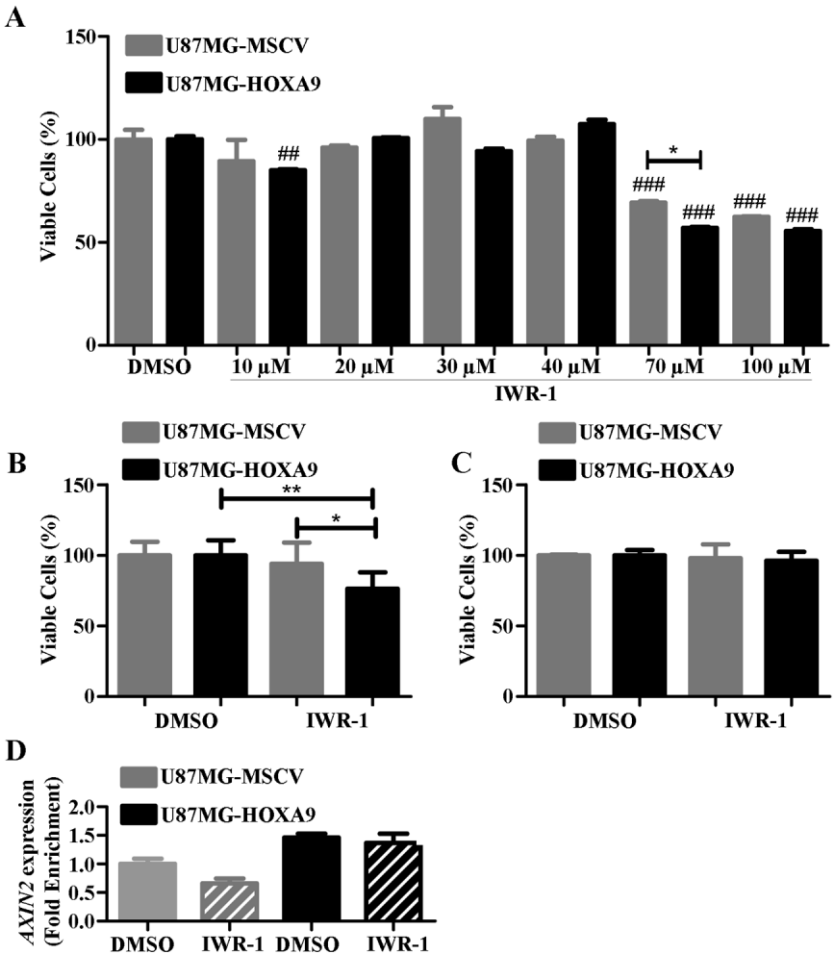

Figure 3.6| Wnt pathway inhibition affects viability of U87MG cells.
Human glioma cell lines U87MG-MSCV and U87MG-HOXA9 were treated with IWR-1, an inhibitor of the Wnt pathway, for 3 days, and cell viability was measured by **(A)** MTT, **(B)** Trypan Blue and **(C)** MTT. **(A)** This result represent 1 assay (mean ± standard deviation) and demonstrates that the response to IWR-1 treatment is dose-independent. **(B)** This result represents 2 independent assays (mean ± standard deviation) and shows that only HOXA9-expressing cells were significantly sensitive to treatment with IWR-1 ($p<0.01$). Moreover, the viability of both treated cell lines was statistically different ($p<0.05$). **(C)** MTT assay (1 assay; mean ± standard deviation) shows no differences in the metabolic viability between treated and non-treated cells. **(D)** *AXIN2* expression was evaluated by qPCR in U87MG GBM cells before and after treatment with IWR-1 showing a trend to decreased expression after treatment in both cell lines. *$p<0.05$, **$p<0.01$; ##$p<0.01$ and ###$p<0.001$ when compared to the control condition (One-way ANOVA, Tukey's post hoc test).

