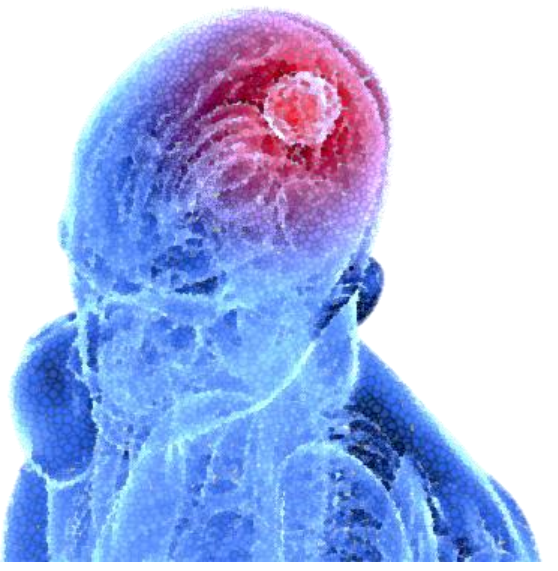
DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
DA UNIVERSIDADE DE COIMBRA

Evaluation of the diagnostic and prognostic value of IDH1 and Progranulin in patients with Gliomas



Marta Alexandra Lopes Ribeiro

2015

Evaluation of the diagnostic and prognostic value of IDH1 and Progranulin in patients with Gliomas

Marta Alexandra Lopes Ribeiro

2015

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA DA
UNIVERSIDADE DE COIMBRA

Evaluation of the diagnostic and prognostic value of IDH1 and Progranulin in patients with Gliomas

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em 2015, realizado sob orientação científica da Professora Doutora Maria do Rosário Almeida (Centro de Neurociências e Biologia Celular da Universidade de Coimbra) e da Professora Doutora Emília Duarte (Departamento de Ciências da Vida, da Universidade de Coimbra)

Marta Alexandra Lopes Ribeiro

2015

"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein

Agradecimentos

Agradecer (v) = mostrar gratidão (por); dar graças (por); retribuir de modo equivalente; compensar

É com muita satisfação que expresso aqui o mais profundo agradecimento a todos aqueles que, que directa ou indirectamente, tornaram a realização deste trabalho possível.

Gostaria antes de mais de agradecer à Professora Doutora Rosário Almeida, orientadora desta tese, pelo apoio, incentivo e disponibilidade demonstrada em todas as fases que levaram à concretização deste trabalho. Agradeço não só por todo o conhecimento transmitido ao longo deste percurso, mas também pelo sentido de responsabilidade que me incutiu durante o mesmo. Para além disso, muito obrigada pela amizade e sobretudo por acreditar/confiar em mim.

Quero ainda agradecer:

À Faculdade de Ciências e Tecnologia da Universidade de Coimbra, ao Centro de Neurociências e Biologia Celular de Coimbra, ao Laboratório de Neurogenética pelas condições proporcionadas para a realização deste trabalho.

À "vasta equipa" do Laboratório de Neurogenética, Ana Santos e Maria Helena Ribeiro. Obrigada pela forma como me receberam, pelo ambiente que me proporcionaram e por tudo o que me ensinaram. O vosso companheirismo e amizade foram determinantes na realização deste trabalho. Jamais esquecerei os momentos divertidos que vivemos.

À Dra. Olinda Rebelo, Neuropatologista do CHUC, pela amabilidade, disponibilidade e colaboração ao longo deste projecto. A sua sabedoria e interesse foram cruciais para a realização do mesmo.

Ao Dr. Hermínio Tão, Neurocirurgião do CHUC, pela disponibilidade e prontidão que possibilitaram a realização deste projecto.

À Graça e Sandra, Técnicas do Serviço de Neuropatologia do CHUC, pela ajuda e apoio prestados ao longo destes meses.

À Professora Doutora Inês Baldeiras pela ajuda, disponibilidade e apoio prestados, sobretudo em momentos de maior aflição. A sua colaboração foi essencial para a realização desta tese.

À Mª João (Jajão) pela ajuda, carinho e amizade manifestados, além da enorme preocupação nos momentos menos bons.

À Dona Arminda, Lurdes e Rui por toda a simpatia nos vários momentos.

À Dona Fernanda pelas inúmeras vezes que me abriu o laboratório e por toda a simpatia.

Aos meus colegas de mestrado, em especial à Isabella Boux e Sara Vaz, que de alguma forma contribuíram para a realização desta tese.

Às minhas amigas de infância e de licenciatura pelas palavras amigas e partilha de momentos.

À Carla Ferreira por me ouvir e aliviar o *stress* o que foi fundamental, sobretudo nesta fase final.

À Verinha e ao pequeno Salvador por todo o carinho, amizade, apoio e palavras de incentivo.

Aos meus avós Lópão e Mari Lopes por todo o mimo, amor e orgulho que sentem em mim.

Ao Carlitos por todo o amor, apoio e companheirismo incondicionais. Jamais esquecerei as palavras doces e transmissão de força e confiança nos momentos de desânimo. Obrigada por acreditares em mim e em nós.

Aos meus pais, Paula e Tó-Zé, um ENORME obrigada pelo amor incondicional, por todos os ensinamentos de vida e por acreditarem em mim. Tudo o que sou hoje reflecte, na grande maioria, a educação que me deram. Esta etapa que agora termino apenas foi possível graças a vocês. Espero um dia poder retribuir parte daquilo que constantemente me oferecem.

De uma forma simples, mas sincera, muito obrigada por me terem acompanhado nesta jornada, que por vezes pareceu tão dura e difícil!

Esta tese é-vos dedicada!

Table of Contents

Illustrations Index

Tables Index

Abbreviations

UNITS:	
ºC	Celsius degrees
µl	Microliter
ml	Milliliter
M	Molar
µM	Micromolar
mM	Milimolar
µm	Micrometer
nm	Nanometer
g	Gram
ng	Nanogram
min	Minutes
sec	Seconds
U	Enzyme unit
bp	Basis pairs
Da	Daltons
KDa	Kilodaltons

A-Z:	
3'UTR	3' untranslated region
5'UTR	5' untranslated region
ApoA-1	Apolipoprotein a-1
CHUC	Centro Hospitalar e Universitário de Coimbra
CNS	Central nervous system
D-2-HG	D-2-hydroxyglutarate
ddNTPs	Dideoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ECL	Chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FFPE	Formalin-fixed paraffin-embedded
FTLD	Frontotemporal lobar degeneration
Fwd	Forward
GBM	Glioblastoma multiform
GBMs	Glioblastomas multiform
GEP	Granulin-epithelin precursor
GSC	Glioma stem cells
HDL	High density lipoprotein
Hif-1 α	Hypoxia-inducible factor-1 α
IDH	Isocitrate dehydrogenase
IDH1	Isocitrate dehydrogenase 1
IHC	Immunohistochemistry
L-2-HG	L-2-hydroxyglutarate
MGMT	O(6)-methylguanine dna methyltransferase
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NADP	Nicotinamide adenine dinucleotide phosphate oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate reduced

PCDGF	PC cell-derived growth factor
PCR	Polymerase chain reaction
PEPI	Proepithelin
PGRN	Progranulin
PHD	Prolylhydroxylases
PI3K	Phosphatidylinositol 3-kinase
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride
R-2-HG	R-enantiomer of 2-hydroxylglutarate
RB	Retinoblastoma protein
Rev	Reverse
RIA	Radioimmunoassay
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SDS–PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLPI	Secretory leukocyte protease inhibitor
SS	Sanger sequencing
TBE	Tris borate ethylenediaminetetraacetic acid
TBST	Tris buffered saline and tween
TCGA	The cancer genome atlas
TGFe	Epithelial transforming growth factor
TNF-α	Tumour necrosis factor- α
WB	Western blot
WHO	World health organization
α-KG	A-ketoglutarate

Abstract

Gliomas are the most common primary brain tumours that arise in the central nervous system (CNS). According to the World Health Organization (WHO) their classification, is based on cell origin, glial cells and are graded according to their histological degree of malignancy, ranging from the least aggressive (Grade I) to the most aggressive (Grade IV), also known as Glioblastomas (GBM). This latter one, arise from astrocytes and are considered the most lethal adult brain tumour, representing 20% of all primary brain tumours.

Unfortunately, the prognosis of most gliomas, especially glioblastoma, continues to be dismal and the median survival has remained at 9 to 15 months for decades. Despite all the progresses in understanding the molecular mechanisms involved in biology of this brain tumours, the discovery of successful strategies for diagnosis, prognosis and therapeutic treatments are urgently needed.

Over the past few years, a few promising molecular markers have been described conferring diagnostic, prognostic and predictive value to therapy response. This study focused on the investigation of the value of IDH1 as diagnostic and prognostic marker in glioma patients. In addition, we also aim to explore the progranulin (PGRN) levels in glioblastoma patients to further correlate with their overall survival.

Several mutations have been described in IDH1 gene, however the most frequent (>95%) is p.R132H. Ninety-six patients assisted in the Centro Hospitalar e Universitário de Coimbra (CHUC) were screened for the presence of somatic mutations in IDH1 gene using two different methods in parallel, Sanger sequencing and Immunohistochemistry (IHC). Gathering the results, we concluded that the combination of the two techniques increased the specificity and sensitivity of mutation detection. Furthermore, in a daily practice, we advocate the use of IHC as a first line method, followed by sequencing in IHC-negative cases with dubious clinical or morphological features. Moreover, the patient's tumour harbouring *IDH1* mutations showed a better outcome than patients with non-mutated tumours.

Recently, it has been shown that PGRN and PGRN mRNA expression is highly increased in several human tumour types. In addition, this year it was demonstrated that PGRN promotes temozolomide resistance, the current first-line chemotherapeutic agent for

glioblastoma. The level of PGRN has been assessed in 40 glioblastoma patients by ELISA technique. Surprisingly, the plasma levels of PGRN in the patients with GBM were significantly decreased compared with healthy control ($p<0.001$). Furthermore, patients with decreased levels of PGRN show a shorter survival than patients with normal levels.
These results suggested that PGRN might be used as a potential prognostic marker. However, further studies should be performed in order to establish a correlation between plasma level and tumour tissue PGRN expression as well as patient prognosis.

Keywords: Glioma, glioblastoma, IDH1, PGRN and predictive markers.

Resumo

Os gliomas são os tumores cerebrais primários mais comuns que surgem no sistema nervoso central (SNC). Segundo a Organização Mundial de Saúde (OMS) a sua classificação é baseada na célula de origem, células gliais, e são classificados de acordo com seu grau histológico de malignidade, do menos agressivo (Grau I) para o mais agressivo (Grau IV), também conhecido como Glioblastoma (GBM). Este último tem origem em astrócitos e é considerado o tumor cerebral mais letal na idade adulta, representando 20% de todos os tumores cerebrais primários.

Infelizmente o prognóstico da maioria dos doentes com gliomas, e especialmente com glioblastomas, continua a ser muito reservado. E há décadas que a sobrevida se mantém entre 9 a 15 meses. Apesar de todos os avanços na compreensão dos mecanismos moleculares envolvidos na biologia destes tumores cerebrais, é urgente a descoberta de estratégias eficazes no diagnóstico, prognóstico e tratamentos terapêuticos.

Ao longo dos últimos anos, foram descritos alguns marcadores moleculares promissores para o diagnóstico, prognóstico e resposta à terapêutica. O presente estudo focou-se na investigação do valor do marcador IDH1 no diagnóstico e prognóstico de doentes com glioma. Além disso, também teve como objectivo explorar os níveis da progranulina (PGRN) em doentes com glioblastomas correlacionando-os com a sua sobrevida.

Várias mutações têm sido descritas no gene IDH1, no entanto, a mais frequente (> 95%) é p.R132H. Noventa e seis doentes seguidos no Centro Hospitalar e Universitário de Coimbra (CHUC) foram estudados para a presença de mutações somáticas no gene IDH1 usando em paralelo dois métodos diferentes, sequenciação de Sanger e imunohistoquímica (IHQ). Dos resultados obtidos podemos concluir que a combinação das duas técnicas aumenta a especificidade e sensibilidade na detecção de mutações. Assim, no dia-a-dia aconselhamos o uso de IHQ como método de primeira linha, seguida de sequenciação para casos em que a IHQ tenha sido negativa ou que apresentem características clínicas/morfológicas duvidosas. Além disso, doentes com mutações no *IDH1* apresentaram um melhor prognóstico do que os doentes com tumores não mutados.

Recentemente, foi demonstrado que a PGRN e expressão do mRNA estão aumentadas em vários tipos de tumores. Também, este ano foi descoberto que a PGRN torna as células de glioblastoma resistentes à temozolomida, agente quimioterapêutico de primeira linha no

tratamento destes tumores. O nível da PGRN foi avaliado em 40 doentes com glioblastoma através da técnica de ELISA. Surpreendentemente, plasmas de doentes com GBM apresentaram níveis da PGRN significativamente diminuídos quando comparados com os indivíduos controlo ($p<0.001$). E ainda, os doentes com níveis diminuídos de PGRN apresentaram uma sobrevida menor em relação aos doentes com níveis normais. Estes resultados sugeriram que a PGRN poderá ser usada como um possível marcador de prognóstico. Contudo, estudos adicionais deverão ser realizados de modo a estabelecer uma correlação entre o nível da PGRN no plasma e a sua expressão no tecido tumoral.

Palavras-Chave: Glioma, glioblastoma, IDH1,PGRN e marcadores preditivos.

CHAPTER 1 Introduction

1. Introduction

1.1. Cancer and general concepts

Cancer is one the most devastating diseases in the world. In 2012 worldwide, over 14 million of new cancer cases were reported, and about 8.2 million of deaths were estimated. Actually, cancer is the third leading cause of death. Men and women were similar affected but by different types each group. For instances, in 2012 lung cancer was the most common cancer worldwide in both sexes, as well as in men. Nevertheless, in women breast cancer was the most common cancer worldwide. In Portugal about 500.000 new cancer cases and over 121.000 deaths have been reported in 2012 (Ferlay et al., 2013).

The term cancer do not refer just to one disease but many diseases, in which abnormal cells divide at high and rapid rates and become able to invade other tissues. At high stages, cancer cells can be spread for whole body through the blood or lymph systems. There are several types of cancer take into account the cell which originate it. For instances, carcinoma is a cancer that begins in epithelial cells, others main categories of cancers are: sarcoma, leukemia, lymphoma, myeloma and central nervous system cancers.

In addition, cancer resulted from several mutational events in which accumulations of genetic and epigenetic changes take place at somatic level and are responsible for turning a normal cell into a cancer cell. However, not only somatic alteration leads to cancer there are also inherited alterations which drive to the carcinogenic process (Ogino et al. 2012).

