IDH1/2 (isocitrate dehydrogenase 1/2) Mutations in Gliomas : genotype-Phenotype Correlation, Prognostic impact, and Response to Irradiation

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UNIVERSITE PARIS XI

FACULTE DE MEDECINE PARIS-SUD

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Thèse réalisée dans le laboratoire de Neuro-Oncologie expérimentale du Pr. Jean-Yves DELATTRE. UPMC- INSERM U 975- CNRS, UMR 7225. Groupe Hospitalier Pitié-Salpetrière. 47-83 Bd de l'Hopital. 75013 Paris.

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ABSTRACT

TITLE IN ENGLISH:

***IDH1/2* (isocitrate dehydrogenase 1/2) mutations in gliomas: genotype-phenotype Correlation, Prognostic impact, and Response to irradiation**

TITLE IN FRENCH:

Les mutations *IDH1/2* (isocitrate déshydrogénase 1/2) dans les gliomes: Corrélation au profile génomique, facteur pronostique, et Implication dans la réponse à l'irradiation.

ABSTRACT IN ENGLISH:

Since Parsons et al. (2008) found the frequent mutations of *IDH1* (12%) in GBMs, various reports have studied the prevalence and characteristic of *IDH1* and *IDH2* mutations.

The mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene occur in nearly 40% of gliomas. Analogue *IDH2* mutations are mutually exclusive with *IDH1* mutations and much less frequent (~5%) in gliomas. However, *IDH3* is not mutated in gliomas. Up to now, 7 different types of *IDH1* mutations and 5 different types of *IDH2* mutations were found. The *IDH1*R132H mutation is the most common (>90%) in gliomas. The frequency of *IDH1* mutations are inversely connected with grade II (~80%), III (~50%), and IV (~ 10%) gliomas. Importantly, the status of *IDH1* mutations is associated with a better outcome and demonstrated a diagnostic value. We analyzed also these mutations in distribution, association with tumor-derived other genetic alterations and the diagnostic and prognostic value in a cohort of 1332 glioma patients.

A synonymous single nucleotide polymorphism [SNP rs 11554137; C (cytosine) substituted by T (thymin)] has been studied in gliomas patients. The SNP rs 11554137 (in codon 105) are located in the same exon with the *IDH1* R132 mutations (in codon 132). And gliomas patients with SNP rs 11554137: C>T had a poorer outcome than patients without

SNP rs 11554137. This was observed a similarly adverse effect in survival in patients with AML.

Mutations in codon 132 can cause a decrease of *IDH1/2* activity and also gain a new enzyme function for the NADPH dependent reduction of alpha-ketoglutarate to 2-hydroxyglutarate. High 2HG and low NADPH levels might sensitize tumors to oxidative stress, potentiating response to radiotherapy, and may account for the prolonged survival of patients harboring the mutations. So we studied further the alterations of function in IDH1R132H mutant cells in vitro. Based on the decrease of defence and the increase of impairing factors in tumor cells, we found that the tumors harbouring *IDH1* mutations may have an elevated radiosensitivity.

In the present study, we described the impact of *IDH1* mutations in gliomas and search for new perspectives for the treatment strategy.

KEY WORDS:

Gliomas, isocitrate dehydrogenase 1 (*IDH1*), single nucleotide polymorphism (SNP), irradiation

ABSTRACT IN FRENCH :

Depuis que Parsons et col. ont découvert en 2008 que le gène de l'isocitrate déshydrogénase 1 (*IDH1*) est fréquemment muté dans les glioblastomes (12%), de nombreuses équipes ont étudié la prévalence et les caractéristiques des mutations des gènes *IDH1 et 2* dans les gliomes.

Les mutations du gène *IDH1* sont observées dans environ 40% des gliomes. Des mutations analogues sur l'isoforme mitochondriale de ce gène, *IDH2*, ont été observées dans environ 5% des gliomes et sont mutuellement exclusives avec les mutations du gène *IDH1*. Le gène *IDH3*, autre isoforme mitochondriale, n'est quant à lui pas muté dans les gliomes. A ce jour, 7 mutations différentes d'*IDH1* et 5 d'*IDH2* ont été découvertes. La mutation d'*IDH1* la plus fréquentes dans les gliomes (>90% des cas) est la mutation R132H. La fréquence des mutations *IDH1* et 2 est inversement corrélée au grade des gliomes (grade II ~80%, III ~50%, and IV ~10%). Les mutations *IDH1/2* ont une valeur diagnostique ainsi que pronostique (associées à une meilleure survie).

Pendant ce travail de thèse nous avons dans une première partie analysé la distribution de ces mutations *IDH1/2* dans les différents gliomes, leur association avec d'autres altérations génétiques, ainsi que leur valeur diagnostique et pronostique dans une cohorte de 1332 patients atteints de gliomes. Nous confirmons sur cette très grande cohorte les données de la littérature et affinons la valeur pronostique des mutations *IDH1/2*.
Dans une seconde partie, nous avons mis en évidence dans les gliomes un polymorphisme (SNP) du gène *IDH1* (SNP rs 11554137; C (cytosine) substituted by T (thymin)) précédemment observé dans les leucémies myéloïdes aigues. Ce SNP, codon 105, est localisé dans le même exon que le codon 132 fréquemment muté, et nous avons montré qu'il est associé à une moins bonne survie des patients atteints de gliomes.

Les mutations du codon 132 causent une baisse de l'activité enzymatique normale d'IDH1/2 qui est remplacé par le gain d'une nouvelle. Les protéines IDH1/2 mutés, au lieu de produire de l'alpha-cétoglutarate de façon NADP dépendante, réduisent de façon NADPH dépendante l'alpha-cétoglutarate en 2-hydroxyglutarate (2HG). Une forte concentration de 2HG et une baisse de la quantité de NADPH peuvent sensibiliser les tumeurs au stress oxidatif et donc potentialiser l'effet de la radiothérapie, ce qui pourrait expliquer la meilleure survie de ces patients. Nous avons donc dans une troisième partie étudié *in vitro* l'impact de la mutation

IDH1R132H sur la survie après radiothérapie de cellules tumorales exprimant de façon stable ce gène muté. Les résultats obtenus montrent que dans certaines conditions ces cellules pourraient être plus radiosensibles que les mêmes cellules exprimant le gène IDH1 non-muté.

Dans ce travail de thèse, nous avons donc étudié le gène *IDH1* dans les gliomes de patients et tenté par une approche fonctionnelle *in vitro* d'évaluer l'impact de la mutation *IDH1*R132H sur la radiosensibilité des cellules tumorales.

MOTS CLES:

Gliomes, isocitrate déshydrogénase 1 (*IDH1*), polymorphism nucléotide (SNP), radiothérapie, radiosensibilité.

ABBREVIATIONS

2HG	2-Hydroxyglutarate
AML	Acute Myeloid Leukemia
CDK4	Cyclin-Dependent Kinase 4
CIC	*Capicua gene*
CNS	Central Nervous System
COLD PCR	coamplification at lower temperature-PCR
CT	X-ray Computed Tomography
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
EORTC	European Organisation for Research and Treatment of Cancer
FUBP1	Far Upstream Element [FUSE] Binding Protein 1
GBM	Glioblastoma
HIF	Hypoxia Inducible Factor
HRM	High-resolution melting
*IDH*s	Isocitrate Dehydrogenases
LGG	Low-Grade Gliomas
LOH	Loss of Heterozygosity
MDM	Murine Double Minute
MGMT	O6-Methylguanine-DNA Methyltransferase
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCIC	National Cancer Institute of Canada Clinical Trials Group
NF1	Neurofibromin 1
OS	Overall survival
PA	Pilocytic Astrocytomas
PCV	Procarbazine, Lomustine, and Vincristine
PDGFRA	Alpha-type Platelet-Derived Growth Factor Receptor
PET	Positron Emission Tomography
PFS	Progression Free Survival
PI3K	Phosphatidylinositol 3-Kinases
PTEN	Phosphatase and Tensin Homolog

RB1	Retinoblastoma Protein
ROS	Reactive Oxygen Species
RTKs	Receptor Tyrosine Kinases Signaling
SNP	Single Nucleotide Polymorphism
TCA	Tricarboxylic Acid Cycle
TCGA	The Cancer Genome Atlas
TMZ	Temozolomide
TP53	Tumor Protein 53
WHO	World Health Organization
α-KG	α-Ketoglutarate

TABLE OF CONTENTS

INTRODUCTION 1
Chapter I. Gliomas: Epidemiology, Histo prognostic classification and Treatment options 2
I. Epidemiology 2
II. Histoprognostic classification of gliomas 5
III. Treatment options 7
Chapter II: Molecular classification of human gliomas 10
I. Genomic classification of gliomas 12
II. Epigenomic classification of gliomas 17
III. Transcriptomic classification of gliomas 18
Chapter III: Normal function of IDHs and *IDH1/2* mutations in gliomas 19
I. Isoenzymes and roles in normal cell metabolism 20
II. *IDH1* and *IDH2* mutations in gliomas 24

OBJECTIVES 26

RESULTS 29
Publication n°1 "*IDH* mutations: genotype-phenotype correlation and prognostic impact" 30
Publication n°2 "Prognostic impact of *IDH1* SNP rs11554137 in malignant gliomas" 48
Publication n°3 "Isocitrate dehydrogenase 1 mutation (IDH1 R132H) increases U87 glioma cell sensitivity to radiation therapy in hypoxia" 66

DISCUSSION 82
I. Description of *IDH1/IDH2* mutations in gliomas 83
II. Diagnostic value of *IDH1/2* mutations and new methods for IDH status determination 101
III. Prognostic/ predictive value of *IDH1/2* mutations 104

PERSPECTIVES 107

REFERENCES 109

ANNEXES 118
Article 4"Chromosome 7p11.2 (EGFR) variation influences glioma risk." 119
Article 5 "All the 1p19q codeleted gliomas are mutated on *IDH1* or *IDH2*." 128
Article 6 "*IDH1* and *IDH2* mutations in gliomas." 134
Article 7 "COLD PCR HRM: a highly sensitive detection method for *IDH1* mutations." 137
Article 8 "*IDH1* or *IDH2* mutations predict longer survival and response to temozolomide in low-grade gliomas." 144

INTRODUCTION

Chapter I. Gliomas: Epidemiology, Histo prognostic classification and Treatment options

Chapter I. Gliomas: Epidemiology, Histopronostic classification and Treatments

I. Epidemiology

1. Brain tumors

There are two types of brain tumors: a) primary brain tumors that originate in the brain, normally in the brain itself, in the cranial nerves, in the meninges, etc; and b) metastatic brain tumors that originate from cancer cells that have migrated from other organs of the body. Brain tumors account for 5% of all adult cancers, 70% of pediatric cancers. Primary brain tumors account for 50% of intracranial tumors. The incidence of brain tumors is 14.8 per 100,000 person-years, with approximately half being histologically benign. Females have a slightly higher incidence (15.1/100,000 person-years) than males (14.3/100,000 person-years) (1–3).

2. Gliomas

Gliomas are named according to histological features as Astrocytomas, Oligodendrogliomas and mixed gliomas (also called oligoastrocytomas) contain cells from different types of glia. Malignant gliomas account for approximately 70% of primary malignant brain tumors in adults (Figure 1). Glioblastomas account for approximately 60 to 70% of malignant gliomas, anaplastic astrocytomas for 10 to 15%, and anaplastic oligodendrogliomas and anaplastic oligoastrocytomas for 10%. Malignant gliomas are 40% more common in men than in women. Malignant gliomas are associated with high morbidity and mortality. The median survival is only 12 to 15 months for patients with glioblastomas and 2 to 5 years for patients with anaplastic gliomas (4). Additional survival data are given below (part II. Histoprognostic classification of gliomas).

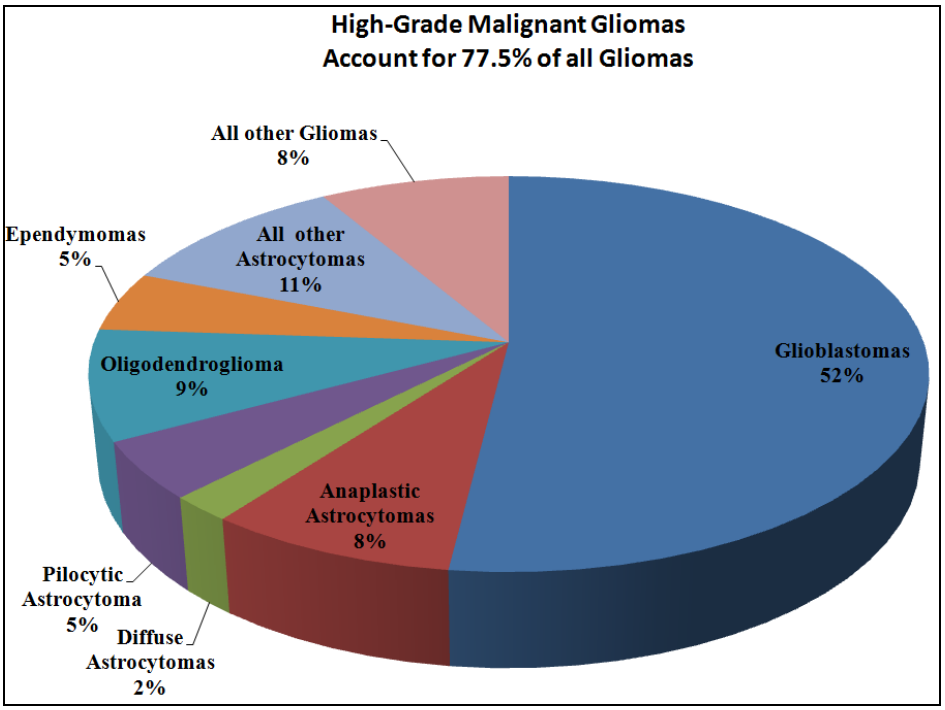

Figure 1: The prevalence of gliomas. Adapted from Central Brain Tumor Registry of the United States (CBTRUS) 2002-2003. Statistical report 1995-1999.

3. Environmental and genetic risk factors for gliomas

It is difficult to identify underlying cause for malignant gliomas. The following risk factors have been discovered for brain tumors by a great number of studies:

1) ***Ionizing radiation:*** therapeutic or high-dose radiation has been firmly identified as a rare cause of primary brain tumors. For example, cranial irradiation for children with acute lymphocytic leukemia has been associated with an increased risk of subsequent brain tumors, especially meningiomas, but also gliomas (5) In a recent retrospective study, cumulative radiation doses provided by CT scans significantly associate and triple the risk of leukaemia (~50 mGy) and brain tumors (~60 mGy) in children (6).

2) ***Genetic factors:*** While Li-Fraumeni, Turcot, and the melanoma/glioblastoma syndromes are associated with substantive risks of glioma, all are rare and collectively account for less than 5% of glioma cases (4). The hypothesis that common genetic variation is a determinant of glioma risk has recently been vindicated by genome-wide association studies (GWASs) which have identified single nucleotide polymorphisms (SNPs) at seven loci influencing glioma risk - TERT, EGFR (2 loci), CCDC26, CDKN2A/B, PHLDB1, RTEL1.(7,8). TERT and RTEL risk variants are associated with high-grade disease whereas the CCDC26 and PHLDB1 risk variants are associated with low-grade disease, and EGFR and CDKN2A/B risk variants appear to have a generic effect on tumor risk (9,10) (**Article 4**).

3) ***Immunologic factors:*** an association between immunologic factors and gliomas has been suggested by several studies. Indeed, patients with atopy have a reduced risk of gliomas (11); and patients with glioblastoma who have elevated IgE levels have a longer survival than those with normal levels (12). However, the importance of these associations remains unclear.

Environmental factors associated with primary brain tumors are difficult to identify. Evidence for an association with cell phones, head injury, foods containing N-nitroso compounds, and exposure to certain chemicals at work or electromagnetic fields is inconclusive, but additional studies are needed (2).

II. Histoprognostic classification of gliomas

The WHO Classification of Tumors of the Central Nervous System is the universal standard for classifying and grading brain neoplasms (13). The aim is to define the histological type of glioma and the grade in order to classify the patients and give them an accurate treatment. According to the presumed cell of origin, gliomas have been classified into three major groups: astrocytomas, oligodendrogliomas and mixed oligoastrocytomas. Based on the presence or absence of malignant features: cell density, nuclear atypia, mitosis, microvascular proliferation and necrosis, the WHO classification distinguishes grade I, II (LGG), III (anaplastic), IV (glioblastomas, GBM) (14) (Table 1).

Grade I gliomas are pilocytic astrocytomas, which are benign and curable and occur primarily in children. Astrocytomas, oligodendrogliomas and oligoastrocytomas correspond to low-grade (II) or high-grade (III and IV), which are invasive tumors and often progress to glioblastoma. Grade IV gliomas include primary and secondary GBM: *de novo* glioblastomas are grade IV astrocytomas without of a prior history of brain tumor; secondary glioblastomas occur in the context of a pre-existing low-grade glioma (15).

Based on histology and imaging data, Daumas-Duport et al. have proposed another classification, which distinguishes oligodendrogliomas and mixed gliomas of grade A (without endothelial proliferation and/or contrast enhancement), oligodendrogliomas and mixed gliomas of grade B (with endothelial proliferation or contrast enhancement), glioblastomas and glioneuronal malignant tumors (16). However, both classifications lack reproducibility.

Table 1: Histological classification of human gliomas depending on WHO (2007) (14)

	Histologic types (grades)	Differentiation	Necrosis	Vascular Proliferation	Cytogenetics/ Molecular genetics	Age at diagnosis (years)	Survival time (years)
Astrocytic tumors	Pilocytic astrocytoma (I)	Well differentiated	absent	absent	*NF1*	children	>20
	Diffuse astrocytoma (II)	Well differentiated	absent	absent	*TP53*; 17p13.1	young adults	4-10
	Anaplastic astrocytoma (III)	Focal or diffuse anaplasis	absent	absent	*TP53*	~41	2-5
	Glioblastoma (IV)	Poorly differentiated	present	present	*EGFR; PTEN*; LOH 10	45-75	1-2
Oligodendroglial tumors	Oligodendroglioma (II)	Well differentiated	absent	absent	1p19q	50-60	8-20
	Anaplastic Oligodendroglioma (III)	Focal or diffuse anaplasis	possible	possible	1p19q	50-60	2-10
Mixed gliomas	Oligoastrocytoma (II)	Well differentiated	absent	absent	-	35-45	5-12
	Anaplastic oligoastrocytoma (III)	-	possible	possible	-	~45	2-8

III. Treatment options

Treatment of gliomas involves a multidisciplinary team effort to provide an integrated model of care. Current treatment options for gliomas include surgery, radiation therapy, chemotherapy, medications and experimental therapies. The goals of gliomas treatments are to: a) remove as many tumor cells as possible (with surgery); b) kill as many of the cells left behind after surgery as possible (with radiation and chemotherapy); c) put remaining tumor cells into a nondividing, quiescent state for as long as possible (with radiation and chemotherapy). However, treatment options vary depending on the type, the size, the location and the grade of malignancy of the tumor, as well as the overall health of patients.

1. Surgery

Surgical resection is recommended for most types of brain tumors (40%-50%) in most locations. Primary goals of surgery include: (a) establishing an accurate histologic diagnosis; (b) resulting in symptomatic relief and neurological improvement; and (c) leading to oncologic reduction to increase the efficiency of adjuvant therapy and improve survival outcome (17,18). Surgical treatment included biopsy only, incomplete resection or complete resection. If tumors are small and easy to separate from surrounding brain tissue, complete surgical resection (radical resection) is possible. If tumors can't be separated from surrounding tissue or they're located near sensitive areas in brain, the neuro surgeon will remove as much of the tumor as is safe (partial resection). Stereotaxic biopsy can be used for lesions that are difficult to reach and resect. However, there is a general consensus, based on retrospective studies, that surgical resection improves survival outcome (17).

2. Radiation therapy

Post-operative external beam radiotherapy is recommended as standard therapy for patients with malignant glioma. Radiation therapy usually comes from a device outside the body (external beam radiation) and uses beams of high-energy particles, such as X-rays, to

kill tumor cells. Standard treatment planning relies on a planning CT coregistered with an MRI and incorporates the enhancing tumor plus a limited margin (e.g. 2 cm) for the planning target volume, with a total dose of 54–60 Gy in daily fractions of 1.8–2.0 Gy, in 30 fractions for 6 weeks (19,20). Incorporation of amino acid PET information seems to be a valuable tool to optimize clinical results (21). Then, radiation therapy is an essential part of treatment for patients with gliomas and can increase the cure rate or prolong survival (22).

3. Chemotherapy

Chemotherapy uses drugs to kill or alter dividing cells. Cell division is preceeded by duplication and recombination of DNA. During this process chemotherapy may introduce errors into the DNA of tumor cells, leading to damages to the DNA and subsequent cell death. As a rule, chemotherapy for brain tumors is administered following surgery or radiation therapy. Standard chemotherapy drugs which may be used to treat primary brain tumours include: temozolomide, carmustine (BCNU), lomustine (CCNU), procarbazine, vincristine and platinum-based drugs (cisplatin and carboplatin). Sometimes lomustine, procarbazine and vincristine are used together and this combination of chemotherapy drugs is known as PCV (23). Chemotherapy has been reported to prolong survival in patients with gliomas. *MGMT* promoter methylation status identifies patients most likely to benefit from the addition of temozolomide (24,25).

4. Multidisciplinary approach

Due to the particularly invasive properties, gliomas are difficult to treat. Therefore, treatment is often a combined approach, using surgery, radiation therapy, and chemotherapy. Surgery and radiation therapy are the primary modalities used to treat gliomas. In typical situations, patients begin radiation treatments within 2 to 4 weeks after tumor resection (26). Although chemotherapy is not an effective initial treatment for low-grade brain tumors, it is helpful in controlling high-grade gliomas. The standard treatment for glioblastoma (GBM) patients is considered to be neurosurgical resection, followed by concomitant irradiation and chemotherapy with temozolomide (TMZ) (27). In 2009, the European Organisation for Research and Treatment of Cancer (EORTC) and National Cancer Institute of Canada

Clinical Trials Group (NCIC) reported a randomised phase III trial comparing radiotherapy alone with radiotherapy and concomitant treatment with temozolomide, followed by adjuvant temozolomide therapy: improved median and 5-year survival for patients with glioblastomas. Overall survival was 27.2% at 2 years, 16.0% at 3 years, 12.1% at 4 years, and 9.8% at 5 years with temozolomide and radiotherapy, *versus* 10.9%, 4.4%, 3.0%, and 1.9% with radiotherapy alone (24). This protocol is currently the standard of care for primary glioblastoma patients.

5. New treatments

Currently, new treatment strategies are emerging that target steps in the molecular pathogenesis of tumors. Targeted therapies work on a molecular level by blocking specific mechanisms associated with cancer cell growth and division. These biologic drugs may induce less severe side effects, as they selectively target cancerous cells. In addition, these targeted drugs hold the promise of creating options for more individualized cancer treatment based on a patient's genotype. Many targeted drug therapies for brain tumors, including antisense oligonucleotides, gene therapy, and angiogenesis inhibitors, are very new and still undergoing careful study in clinical trials (28,29). Bevacizumab (Avastin®), a monoclonal antibody targeting VEGF, that blocks the formation and growth of new blood vessels, cutting off blood supply to a tumor and killing the tumor cells, is the first targeted therapy approved for brain tumors, and the first new treatment for glioblastoma in more than a decade (30,31).

Some patients under regular treatment may choose to receive experimental therapies. For example, vaccine and viral therapies approaches include the alteration of the immune system to attempt to control the glioma with vaccines, infusion of immune cells into the body and the use of modified viruses to attack the tumor.

INTRODUCTION

Chapter II: Molecular classification of human gliomas

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Human cancer is driven by an accumulation of genome alterations over time. DNA sequence changes, copy number aberrations, chromosomal rearrangements, and modification in DNA methylation together result in the formation, development and progression of human malignancies.

The alterations in proto-oncogenes, tumor suppressor genes, or DNA repair genes will develop cancer. Proto-oncogenes activated through mutation, amplification or translocation become oncogenes and then can promote tumor formation or growth. Tumor suppressor genes are inactivated through mutation, deletion or methylation to cause a loss or reduction in its function, then the cell can undergo malignant transformation in combination with other genetic changes. DNA repair proteins are usually classified as tumor suppressors as well, as mutations in such their genes increase inactivation of other tumor suppressors and activation of oncogenes and then lead to increase the risk of cancer (32).

Many studies have focused on a molecular classification of gliomas. Only, few molecular alterations have a prognostic value and affect the response to treatment. Molecular classifications have been proposed at the genomic, epigenomic and transcriptomic levels.

I. Genomic classification of gliomas

1. The alteration of three major pathways in glioblastomas

The Cancer Genome Atlas (TCGA) pilot project analyzed integratively common genetic alterations in the GBM genome and found that three major pathways are affected in the majority of primary glioblastomas. These pathways include (Figure 2): (i) receptor tyrosine kinases (RTKs) signaling (EGFR/RAS/NF1/PTEN/PI3K pathway), (ii) the p53/RB tumor suppressor pathways (*TP53*/MDM2/MDM4/$p14^{ARF}$ pathway and (iii) $p16^{INK4a}$/CDK4/RB1 pathway) (33).

a. EGFR/RAS/NF1/PTEN/PI3K pathway

The receptor tyrosine kinases (RTKs) signaling pathway is a key signaling pathway in cell proliferation and increased cell survival of primary glioblastomas (Figure 2) (34). The TCGA pilot project showed that the overall frequency of genetic alterations in the EGFR/RAS/NF1/PTEN/PI3K pathway in glioblastomas was 88%, through *EGFR* mutation or amplification (45%), *RAS* mutation (2%), *NF1* mutation or homozygous deletion (18%), *PTEN* mutation or homozygous deletion (36%), *PI3K* mutation (15%) (33).

PI3K pathway is a progressive activation pattern with glioma grade (35). The activated frequency is significantly higher in GBM than non-GBM gliomas. Among non-GBM gliomas, the PI3K pathway is more frequent in anaplastic gliomas compared to non-anaplastic gliomas. The activation of PI3K is inversely associated with outcome.

b. TP53/MDM2/MDM4/$p14^{ARF}$ pathway

The TCGA project identified that 87% of the overall frequency of genetic alterations was observed in the *TP53/MDM2/MDM4/*$p14^{ARF}$ pathway in primary glioblastomas, through *TP53* mutations or homozygous deletion (35%), *MDM2* amplification (14%), *MDM4* amplification (7%), or $p14^{ARF}$ homozygous deletion or mutation (49%) (33) (Figure 2).

c. p16^{INK4a}/CDK4/RB1 pathway

The p16^{INK4a}/CDK4/RB1 pathway seems to be important in pathways to both primary and secondary glioblastomas (Figure 2). The TCGA project demonstrated that the overall frequency of genetic alterations in the RB1 signaling pathway in primary glioblastomas was 78%, through p16^{INK4a} homozygous deletion or mutations (52%), p15^{INK4b} homozygous deletion (47%), *CDK4* amplification (18%), *RB1* mutation or homozygous deletion (11%) (33).

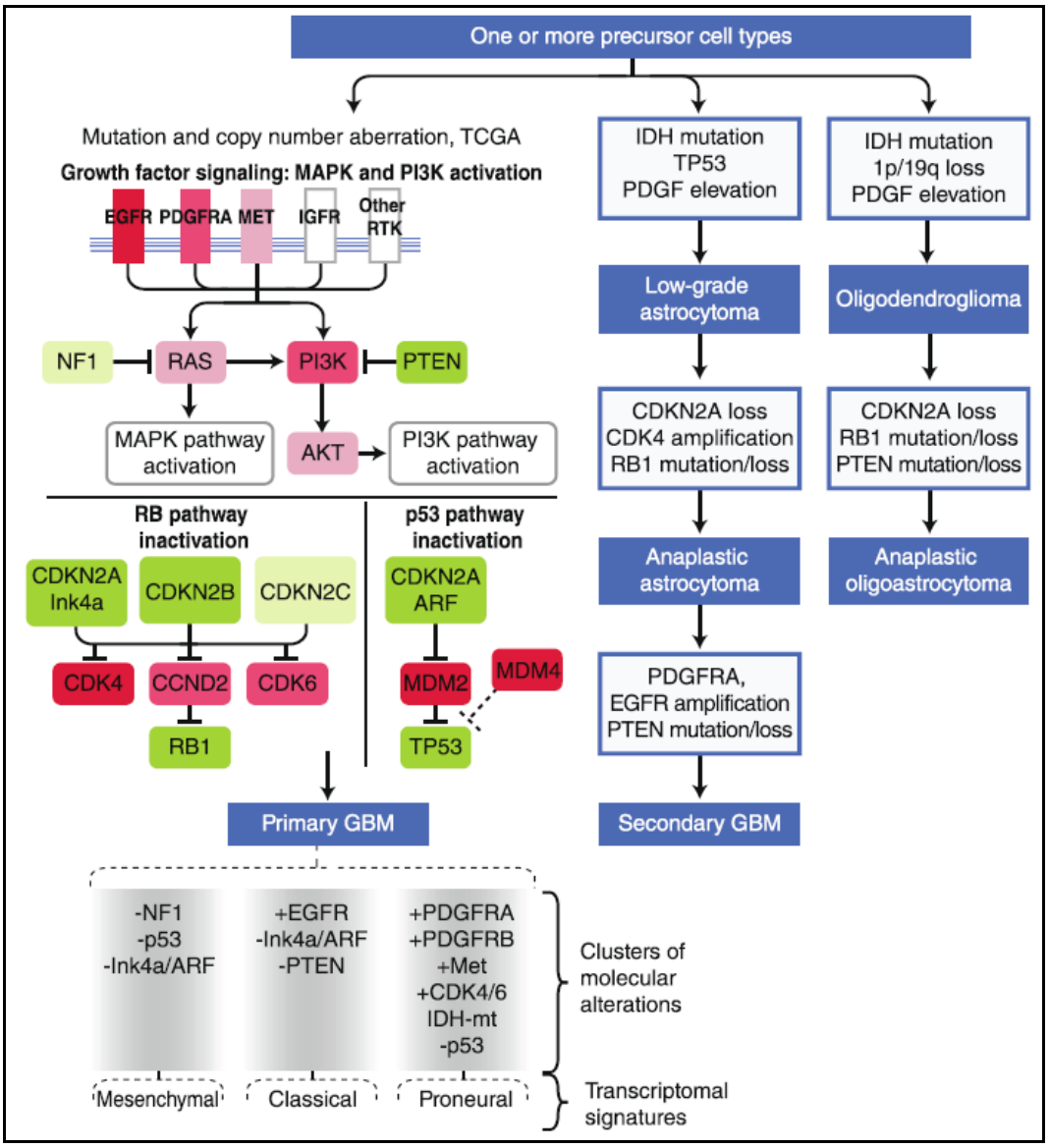

Figure 2: The pathogenesis of gliomas and four transcriptomal subclasses of GBM. Brennan et al. 2011 (36)

2. Others glioma subtypes

In contrast to primary GBM, lower-grade gliomas and secondary GBM have not yet been profiled and subclassed at the resolution of TCGA project. Gliomas are characterized by heterogeneity of clinical, histologic and molecular features in the WHO classification system (13,37). Astrocytomas are associated with *TP53* mutation whereas oligodendrogliomas are associated with 1p/19q co-deletion, these molecular events being largely mutually exclusive (38).

a. TP53 mutation

Tumor protein *p53* encoding by the *TP53* gene acts as a tumor suppressor. When the DNA is damaged by agents such as toxic chemicals or radiation, p53 plays a critical role in determining whether the DNA will be repaired or the damaged cell will undergo apoptosis (39). *TP53* mutation is a common finding in all grades of infiltrating astrocytomas and is the first detectable genetic alteration in two-thirds of low-grade diffuse astrocytomas; a similar frequency is found in anaplastic astrocytomas and secondary glioblastomas (40), and is higher than in primary glioblastomas (65% *vs.* 28%) (41) (Figure 4).

b. Loss of 1p and 19q

Chromosome 1p/19q codeletion is the consequence of an unbalanced translocation between the entire arms of 19p to 1q, yet the mechanism of codeletion is unexplained (42). Nearly 85% of low-grade oligodendrogliomas and 65% of anaplastic oligodendrogliomas harbor 1p19q co-deletion (43), suggesting 1p19q deletion is an early event and is required for the development of the majority of oligodendrogliomas (42) (Figure 4). Recently, both EORTC 26951 (van den Bent et al.) and RTOG 9402 (Caimcross et al.) in ASCO 2012 showed that patients with 1p/19q codeleted oligodendrogliomas had a better prognosis in combination of chemotherapy and radiation therapy (RT) vs. RT alone. 1p19q was confirmed to be a predictive molecular marker by both studies for treatment decisions.

c. IDH1/IDH2 mutations

Mutations in the gene encoding NADP+-dependent isocitrate dehydrogenase enzyme isoforms 1 (*IDH1*) and 2 (*IDH2*) have recently been identified in a large proportion of glial tumors of the CNS (44–50). *IDH1*/*2* mutations occur in about 40% of gliomas (41). Most

notably, *IDH1* mutations occurred preferentially in younger patients and in most patients with secondary GBMs and were associated with a more favorable outcome (44). Additional mutations in the *IDH2* gene affecting the analogous amino acid (R172) were then discovered in gliomas without IDH1 mutations (47) (Figure 3). *IDH3* is not mutated in gliomas.

An *IDH1*/*IDH2* mutation is virtually always associated with a complete 1p/19q codeletion, and the majority (>60%) of low-grade astrocytomas have *TP53* mutations plus *IDH1* mutations (51). Therefore, *IDH1* mutation is an early event in both oligodendroglial and astrocytic tumors (Figure 4). Based on IDH1 and 1p19q status alterations, three prognostic subtypes of grade II and grade III can be determined: 1) *IDH*mut/1p19qdel, 2) *IDH*mut/1p19qnon del, 3) *IDH*non mut/1p19qnon del (52) (**Article 5**). Interestingly most of the *TP53* mutation is found in the *IDH*mut/1p19qnon del group, whereas the group 1 are 3 are mostly *TP53* wildtype. The group 3 corresponds mostly to the triple negative subtype defined by Metellus et al, highly infiltrative, and carrying the worst prognosis (53).