significantly lower after treatment ($p<0.01$), while their negative counterpart were not affected [Figure 3.6B]. In contrast, this effect was not observed by MTT [Figure 3.6C]. Although it is well recognized that Wnt targets are tissue-specific, *AXIN2* gene expression is commonly used to assess Wnt pathway activity. Thus, in order to evaluate if this concentration of IWR-1 was efficient to inhibit the activity of the Wnt pathway, qPCR to *AXIN2* was performed [Figure 3.6D]. A slight decrease in *AXIN2* (higher in U87MG-MSCV cells) was observed for both cells lines after IWR-1 treatment, suggesting that the Wnt pathway is barely inhibited in these cells at 1µM of IWR-1.

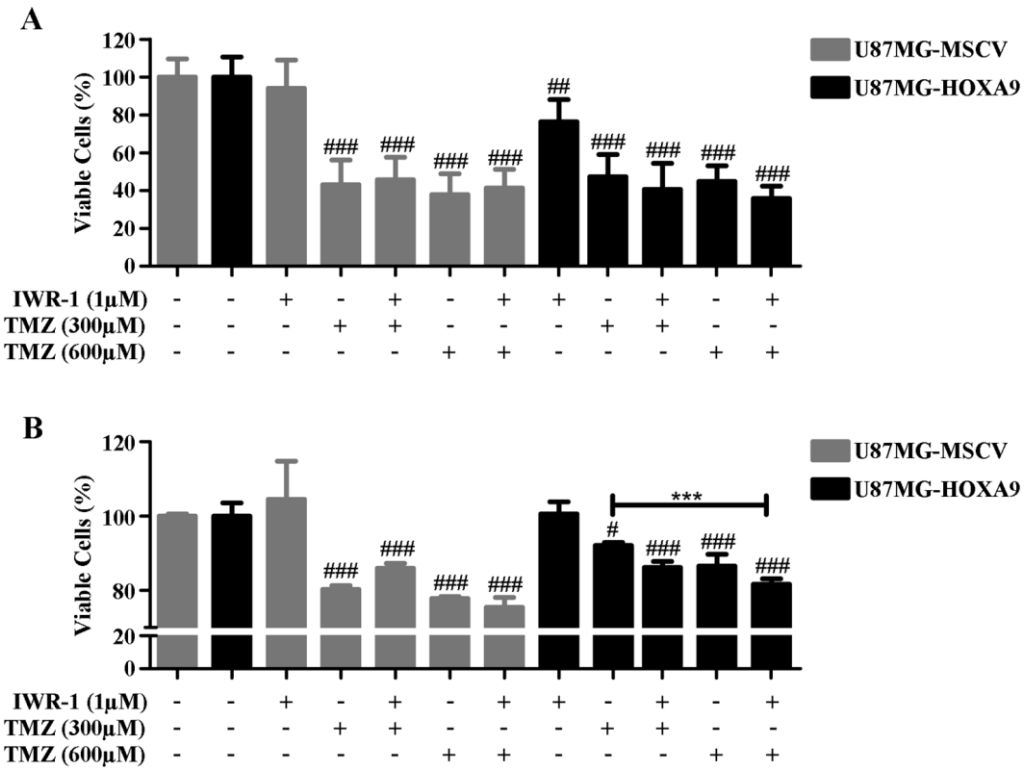

Figure 3.7| Combinatorial treatment with TMZ and IWR-1 presents a trend to decrease the viability of HOXA9-positive cells.

Human glioma cell lines U87MG-MSCV and U87MG-HOXA9 were treated with IWR-1 at a fixed concentration of 1µM, alone or in combination with TMZ at the concentrations of 300 µM or 600 µM (IC_{50} value), for 3 days, and cell viability was measured by **(A)** Trypan Blue and **(B)** MTT assays. **(A)** This result represents 2 independent assays (mean ± standard deviation) and shows that TMZ, alone or in combination with IWR-1, is effective in decreasing the viability of both cell lines. However, as expected, IWR-1 alone was only effective for HOXA9-positive cells. Moreover, a modest trend to decreased viability in these cells was observed when IWR-1 was added to TMZ. **(B)** By MTT assay (1 assay; mean ± standard deviation), the same tendency was observed. However, IWR-1 alone does not decrease the viability of HOXA9-positive cells, as observed in [Figure 3.6]. ***p<0.001; #p<0.05 and ###p<0.001 when compared to the control condition (One-way ANOVA, Tukey's post hoc test).