There are two main types of genes involved in cancer origin which are: proto-oncogenes and tumour suppressor genes. The proto-oncogenes encode proteins responsible for controlling cell proliferation, apoptosis or both. When proto-oncogenes are mutated they become known as oncogenes. Typically, these oncogenes have a gain of function which leads to an increase of cell division, decrease of cell differentiation and inhibition of cell death (Croce 2008). Conversely, the tumour suppressor genes encode proteins which slow down cell division, repair DNA mistakes or lead to apoptosis. Once mutated, these genes lost the function and cells grow out of control (Sherr 2004).

Each cancer has certain characteristics which make it unique. However, all cancers have common features which enhance their fitness, for example influencing the cell cycle

checkpoints, angiogenesis, apoptosis, telomere synthesis and others (Aktipis & Nesse 2013). One way to get understanding about the remarkable diversity of neoplastic diseases was to propose hallmarks for cancer. The capabilities acquired during cancer development include: sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, deregulating cellular energetics, resisting cell death, enabling replicative immortality, genome instability and mutation, inducing angiogenesis, activating invasion and metastasis and tumour-promoting inflammation (Figure 1) (Hanahan & Weinberg 2011).

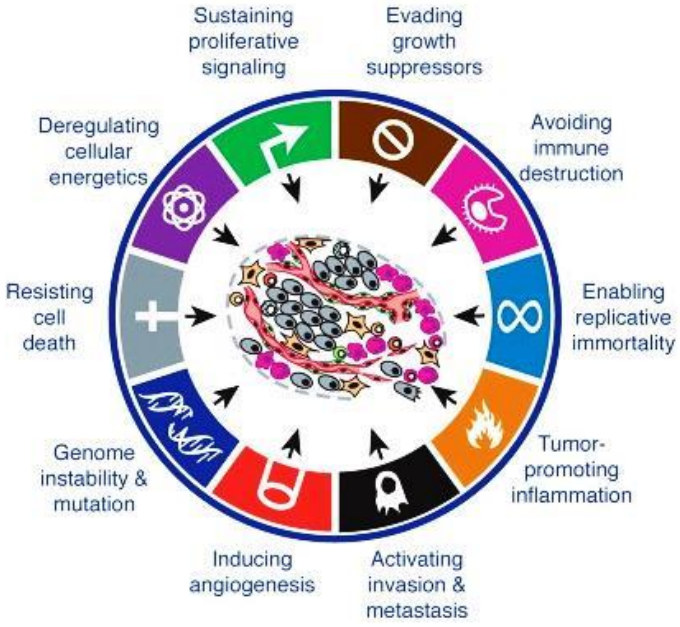


Figure 1- The Hallmarks of Cancer. Adapted from (Hanahan & Weinberg 2011).

Despite the various hallmarks previously reported there is, indeed other key factor in cancer initiation. The normal microenvironment of cells is extremely important to avoid cancer, its progression and also response to treatment. In cancer, cells are highly social in order to get advantages to grow and disseminate. Tumour microenvironment includes tumour cells as well as the nearby normal cells. These normal cells can be manipulated by tumour cells to provide them growth signals or other fitness enhancing factors. A variety of changes to neighbouring cells such as fibroblasts, vasculature, immune cells and interstitial extracellular matrix, increase the risk of cancer (Figure 2) (Bissell & Hines 2011).

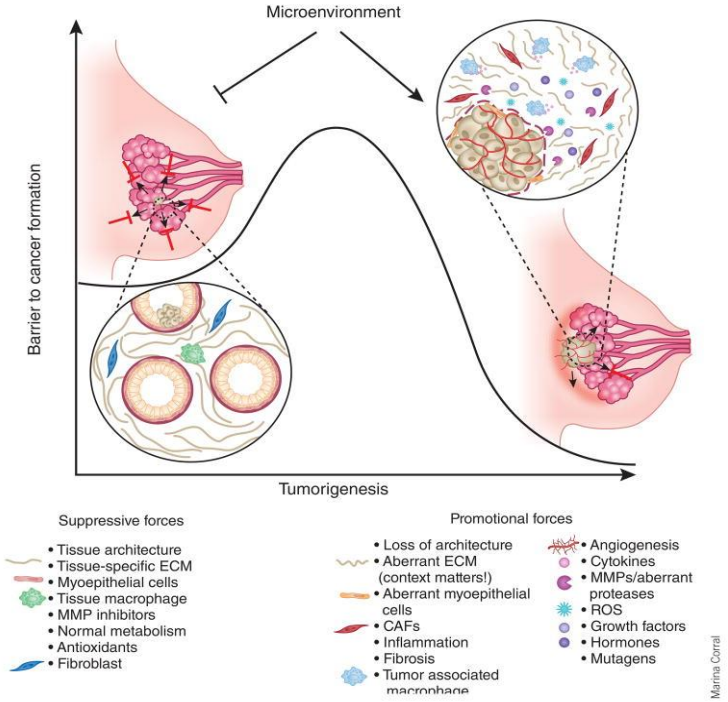


Figure 2 - The importance of the microenvironment to induce tumorigenesis. Adapted from (Bissell & Hines 2011).

1.2. Tumours in Central Nervous System

The nervous system is divided into two main systems: the central nervous system (CNS) which contains the brain and spinal cord; and the peripheral nervous system (PNS) which is consisted mainly by nerves. The CNS is responsible for controlling most functions of the body and mind by processing information from all parts of the body. The PNS connects the CNS with limbs and organs, carrying information back and forward between the body and the brain.

CNS tumours could be either benign or malignant depending on the capacity to invade others tissues and spread for all body. While a benign tumour is restricted to certain area, a malignant tumour surrounds several tissues.

There are several types of CNS tumours depending on the part of the brain they appear. Some are really aggressive with high grow rate and invasion named high-grade. Others are less aggressive and often called low-grade. According to WHO, several new entities are listed to the classification of the CNS every year. However, the most common are gliomas,

representing more than 80 percent of all primary brain tumours in both children and adults (Louis et al. 2007; Horbinski 2012; Wang et al. 2015).

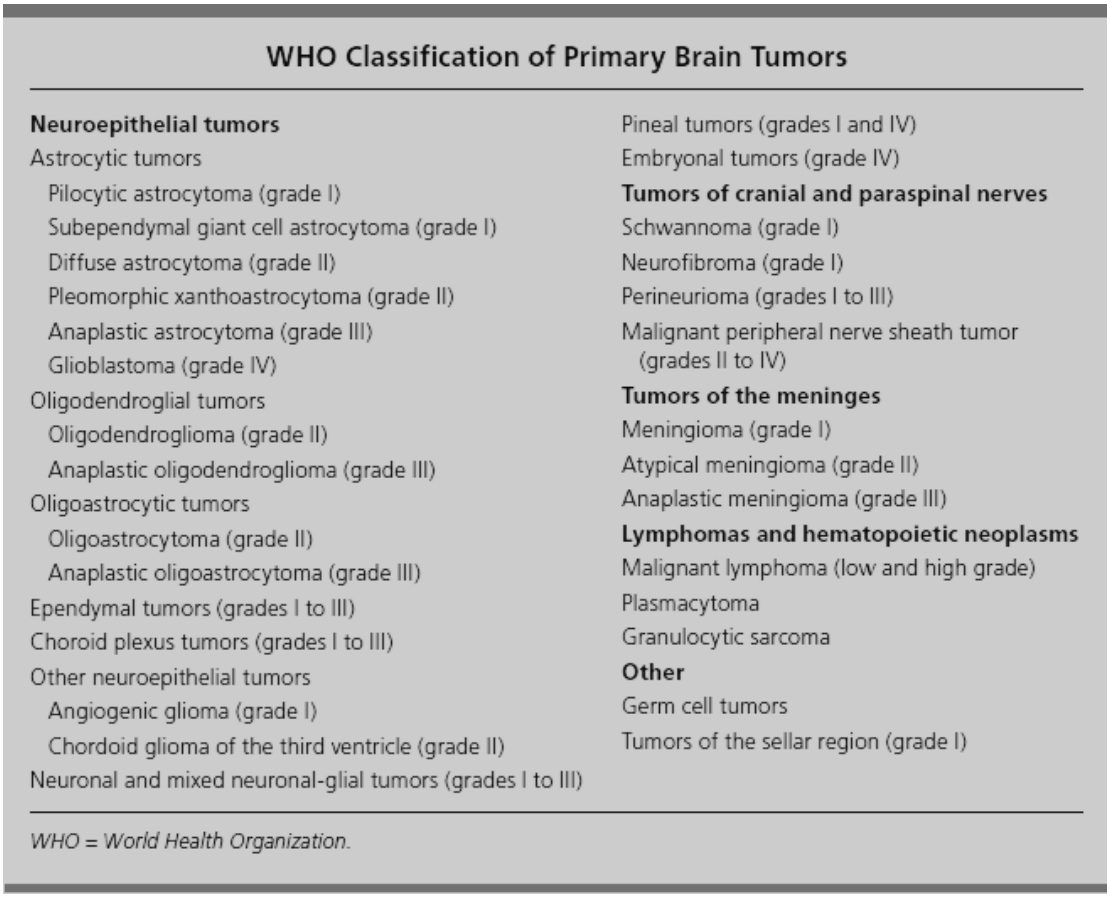
The incidence of brain and CNS tumours in the Europe is 6.6 per 100,000 persons with an estimated mortality of 4.9 per 100,000 persons for both genders (http://globocan.iarc.fr).

In Portugal, men and women with CNS tumours have an estimated incidence of 7.1 and a mortality of 5.0 per 100,000 persons (Ferlay et al. 2013).

1.2.1. Gliomas

1.2.1.1. Classification

All tumours are classified according to WHO classification which is based on cellular origin and histological appearance (Figure 3). Gliomas derive from three types of cells: astrocytes, oligodendrocytes or ependymal cells, therefore, the major representative forms are: astrocytoma, oligodendroglioma and ependymoma, respectively. Moreover, take into account the tumour malignancy, gliomas are divided in four grades. Grade I and II are the lowest grades, while grade III and IV are the highest ones (Chandana et al. 2008). Lesions of grade I usually have low proliferative potential and they are curable with surgical resection alone. On the other hand, grade II neoplasms are commonly infiltrative, besides the low proliferation, and often reappear. Frequently, some types of grade II tumour are able to become more aggressive, for example a diffuse astrocytoma can progress into anaplastic astrocytoma. The classification grade III is attributed to tumour with histological evidence of malignancy such as abnormal nucleus and uncontrolled mitotic activity. Ultimately, tumours with grade IV should have certain features like cytological malignancy, high mitotic activity, necrotic cells, fast disease progression and poor prognosis. Glioblastoma Multiform (GBM) is a grade IV astrocytoma, the most common and aggressive type of glioma (Louis et al. 2007).



WHO Classification of Primary Brain Tumors

Neuroepithelial tumors
Astrocytic tumors
- Pilocytic astrocytoma (grade I)
- Subependymal giant cell astrocytoma (grade I)
- Diffuse astrocytoma (grade II)
- Pleomorphic xanthoastrocytoma (grade II)
- Anaplastic astrocytoma (grade III)
- Glioblastoma (grade IV)

Oligodendroglial tumors
- Oligodendroglioma (grade II)
- Anaplastic oligodendroglioma (grade III)

Oligoastrocytic tumors
- Oligoastrocytoma (grade II)
- Anaplastic oligoastrocytoma (grade III)

Ependymal tumors (grades I to III)
Choroid plexus tumors (grades I to III)
Other neuroepithelial tumors
- Angiogenic glioma (grade I)
- Chordoid glioma of the third ventricle (grade II)

Neuronal and mixed neuronal-glial tumors (grades I to III)
Pineal tumors (grades I and IV)
Embryonal tumors (grade IV)

Tumors of cranial and paraspinal nerves
Schwannoma (grade I)
Neurofibroma (grade I)
Perineurioma (grades I to III)
Malignant peripheral nerve sheath tumor (grades II to IV)

Tumors of the meninges
Meningioma (grade I)
Atypical meningioma (grade II)
Anaplastic meningioma (grade III)

Lymphomas and hematopoietic neoplasms
Malignant lymphoma (low and high grade)
Plasmacytoma
Granulocytic sarcoma

Other
Germ cell tumors
Tumors of the sellar region (grade I)

WHO = World Health Organization.

Figure 3 – Classification of primary brain tumours according to WHO. Adapted from (Chandana et al. 2008).

1.2.1.2. Primary and secondary tumours

There are two types of tumours depending on its progression. When a tumour arises for the first time in certain part of the body is known as primary tumour. However, due to blood and lymphatic system tumour cells from a primary tumour can be spread to other parts of the body and through several divisions a new tumour called secondary tumour or metastasis is formed (Bernstein & Berger, 2008).

Noteworthy, primary and secondary brain tumours are highly devastating and they have poor outcomes (Jadin et al. 2015). About 45-50% of all primary brain tumours are GBMs, a genetically and phenotypically heterogeneous glioma. The vast majority of GBMs, approximately 90% of the cases, arising *de novo* as primary GBM which grows within 3 months through a multistep tumorigenesis from normal glial cells. The remaining 10% of GBMs are named as secondary GBM and develop by the progression from a previous II or

III WHO grade glioma, taking 4-5 years (Aldape et al. 2015; Urbańska et al. 2014). In histologic terms, primary and secondary GBMs are indistinguishable having the same rates of proliferation and also invasion (Kleihues & Ohgaki 1999). However, these two types of GBMs are associated to different genetic basis and also molecular pathways, and affecting different aging groups. Generally, all GBMs are almost incurable with a median survival of nine to fifteen months (Karcher et al. 2006; Aldape et al. 2015).

1.2.1.3. Development and progression

In general, tumours are complex multifactorial diseases which underlie complex carcinogenesis processes, some of them still unknown (Ogino et al. 2012). Regarding gliomas, there are only two risk factors reported such as: exposure to high doses of radiation and genetic susceptibility caused by inherited mutations associated with rare syndromes. Some examples of inherited syndromes associated with increased risk for gliomas are neurofibromatosis 1 and 2, tuberous sclerosis, retinoblastoma, Li–Fraumeni syndrome, and Turcot's syndrome. However, these syndromes account only for a small part of the gliomas cases since the vast majority are sporadic (Schwartzbaum et al. 2006; Ichimura et al. 2009).

Over the last years advances in understanding the molecular pathology and progression of gliomas have occurred and basic features of gliomagenesis have been unrevealed. In order to have an uncontrolled cellular growth, proliferation and invasion, normal cells need to become neoplastic by accumulating somatic genetic and epigenetic changes in a sequential fashion. Studies using classic cytogenetic and arrays to compare genomic hybridization have identified copy number changes such as gains, deletions and amplifications in several genomic regions. Through these studies numerous candidate genes involved in tumour suppression and in tumour initiation or progression could be pointed (Schwartzbaum et al. 2006). However, the cells which originate gliomas have generated a lot of disagreement for many years and continue under strong research. Virchow and colleagues were the first to introduce the concept of glioma stem cells (GSC) in brain tumours. Actually, there are three theories related with cells responsible for gliomagenesis: the de-differentiation theory, the precursor cell theory, and the stem cells theory, with astrocytes, glial precursors and stem cells as original cells, respectively (Germano & Binello 2014).