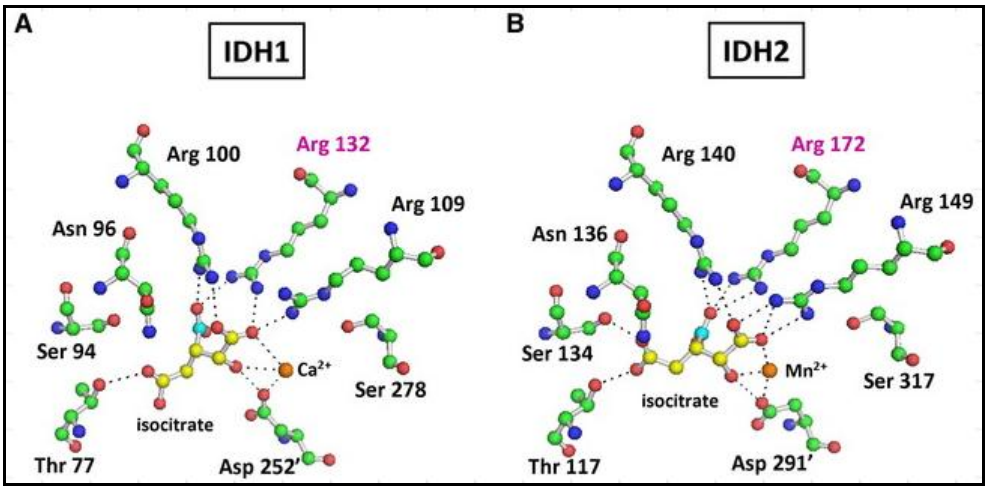

Figure 3: Mutations affect analogous residues in *IDH1* and *IDH2*. Ward et al. 2010 (54).

d. Mutations in CIC and FUBP1

Recently, Bettegowda et al. (2011) (55) found the *CIC* gene (homolog of the *Drosophila* gene *capicua*) on chromosome 19q and the *FUBP1* gene [encoding far upstream

element (FUSE) binding protein] on chromosome 1p mutated in oligodendrogliomas. Mutations affect splice sites, produce stop codons, or generate out-of-frame insertions or deletions and are likely to inactivate. They are unfrequent in other tumors, including non codelted gliomas and are therefore likely to play a specific role in oligodendrogliomagenesis.

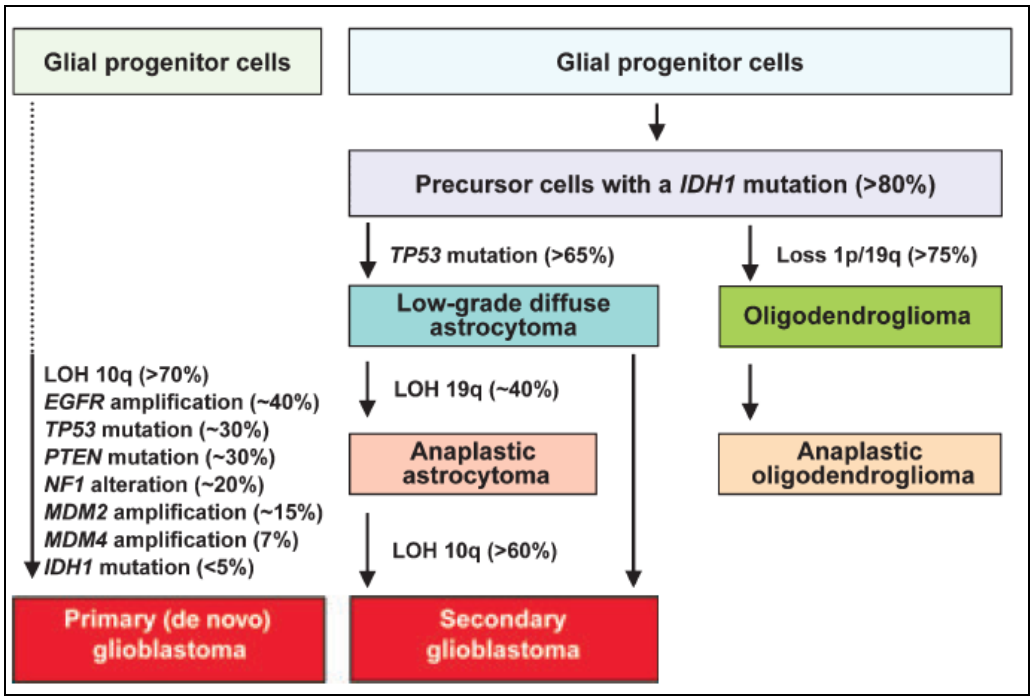

Figure 4: Genetic pathways to gliomas. Ohgaki et al. 2009 (41)

II. Epigenomic classification of gliomas

1. Hypermethylated phenotypes

Epigenetics is related to DNA methylation and histone modification (acetylation or methylation). These changes only affect the regulation and level of gene expression, but do not alter the nucleotide sequence (56).

5-methylcytosine (5mC) at CpG dinucleotides are the most important DNA methylation sites. 5mC may be converted into 5-hydroxymethylcytosine (5hmC) by TET2 (TET family of 5-methylcytosine hydroxylases), resulting in the DNA demethylation. This mechanism may explain why *IDH* mutations are associated with CIMP phenotype in gliomas.

Several studies have reported the changes of the intermediate products of DNA methylation: (i) 5mC is elevated in *IDH1*R132H and *IDH2*R172K mutated 293T cells (57); (ii) Turcan et al. showed that 5hmC is decreased in mutant *IDH1*-transduced astrocytes, and increased when *TET2* was expressed in the astrocytes. Importantly, they confirmed that cells with *IDH1* mutation developed glioma-CpG island methylator phenotype (G-CIMP), suggesting that *IDH1* mutation is the molecular basis of CIMP in glioma. (58).

Histone lysine methyltransferases and *TET2* (TET family of 5-methylcytosine hydroxylases) are αKG-dependent dioxygenase enzymes. D-2-HG is a competitive inhibitor of the α-ketoglutarate-dependent histone lysine methyltransferases and TEF 5-methylcytosine hydroxylases (59,60). Thus, because of the competitive inhibition by 2HG, *IDH* mutation prevents DNA and histones demethylation and result in hypermethylated phenotypes. These hypermethylated phenotypes decrease globally the gene expression, and probably contribute to the formation and development of tumors.

2. MGMT promoter methylation

O6-Methylguanine-DNA methyltransferase (*MGMT*) is a DNA repair enzyme that removes alkylating lesions induced by chemotherapeutic agents. Loss or reduction of *MGMT* activity through promoter methylation decreases DNA repair activity, increasing sensitivity to alkylating agents. A methylated *MGMT* promoter is observed in low-grade gliomas (60-93%)

and GBM (45%) (61,62). Methylation of *MGMT* was the strongest predictor of outcome and benefit from temozolomide chemotherapy (62,63).

III. Transcriptomic classification of gliomas

Clinically relevant subtypes of GBM can be characterized by alterations and abnormal expression of *EGFR*, *NF1*, *PDGFRA*, and *IDH1* genes. Verhaak et al. reported four transcriptomal subclasses of GBM based on transcriptomic profile: the *Proneural*, *Mesenchymal*, *Classical*, and *Neural* (29,58) (Figure 2). Gain of chromosome 7 and chromosome 10 deletion are almost constant in the **Classical** subtype, with frequent *EGFR* gene amplifications or mutations. **Proneural** subtype harbors frequent mutations of *TP53* and alterations (mutations and amplifications) of *PDGFRA* gene. *IDH1* mutations are found exclusively in the proneural subclasse (65). The **Mesenchymal** subtype is characterized by frequent deletions/mutation of *NF1* gene, with overexpression of mesenchymal genes such as YKL40, IGFBP (66).The **Neural** subtype is characterized by neuron-related gene expression.

These four subtypes may have different benefit from combined radio-chemotherapy (65): ie low or no benefit in the **Proneural** subtype and greatest benefit in the **classical** subtype, but this need to be confirmed.

INTRODUCTION

Chapter III: Normal function of IDHs and *IDH1/2* mutations in gliomas

Chapter III: Normal function of isocitrate dehydrogenase and *IDH1/2* mutations in gliomas

In this work, we have focused on the most frequent alteration found in gliomas: mutations of isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*).

I. Isoenzymes and roles in normal cell metabolism

In humans, five genes encode three isoforms of *IDH* in humans: IDH1, IDH2 and IDH3. These enzymes are distinguished by their cofactor NADP or NAD.

1. IDH isoenzymes

a. NADP+ dependent

IDH1 (chromosome 2q33.3) and *IDH2* (chromosome 15q26.1) are NADP+-dependent homodimeric isozymes that share considerable sequence similarity and an almost identical protein structure, each with two identical active sites per homodimer (Figure 5). They catalyze reversible reactions in the tricarboxylic acid (TCA) cycle (67). *IDH1* is in the cytoplasm and peroxisomes; it is highly expressed in the liver and moderately expressed in other tissues. *IDH2* is exclusively localized to the mitochondria and highly expressed in heart, muscle, activated lymphocytes and to a lesser level in other tissues (68).

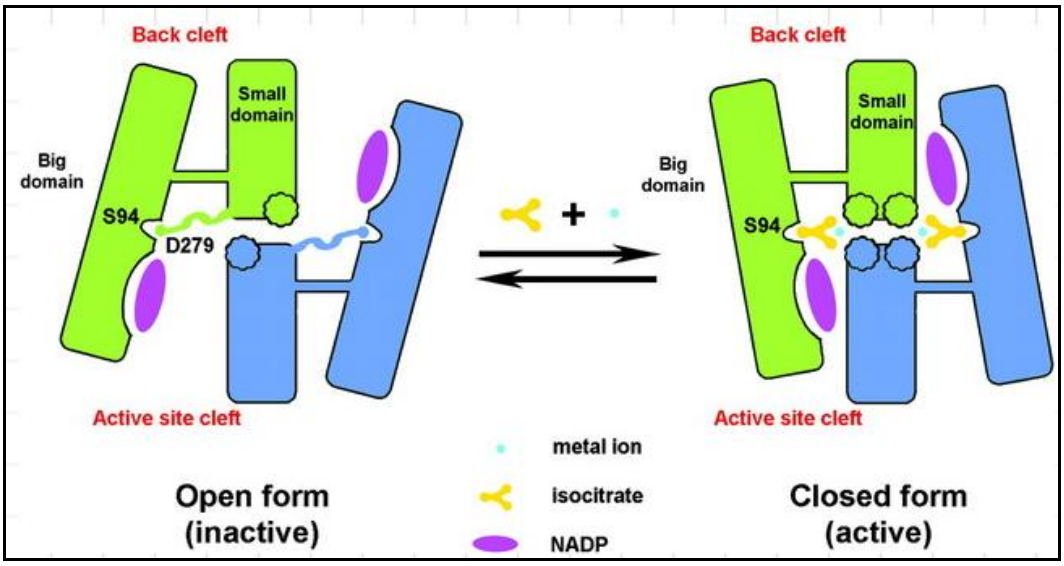

Figure 5: Homodimer IDH with two identical active sites and the conformation regulates the activity (67)

b. NAD+ dependent

IDH3 (211) is a NAD+-dependent heterotetrameric enzyme composed of two alpha subunits, which contain the two active sites, together with two regulatory subunits, beta and gamma (subunit alpha chromosome 15q25.1–q25.2; subunit beta chromosome 20p13; subunit gamma chromosome Xq28). *IDH3* is also localized to the mitochondria and plays a central role in the TCA cycle. The reaction catalyzed by *IDH3* is irreversible (68).

2. Enzymatic reactions catalyzed by IDHs

Isocitrate dehydrogenases are important enzymes in the tricarboxylic acid cycle. They catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate while converting NAD(P)+ to NAD(P)H. The reaction is a two-step process, which involves the oxidation of isocitrate to the intermediate oxalosuccinate, reducing NAD(P)+ to NAD(P)H, followed by decarboxylation of oxalosuccinate to form α-ketoglutarate (69) (Figure 6).

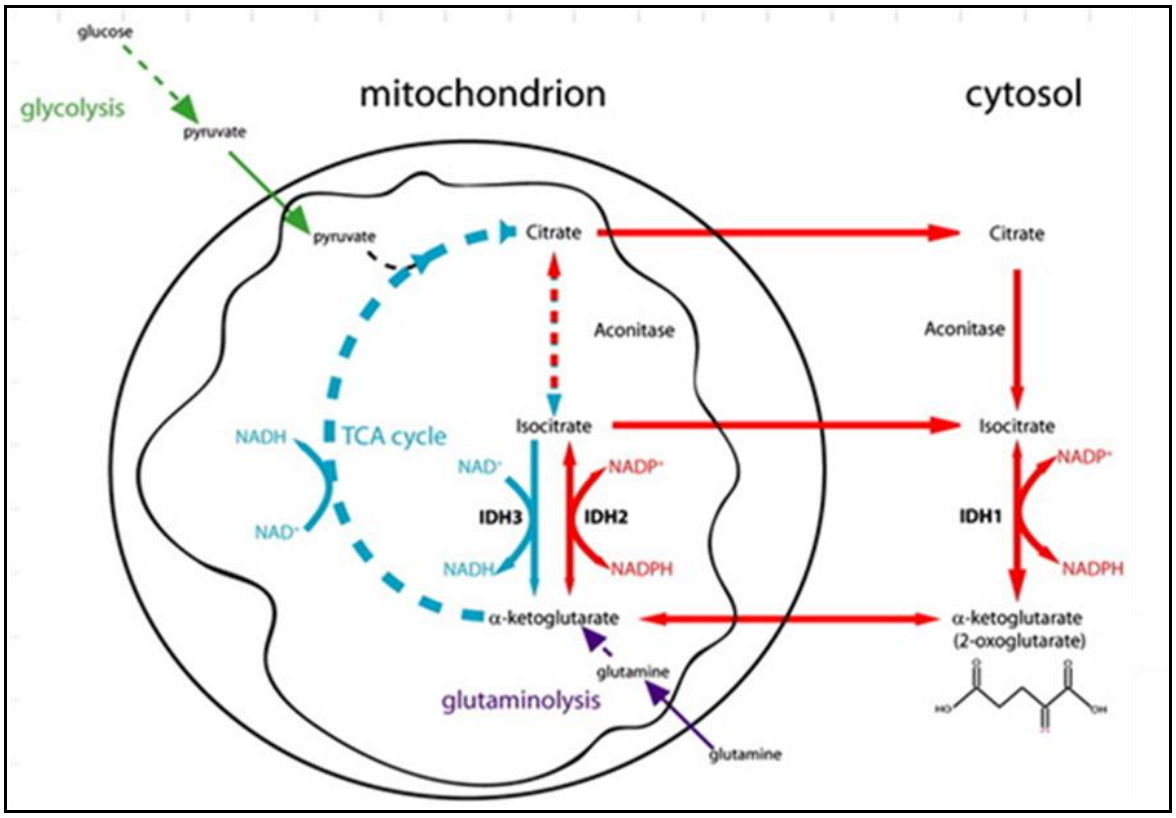

Figure 6: Metabolic reactions catalyzed by IDH family enzymes, from Dang et al. 2010 (70)

3. Fonctional roles of IDHs

IDH1 catalyzes the reaction of oxidative decarboxylation in the cytosol and peroxisome with the production of the NADPH. IDH1 is involved in lipid synthesis and cholesterol synthesis (71,72) by supplying NADPH. NADPH also can keep the reductive status of Glutathione for mediating a variety of cellular housekeeping functions against oxidative stress (73,74).

IDH2 and IDH3 catalyze a step in the tricarboxylic acid (TCA) cycle to play a central role in aerobic energy production, such as oxidative respiration, and signal transduction (68).

a. Participation in the response to anti oxidative stress

The IDHs have been reported as a major source for NADPH, which is essential for the reduction of glutathione by glutathione reductase and the thioredoxin antioxidant system, both of which protect cell against oxidative damages to lipids, proteins and DNA (75,76) (Figure 7). Enhanced productions of α-KG (a powerful antioxidant) and NADPH by IDH1/2 were found under oxidation stress condition throught decreasing the utilization of IDH3 in TCA cycle and α-KG dehydrogenase activity. This mechanism evidences the role of IDH1/2 in response to oxidative damage (77).

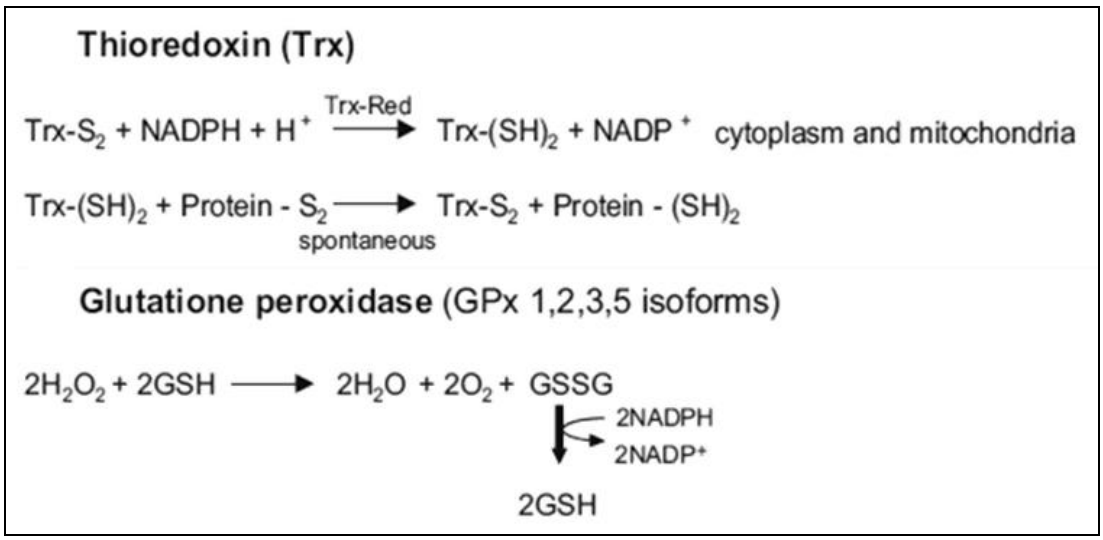

Figure 7: NADPH is a reducing equivalent essential for the reduction of glutathione and the thioredoxin antioxidant system

b. Roles of αKG

α-KG is a key metabolic product in TCA cycle and often serve as co-substrate of a number of enzymes. α-KG as the substrate of HIF prolyl hydroxylase (PHD) induces the hydroxylation and subsequent degradation of hypoxia-inducible factor 1 (HIF-1α) by von Hippel Lindau protein (vHL) (78). α-KG is a very important cosubstrate for dioxygenase enzymes, which promote the demethylation of DNA and histone proteins (60), and result in the degradation of HIF-1 under normoxic conditions

II. *IDH1* and *IDH2* mutations in gliomas

1. *IDH1/2* mutations results in the loss of normal enzymatic activity and the gain of a new enzymatic activity

Heterozygous *IDH1* mutations impair the enzyme's affinity for the Mg^{2+}-isocitrate substrate complex and enzymatic activity (47,46,79). Dang et al. (80) first showed that the mutant *IDH1* enzymes have not only (i) a loss of enzyme's function to convert isocitrate to alpha-KG, but also (ii) a gain of enzyme's function to catalyze the NADPH-dependent reduction of alpha-KG to R(-)-2 hydroxyglutarate (2HG) (Figure 8).

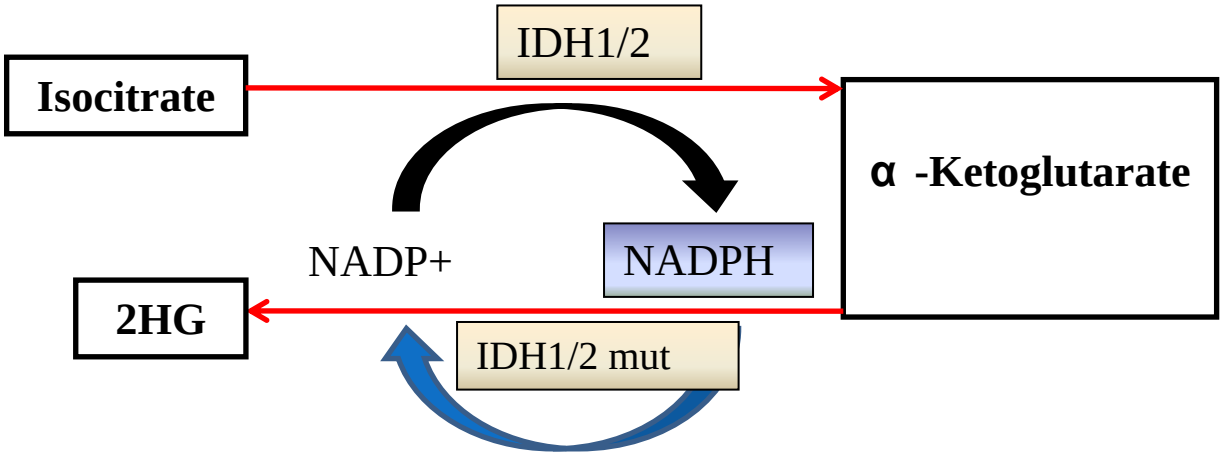

Figure 8: Abnormal metabolic mechanism catalyzed by *IDH1* and *IDH2* mutations. Novel enzymatic ability was gained in *IDH1/2* mutated tumors.

As a result, R(-)-2HG is markedly elevated in IDH-mutated cancer tissues, and this 'onco-metabolite' may contribute to the oncogenesis of gliomas (80). Several hypotheses how 2HG drives the formation and progression of gliomas have been raised:

a. Stabilization of HIF1α

The high levels of 2HG and structural similarities between 2HG and α-KG were first thought to inhibit prolyl hydroxylase (PHDs), contributing to the stabilization of HIF1α (79,81). However, Koivunen et al. (82) found recently that accumulation of R-2HG acts as an

agonist of EGLN (HIF prolyl 4-hydroxylases) activity, diminishes HIF levels and enhances the proliferation of astrocyte line. *IDH1/2* mutations-associated HIF-1α response and its consequence are still intriguing.

b. Alteration of DNA and histone methylation patterns

The balanced activities of histone lysine methyltransferases and TET family of 5-methylcytosine hydroxylases play an important role in building and keeping the methylation of histone and DNA proteins. D-2-HG is a competitive inhibitor of the α-ketoglutarate-dependent histone lysine methyltransferases and TEF 5-methylcytosine hydroxylases (81,83). Thus, because of the 2HG competitive inhibition, *IDH1/2* mutations result in an hypermethylated phenotypes, decreasing globally the gene expression, blocking the lineage-specific progenitor cell differentiation and developing glioma-CpG island methylator phenotype (G-CIMP) to the formation and development of tumors (58,84).

c. D-2HG and glutamate-structural similarities

High concentration of glutamate in neurons acts as a neurotoxin. Glutamate neurotoxicity plays an important role in the pathogenesis of many neurological diseases (85). D-2HG and glutamate have similar structure. 2HG could lead to excitotoxicity through the activation of the glutamate receptor (N-methyl-D-asparticacid-NMDA-receptor) in neurons (86).

d. Accumulation of reactive oxygen species (ROS).

Latini et al. (87) have observed that treatment with 2HG may generate the accumulation of ROS by stimulating specific NMDA (N-methyl-D-aspartate) glutamate receptors in rat brains in vivo. Large amounts of ROS accumulation can drive the pathogenesis of gliomas.

Taken together these, high levels of 2HG in tumours harbouring *IDH* mutations could play a key role in tumorigenesis. The exact effects of mutant *IDH* and 2HG will be studied further.

OBJECTIVES

Objectives

1. Analysis of *IDH1*/*IDH2* mutations in gliomas: frequency, correlation with genomic profile and impact on outcome

To better understand the impact of *IDH1/IDH2* mutations in gliomas, we have studied the frequency of these mutations in a large cohort of 1238 gliomas. Thanks to the molecular and clinical database of the neuro oncology department, we were able to study not only the association of *IDH* mutations with the genomic profile of gliomas (*EGFR* amplification *MGMT* promoter methylation, 1p19q codeletion chromosome 10q loss…), but also with survival data, demonstrating the prognostic impact of *IDH* mutations.

2. Analysis of IDH1 polymorphism (SNP rs 11554137) in gliomas: correlation with genomic profile and impact on outcome

During the analysis of *IDHs* mutation in gliomas, Wagner K et al. (88) have described a synonymous single nucleotide polymorphism [SNP rs 11554137; C (cytosine) substituted by T (thymine)] in acute myeloid leukemia patients. The SNP rs 11554137 (in codon 105) and the *IDH1* mutations (in codon 132) are located in the same exon. AML patients with SNP rs 11554137 had a poorer outcome than patients without SNP rs 11554137. However, SNP rs 11554137: C>T has not been previously investigated in gliomas. We first studied *IDH1* SNP rs 11554137: C>T in 952 gliomas samples. We analyzed the associations of *IDH1* SNP rs 11554137: C>T with sex, age, genetic alterations (*IDH1* R132 mutations, *EGFR* amplification, chromosome 10q loss, 1p19q codeletion, *MGMT* promoter methylation, and *P16* homozygous deletion), *IDH1* mRNA expression, and prognosis. In addition, we studied two independent patient series (Paris validation series: 309 glioblastomas, Bonn series: 591 WHO grade II-IV gliomas) in order to confirm a potential prognostic role for rs11554137: C>T.

3. The impact of *IDH1* R132H mutation on cell growth and response to radiotherapy.

It is believed that *IDH1* can defend cells against oxidative stress through the production of NADPH which is a reducing equivalent for the reduction of glutathione and the thioredoxin antioxidant system. Moreover, α-ketoglutarate, being the cofactor of several dioxygenases, may be involved in the regulation of multiples biological processes.

IDH1 and *IDH2* mutations lead to the reduction of the enzyme's activity and to a decrease of the production of both NADPH and α-ketoglutarate, suggesting that the cell defense mechanisms will be altered and that *IDH* mutated cells will be more vulnerable against oxidative stress. Since ionizing radiations provoke an acute increase in the concentration of reactive oxygene species (ROS), we hypothetized that tumor cell harboring *IDHs* mutations might have an increased sensitivity to radiotherapy, as compared to *IDHs* wild type cells.

RESULTS

RESULTS

Publication n°1

IDH mutations: genotype-phenotype correlation and prognostic impact

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***IDH* mutations: genotype-phenotype correlation and prognostic impact**

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Running head: *IDH1* mutation, genomic profile and prognosis of gliomas

This study has not been presented previously.

Conflict of interest statement: We declare that we have no conflict of interest.

All authors had full access to the original data, reviewed the data analyses, read and approved the final manuscript.

ABSTRACT

IDH1/2 mutation is the most frequent genomic alteration found in gliomas, affecting 40% of these tumors and is one of the earliest alterations occurring in gliomagenesis. We investigated a series of 1332 gliomas and showed that *IDH* mutation is almost constant in 1p19q codeleted tumors. We found that the distribution of $IDH1^{R132H}$, $IDH1^{nonR132H}$ and *IDH2* mutations differed between astrocytic, mixed and oligodendroglial tumors, with an over representation of *IDH2* mutations in oligodendroglial phenotype and an over representation of $IDH1^{nonR132H}$ in astrocytic tumors. We stratified grade II and grade III gliomas according to the codeletion of 1p19q and *IDH* mutation to define three distinct prognostic subgroups: 1p19q and *IDH* mutated, *IDH* mutated –which contains mostly P53 mutated tumors-, and none of these alterations. We confirmed that IDH mutation with a hazard *ratio* = 0.688 is an independent prognostic factor.

These data refine current knowledge on IDH prognostic impact and genotype-phenotype associations

Keywords: *IDH1* mutation, gliomas, *TP53* mutations, astrocytic and oligodendoglial phenotype

INTRODUCTION

The WHO Classification of Tumors of the Central Nervous System is the universal standard for classifying and grading brain neoplasms (1). According to the presumed cell of origin, gliomas have been classified into three major groups: astrocytomas, oligodendrogliomas and mixed oligoastrocytomas. Based on the presence or absence of malignant features: cell density, nuclear atypia, mitosis, microvascular proliferation and necrosis, the WHO classification distinguishes grade I, II (LGG), III (anaplastic), IV (glioblastomas, GBM) (2). However, this classification suffers from a lack of reproducibility, with a high inter-observer variability, often leading to discordant results between centers (3-5).

In these settings, there is a need for the identification of additional prognostic markers to refine the WHO classification in order to define more homogeneous subgroups. Mutations in the *IDH1* (isocitrate dehydrogenase 1) gene has been first reported in 2008 (6). Since then, the *IDH1* mutation has been recognized as the most frequent alterations in gliomas, occurring in 40% of glial tumors (7-9) and is the most powerful prognostic factor ever described in gliomas (10, 11). Less frequently the mitochondrial isoform *IDH2* is mutated.

We have investigated the mutational status of *IDH1* and *IDH2* in a cohort of 1332 glioma patients and correlated it with the genomic profile and the outcome.

PATIENTS AND METHODS

Patients and tissue samples

Patients were selected according to the following criteria: histologic diagnosis of primary brain tumor; clinical data and follow-up available in the neuro-oncology database; and written informed consent. Tumor DNA was extracted from both frozen and paraffin embedded formalin fixed tumors, when available, using the QIAmp DNA minikit, as described by the manufacturer (Qiagen). CGH-array analysis, LOH (loss of heterozygosity) analysis, *EGFR* amplification and *P16* deletion assessment were performed as previously described (12).

Determination of *IDH1* and *IDH2* mutational status

The genomic regions spanning wild-type R132 of *IDH1* and wild-type R172 of *IDH2* were analyzed by direct sequencing using the following primers: IDH1f 5-TGTGTTGAGATGGACGCCTATTTG, IDH1r 5-ACTGAACCAGCAACCACCGT, IDH2f 5-GCCCGGTCTGCCACAAAGTC and IDH2r 5-TTGGCAGACTCCAGAGCCCA, as previously described (10). For both genes, forward and reverse chains were analyzed on an ABI prism 3730 DNA analyzer (Perkin Elmer).

MGMT status and *TP53* mutations determination

DNA methylation status of the *MGMT* promoter was determined by bisulfite modification and subsequent nested MSP, a two-stage PCR approach, as previously described (13).

TP53 gene mutations were screened for exons 5–8 by using previously reported primers and methods (14).

Statistical analysis

The χ^2 test (or Fisher's exact test when one subgroup was <5) was used to compare the genotype distribution. The association with continuous variables was calculated with a Mann-Whitney test.

Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Progression-Free Survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up.

Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at last follow up were considered as a censored event in analysis. Progression free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at last follow up were considered as a censored event in analysis. To find clinical and/or genomic factors related to OS (or PFS), survival curves were calculated according to the Kaplan-Meier method and differences between curves were assessed using the log-rank test. Variables with a significant *p*-value were used to build multivariate Cox model.

RESULTS

We have screened for the presence of codon-132 mutations in the *IDH1* gene in a large cohort of 1838 brain tumors including 1546 gliomas and 292 others CNS tumors. The presence of *IDH2* mutation was investigated in a cohort of 1276 brain tumors. Taken together we found 809 *IDH1* and 37 *IDH2* mutations (global mutation rates of 44.0% and 2.9%, respectively) (**Supplementary Table I**). No tumor harbored both *IDH1* and *IDH2* mutations.

Genotype-phenotype correlations

We then focused our analysis on grade II to IV gliomas with complete clinical follow-up (1332 patients), including 442 WHO grade II, 405 WHO grade III and 485 WHO grade IV gliomas. The presence of *IDH2* mutation was investigated in a cohort of 996 gliomas (383 grade II, 296 grade III, 317 grade IV). In the whole cohort, sex ratio was 1.3 and median age at diagnosis was 48.9 years (range, 16.1 to 89.1 years). The characteristics of the population are indicated in **Table I.**

Taken together we found 619/1332 *IDH1* and 31/996 *IDH2* mutations (global mutation rates of 46.5% and 3.1%, respectively). The majority (581/619= 93.9%) of *IDH1* mutation were G395A (Arg132His), followed by 15 C394T (Arg132Cys, 2.4%), 12 C394G (Arg132Gly, 1.9%), 7 C394A (Arg132Ser, 1.1%), and 4 G395T (Arg132Leu, 0.6%) (**Supplementary Table II**). *IDH2* mutational status was determined by Sanger sequencing and by PCR HRM. The latter approach allowing only the detection of an *IDH2* mutation presence, we have only determined the *IDH2* mutation subtype in 15 tumors, including 11 G515A (Arg172Lys, 73.3%), 3 G516T (Arg172Ser, 20.0%) and 1 G515T (Arg172Met, 6.7%). Patients with mutations were younger for the whole series (median age 40.5 years for *IDH1* mutated patients *vs*. 55.4 years; $p<.0001$) and also for grade III and IV separately (median age at diagnosis 43.8 and 46.9 years for grade III and IV *IDH* mutated tumors, *vs.* 50.1 and 59.0 years for grade III and IV non mutated gliomas; $p= 0.0011$ and $p< 0.0001$, respectively).

IDH1 mutations affected 71.9% (318/442) grade II, 63.2% (256/405) grade III and 10.2% (45/440) grade IV gliomas. We looked then for association between glioma subtypes (astrocytic, mixed and oligodendroglial tumors) and $IDH1^{R132H}$, $IDH1^{non\ R132H}$ mutations, and *IDH2* mutations (**Table II**). In grade II and III gliomas, we found that $IDH1^{non\ R132H}$ mutations were more frequent in astrocytic (6/67, 8.9% *IDH* mutated tumors) and mixed tumors (15/174, 8.6%), compared to oligodendroglial tumors (16/347, 4.6%, $p= 0.025$). In contrast, the ratio of

IDH2 mutation/*IDH1* mutation was 2/65 (3.2%) in astrocytic and 6/168 (3.5%) in mixed versus 22/332 (6.6%) in oligodendroglial tumors indicating an over representation of *IDH2* mutation in oligodendrogliomas, compared to astrocytomas and mixed gliomas (p= 0.045).

IDH mutations are associated with tumor genomic profile

MGMT promoter methylation status was available in 590 tumors. *IDH1* mutation was significantly associated with the methylation of *MGMT* promoter: 196/257 (76.3%) of *IDH* mutated tumors were *MGMT* promoter methylated, whereas 52.0% (173/333) of non mutated gliomas harbored an *MGMT* promoter methylation (p< 0.0001) (**Suppl Table III**). Genomic profiling data were available for 774 gliomas. In our series, only 9 out of 621 (1.4%) *IDH* mutated tumors presented an *EGFR* amplification, whereas we found 197 (30.1%) *EGFR* amplified gliomas among 655 non mutated tumors (p< 0.0001). Moreover, there was a strong association between the absence of *IDH* mutation and complete loss of chromosome 10q. Indeed, only 59 (20.9%) tumors out of 582 *IDH* mutated tumors harbored a complete loss of chromosomes 10q, *vs*. 61.0% (362/590) for the non mutated gliomas (p< 0.0001). A similar association was evidenced with *P16* deletion present in 10.4% (63/606) of *IDH* mutated tumors, whereas 205/653 (31.4%) of *IDH* wild type gliomas were *P16* deleted.