So far, the cancer stem cell (CSC) theory envisages that these cells are more resistant to radiochemotherapy, including TMZ [17]. This hypothesis postulates that the selective elimination of these cells would arrest the uninterrupted growth and tumorigenicity of the tumor. However, some authors report that differentiated tumor cells may acquire CSC properties, suggesting a novel mechanism of resistance [17]. Thus, it is imperative to use targeted therapies based on the genetic, molecular and cellular information of the tumor, in an attempt to eliminate all tumor cells (non-CSC and CSC). In this context, we decided to evaluate the efficacy of a new combinatorial treatment approach, consisting of TMZ and IWR-1, in order to understand if this approach can be more effective to target HOXA9-positive cells. For this, cells were treated with 600µM (IC_{50} value) or 300µM of TMZ, alone or in combination with IWR-1 (1µM). As expected, TMZ significantly affected cell viability of both cell lines [Figure 3.7], as observed by Trypan Blue

and MTT assays. Additionally, a trend to decrease the viability of HOXA9-positive cells was observed for the combinatorial treatment as compared with TMZ alone, either by Trypan Blue [Figure 3.7A] or MTT assay [Figure 3.7B]. Interestingly, although no statistical differences were observed between TMZ 300μM and 600μM, a statistical difference was observed between TMZ 300μM and the combinatorial treatment with TMZ 600μM and IWR-1. These results suggest that HOXA9-positive cells may be more sensitive to IWR-1 alone or in combination with TMZ. However, no statistical differences were observed and more studies are necessary to better understand the relation between Wnt pathway inhibition and *HOXA9* expression.

4. DISCUSSION

4| Discussion

Glioblastoma multiforme (GBM) is the most common and lethal tumor of the CNS and presents a median survival, after diagnosis, of approximately 15 months. Although the clinical response of GBM patients is poor and unpredictable, they are equally treated with a standardized approach that includes surgery, radiotherapy and chemotherapy, mostly with temozolomide. To overcome this problem of a universal therapy to strikingly heterogeneous tumors, there has been an increasing effort to identify and use molecular markers for the stratification of GBM patients. Nowadays, several molecular markers have been described to have clinical potential but none of them is currently used in the clinics. In this context, our group has shown recently that *HOXA9* overexpression is associated with poor prognosis in GBM patients. Functionally, overexpression of *HOXA9* in GBM *in vitro* models associates with pro-proliferative and anti-apoptotic features.

Since HOXA9 is a transcription factor, its targets can be the true biological effectors of its aggressiveness. To obtain the transcriptome of HOXA9 in GBM, genome-wide expression microarrays were performed in U87MG cells previously [52] retrovirally infected to overexpress HOXA9. Although genome-wide expression microarrays are routinely used in genomic research, the extraction of biological insights from the massive information is manually challenging. Thus, given the vast number of differentially expressed genes obtained, we used bioinformatics tools to analyze the transcriptome. Here, we show results from GSEA (Gene Set Enrichment Analysis) that evaluates the expression levels of all transcripts in the experiment in an integrated manner [142]. In this analysis, we found that the HOXA9-transcriptome is enriched not only in the Wnt pathway [Figure 3.1], but also in several stem-cell and cell cycle signatures (data not shown). Moreover, GSEA analysis also reveals an enrichment of the down-regulated genes in cell adhesion (data not shown), reflecting a decrease in cell adhesion after *HOXA9* overexpression. In a normal condition, the Wnt pathway controls cell fate, proliferation, migration, polarity and death [154]. Deregulated Wnt pathway has been reported in several cancers, including GBM, and has been implicated in promoting resistance to the current therapy. This increased resistance has been attributed to stem-cell features sustained by Wnt signaling, such as increased proliferation, migration and decreased adhesion [56,57,59,60].