Besides that, several cellular pathways which are involved in the oncogenesis and progression of gliomas have been identified. According to The Cancer Genome Atlas (TCGA) research network, the three core cellular pathways involved in gliomagenesis include the receptor tyrosine kinase (RTK)/RAS/ phosphatidylinositol 3-kinase (PI3K), the p53, and the retinoblastoma protein (RB) signaling pathways (Wang et al. 2015; Ichimura et al. 2004).

Mutations and copy number changes (insertion/deletion) are responsible for activation or inactivation of components in RTK/RAS/ PI3K, RB, and p53 pathways. In each pathway, the altered components could lead to proliferation translation survival, senescence apoptosis or G1/S progression (Figure 4) (Wang et al. 2015).

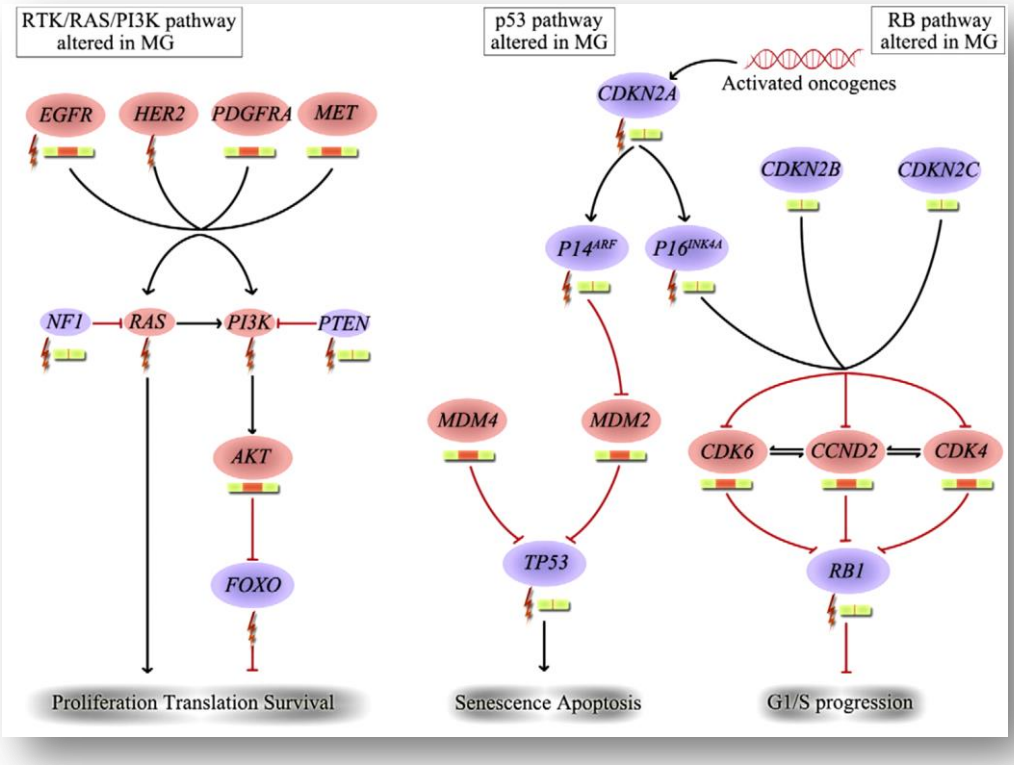


Figure 4 – The three core cellular pathways involved in gliomagenesis. Depending on the type of each alteration in certain gene we have activation or inactivation of specific pathway. MG indicates malignant glioma (Wang et al. 2015).

1.2.1.4. Diagnosis

Currently, the diagnosis of gliomas is based on clinical examination, imaging and histopathology. At the present, gliomas are classified and graded based on histological features and degrees of malignancy after hematoxylin and eosin staining according to WHO classification. This classification stratifies gliomas in four grades (Komori 2015). By itself, WHO classification is very clear in diagnosis gliomas circumscribed (grade I) and the most aggressive diffuse glioma, GBM (grade IV). However, the interpretation of WHO classification for diffuse gliomas with grade II and grade III generate a huge variability among neuropathologists. Besides that, the techniques available are not sufficient informative to predict diagnosis, prognosis and treatment of most glioma patients (van den Bent 2010; Coons et al. 1997).

Therefore, in order to improve the glioma diagnosis, recently efforts have been performed to combine the use of WHO classification with molecular markers of the tumour (Olar & Sulman 2015) (Vigneswaran et al. 2015).

1.2.1.5. Importance of Markers in Gliomas

Over last years, the improvements in genomics, proteomics, molecular pathology and modern imaging techniques have provided many candidate biomarkers. The term biological marker (biomarker) is defined by National Institutes of Health as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention*" (Anon 2001).Thus, a biomarker is a potential tool for different stages of the disease process. For instance, before the diagnosis a biomarker could be used for screening and risk assessment. It can be also used during diagnosis to determine the stage, grade and to select the therapy. Moreover, a biomarker can be used during a treatment to control therapy, allowing the selection of additional therapy (Kumar & Sarin 2010).

Numerous potential glioma biomarkers have been recently discovered such as: IDH1 and IDH2 mutations, 1p and 19q deletions, MGMT promoter methylation and level of progranulin (PGRN). However, their ability to predict the clinical outcome in terms of risk of

tumour relapse, treatment failure, adverse events and to allow an accurate prognosis as well as clinical decision making, required further studies (Towner et al. 2013) .

1.2.1.5.1. Analysis of *IDH1 mutations*

The oxidation of isocitrate to α-ketoglutarate (α-KG) is performed by three enzymes in human cells: isocitrate dehydrogenase 1 (IDH1); isocitrate dehydrogenase 2 (IDH2); isocitrate dehydrogenase 3 (IDH3). Even though the similarities of their names these enzymes have different subcellular locations, composition and functions (Horbinski 2013).

The IDH1 gene is located on 2q33 and encodes a homodimeric enzyme that converts isocitrate into α-ketoglutarate with the simultaneous reduction of NADP+ to NADPH. Mammalians have this enzyme present mainly in the cytoplasm and also reduced quantities in peroxisomes (Bogdanovic et al. 2014).

Of all IDH enzymes, it is believed that only IDH3 participates in Krebs cycle being a unidirectional enzyme which only converts isocitrate to α-KG. Nonetheless, IDH1 and also IDH2 are bidirectional enzymes during adverse conditions such as hypoxia or mitochondrial dysfunction. Thus, through the reduction of α-KG into isocitrate they help the cell reload Krebs cycle intermediates and fatty acid precursor acetyl- CoA (Yen et al. 2010);(Wise et al. 2011).

Somatic mutations in IDH1 gene were first discovered during a sequencing effort across colorectal cancers (Sjöblom et al. 2006). More recently, in 2008, *IDH1* mutations were identified by whole genome analysis in a small subset of glioblastomas (Parsons et al. 2008). After that, several studies in this field have been published reporting that the majority of low grade gliomas and also secondary glioblastomas harbour mutations in IDH1.

Secondary glioblastomas develop from a lower grade tumour, while primary glioblastoma develop de novo without known precursor lesions (Ichimura et al. 2009; Balss et al. 2008). These mutations tend to occur in both younger adults and adults comprising the age of 20-60 years.

All reported *IDH1* mutations are missense mutations which are almost always presented in heterozygosity. About 90% of these mutations are CGT>CAT transitions in codon 132, resulting in the replacement of an arginine for a histidine (R132H IDH1). Although less

frequent, other mutations occur at codon 132 leading to other substitutions like R132C, R132S, R132G, R132L, whereas R132P and R132V mutations are extremely rare (Horbinski 2013; Arita et al. 2015) (Fig.5).

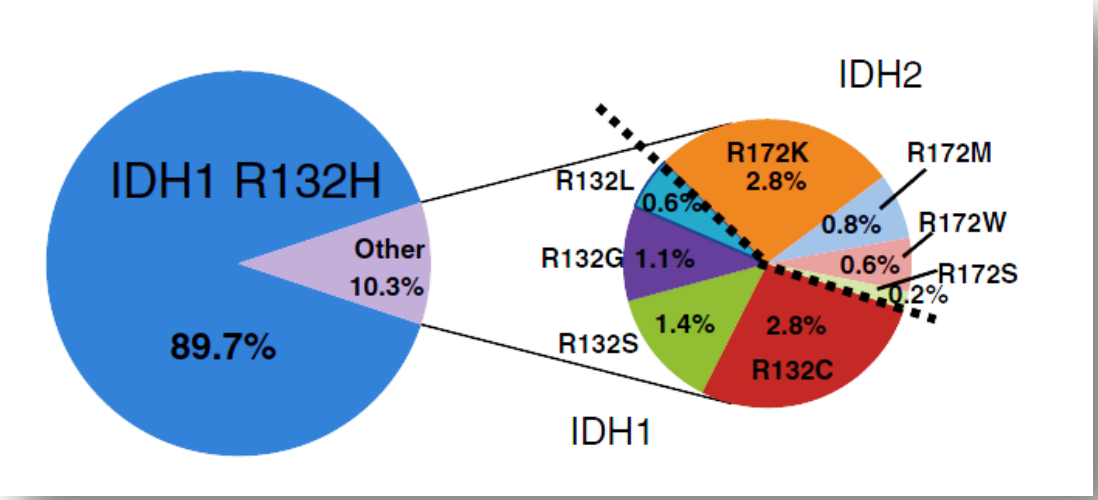


Figure 5 – Graphic representation of frequency of IDH1 R132H mutation and non-R132H IDH1/2 mutations. Adapted from (Arita et al. 2015)

Regarding these *IDH1* mutations, they are known as targeting the arginine codon involved in the binding of isocitrate. Thus, tumours with *IDH1* mutated have impaired affinity for isocitrate. In 2009, Zhao and colleagues believed the occurrence of mutations in IDH1 acted as a dominant negative inhibitor of wild-type IDH1 which leaded to tumorigenesis. The hypoxia-inducible factor-1 α (Hif-1 α) degradation by prolylhydroxylases (PHD) requires α-KG, IDH1 mutants presented increased levels of Hif-1. This finding suggested the presence of the catalytically inactive heterodimer was responsible for controlling the levels of α-KG (Zhao et al. 2009). However, this hypothesis was declined once other studies have shown no difference in either Hif-1 α or α-KG levels between mutant and wild-type gliomas (Williams et al. 2011; Dang et al. 2009).

In order to investigate the *IDH1* mutations tumorigenic role, Dang and collaborates focus on the IDH1 ability to act on α-KG as a substrate which has not been explored, yet. The study showed that R132H mutation confers a neoenzymatic activity on IDH1, catalysing the NADPH-dependent conversion of α-KG to D-2-hydroxyglutarate (D-2-HG) (Dang et al.

2009)(Fig.6). This neoenzyme is capable to produce 10 to 100-fold higher levels of 2-HG than the wild-type IDH1, reaching concentrations ranging from 10 to 30 mM (Horbinski 2013). Noteworthy, the production of 2-HG by wild-type IDH1 also exists in the reverse reductive reaction under hypoxic conditions however the levels produced are much lesser than mutants levels (Wise et al. 2011).

Under normal physiological conditions the R-enantiomer of 2-hydroxylglutarate (R-2-HG) is present in low levels (Ye et al. 2013). Prior to 2009, the accumulation of high levels of R-2-HG was associated with a rare autosomal recessive neurometabolic disorder called R-2-HG aciduria. This disorder was first described in 1980 and was recognized as existing in two forms: L-2-hydroxyglutarate (L-2-HG) and D-2-HG, depending on the type of isomer. Besides L-2-HG aciduria is more common and severe than the other form, each isomer producing its own phenotypes. L-2-HG aciduria particularly affects the cerebellum in early childhood leading to progressive ataxia; other features are mental retardation, seizures, speech difficulties and macrocephaly. In contrast, patients with D-2-HG aciduria have mental retardation, seizures, hypotonia and abnormalities on the cerebrum affecting several functions (Yang et al. 2012; Rakheja et al. 2013).

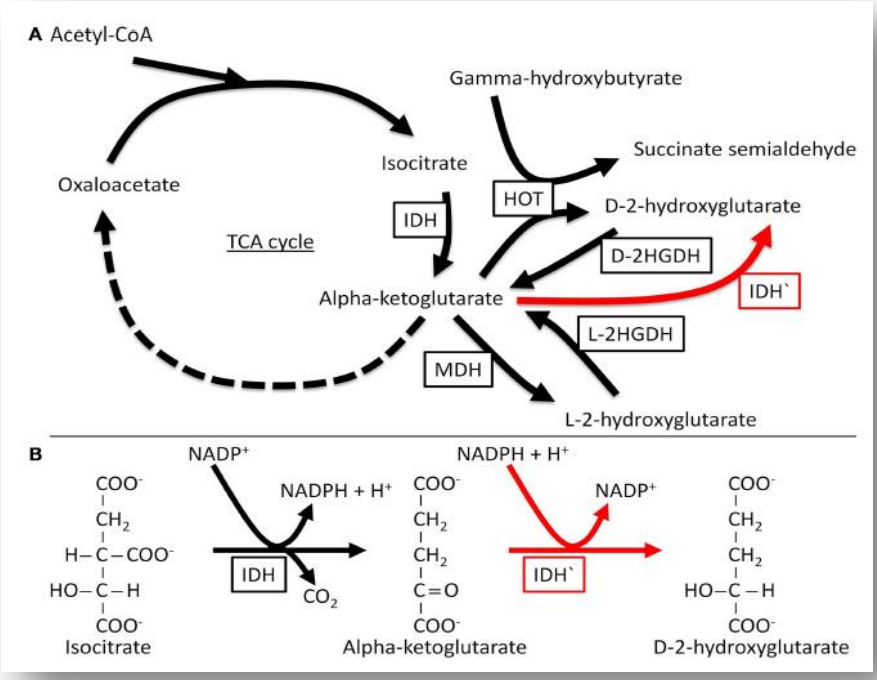


Figure 6 – The normal and neomorphic reactions catalysed by IDH1. Adapted from (Rakheja et al. 2013).

The normal activity of wild-type IDH1 has three main phases: 1) the open state for isocitrate binding; 2) the closed pre-transition state; 3) the catalysis state. When R132H mutation is present the normal activity is blocked preventing the shift to the closed pre-transition state. Even though, isocitrate cannot binding and its catalysis is impeded, additionally the reduction of α-KG to 2-HG is favored (Yang et al. 2010). Co-precipitation experiments showed mutant *IDH1* form heterodimer with wild-type IDH1 instead of homodimers. These heterodimers work in a process known as substrate channeling, where the wild-type IDH1 convert isocitrate into α-KG, which is immediately catalyzed into 2-HG by the mutant *IDH1* (Pietrak et al. 2011).

Although all knowledge regarding mutant IDH1 and its neomorphic activity which leads to increased levels of 2-HG, theirs effects in glioma tumorigenesis have yet to be fully revealed. Recent studies have shown histone and DNA hypermethylation phenotype of gliomas can be coordinate by 2-HG, leading to epigenetic changes and driving the cells for malignant transformation (Guo et al. 2011).