Complete 1p19q codeletion was found in 150 gliomas: the *IDH1* gene was mutated in 135 cases (90.0%) and the *IDH2* gene was mutated in 11 of the 15 remaining tumors. Taken together, the *IDH* genes were altered in 97.3% (146/150) of the 1p19q codeleted tumors. The repartition of *IDH* mutations also did not differ between 1p19q codeleted and non codeleted tumors: the *IDH2* /*IDH1* mutation ratio was 11/135 (7.5%) for 1p19q codeleted tumors, *vs*. 7/186 (3.6%) in non codeleted tumors (p=0.09). $IDH1^{nonR132H}$ mutations represented 8 out of 146 *IDH* mutations (5.5%) in 1p19q codeleted *vs*. 23/193 (11.9 %) in non codeleted tumors (p= 0.03). We also compared the *ratio* of the minor *IDH* alterations, i.e. *IDH2 vs*. $IDH1^{nonR132H}$, in the codeleted and non codeleted IDH mutated tumors. The ratio was 8 $IDH1^{nonR132H}$ /11 *IDH2* for the codeleted *vs*. 22 $IDH1^{nonR132H}$ /7 *IDH2* for the non codeleted (p= 0.015)

P53 mutation was analyzed by Sanger sequencing in 339 tumors: 64/148 (43.2%) *IDH* mutated tumors were also mutated on *P53*, *vs.* 84/191 (43.9%) of the non mutated tumors. *P53* mutation was correlated with astrocytic histology: 122 tumors out of 135 (90.4%) astrocytic and mixed gliomas were *P53* mutated, whereas only 27.7 (26/94) of

oligodendrogliomas were mutated on *P53* ($p< 0.0001$). *P53* mutation was also mutually exclusive with 1p19q codeletion: 1p19q codeleted gliomas were less frequently *P53* mutated (4/54, 7.4%), as compared to the non codeleted tumors (126/249, 50.6%; $p< 0.0001$). When excluding 1p19q codeleted tumors (considered as the hallmark of oligodendrogliomas), *P53* mutation was correlated with *IDH* mutation: 50/76 (65.8%) of *IDH* mutated tumors was also mutated on *P53*, *vs.* 76/173 (43.9%, $p= 0.002$) in non mutated gliomas.

IDH1 mutation is an independent prognostic factor of good outcome

We investigated the prognostic impact of *IDH* status in grade II, grade III and grade IV gliomas. For each grade, *IDH* mutated patients have significantly longer overall survival and progression free survival than *IDH* normal patients (**Figure 1 and Supplementary Table 4**).

We then entered the following factors as candidate variables in the multivariate Cox proportional hazards regression model analysis: WHO grade, *IDH* mutation, *P16* deletion, 1p19q codeletion, and age at diagnosis (**Table III**). *IDH* mutation was a strong and independent predictor of a better outcome (hazard *ratio* = 0.688; 95% CI, 0.521 to 0.907; $p =0.008$).

Moreover, as previously described (15), we stratified the grade II and grade III tumors according to 1p19q codeletion and *IDH* status, thus defining three prognostic groups: 1p19q codeleted (and *IDH* mutated), *IDH* mutated and others (**Figure 2 and Supplementary Table V**). Whatever the grade, patients harboring the 1p19q codeletion have a significantly longer survival (median OS: 150.9 months) than patients only harboring *IDH* mutation (121.9 months) or none of these alterations (30.6 months). We looked then at *P53* mutation in these three prognostic groups and found *P53* mutation strongly associated with group 2 in both grade II and III (**Table IV**). For example in grade II gliomas, *P53* was mutated in 47.9% in group 2, *vs*. 8.0% and 17.4% in group 1 and 3, respectively ($p= 0.002$ and $p= 0.035$, respectively).

DISCUSSION

In this large series, we investigated the place of *IDH1/IDH2* mutation in gliomas, in particular in different genotypes and phenotypes. As a first result, we confirmed the strong association of *IDH* mutations with the tumor genomic profile (10): virtually all 1p19q codeleted tumors are *IDH* mutated (16, 17) whereas *IDH* mutation is extremely rare in gliomas with *EGFR* amplification. Secondly, we showed that the type of mutation is related to the molecular profile. The $IDH1^{R132H}$ mutation represents 90% of all *IDH* mutations. However, we found here that $IDH1^{nonR132H}$ mutations are associated with astrocytic tumors (18), whereas *IDH2* mutations are associated with oligodendrogliomas. The 1p19q codeletion is a hallmark of oligodendroglial phenotype and we found similar results when tumors are stratified according to histological subtype. In contrast, the rate of $IDH1^{nonR132H}$ mutation is over represented in astrocytic and mixed tumors (18).

The association of *IDH* mutation with *P53* mutation has been widely studied in literature and has led to contradictory results. *IDH* mutation was found associated with *P53* mutation in several studies (11, 17, 19-23) but other authors did not found such an association (10, 24). We found no association between *IDH* and *P53* mutations, but we found *P53* mutation correlated with astrocytic phenotype, in contrast with *IDH* mutation more associated with the oligodendroglial phenotype. Therefore, when excluding 1p19q codeleted tumors, mostly oligodendroglial, and not *P53* mutated, we found a positive association between *IDH* and *P53* mutations. This result is concordant with the data of Gravendeel at al. who found a correlation between *P53* mutation and $IDH1^{nonR132H}$ mutation (25).

Confirming previous data obtained on smaller cohorts (10, 15), our findings showed that gliomas patients harboring an *IDH1* mutated tumor present an improved outcome, compared to patients with an *IDH1* normal tumor. The multivariate analysis shows that *IDH* status is an independent prognostic factor in a 1332 glioma patients cohort. To further explore the prognostic impact of *IDH1* mutation, we subdivided both grade II and III gliomas patients in three prognostic subgroups, based on the 1p19q codeletion and *IDH1* mutation status (i. *IDH*mut/1p19qdel, ii. *IDH*mut/1p19qnon del, iii. *IDH*non mut/1p19qnon del.). In line with a recent study (21), we found that *P53* mutation characterizes the group 2 (*IDH* mut non 1p19q codeleted). The third group with the worst prognosis contains mainly triple negative gliomas (non 1p19q codeleted, non *IDH* mutated, non *P53* mutated) (21).

Taken together, our results show that *IDH* mutation, combined with other genomic marker can be used to refine the prognostic classification of gliomas, independently of tumor grade.

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TABLES

Table I. Patients demographics and clinical characteristics

	Glioma by grade		
Characteristics	II	III	IV
	(n=442)	(n=405)	(n=485)
Age, years			
Median	38.1	47.4	58.4
Range	16.1-77.0	19.1-89.1	18.2-89.1
KPS			
Median	90	90	80
Range	50-100	60-100	40-100
Biopsy (%)	26.5	29.2	26.6
Tumor removal (%)	73.5	70.8	73.4
Median survival, months	121.0	42.1	14.5
Median PFS, months	35.9	22.9	9.8

KPS: Karnofsky performance score; PFS: progression-free survival, not determined.

Table II: Distribution of *IDH1*^{R132H} mutations, *IDH1*$^{non\ R132H}$ mutations and *IDH2* mutations among the different histologic subtypes of gliomas

		***IDH1* R132H mutations**	**Non *IDH1* R132H mutations**	***IDH2* mutations**
Astrocytic tumors		**85**	**7**	**3**
	AII	41	2	1
	AIII	18	4	1
	GBM	26	1	1
Oligodendroglial tumors		**331**	**16**	**22**
	OII	172	10	15
	OIII	145	5	7
	GBMO	14	1	0
Mixed tumors		**153**	**15**	**6**
	OAII	78	6	5
	OAIII	75	9	1

Table III: Multivariate Cox proportional hazards regression model analysis of survival the 1332 glioma patients cohort

Parameter	Value	Multivariate					
		Est.	SE	HR	95% CI for HR		p
Grade	2	0.00		1.000			-
	3	0.78	0.17	2.175	1.568	3.015	<.0001
	4	1.67	0.18	5.318	3.734	7.574	<.0001
IDH mutation		-0.37	0.14	0.688	0.521	0.907	0.0081
P16 deletion		0.27	0.10	1.309	1.071	1.600	0.0086
1p19q co deletion		-1.02	0.31	0.361	0.197	0.662	0.0010
Age at diagnosis	< 40	0.00		1.000			-
	40-49	0.32	0.16	1.380	1.017	1.873	0.0385
	50-59	0.59	0.16	1.801	1.318	2.461	0.0002
	≥ 60	0.94	0.16	2.552	1.877	3.471	<.0001
Surgery	Biopsy *vs.* surg	0.65	0.11	1.920	1.558	2.366	<.0001

Table IV: Association of *P53* mutation with 1p19q codeleted tumors and *IDH* mutated tumors

		P53			
		Mutated	Normal	Percentage	Difference to IDH group (*p*)
Grade II	1p19q	2	23	8.0%	*0.0019*
	IDH	34	37	47.9%	-
	others	4	19	17.4%	*0.0349*
Grade III	1p19q	2	11	15.4%	*0.0085*
	IDH	20	10	66.7%	-
	others	21	17	55.3%	*NS*

SUPPLEMENTARY TABLES

Supplementary Table I. *IDH1* and *IDH2* mutations according to histological subtypes

	IDH1			*IDH2*		
	N mut	N total	Mutation rate (%)	N mut	N total	Mutation rate (%)
Astrocytoma I	4	27	14.8	0	20	0.0
Astrocytoma II	55	73	75.3	1	68	1.5
Gemistocytic astrocytoma	8	12	66.7	1	12	8.3
AIII	27	45	60.0	0	36	0.0
DNET	2	4	50.0	0	4	0.0
Ependymoma	1	74	1.4	0	11	0.0
Ganglioglioma	13	31	41.9	0	28	0.0
Glioblastoma	38	387	9.8	1	249	0.4
Secondary glioblastoma	12	32	37.5	0	32	0.0
GBMO	22	138	15.9	0	95	0.0
OAII	99	133	74.4	5	113	4.4
OAIII	113	181	62.4	1	137	0.7
OII	204	269	75.8	16	234	6.8
OIII	205	291	70.4	12	218	5.5
Medulloblastoma	1	6	16.7	0	5	0.0
PNET	2	5	40.0	0	5	0.0
Meningioma	0	69	0.0	0	3	0.0
CNS lymphoma	0	15	0.0	*nd*		*Nd*
Non classified glial tumors	3	20	15.0	0	6	0.0
Others CNS tumors*	0	26	0.0	*nd*		*nd*

** including 2 hemangioblastomas, 6 hemangiopericytomas, 9 metastasis, 6 neurocytomas, and 3 germinal tumors. nd: not determined*

Supplementary Table II: Frequency of *IDH1* and *IDH2* mutations in the 1332 glioma patients cohort

	Nucleotide change	Amino acid change	N (%)
IDH1			**619/1332 (46.5%)**
	G395A (CAT)	Arg132His	58/619 (93.9%)
	C394T (TGT)	Arg132Cys	15/619 (2.4%)
	C394G (GGT)	Arg132Gly	12/619 (1.9%)
	C394A (AGT)	Arg132Ser	7/619 (1.1%)
	G395T (CTT)	Arg132Leu	4/619 (0.6%)
IDH2			**31/996 (3.1%)**
	G515A (AAG)	Arg172Lys	
	G516T (AGT)	Arg172Ser	
	G515T (ATG)	Arg172Met	
	A514T (TGG)	Arg172Try	

Supplementary Table III: Association of *IDH* mutations with common alterations found in gliomas

	n	***IDH* mutated tumors (%)**	***IDH* non mutated tumors**	***p***
MGMT promoter methylation	590	196/257 (76.3%)	173/333 (52.0%)	*< 0.0001*
EGFR amplification	1276	9/621 (1.4%)	197/655 (30.1%)	*< 0.0001*
Complete 10q loss	1172	59/582 (20.9%)	362/590 (61.0%)	*< 0.0001*
P16 deletion	1259	63/606 (10.4%)	205/653 (31.4%)	*< 0.0001*
P53 mutation	339	64/148 (43.2%)	84/191 (43.9%)	*0.9124*

Supplementary Table IV: Prognostic impact of *IDH* status on overall survival and progression free survival in grade II to IV gliomas

	Median overall survival (months)			Median progression free survival (months)		
	IDH mutated	*IDH* normal	*p*	*IDH* mutated	*IDH* normal	*p*
Grade II	136.5	67.0	<0.0001	41.3	28.5	0.0199
Grade III	136.9	20.1	<0.0001	34.6	10.4	<0.0001
Grade IV	27.4	14.3	0.0002	10.6	8.1	0.0001

Supplementary Table V: Prognostic impact of 1p19q codeletion and *IDH* mutation on overall survival (OS) and progression free survival (PFS) in grade II and III gliomas patients

		1p19q co deleted	***IDH* mutated**	**Others**	***p***
Grade II + Grade III	OS (months)	150.9	121.9	30.6	*p< 0.0001*
	PFS (months)	51.1	36.1	13.9	*p<0.0001*

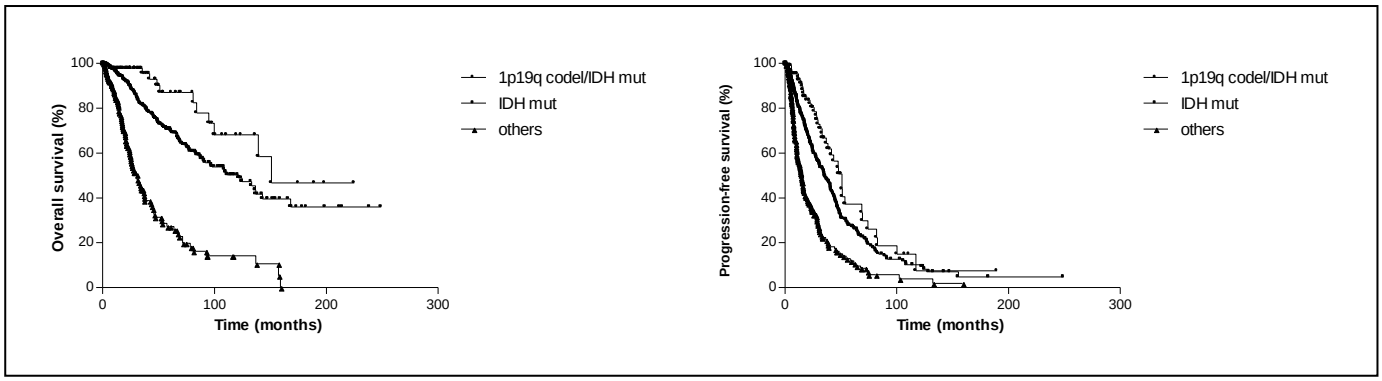
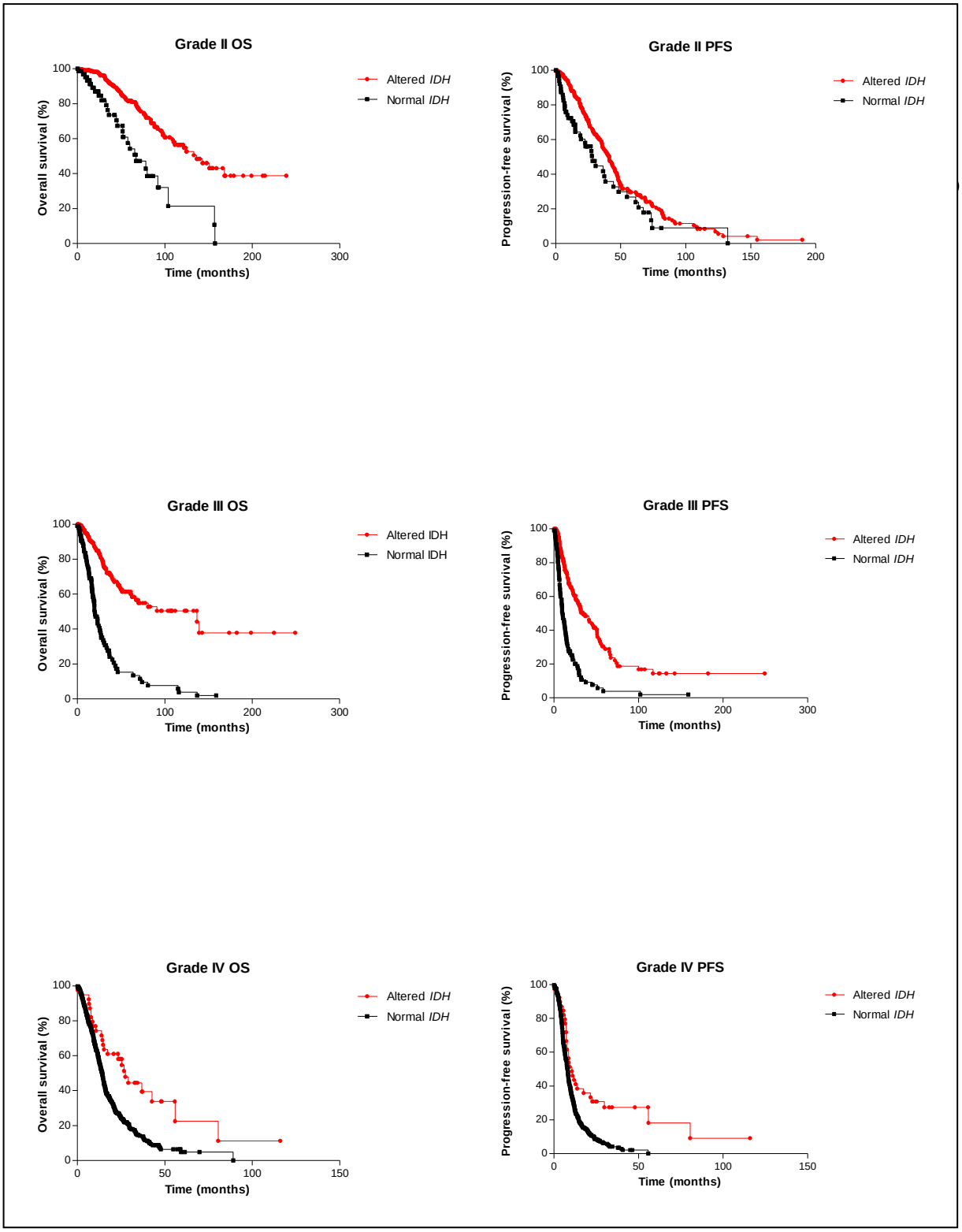


Figure 2: Overall survival (OS, left panel) and progression free survival (PFS, right panel) for grade II and III gliomas patients stratified according to 1p19q codeletion and presence of *IDH* mutations

Figure 1: Prognostic impact of *IDH* status on overall survival and progression free survival in grade II to IV gliomas



RESULTS

Publication n°2

"Prognostic impact of *IDH1* SNP rs11554137 in malignant gliomas"

XiaoWei Wang, Blandine Boisselier, Marta Rossetto, Yannick Marie, Ahmed Idbaih, Karima Mokhtari, Konstantinos Gousias, Khê Hoang-Xuan, Jean-Yves Delattre, Matthias Simon, Marianne Labussière, Marc Sanson

Prognostic impact of *IDH1* SNP rs11554137 in malignant gliomas

Runing Title: *IDH1*^{105} SNP and prognosis of gliomas

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ABSTRACT

Background. The *IDH1* gene encoding the isocitrate dehydrogenase 1 is frequently mutated in gliomas and acute myeloid leukemia. The SNP rs11554137:C>T polymorphism, located on *IDH1* codon 105, has been associated with poor outcome in acute myeloid leukemia, but has not been investigated in gliomas. **Methods**. The *IDH1* codon 105 was genotyped first in a series of 952 patients with grade II to IV gliomas and correlated with outcome and tumor genomic profile, and then in two validations sets of 306 glioblastoma (GBM) patients, and 591 glioma patients. **Results**. The minor allele $IDH1^{105GGT}$ was found in 98/952 patients (10.3%) and was not associated with $IDH1^{132}$ mutation. GBM patients with the $IDH1^{105GGT}$ variant had a poorer outcome than patients without the variant (median OS 10.7 *vs.* 15.5 months $p = .001$; median PFS 6.4 *vs.* 8.5 months $p = .003$), and expressed higher levels of *IDH1* mRNA. The prognostic impact was confirmed in an independent validation set of 306 GBM from the same center (med. PFS: 6.8 *vs*. 9.7 months, $p = .006$, and med. OS: 13.9 *vs*. 18.8 months, $p = .0187$). The second validation cohort (591 grade II to IV gliomas) found a significant association between $IDH1^{105GGT}$ and an adverse prognosis in the overall series, and for WHO grade III gliomas, but the difference did not reach significance in GBM. **Conclusion.** Taken together, this data strongly suggest an association between SNP rs11554137:C>T polymorphism and adverse outcome of malignant gliomas.

Condensed abstract. We analyzed the SNP rs11554137 located on the codon 105 of the IDH1 gene in three independent series of patients with gliomas. The SNP rs11554137 is independent of the occurrence of somatic mutation on IDH1 codon 132, but has *per se* a prognostic impact in malignant gliomas.

Keywords: *IDH1*, glioma, glioblastoma, prognostic markers, SNP

INTRODUCTION

Mutations in the isocitrate dehydrogenase 1 (*IDH1*, MIM #147700) gene affect nearly 40% of gliomas and are associated with a better outcome [1-3]. *IDH1* encodes isocitrate dehydrogenase 1, which catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate. Mutations affecting codon 132 lead not only to a dramatic decrease of IDH1 activity, but also to a gain of enzyme function for the NADPH dependent reduction of alpha-ketoglutarate to 2-hydroxyglutarate, which accumulates in the *IDH1* mutated cells[4]. *IDH1* mutations are inversely associated with grade, affecting nearly 75% of WHO grade II, half of grade III and only 5% of primary grade IV gliomas [2], whereas 80% of secondary glioblastomas are *IDH1* mutated [1, 5].

Beside gliomas, *IDH1* mutations affect also 8%-15% of acute myeloid leukemias (AML), mostly with normal karyotype [6, 7], cartilaginous neoplasms (chondromas and chondrosarcomas)[8], and cholangiocarcinomas[9]. In contrast to glioma patients, *IDH1* mutation in AML patients with normal karyotype is associated with a poor prognosis [7]. Recently, a synonymous single nucleotide polymorphism, SNP rs11554137: C>T, located in codon 105 in the same exon than the *IDH1*^{R132} mutation, has been described in AML patients and has been demonstrated to be an adverse prognostic factor[10]. In the present study, we screened 952 gliomas samples for the presence of the *IDH1* SNP rs11554137:C>T, we correlated the findings with *IDH1* mutation status, *IDH1* mRNA expression, genomic profile (*P16* deletion, *EGFR* amplification, chromosome arm 10q loss, 1p/19q codeletion), *MGMT* promoter methylation status, and outcome. In addition, we investigated the prognostic role of the rs11554137:C>T polymorphism in two independent patient cohorts (Paris validation series: 309 glioblastomas, Bonn series: 591 WHO grade II-IV gliomas).

PATIENTS AND METHODS

Patients

Two series of glioma patients were selected from the neuro-oncology database at the Pitié Salpêtrière Hospital Paris, France: the first one included 952 patients with a histologic diagnosis of glioma grade II to IV, and the second was an additional series of 309 GBM patients. Secondly, we analyzed an independent data set of 591 grade II to IV gliomas collected in the Department of Neurosurgery at the University of Bonn (Germany) from 1996-2007. Collection of blood samples and clinico-pathological information was undertaken with informed consent and relevant ethical board approval in accordance with the tenets of the Declaration of Helsinki.

Tumor and blood DNA analysis

Tumor and blood DNA were extracted from frozen tumors using the QIAmp DNA minikit, as described by the manufacturer (Qiagen).

IDH1 SNP rs11554137:C>T was characterized in blood DNA using a Taqman SNP Genotyping Assay (assay ID: C_42648573_10, Applied Biosystems).

The genomic region of the *IDH1* gene (exon 4) containing the mutation hotspot codon R132 and the SNP rs11554137:C>T was amplified from tumor DNA using PCR with the following primers: IDH1f 5- TGTGTTGAGATGGACGCCTATTTG and IDH1r 5-ACTGAACCAGCAACCACCGT, as previously described [2]. Purified PCR fragments were directly sequenced using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems) and both forward and reverse chains were analyzed on an ABI prism 3730 DNA analyzer (Perkin Elmer).

CGH-array was performed as previously described [11], or -when CGH-array was not available- LOH analysis (for 10q loss assessment) and quantitative PCR (for the determination of *P16* and *EGFR* status) [12]. *MGMT* promoter methylation determination was performed as previously described [13].

Expression of *IDH1* mRNA

Total RNA extraction was performed using RNeasy Lipid Tissue Mini kit (Qiagen). Random hexamers primers and MMLV reverse transcriptase (SuperScript™ III, Invitrogen) were used to generate cDNA. Gene expression was quantified by quantitative PCR using 5x HOT Pol EvaGreen® qPCR Mix (Euromedex). Real time qPCR cycling conditions consisted of an initial denaturation step at 95 ℃ for 15 min; 40 cycles of 95 ℃ for 30s and 60 ℃ for 1 min; and one cycle of 95 ℃ for 1 min; 55 ℃ for 30s and 95 ℃ for 30s. The reactions were carried out using a Mx3000P™ apparatus (Stratagene). *ALAS* (human erythroid 5-aminolevulinate synthase) was quantified as a control gene. We used Quantitect primers (*IDH1* QT00003983; *ALAS* QT00073122) for both genes. The $2^{-\Delta\Delta CT}$ method was used to determine the relative expression of *IDH1* mRNA in tumor samples as compared with non tumor tissues.

Independent data sets

After the analysis of these 952 cases, we studied two independent patient series in order to confirm a potential prognostic role for rs11554137: C>T. In both series, *IDH1* SNP rs11554137:C>T was characterized using a Taqman SNP Genotyping Assay (assay ID: C_42648573_10, Applied Biosystems).

Statistical analysis

The independence of alleles (Hardy-Weinberg equilibrium) was confirmed using the χ^2 test at one degree of freedom for each polymorphism. The χ^2 test (or Fisher's exact test when one subgroup was <5) was used to compare the genotype distribution. Comparison of *IDH1* expression was performed using unpaired t-test.

Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at last follow up were considered as a censored event in analysis. Progression free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at last follow up were considered as a censored event in analysis. To find clinical and/or genomic factors related to OS (or PFS), survival curves were calculated according to the Kaplan-Meier method and differences between curves were assessed using the log-rank test. Variables with a significant *p*-value (<0.05) were used to build multivariate Cox model.

RESULTS

Mutational analysis of *IDH1*

Tumor DNA samples were analyzed from 952 adult glioma patients (239 WHO grade II, 264 grade III and 449 grade IV) for the presence of *IDH1* rs11554137: C>T. Sex ratio was 1.3 (533 men and 419 women), and median age was 50.5 years (range: 16.1 to 86.5 years) **(Table I).**

101 patients were SNP positive including 98 heterozygous (10.3%) and three (0.3%) homozygous for the minor allele $IDH1^{105GGT}$ **(Table II)**. The distribution of allele frequencies in our population met Hardy-Weinberg equilibrium (p= .92). Allele frequencies did not differ between the three grades: heterozygous *IDH1* SNP rs11554137:C>T was identified in 25 (10.4%) WHO grade II, 27 (10.2%) grade III and 46 (10.2%) grade IV gliomas. The variant allele was more frequent in female patients (41 men and 57 women, sex ratio = 0.7 *vs.* 1.4; p= .004).

330 tumors out of 946 (34.9%) were mutated on codon 132, including 157 (65.7%). WHO grade II, 149 (56.4%), grade III and 24 (5.3%) and grade IV gliomas. The Arg132His mutation (CGT→CAT) was found in 300 cases (91.0%) and other mutations in 30 cases (9.0%). We found no association between SNP rs11554137:C>T polymorphism and the global incidence of codon 132 mutation: 33 of the 327 mutated tumors were heterozygous for the rs11554137:C>T minor allele (10.0%) *vs.* 65/619 (10.5%) of the non mutated tumors. We then analyzed the relation between SNP rs11554137:C>T and each type of *IDH1* mutation: 30 out of the 300 Arg132His (CAT) cases had the variant SNP rs11554137:C>T *vs.* six out of the 30 cases with other $IDH1^{R132}$ mutations (10.0% *vs.* 20.0%; p= .12), including 1/5 CGT→AGT (Arg132Ser), 1/3 CGT→CTT (Arg132Leu), 2/12 CGT→GGT (Arg132Gly), 2/10 CGT→TGT (Arg132Cys). Out of the 26 *IDH2* mutations found in the whole series, only one had the variant SNP rs11554137: C>T.

Genomic profiling

Genomic analysis was performed on the 896 samples for which all data were available, including 643 cases analyzed by CGH array. *MGMT* promoter methylation was determined in 499 cases (**table II**). There was an inverse correlation between SNP rs11554137:C>T polymorphism and *EGFR* amplification (11/94 *vs*. 174/802, p= .02) but no correlation with *P16* deletion, chromosome 10q loss, 1p19q codeletion and *MGMT* methylation.

Prognostic impact of *IDH1* SNP rs11554137

We investigated the prognostic impact of *IDH1* SNP rs11554137:C>T in grade II, grade III and grade IV gliomas **(Table II; Figure 1)**. In grade IV gliomas, both progression-free survival (PFS) and overall survival (OS) were shorter for SNP positive patients compared to SNP negative patients (median OS 10.7 *vs.* 15.5 months *p*= .001; median PFS 6.4 *vs.* 8.5 months *p*= .003). We observed a similar trend in grade III (median OS 31.6 *vs.* 36.5 months and median PFS 14.5 *vs.* 16.5 months, p=NS) but not in grade II gliomas.

The following parameters were entered as candidate variables in a multivariate Cox proportional hazards regression model analysis of overall survival for the glioblastoma patients: age, *EGFR* amplification, $IDH1^{132}$ mutation and *IDH1* SNP rs11554137:C>T **(Table III)**. For both PFS and OS, *IDH1* SNP rs11554137:C>T was a strong and independent predictor of outcome (hazard *ratio* = 1.64; 95% CI, 1.1873 to 2.2651; *p* =.003 and hazard *ratio*= 1.65; 95% CI, 1.1725 to 2.3342; *p*= .004, respectively).

Expression of *IDH1* mRNA

We then evaluated the relation between *IDH1* SNP rs11554137:C>T and *IDH1* mRNA level by quantitative RT-PCR in grade IV gliomas. cDNA was available for 16 SNP positive patients and 110 SNP negative patients. SNP positive patients had a higher *IDH1* expression compared to the SNP negative patients (mean ± SD of *IDH1* mRNA level: 7.7 ± 1.3 *vs*. 4.5 ± 0.3, respectively; *p*= .03) **(Figure 2A)**. *IDH1* expression increased from grade II to IV **(Figure 2B)**, tended to be higher in *IDH1* wild type tumors (*p*= 0.18), in gliomas with *EGFR* amplification and lower in gliomas with 1p19q codeletion **(Figure 2C)**, but was not correlated with survival in glioblastomas: median OS was 14.4 months for the low IDH1 expression group (<median RT-PCR value) *vs.* 15.8 months for the high IDH1 expression group (>median RT-PCR value); *p*= .3. Therefore, differences of the *IDH1* mRNA levels do not explain *per se* the difference of survival.

Impact of *IDH1* SNP rs11554137:C>T on prognosis in an independent data set of 306 glioblastomas.

In order to confirm the apparent major prognostic impact of *IDH1* SNP rs11554137:C>T in glioblastomas, we analyzed this polymorphism in an independent series of 306 glioblastomas patients treated at the Pitié-Salpêtrière Hospital **(Table IV)**.

In this validation series, the presence of *IDH1* SNP rs11554137:C>T was identified in 35 patients (11.4%) including 33 heterozygotes and 2 homozygotes, considered as a single group for subsequent survival analysis. Both PFS (6.8 *vs*. 9.7 months, *p*= .006) and OS (13.9 *vs.* 18.8 months *p*= .02) were significantly reduced for SNP positive patients **(Figure 3)**. In 125 of these cases with available tumor DNA, the inverse association between *IDH1* SNP rs11554137:C>T variant and tumor EGFR amplification was not confirmed (4/15 *vs.* 32/110; p=NS).

Impact of *IDH1* SNP rs11554137:C>T on prognosis in an independent data set of 591 grade II to IV gliomas.

IDH1 SNP rs11554137 was analyzed in a cohort of 591 gliomas patients (125 grade II, 129 grade III and 337 grade IV) treated at Bonn University Hospital for which survival data were available **(Table V)**. The *IDH1* SNP rs11554137:C>T was present in 47 patients (8.0%, all heterozygous): 8/125 grade II (6.4%), 11/130 grade III (8.5%) and 28/361 (8.3%) grade IV. The C>T variant was associated with poorer outcome in grade III gliomas, but the trend was not significant in WHO grade II and GBM patients **(Tables VI and VII).**

Impact of *IDH1* SNP rs11554137:C>T on the response to radiotherapy and radio-chemotherapy

In order to determine whether the impact of IDH1 SNP rs11554137:C>T had any correlation with treatment we focused on homogeneously treated patients in the French series. Again we found a difference in PFS for patients treated with radiotherapy (RT) (n= 475 patients; 8.2 months for SNP positive patients vs. 10.2 for SNP negative patients, p= .0001; **Figure 4A**) and for patients treated with RT combined with temozolomide (n= 225 patients; 8.2 months for SNP positive patients vs. 11.5 for SNP negative patients, respectively, p= .0003; **Figure 4B**).

DISCUSSION

The synonymous SNP rs11554137:C>T (*IDH1*^{105GGT}) is located in the 5' region of exon 4 and thus very close to the *IDH1* codon 132. It has been identified as an adverse prognostic factor in acute myeloid leukemia [14]. In line with this result, we show in a cohort of 952 glioma patients that SNP rs11554137:C>T also has a negative impact in patients with glioblastoma on both the PFS and overall survival. We confirmed these findings in an independent series of 306 GBM patients from the same center with similar results. Our data also suggest that SNP rs11554137:C>T is associated with a poor response to either RT alone or RT-temozolomide combined therapy[15].

In the second independent cohort from Germany we also found a negative impact of rs11554137:C>T on OS. However this trend was significant when considering the whole series and the WHO grade III gliomas subset, but not in glioblastoma and WHO grade II patients. Taken together these findings generally support the negative prognostic impact of rs11554137:C>T. Possible explanation for the discrepancy between the two French series and the German series include the lack of a central neuropathological review, particularly critical for grade III gliomas, and for glioblastomas, the fact that the German series is a surgical series that includes a substantial rate of early death (mostly patients in poor condition that were not treated after surgery).

The biological consequences of this synonymous polymorphism remain speculative. One possibility is that SNP rs11554137:C>T may impact mRNA stability. Consistently with this hypothesis we found higher *IDH1* mRNA by RT-PCR in the *IDH1*^{105GGT} variant samples. A similar result was obtained in adult, but not pediatric AML [14, 16]. *IDH1* mRNA is higher in glioma compared to normal tissue, and increases with tumor grade, but was not related to outcome in glioblastoma and is therefore unlikely to explain the prognostic impact of SNP rs11554137:C>T. However, it is possible that a silent SNP may slow down the rate of protein translation, resulting in altered protein folding and, ultimately, decreased protein function [17]

Despite SNP rs11554137:C>T is very close to codon 132, we found no correlation between SNP rs11554137:C>T and the overall occurrence of *IDH1* codon 132 mutations. Similarly we found no association between the SNP rs11554137:C>T and the main other genetic alterations found in gliomas (the inverse relation with *EGFR* amplification was not confirmed by the independent series).