Looking in detail to all altered genes of the HOXA9-transcriptome that belong to the Wnt pathway and crossing this information with a list of putative HOXA9 direct targets obtained *in silico*, we found *WNT6* to be simultaneously a crucial gene of this pathway and a putative direct-target of HOXA9. *WNT6* overexpression was observed in several tumor types, namely in

colorectal, cervical [151], and gastric cancers [152], as well as in GBM [60], and was associated with chemotherapy resistance [152]. Although some transcription factors, namely GATA6 and FOXA2, have been described as transcriptional regulators of *WNT6* in F9 teratocarcinoma cells [156], the ability of HOXA9 to bind to the *WNT6* promoter has never been studied. In this context, *WNT6* gene overexpression observed in HOXA9-positive cells in our microarray data was firstly confirmed by RT-PCR [Figure 3.2A] and next at the protein level by western blot [Figure 3.2B] and immunofluorescence [Figure 3.2C]. Importantly, we confirmed the direct binding of HOXA9 to the promoter region of *WNT6* in A172 cells by ChIP, demonstrating for the first time the transcriptional regulation of *WNT6* expression by HOXA9 [Figure 3.2D and E].

Due to its critical function during embryogenesis, inappropriate expression of *WNT6* in the adult may present an important role in tumorigenesis. Since *WNT6* overexpression was observed in our *in vitro* models, we intend to assess if this altered expression of *WNT6* is observed in patients' clinical samples and other glioma cell lines. In this context, *WNT6* expression was evaluated in a panel of glioma patients' samples from the Hospital of Braga, glioma pediatric and adult cell lines [Figure 3.3]. All glioma patients' samples and the majority of glioma pediatric and adult cell lines presented *WNT6* expression. Interestingly, high levels were found in a subset of grade IV adult glioma cell lines and primary tumors [Figure 3.3]. To confirm this, the publicly available dataset from TCGA was used to evaluate the molecular status of *WNT6* in normal, low-grade gliomas (LGGs) and GBM samples, as well as to investigate the clinical importance of this gene in gliomas. As demonstrated in [Table 3.1] and [Figure 3.4A], a subset of GBM patients present overexpression of *WNT6*, while no overexpression was observed in LGG patients. Indeed, *WNT6* expression was lower than normal samples in practically all LGG and in a subset of GBM patients. However, due to the dual action of WNTs as differentiation factors [157,158] or in the aggressiveness of tumors [57,58,152,159,160], depending on the patient age or the progression stage of the tumor, *WNT6* expression in gliomas, similarly to what was described for gastric cancer, may be maintained throughout tumor progression or re-acquired after initial loss due to epigenetic silencing [152]. Indeed, Yuan *et al.* [152] recently described in gastric cancer that, at early stages of tumorigenesis, *WNT6* may block or slow down the rapid proliferation of the tumor and its expression may be silenced, for example, by gene DNA methylation [152]. On the other hand, they suggested that in advanced disease stages and under stress conditions, *WNT6* may be re-expressed to protect cancer cells against apoptosis [152].

These results suggest that the expression of *WNT6* in gliomas is grade-specific and associated with the increased malignancy of gliomas.