1.2.1.5.2. PGRN levels

PGRN gene is located on chromosome 17q21.32 and encodes a 68-kDa secreted protein that is extensively glycosylated to 88 kDa with seven and one-half tandem repeats (granulins A-G and paragranulin) of a unique 12 cysteine sequence (He & Bateman 2003). Human PGRN has 2 isoforms.

After secretion, in peripheral tissues, the full-length of PGRN can be cleaved between the granulin-like domains by extracellular proteases such as: elastases and metalloproteinases. This complete proteolytic cleavage converts the full-length of PGRN into granulins peptides with 6kDa each. Both intermediaries, the full-length of PGRN and the granulins peptides, have biological activities, but it remains unknown if their functions are distinct or overlapping. However, this proteolytic process can be inhibited by secretory leukocyte protease inhibitor (SLPI) or high density lipoprotein/apolipoprotein A-1 (HDL/ApoA-1). One of these entities binds to the full-length PGRN preventing its cleavage (Demorrow 2013b)(Fig.7).

PRGN is also known as granulin-epithelin precursor (GEP), proepithelin (PEPI), PC cell-derived growth factor (PCDGF), acrogranin and epithelial transforming growth factor

(TGFe). These different nomenclatures emphasize the several actions of PGRN (Bateman & Bennett 2009). Structurally, PGRN belongs to none of the well-established growth factor families. It is a pluripotent growth factor which mediates several events such as cellular proliferation, differentiation, development and pathological processes (He & Bateman 2003).

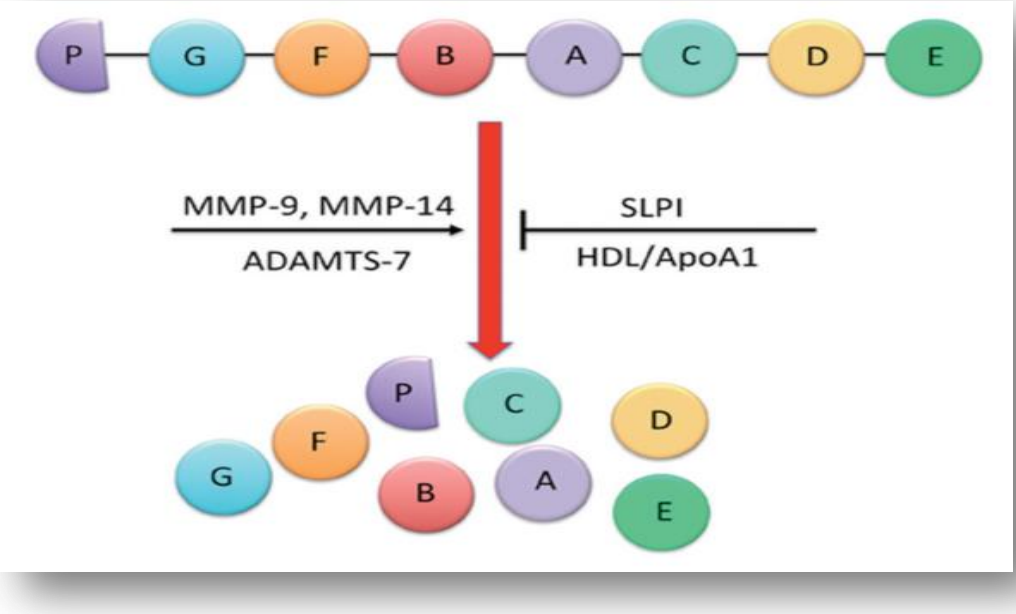


Figure 7 – Structure and cleavage of PGRN. After secretion, the full-length PGRN can be cleaved by MMP-9, MMP-14 or ADAMTS-7. But the cleavage can be prevented by SLPI, HDL/ApoA-1.

This protein is expressed in many tissues throughout the body. Among others, PGRN is prominent in epithelial and myeloid cells, where it is involved in cellular proliferation, wound healing and inflammation (Suh et al. 2012). During wound healing, PGRN expression is highly increased in order to stimulate the proliferation of fibroblasts and endothelial cells. Besides that, PGRN regulate inflammation inhibiting the inflammatory cytokine tumour necrosis factor- α (TNF-α) (Bateman & Bennett 2009).

Beyond the relationship between PGRN and tissue remodelling, the interest in its function is also related with neurodegeneration and tumorigenesis. PGRN is a double-edge sword. Both low levels of PGRN as high levels of PGRN are associated with pathological outcomes. It is known that PGRN protects neurons from premature death and mutations in PGRN gene result in a decrease of PGRN function leading to front temporal lobar degeneration (FTLD) (Toh et al. 2011).. On the other hand, in a number of tumour types including

ovarian, breast, prostate, renal, liver and esophageal, an increased level of PGRN has been demonstrated. In most cases of cancer there is a relationship between PGRN levels and tumour progression, being the high grade tumours the ones that exhibit high levels of PGRN (Demorrow 2013; Pickford et al. 2011).

The aggressive growth and intense vascularity of high grade astrocytomas are associated with their ability to produce a variety of growth factors. In 2012, Wang and colleagues have shown that PGRN and PGRN mRNA expression is highly increased in glioblastoma. Moreover, it is thought to confer a more aggressive phenotype since it stimulates the cell growth, increases proliferation, confers resistance to apoptosis and adversely impacts therapeutic responses as it confers resistance to temozolomide. Recently, Bandey and colleagues showed that PGRN had a critical role in the pathogenesis and chemoresistance of GBM and functioned at the top of the hierarchy of cellular machinery that modulates both DNA repair pathways and cancer stemness. Therefore, a new strategy combining current regimens with compounds targeting PGRN/AP-1 loop like curcumin may significantly improve the therapeutic outcome of GBM (Bandey et al. 2015).

At the present, is still unknown how PGRN is up-regulated in tumours, however, several miRNAs studies demonstrated their ability to regulate PGRN expression. Therefore, translational regulation by miRNAs may represent a common mechanism underlying PGRN-associated diseases (Wang et al. 2012; Rademakers et al. 2008).

1.2.1.6. Gliomas Treatment

The current therapy to treat gliomas only slightly prolongs the survival of these patients, being more a palliative care to improve the life quality. According to the present guidelines the management options for malignant gliomas involved total resection or biopsy followed by radiotherapy or chemotherapy (or a combined modality treatment) (Weller et al. 2014).

CHAPTER 2 Objectives

2. Objectives

The current diagnostic techniques based on clinical examination, neuro-imaging and neuropathology are not sufficiently informative to predict the prognosis of most glioma patients. One of the main aims of this project is to evaluate the diagnostic and prognostic value of the IDH1 marker in a patients cohort diagnosed with gliomas. This evaluation will be performed by two different techniques: Immunohistochemistry and Sanger Sequencing, in order to improve IDH1 analysis routinely in a clinical setting. The comparison of both techniques in terms of mutation detection rate will be also studied.

In addition, we aim to investigate the level of PGRN in the plasma of glioblastoma patients using a sandwich enzyme immunoassay technique (ELISA). Ultimately, we also aim to correlate the PGRN levels with patients overall survival.

CHAPTER 3 Material and Methods

3. Material and Methods

3.1. Sample Set

3.1.1. Detection of *IDH1* Mutations

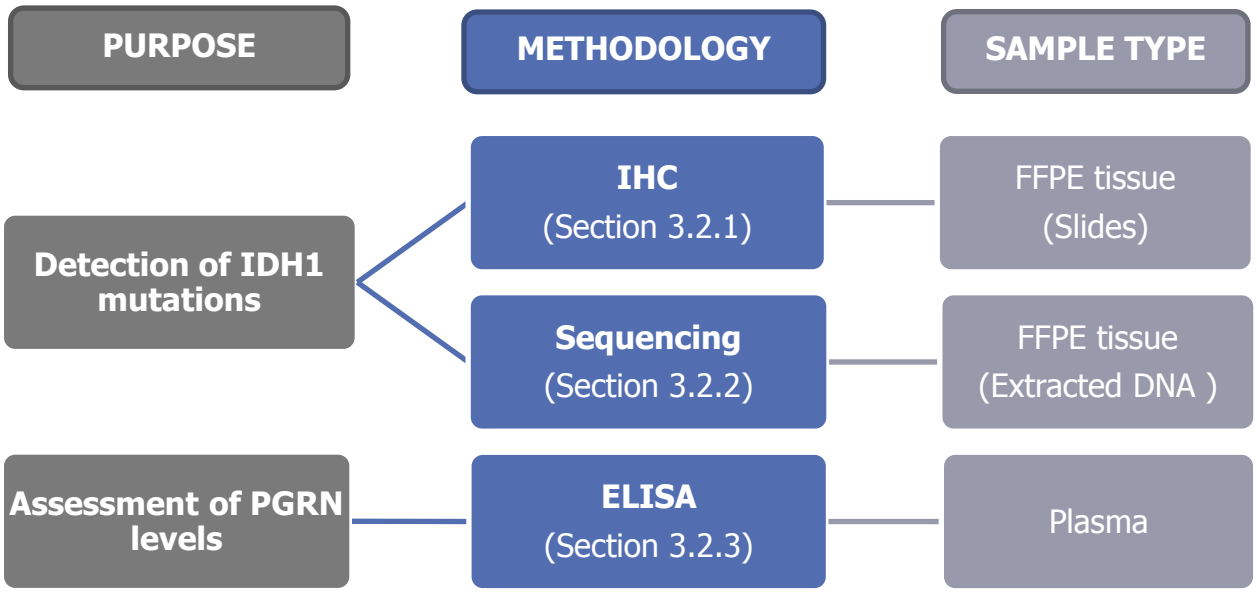
A total of 96 formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from operated glioma patients at the Department of Neurosurgery, CHUC from 2006 to 2014. The clinicopathological information was obtained from medical records. At the Neuropathology service, the tumour histology was confirmed. All participants had provided a written informed consent.

3.1.2. Assessment of PGRN Levels

A total of 40 plasma samples from glioblastoma patients were collected from Tumour Bank at CHUC after submitted an appropriate request form.

3.2. Sample Circuit

In this study, we used two types of samples from glioma patients: FFPE tissue and plasma, which were treated according to the different purposes using the methodologies illustrated in the following flow chart:



3.2.1. Immunohistochemistry for Detection of R132H *IDH1* Mutation

PRINCIPLE:

Immunohistochemistry (IHC) is an important tool for scientific research and also for the detection of biomarkers in clinical practice. This technique allows to identify distinct tissue components through the interaction of specific antibodies previously labelled with the target antigens. IHC encompass three main steps: 1) slide preparation (specimen fixation and tissue processing) 2) sample labelling (antibody incubation and its detection); and (3) analysis and quantification of the obtained expression (Matos et al. 2010).

PROCEDURE:

All samples used in IHC were previously fixed, place on a cassette and embedded with paraffin. We performed tissue sections of 4 µm thickness using a rotary microtome and floated the sections in a 40ºC water bath. After that, we mounted the sections onto gelatin-coated slides and dry overnight at room temperature. Slides pre-coated help the section to adhere to the slide.

After slides preparation, they were introduced in an automated IHC staining instrument (BenchMark GX, Roche, Switzerland). This device performs all steps from a manual IHC such as: 1) Deparaffinization and rehydration of the section; 2) Antigen retrieval; 3) Immunohistochemical staining; 4) Dehydration and stabilization with a mounting medium.

The detection of IDH1 R132H mutation, which occurs in more than 80% of astrocytomas tumours, was performed using the antibody clone H09 (Dianova, Germany) which reacts specifically with this point mutation.

3.2.2. Sequencing for Detection of *IDH1* Mutations

PRINCIPLE:

Sanger sequencing is the most commonly used method of DNA sequencing based on modified bases called dideoxy bases. When a certain gene is being replicated and a dideoxy base is incorporated into the new chain, the replication reaction is terminated. Thus, a set of fragments that differ in length from each other by a single base are generated. Then, the fragments are separated by size and the bases at the end identified. The original sequence of the DNA is recreated by determining the exact order of the bases.

PROCEDURE:

The Sanger sequencing comprises 7 main steps: 1) DNA Extraction; 2) DNA Quantification; 3) DNA Amplification by Polymerase Chain Reaction (PCR); 4) Electrophoresis in agarose gel; 5) Purification of PCR Products; 6) Sequencing Reaction; 7) Purification of the Sequencing Reaction Products.

3.2.2.1. DNA Extraction

Genomic DNA from formalin-fixed paraffin-embedded samples was extracted by use of the QIAamp® DNA Mini Kit (Qiagen, Germany), according to the manufacturer's instructions.

QIAamp® DNA Mini Kit is commercially available for an easy and fast purification of genomic DNA. This kit is based on spin columns which use the silica-membrane technology. The QIAamp® DNA extraction procedure comprises 4 steps: lysis with a proteinase k at 56ºC, binding of the DNA to the membrane, washes to remove residual contaminants and elution of pure nucleic acids.

Briefly, for each sample 10 µm thick consecutive tissue sections have been performed (varying in number depending on the size of the sample). The paraffin has been removed by extraction with xylene and incubated in Buffer ATL with proteinase k at 56ºC, overnight. Once the tissues were completely lysed, the samples were incubated 10 minutes at 70ºC with Buffer AL. After that, we added ethanol to the tubes, performed a brief centrifugation and transferred the content to the QIAamp Mini spin columns, where the DNA binds to the

membrane. Series of centrifugation were done with buffers AW1 and AW2 in order to remove all contaminants.

Finally, 50 µl Buffer AE (elution buffer) was applied in the column membrane, incubated at room temperature during 5 minutes and then centrifuged to elute the DNA in a 1.5 ml tube. The DNA was stored at 4ºC. The figure 8 represents all procedure.