In conclusion, our data suggest that the *IDH1*^{105GGT} variant is associated with a negative prognostic impact in high grade gliomas -particularly glioblastomas- and a higher expression of *IDH1* mRNA. Further independent studies are warranted to define this potential

prognostic role more clearly. *In vitro* analysis is required to better understand the functional impact of the *IDH1*^{105GGT} variant on IDH1 expression, not only at the RNA, but also at the protein level.

Tables

Table I. Tumor population characteristics

	Glioma by grade		
	II	III	IV
n	239	264	449
Sex			
Male-to-female	1.2	1.3	1.3
Male	132	149	252
Female	107	115	197
Age at diagnosis, years			
Median	38.1	47.0	58.5
Range	16.1-76.7	19.1-84.1	18.2-86.5
Median OS, months	110.0	36.3	15.0
Median PFS, months	37.9	19.8	9.8

OS: overall survival, PFS: progression-free survival, n: number of patients

Table III. Multivariate Cox proportional hazards regression model analysis of survival in glioblastoma patients

	Variable	**Hazard ratio**	**95% CI**	***p***
Progression free survival (PFS)	Age	1.41	1.15-1.7	*0.0008*
	IDH1 SNP rs11554137	1.64	1.19-2.27	*0.003*
	IDH1 mutation	0.61	0.39-0.97	*0.04*
Overall survival (OS)	Age	1.60	1.28-2.00	*0.0000*
	IDH1 SNP rs11554137	1.65	1.17-2.33	*0.004*
	IDH1 mutation	0.71	0.43-1.18	*0.19*

PFS: progression free survival, OS: overall survival, EGFR: epidermal growth factor receptor, *IDH1*: isocitrate dehydrogenase 1.

Table II. Characteristics of glioma patients with or without *IDH1* SNP rs11554137:C>T according to grade.

	Grade II		Grade III		Grade IV	
***IDH1* rs11554137 status**	**GGC/GGC**	**GGC/GGT and GGT/GGT**	**GGC/GGC**	**GGC/GGT and GGT/GGT**	**GGC/GGC**	**GGC/GGT and GGT/GGT**
n	213	26	235	29	403	46
Sex						
Male-to-female	1.4	0.5	1.4	0.8	1.4	0.8
Male	123	9	136	13	232	20
Female	90	17	99	16	171	26
Age at diagnosis, years						
Median	38.2	36.8	47.0	52.2	58.5	59.8
Range	16.1-77.0	17.3-64.8	19.1-84.1	23.7-73.7	18.2-86.5	23.4-79.7
Prognosis						
Median OS, months	110.0	NR	36.5	31.6	15.5	10.7
Median PFS, months	33.3	35.9	16.5	14.5	8.5	6.4
Genetic alterations (%)						
IDH1 R132 mutation	65.1 (138/212)	72.0 (18/25)	56.4 (132/234)	55.6 (15/27)	6.0 (24/402)	0.0 (0/46)
Arg132His	91.3 (126/138)	88.9 (16/18)	92.4 (122/132)	80.0 (12/15)	91.7 (22/24)	0.0 (0/46)
Other 132 mutations	8.7 (12/138)	11.1 (2/18)	7.6 (10/132)	20.0 (3/15)	8.3 (2/24)	0.0 (0/46)
EGFR amplification	0.5 (1/205)	0.0 (0/25)	14.7 (34/232)	7.4 (2/27)	38.1 (139/365)	21.4 (9/42)
Chromosome 10q loss	15.1 (30/199)	16.7 (4/24)	36.3 (81/223)	36.0 (9/25)	80.4 (263/327)	81.1 (30/37)
1p 19q codeletion	27.3 (36/132)	31.6 (6/19)	35.6 (67/188)	20.0 (4/20)	0.0 (0/254)	0.0 (0/28)
MGMT methylation	87.0 (80/92)	92.3 (12/13)	65.3 (79/121)	53.8 (7/13)	53.2 (124/233)	51.8 (14/27)
P16 homozygous deletion	6.3 (12/192)	20.0 (5/25)	12.1 (28/232)	19.2 (5/26)	39.4 (143/363)	31.0 (13/42)

OS: overall survival, PFS: progression-free survival, n: number of patients, *IDH1*: isocitrate dehydrogenase 1, *EGFR*: epidermal growth factor receptor, *MGMT*: methyl guanine methyl transferase. NR: not reached.

Table IV: Characteristics of the patients included in the first validation series (Paris)

	Genotype		
	GGC/GGC	GGC/GGT or GGT/GGT	*p*
n	271	35	
Sex			
Male-to-female	1.7	1.9	*NS*
Male	169	23	
Female	102	12	
Age at diagnosis, years			
Median	57.8	60.0	*NS*
Range	19.9-86.5	36.5-84.6	
Median OS, months	14.5	10.1	*.02*
Median PFS, months	8.7	6.3	*.006*

OS: overall survival, PFS: progression-free survival, n: number of patients

Table V: Characteristics of the patients included in the second validation series (Bonn series)

Grades	II	III	IV
n	125	129	337
Sex			
Male-to-female	1.45	1.30	1.57
Male	74	73	206
Female	51	56	131
Age at diagnosis, years			
Median	34	41	61
Range	13-74	17-80	13-83
Mean KPI	84.6	85.5	75.5
SD	13.5	11.7	13.5
Degree of resection			
Bx/STR/GTR (%)	8.0/52.0/40.0	2.4/40.9/56.7	3.9/38.1/58.0
Postop. Radiotherapy (%)	24.8	89.9	91.4
Postop. Chemotherapy (%)	16.8	75.2	37.1
Median OS, months	178.0	103.0	11.0
95% CI	155.4-200.6	71.2-134.8	10.4-13.6
Median PFS, months	60.0	42.0	8.0
95% CI	46.0-70.0	39.0-62.0	6.0-11.0

OS: overall survival, n: number of patients

Table VI: *IDH1* SNP rs11554137:C>T genotype and patient characteristics (Bonn series).

	Genotype		
	GGC/GGC	GGC/GGT	
n	544	47	
WHO II/III/IV (%)	93.6/91.5/91.7	6.4/8.5/8.3	NS
Sex			
Male-to-female	1.45	1.94	NS
Male	322	31	
Female	222	16	
Age at diagnosis, years			
Median	50	52	NS
Range	17-83	19-81	
Mean KPI	79.9	75.7	NS
SD	13.9	13.9	
Degree of resection			
Bx/STR/GTR (%)	4.1/41.6/54.4	8.7/43.5/47.8	NS
Postop. Radiotherapy (%)	76.7	80.9	NS
Postop. Chemotherapy (%)	41.7	34.0	NS
Median OS, months	33.0	16.0	.037
95% CI	18.0-48.0	7.4-24.6	
Median PFS, months	13.0	10.0	NS (.093)
95% CI	11.0-15.0	6.0-19.0	

Table VII: Overall and progression-free survival according to *IDH1* SNP rs11554137:C>T (Bonn series).

	Genotype		
	GGC/GGC	GGC/GGT	*p*
n	544	47	
OS (months, 95% CI)			
Grade II	184.0 147.8-220.2	160.0 NR-339.0	NS (*p*= .072)
Grade III	123.0 70.0-176.0	16.0 13.1-18.9	.001
Grade IV	12.0 10.3-13.7	10.0 3.6-16.4	NS
PFS (months, 95% CI)			
Grade II	61.0 50.0-74.0	37.0 21.0-53.0	NS
Grade III	49.0 48.0-71.0	13.0 NR-24.0	NS (*p*= .066)
Grade IV	10.0 9.0-11.0	10.0 6.0-19.0	NS

OS: overall survival, PFS: progression-free survival, n: number of patients.

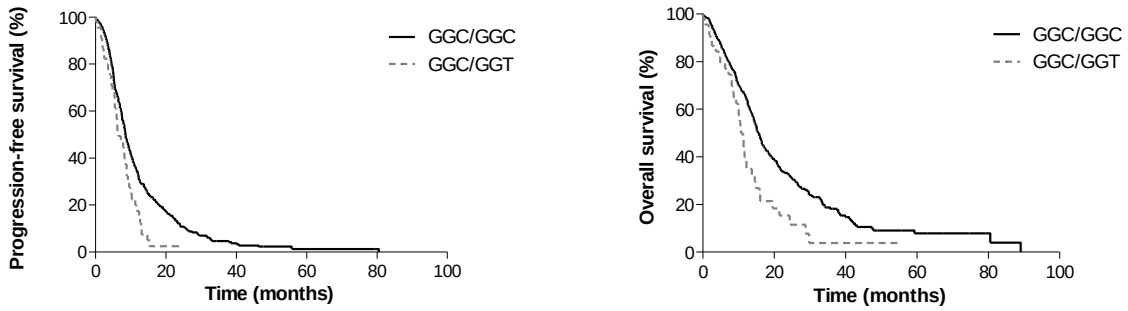

Figure 1: Progression-free survival (PFS) curves and overall survival (OS) curves in glioblastoma patients according to *IDH1* SNP rs11554137:C>T

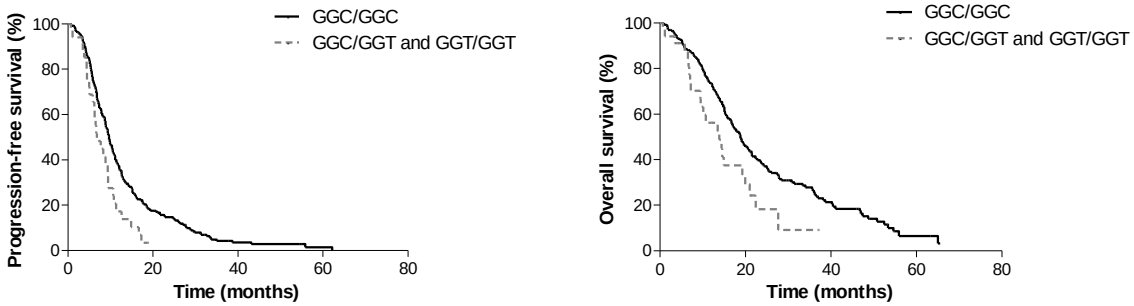

Figure 3: Progression-free survival (PFS) curves and overall survival (OS) curves in the validation series of 306 glioblastoma patients according to *IDH1* SNP rs11554137:C>T.

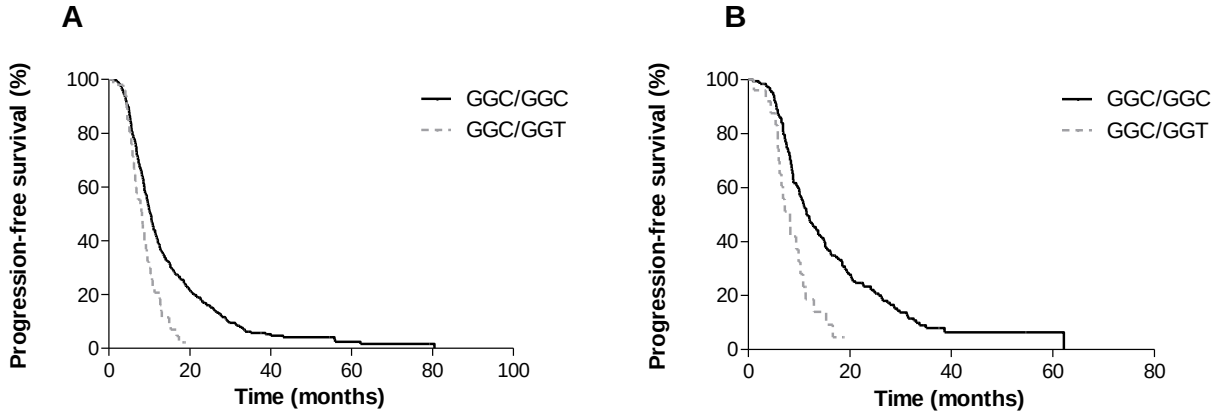

Figure 4: Progression-free survival (PFS) curves in glioblastoma patients treated with RT (4A) or RT-temozolomide regimen (4B) according to *IDH1* SNP rs11554137:C>T.

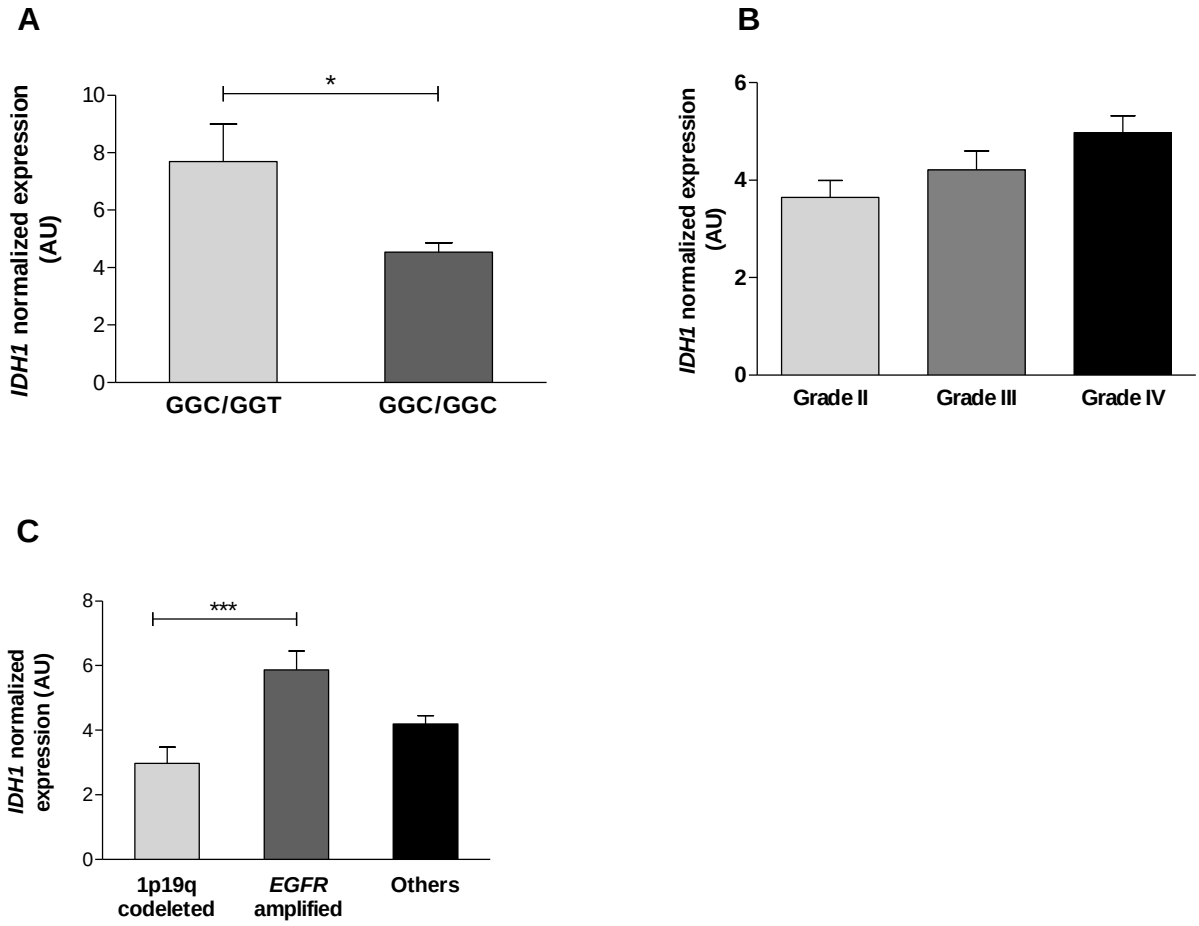

Figure 2: *IDH1* mRNA level. 2A: Impact of *IDH1* SNP rs11554137:C>T on *IDH1* mRNA level. *IDH1* expression was normalized both to the expression of *ALAS* mRNA and to the expression of non tumoral brain tissue. Results are expressed as the mean (± SD) *IDH1* mRNA expression. GGC/GGT patients, n= 16; GGC/GGC patients n= 110. AU: arbitrary units. * $p < .05$ (t-test). **2B: *IDH1* mRNA level according to glioma grade.** IDH1 expression was normalized both to the expression of *ALAS* mRNA and to the expression of non tumoral brain tissue. Results are expressed as the mean (± SD) *IDH1* mRNA expression. Grade II patients, n= 39; Grade III patients n= 46, Grade IV patients n= 126. AU: arbitrary units. **2C: *IDH1* mRNA level according to glioma genetic subtype.** *IDH1* expression was normalized both to the expression of *ALAS* mRNA and to the expression of non tumoral brain tissue. Results are expressed as the mean (± SD) *IDH1* mRNA expression. 1p19q codeleted patients, n= 24; *EGFR* amplified patients n= 54, other patients n= 116. AU: arbitrary units. *** $p < .001$ (t-test with Welch's correction).

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RESULTS

Publication n°3

“Isocitrate dehydrogenase 1 mutation (IDH1 R132H) increases U87 glioma cell sensitivity to radiation therapy in hypoxia”

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Isocitrate dehydrogenase 1 mutation (IDH1 ^{R132H}) increases U87 glioma cell sensitivity to radiation therapy in hypoxia

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ABSTRACT

Objectives

IDH1 codon 132 mutation (mostly Arg132His) is frequently found in gliomas and is associated with longer survival. However, it is still unclear whether *IDH1* mutation makes the cell more vulnerable to current treatment, radio- and chemotherapy.

Materials and Methods

We transduced U87 with wild type *IDH1* or U87-*IDH1*^{R132H} expressing lentivirus, and analyzed the radiosentivity (dose ranging 0 to 10Gy) under normoxia and moderate hypoxia

Results

We observed that U87-*IDH1*^{R132H}cells grow faster in hypoxia, and were more sensitive to radiotherapy (in terms of cell mortality and colony formation assay) compared to non transduced U87 and *IDH1*^{WT}cells. This difference was not observed in normoxia.

Conclusion

These data suggest that *IDH1*^{R132H} mutation trigger radiosensitivity in hypoxic condition.

Keywords: Isocitrate dehydrogenase 1; U87; Radiosensitivity; Radiation therapy; Antioxidant enzyme

INTRODUCTION

The *IDH1* gene encoding the cytoplasmic NADP+-dependent isocitrate dehydrogenase (more rarely *IDH2*) is frequently mutated in gliomas, especially low grade gliomas and secondary glioblastomas (1–6). *IDH1/IDH2* mutation is associated with good clinical outcome, whatever the grade, but it is still not clear whether it is merely a prognostic marker, or a predictor of the response to radiotherapy or chemotherapy (7–10). *IDH1*/*IDH2* mutation results in a new enzyme function catalyzing the NADPH-dependent reduction of a-ketoglutarate to 2-D-hydroxyglutarate (2HG) (11). *IDH1* and *IDH2* mutations results in 2HG accumulation and lowering NADPH levels (11,12). On one hand 2HG inhibits various a-ketoglutarate dependant reactions, including histone and DNA demethylation (13,14). On the other hand, low NADPH levels might sensitize tumors to oxidative stress, potentiating response to radiotherapy, and may account for the prolonged survival of patients harboring the mutations.

Since the majority of gliomas are poorly responsive to current treatment regimens, the ability to enhance cell radio-chemosensitivity would be of clinical benefit. In this study, we characterized the impact of *IDH1* mution on U87 glioma cell growth, and radiosensitivity.

METHODS AND MATERIALS

Cell culture and hypoxia treatment

The human glioblastoma cell line U87 MG (HTB14) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin. Normoxic cells (21% O_2) were grown in a humidified-air atmosphere incubator containing 95% air/5% CO_2 at 37 ℃. Hypoxia experiments were performed in a controlled atmosphere chamber (INVIVO2 1000, 3M, France) set at 1%O_2, 94%N_2 and 5%CO_2 at 37 ℃.

Production of recombinant expression lentiviruses

A recombinant pLenti7.3/V5-TOPO expression vector (Invitrogen's ViraPowerTM HiPerformTM Lentiviral Expression Systems; Catalog no: K5320-00) containing the human *IDH1* wild type and *IDH1*^{R132H} cDNA was generated. Cotransfect the expression clones and the ViraPower Packaging Mix into the 293FT Cell Line to produce lentiviral stocks. Use the lentiviral stocks to transducer the mammalian U87 cell line. U87-*IDH1*wt and U87-*IDH1*^{R132H} stable cell lines were acquired with a using EmGFP selction by flow cytometry. The constructs was verified by DNA sequencing and RT-qPCR analysis.

Cell proliferation assay in normoxia and in hypoxia

To evaluate the impact of *IDH1* mutation on cell spontaneous growth in normoxia and in hypoxia by trypan blue dye exclusion method. U87, U87-IDHwt and U87-IDH^{R132H} cells (4000/well) plated in 24-well plates (6 plates in total) were incubated at 37 ℃ for six hours in nomoxia to adhere; then 3 plates were removed at 37 ℃ in the controlled atmosphere chamber overnight. At 1, 3, and 7 days after exposure to normoxia and hypoxia, the cells were trypsinized, and the viable cell number per well was determined by counting with trypan blue. The experiment was performed three times in triplicate each.

Cell viability assay with increased doses of radiation at 120h in normoxia and in hypoxia

To evaluate the cytotoxicity of radiation, 4000 of U87 and U87 transduced with *IDH1*wt and *IDH1*^{R132H} cells were plated per well in 24-well plates. Six hours later at 37 ℃ in normoxia, cells were incubated in the controlled atmosphere chamber overnight. The next day,

cells were irradiated with doses ranging from 0 to 10 Gy; Five days later, Fix Cells by Paraformaldehyde (PFA) 4%, then cells were stained by Hoechst 33342 (10µg/ml in PBS, Sigma-Aldrich, France) and photographed in a blinded fashion under fluorescence (4 wells per condition; 4 photographs per well). Cell number was obtained by automated counting with ImageJ software (Rasband, W.S., ImageJ, U.S.N.I.H.).

Cell viability assay irradiated at 8Gy at 24/48/120h in normoxia and in hypoxia

To evaluate the effect of *IDH1*^{R132H} in glioma radioresponse, 4000 of U87 and U87 transduced with *IDH1*wt and *IDH1*^{R132H} cells were plated per well in 24-well plates. Six hours later at 37℃ in normoxia, half of plates were incubated in the controlled atmosphere chamber overnight. The next day, cells were irradiated with doses 8Gy. Fix Cells by Paraformaldehyde (PFA) 4%, then cells were stained by Hoechst 33342 (10µg/ml in PBS, Sigma-Aldrich, France) and photographed in a blinded fashion under fluorescence (4 wells per condition; 4 photographs per well) at 24h, 48h, and 120h, respectively. Cell number was obtained by automated counting with ImageJ software (Rasband, W.S., ImageJ, U.S.N.I.H.). Adhesion data represent the mean and s.e.m. of two independent experiments, measured in quatriplicate samples.

Colony-formation assay in normoxia and in hypoxia

To evaluate radiosensitization by *IDH1*^{R132H}. Prepare 1% base agar layer. 4000 of U87, U87-*IDH1*wt and U87-*IDH1*^{R132H} cells were plated per well of 6-well plates in 0.6% top agar. Six hours later, cells were incubated in the hypoxic chamber overnight. The next day, Transduced cells were treated by radiotherapy in the Radiotherapy Department of Central Universital Hospital of Caen using an X-ray generator with doses ranging 0-8Gy. The irradiated cells were returned to the incubator for colony formation. Three weeks later, the colonies were fixed in 20% ethanol and stained with 0.05% crystal violet. Colonies that contained more than 50 cells were counted. Survival was calculated as the average number of colonies counted divided by the number of cells plated times the plating efficiency (PE), where PE was the fraction of colonies counted divided by cells plated without radiation. The clonogenic survival data were generated using JMP software (kindly provided by S. Valable, Université de caen basse-normandie, Caen, France). The experiment was performed five

times in triplicate each.

Statistical analysis

Results obtained in vitro are expressed as mean±s.e.m. Image analysis was performed with in-house macros under the ImageJ Software (Rasband, W.S., ImageJ, U.S. NIH). All statistical analyses were determined using post-hoc tests after significant ANOVA. Values of $p<0.05$ were considered statistically significant.

RESULTS

1. Transduced cells express high quantities of IDH1wt and IDH1^{R132H}

The presence of *IDH1*^{R132H} transduced gene was confirmed by DNA sequencing (Figure 1). Real time PCR showed a high expression of gene *IDH1*wt (four-fold increase compared to non transduced cell) and *IDH1*^{R132H} (two-fold increase) in transduced U87cells (Figure 2).

2. IDH1^{R132H} expressing U87 glioma cells grow faster in hypoxia

We determined whether *IDH1*^{R132H} expression directly influences cell growth in normoxia and in hypoxia. The viable cell number per well was determined by counting with trypan blue at 1, 3, and 7 days after incubation in normoxia and in hypoxia. In normoxia, U87, U87-*IDH1*wt and U87-*IDH1*^{R132H} cells grew at the same rate (Figure 3A), whereas U87-*IDH1*^{R132H} grew faster than U87 and U87-*IDH1*wt at D7 in hypoxia (Figure 3B). Comparing to U87-*IDH1*^{R132H} cell growth in normoxia and hypoxia, the rate of proliferation of U87-*IDH1*^{R132H} cells in normoxia (about 20 times at 7 days) was significantly higher than that observed in cells incubated in hypoxia (about 6 times at 7 days).

3. Effect of transduced IDH1^{R132H} on cell viability upon exposure to doses (0-10Gy) in normoxia and in hypoxia

To evaluate the role of *IDH1*^{R132H} in the response to radiotherapy, U87, U87-*IDH1*wt and U87-*IDH1*^{R132H} were exposed to different doses (range: 0-10Gy) in normoxia..the three cell lines showed the same radiosensitivity profile (Figure 4A), whereas in hypoxia, the viability of U87-*IDH1*^{R132H} cells was significantly lower after 5 days compared to control cells and *IDH1*wt cells (Figure 4B)(13% vs 23% and 22% for a dose of 10Gy, $p<0.001$). This result suggests that *IDH1*^{R132H} makes the cells more radiosensitive in hypoxic, but not normoxic conditions.

4. Effect of transduced IDH1^{R132H} on cell mortality over time following 8Gy irradiation in normoxia and in hypoxia

We quantified then cell death 24h, 48h, and 120h after 8 Gy irradiation. There was no substantial cell death after 24h. The effect appeared at 48h in both normoxia and in hypoxia

(data not shown), and was maximal after 5 days (Figure 5A, B). Cell death was significantly higher for *IDH1*^{R132H} transduced cells in hypoxia but not in normoxia (figure 5C, 5D).

5. Radiosensitivity of U87 IDH1^{R132H} in hypoxia is confirmed by colony-formation assay

A colony-formation assay was used to confirm the effect of *IDH1*^{R132H} on the response to radiotherapy. Cells were treated with graded doses of radiation (0, 2, 4, 6, and 8Gy). Colony-forming efficiency was determined 1 month (Figure 7) later and surviving fractions were calculated. In normoxia, U87, U87-*IDH1*wt and U87-*IDH1*^{R132H} have the same colony-formation capacity after radiotherapy (Figure 6A). In hypoxia, the colony number of U87-*IDH1*^{R132H} after radiotherapy was significantly lower than U87 and U87-*IDH1*wt (Figure 6B). Thus, *IDH1*^{R132H} indeed significantly sensitized U87 glioma cells to radiation.

DISCUSSION

We observed here that *IDH1* mutated U87 grew faster in moderate hypoxic conditions (1%) than in normoxia (20%) and were more sensitive to radiotherapy in hypoxic condition.

A first statement is that high rate of cell proliferation is *per se* a sensitive factor of the radiation therapy response. The second point is that IDH mutated cells may be more sensitive to oxidative stress. The role of *IDH1* and *IDH2* in cellular defense against oxidative stress has been demonstrated (15–24). The α-KG may participate to the detoxification of ROS(25). In addition the elevated levels of 2HG provokes oxidation of lipids and proteins by reducing the brain capacity to modulate efficiently the damage associated with an enhanced generation of free radicals (26). Furthermore, structural similarities between 2HG and glutamate suggest that 2HG could affect excitotoxicity via the activation of the NMDA glutamate receptor in neurons (11). *IDH1*/*IDH2* serves as a major source of cytosolic and mitochondrial NADPH production necessary to regenerate reduced glutathione (GSH) by glutathione reductase and for the activity of NADPH-dependent thioredoxin system, both important in the protection of cells from oxidative damage (17). Thus, the decrease of NADPH in IDH mutated cells, might result in an increase of reactive oxygen species that can damage DNA.

Despite hypoxia is considered as a factor of radioresistance, we observed here a radiosensitizing effect of $IDH1^{R132H}$ in glioblastoma cell line in hypoxic but not normoxic condition. Until recently, *IDH1*/2 mutation were believed to result in the stabilization of HIF (12,27). Interestingly Koivunen et al. (28) showed that R-2HG (but not L-2HG) instead of being an inhibitor of EGLN (HIF prolyl 4-hydroxylases) activity, was an agonist and promotes the degradation of HIF. Because HIF protects cells from irradiation therapy under hypoxic condition, we may hypothesize that IDH mutation, by inducing an inappropriate degradation of HIF, could make the mutated cell more vulnerable to RT.

In conclusion, this study showed a radiosensitizing effect of $IDH1^{R132H}$ in glioblastoma cell lines U87 grown in hypoxic condition. We need, to confirm this hypothesis, to test additional cell lines, and more importantly to confirm it on clinical setting. The ongoing EORTC trial on low grade gliomas, which randomize radiotherapy vs chemotherapy, and include also a prospective observational cohort, will be pivotal to answer this question.

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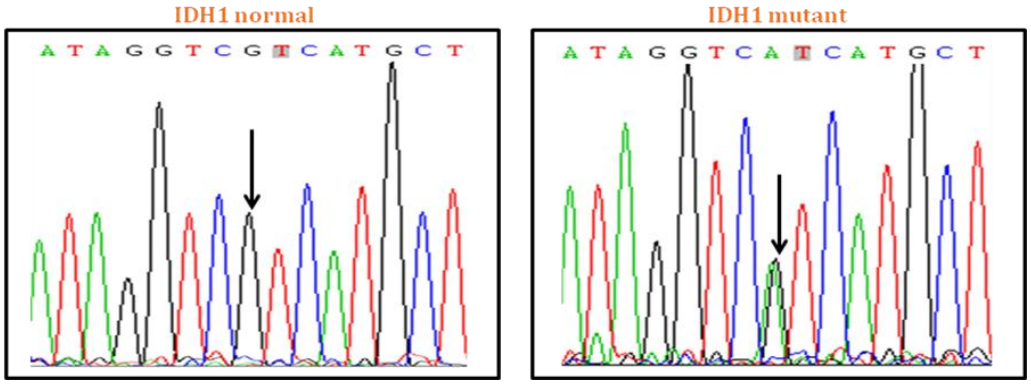

Figure 1: The presence of the *IDH1*wt and *IDH1*^{R132H} transduced genes was confirmed by DNA sequencing in cells U87.

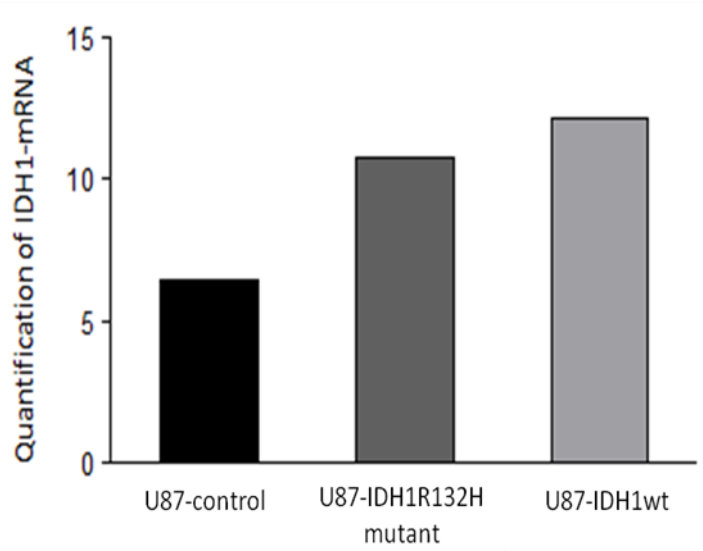

Figure 2: Real time PCR quantificated the expression of the *IDH1*wt and *IDH1*^{R132H} transduced genes.

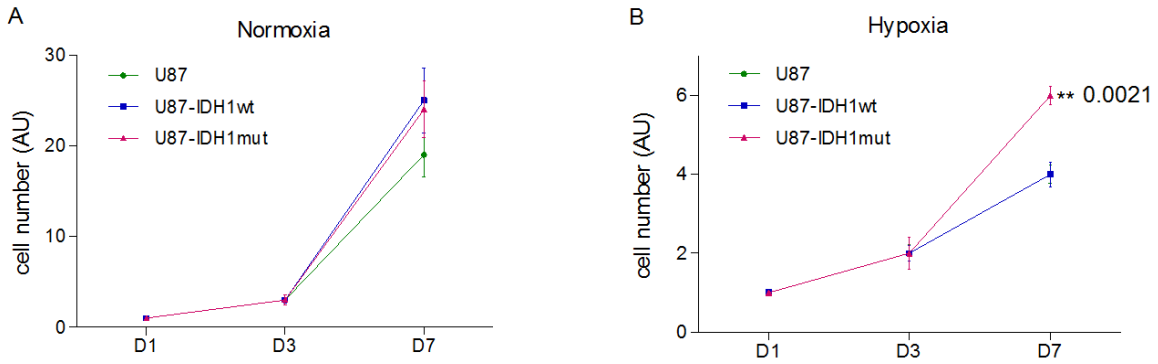

Figure 3: Effect of *IDH1*^{R132H} on U87 cell proliferation. U87, U87-*IDH1*wt and U87-*IDH1*^{R132H} cells were incubated in normoxia 20% (left) or hypoxia 1% (right) and cells were counted after 1, 3 and 7 days.

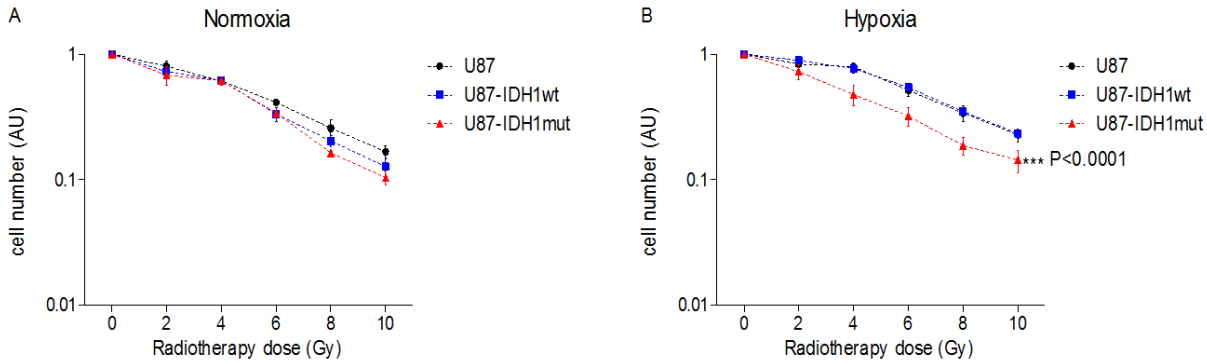

Figure 4: Effect of *IDH1*^{R132H} on U87 cell viability after irradiation. Transduced cells were plated and then irradiated with doses ranging from 0 to 10Gy, in normoxia (20%) (left) and in hypoxia (1%) (right). Cells were counted 5 days later.