Recently, Verhaak *et al.* [69] used the TCGA data to successfully classify GBM into four subtypes. Our study provides the first evidence of the association between high *WNT6* expression and the proneural subtype [Table 3.2]. Even though this subtype was linked to younger age and longer survival, it is one of the subtypes (like the neural subtype) that did not benefit from more intensive treatment (defined as concurrent radiochemotherapy or more than three subsequent cycles of chemotherapy) [69]. Moreover, one of the characteristics of the proneural subtype is the high expression of *TCF4*, the β-catenin partner, recently reported as increased in higher glioma grades [161], similarly to what we observed for *WNT6*. In addition, Zhang *et al.* reported that the reduction of TCF4/β-catenin activity leads to decreased glioma growth *in vitro* and *in vivo* [161]. These authors also showed that TCF4/β-catenin binds directly to the *AKT2* promoter activating its transcription [161], and that the inhibition of the PI3K pathway results in tumor growth inhibition, through negative modulation of β-catenin activity [162]. Interestingly, another report shows that PI3K pathway inhibition negatively affects the Wnt signaling (through inhibition of GSK-3β and decreasing the expression of *cyclin D1* and *MYC*) in medulloblastoma [163]. In this context, and taking into account that the PI3K pathway is known to be activated in GBMs [164] and that its inhibition negatively affects the Wnt signaling, our results reinforce the importance of the Wnt pathway in GBM, in particular for those belonging to the proneural subtype. Although further studies are necessary to fully understand the PI3K-Wnt pathways interaction, our study, in combination with others showing the link between HOXA9 and the PI3K pathway [52,53], or Wnt and PI3K pathways [161,162], sheds light into the possible existence of a PI3K-HOXA9-Wnt axis in GBM.

Although we observed that HOXA9 transcriptionally regulates the expression of *WNT6* in GBM [Figure 3.2D and E], due to its apparent importance in these tumors, we searched for other possible molecular mechanisms that can be regulating its expression. Regarding copy number aberrations, we found that they are rare in GBM [Figure 3.4B]. While different patterns of DNA methylation were observed along the *WNT6* locus [Figure 3.4C], only one specific CpG dinucleotide located in the first CpG island was inversely associated with the expression of this gene [Figure 3.4D]. These results suggest that DNA methylation, together with HOXA9 activation status, can sustain the aberrant expression of *WNT6* in GBM. Thus, to understand if *WNT6* promoter should be unmethylated for its transcriptional activation and/or if the presence of

HOXA9 is fundamental, our *in vitro* GBM models positive and negative for *HOXA9* were treated with 5-Aza-2′-Deoxycytidine (5-Aza). This drug is a well characterized DNA demethylating agent that acts by incorporation into the DNA, consequently disrupting the association between the DNA and DNMTs (DNA Methyltransferases) [146]. Therefore, querying the expression of *WNT6* by RT-PCR (before or after 5-Aza treatment), we observe a relevant increase in *WNT6* expression in both cell lines after 5-Aza treatment [Figure 3.5A]. Interestingly, after 5-Aza treatment, U87MG-MSCV cells, that present normally undetectable levels of *HOXA9* expression, present a faint band corresponding to *HOXA9* [Figure 3.5A]. These results suggest that *WNT6* expression increases after 5-Aza treatment as a result of (i) demethylation of its promoter region, (ii) HOXA9 activation that may in turn bind *WNT6* promoter and activate its transcription; or (iii) both. In this context, we cannot rule out the possibility that HOXA9 depends on DNA methylation levels to activate the transcription of *WNT6*.