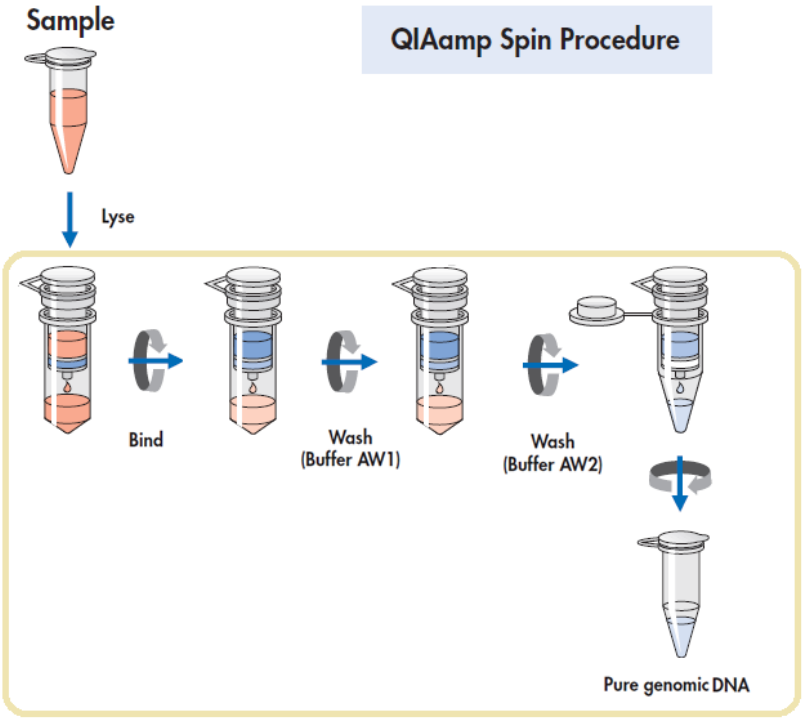


Figure 8 – Schematic representation of QIAamp Spin Procedure. Adapted from QIAamp® Handbook

3.2.2.2. DNA Quantification

DNA concentration and quality control were estimated by spectrophotometry using NanoDrop ND-1000 Spectrophotometer® (Thermo Fisher Scientific, USA), version 3.5.3 and respective software. The concept of the spectrophotometer is based on the absorbance/transmission of light through a liquid to determine the concentration of a particular substance in that liquid. The DNA readings were taken at wavelengths of 260 nm and 280 nm, which allow calculation of the concentration of nucleic acid (ng/µl) and the amount of protein in the sample, respectively. Thus, as an indicator of the purity of DNA samples, we generally use the ratio of nucleic acid to protein (260/280).

3.2.2.3. DNA Amplification by PCR

The DNA amplification was performed by PCR using a set of primers previously designed in the laboratory. Table 1 shows the primers sequence and also the respective length of the PCR product.

Name	Sequence (5'-3')	Product Length (bp)
IDH1 Forward	ACGGTCTTCAGAGAAGCCA	133
IDH1 Reverse	CATGCAAAATCACATTATTGCC	

Table 1 – Primers sequence and PCR product length.

The mixture of reagents to amplify the IDH1 region by PCR reaction was prepared for a final volume of 24 µl, according to the table 2.

Reagents	Final Concentration	Volume used by each sample (µl)
H_2O	-	14,4
Taq Buffer 5x (Promega)	1x	5
$MgCl_2$ 25mM (Promega)	1,5 mM	1,5
dNTPs 5mM (Nzytech)	0,2 mM	1
Primer Forward 10 µM	0,5 µM	1
Primer Reverse 10 µM	0,5 µM	1
Taq DNA polymerase 5U/ µl (Promega)	0,5U	0,1

Table 2 – Reagents used in the PCR mixture to amplify IDH1.

After we pipetted all PCR reagents into a 0.2ml PCR tube according to table 3, we added 1µl of DNA to the final volume of 25 µl. All PCR reactions done had included a negative control. Immediately after adding the DNA, the tubes were briefly centrifuged and placed into Biometra thermal cycler to begin the PCR.

The amplification conditions, using the primers described in table 2 were the following:

1. Initial Denaturation for 5 minutes at 96ºC (the double-stranded DNA starts denaturing and the hydrogen bonds between the nucleotide base pairs are broken);
2. Denaturation for 45 seconds at 96ºC (DNA continues to be denatured) ;
3. Primers annealing for 45 seconds at 60ºC (the forward and reverse primers anneal to each DNA strand. The DNA polymerase is also stable to bind to the primer DNA sequence);
4. DNA extension for 45 seconds at 72ºC (optimal temperature for Taq polymerase to synthesize and elongate the new DNA strand);
5. Repeat steps 2-4 29 times;
6. Final Extension for 10 minutes at 72ºC (a final extension is often recommended to promote complete synthesis of all PCR products).

3.2.2.4. Electrophoresis in Agarose Gel

The resulted PCR products were loaded into wells of an agarose gel and subjected to an electric field, causing the movement of nucleic acids (negatively charged) toward the positive electrode (Figure 9).

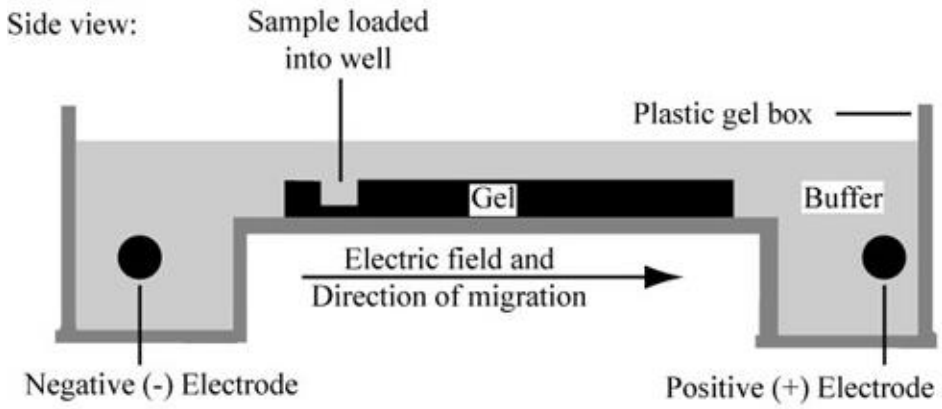


Figure 9 – Representative diagram of electrophoresis. Adapted from *MIT OpenCourseWare*.

This technique allows the separation of the fragments based on their size, the shorter DNA fragments will migrate more rapidly and far than the longer DNA fragments which remain closer to the well. Electrophoresis is also used to check if the PCR products are free of contamination.

The electrophoresis was performed according to the following procedure:

1. We prepared a 2% agarose gel by first boiling in a microwave a mixture of agarose and TBE 1x Buffer in an Erlenmeyer flask until agarose melting. When the mixture was cooled to about 65ºC, we added EtBr (0,5µg/mL) and we placed the mixture into a gel mould with a comb. At room temperature, the agarose gel was polymerized (Figure 10).
2. Once the gel was polymerized the comb was removed and the agarose gel was placed into an electrophoresis apparatus with TBE 1x Buffer.
3. After that, we loaded 5µl of PCR product in each well and turned on the electric field by a power supply for 30 minutes at 100 volts.
4. Once electrophoresis finished, a photograph was taken using a UV transilluminator, Gel doc_XR® (BIO-RAD, USA) with its specific software.

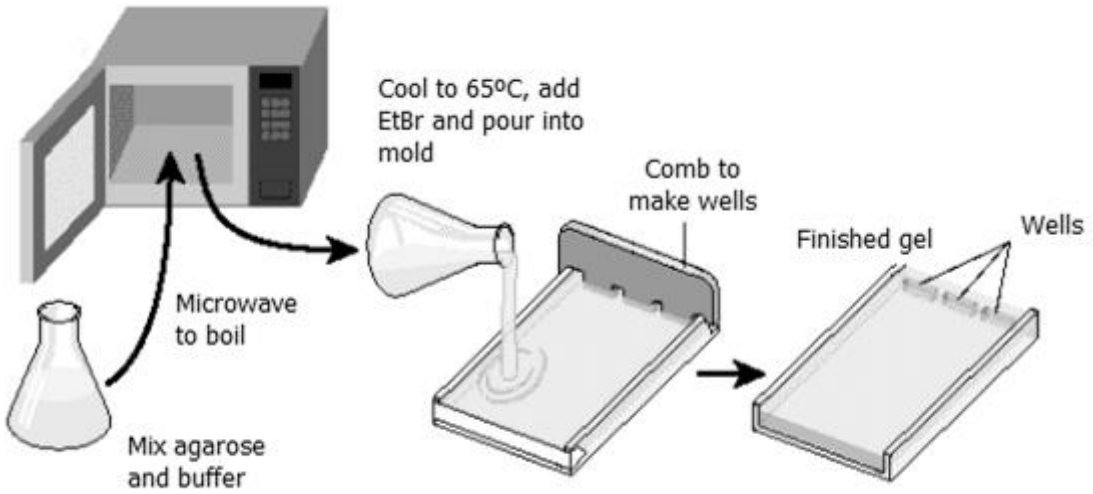


Figure 10 - Diagram illustrating making an agarose gel. Adapted from http://www.nslc.wustl.edu/courses/bio2960/labs/07DNA/Gel/index.html

3.2.2.5. Purification of PCR Products

After electrophoresis, the PCR products were purified to remove salts, dNTPs, primers and polymerases which have not been incorporated during the PCR reaction. We used the NZYGelpure® kit which utilizes a silica-gel based membrane where the amplified fragments were absorbed in the presence of appropriate binding buffers. The others impurities did not bind to the membrane and were washed away by a wash buffer. Between steps, all the columns were centrifuged for 1 minute at 1,3000g and the solutions in the collection tubes were discarded. Once replaced the collection tubes by 1,5ml eppendorf tubes, the purified DNA was eluted by an elution buffer through the column after a final centrifugation at the same conditions.

3.2.2.6. Sequencing Reaction

The direct sequencing reaction was performed using the GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit, based on Sanger sequencing (Sanger et al. 1977), as mentioned above in 3.2.2. This kit has a master mix which contains DNA polymerase, dNTPs dye terminators (ddUTP, ddGTP, ddCTP and ddATP) and reaction buffer. During a sequencing reaction, the dNTPs and also ddNTPs were incorporated on the 3' end of DNA strand by DNA polymerase. Every time that one ddNTP is added to the DNA strand, the elongation is finished. Thus, several DNA molecules with different size and bases were generated.

Each reaction was prepared in a 0,2 ml tube using just one primer, however the presence of a mutation was always confirmed on a separate amplification with subsequent direct sequencing of both DNA strands. After tubes identification, the sequencing reactions were prepared for a final volume of 20 µl adding all components listed in table 3.

Reagent	Quantity used (µl)
dH_2O (to adjust total volume to 20,0 µl)	0-9,5 µl
DNA template	0,5-10,0 µl*
Sequencing Primer (Fwd or Rev)	1,0 µl
DTCS Quick Start Master Mix	3,0 µl
Total	20,0 µl

Table 3 – All the reagents used in sequencing reactions.

***Note:** The volume pipetted was defined taken into account the band intensity observed on the agarose gel.

After preparing the sequencing reactions, all tubes were briefly centrifuged in order to mix all components before placed in thermocycler according to the following conditions:

Temperature	Time	
96°C	20 sec.	x 30 cycles
50°C	20 sec.	
60°C	4 min.	
4°C	+∞	

3.2.2.7. Purification of Products of Sequencing Reaction

The products resultant of sequencing reactions were immediately purified in order to remove any residual salts, ddNTPs and other components which were not incorporated during the reaction and could interfere with the sequencing analysis. The purification of sequencing reaction was based on ethanol precipitation and the main steps were:

1. For each sequencing reaction, a fresh Stop Solution has been prepared (3 µL of 3M Sodium Acetate (pH 5.2), 14,5 µL of sterile water and 62,5 µL of absolute ethanol; all reagents were stored at 4ºC).

2. 80 µL of stop solution were added to each sequencing reaction and transferred to an appropriate plate of 96 wells. After that the plate was sealed with parafilm and placed at -20°C for 10 minutes.
3. Immediately the plate was centrifuged at 6100 g at 4°C for 30 minutes. Carefully the supernatant has been removed by inverting the plate on the absorbent paper.
4. Then the pellet was rinsed 2 times with 200 µL 70% (v/v) ethanol/dH2O at -20°C through a centrifugation at 6100 g at 4°C firstly for 10 minutes and secondly for 5 minutes. After these two centrifugations, the plate has been inverted carefully on absorbent paper to discard the supernatant.
5. Next we centrifuged the inverted plate on absorbent paper at 100 g during 10 seconds to remove all supernatant.
6. After that, we resuspended the sample in 25 µL of the Sample Loading Solution (provided in the kit) and vortex the plate carefully.
7. Each resuspended sample was overlaid with one drop of light mineral oil (provided in the kit) to avoid the evaporation during the run in the sequencer.
8. Finally, we loaded the sample plate into the sequencer CEQ 8000 Genetic Analysis System, (Beckman Coulter, USA) and started the run. Once finished the analysis was performed by Sequencher Demo Software, version 4.7 from Genes Code Corporation.

3.2.3. ELISA for PGRN Assessment

PRINCIPLE:

ELISA was introduced by two Swiss researchers Engvall and Perlamann in 1971. ELISA was based on radioimmunoassay (RIA) previously used by Yalow and Berson in 1960s (Engvall & Perlmann 1971; Yalow & Berson 1960) . This quantitative technique uses a 96 well plate which has a specific antibody adsorbed for detecting certain protein, peptide, hormone and other substances.

In this work, we used the Quantikine® ELISA Human Progranulin Immunoassay (R&D Systems, USA). The microplates pre-coated with a monoclonal antibody specific for human PGRN allowed the detection of this protein in plasma samples through the bound antigen-antibody. Using a calibration curve, this kit provided the concentration of PGRN in each well after the calculation of the results.

PROCEDURE:

The plasma provided by the Tumour Bank at CHUC were firstly diluted (50 µl of sample + 150 µl of Calibrator Diluent RD6-23).100 µl of Diluent RD1-5 was added to each well. And after that we added 50 µl of plasma samples, previously diluted, standards (1,56-100ng/ml) and controls to each well and incubated for 2 hours at room temperature.

Secondly, the microplate was aspirated and washed with Wash Buffer (400 µl) four times using an auto washer. After the last wash, the microplate was aspirated and we added 200 µl of Progranulin Conjugate to each well. The incubation was for 2 hours at room temperature.

Thirdly, we repeated the aspiration/wash steps four times and added 200 µl of Substrate Solution to each well. The incubation was for 30 minutes at room temperature, protected from the light.

Finally, we added 50 µl of Stop Solution to each well and then determined the optical density of each well using a microplate reader of 450 nm. In order to have more accurate results a wavelength correction, set to 570 nm, was performed.

A linear standard curve was created through regression analysis and PGRN concentration was extrapolated from this curve. The concentration read from the standard curve was multiplied by the dilution factor.

3.3. Statistical Analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences 20.0 software (IBM SPSS, Inc., Chicago, Ill., USA).

Normal distribution of continuous variables was first assessed by the Kolmogorov-Smirnov test. Differences between groups were then examined using the non-parametric Mann-Whitney U test for continuous variables and the Chi-square test was used to assess differences between categorical variables. Pearson correlation coefficient was used for bivariate correlation analyses.

The Kaplan-Meier survival curves were plotted and the survival distributions according to the status of IDH1mutation and PGRN levels were compared by the log-rank test. The overall survival was defined as the time between the diagnosis and death or last follow-up.

All reported P values <0.05 were considered statistically significant. All results were presented as mean $\pm$ SD.

CHAPTER 4 Results and Discussion

4. Results and Discussion

4.1. Detection of *IDH1* mutations

A total of 96 tumour samples from patients with diverse types of gliomas have been studied for the presence of *IDH1* mutations using two different techniques, IHC and DNA sequencing. They were classified according to the WHO, based on their cell origin, glial cells, and were graded according to their histological degree of malignancy from Grade I to Grade IV. As illustrated in Figure 11, our gliomas cohort was a heterogeneous group, encompassing many different histological types and malignancy grades. The major representative form was glioblastoma (65%) whereas the less frequent was low grade gliomas like ependymomas and diffuse gliomas (5%). The mean age of the patients included was 57± 16 years, being 35 females and 61 males.