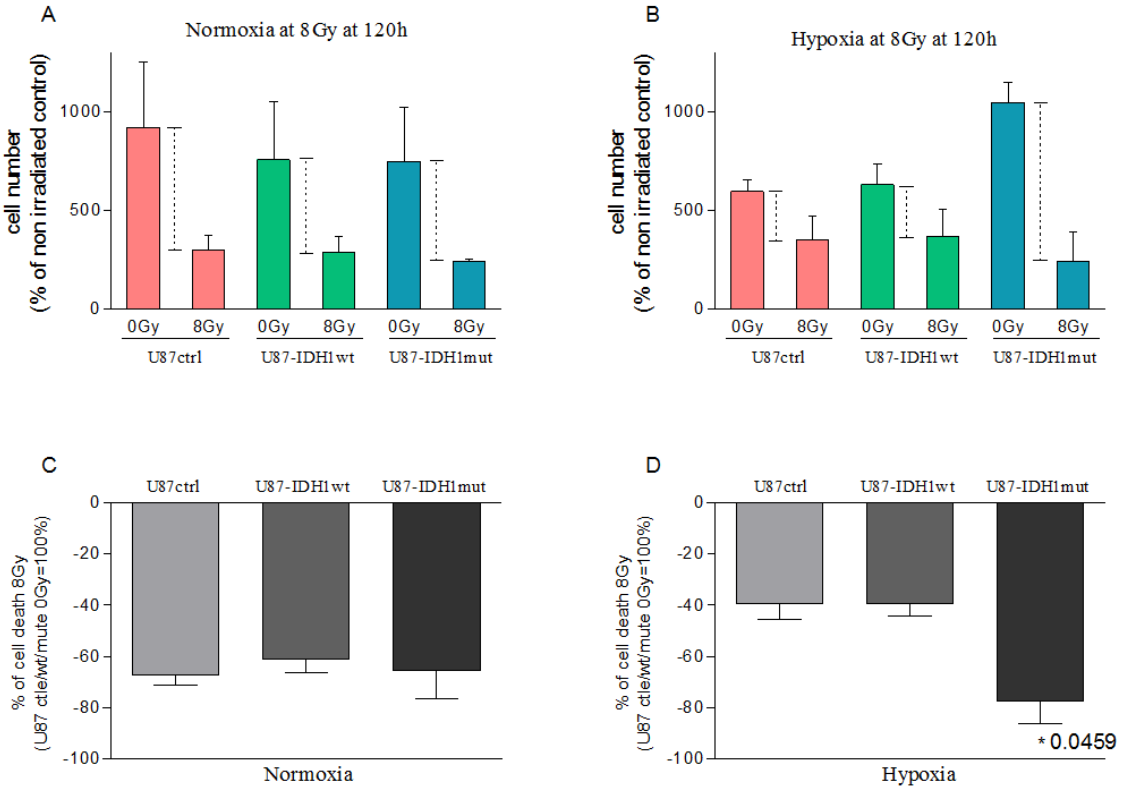

Figure 5: Cell viability after 8Gy irradiation. (A-B) Cells were counted before 8Gy irradiation and 5 days after, in normoxia (20%) (left) and in hypoxia (1%) (right). (C-D) Proportion of cellular shrinkage after irradiation, in normoxia (20%) (left) and in hypoxia (1%) (right).The percent of cell death following 8Gy of irradiation was compared with the non-irradiated cells.

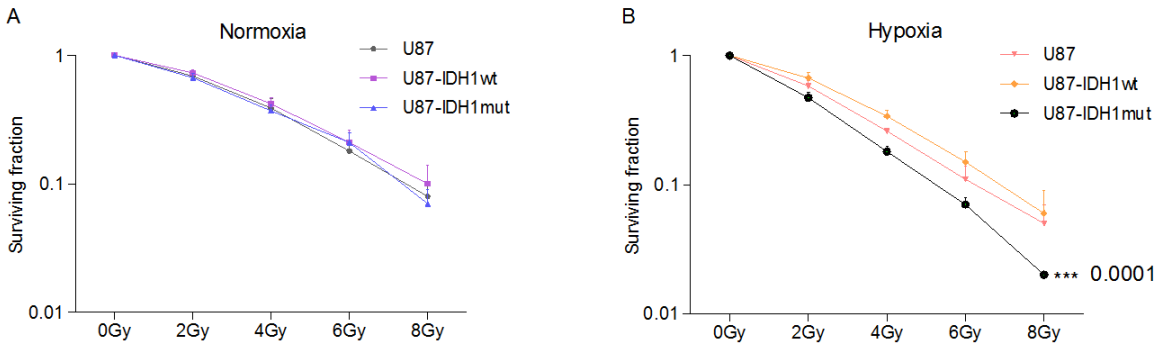

Figure 6: *IDH1*^{R132H} cells have a reduced colony forming cell ability after irradiation in hypoxia. U87, U87-*IDH1*wt and U87-*IDH1*^{R132H} cells were plated 24 h before irradiation in 6-well plates containing 0.3% base agar layer. The cells were irradiated 0-8Gy and incubated for one month in normoxia (20%) and in hypoxia (1%). One month later, the colonies were fixed in ethanol and stained with 0.05% crystal violet. Colonies that contained more than 50 cells were counted. Survival rate was estimated by the ratio between the colonies count and the number of cells plated, multiplicated by the plating efficiency (PE= ratio between the colonies count and the number of cells plated, without radiation).

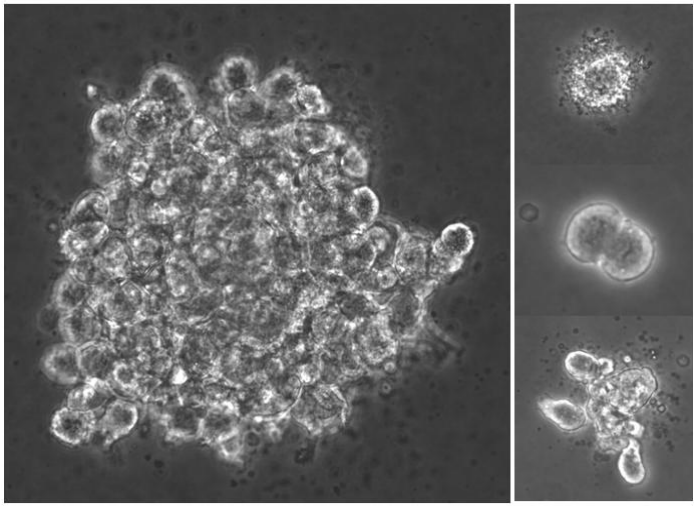

Figure 7: colony-forming assay. The numbers of cells are superior to 50 cells in a formed colony (left photo); the dead or stopping divised cells were not able to form a true colony (right photos).

DISCUSSION

Discussion

IDH1/IDH2 mutations are important biomarkers in gliomas. We compare here our results to other studies for *IDH1*/*IDH2* mutations types and frequency, distribution in gliomas subtypes and grades, correlation with age, other genetic alterations in gliomas, and outcome.

IDH1/IDH2 mutations possess diagnostic, prognostic value in gliomas. Several studies also have analyzed that the gliomas with *IDH1* mutations response to treatment (chemo- and radiotherapy).

I. Description of *IDH1/IDH2* mutations in gliomas

1. *IDH1* and *IDH2* mutations types and frequency

Only *IDH1* and *IDH2* mutations but no mutations in *IDH3* have been found in human tumors. The *IDH* mutations are somatic because *IDH1* and *IDH2* mutations are not found in peritumoral normal brain tissue or peripheral blood samples. Somatic, missense, and heterozygous mutations were demonstrated in the homologous residues *IDH1* R132 and *IDH2* R172 (Figure 9). *IDH2* R140 mutations occur in AMLs but have not been reported in gliomas so far. Figure 9 presents the different mutation sites in *IDH1* and *IDH2* genes.

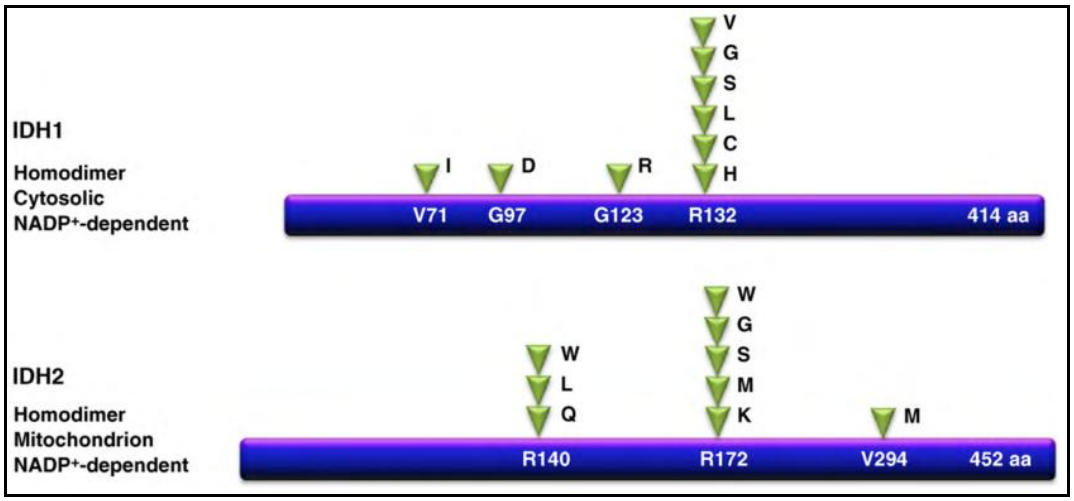

Figure 9: *IDH1* and *IDH2* mutation sites in tumors. Adapted from Dang et al. (2010) (70).

Our study was focused on mutations affecting codon R132 of *IDH1* and codon R172 in *IDH2* gene. *IDH1* and *IDH2* mutations are mutually exclusive in our study. The conserved residue *IDH1* R132 and *IDH2* R172 are located in the substrate-binding site of *IDH1*/*IDH2*, and all mutations found were heterozygous, leaving one unaffected parental allele.

We found 5 types of *IDH1* R132 mutations and 4 types of *IDH2* R172 mutations. The proportion of various types of *IDH1* and *IDH2* mutations is similar to other studies (Figure 10-11, Tables 2 and 3).

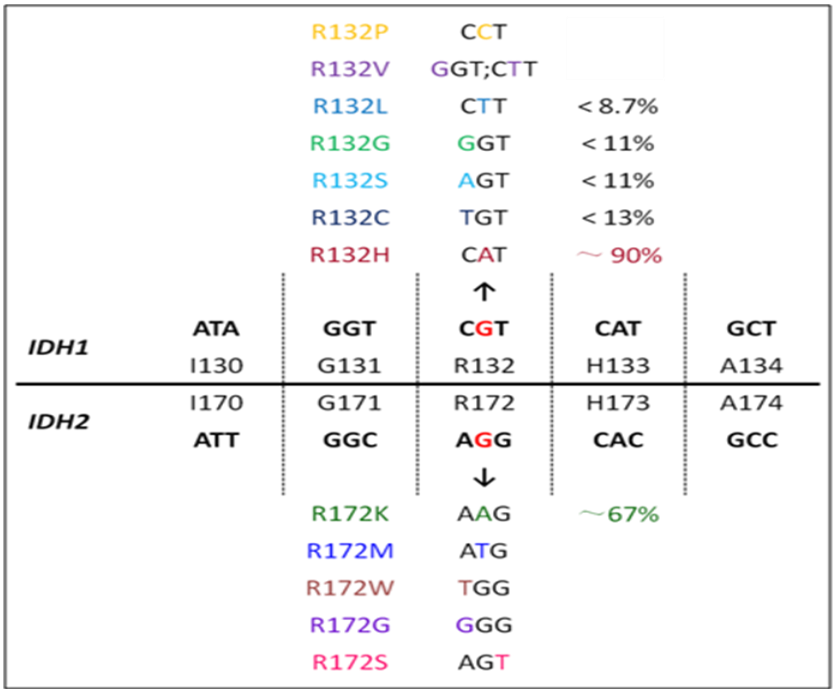

Figure 10: *IDH1* and *IDH2* mutations types and frequency in gliomas in literature.

To date, seven types of *IDH1* R132 mutations have been discovered in gliomas. The *IDH1* (R132H) mutation is the most common (>90%) in gliomas, followed by R132C (<13%), R132S (<11%), R132G (<11%), R132L (<8.7%), and single R132V (1/221) and R132P (1/246) (Table 2).

R172K is the most common *IDH2* mutation (up to 67%). Five types of *IDH2* R172 mutations have been reported in gliomas: substitution of arginine by lysine (R172K), methionine (R172M), glycine (R172G), serine (R172S) and tryptophan (R172W) (Table 2).

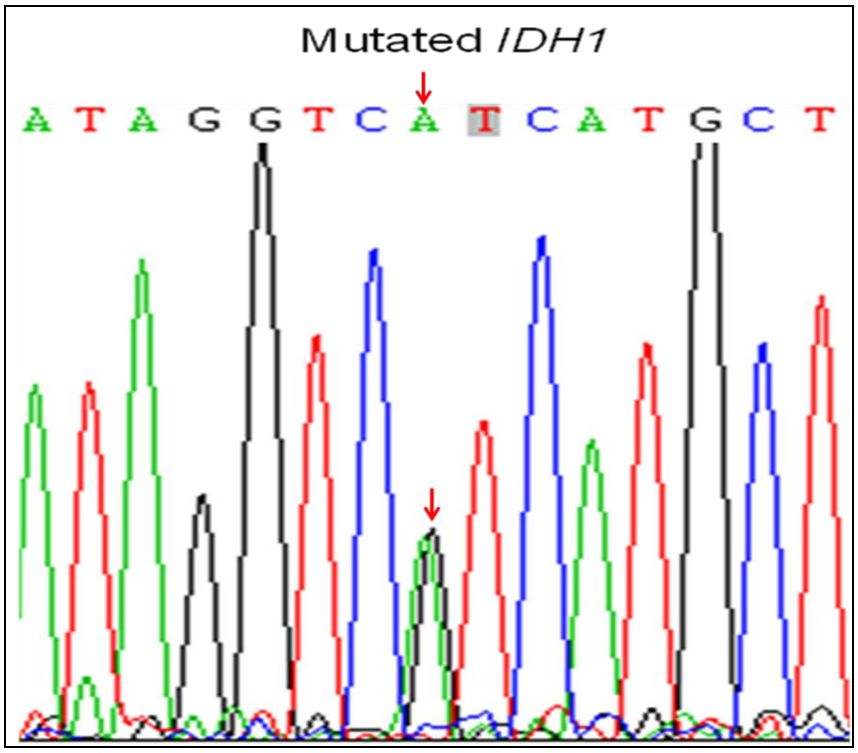

Figure 11: Mutations in *IDH1* R132 and in *IDH2* R172 in our study

Table 2: Type and frequency of *IDH1* mutations in CNS tumors

Author (year)	Cases	Number of cases	Type and frequency of *IDH1* mutations in CNS tumors. % (Number of cases)							
			p.R132H G395A	p.R132C C394T	p.R132S C394A	p.R132G C394G	p.R132L G395T	p.R132V C394G G395T	p.R132P G395C	Total
Wang XW	Gliomas	1332	93.9% (581/619)	2.4% (15/619)	1.1% (7/619)	1.9% (12/619)	0.6% (4/619)	-	-	46.5% (619/1332)
Parson, 2008 (44)	GBMs	149	88.9% (16/18)	-	11.1% (2/18)	-	-	-	-	12% (18/149)
Balss, 2008 (86)	Brain tumors	685	92.7% (205/221)	3.6% (8/221)	1.8% (4/221)	0.9% (2/221)	0.5% (1/221)	0.5% (1/221)	-	32.3% (221/685)
Bleeker, 2009 (50)	High grade gliomas	113	73.9% (17/23)	13% (3/23)	4.3% (1/23)	4.3% (1/23)	4.3% (1/23)	-	-	20.3% (23/113)
Watanabe, 2009 (48)	Gliomas	321	90.8% (118/130)	4.6% (6/130)	0.8% (1/130)	3.8% (5/130)	-	-	-	40.5% (130/321)
Hartmann, 2009 (89)	Gliomas	1010	92.7% (664/716)	4.2% (29/716)	1.5% (11/716)	1.4% (10/716)	0.2% (2/716)	-	-	70.9% (716/1010)
Ichimura, 2009 (46)	Intra-cranial tumors	596	92.4% (110/119)	3.4% (4/119)	0.8% (1/119)	3.4% (4/119)	-	-	-	20% (119/596)
Yan, 2009 (47)	CNS tumors	445	88.2% (142/161)	4.3% (7/161)	2.5% (4/161)	0.6% (1/161)	4.3% (7/161)	-	-	36.2% (161/445)
Sanson, 2009 (49)	Gliomas	404	89% (138/155)	3.2% (5/155)	1.9% (3/155)	4.5% (7/155)	1.3% (2/155)	-	-	38% (155/404)
Horbinski, 2009 (90)	Intra-cranial tumors	75	85.3% (29/34)	2.9% (1/34)	5.9% (2/34)	2.9% (1/34)	2.9% (1/34)	-	-	45.3% (34/75)
Korshunov, 2009 (92)	PA I and A II	120	89.5% (34/38)	7.9% (3/38)	-	2.6% (1/38)	-	-	-	76% (38/50)
Nobusawa, 2009 (92)	Glioblastomas	407	83%	2.8%	2.8%	11.1%	-	-	-	8.8% (36/407)
Sonoda, 2009 (93)	Gliomas	125	31.2% (39/125)	-	-	-	-	-	-	31.2% (39/125)
Dubbink, 2009 (94)	Low-grade astrocytomas	49								86% (42/49)
Wick, 2009 (95)	Anaplastic glioma	195	94.5% (121/128)	3.9% (5/128)	0.8% (1/128)	0.8% (1/128)				65.6% (128/195)
Gravendeel, 2010 (96)	Gliomas	496	92.3% (227/246)	3.7% (9/246)	2.0% (5/246)	1.2% (3/246)	0.4% (1/246)	-	0.4% (1/246)	49.6% (246/496)
Felsberg, 2010 (97)	Gliomas	262	90.5 (57/63)	1.6% (1/63)	4.8% (3/63)	3.2% (2/63)				24% (63/262)
Van den bent, 2010 (98)	Anaplastic oligodendroglioma	159								46% (73/159)
Metellus P, 2010 (99)	low-grade gliomas (LGGs)	47	92.1% (35/38)	5.3% (2/38)	2.6% (1/38)					80.9% (38/47)
Jha, 2011 (100)	Gliomas	100	84.8% (39/46)	4.3% (2/46)		2.2% (1/46)	8.7% (4/46)			46% (46/100)

Table 3: Type and frequency of *IDH2* mutations in CNS tumors

Author (year)	Cases	Number of cases	Type and frequency of *IDH2* mutations in CNS tumors. % (Number of cases)					
			p.R172K G515A	p.R172M G515T	p.R172W A514T	p.R172G A514G	p.R172S G516T	Total
Wang XW	Gliomas	996	73.3% (11/15)	6.7% (1/15)			20.0% (3/15)	3.1% (31/996)
Hartmann, 2009 (89)	Gliomas	1010	64.5% (20/31)	19.3% (6/31)	16.2% (5/31)			3% (31/1010)
Yan, 2009 (47)	CNS tumors	426	44.4% (4/9)	33.3% (3/9)	-	22.2% (2/9)		2.1% (9/426)
Horbinski, 2009 (90)	Intra-cranial tumors	75		100% (1/1)				1.3% (1/75)
Nobusawa, 2009 (92)	Glioblastomas	367						0% (0/367)
Sonoda, 2009 (93)	Gliomas	63					100% (1/1)	1.6% (1/63)
Felsberg, 2010 (97)	Gliomas	210	66.7% (4/6)	16.7% (1/6)	16.7% (1/6)			2.9% (6/210)
Van den bent, 2010 (98)	Anaplastic oligodendroglioma	159						0.6% (1/159)
Dubbink, 2009 (94)	Low-grade astrocytomas	49						0% (0/49)
Wick, 2009 (95)	Anaplastic glioma	195						3.1% (6/195)
Metellus P, 2010(99)	low-grade gliomas (LGGs)	47		100% (2/2)				4.3% (2/47)

2. Distribution of *IDH1*/*IDH2* mutations in subtypes and grades gliomas

a. Distribution of IDH1 mutations in gliomas

- **PA I and Ependymal tumors**

The rates of *IDH1* mutations in pilocytic astrocytomas (0%-10%; grade I gliomas) and ependymal tumors (0%) are very rare, providing a diagnostic utility. Similarly, we did not find *IDH1* mutations in pilocytic astrocytomas and only 1 case (1/74) *IDH1* mutations in ependymal tumors in our study (Table 4).

- **Grade II and III gliomas**

IDH1 is commonly mutated in grade II and III gliomas and our study is in line with data of the literature. *IDH1* mutations were present in 75.3% of diffuse astrocytomas (literatures:59-88%), 75.8% of oligodendrogliomas (68-82%), 74.4% of oligoastrocytomas (50-100%), 60.0% of anaplastic astrocytomas (50-78%), 70.4% of anaplastic oligodendrogliomas (49-75%), and 62.4% of anaplastic oligoastrocytomas (63-100%) (Table 4).

The frequency of *IDH1* mutations in astrocytic and oligodendroglial tumors is almost similar. Several studies did not find different frequencies of *IDH1* mutation between WHO grade II and WHO grade III gliomas. However, a few studies found *IDH1* mutations more frequent in grade II gliomas compared to grade III gliomas (46,48,93,96). In our series, we found that *IDH1* mutations affected 71.9% grade II and 63.2% grade III.

- **Primary GBM and secondary GBM**

In our study, *IDH1* mutations were identified in 9.8% of primary glioblastomas (Table 4). *IDH1* mutations have been identified in 3-7% of primary glioblastomas, and 50-88% of secondary glioblastomas that develop from grade II and III astrocytomas, oligodendrogliomas.

Table 4: Frequency of *IDH* mutations in different subtypes of gliomas

Authors (year)	*IDH* status	WHO grade I-III	WHO grade I	WHO grade II			WHO grade III			WHO grade IV	
		E	PA% (n)	A% (n)	OA% (n)	O% (n)	AA% (n)	AOA% (n)	AO% (n)	prGBM% (n)	sGBM% (n)
Wang XW	*IDH1* mut	1.4 (1/74)		75.3 (55/73)	74.4 (99/133)	75.8 (204/269)	60.0 (27/45)	62.4 (113/181)	70.4 (205/291)	9.8 (38/387)	37.5 (12/32)
	IDH2 mut	0 (0/11)		1.5 (1/68)	4.4 (5/113)	6.8 (16/234)	0 (0/36)	0.7 (1/137)	5.5 (12/218)	0.4 (1/249)	0 (0/32)
Parson, 2008 (44)	*IDH1* mut									7 (7/99)	83 (5/6)
Bleeker, 2009 (50)	*IDH1* mut						0 (0/2)		50 (1/2)	12 (11/94)	73 (11/15)
Balss, 2008 (45)	*IDH1* mut	0 (0/31)	2 (1/41)	74 (34/46)	78 (36/46)	71 (36/51)	62 (29/47)	78 (29/37)	67 (36/54)	7 (7/99)	88 (7/8)
Watanabe, 2009 (48)	*IDH1* mut	0 (0/24)	10 (3/31)	88 (46/68)	94 (16/17)	79 (31/39)	78 (21/27)	71 (10/14)	75 (6/8)	5 (3/59)	82 (28/34)
Hartmann, 2009 (89)	*IDH1* mut			73 (165/227)	82 (62/76)	82 (105/128)	64 (146/228)	66 (117/177)	70 (121/174)		
	IDH2 mut			1 (2/227)	1 (1/76)	5 (6/128)	1 (2/228)	6 (11/177)	5 (9/174)		
Yan,2009 (47)	*IDH1* mut	0 (0/30)	0 (0/21)	83 (25/30)	100 (3/3)	80 (41/51)	69 (36/52)	100 (7/7)	86 (31/36)	5 (6/123)	85 (11/13)
	IDH2 mut	0 (0/30)	0 (0/21)	7 (2/30)		4 (2/51)	4 (2/52)		8 (3/36)	0 (0/123)	0 (0/13)
Ichimura, 2009 (46)	*IDH1* mut	0 (0/50)	0 (0/38)	59 (13/22)	50 (10/20)	68 (23/34)	52 (32/62)	78 (18/23)	60 (12/20)	3 (6/173)	50 (5/10)
Kang, 2009 (101)	*IDH1* mut									16 (4/25)	
Sanson, 2009 (91)	*IDH1* mut			83 (10/12)	76 (26/34)	76 (41/54)	50 (9/18)	63 (34/54)	49 (24/49)	6 (11/183)	77 (10/13)
Horbinski, 2009 (90)	*IDH1* mut	0 (0/1)	0 (0/3)	33 (3/9)	100 (1/1)	80 (16/20)	44 (4/9)		89 (8/9)	17 (1/6)	
	IDH2 mut	0 (0/1)	0 (0/3)	0 (0/9)	0 (0/1)	5 (1/20)	0 (0/9)		0 (0/9)	0 (0/6)	
Korshunov, 2009 (91)	*IDH1* mut		0 (0/70)	76 (38/50)							
Nobusawa, 2009 (92)	*IDH1* mut									4 (14/377)	73 (22/30)
Sonoda, 2009 (93)	*IDH1* mut			0 (0/2)		67 (6/9)	57 (12/21)	75 (3/4)	50 (7/14)	5 (3/59)	67 (2/3)
	IDH2 mut						5 (1/21)				
Weller, 2009 (102)	*IDH1* mut									6 (16/286)	

Authors (year)	*IDH* status	WHO grade I-III	WHO grade I	WHO grade II			WHO grade III			WHO grade IV	
		E	PA% (n)	A% (n)	OA% (n)	O% (n)	AA% (n)	AOA% (n)	AO% (n)	prGBM% (n)	sGBM% (n)
Gravendeel, 2010 (96)	*IDH1* mut			74 (54/73)	79 (22/28)	79 (34/43)	59 (19/32)	49 (19/39)	60 (64/106)	19 (34/175)	
Jha, 2011 (100)	*IDH1* mut			83 (15/18)	57 (4/7)	43 (3/7)	88 (7/8)	75 (3/4)	89 (8/9)	5 (2/41)	67 (4/6)
Felsberg, 2010 (97)	*IDH1* mut			67 (10/15)		63 (5/8)	84 (16/19)		80 (8/10)	7 (11/152)	81 (13/16)
	IDH2 mut			20 (1/5)		67 (2/3)	0 (0/3)		100 (2/2)	0 (0/152)	33 (1/3)
Van den bent, 2010 (98)	*IDH1* mut								46 (73/159)		
	IDH2 mut								1 (1/159)		
Wick, 2009 (95)	*IDH1* mut						57 (51/89)	73 (55/75)			

E: Ependymal tumors; PA: Pilocytic astrocytoma; A: Astrocytoma; O: Oligodendroglioma; OA: Oligoastrocytoma; AA: Anaplastic astrocytoma; AO: Anaplastic oligodendroglioma; AOA: Anaplastic oligoastrocytoma; pGBM: Primary Glioblastoma; sGBM: Secondary Glioblastoma

b. Distribution of IDH1R132H mutation, IDH1-nonR132H mutations among the different histologic subtypes of gliomas (analysis of 5 studies from different research centers)

In total of 3200 cases (Wang et al. 1332 cases, Hartmann et al. 1010 cases, Gravendeel et al. 496 cases, Felsberg et al. 262 cases, and Jha et al. 100 cases) from 5 different research centers were analysed for distribution of IDH1R132H mutation, IDH1-nonR132H mutations and IDH2 mutations in different histologic subclasses of gliomas (Table 6). The frequency of IDH1-nonR132H mutations was significantly more prevalent in astrocytic tumors (AII and AIII) than in oligodendroglial ($p<0.0001$) and mixed tumors ($p=0.0018$) (Figure 12).

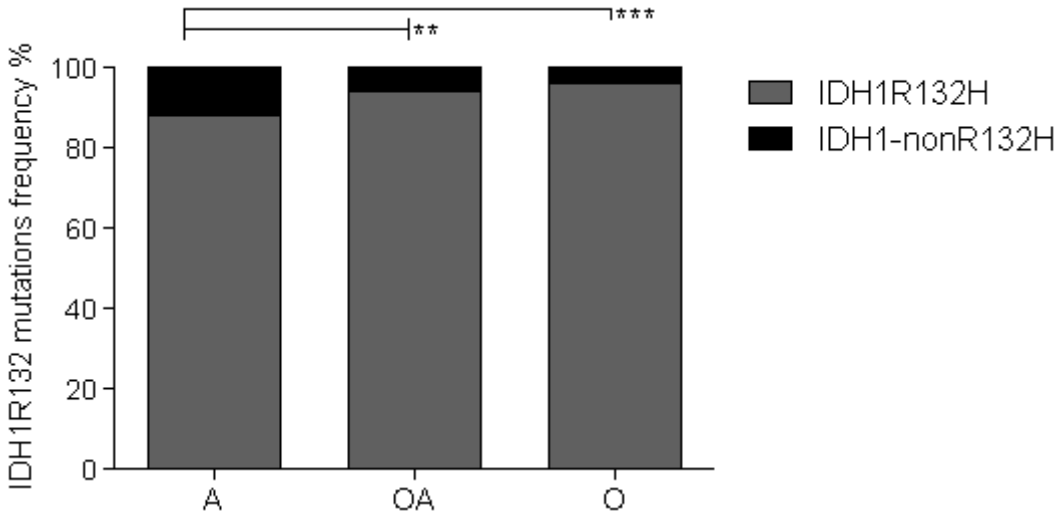

Figure 12: Prevalence of IDH1-nonR132H mutations in different histological gliomas by synthesizing 5 studies of gliomas. IDH1-nonR132H mutations are more frequent in astrocytomas compared to oligodendrogliomas and mixed gliomas. **: p=0.0018, ***: p<0.0001, Fishers' exact test.

Table 5: Distribution of IDH1/2 mutations in different histologic subtypes of gliomas by synthesizing 5 studies of gliomas from different research centers

		IDH1 R132H mutations	Non *IDH1* R132H mutations	*IDH2* mutations
Astrocytic tumors	AII	253 (86.9%)	34 (11.7%)	4 (1.4%)
	AIII	183 (85.9%)	27 (12.7%)	3 (1.4%)
Mixed tumors	OAII	163 (91.6%)	9 (5.1%)	6 (3.4%)
	OAIII	208 (88.5%)	15 (6.4%)	12 (5.1%)
Oligodendroglial tumors	OII	317 (90.1%)	12 (3.4%)	23 (6.5%)
	OIII	333 (79.1%)	18 (4.3%)	18 (4.3%)

c. Distribution of IDH2 mutations in gliomas

IDH2 mutations (<8%) are much less frequent than *IDH1* mutations and are associated with oligodendrogliomas (89). *IDH2* mutations are present in 1-7% of diffuse astrocytomas, 4-5% of oligodendrogliomas, ≤1% of oligoastrocytomas, 1-4% of anaplastic astrocytomas, 5-8% of anaplastic oligodendrogliomas, and ≤6% of anaplastic oligoastrocytomas, but are not observed in either primary or secondary glioblastomas (Table 4). In our series, only 3.1% of *IDH2* mutations were observed in 996 gliomas. The *IDH2* mutations were over-present in oligodendrogliomas (6.3%), compared to astrocytomas and mixed gliomas (3.5%; p= 0.045).

Analysis of 5 studies from different research centers (Table 6) shows that *IDH2* mutations were significantly more frequent in oligodendrogliomas (p<0.0001) and in mixed gliomas (p=0.0074) compared to astrocytomas (Figure 13).

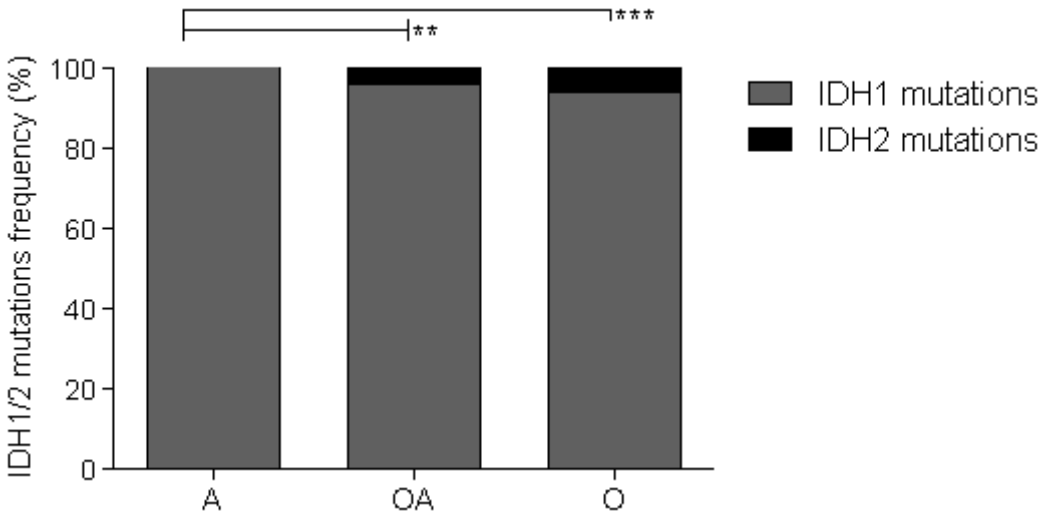

Figure 13: Distribution of *IDH2* mutations in different histological subclasses of gioma by synthesizing 5 studies of gliomas. *IDH2* mutations are rarely observed in astrocytomas and more frequent in oligodendrogliomes and mixed gliomas. **: p=0.0074*: p<0.0001, Fishers' exact test.**

d. Distribution of IDH1/IDH2 mutations in Gangliogliomas

The frequency of *IDH1* mutations in gangliogliomas (38%), anaplastic gangliogliomas (60%) (93) is noteworthy. Gangliogliomas are constituted by neoplastic ganglion and glial cells. Sometime, it is difficult to distinguish these tumors from infiltrative gliomas. 16.3% *IDH1/2* mutations (8 *IDH1* mutations and 8 *IDH2* mutations) were found in 98 gangliogliomas by Horbinski et al. Higher age, increased risk of recurrent, worse outcome were showed in patients with *IDH1/2* mutated gangliogliomas. They suggested that *IDH1*/*2* mutations had the value of diagnosis in gangliogliomas to differentiate from infiltrative gliomas (103) (Table 5).

e. Distribution of IDH1/IDH2 mutations in other CNS tumors

In contrast to diffuse gliomas, *IDH* mutations are very rare or absent in the other central nervous system tumours (Table 5). *IDH* mutations have not been detected in medulloblastomas, schwannomas, meningiomas, dysembryoplastic neuroepithelial tumors, or pleomorphic xanthoastrocytomas.

f. Distribution of IDH1/IDH2 mutations in pediatric gliomas

IDH1 and *IDH2* mutations which are exceedingly rare in pediatric gliomas support the concept that the pediatric tumors and their adult counterparts have an essentially different origin. Rare studies determined that *IDH* mutations occur preferentially in the older patients and demonstrated better outcome among pediatric patients with gliomas (100,104,105) (**Article 6**).