Since 5-Aza acts at a global level and can be re-expressing several transcription factors, possibly altering the *WNT6* expression, we intend to clarify the relation between *WNT6* expression, the levels of DNA methylation and *HOXA9* expression. Thus, we designed MSP primers for the same region detected previously and inversely correlated with the expression of this gene in GBM patients [Figure 3.4C and D]. As expected, lower methylation levels in both cell lines were observed after 5-Aza treatment, while the difference was less evident in HOXA9-positive cells [Figure 3.5B]. The less notorious effect of 5-Aza in HOXA9-positive cells can be explained by the fact that in basal conditions, these cells already present lower levels of *WNT6* methylation. Interestingly, in basal conditions, the levels of *WNT6* methylation were dependent on *HOXA9* expression [Figure 3.5B], being lower in U87MG-HOXA9 cells, which may partly explain the higher levels of *WNT6* expression observed in HOXA9-positive cells. Together, these results suggest that although *WNT6* activation after 5-Aza treatment in U87MG-MSCV cells may be due to the activation of *HOXA9* expression, the methylation level of this region may also explain *WNT6* higher expression after *HOXA9* overexpression and/or after 5-Aza treatment. Interestingly, no correlation between HOXA9 levels and DNA methylation status of other genes was reported so far. However, GSEA analysis revealed that downregulated genes after *HOXA9* overexpression are enriched in gene sets related to genes frequently methylated in cancer (data not shown), suggesting that HOXA9 may alter the DNA methylation level of genes. In the future, it is essential to quantify by quantitative-MSP the levels of DNA methylation, and, more importantly, this experiment should be repeated using a GBM cell line silenced for *HOXA9*. Such experiments

would allow us to assess the levels of expression and DNA methylation of *WNT6* without any putative influence from *HOXA9* expression.

Given the apparent importance of *WNT6* in glioblastoma, we tried to understand whether *WNT6* expression has clinical value. In GBM patients from TCGA, higher levels of *WNT6* expression were associated with shorter survival, independently of other well characterized prognostic factor, such as patients' age, KPS, gender and treatment [Table 3.3]. This results was further confirmed in an independent dataset from Rembrandt (data not shown), suggesting that *WNT6* expression could represent a new potential prognostic factor for GBM patients. In this context, future work should focus on the potential predictive response of *WNT6* expression for treatments using inhibitors of Wnt-related pathways. Moreover, it is crucial to validate this finding in other independent datasets and evaluate the dependency of *WNT6* prognostic value to other known prognostic biomarkers.

When aberrant activation of the Wnt pathway was reported to be associated with colon cancer aggressiveness, an increasing interest in developing pharmaceutical inhibitors of this pathway was observed. Due to *HOXA9* overexpression, the expression of several genes of this pathway is altered [Figure 1.6], including *APC*, which is downregulated in our microarray. This gene encodes a tumor suppressor gene frequently mutated in colorectal cancer that in combination with other proteins, like AXIN2, forms the destruction complex responsible for the degradation of β-catenin. Taking these facts into account, the newly described Wnt pathway inhibitor [147], IWR-1, was used in this study to evaluate the effects of the Wnt pathway inhibition in HOXA9-positive and -negative cells. This inhibitor was reported as efficient in inhibiting the Wnt pathway in mouse fibroblast cells, even when *APC* is silenced using an siRNA (small interfering RNA) approach, and in colorectal cancer cells harboring an inactivating mutation in *APC* [147]. Although in this report, Wnt pathway inhibition was dose-dependent, in our GBM cell model we mainly observed a dose-independent inhibition of the cell viability [Figure 3.6A]. This result restrains the determination of IC_{50} values for these cell lines and can be due to compensatory feedbacks of the cells. Thus, to evaluates the effects on cell viability, we decided to use in our cells the minimal dose (1μM) reported by Chen *et al.* [147] that shows a significant inhibition of the pathway. Using Trypan Blue assay, we observed a statistical significant decrease in the cell viability of HOXA9-positive cells after IWR-1 treatment, suggesting that for this concentration, HOXA9-positive cells are more sensitive to Wnt inhibition [Figure 3.6B]. However, this was not corroborated by MTT assay [Figure 3.6C]. This difference can be explained by the differences in

the principles of each method to evaluate cell viability. As stated above in the section 2.9, the Trypan Blue assay is an exclusion dye assay that allow for viable cells to exclude the dye, while cells whose membrane is compromised will not. On the other hand, the MTT assay is based on the metabolic viability of the cells, since the compound added to the cells needs to be metabolized by a mitochondrial reductase in viable cells to produce formazan, which is measured by spectrometry. In short, since MTT assay depends on the activity of one specific enzyme and Trypan Blue depends on the membrane integrity, the different results can be explained by the possibility of an intact membrane with an affected metabolic viability, or vice-versa.