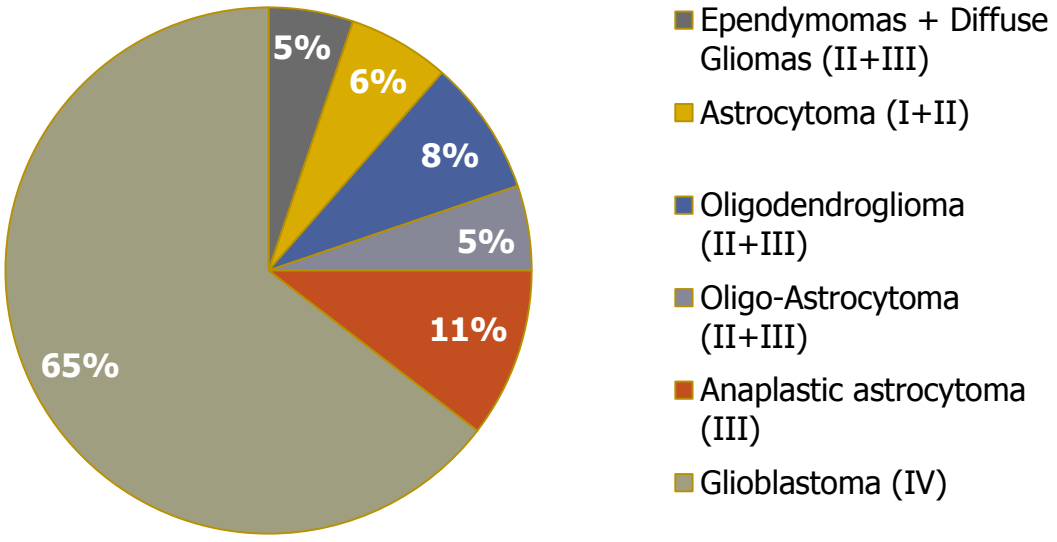


Figure 11 – The distribution of gliomas types.

4.1.1. Analysis of R132H *IDH1* mutation by Immunohistochemistry

As described previously (section 3.2.1), the IHC assay was optimized using a mouse monoclonal antibody which detects the most common R132H mutation. Under the microscope, each studied sample slide was carefully analysed comparing with a positive control for R132H *IDH1* mutation. If the tumour tissue has a staining similar to the positive control, it is positive for R132H *IDH1* mutation, otherwise it is negative if cells presenting no staining (Figure 12).

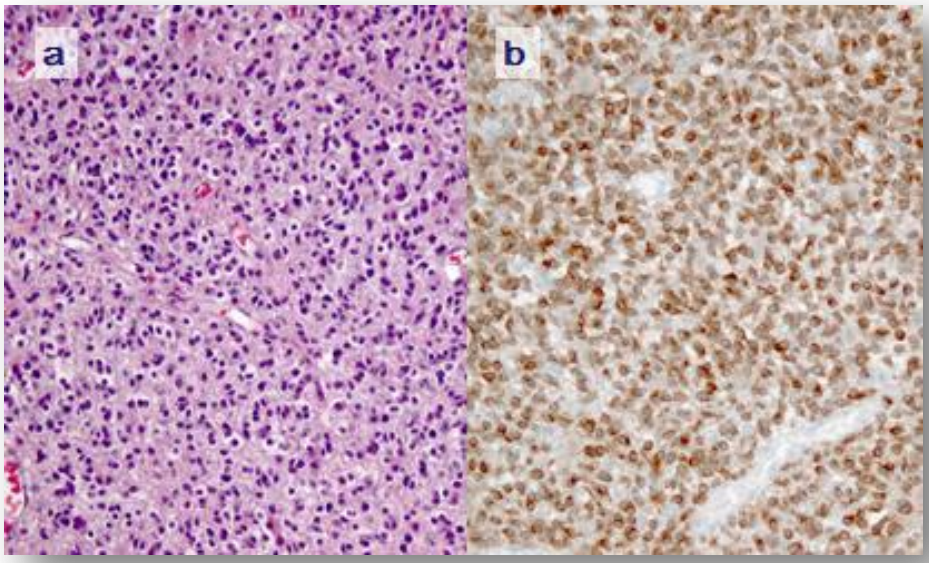
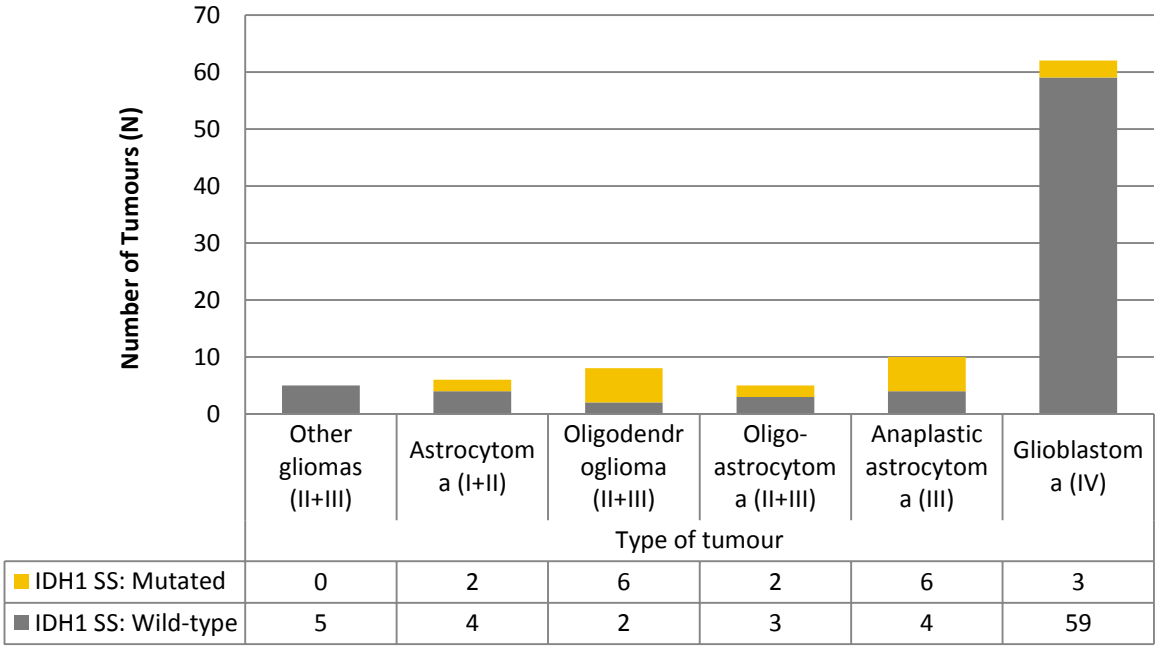


Figure 12 – a) Oligodendroglioma. Hematoxylin and eosin staining. b) Oligodendroglioma. R132H IDH1 immunohistochemistry. Positive cytoplasmatic immunostaining. a-b) Original magnification 200x

Of the 95 tumours analysed by IHC, 17 (18%) were positive for the R132H IDH1 (Figure 13). This mutation was found in all type of tumours except in ependymomas and diffuse gliomas (II and III). It was more common in oligodendroglioma (II and III) (88%), anaplastic astrocytoma (III) (50%), astrocytoma (I and II) (33%), oligo-astrocytoma (II and III) (20%) whereas in the glioblastomas (IV) was found in only 3% of the cases. This later group as well as ependymomas and diffuse gliomas (II and III) group have none or few mutated *IDH1*. In contrast, gliomas of grade II and III often carried *IDH1* mutation (Watanabe et al. 2009). These findings corroborate the results of Yan and colleagues who

showed that IDH1 is involved in the pathogenesis of malignant tumours, allowing the clinical and genetic distinction between these different entities such as grade II and III gliomas and secondary glioblastomas(Yan et al. 2009).

In addition, in the present study, the mean age of patients with and without *IDH1* mutation was 47 ± 16 years and 59 ± 16 years, respectively. This age distribution showed statistically significant differences between these two groups of patients (p<0,005)



	Other gliomas (II+III)	Astrocytoma (I+II)	Oligodendroglioma (II+III)	Oligo-astrocytoma (II+III)	Anaplastic astrocytoma (III)	Glioblastoma (IV)
IDH1 SS: Mutated	0	2	6	2	6	3
IDH1 SS: Wild-type	5	4	2	3	4	59

Figure 13 – *IDH1* mutation status of the 95 gliomas studied by IHC.

4.1.2. Analysis of *IDH1* mutations by Sequencing

As previously described in section 3.2.2, all the 96 gliomas samples were studied in parallel by sequencing analysis for the presence of *IDH1* mutations. The comparison between the informativeness of both techniques in terms of mutation detection rate as well as other parameters such as time consuming and costs, enabled us to improve the laboratory assessment of IDH1 routinely in a clinical setting. This approach consisted with the PCR amplification of the codon 132 in exon 4 of the IDH1 gene, since all the mutations described thus far are located in this region (Figure 14), followed by direct sequencing which is illustrated in the electropherogram depicted in Figure 15.

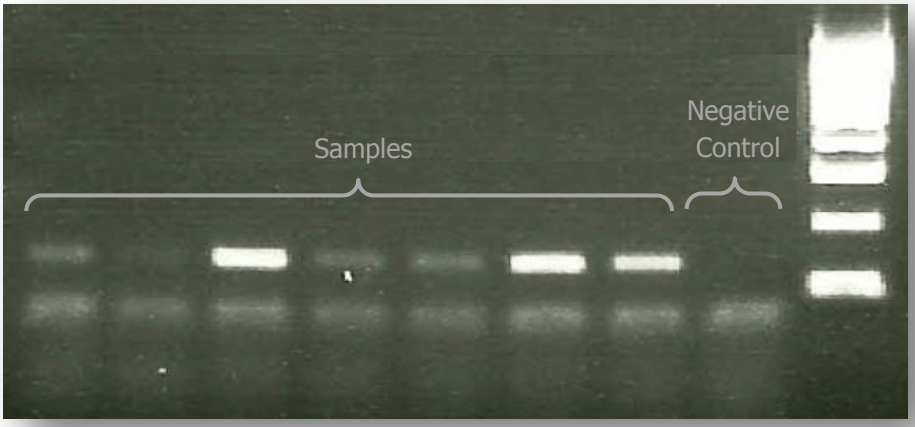


Figure 14 – PCR products after electrophoresis on 2% agarose gel. The bands represent the amplification of IDH1 gene in different samples. The clean negative control indicates that there is no contamination.

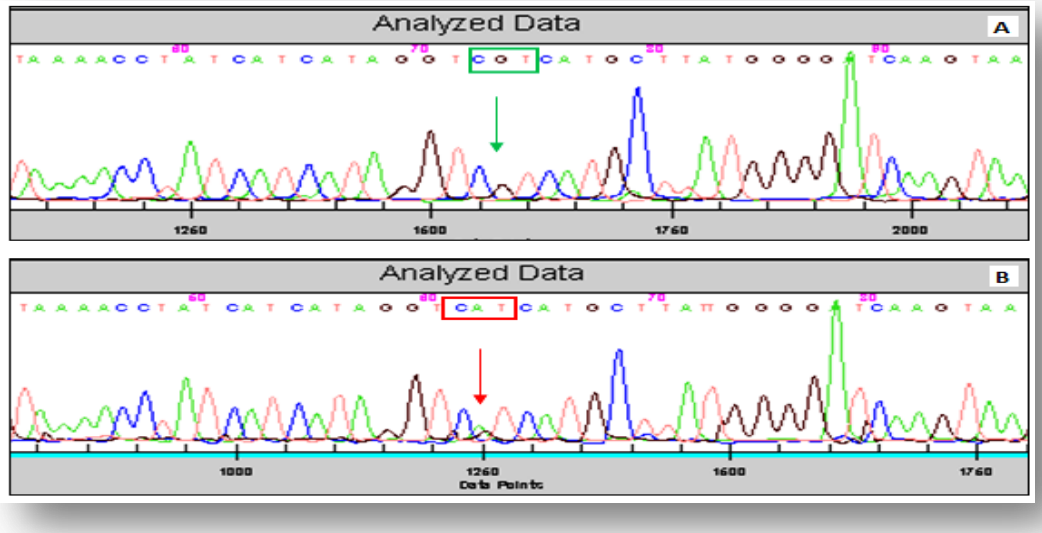


Figure 15 - Electropherograms of sequencing of IDH1, codon 132, exon 4. A) Electropherogram of normal sample, with no mutation in codon 132 (CGT). B) Electropherogram of sample with mutation in codon 132 (CGT>CAT, R132H).

The sequence analysis revealed 19 somatic mutations at codon R132, of which 16 were the most common one, R132H, and the other three included less common mutations such as R132S, R132L and R132G. All the mutations identified were missense mutations because the change of a single base pair (c.394G>A, c.394C>A, c.394G>T and c.394C>G) caused

the substitution of a different amino acid (R132H, R132S, R132L and R132G, respectively). In terms of frequency, R132H occurred in 84% of the cases while R132S, R132L and R132G only occurred in 5% of the cases. Besides that, all of them were heterozygous and have been already described in literature (Guo et al. 2011; Horbinski 2013). The Figure 16 and table 4 showed the total *IDH1* mutations detected in the different types of tumours.

As for IHC, with sequencing analysis the mutations in codon 132 were also found in all type of tumours except in ependymomas and diffuse gliomas (II and III). The major representative groups were oligodendroglioma (II and III) with 75% of the cases mutated, followed by anaplastic astrocytoma (III) (60%), oligo-astrocytoma (II and III) (40%) and astrocytoma (I and II) (33%). Glioblastomas (IV) was the less representative group, with only 5% of the cases mutated.

Similarly to IHC, the results of sequencing were correlated with patient's age ($p<0,001$). The mean age in patients with and without IDH1 mutation was 46 $\pm$ 15 years and 59 $\pm$ 16 years, respectively.