Table 5: Frequency of *IDH* mutations in other CNS tumors

Other gliomas	*IDH1/2* mut	Authors (year)
Ganglioglioma	16.3% (16/98)	Horbinski, 2010(103)
	38% (3/8)	Sonoda, 2009(93)
	41.9% (13/31)	WANG XW
Anaplastic ganglioglioma	60% (3/5)	Sonoda, 2009(93)
Other brain tumors		
Medulloblastoma	0% (0/58)	Balss, 2008(45)
	0% (0/55)	Yan, 2009(47)
	0% (0/36)	Ichimura, 2009(46)
PNET	33% (3/9)	Balss, 2008(45)
	0% (0/8)	Ichimura, 2009(46)
DNET	0% (0/4)	Ichimura, 2009(46)
Vestibular schwannoma	0% (0/17)	Balss, 2008(45)
	0% (0/48)	Ichimura, 2009(46)
Meningioma	0% (0/72)	Balss, 2008(45)
	0% (0/29)	Kang, 2009(101)
	0% (0/48)	Ichimura, 2009(46)
Gliosarcoma	0% (0/5)	Balss, 2008(45)
Pleomorphic xanthoastrocytomas	0% (0/42)	Felsberg, 2010(97)
Gliomatosis cerebri	29% (10/35)	Seiz, 2010(106)
	40% (17/40)	Desestret, 2011 (107)
Chordoid glioma	0% (0/1)	Horbinski, 2009(90)
Hemangioblastoma	0% (0/1)	Horbinski, 2009(90)
Hemangiopericytoma	0% (0/1)	Horbinski, 2009(90)

3. Correlation of *IDH1*/*IDH2* mutations with age at diagnosis

Several studies showed that patients with *IDH1* mutated grades II, III and IV glioma were younger than those without *IDH1* mutations (Table 6). A few reports compared the mean age at diagnosis to the types of *IDH1* mutations. Hartmann et al. (89) observed that patients harbouring the most common R132H mutation were older than those harbouring rare non-R132H mutations (mean age of 42.9 years vs 34.9 years; $p<0.01$), R132G mutation (37.9 years; not significant), and R132S mutation (36.2 years; $p<0.01$). Notably, Jha et al. (100) also confirmed this observation (R132H mutation 36.8 years *vs*. non-R132H mutation 29.3 years; $p=0.047$). Noteworthy, multivariate analyses have confirmed the independent prognostic value of *IDH1* mutations, despite age is *per se* an independent prognostic factor in gliomas. However, despite the lower mean age at diagnosis of patients with *IDH* mutations, *IDH*-mutated cancers occur rarely in glioma patients aged 18 years or younger (47,69,89,104).

Table 6: *IDH1* mutations with age in different subtypes of gliomas

Authors, year	Mean age with/without mutation (years)						
	A (II)	O (II)	OA (II)	AA (III)	AO (III)	AOA (III)	GBM (IV)
Parsons, 2008 (44)							33/53 (p<0.001)
Balss, 2008 (45)	N.S.	N.S.	N.S.	35.0/44.4 (p<0.05)	47.7/54.8 (N.S.)	44.3/63.5 (p<0.0005)	40.3/52.6 (p<0.005)
Watanabe, 2009 (48)	36.2/51.6 P=0.0004	42.6/37.1 P=0.307	-	-	-	-	44.2/56.4 (p=0.0001)
Hartmann, 2009 (89)	41.3/42.8 (not significant)				43.9/50.6 (P<0.0001)		
				P<0.01	N.S.	P<0.01	
Ichimura, 2009 (46)							41/56 (p=0.002)
Yan, 2009 (47)	35/5	37/13.5		34*/56* P<0.001			32*/59* P<0.001
Korshunov, 2009 (91)	36.5/37.4						
Nobusawa, 2009 (92)							47.9/60.6 P<0.0001
Sonoda, 2009 (93)							35/57 (p=0.0158)
Felsberg, 2010 (97)							42*/60*
Van den bent, 2010 (98)					Younger age P=0.0021		
Jha, 2011 (100)	30.1/31 P=0.8003	37.9/29.4 P=0.012					46.7/53.2 (p=0.2185)

A: Astrocytoma; O: Oligodendroglioma; OA: Oligoastrocytoma; AA: Anaplastic astrocytoma; AO: Anaplastic oligodendroglioma; AOA: Anaplastic oligoastrocytoma; pGBM: Primary Glioblastoma; sGBM: Secondary Glioblastoma; *median

4. Association of *IDH* mutations with other genetic alterations in gliomas

We found an association of *IDH1/2* mutations with 1p19q codeletion, *MGMT* promoter methylation, *EGFR* amplification, chromosome 10 loss, but not with *TP53* mutation. These findings are mostly in line with published studies that found *IDH1*/*IDH2* mutations positively associated with 1p19q codeletion, *MGMT* promoter methylation, but also with *TP53* mutation, and inversely associated with *EGFR* amplification, *PTEN* mutations, *CDKN2A/2B* deletion, Chromosome 10 loss and *BRAF* gene fusion (Table 7).

a. Correlation with 1p19q codeletion

1p19q codeletion is commonly found in oligodendroglial tumors and is absent in *IDH* wild-type tumors. *IDH* mutation is a constant feature in 1p19q codeleted gliomas (52). Gravendeel et al. (96) studied 496 gliomas and demonstrated that non-R132H mutations are virtually absent in tumors with 1p/19q codeletion.

b. Correlation with TP53 mutations

TP53 mutations (>65%) are more frequent in astrocytic tumors. Like Dubink et al but in contrast to most studies, we did not find *TP53* mutation correlated with *IDH* mutation. We found the majority of *TP53* mutations in the group *IDH1/2* mutated- 1p19q non codeleted. When excluding 1p19q codeleted tumors (mostly oligodendrogliomas), *TP53* mutation was correlated with *IDH* mutation (p= 0.0016) (91,96) (Table 7).

Gravendeel et al. (96) found that non-R132H *IDH1* mutations occur at significantly higher frequency in gliomas with *TP53* mutations. Five of patients with astrocytomas with Li-Fraumeni syndrome had an *IDH1*R132C mutation (48). This may suggest that gliomas cells with IDH1-nonR132H mutations have an increased risk to acquire a *TP53* mutation.

The *IDH1/2* wild type grade II gliomas are unfrequent. None are 1p19q codeleted, a few are *TP53* mutated, and the majority are therefore “triple negative” This subgroup represent only 7% of low-grade gliomas and is characterized by a poor prognosis and distinctive radiologic characteristics (infiltrative pattern, larger axial diameter, higher frequent in frontotemporo-insular location) **(99)**. Kim, et al. **(108)** recently showed that the alterations of the RB1 pathway commonly occured in low-grade gliomas without *IDH1/2* mutations,

TP53 mutations and 1p19q codeletion. Patients with RB1 pathway altered low-grade gliomas have a poor prognosis.

c. Association with MGMT promoter methylation

IDH mutations are associated with *MGMT* promoter methylation. A methylated *MGMT* promoter is observed in low-grade gliomas, anaplastic gliomas (60-93%) and GBM (45%) (61,62). Methylation of *MGMTP* is associated with chemosensitivity (62,63) . These features may be related to the CpG hypermethylation induced by 2HG (96,109,110) .

Table 7: Correlation of *IDH* mutations with other genetic alterations in gliomas

Authors, year	*IDH*status	*TP53* mutation	1p19q codeletion	*EGFR* amplification	*MGMT* methylation	*PTEN* mutations	*CDKN2A/2B* deletion;	Chromosome 10 loss;	*BRAF* fusion
Wang XW		Not significant P=0.9124	Positive correlation P< 0.0001	Inverse correlation P<0.0001	Positive correlation P<0.0001			Inverse correlation P<0.0001	
Watanabe, 2009 (48)	*IDH1* mut	Positive correlation P=0.015	Not significant P=0.658	-		-			
Ichimura, 2009 (46)	*IDH1* mut	Positive correlation P<0.001	Positive correlation P<0.001	Inverse correlation P<0.001		Inverse correlation P<0.001	Inverse correlation P<0.001	Inverse correlation P<0.001	
Yan, 2009 (47)	*IDH1/2* mut	Positive correlation P<0.001	Positive correlation P<0.001	Inverse correlation P<0.001		Inverse correlation P<0.001	Inverse correlation P<0.001	Inverse correlation P<0.001	
Sanson, 2009 (91)	*IDH1* mut	Not significant P=0.5	Positive correlation $P=10^{-6}$	Inverse correlation P<0.004	Positive correlation P<0.001			Inverse correlation $P<10^{-15}$	
Korshunov, 2009 (91)	*IDH1* mut								Inverse correlation P<0.0001
Nobusawa, 2009 (92)	*IDH1* mut	Positive correlation P<0.0001	Positive correlation (LOH 19q) P<0.0001	Inverse correlation P=0.005		Not significant		Not significant (LOH 10q)	
Weller, 2009 (103)	*IDH1* mut	Positive correlation P=0.005		Inverse correlation P=0.0003				Inverse correlation (LOH 10q) P<0.0006	
Wick, 2009 (95)	*IDH1* mut		Positive correlation		Positive correlation				
Gravendeel, 2010 (96)	*IDH1* mut	Positive correlation (non R132H mut) P<0.05	Inverse correlation (non R132H mut) P<0.01						
Van den bent, 2010 (98)	*IDH1/2* mut		Positive correlation P=0.001	Inverse correlation P=0.0007	Positive correlation P<0.0001			Inverse correlation P<0.001	
Jha, 2011 (100)	*IDH1* mut	Positive correlation P=0.004	Not significant	Not significant		Not significant			
Dubbink, 2009 (95)	*IDH1/2* mut	Not significant							
Metellus, 2010(99)	*IDH1/2* mut	Positive correlation P=0.014	Positive correlation P=0.031						

II. Diagnostic value of *IDH1/2* mutations and new methods for IDH status determination

1. Sensitive methods for IDH status dermination

These methods are either based on direct *IDH* mutation determination or detection of 2-HG

Immunohistochemical analysis of gliomas with an *IDH1 R132H* mutation-specific monoclonal antibody (DIANOVA, clone H09) is easy and effective to test *IDH* mutations in a clinical setting. The anti-IDH1 R132H antibody can even detect single glioma cells with *IDH1 R132H* mutation which cannot be detected by sequencing. Thisantibody allows to differentiate the glioma cells with *IDH R132H* mutations from non-neoplastic glial cells (111,112).

Double COLD PCR HRM assay is a fast, powerful sensitive method to find *IDH1* R132H mutation in samples with very few glioma cells. We **(113)** have reported that this assay is more sensitive than *IDH1* R132H mutation-specific monoclonal antibody in biopsies of tumor edges (**Article 7**). In the future, this approach might be used for glioma diagnosis by detection of mutation on free plasmatic DNA (see below).

2. Diagnostic value

Since *IDH1/2* mutations are restricted to gliomas and particularly specific subtypes, it is helpful in the following diagnostic situations:

a -to recognize diffuse gliomas (astrocytomas or oligodendroglioma) (45,48);

b -to distinguish diffuse astrocytomas (WHO grade II) from pilocytic astrocytomas (WHO grade I) (91);

c -to differentiate between prGBM and sGBM, which have indistinguishable histological features (114);

d –to differentiate oligodendrogliomas from glioneuronal tumors, such as central neurocytomas, oligodendroglial-like PA I(47,45);

e –to distinguish diffuse gliomas from reactive gliosis (111);

f –to detect the presence of few single cells with anti-*IDH1*R132H antibody, or with COLD PCR in small samples higly contaminated with normal tissu (111–113) ;

g –to identify residual glioma cells in gliomas with post-therapy evolution (114).

3. Non invasive detection of *IDH1/2* mutations-Detection of *IDH* mutation from free plasmatic DNA

The identification of a reliable biomarker in accessible bodily fluids would be useful for diagnostic, prognostic and follow-up purposes (115). Tumor-free DNA, as evidenced by the presence of a specific mutation, provides an optimum marker. *IDH1/2* mutations are frequent, R132 specific in genomic site, and have a strong diagnostic, prognostic or predictive value in gliomas (44,50,49,92,98).

Correlated COLD PCR with MRI data can detect IDH^{R132H} mutations in free plasma DNA from glioma patients. The small-size DNA (150bp to 250 bp, ssDNA) concentration was markedly associated with grade and enhancing tumor volume. Importantly, IDH^{R132H} mutation in plasma DNA samples from patients with mutated gliomas is feasible, confirming that ssDNA originates from tumors cells. Because *IDH1* mutations are rare in other CNS tumors, a positive $IDH1^{R132H}$ mutation in plasma DNA has a high non-invasive diagnosed value for gliomas.

4. Detection of 2-HG as a biomarker in gliomas

Gliomas with *IDH1*/*IDH2* mutations have a common feature, elevated levels of 2HG. 2HG are over 100-fold higher in gliomas with *IDH1/2* mutations versus gliomas with wild-type *IDH1/2* and normal brain tissue.

a. 2-HG detection

The quantity of 2-HG in urine was significantly higher in patients with *IDH* mutations (Figure 14).

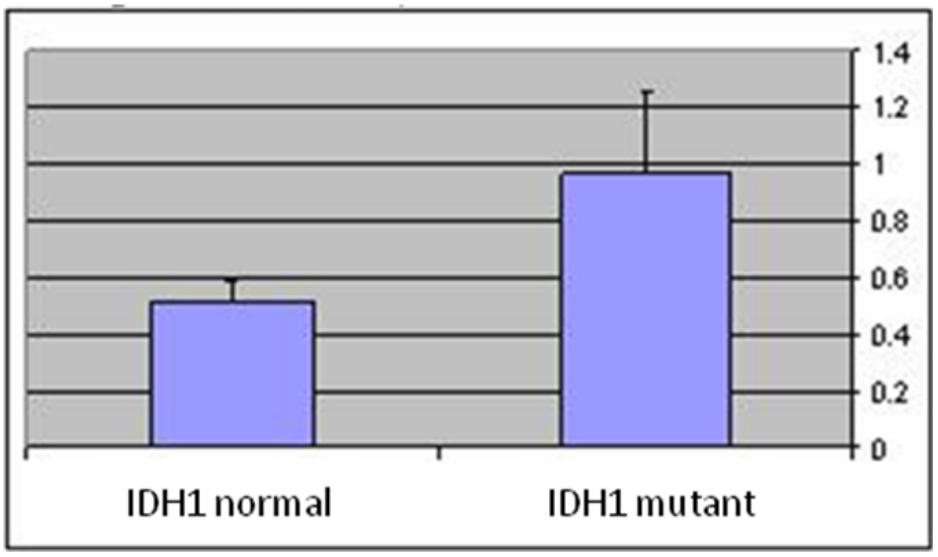

Figure 14: Determination of D 2 hydroxyglutarate in urine from glioma patients under treatment. N=15, mean +s.e.m. (collaboration Service de Biochimie Métabolique, Hôpital Necker – Dr C. Ottolenghi).

b. 2-HG detection by spectro-MRI

No invasive detection of 2HG by optimized MRS methods has been reported in patients with gliomas. The accumulation of 2HG levels was concordant with *IDH1/2* mutations in tumor tissue. The signal overlaps of 2HG with GABA, glutamate and glutamine were overcome with optimizing management. Until date, it is the only direct metabolic consequence that can be identified through noninvasive imaging in a genetic mutant cancer cell. 2HG is regarded as an important biomarker to guide diagnosis, prognosis and clinical follow-up post treatment in gliomas and may guide adapted therapy in patients with gliomas (116,117).

III. Prognostic/ predictive value of *IDH1/2* mutations

The vast majority of studies including our study have confirmed that *IDH1/2* mutation is a major prognostic factor in gliomas of grades II-IV and in both astrocytic and oligodendroglial gliomas (Table 8). However it is still unknown wether IDH1 status may predict the response to treatment

Previous data from literature demonstrated that a clear inverse relationship was observed between the level of *IDH1* and *IDH2* expressed in target cells and their susceptibility to apoptosis after exposure to ROS (118), ultraviolet B-induced phototoxicity (73), singlet oxygen species (119), high glucose (120), heat shock (121), tumor necrosis factor-α (122), ionizing radiation (123) or gamma irradiation (124). We hypothesize that IDH mutation could make the cell more sensitive to the oxidative stress, and particularly to radiotherapy.

1. Response to temozolomide (TMZ)

The NOA-04 (95), EORTC 26951 trial (98) and a GBMs group (102) failed to show that *IDH1* mutation status predict the response to treatment. Dubbink et al. (94) studied the response to temozolomide (TMZ) in patients with dedifferentiated low-grade astrocytomas treated at progression after radiotherapy, and found no difference between *IDH1* mutant and wild-type tumor patients.

The German NOA-04 trial studied WHO grade III glioma patients which received primary radiotherapy or primary chemotherapy and followed these treatments at progression. Patients with anaplastic oligodendroglial tumors in EORTC 26951 were treated with radiotherapy alone or combined with PCV (procarbazine, lomustine, and vincristine). None of these two trials found any predictive value of *IDH1* mutations to PCV treatment.

However, our retrospective study suggested an influence of *IDH1* mutations on the response to chemosensitivity of low-grade gliomas, treated at progression (125) (**Article 8**).

2. Response to radiotherapy

Ionizing radiation plays a major role in malignant tumor control through the induction of mitotic cell death and inhibition of proliferation (126). However, resistance of many tumors to consecutive radiation constitutes a major problem in the cancer therapy. It has been recognized that tumorigenesis and development of radioresistance are both related to change in tumor environment by hypoxia and acidosis and the dysregulation of oncogenes and tumor suppressor genes (127,128). Unexpectedly, our study suggests a radiosensitizing effect of *IDH1*R132H in glioblastoma cell line in hypoxic but not normoxic condition. *IDH1* and *IDH2* mutations result in the reduced production of α-KG, low NADPH levels, and elevated levels of 2-hydroxyglutarate (2HG) (47,80). Until recently, *IDH1/2* mutation were believed to upregulate HIF first because of low α-KG (79) and secondly because 2-HG was believed to act as a inhibiting competitor of α-KG (81), resulting in the inhibition of HIF degradation. Recently Koivunen et al. (82) showed that R-2HG (but not L-2HG) instead of being an inhibitor of EGLN (HIF prolyl 4-hydroxylases) activity, was an agonist and promotes the degradation of HIF. Because HIF protects cells from irradiation therapy under hypoxic condition, we may hypothesize that *IDH* mutation, by inducing an inappropriate degradation of HIF, could make the mutated cell more vulnerable to RT.

Elevated reactive oxygen species (ROS) induced by hypoxia (129), ionizing radiation (130) and 2-HG (87) result in the damage of DNA in cells. Mailloux et al. (77) found that the production of α-KG and NADPH by IDH1/2 increased in oxidative stress. The reduced production of α-KG and NADPH and markedly increased concentration of 2-HG may lead to the reduction of antioxidants (α-KG, GSH) which can protect cells from oxidative stress. So the impact of ROS to damage DNA in IDH1/2 mutant cells may be higher than in IDH1/2wt cells. In several studies, the role of *IDH1* and *IDH2* in cellular defense against oxidative stress was investigated by comparing the cellular responses after stable transfection of *IDH1*/*IDH2* cDNA into the target cells in sense and antisense orientations. These studies demonstrate an inverse relationship between the level of *IDH1* and *IDH2* expressed in target cells and their susceptibility to apoptosis after exposure to different treatments (73,74,118–124,131). These results indicate that an important role of *IDH1* and *IDH2* in protection against oxidative damage.

In conclusion, this study suggests a radiosensitizing effect of *IDH1*R132H in glioblastoma cell lines under hypoxic condition. Such effect need however to be confirmed on clinical setting.

Table 8: *IDH1* mutations with survival in different subtypes of gliomas

Authors, year	Median PFS and OS with/without mutation (months)							
	A (II)	**O (II)**	**OA (II)**	**AA (III)**	**AO (III)**	**AOA (III)**	**prGBM (IV)**	**sGBM (IV)**
Wang XW	OS: 136.5 vs 67.0 (p< 0.0001) PFS: 41.3 vs 28.5 (p=0.0199)			OS: 136.9 vs 20.1 (p<0.0001) PFS: 34.6 vs 10.4 (p<0.0001)			OS: 27.4 vs 14.3 (p=0.0002) PFS: 10.6 vs 8.1 (p=0.0001)	
Parsons, 2008 (44)							OS: 45.6 *vs* 13.2 (p<0.001)	
Yan, 2009 (47)				OS: 65 *vs* 20 (p<0.001)			OS: 31 *vs* 15 (p=0.002)	
Sonoda, 2009 (93)				OS: 50 *vs* 22 (p<0.001)			OS: 66 *vs* 17	
Weller, 2009 (102)							OS: 30.2 *vs* 11.2 (p=0.002) PFS: 16.2 *vs* 6.5 (p<0.001)	
Dubbink, 2009 (94)	OS: 98 *vs* 48 (p=0.003)							
Gravendeel, 2010 (96)							OS: 24 vs 8.6 (p<0.001)	
Felsberg, 2010 (97)							OS: longer (p=0.012)	
Van den bent, 2010 (98)					OS: non reached *vs* 16 PFS: 50 *vs* 7.8			
Metellus P, 2010 (99)	OS (5-year): 93% *v* 51% (p = 0.000001)							
Sanson, 2009 (49)	OS: 150.9 *vs* 60.1 (p=0.01) PFS: 35.2 *vs* 44.5 (NS)			OS: 81.1 *vs* 19.4 (p<0.001) PFS: 37 *vs* 9.2 (p<0.001)			OS: 27.4 *vs* 14 (p<0.01) PFS: 55 *vs* 8.8 (p=0.01)	
Nobusawa, 2009 (92)							OS: 31.6 *vs* 10 OS: 24 *vs* 9.9 (p<0.0001)	
Ichimura, 2009 (46)	OS: longer in patients with *IDH1* mutations in all subtypes							

PFS: progression free survival; OS: overall survival; NS: not significant

PERSPECTIVES

Search for *IDH1* mutation is now routinely performed in most neuro-oncological centers using antibodies specific for IDH1R132H. Based on COLD-PCR technique, *IDH1/2* mutations can be detected from plasma in patients not amenable to biopsies. Since all *IDH1/2* mutations produce 2-HG in tumors, the detection of D-2-HG in biological liquid appears an attractive method, but urinary and plasmatic dosages have shown unsufficient specificity and sensiticity, unlike LAM **(132)**. In contrast, MRS appears a very promising method to detect 2-HG on MRI for both the diagnosis and the follow-up **(116,117)**.

IDH1/2 mutations demonstrate a favorable clinical outcome in all types of gliomas. It is still unclear whether it is also a predictor of chemo(radio)sensitivity. Additional in vitro and in vivo experiments are needed, and IDH1 assessement in ongoing prospective trials is warranted to answer this question.

Targeted therapeutic approaches would include altering the expression of IDH1/2 mutant enzymes, developing inhibitors of neomorphic enzyme activity, targeting certain downstream effects (hypermethylation phenotype), or using glutaminase inhibitor to block αKG supply by glutamine pathway, as recently shown in vitro **(133)**.

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ANNEXES

Article 4

Sanson M, Hosking FJ, Shete S, Zelenika D, Dobbins SE, Ma Y, Enciso-Mora V, Idbaih A, Delattre JY, Hoang-Xuan K, Marie Y, Boisselier B, Carpentier C, **Wang XW**, Di Stefano AL, Labussière M, Gousias K, Schramm J, Boland A, Lechner D, Gut I, Armstrong G, Liu Y, Yu R, Lau C, Di Bernardo MC, Robertson LB, Muir K, Hepworth S, Swerdlow A, Schoemaker MJ, Wichmann HE, Müller M, Schreiber S, Franke A, Moebus S, Eisele L, Försti A, Hemminki K, Lathrop M, Bondy M, Houlston RS, Simon M.

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Chromosome 7p11.2 (*EGFR*) variation influences glioma risk

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While gliomas are the most common primary brain tumors, their etiology is largely unknown. To identify novel risk loci for glioma, we conducted genome-wide association (GWA) analysis of two case–control series from France and Germany (2269 cases and 2500 controls). Pooling these data with previously reported UK and US GWA studies provided data on 4147 glioma cases and 7435 controls genotyped for 424 460 common tagging single-nucleotide polymorphisms. Using these data, we demonstrate two statistically independent associations between glioma and rs11979158 and rs2252586, at 7p11.2 which encompasses the *EGFR* gene (population-corrected statistics, $P_c = 7.72 \times 10^{-8}$ and 2.09×10^{-8}, respectively). Both associations were independent of tumor subtype, and were independent of *EGFR* amplification, *p16INK4a* deletion and *IDH1* mutation status in tumors; compatible with driver effects of the variants on glioma development. These findings show that variation in 7p11.2 is a determinant of inherited glioma risk.

INTRODUCTION

Gliomas account for ~40% of all primary brain tumors and are responsible for around 13 000 deaths in the USA each year. Gliomas are heterogeneous and different tumor subtypes defined in part by malignancy grade [e.g. pilocytic astrocytoma WHO grade I, diffuse 'low grade' glioma WHO grade II, anaplastic glioma WHO grade III and glioblastoma (GBM) WHO grade IV] can be distinguished. Most gliomas are associated with a poor prognosis irrespective of clinical care, with the most common type of glioma, GBM, having a median overall survival of 10–15 months (1–3).

While glioma subtypes have distinct molecular profiles resulting from different etiological pathways, no lifestyle exposure have, however, consistently been linked to glioma risk except for ionizing radiation, which only accounts for a very small number of cases (4). Direct evidence for inherited predisposition to glioma is provided by a number of rare inherited cancer syndromes, such as Turcot's and Li–Fraumeni syndromes, and neurofibromatosis (4,5). However, collectively, these diseases account for little of the 2-fold increased risk of glioma seen in first-degree relatives of glioma patients (6), and much of the excess familial risk is likely to be a consequence of the co-inheritance of multiple low-risk variants.

To search for genetic risk variants influencing glioma, we have previously conducted genome-wide association (GWA) studies of UK and US glioma patients with replication of the most significantly associated single-nucleotide polymorphisms (SNPs) in independent case–control series from France, Germany and Sweden. This analysis robustly demonstrated that common variants mapping to 5p15.33 (*TERT*), 8q24.21 (*CCDC26*), 9p21.3 (*CDKN2A/CDKN2B*), 20q13.33 (*RTEL1*) and 11q23.3 (*PHLDB1*) contribute to heritable risk of glioma (7). Confirmation of the 9p21.3 and 20q13.33 loci as risk factors for GBM was provided by a contemporaneous study (8).

GWA studies are not contingent on prior information concerning candidate genes or pathways, and thereby have the ability to identify important variants in hitherto unstudied genes. However, the effect sizes of individual variants and the need for stringent thresholds for establishing statistical significance inevitably constrain study power. To increase our power to identify novel genetic risk loci for glioma, we have performed GWA scans on augmented series based on the aforementioned French and German cases and combined these data with our GWA scans of US and UK glioma patients. This analysis identifies genetic variation at 7p11.2 as risk factors for the development of glioma and provides further insight into the biological basis of tumor development.

RESULTS

The four GWA studies collectively provided data on 4147 glioma cases and 7435 controls genotyped for 424 460 common tagging SNPs. As the quantile–quantile (Q–Q) plots for the German and US series showed some evidence of inflation [inflation factor $\lambda = 1.16$ and 1.11, respectively, based on the 90% least significant SNPs (9)], we corrected for population substructure using principal-components analyses as implemented in Eigenstrat (Supplementary Material, Fig. S1). This analysis reduced all four inflation factors to <1.05.

Meta-analyses of these data resulted in 37 SNPs showing an association with glioma risk at $P < 5.0 \times 10^{-7}$; 35 of these map to the five loci we have previously shown (7) to influence disease risk namely, 5p15.33, 8q24.21, 9p21.3, 20q13.33 and 11q23.3 (Supplementary Material, Table S1). The strongest evidence for a novel association was provided by two SNPs mapping to 7p11.2.

The two SNPs on 7p11.2, rs11979158 and rs2252586, mapping to 55 126 843 and 54 946 418 bp, yielded *P*-values of 7.03×10^{-8} and 7.89×10^{-8}, population-corrected *P*-values using EIGENSTRAT software, $P_c = 7.72 \times 10^{-8}$ and 2.09×10^{-8}, respectively [odds ratio (OR) = 1.23, 95% confidence interval (CI): 1.15–1.35 and OR = 1.18, 95% CI: 1.11–1.25, respectively; Table 1, Fig. 1]. For completeness, we also derived population-corrected *P*-values using PLINK software (*P*-values for rs11979158 and rs2252586 were 3.11×10^{-7} and 4.55×10^{-7}). Although only one of the associations when corrected for population stratification attained the conventional threshold for genome-wide significance (i.e. $P \leq 5.0 \times 10^{-8}$), both associations were consistent across the four GWA studies ($P_{het} = 0.44$ and 0.27, respectively; Table 1). The SNP rs11979158 maps within intron 1 of the Epidermal Growth Factor Receptor 1 (*EGFR*) gene and rs2252586 lies 107 kb telomeric from *EGFR* (Fig. 1). Several lines of evidence support the two SNPs defining independent disease loci. First, there is low linkage disequilibrium (LD) between the two SNPs ($D' = 0.62$, $r^2 = 0.03$).

Table 1. Novel loci with single SNP significance of $P < 5.0 \times 10^{-7}$ for association with glioma on 7p11.2

	Study	Case genotypes			Control genotypes			Case MAF	Control MAF	OR (95% CI)*	*P*-value	Population-corrected *P*-value
		Common homozygotes	Heterozygotes	Rare homozygotes	Common homozygotes	Heterozygotes	Rare homozygotes					
rs11979158; 55 126 843 bp; minor allele = G; risk allele = A	France	1091	310	22	858	313	18	0.124	0.147	1.22 (1.03–1.43)	0.017	6.40×10^{-3}
	Germany	635	193	17	893	371	46	0.134	0.177	1.36 (1.16–1.64)	2.70×10^{-4}	7.75×10^{-3}
	UK	471	142	18	1836	776	85	0.141	0.175	1.28 (1.09–1.54)	3.75×10^{-3}	2.75×10^{-3}
	USA	908	312	27	1564	601	71	0.147	0.166	1.15 (1.01–1.32)	0.036	0.031
	Combined									1.23 (1.15–1.35)	7.03×10^{-8}	7.72×10^{-8}
rs2252586; 54 946 418 bp; minor allele = T; risk allele = T	France	601	636	185	580	496	114	0.354	0.304	1.25 (1.11–1.41)	1.84×10^{-4}	7.09×10^{-5}
	Germany	366	374	106	652	536	120	0.346	0.297	1.25 (1.10–1.43)	7.24×10^{-4}	8.05×10^{-4}
	UK	285	278	68	1335	1131	232	0.328	0.296	1.16 (1.02–1.33)	0.024	0.020
	USA	580	551	116	1091	962	183	0.314	0.297	1.09 (0.97–1.20)	0.133	0.103
	Combined									1.18 (1.11–1.25)	7.89×10^{-8}	2.09×10^{-8}

*ORs and 95% CI per copy of the risk allele.

Figure 1. Plot of association results and recombination rates for the 7p11.2 (*EGFR*) region. $-\log_{10}P$-values (*y*-axis) of the SNPs are shown according to their chromosomal positions (*x*-axis). The top two genotyped SNPs, rs11979158 and rs2252586, are labeled. The color intensity of each symbol reflects the extent of LD with the top genotyped SNP; $r^2 > 0.8$ being represented with blue (with rs11979158) and red (with rs2252586) through to white ($r^2 < 0.2$). Genetic recombination rates (cM/Mb), estimated using HapMap CEU samples, are shown with a light blue line. Physical positions are based on build 36 (NCBI) of the human genome. Also shown is the relative position of *EGFR*.

Secondly, adjusting rs11979158 through conditional logistic regression for rs2252586 provided evidence of an association (OR = 1.20, 95% CI: 1.10–1.30; $P = 1.36 \times 10^{-5}$). Similarly, adjusting rs2252586 for rs11979158 also provided evidence of an association (OR = 1.14, 95% CI: 1.07–1.22; $P = 2.04 \times 10^{-5}$). Thirdly, there was also an increasing trend in OR with an increasing number of risk alleles (Fig. 2; Supplementary Material, Table S2). To examine the possibility that these SNPs may be correlated with an untyped variant, we made use of HapMap3 and 1000 Genomes data to impute additional variants localizing to 54 850 000–55 200 000 bp region. This analysis provided no SNPs with significantly superior evidence for an association and provides further support for the existence of two independent risk loci at 7p11.2 (Supplementary Material, Fig. S2).

We found no evidence for a pairwise interaction between rs2252586, rs11979158 and the five previously identified risk variants for glioma at 5p15.33 (rs2736100), 8q24.21 (rs4295627), 9p21.3 (rs4977756), 20q13.33 (rs6010620) and 11q23.3 (rs498872) ($P > 0.1$ after correction for multiple testing; Supplementary Material, Table S3). These data are consistent with each variant having an independent role in defining glioma risk whereby the risk increases with increasing number of variant risk alleles ($OR_{per\ allele} = 1.24$, 95% CI: 1.21–1.27, $P = 2.89 \times 10^{-72}$; Supplementary Material, Table S2).

To examine whether the implicated SNPs at 7p11.2 loci were differentially associated with tumor subgroup, we analyzed their prevalence according to histology across all cases where such data were available ($n = 4002$). The carrier frequencies of the risk alleles for the 7p11.2 SNPs showed no correlation with stratification of tumors by WHO grade in any of the four patient cohorts (Supplementary Material, Table S4). These observations contrast with the strong relationship between genotype and histology seen for 5p15.33 (*TERT*; rs2736100), 8q24.21 (*CCDC26*; rs4295627),

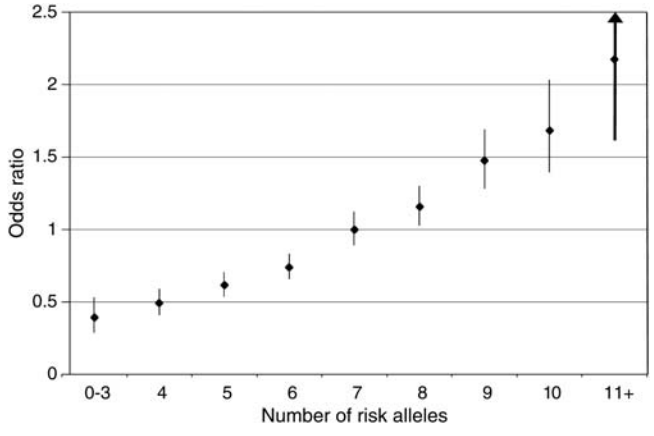

Figure 2. Cumulative effect of glioma risk alleles. Plot of increasing ORs for glioma for increasing number of risk alleles. The ORs are relative to the median number of eight risk alleles; vertical bars correspond to 95% CIs. Horizontal line marks the null value (OR = 1.0).