So far, it is not proved that "universal" Wnt targets exist, being the majority of Wnt targets described as cell-type specific, which include, for example, *AXIN2* and *SP5* in embryonic tissues. Thus, in order to understand if this concentration was inhibiting the pathway in our model, the expression levels of *AXIN2* were measured by qRT-PCR [Figure 3.6D]. However, only a slight decrease in *AXIN2* expression was observed after 24h of IWR-1 treatment. As stated above (see section 2.9), IWR-1 should be renewed every 24h to ascertain that the compound is bioavailable; in this sense, this result can be a consequence of (i) the recovery of the cells to the inhibition, suggesting that the measurement should be performed at earlier time points; or (ii) the levels of *AXIN2* expression are not a good indicator of Wnt pathway activity in our GBM *in vitro* model, emphasizing the cell type-specific targets of Wnt signaling. In the future, the expression of other putative targets of this pathway should be evaluated and/or a luciferase activity assay should be performed to assess the transcriptional activity of the pathway.

The efficacy of a new combinatorial treatment based on TMZ and IWR-1 was evaluated to understand whether this approach can be more effective in targeting HOXA9-positive cells [Figure 3.7]. As expected, TMZ alone was significantly cytotoxic in both cell lines, and the combination with IWR-1 does not alter this effect. Interestingly, a trend to lower viability upon dual treatment was observed in HOXA9-positive cells, compared to TMZ alone, both by Trypan Blue and MTT assays [Figure 3.7]. By MTT, although no statistical differences, at day 3 of treatment, were observed between TMZ 300μM and 600μM, the combination of TMZ 600μM and IWR-1 was more cytotoxic than TMZ 300μM alone in HOXA9-positive cells [Figure 3.7B]. Even though, no statistical differences were observed between TMZ alone or TMZ in combination with IWR-1 at day 3, a trend was observed suggesting that more studies at higher time points and a more extended range of IWR-1 concentration should be tested [Figure 3.7]. Moreover, TMZ alone was

already highly cytotoxic, suggesting that lower concentrations of TMZ should be tested to search for a more efficient addictive effect.

Although more studies are necessary to confirm our results, here we present some clues of a potential therapy to target effectively HOXA9-positive cells. In the future, we intend to confirm this trend using different concentrations of the same inhibitor and other potential drugs, but also to modulate *WNT6* expression (e.g. using shRNA) in GBM cell lines to observe the specific effects of *WNT6* inhibition/overexpression. Additionally, more studies are essential to unveil the influence of the Wnt pathway in other cellular features, such as migration, invasion and cell cycle, since this pathway is associated with increased migration and decreased adhesion of the cells.

5. CONCLUSIONS

5| Conclusions

This work aimed to study the relevance of the Wnt signaling activation by HOXA9 in GBM.

Throughout this study, we demonstrated that (i) the transcriptome of HOXA9 is enriched for the Wnt pathway; (ii) HOXA9 directly binds to *WNT6* promoter and transcriptionally activates *WNT6*; (iii) *WNT6* expression in glioma seems to be grade specific; (iv) the proneural GBM subtype holds the higher percentage of patients with high *WNT6* expression; (v) *WNT6* expression in GBM can be regulated by DNA methylation; (vi) *WNT6* expression can be a new prognostic factor in GBM; and (vii) the inhibition of the Wnt pathway may be a potential therapy to effectively target HOXA9-positive cells.

In summary, we provide significant contributions to the oncology field, namely for the basic sciences, profiting from the interaction between HOXA9 and *WNT6*, and for the clinical context, with new insights for the prognosis of GBM patients.

Future work is warranted to further elucidate the relevance of the presented mechanistic, prognostic and therapeutic insights of Wnt pathway in GBM in order to ultimately obtain clinical value.

6. REFERENCES

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