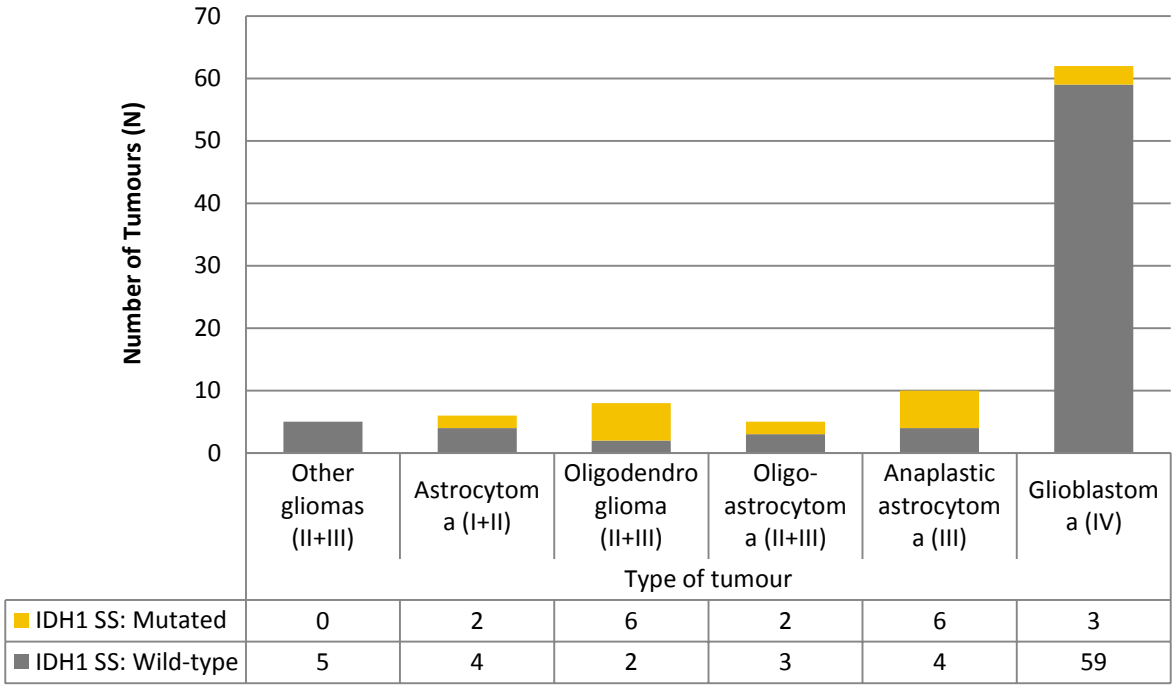


Figure 16 – *IDH1* mutation status of the 95 gliomas studied by Sanger sequencing.

Type of Tumour	Other Gliomas (II+III)	Astrocytoma (I+II)	Oligodendroglioma (II+III)	Oligo-astrocytoma (II+III)	Anaplastic Astrocytoma (III)	Glioblastoma (IV)
***IDH1* Mutation**						
R132H	-	2	6	1	5	2
R132L	-	-	-	-	-	1
R132S	-	-	-	-	1	-
R132G	-	-	-	1	-	-
Total	5	6	8	5	10	62

Table 4 – *IDH1* mutations identified in the different type of tumour.

4.1.3. Comparison between Immunohistochemistry and Sequencing analysis

Ninety-six gliomas samples were studied to the presence of mutations in IDH1 gene using two different methods. Noteworthy, that both methods as well as the yielded results have been analysed independently.

As expected, Sanger sequencing (SS) allowed the identification of other types of *IDH1* mutations (p.R132L, p.R132S and p.R132G), rather than the most common one, p.R132H. However, sequencing failed to detect two cases with R132H mutation previously detected by IHC. One possible explanation is related with the quantity of tumour cells of each sample and the lower sensitivity of this method comparing with IHC. As reported by others samples containing less than 20% of mutant allele could lead to false negatives due to the number of normal cells being higher than mutant cells (Arita et al. 2015). Therefore, it seems crucial to select samples to be analysed according to the percentage of tumour cells. Oppositely, IHC seemed to be more specific for detection of p.R132H than SS. One of the causes is the fact that there is no need to select a high level of tumours cells since IHC is able to stain samples containing only 6-9% of mutant allele (Arita et al. 2015). Obviously, IHC failed to detect p.R132L, p.R132S and p.R132G. Surprisingly, one p.R132H was not

detected by IHC. When this case was re-evaluated we could observed that the tumour tissue was extinct due to several cuts.

Take together all data from both techniques we concluded that detection of p.R132H is very consistent through them (Table 8). However, looking at the failed detection of the most common *IDH1* mutation it seems that IHC (1 failed) is more accurate than sequencing (2 failed).

In conclusion, because IHC is easy to execute and it has low costs comparing with sequencing it should be used as the first analysis in a daily practise. However, SS should be performed whenever the clinics required based on the clinical features of the patients or negative cases.

IHC vs. Sequencing

IHC	N	IDH1 Status	Sequencing	N
Positive (+)	17	R132H	R132H	16
Negative (-)	0	R132L	R132L	1
Negative (-)	0	R132S	R132S	1
Negative (-)	0	R132G	R132G	1
Negative (-)	78	Wild Type	Wild Type	77
Total	95		Total	96

Table 5 – Comparison between IHC and Sequencing.

4.1.4. Survival Analysis

In this study, patients with mutation in IDH1 had longer survival time than patients without *IDH1* mutation (127±71 weeks and 56±69 weeks, respectively) ($p<0,001$).

To evaluate the potential role of *IDH1* mutation in determining the prognosis of glioma patients, survival analysis using Kaplan-Meier was performed (Figure 17).

The results showed that presence of *IDH1* mutation is associated with longer survival ($p<0,001$).

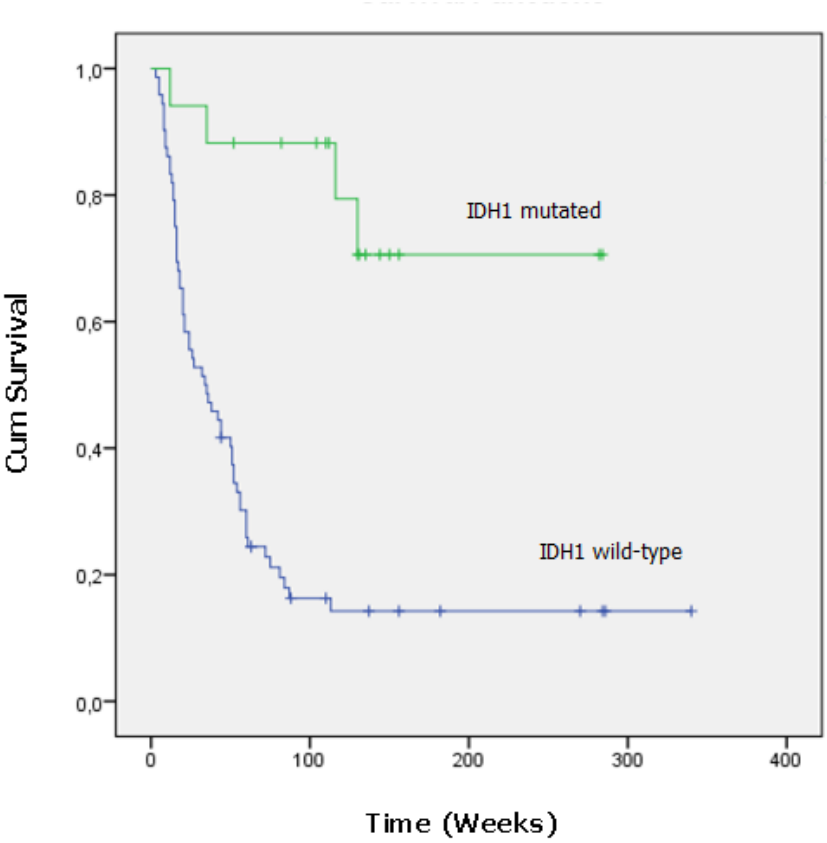


Figure 17 – Kaplan-Meier curve comparing survival of gliomas patients according to the IDH1 status.

Over the last years, several studies have been emphasized the importance of IDH1 evaluation as diagnostic and prognostic biomarker in clinical practice (Toedt et al. 2011; Appin & Brat 2015; Sanson et al. 2009). Studies various demonstrated that *IDH1* R 132 mutations range from 55% to 80% in grade II and III oligodendroglioma and astrocytoma but they are rare in primary GBM (5%) and other primary brain tumours such as pilocytic astrocytoma (Watanabe et al. 2009). Diagnostically, this marker is useful to distinguish between secondary GBM and primary GBM (Parsons et al. 2008). Despite that, it has been reported that IDH1 mutations are associated with a better prognosis due to the increased survival of mutant patients. However, this gene does not predict the outcome of chemotherapy (van den Bent et al. 2010).

According to the literature, in this project we verified that gliomas patients harbouring *IDH1* mutations had an increased survival compared with the patients with wild-type IDH1. These observations allowed us to confirm the prognostic value of IDH1 in daily-routine. Besides that, this gene is still important as diagnostic biomarker in dubious cases. Thus,

when histologic analysis is unclear this molecular signature could help to distinguish between primary and secondary GBM, and also between grade II and III gliomas and grade I like pilocytic astrocytoma.

When the patient sample was divided into two main groups by the type of tumour (glioblastoma and other all gliomas) it was observed a correlation between age and type of tumour ($p<0.001$). The mean age for patients with glioblastomas and other gliomas was 63 $\pm$ 11 years and 44 $\pm$ 17 years, respectively. Many studies that have been published are in agreement with these data (Nobusawa et al. 2009; (Bleeker et al. 2012); (Yan et al. 2009).

In spite of our intention to study the survival time according to IDH1 status only in glioblastomas patients, this analysis was not possible due to the reduced number ($n=3$) of mutated glioblastomas.

In summary, our findings strongly suggested that this biomarker should be introduced as an additional tool with potential diagnostic and prognostic value for glioma in a routine setting.

4.2. PGRN plasma levels assessment

As described previously (section 3.1.2), plasma PGRN levels were measured in 92 subjects (40 patients with GBM and 51 healthy controls) by a commercial ELISA kit. The mean age of GBM patients was 65 $\pm$ 10 years, being 19 females and 21 males. Regarding healthy controls, the mean age was 56 $\pm$ 8 years, being 22 females and 29 males. Age in GBM group and control individuals showed statistically significant differences ($p<0.001$). Analysis of gender distribution showed no statistically differences between groups.

Normal plasma PGRN levels were established using the mean$\pm$ 2SD levels of our control population. Thus, PGRN levels between 20,28 and 57,84 ng/ml were considered normal. In this study, the plasma levels of PGRN in patients with glioblastoma were significantly lower than healthy controls (24.85 $\pm$ 7.23 and 39.06 $\pm$ 9.39 ng/ml, respectively; $p<0,001$). Thirteen out of forty patients had PGRN levels inferior than the lower cut-off (<20,28ng/ml) (Fig.18).

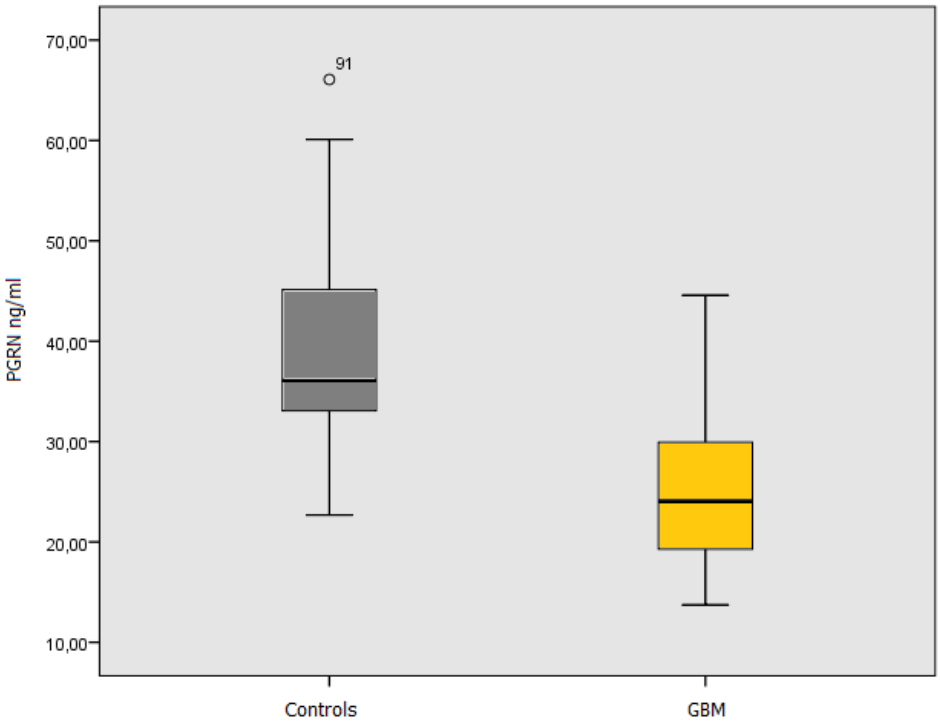


Figure 18 – Box plot of plasma levels of PGRN in the patients with GBM (n=40) and healthy control (n=51).

4.2.1. Survival Analysis

In this study, patients with normal PGRN levels had longer survival than patients with decreased levels of PGRN (50± 47 weeks and 24± 19 weeks, respectively), but this difference did not reach statistical significance (p= 0,087).

However, survival analysis using Kaplan-Meier showed that decreased levels of PGRN (<20,28ng/ml) were associated with shorter survival (p<0,05) (Figure 19).

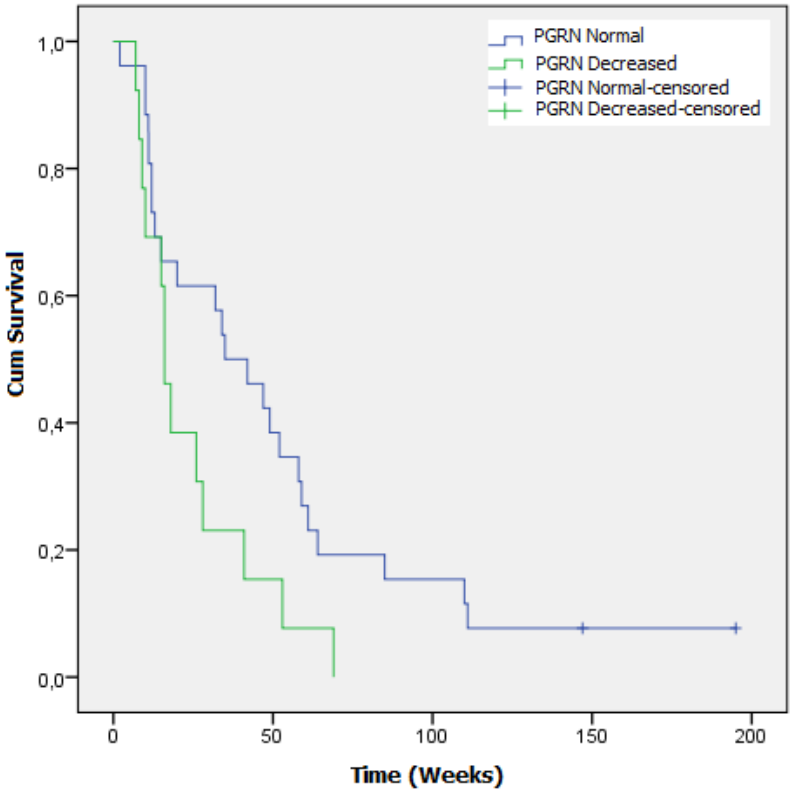


Figure 19 - Kaplan-Meier curve comparing survival of GBM patients according to the levels of PGRN.