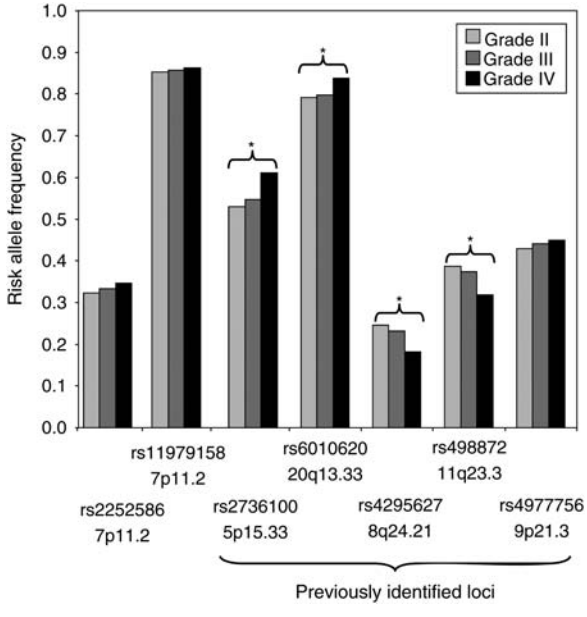

Figure 3. Relationship between 7p11.2 genotype and WHO tumor grade. Also shown are the relationships between histology and 5p15.33 (rs2736100), 8q24.21 (rs4295627), 9p21.3 (rs4977756), 20q13.33 (rs6010620) and 11q23.3 (rs498872) genotypes. *Significant association at $P < 0.05$.

20q13.33 (*RTEL1*; rs6010620) and 11q23.3 (*PHLDB1*; rs498872) risk variants (Fig. 3).

Primary and secondary forms of GBM are recognized; with secondary GBM developing through progression from low-grade diffuse astrocytomas or anaplastic astrocytomas. While usually indistinguishable histologically, distinct molecular pathways characterize the primary and secondary forms. Notably, *IDH1* mutations are commonly detectable in low-grade glioma and secondary GBM (>70% of cases) but are rare in primary GBMs (10). In addition, *EGFR* amplification and *p16INK4a* deletion are more frequent in primary disease (11). Tumor DNAs were available for a subset of the French cases ($n = 761$) permitting us to examine the relationship between SNP genotype and *EGFR* amplification, *p16INK4a* deletion and *IDH1* mutation status in glioma. For both 7p11.2 SNPs, no association was shown with *EGFR* amplification, *p16INK4a* deletion or *IDH1* mutation (Supplementary Material, Table S5) contrasting with the associations seen between rs2736100, rs4295627, rs498872 and *IDH1* status as well as rs4295627and *p16INK4a* status (Supplementary Material, Table S5).

To explore the possibility that 7p11.2 SNP genotype may influence tumor progression, we examined the relationship between genotype and patient outcome in both the French ($n = 1126$) and German ($n = 614$) case series. Survival analysis stratified by histology and adjusting for age at diagnosis, sex, preoperative Karnofsky performance index (KPI), degree of resection, chemotherapy and radiotherapy provided no evidence for an independent relationship between rs11979158 or rs2252586 SNP genotype and overall survival within each of the histological categories in either of patient cohort (Supplementary Material, Table S6).

DISCUSSION

This analysis has shown that the risk of developing glioma is influenced by genetic variation at 7p11.2. Our data support a previous report of an association between rs11979158 and GBM risk based on analysis of a smaller data set (12). These associations are a priori biologically plausible, since EGFR is the prototypical member of the ErbB/EGFR family, which is involved in multiple cellular processes, including cell division, migration, adhesion, differentiation and apoptosis. *EGFR* is well established to be pivotal in both initiation of primary GBM and progression of lower-grade glioma to grade IV (13); hence genetic variation in *EGFR* is an attractive basis for predisposition to glioma. Furthermore, the Cancer Genome Atlas (TCGA) consortium reported *EGFR* as the fourth most highly mutated gene in a compendium of common cancer genes sequenced in GBM (14). While *EGFR* amplification characterizes 40–70% of primary GBM, it is rarely seen in lower-grade astrocytomas, thereby implicating EGFR activation as a driver of glial tumorigenesis (13). There are multiple mechanisms by which EGFR mediates tumor initiation and progression, all of which are seen in primary glioma. Increased EGFR is common in primary GBM as a consequence of gene amplification and is often associated with exon 2–7 truncation resulting in constitutive receptor activation (EGFRVIII), or less frequently, an activating missense mutation of the EGFR extracellular domain (15–17). While speculative at this juncture, it is entirely plausible that the influence of SNP-mediated risk on glioma development is a consequence of a subtle effect on *EGFR* expression, thereby having the potential to impact on all histological forms of glioma.

We have previously shown that the *TERT* and *RTEL1* risk variants are primarily associated with high-grade disease and *CCDC26* and *PHLDB1* with the development of less aggressive glial tumors (18). Moreover, we have now shown that there are differences in the molecular phenotype with respect to *p16INK4a* and *IDH1* status for some of these associations. In contrast, *CDKN2A/CDKN2B* variation appears independent of the expression of a malignant phenotype,

compatible with a driver impact on tumorigenesis (18). Here we have implicated variation at *EGFR* as risk factors for all forms of glioma which is also reflected in no differences in molecular phenotype as defined by *EGFR*, *p16INK4a* or *IDH1* status. While these risk variants do not impact on disease outcome, they provide evidence that the glioma subtypes result from different etiological pathways.

As the SNPs genotyped during GWA studies are generally not themselves strong candidates for causality, enumeration of the causal variant at 7p11.2 will involve fine mapping and functional analyses to elucidate the causal basis of the association. While partly speculative as these SNPs are not correlated with known polymorphisms in the coding sequence of *EGFR*, the glioma association may be mediated through a change in gene expression rather than a sequence change in the expressed protein or through LD with low-frequency variants not catalogued. Although the risk of glioma associated with the 7p11.2 SNPs is modest, the carrier frequency of these risk alleles is high in the European population and therefore, irrespective of the causal basis of the association, these loci make a substantial contribution to the overall development of glioma.

In conclusion, this large study provides unambiguous evidence that common genetic variation in 7p11.2 influences the risk of developing glioma. Furthermore, our findings provide support for the notion that the glioma subtypes result from different etiologic pathways, rather than different stages of tumor evolution within a common carcinogenic pathway. The impact of the currently identified SNPs on glioma risk is small in isolation and hence individually they do not have immediate clinical application. However, the observed differences provide insight into the biological mechanisms that underscore inherited susceptibility to glioma.

MATERIALS AND METHODS

Ethics

Collection of blood samples and clinico-pathological information from patients and controls was undertaken with informed consent and relevant ethical review board approval in accordance with the tenets of the Declaration of Helsinki. Ethical committee approval for this study was obtained from relevant study centers [UK: South East Multicentre Research Ethics Committee (MREC) and the Scottish Multicentre Research Ethics Committee; France: APHP ethical committee-CPP (comité de Protection des Personnes); Germany: Ethics Commission of the Medical Faculty of the University of Bonn and USA: University of Texas MD Anderson Cancer Institutional Review Board].

Subjects

The present study is based on pooling data from GWA studies of UK, US, French and German case–control series. The UK and US GWA studies have been the subject of a previous publication (7). Briefly, the UK study was based on 636 cases (401 male; mean age 46 years) ascertained through the INTERPHONE Study (19). Individuals from the 1958 Birth Cohort served as a source of controls. The US study was based on 1281 cases (786 male; mean age 47 years) ascertained through the MD Anderson Cancer Center, Texas, between 1990 and 2008. Individuals from the Cancer Genetic Markers of Susceptibility (CGEMS) studies served as controls (20,21). The French GWA study comprised 1495 patients with glioma ascertained through the Service de Neurologie Mazarin, Groupe Hospitalier Pitié-Salpêtrière Paris. The controls were ascertained from the SU.VI.MAX (SUpplementation en VItamines et Mineraux AntioXydants) study of 12 735 healthy subjects (women aged 35–60 years; men aged 45–60 years) (22). The German GWA study comprised 880 patients who underwent surgery for a glioma at the Department of Neurosurgery, University of Bonn Medical Center, between 1996 and 2008. Control subjects were taken from three publicly available studies: KORA (Co-operative Health Research in the Region of Augsburg; $n = 488$) (23,24), POPGEN (Population Genetic Cohort; $n = 678$) (25) and from the Heinz Nixdorf Recall study ($n = 380$) (26).

Genotyping and molecular analysis

Genotyping of cases were conducted using Illumina Infinium HD Human610-Quad BeadChips according to the manufacturer's protocols (Illumina, San Diego, CA, USA; Supplementary Material S1). Descriptions of genotyping are included in Supplementary Material S1. To determine amplification, *p16INK4a* deletion and *IDH1* mutation status in gliomas, DNA was extracted from frozen tumors using a standard protocol. CGH-array analysis, *EGFR* amplification, *p16INK4a* deletion assessment and sequencing of *IDH1* were performed as previously described (2,27).

Statistical analysis

Genotype data from each of the four studies were filtered on the basis of pre-specified quality-control measures (Supplementary Material, Text S1). Individual SNPs were excluded from further analysis if they showed deviation from the Hardy–Weinberg equilibrium with a *P*-value of $<1 \times 10^{-5}$, an individual SNP genotype yield of <98% or a minor allele frequency of <5%. This filtering resulted in the use of 424 460 SNPs, common to the four case–control series. A total of 363 samples were removed during quality-control steps for reasons including a failure to genotype, unknown duplicates, closely related individuals and sex discrepancies (Fig. 4). Furthermore, for the UK control group, we made the exclusions as proposed by the Wellcome Trust Case Control Consortium (WTCCC; $n = 65$). For the other controls, we removed people with a history of cancer as well as those whose parents/grandparents were not born in the study regions ($n = 216$; Fig. 4). We then performed principal-components analyses to identify outlier samples to reduce the effects of population stratification (Supplementary Material, Fig. S1). These analyses resulted in the removal of 644 samples (145 cases and 499 controls), resulting in a total of 4147 patients and 7435 control subjects (Fig. 4).

The association between each SNP and risk of glioma was assessed by the Cochran–Armitage trend test. ORs and associated 95% CIs were calculated by unconditional logistic regression using R software. Relationships between multiple

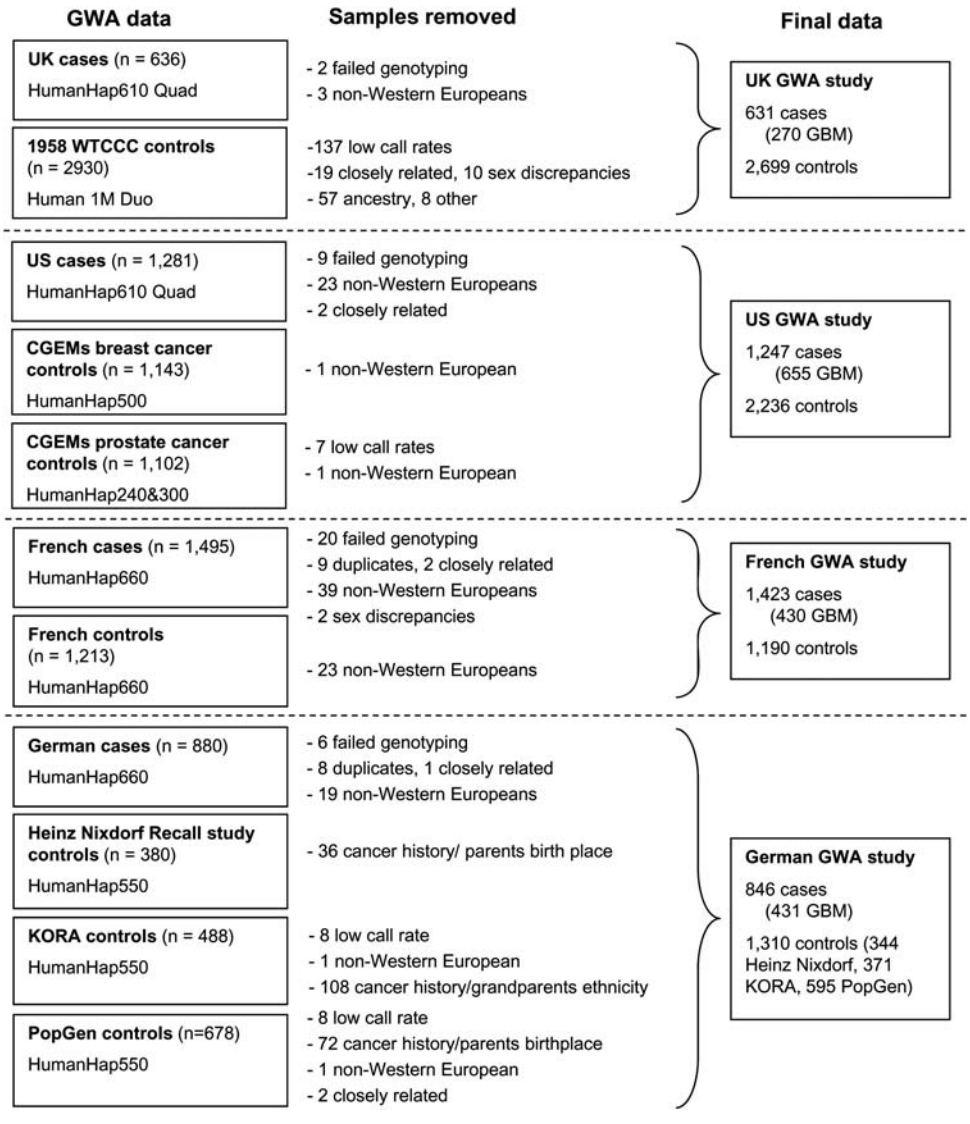

Figure 4. Patient exclusion schema for the genome-wide studies.

SNPs showing association with glioma risk in the same region were investigated using logistic regression analysis, and the impact of additional SNPs from the same region was assessed by a likelihood ratio test.

To further control for the potentially confounding influence of population stratification, we utilized the program Eigenstrat (28). The Eigenstrat algorithm adjusts genotypes and phenotypes by amounts attributable to ancestry using the calculated principal components of variation. These adjustments were applied to each of the four GWA studies correcting for the top 10 principal components of variation. Figure S3 (Supplementary Material) shows Q–Q plots before and after correction. Adjusted data from each of the four data sets were used to perform a meta-analysis using a modified Cochran–Armitage trend test. The results are reported as Eigenstrat *P*-values (P_c). The Eigenstrat algorithm involves the adjustment of genotypes and phenotypes by amounts attributable to ancestry using the calculated axes of variation. We also corrected for population structure using PLINK software (http://pngu.mgh.harvard.edu/~purcell/plink/) which implements a logistic regression analysis using 10 principal components as covariates.

Prediction of the untyped SNPs was carried out using IMPUTEv2, based on HapMapIII Release27 (February 2009, NCBI B36, dbSNP26) and the 1000 Genomes Project. Imputed data were analyzed using SNPTESTv2 to account for uncertainties in SNP prediction. LD metrics between HapMap SNPs were based on HapMapIII Release27, viewed using Haploview (v4.2) and plotted using SNAP.

Of the 4147 patients, clinical (age at diagnosis, sex, preoperative KPI , degree of resection, chemotherapy and radiotherapy) and histological covariate data obtained at diagnosis were available for most. Complete survival data were available for 1740 patients (77%) in the French and German cohorts, with a median follow-up interval of 8.9 years for patients without an event. Association analyses of 7p11.2 SNPs with clinical characteristics were performed with the χ^2 test on allele and genotype counts. Association with

overall survival was performed by comparing the Kaplan–Meier survival curves by means of the log-rank test in a pairwise fashion.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Article 5

Labussière M, Idbaih A, **Wang XW**, Marie Y, Boisselier B, Falet C, Paris S, Laffaire J, Carpentier C, Crinière E, Ducray F, El Hallani S, Mokhtari K, Hoang-Xuan K, Delattre JY, Sanson M.

"All the 1p19q codeleted gliomas are mutated on IDH1 or IDH2."

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All the 1p19q codeleted gliomas are mutated on *IDH1* or *IDH2*

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ABSTRACT

Background: Recently, the gene encoding the human cytosolic NADPH-dependent isocitrate dehydrogenase (*IDH1*) was reported frequently mutated in gliomas. Rare mutations were also found in the sequence of the mitochondrial isoform *IDH2*.

Methods: In a series of 764 gliomas genome-wide characterized, we determined the presence of mutations in the sequences of both *IDH1* and *IDH2* genes by direct sequencing.

Results: We found that all tumors with complete 1p19q codeletion (n = 128) were mutated in the *IDH1* (118) or *IDH2* (10) gene. This 100% mutation rate contrasted strikingly with other gliomas exhibiting either variable 1p and 19q alterations (n = 159, *IDH1/IDH2* mutation rate of 33%) or no 1p19q alteration (n = 477, *IDH1/IDH2* mutation rate 32%). Our data also confirm the prognostic impact of *IDH1/IDH2* mutation in gliomas whatever grade considered: patients harboring mutations of *IDH1/IDH2* have an improved median overall survival. Moreover, in WHO grade II and III gliomas, 3 groups with significantly different outcomes were identified according to their 1p19q and *IDH1/IDH2* statuses. Tumors carrying both alterations had longer overall survival than their nonmutated counterpart.

Conclusions: This exclusive association suggests a new mechanism of tumorigenesis. Perhaps the *IDH1/IDH2* mutation is a prerequisite for the occurrence of the t(1;19) translocation, or it is required for the 1p19q codeleted cells to acquire a tumor phenotype. ***Neurology*® 2010;74:1886–1890**

GLOSSARY

A = astrocytoma; **AI, AII, AIII** = astrocytoma of WHO grade I, II, and III; **aCGH** = array-based comparative genomic hybridization; **CI** = confidence interval; **GBM** = glioblastoma; **GBMO** = glioblastoma with oligodendroglial component; **IDH** = isocitrate dehydrogenase; **LOH** = loss of heterozygosity; **MS** = median survival; **O** = oligodendroglioma; **OII and OIII** = oligodendroglioma of WHO grade II and III; **OA** = oligoastrocytoma; **OAII and OAIII** = oligoastrocytoma of WHO grade II and III; **OS** = overall survival; **PFS** = progression-free survival.

Chromosome arms 1p/19q codeletion characterizes a subtype of oligodendroglial tumors with better prognosis and higher chemosensitivity.[1,2] Some widely used techniques such as fluorescent in situ hybridization (especially when testing the 1p36 locus alone) or loss of heterozygosity (LOH) analysis may fail to distinguish this complete 1p/19q codeletion (referred to here as a "true" 1p19q signature) from variable 1p (typically 1p36) and 19q partial deletions. The LOH technique may even fail to separate the true 1p19q signature from the variable 1p and 19q gain (both referred to here as a "false" 1p19q signature).[3]

Recurrent point mutations in codon 132 of the gene encoding human cytosolic NADPH-dependent isocitrate dehydrogenase (*IDH1*) have been described in nearly 40% of gliomas. This mutation results not only in a dramatic decrease of IDH1 activity,[4-6] but also in a gain of enzyme function for the NADPH-dependent reduction of α-ketoglutarate to 2-hydroxyglutarate, which accumulates in the IDH1 mutated cells, as shown very recently.[7]

Editorial, page 1848

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From UMR 975 INSERM-UPMC (M.L., A.I., X.-W.W., Y.M., B.B., C.F., S.P., J.L., C.C., E.C., F.D., S.E.H., K.H.-X., J.-Y.D., M.S.), Groupe Hospitalier Pitié-Salpêtrière, Paris; AP-HP (A.I., F.D., S.E.H., K.X.-H., J.-Y.D., M.S.), Groupe Hospitalier Pitié-Salpêtrière, Service de Neurologie Mazarin, Paris; AP-HP (K.M.), Groupe Hospitalier Pitié-Salpêtrière, Laboratoire de Neuropathologie R. Escourolle, Paris; and Université Pierre et Marie Curie (K.X.-H., J.-Y.D., M.S.), Faculté de Médecine, Paris, France.

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Table Type of 315 *IDH1* and 11 *IDH2* mutations and frequency among mutations in 764 human gliomas

Gene	Nucleotide change	Amino acid change	True 1p19q with mutation	False 1p19q with mutation	No 1p19q alteration with mutation	Total of mutation (%)
IDH1	G395A	Arg132His	109	42	128	279 (88.6)
	C394G	Arg132Gly	5	5	4	14 (4.4)
	C394T	Arg132Cys	3	2	7	12 (3.8)
	C394A	Arg132Ser	0	0	5	5 (1.6)
	G395T	Arg132Leu	1	2	2	5 (1.6)
IDH2	G515A	Arg172Lys	7	1	4	12 (75.0)
	G516T	Arg172Ser	1	0	1	2 (12.5)
	G515T	Arg172Met	1	0	0	1 (6.25)
	A514T	Arg172Try	1	0	0	1 (6.25)

IDH1 mutation rate was highly variable among glioma histologic subtypes, from 5% in adult primary glioblastoma up to 86% in anaplastic oligodendroglioma.[6] In addition, mutations of the mitochondrial isoform *IDH2* were found in 5% of gliomas.[6] We recently reported the impact of *IDH1* mutation on glioma patients' outcome and showed that *IDH1* mutation constitutes a major favorable prognostic marker in grade II to IV gliomas.[5]

We identified the *IDH1* and *IDH2* mutation status of a large series of gliomas analyzed by array-based comparative genomic hybridization (aCGH). We investigated whether the occurrence of *IDH1* or *IDH2* mutation correlates with 1p19q status (i.e., true 1p19q signature, false 1p19q signature, or intact 1p and 19q).

METHODS **Patients.** Patients were selected, as previously described,[5] according to the following criteria: histologic diagnosis (performed by K.M.) of WHO grade I, II, III, or IV glioma; available frozen tumor and blood DNA; available bacterial artificial chromosome array-based comparative genomic hybridization profiling (CGH array); clinical data and follow-up available in the neuro-oncology database; and written informed consent.

***IDH1/2* sequencing.** Glioma samples were snap frozen in liquid nitrogen. Tumor DNA was extracted from frozen tumors using the QIAmp DNA mini-kit, as described by the manufacturer (Qiagen, Courtaboeuf, France). The genomic region spanning wild-type R132 of *IDH1* and wild-type R172 of *IDH2* were analyzed by direct sequencing using the following primers: IDH1f 5- TGTGTTGAGATGGACGCCTATTTG, IDH1r 5-ACTGAACCAGCAACCACCGT, IDH2f 5-GCCCGGTCTGCCACAAAGTC, and IDH2r 5-TTGGCAGACTCCAGAGCCCA. For both genes, forward and reverse chains were analyzed on an ABI prism 3730 DNA analyzer (Perkin Elmer).

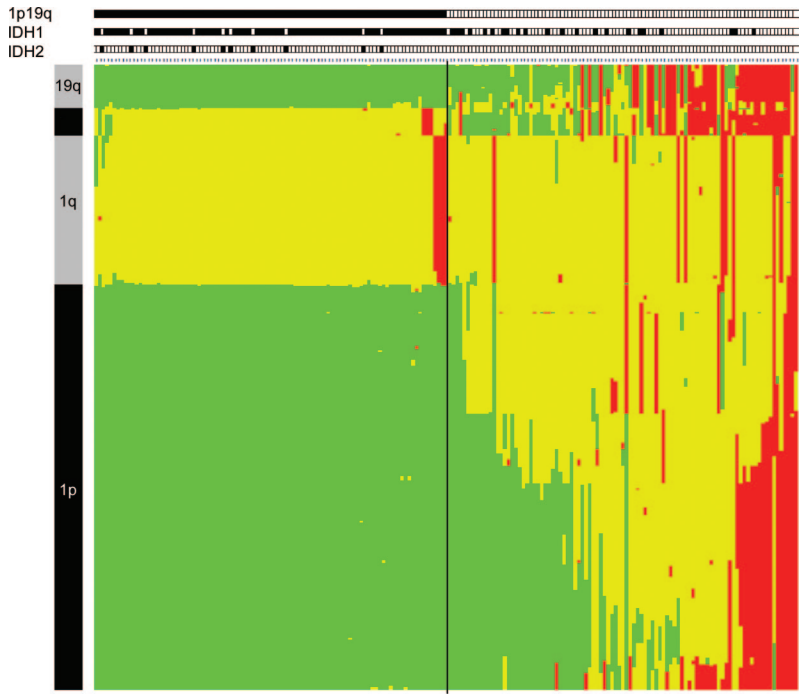
Statistical analysis. Overall survival (OS) was defined as the time between the diagnosis and death or last follow-up. Patients who were still alive at last follow-up were considered as a censored event in analysis. Progression-free survival (PFS) was defined as the time between the diagnosis and recurrence or last follow-up. Patients who were recurrence-free at last follow-up were considered as a censored event in analysis. To find clinical and genomic factors related to OS (or disease-free survival), survival curves were obtained according to the Kaplan-Meier method (function surv, *R* package survival, V2.29), and differences between curves were assessed using the log-rank test (function survdiff, *R* package survival). Two-sided $p = 0.05$ was considered significant.

RESULTS Tumor DNA from 764 gliomas (90 OII, 119 OIII, 61 OAII, 88 OAIII, 13 AI, 26 AII, 30 AIII, 240 GBM, 97 GBMO) was analyzed by aCGH, as previously described.[3] We found 128 gliomas (47 OII, 64 OIII, 7 OAII, 8 OAIII, 1 AIII, 1 GBMO) with true 1p19q signatures, 159 gliomas (12 OII, 15 OIII, 9 OAII, 27 OAIII, 5 AI, 3 AII, 11 AIII, 59 GBM, 18 GBMO) with false 1p19q signatures (variable partial deletions or gains involving 1p and 19q), including 79 samples with combined partial loss of 1p and 19q (10 OII, 7 OIII, 7 OAII, 17 OAIII, 4 AI, 1 AII, 2 AIII, 21 GBM, 10 GBMO).

Of the whole series of 764 gliomas, mutation of the *IDH1* gene was found in 315 cases (41%). All mutations were located at amino acid residue 132, and nearly 90% were G395A (Arg132His) (table). Considering the 1p19q status, *IDH1* mutations were distributed as follows: 118 mutations were found in the group of tumors with a true 1p19q signature (118/128; 92%). In contrast, only 32% (51/159) of the tumors with a false 1p19q signature harbored an *IDH1* mutation, not differing from the rest of the series (146/477; 31%).

We next determined *IDH2* codon 172 mutation in the whole series. We found a total of 16 *IDH2* mutations, corresponding to a mutation rate of 2.1% (16/764). Strikingly, the 10 tumors with a true 1p19q signature and no *IDH1* mutation were all mutated on *IDH2* (figure 1). In contrast, only 1 *IDH2* mutation was found in the series of tumors with a false 1p19q signature (1/159; 0.6%), and 5

Figure 1 Genomic alterations of 1p and 19q chromosomes found in 287 gliomas and *IDH1* and *IDH2* mutations



Each column corresponds to a sample. Lines correspond to BACs located on chromosomes 19q, 19p, 1q, and 1p. Yellow indicates normal genomic copy number; green indicates a loss and red indicates a gain of copy number. The top of the figure indicates the mutational status for *IDH1* (first line) and *IDH2* (second line): a black box indicates a mutation and a white box indicates the absence of mutation. The order of the samples, from left to right, is as follows: gliomas with complete 1p19q codeletion (true 1p19q signature), gliomas with partial 1p19q codeletion, gliomas with combined gain and loss affecting 1p19q (both corresponding to false 1p19q signature). The vertical black line separates the "true 1p19q" from the "false 1p19q" signatures.

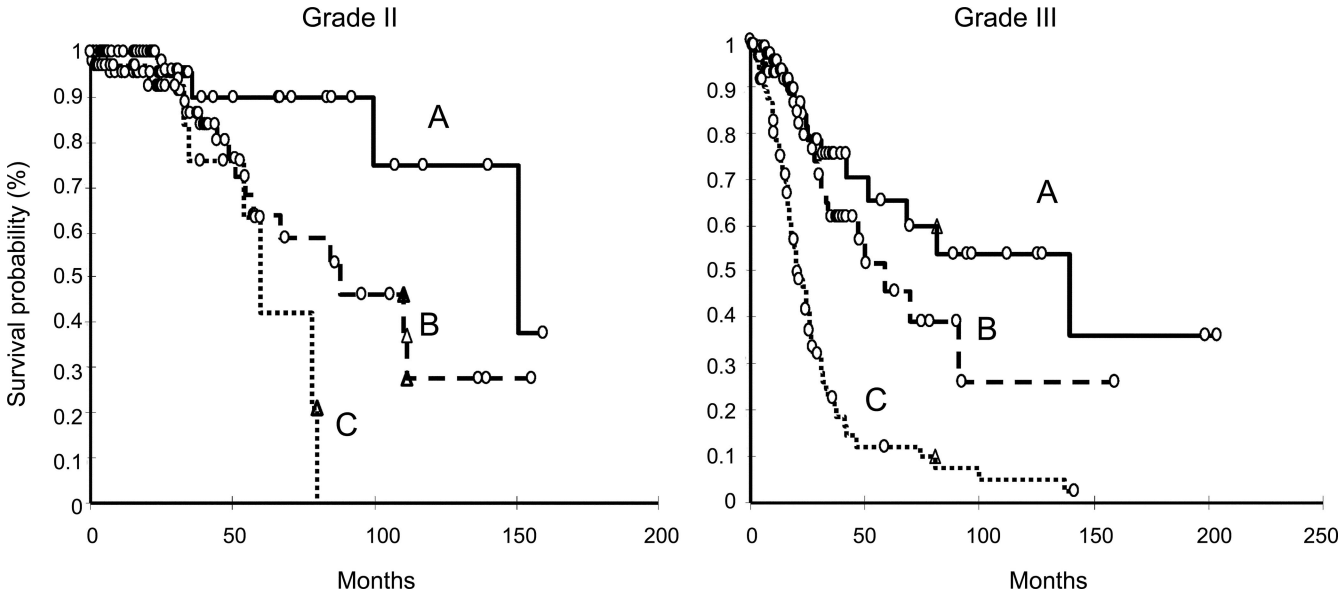
IDH2 mutations were identified in the rest of the series (5/477; 1%). No tumor was mutated in both *IDH1* and *IDH2*. The table recapitulates the different types of *IDH1* and *IDH2* mutations in our series.

We next investigated the prognostic impact of *IDH1/IDH2* mutations in grade II, III, and IV gliomas. Consistent with our previous results,[5] *IDH1/IDH2* mutation was correlated with better survival in GBM (median survival [MS] = 18 months [confidence interval (CI) 14–63] vs 13 months [CI 12–14.5]; $p = 0.015$), grade III patients (MS = 70 months [CI 50–∞] vs 20 months [CI 17–26]; $p < 10^{-4}$), and grade II patients (MS = 111 months [CI 88–∞] vs 60 months [CI 54–80]; $p = 0.015$). When tumors were evaluated according to both 1p19q and *IDH1/1DH2* status, 3 prognostic subtypes were identified in grade II and III (true 1p19q signature was found in one grade IV tumor only): A) true 1p19q signature, B) no true 1p19q signature but *IDH1/IDH2* mutation, C) no true 1p19q signature and no *IDH1/IDH2* mutation (figure 2). Whatever grade considered, patients harboring both *IDH1/IDH2* mutations and complete 1p19q codeletion (group A) survived longer than patients with either *IDH1/IDH2* mutations with no complete 1p19q codeletion (group B) or no alterations on these 2 markers (group C). As an example, in grade III tumors, MS was 139 months (51–∞) for group A, 59 months (33–∞) for group B, and 20 months (17–26) for group C ($p < 10^{-4}$).

DISCUSSION Based on a large series, our results clearly demonstrate that *IDH1/IDH2* mutation is a constant feature in gliomas with complete 1p19q codeletion. Because previous studies were based on less comprehensive and powerful methods than aCGH, they were unable to find this result.[6] For the clinician, this intriguing finding has direct diagnostic significance, since a glioma without an *IDH1/IDH2* mutation is extremely unlikely to have a true 1p19q signature. Secondly, our data confirm that *IDH1/IDH2* mutation is a highly favorable prognostic factor for gliomas of any grade. However, the improved prognosis of true 1p19q codeleted gliomas cannot merely be the consequence of an *IDH1/IDH2* mutation because in the *IDH1/IDH2* mutated tumor group, true 1p19q codeleted gliomas do clearly better than non-true codeleted ones (figure 2). This indicates that both alterations contribute to the favorable outcome. IDH1/IDH2, by producing NADPH that reduces glutathione, protects the cell against cellular damage. Thus cells with mutated *IDH1/IDH2* alleles could be more sensitive to treatment-induced oxidative cellular damage.

Unlike other common genetic alterations found in gliomas, such as *TP53* mutations or *EGFR* amplification, both complete 1p19q codeletions and *IDH1/IDH2* mutations seem to be restricted to glial tumors (with the exception of a 10% rate of *IDH1* mutation in acute myeloid leukemia[8]); in addition, both are related to good outcome, and both are tightly associated with a proneural pattern of expression.[9,10] The chromosome arms 1p and 19q codeletion is the consequence of an unbalanced t(1q;19p) translocation.[11] All previous efforts to identify the putative gene involved in the translocation—including high-density genome mapping—have failed, and the mechanism driving this translocation remains unknown.[12] In this setting, the 100% *IDH1/IDH2* mutation found in this subset of glioma is particularly intriguing. This observation suggests a synergistic association of these 2 alterations. This synergy may play a key role in the oncogenesis of a restricted population of glial cells or of their precursors through an unknown mechanism. One hypothesis is that IDH1/IDH2 impairment favors DNA double-strand break occurrence and more specifically, pericentromeric 1p19q codeletion/translocation. At least

Figure 2 Overall survival in grade II and III gliomas according to 1p19q and IDH1/IDH2 status identifies 3 prognostic subtypes: Complete 1p19q codeletion (A), no complete 1p19q codeletion but *IDH1/2* mutation (B), no complete 1p19q codeletion and no *IDH1/2* mutation (C)



In these 3 categories, median overall survival is as follows: for grade II, 151 months (100–∞), 88 months (58–∞), 60 months (54–80), $p = 0.012$; and for grade III, 139 months (51–∞), 59 months (33–∞), 20 months (17–26), $p < 10^{-4}$.