Unexpectedly the plasma levels of GBM versus controls were different from the previous one reported by Wang and colleagues in 2012. These authors have shown an increased serum levels of PGRN in 17 GBM patients and 8 healthy controls (Wang et al. 2012). In order to confirm these controversial results, a larger number of GBM patients might be necessary to evaluate, from whom the age should be considered in order to match the control group.

Nevertheless, in literature increased levels of PGRN has been reported in many type of tumours, in our study this association at peripheral level was not observed. Several possible explanations to the striking different observed can be ruled out i) an age related downregulation of PGRN since GBM group was older than controls; ii) an increase of circulating of elastases secreted by neutrophils associated with dysregulated inflammation in GBM group; iii) genetic variations located in the 3'-UTR region of PGRN in binding sites

for miRNAs. Indeed, a common genetic variant (rs5848), which is a binding site for miR-659 might result in augmented translational inhibition of PGRN.

Interestingly, the presence of decreased levels of PGRN was associated with shorter survival. Although, a larger number of patients needed to be studied to confirm this finding, it suggests that PGRN level in plasma can be used as a prognostic marker. Importantly, it is crucial to evaluate the PGRN expression in the tumour tissues of these patients. According to the literature, an overexpression of PGRN in tumour tissues is expected positively graded. Moreover, it is important to explore its association with overall patients' survival.

Thereby, further studies on the correlation between plasma level and tumour tissue PGRN expression as well as a patient prognosis will be imperative.

CHAPTER 5

Conclusion & Future Perspectives

5. Conclusion & Future Perspectives

In the present study, we evaluated the diagnostic and prognostic value of IDH1 marker in a cohort of 96 patients with gliomas. The IDH1 analysis was performed using two different techniques, sequencing and IHC. Somatic mutations at codon R132 have been identified in 20 out of the 96 patients analysed (21%), the most common one, R132H (18%), and other rare ones, R132S (1%), R132L (1%) and R132G (1%), all of them previously reported.

In this study, the IDH1 analysis confirmed its usefulness to distinguish between grade II and III gliomas plus secondary glioblastomas from grade I gliomas plus primary GBM. Thus, this finding showed the predictive value of IDH1 as a diagnostic marker, suggesting that IDH1 status should become part of the standard diagnostic assessment of gliomas.

In addition, we observed that patients who carried *IDH1* mutations were younger than those who were wild-type for IDH1 ($p<0,001$). This finding was also corroborated by other authors.

The comparison between sequencing and IHC, to detect *IDH1* mutations, has revealed that both assays were similar in terms of detection rate of p.R132H, however the IHC failed to detected other less common mutations. Therefore, we advocate that IHC should be used as a first line method followed by sequencing for IHC-negative and cases with dubious clinical or morphological features. Nowadays, in our clinical daily practice we have both techniques available in the laboratory to increase the accuracy of the IDH1 analysis in a rapid and cost effective manner.

We also have shown that gliomas patients with *IDH1* mutations presented longer survival than wild-type patients, confirming the prognostic value of this marker.

In the present study, we also investigated the levels of PGRN in the plasma of 40 patients with GBM and 52 healthy controls and subsequently we explored its prognostic value.

We observed a lower level of PGRN in plasma of patients with glioblastoma compared with healthy controls ($p<0,001$). This data did not agree with the previous study reported by Wang and colleagues in 2012 who evaluated only 17 GBM patients and 8 healthy controls (Wang et al. 2012). Thus, in order to clarify this discrepancy, we intend to expand the size of our cohort to confirm this result.

To understand how PGRN plasma levels are downregulated, we also aim to investigate the role of miRNA-659 in translational regulation of PGRN. In addition, to address this issue, we plan to assess the circulating levels of SLPI, a potent inhibitor of many proteolytic enzymes including elastase.

Regarding the prognostic value of PGRN, we verified that GBM patients with decreased levels of PGRN were associated with a shorter survival ($p<0,05$). Therefore, we intend to perform further studies to establish a correlation between plasma level and tumour tissue PGRN expression by IHC and Western blot (WB) using Goat Anti-Human Progranulin Antigen Affinity-purified Polyclonal Antibody (R&D Systems, USA).

CHAPTER 6 References

6. References

Aktipis, C.A. & Nesse, R.M., 2013. Evolutionary foundations for cancer biology. *Evolutionary Applications*, 6(1), pp.144–159.

Aldape, K. et al., 2015. Glioblastoma: pathology, molecular mechanisms and markers. *Acta Neuropathologica*, 129(6), pp.829–848.

Anon, 2001. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical pharmacology and therapeutics*, 69(3), pp.89–95.

Appin, C.L. & Brat, D.J., 2015. Biomarker-driven diagnosis of diffuse gliomas. *Molecular aspects of medicine*.

Arita, H. et al., 2015. IDH1/2 mutation detection in gliomas. *Brain Tumor Pathology,* 32(2), pp. 70-89.

Balss, J. et al., 2008. Analysis of the IDH1 codon 132 mutation in brain tumors. *Acta Neuropathologica*, 116(6), pp.597–602.

Bandey, I. et al., 2015. Progranulin promotes Temozolomide resistance of glioblastoma by orchestrating DNA repair and tumor stemness. *Oncogene*, 34(14), pp.1853–64.

Bateman, A. & Bennett, H.P.J., 2009. The granulin gene family: From cancer to dementia. *BioEssays*, 31(11), pp.1245–1254.

Bernstein, M. & Berger, M., 2008 Neurooncology – the essential, 2nd edition. *Thieme*.

Van den Bent, M.J. et al., 2010. IDH1 and IDH2 mutations are prognostic but not predictive for outcome in anaplastic oligodendroglial tumors: a report of the European Organization for Research and Treatment of Cancer Brain Tumor Group. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 16(5), pp.1597–604.

Van den Bent, M.J., 2010. Interobserver variation of the histopathological diagnosis in clinical trials on glioma: a clinician's perspective. *Acta neuropathologica*, 120(3), pp.297–304.

Bissell, M.J. & Hines, W.C., 2011. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature medicine*, 17(3), pp.320–9.

Bleeker, F.E., Molenaar, R.J. & Leenstra, S., 2012. Recent advances in the molecular understanding of glioblastoma. *Journal of Neuro-Oncology*, 108(1), pp.11–27.

Bogdanovic, E. et al., 2014. IDH1 regulates phospholipid metabolism in developing astrocytes. *Neuroscience letters*, 582, pp.87–92.

Chandana, S.R. et al., 2008. Primary brain tumors in adults. *American Family Physician*, 77(10), pp.1423–1430.

Coons, S.W. et al., 1997. Improving diagnostic accuracy and interobserver concordance in the classification and grading of primary gliomas. *Cancer*, 79(7), pp.1381–1393.

Croce, C.M., 2008. Oncogenes and cancer. *The New England journal of medicine*, 358(5), pp.502–11.

Dang, L. et al., 2009. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature*, 462(7274), pp.739–44.

Demorrow, S., 2013. Progranulin: a novel regulator of gastrointestinal cancer progression. *Translational gastrointestinal cancer*, 2(3), p.145-151.

Engvall, E. & Perlmann, P., 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, 8(9), pp.871–4.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on day/month/year.

Ferlay, J. et al., 2013. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *European journal of cancer (Oxford, England : 1990)*, 49(6), pp.1374–403.

Germano, I.M. & Binello, E., 2014. Stem cells and gliomas: past, present, and future. *Journal of Neuro-Oncology*, 119(3), pp.547–555.

Guo, C. et al., 2011. Isocitrate dehydrogenase mutations in gliomas. *Current Opinion in Neurology*, 24(6), pp.648–652.

Hanahan, D. & Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell*, 144(5), pp.646–74.

He, Z. & Bateman, A., 2003. Progranulin (granulin-epithelin precursor, PC-cell-derived growth factor, acrogranin) mediates tissue repair and tumorigenesis. *Journal of molecular medicine (Berlin, Germany)*, 81(10), pp.600–12.

Horbinski, C., 2012. Something old and something new about molecular diagnostics in gliomas. *Surgical pathology clinics*, 5(4), pp.919–939.

Horbinski, C., 2013. What do we know about IDH1/2 mutations so far, and how do we use it? *Acta neuropathologica*, 125(5), pp.621–36.

Ichimura, K. et al., 2009. IDH1 mutations are present in the majority of common adult gliomas but rare in primary glioblastomas. *Neuro-oncology*, 11(4), pp.341–347.

Ichimura, K. et al., 2004. Molecular pathogenesis of astrocytic tumours. *Journal of neuro-oncology*, 70(2), pp.137–60.

Jadin, L. et al., 2015. Hyaluronan expression in primary and secondary brain tumors. *Annals of Translational Medicine* , 3(6), pp. 80.

Jiao, J. et al., 2010. MicroRNA-29b regulates the expression level of human progranulin, a secreted glycoprotein implicated in frontotemporal dementia. *PLoS ONE*, 5(5), pp.1–8.

Karcher, S. et al., 2006. Different angiogenic phenotypes in primary and secondary glioblastomas. *International journal of cancer. Journal international du cancer*, 118(9), pp.2182–9.

Kleihues, P. & Ohgaki, H., 1999. Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro-oncology*, 1(1), pp.44–51.

Komori, T., 2015. Pathology and genetics of diffuse gliomas in adults. *Neurologia medico-chirurgica*, 55(1), pp.28–37.

Kumar, M. & Sarin, S.K., 2010. Biomarkers of diseases in medicine. *Current Trends in Science*, 15, pp.403–4017.

Louis, D.N. et al., 2007. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathologica*, 114(2), pp.97–109.

Matos, L.L. de et al., 2010. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomarker insights*, 5, pp.9–20.

Nobusawa, S. et al., 2009. IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 15(19), pp.6002–7.

Ogino, S., Fuchs, C.S. & Giovannucci, E., 2012. How many molecular subtypes? Implications of the unique tumor principle in personalized medicine. *Expert Review of Molecular Diagnostics*, 12(6), pp.621–628.

Olar, A. & Sulman, E.P., 2015. Molecular Markers in Low-Grade Glioma-Toward Tumor Reclassification. *Seminars in radiation oncology*, 25(3), pp.155–163.

Parsons, D.W. et al., 2008. An Integrated Genomic Analysis of Human Glioblastoma Multiforme. *Sience*, 321(5897), pp.1807–12.

Pickford, F. et al., 2011. Progranulin is a chemoattractant for microglia and stimulates their endocytic activity. *American Journal of Pathology*, 178(1), pp.284–295.

Pietrak, B. et al., 2011. A tale of two subunits: how the neomorphic R132H IDH1 mutation enhances production of αHG. *Biochemistry*, 50(21), pp.4804–12.

Rademakers, R. et al., 2008. Common variation in the miR-659 binding-site of GRN is a major risk factor for TDP43-positive frontotemporal dementia. *Human molecular genetics*, 17(23), pp.3631–42.

Rakheja, D. et al., 2013. The emerging role of d-2-hydroxyglutarate as an oncometabolite in hematolymphoid and central nervous system neoplasms. *Frontiers in oncology*, 3(July), p.169.

Sanger, F., Nicklen, S. & Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 74(12), pp.5463–7.

Sanson, M. et al., 2009. Isocitrate Dehydrogenase 1 Codon 132 Mutation Is an Important Prognostic Biomarker in Gliomas. *Journal of Clinical Oncology*, 27(25), pp.4150–4154.

Schwartzbaum, J. a et al., 2006. Epidemiology and molecular pathology of glioma. *Nature clinical practice. Neurology*, 2(9), pp.494–503; quiz 1 p following 516.

Sherr, C.J., 2004. Principles of Tumor Suppression. *Cell*, 116(2), pp.235–246.

Sjöblom, T. et al., 2006. The consensus coding sequences of human breast and colorectal cancers. *Science (New York, N.Y.)*, 314(5797), pp.268–74.

Suh, H.-S. et al., 2012. Regulation of progranulin expression in human microglia and proteolysis of progranulin by matrix metalloproteinase-12 (MMP-12). *PloS one*, 7(4), p.e35115.

Toedt, G. et al., 2011. Molecular signatures classify astrocytic gliomas by IDH1 mutation status. *International journal of cancer. Journal international du cancer*, 128(5), pp.1095–103.

Toh, H. et al., 2011. Structure, function, and mechanism of progranulin; the brain and beyond. *Journal of molecular neuroscience : MN*, 45(3), pp.538–48.

Towner, R. a. et al., 2013. ELTD1, a potential new biomarker for gliomas. *Neurosurgery*, 72(1), pp.77–90.

Urbańska, K. et al., 2014. Review Glioblastoma multiforme – an overview. *Współczesna Onkologia*, 5, pp.307–312.

Vigneswaran, K., Neill, S. & Hadjipanayis, C.G., 2015. Beyond the World Health Organization grading of infiltrating gliomas: advances in the molecular genetics of glioma classification. *Annals of translational medicine*, 3(7), p.95.

Wang, H. et al., 2015. The Challenges and the Promise of Molecular Targeted Therapy in Malignant Gliomas. *Neoplasia*, 17(3), pp.239–255.

Wang, M. et al., 2012. Progranulin overexpression predicts overall survival in patients with glioblastoma. *Medical Oncology*, 29(4), pp.2423–2431.

Watanabe, T. et al., 2009. IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *The American journal of pathology*, 174(4), pp.1149–53.

Weller, M. et al., 2014. EANO guideline for the diagnosis and treatment of anaplastic gliomas and glioblastoma. *The Lancet. Oncology*, 15(9), pp.e395–403.

Williams, S.C. et al., 2011. R132H-mutation of isocitrate dehydrogenase-1 is not sufficient for HIF-1α upregulation in adult glioma. *Acta neuropathologica*, 121(2), pp.279–81.

Wise, D.R. et al., 2011. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of α-ketoglutarate to citrate to support cell growth and viability. *Proceedings of the National Academy of Sciences of the United States of America*, 108(49), pp.19611–6.

Yalow, R.S. & Berson, S.A., 1960. Immunoassay of endogenous plasma insulin in man. *The Journal of clinical investigation*, 39, pp.1157–75.

Yan, H. et al., 2009. IDH1 and IDH2 mutations in gliomas. *The New England journal of medicine*, 360(8), pp.765–73.

Yang, B. et al., 2010. Molecular mechanisms of "off-on switch" of activities of human IDH1 by tumor-associated mutation R132H. *Cell research*, 20(11), pp.1188–200.

Yang, H. et al., 2012. IDH1 and IDH2 mutations in tumorigenesis: mechanistic insights and clinical perspectives. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 18(20), pp.5562–71.

Ye, D. et al., 2013. R-2-hydroxyglutarate as the key effector of IDH mutations promoting oncogenesis. *Cancer Cell*, 23(3), pp.274–276.

Yen, K.E. et al., 2010. Cancer-associated IDH mutations: biomarker and therapeutic opportunities. *Oncogene*, 29(49), pp.6409–17.

Zhao, S. et al., 2009. Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1alpha. *Science (New York, N.Y.)*, 324(5924), pp.261–5.