2 mechanisms may be involved: 1 is the decrease of reduced glutathione that increases oxidative damage and therefore genomic instability. The second is related to the decrease of α-ketoglutarate, the product of IDH1 catalysis, that activates a broad range of dioxygenases including histone demethylase. Histone methylation is now recognized as an important modification linked to both transcriptional activation and repression; it also regulates stability of heterochromatin and thereby genome integrity.[13,14] In this setting, we can speculate that the modification of the pericentric heterochromatin on chromosomes 1 and 19 creates the conditions of 1q19p translocation.[15] Another hypothesis is that 1p19q codeletion has transforming properties only in the context of *IDH1/IDH2* inactivation. Exploring these hypotheses by functional studies will be the next step.

AUTHOR CONTRIBUTIONS

M.L., Y.M., X.-W.W., B.B., C.F., and S.P. performed IDH1 and IDH2 sequencing. A.I., Y.M., J.L., C.C., E.C., F.D., and S.E.H. performed CGH-array analysis and clustering. K.M. performed the histologic diagnosis. Clinical data were collected and analyzed by A.I., Y.M., F.D., K.H.-X., J.-Y.D., and M.S. M.S. designed the study and wrote the manuscript with M.L., A.I., K.H.-X., and J.-Y.D. All the authors read and approved the manuscript.

DISCLOSURE

Dr. Labussière, Dr. Idbaih, Dr. Wang, Y. Marie, B. Boisselier, C. Falet, S. Paris, Dr. Laffaire, C. Carpentier, E. Crinière, Dr. Ducray, Dr. El Hallani, and Dr. Mokhtari report no disclosures. Dr. Hoang-Xuan serves as an Associate Editor for the *Revue Neurologique* and as an unpaid consultant for Roche. Dr. Delattre serves on the editorial boards of the *Journal of Clinical Oncology* and *The Oncologist* and receives research support from the French National Cancer Institute (INCa). Dr. Sanson serves as Neuro-oncology Section Editor for *Current Opinion in Oncology* and has served on an advisory board for Bristol-Myers Squibb.

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Article 6

De Carli E, **Wang X**, Puget S.

"IDH1 and IDH2 mutations in gliomas."

N Engl J Med. 2009 Feb 19; 360(8):765-73.

whether extreme or moderate, do not consistently result in more weight loss than other approaches. Moreover, our findings confirm that despite best efforts, studies that compare diets for weight loss have not shown large differences in dietary macronutrient composition.

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IDH1 and *IDH2* Mutations in Gliomas

TO THE EDITOR: Yan et al. (Feb. 19 issue)[1] found that mutations of genes encoding isocitrate dehydrogenases (*IDH1* and *IDH2*), as compared with no mutations, are associated with younger age and better prognosis in adults with gliomas. Their study and other, similar studies[2-4] prompted us to search for mutations at codon 132 of *IDH1* in children and adolescents with gliomas. In our series, 155 of 404 adults (38%) and 4 of 73 children (5%) with nonpilocytic gliomas had *IDH1* mutations ($P<0.001$). We did not find *IDH2* mutations in tumors in children. We also found that *IDH1* mutations in adults were significantly associated with a lower tumor grade, increased overall survival, and younger age. Children with tumors bearing *IDH1* mutations were older than children with mutation-negative tumors (median age, 16 years vs. 7 years; $P=0.002$). No association with survival was observed in children.

Our results and other studies in children[5] suggest that pediatric and adult gliomas differ biologically, although adolescents may have gliomas resembling those in adults.

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TO THE EDITOR: Yan et al. report that gliomas with *IDH1* mutations have distinctive genetic characteristics (particularly frequent codeletion of 1p and 19q) and are associated with a better outcome than gliomas without these mutations. We studied the gene-expression profile of 100 gliomas (21 World Health Organization [WHO] grade II, 24 grade III, and 55 grade IV gliomas), with the use of Affymetrix U133 plus 2.0 microarrays; 40 gliomas had *IDH1* mutations at codon 132, and 60 did not have such mutations. We found that the association between *IDH1* mutations and a good prognosis was related to the proneural gene-expression profile. After hierarchical clustering with the use of the gene-expression signature reported by Phillips et al.,[1] 36 of 40 mutated tumors (90%) were classified as proneural as compared with only 8 of 60 nonmutated tumors (13%). Conversely, 36 of 44 proneural tumors (82%) had mutated *IDH1*, as compared with only 4 of 56 proliferative and mesenchymal tumors (according to the categories described by Phillips et al.) (7%) ($P<10^{-14}$ according to the chi-square test). Thus,

the proneural signature may explain the good prognosis associated with *IDH1*-mutated gliomas.[1] *IDH1* mutations may occur in a specific cell lineage with metabolic characteristics that favor the occurrence of this mutation.

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1. Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006;9:157-73.

THE AUTHOR AND A COLLEAGUE REPLY: De Carli et al. report the *IDH* mutation status of pediatric gliomas. In fact, some of the gliomas sequenced in our study were from children. We report here that these childhood tumors included 14 WHO grade II gliomas (of which 4 contained *IDH1* mutations and 1 contained an *IDH2* mutation) and 3 WHO grade III gliomas (none of which contained *IDH* mutations). As we reported in our article, 15 pediatric glioblastomas (WHO grade IV) did not contain *IDH* mutations. Children with *IDH*-mutated gliomas were also older than the other patients in our study (median age, 17 years vs. 5 years; $P=0.002$). Our findings complement those of De Carli et al., and we agree with the interpretation that adolescent gliomas may resemble adult gliomas.

Ducray et al. elaborate on an association between the proneural gene-expression signature and *IDH1* mutations in WHO grades II, III, and IV gliomas. A confounder in this analysis is tumor type, which is associated with both the molecular signature[1] and *IDH1* mutation status. For instance, 89% of anaplastic astrocytomas and 31% of glioblastomas in a previous analysis had a proneural signature,[1] and we found that 69% of anaplastic astrocytomas and 11% of glioblastomas in our study contained *IDH1* mutations. Thus, the association reported by Ducray et al. may reflect the known association of molecular subclass with tumor type and not *IDH1* status in particular. Previous studies have shown mixed results for associations between genetic alterations and expression patterns in specific tumor types. For instance, a 1000-gene complementary DNA array analysis of diffuse astrocytomas did not show any patterns associated with p53 alteration,[2] whereas Ducray et al. previously identified a significant association between codeletion of 1p and 19q and the proneural signature in anaplastic oligodendrogliomas, using microarray analysis.[3] Nevertheless, associations among proneural signature, tumor type, *IDH* status, and other genetic alterations may suggest distinct biologic properties in a subgroup of gliomas. We agree that a discrete molecular signature in *IDH1*-mutated gliomas could reflect a cell lineage of origin in these gliomas in which such mutations are favored. However, we cannot discount the non–mutually exclusive possibility that some differences in molecular signature arise later during gliomagenesis, perhaps as a phenotype of the genetic alterations with which they associate.

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Cytochrome P-450 Polymorphisms and Response to Clopidogrel

TO THE EDITOR: On the basis of the results of a genetic association study (Jan. 22 issue),[1] Mega et al. conclude that reduced-function variants of the *CYP2C19* allele are responsible for lower plasma levels of the active metabolite of clopidogrel; these lower levels lead to decreased platelet inhibition and thereby increase cardiovascular risk. However, no direct evidence of the causal involvement of the cytochrome P-450 enzyme CYP2C19 in the biotransformation of clopidogrel to its active metabolite is presented.

To test the hypothesis of Mega et al., we incubated clopidogrel, the inactive metabolic intermediate 2-oxo-clopidogrel,[2] and the known CYP2C19

Article 7

Boisselier B, Marie Y, Labussière M, Ciccarino P, Desestret V, **Wang X**, Capelle L, Delattre JY, Sanson M.

"COLD PCR HRM: a highly sensitive detection method for IDH1 mutations."

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METHODS

Human Mutation

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COLD PCR HRM: A Highly Sensitive Detection Method for *IDH1* Mutations

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ABSTRACT: The p.Arg132His mutation of isocitrate dehydrogenase 1 (*IDH1*^{R132H}) is a frequent alteration and a major prognostic marker in gliomas. However, direct sequencing of highly contaminated tumor samples may fail to detect this mutation. Our objective was to evaluated the sensitivity of a newly described amplification method, coamplification at lower temperature-PCR (COLD PCR), combined with high-resolution melting (HRM) for the detection of the *IDH1*^{R132H} mutation. To this end, we used serial dilutions of mutant DNA with wild-type DNA. PCR-HRM assay detects *IDH1*^{R132H} at an abundance of 25%, similar to the detection limit of direct Sanger sequencing. Introducing a run of COLD PCR allows the detection of 2% mutant DNA. Using two consecutive runs of COLD PCR, we detected 0.25% mutant DNA in a background of wild-type DNA, that mimics a tumor sample highly contaminated by normal DNA. We then analyzed 10 biopsies of tumor edges, considered free of tumor cells by histological analysis, and showed that immunohistochemistry of *IDH1*^{R132H} was positive in three cases (30%), whereas double COLD PCR HRM was positive in the 10 cases studied (100%). In summary, COLD PCR HRM analysis is 100-fold more sensitive than Sanger sequencing, rendering this rapid and powerful strategy particularly useful for samples highly contaminated with normal tissue.

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KEY WORDS: *IDH1*; COLD PCR; high-resolution melting; HRM; HRMA; glioma

Introduction

In recent years, recurrent mutations of the gene encoding isocitrate dehydrogenase 1 (*IDH1*; MIM# 147700) have been described at a high frequency in gliomas [Balss et al., 2008; Bleeker et al., 2009; Hartmann et al., 2009; Ichimura et al., 2009; Parsons et al., 2008; Sanson et al., 2009; Yan et al., 2009]. Mutations in the *IDH1* gene always affect the codon 132, which in more than 90% of cases result in a substitution of arginine by histidine, p.Arg132His (p.R132H or *IDH1*^{R132H}) [Ichimura et al., 2009; Sanson et al., 2009; Yan et al., 2009]. Because it is almost exclusively found in gliomas, *IDH1* mutation has a strong diagnostic value [Yan et al., 2009]. *IDH1* mutation is also a powerful independent prognostic factor in gliomas [Dubbink et al., 2009; Nobusawa et al., 2009; Sanson et al., 2009]: patients with an *IDH1*^{R132H} mutated tumor having a longer survival when compared with those nonmutated for the same grade. Given its diagnostic and prognostic potential, the determination of *IDH1* status in glioma samples is gaining increasing attention in clinical practice.

Conventional polymerase chain reaction (PCR) followed by Sanger sequencing is currently the gold standard in identifying *IDH1* mutations in tumor DNA. Typically, clinical tumor samples are contaminated by normal tissue, thereby diluting the total amount of genetic material that is present, and techniques are often required to identify low level alterations within an excess of wild-type DNA. This is particularly true for gliomas that are highly infiltrating tumors [Furnari et al., 2007]. Conventional PCR enables mutation detection from a very small amount of sample but does not selectively amplify the mutant sequences. Therefore, unless the mutation exceeds a 20–25% abundance relative to wild-type alleles, conventional PCR followed by downstream methods such as Sanger sequencing will fail to detect mutations in clinical samples [Li et al., 2009; Zuo et al., 2009]. Enrichment methods are thus necessary to increase the mutant DNA/wild-type DNA *ratio*. Tumor-cell enrichment methods, such as cell sorting or microdissection, are expensive and time consuming [Zuo et al., 2009], and therefore inadequate for clinical routine use.

Recently, coamplification at lower denaturation temperature PCR (COLD PCR) has been described as a powerful method to identify low-level mutations in the *TP53* gene [Li et al., 2008; Milbury et al., 2009]. This approach uses a critical temperature (T_c) during the PCR process in order to enrich mutations at any position of the amplified sequence. Indeed, a single nucleotide mismatch anywhere along a double-stranded DNA sequence generates a small change in the melting temperature for that

Additional Supporting Information may be found in the online version of this article.

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sequence, with mutated sequences melting at a lower temperature than wild-type sequences [Li et al., 2008]. During COLD PCR, the denaturation temperature is set to T_c; thereby, mutation-containing sequences are preferentially denatured and available for primers binding and subsequent amplification. High-resolution DNA melting (HRM) analysis is a relatively new and rapid method for detecting DNA sequence variants following an initial amplification [Erali et al., 2008; Vossen et al., 2009; Wittwer, 2009]. Gene scanning by HRM depends on the recognition of changes in the shape of the amplicon melting curve that result from heterozygous sequence alterations [Erali et al., 2008]. At the end of PCR amplification, samples are heated to 95°C to ensure a complete denaturation. Next, the temperature is reduced to allow crosshybridization between wild-type and mutant sequences, leading to the formation of mismatched heteroduplexes, which melt at a lower temperature than wild-type and mutant homoduplex [Vossen et al., 2009]. In this study, we evaluated the sensitivity of different COLD PCR HRM assays for the detection of *IDH1*^{R132H} mutation in glioma samples.

Material and Methods

DNA and Tumor Samples

Tumor samples were selected from the Pitié-Salpêtrière brain tumor database. Tumor DNA was extracted from frozen tissues using the QIAmp DNA according to the manufacturer instructions (Qiagen, Chatsworth, CA). Samples used in this work have previously been tested for the presence of *IDH1* mutation (reference sequence NM_005896.2) by direct sequencing, as already described [Parsons et al., 2008; Yan et al., 2009].

Determination of T_c

We applied the fast COLD PCR assays for detecting *IDH1* mutation based on the methodology described in the literature [Li et al., 2008], with several modifications. We first determined the new denaturation temperature T_c for the reaction. We used the same primers as for conventional PCR assay, which produced an amplicon of 172 bp. To identify the optimal critical denaturation temperature T_c, a series of COLD PCR reactions at graded temperatures below T_m were performed. We then set the T_c at 81°C, the lowest temperature that reproducibly yielded a substantial PCR. In fast COLD PCR, the reaction protocol began with 20 cycles of conventional amplification for an initial buildup of all amplicons, followed by 30 COLD PCR cycles to selectively enrich for T_m-reduced mutant sequences (Fig. 1). COLD PCR assays were performed on a LightCycler480 (Roche Diagnostics Corporation, Indianapolis, IN) to ensure a precise control of temperature during the experiments.

Comparison of Sensitivities of the Different Assays to Detect *IDH1* Mutation

To determine if COLD PCR could enhance the sensitivity of *IDH1*^{R132H} mutation detection, we compared the sensitivity of three experimental protocols. Exon 4 of the *IDH1* gene was first amplified either by conventional PCR or by fast COLD PCR. A second round of amplification was then performed either by PCR HRM, or by fast COLD PCR HRM (Fig. 2).

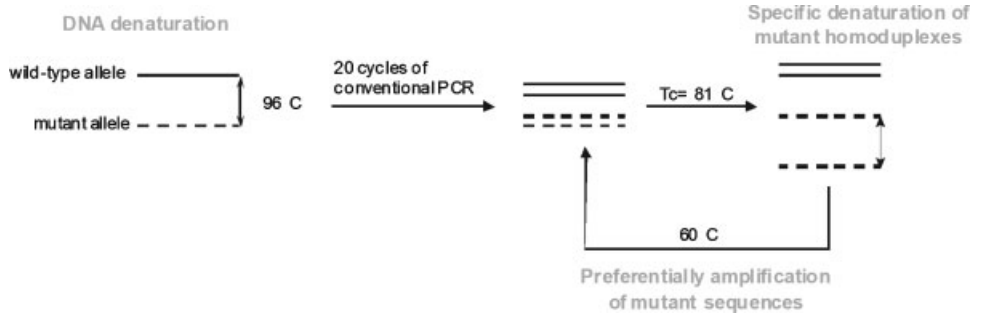

Figure 1. Principle of fast COLD PCR performed for the detection of the *IDH1*^{R132H} mutation (p.Arg132His) in glioma samples.

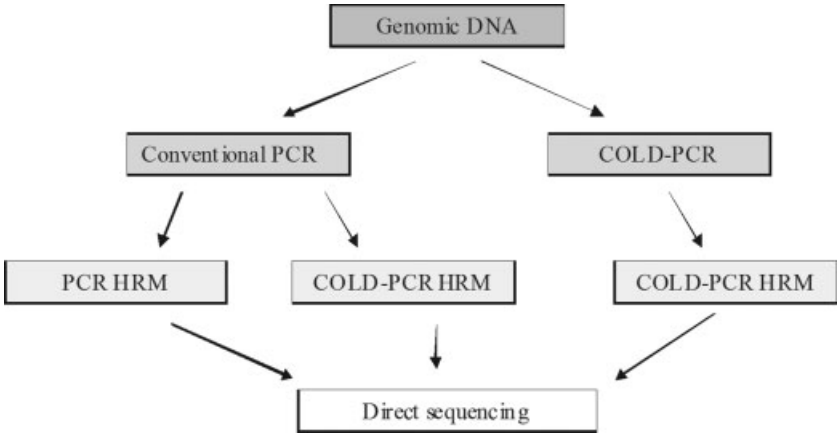

Figure 2. Assays performed to detect *IDH1*^{R132H} mutation in tumor samples.

To compare the sensitivity of the different assays, we performed a serial dilution study using a gDNA sample from a grade II oligoastrocytoma (OAII) patient containing an *IDH1*^{R132H} mutation as the source of the mutant allele. This mutation-containing gDNA sample was serially diluted into wild-type gDNA (i.e., blood constitutive gDNA from the same patient) to the following percentages: 25, 10, 8, 5, 4, 2, 1, 0.5, 0.25, 0.1, and 0.05%. In addition, wild-type gDNAs ($n = 7$) were included in each experiment. The same experiment was reproduced with several other mutated DNA samples: another CGT→CAT mutation (c.395G>A; p.Arg132His), and also with other, less frequent mutations, such as CGT→CTT (c.395G>T; p.Arg132Leu) and CGT→AGT (c.394C>A; p.Arg132Ser).

First Round of Amplification

Each PCR amplification reaction contained 50 ng gDNA. PCR cycling conditions for the first round consisted of an initial denaturation step at 94°C for 5 min; 40 cycles of 94°C for 30 sec; 60°C for 1 min; and 72°C for 1 min 30 sec; and final extension at 72°C for 7 min. The reactions were carried out using a Mastercycler (Eppendorf, Westbury, NY). Conventional PCR reactions contained final reagent concentrations as follows: 1 × PCR Master Mix (Abgene), 0.25 µM forward and reverse primers (Invitrogen, Carlsbad, CA) (Table 1) and DNA template. COLD PCR cycling conditions on LightCycler480 (Roche Diagnostics Corporation) are summarized as follows: 96°C, 10 min; 20 cycles of 95°C, 15 sec; 60°C, 30 sec, then 30 cycles of 81°C, 15 sec; 60°C, 30 sec. COLD PCR assays contained final reagent concentrations as follows: LightCycler480 HRM Master (Roche Diagnostics Corporation), 0.25 µM forward and reverse primers (Invitrogen), 3 mM $MgCl_2$, and DNA template.

Second Round of Amplification

PCR amplification was performed with the LightCycler480 (Roche Diagnostics Corporation). Each reaction contained diluted PCR amplicons (1/1,000), 0.25 µM forward and reverse primers (Invitrogen) (Table 1), 3 mM $MgCl_2$, and LightCycler480 HRM Master (Roche Diagnostics Corporation). PCR HRM cycling conditions were as follows: 96°C, 10 min; 40 cycles of 95°C, 30 sec; 60°C, 30 sec. COLD PCR HRM cycling conditions are summarized as follows: 96°C, 10 min; 20 cycles of 95°C, 15 sec; 60°C, 30 sec, then 30 cycles of 81°C (T_c), 15 sec; 60°C, 30 sec. After amplification, a postamplification melting curve program was initiated by heating to 95°C for 1 min, cooling to 40°C for 1 min, and increasing the temperature to 95°C while continuously measuring fluorescence at 25 acquisitions per degree. Each PCR run contained a negative (no template) control and each amplification was duplicated.

HRM Analysis and Direct Sequencing

At the end of the second round of amplification, fluorescent melting curves were analyzed using LC480 Gene Scanning software V1.2.9 (Roche Diagnostics Corporation). All curves were analyzed following normalization, temperature shifting, automated grouping, and the inspection of difference plots. The grouping software uses a curve shape-matching algorithm in order to identify wild-type from mutant samples. The 0.2 value was chosen for the grouping sensitivity in all experiments.

Products of each assay were then submitted to the sequencing reaction using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Bedford, MA) as previously described [Sanson et al., 2009]. After a purification step using BigDye Xterminator Purification Kit (Applied Biosystems), both forward and reverse sequences were determined on an ABI prism 3730 DNA analyzer (Perkin-Elmer, Norwalk, CT).

Table 1. Primers Used for First and Second Rounds of Amplification

	Primer		Amplicon length
First amplification	Forward	CGGTCTTCAGAGAAGCCATT	172 bp
	Reverse	CACATACAAGTTGGAAATTTCTGG	
Second amplification	Forward	CGGTCTTCAGAGAAGCCATT	129 bp
	Reverse	GCAAAATCACATTATTGCCAAC	

Tumor Sample Selection and Immunohistochemical Detection of *IDH1*^{R132H}

Tumors were retrieved from the Neuropathology Department database according to the following criteria: histological diagnosis of WHO grade II gliomas; presence of *IDH1*^{R132H} was confirmed by Sanger sequencing; availability of frozen and paraffin embedded samples of tumor core and tumor edge, with edge biopsies considered as free of infiltrated tumor cells by standard HE staining.

Immunohistochemical staining for *IDH1*^{R132H} was performed on 4-µm paraffin sections of formalin-fixed tumor samples using mouse monoclonal anti-R132H-IDH1 antibody culture supernatant (a generous gift from Prof. A. von Deimling), as previously published [Capper et al., 2009, 2010]. Local immunohistochemistry protocol was validated on gliomas samples that were previously analyzed by Sanger sequencing for *IDH1*^{R132H} mutation (positive and negative controls). Labeling was defined as positive (at least one cluster of positive tumor cells) or negative (no positive tumor cells detected).

Mutation Nomenclature

Nucleotide numbering reflects the cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference GenBank sequence NM_005896.2, according to the Journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon number 1.

Results

Determination of the Sensitivity with Dilution Studies

To identify the most sensitive method for detecting *IDH1* mutation, we evaluated two techniques after a first stage of conventional PCR: the PCR HRM assay and the COLD PCR HRM assay. In a third assay, we replaced the first conventional PCR by a run of COLD PCR (Fig. 2). The sensitivity of the three assays was compared using a dilution series obtained by mixing DNA from heterozygous positive control carrying the *IDH1*^{R132H} mutation with wild-type DNA from peripheral blood.

Conventional PCR HRM assay detects mutant DNA at a concentration of 25% in a background of wild-type DNA (Fig. 3A). In contrast, the COLD PCR HRM assay detects *IDH1*^{R132H} at a much lower concentration: mutant DNA diluted into wild-type DNA to a 2% abundance was still clearly differentiated from normal sequences (Fig. 3B). Therefore, the COLD PCR HRM assay produced an approximately 10-fold improvement in *IDH1* mutation detection, compared to the conventional PCR HRM analysis. Replacing the first

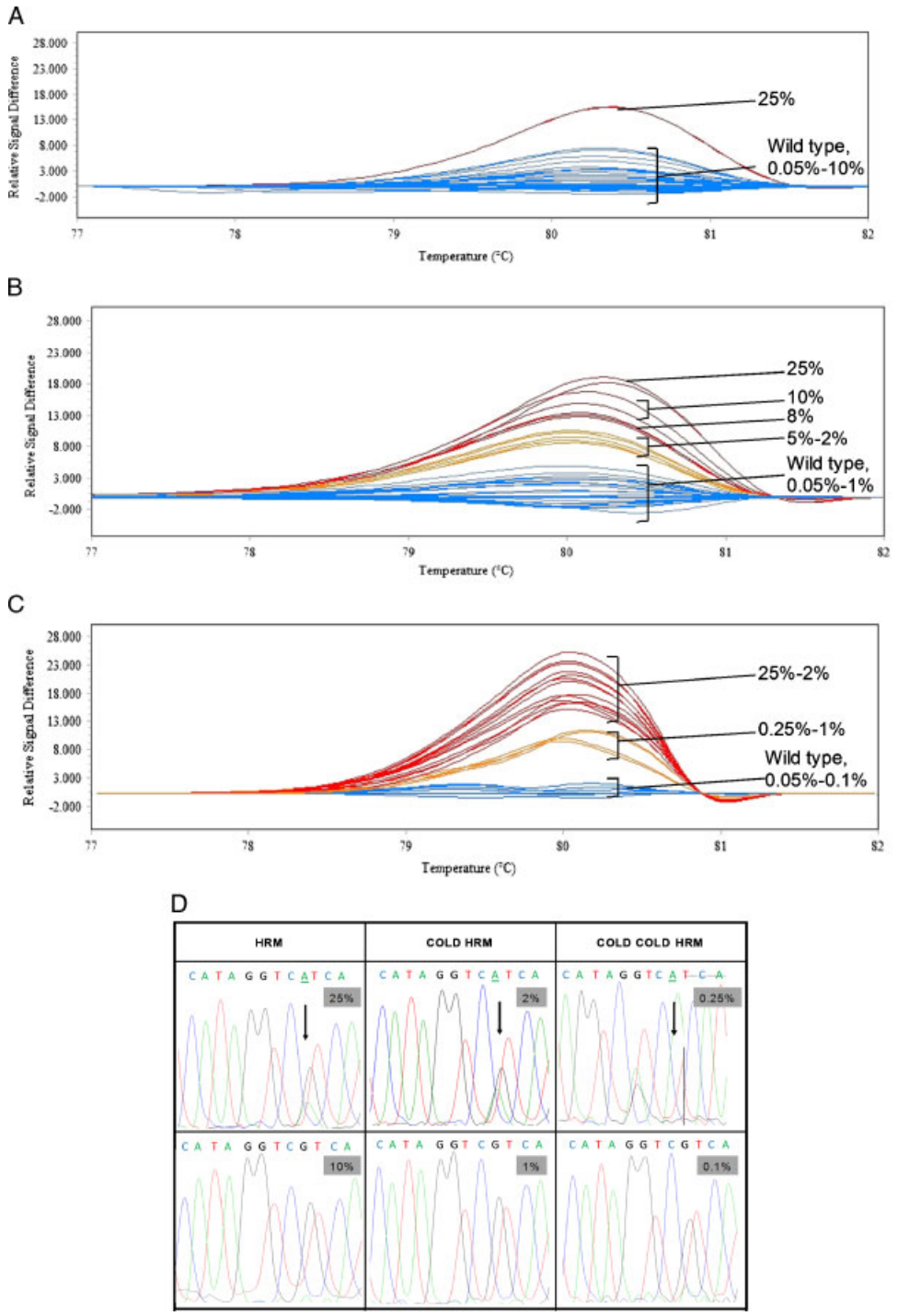

Figure 3. Comparison of conventional PCR HRM (**A**), COLD HRM (**B**), and double COLD HRM (**C**) for the detection of *IDH1*^{R132H} mutation. Mutant DNA was serially diluted with wild-type DNA and each mixture was submitted to the three assays. Sequencing chromatograms after conventional PCR HRM, COLD HRM, and double COLD HRM (**D**).

conventional PCR amplification by a run of COLD PCR further enhanced selective enrichment of the mutant DNA and improved the detection of *IDH1*^{R132H}. In these conditions, we were able to detect mutant DNA at a concentration as low as 0.25% in mixture with wild-type DNA (Fig. 3C). Thus, the double COLD PCR HRM assay for the detection of *IDH1*^{R132H} was 100-fold more sensitive than the PCR HRM assay.

To both confirm the selective mutation enrichment and to exclude false positive results, amplicons produced at the end of the three assays were submitted to direct sequencing using the Sanger method. The sequencing chromatograms are presented in Figure 3D. In all three cases, direct sequencing confirmed the presence of the *IDH1*^{R132H} mutation. After double COLD PCR HRM with 0.25% of mutant DNA/wild DNA, *IDH1*^{R132H} was evident as clearly as for 25% with conventional PCR HRM and 2% with COLD PCR HRM.

The same results were obtained with another glioma with CGT→CAT mutation (p.Arg132His), and also with less-frequent

IDH1 codon 132 mutations CGT→CTT (p.Arg132Leu) and CGT→AGT (p.Arg132Ser) (Supp. Fig. S1).

Comparison of PCR HRM, COLD PCR HRM, and double COLD PCR HRM and *IDH1*^{R132H} Immunohistochemistry in Biopsies of Tumor Edges

To definitely validate the clinical interest of our technique, we selected 10 pairs of tumor core and tumor edge (one astrocytoma [AII], five oligodendrogliomas [OII], and four oligoastrocytomas), and performed *IDH1*^{R132H} mutation detection by both immunohistochemistry and molecular techniques (Table 2). *IDH1*^{R132H} immunohistochemistry was positive in three cases (30%, two grade II oligodendrogliomas, and one grade II oligoastrocytoma) showing a cytoplasmic granular staining as previously described [Capper et al., 2010]. Figure 4A presents the results of immunohistochemistry staining for patient 8 in the tumor core and in the biopsy edge (lower panel). PCR HRM was positive in 8 cases out of 10 (80.0%; Fig. 4B upper panel). In contrast, both COLD PCR HRM (data not shown) and double COLD PCR HRM were positive in all cases (100%) (Fig. 4B, lower panel, and Table 2).

Discussion

Gliomas are characterized by a highly invasive phenotype, with tumor cells invading the brain at a distance far from the bulk of tumor [Furnari et al., 2007]. Therefore, biopsy samples are often

Table 2. Comparison of *IDH1*^{R132} Immunohistochemistry with PCR HRM and Double COLD PCR HRM for the Detection of *IDH1*^{R132H} Mutation in Tumor Edges

		IDH1^{R132} immunohistochemistry		PCR HRM		Double COLD PCR HRM	
Patients	Histology	Tumor core	Tumor edge	Tumor core	Tumor edge	Tumor core	Tumor edge
1	OAII	+	–	+	+	+	+
2	OII	+	–	+	–	+	+
3	AII	+	–	+	+	+	+
4	OAII	+	–	+	+	+	+
5	OAII	+	+	+	+	+	+
6	OII	+	–	+	+	+	+
7	OII	+	+	+	+	+	+
8	OII	+	+	+	+	+	+
9	OAII	+	–	+	+	+	+
10	OII	+	–	+	–	+	+

AII, grade II oligoastrocytoma; OAII, oligoastrocytoma; OII, oligodendroglioma. For all techniques, results are expressed as positive (+) or negative (–). COLD PCR HRM (data not shown) gave the same results as double COLD PCR HRM.

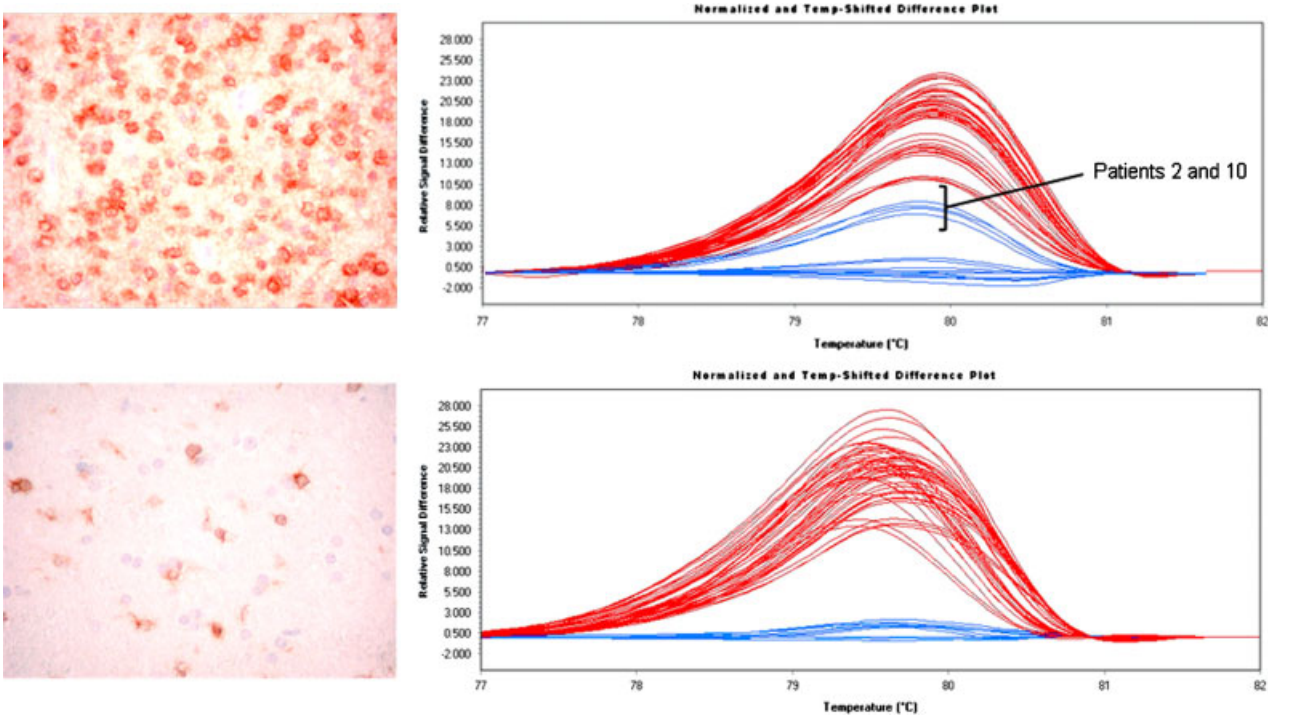

Figure 4. Detection of *IDH1*^{R132H} mutation in grade II gliomas edges. **A:** *IDH1*^{R132H} immunohistochemistry in patient eight tumor core (upper panel) and in tumor edge (lower panel); magnification × 200. **B:** *IDH1*^{R132H} detection by PCR HRM (upper panel) and double COLD PCR HRM (lower panel).

contaminated by normal brain tissue, and mutated DNA is diluted in a background of wild-type DNA from surrounding brain tissue, vasculature, and infiltrating lymphocytes [Dobrowolski et al., 2009]. Because the *IDH1*^{R132H} mutation is almost restricted to gliomas [Yan et al., 2009], it has a major diagnostic potential. Therefore, a fast and reliable scanning technique to detect this mutation in clinical samples with very few tumor cells (and appearing therefore normal at neuropathological analysis) is particularly important. In addition, the *IDH1*^{R132H} mutation is a major prognostic factor [Sanson et al., 2009; van den Bent et al., 2010]. Simple HRM has been very recently reported as a fast and sensitive strategy, detecting IDH1 and IDH2 mutations in a 90% normal DNA background [Horbinski et al., 2010]. In this study, we used fast COLD PCR to selectively amplify *IDH1*^{R132H} mutated DNA and we showed that double COLD PCR HRM assay is a highly reliable method to detect *IDH1* mutation in samples with very few tumor cells (up to 0.25% mutated/non mutated DNA). In addition, this assay is fast (less than 3 hr) and therefore particularly suitable for routine diagnosis purposes in neuropathology.

Despite the fact that *IDH1*^{R132H} accounts for more than 90% of *IDH1* mutations reported in gliomas, other mutations have been described: CGT→AGT, CGT→CTT, CGT→GGT, CGT→TGT [Balss et al., 2008; Gravendeel et al., 2010; Sanson et al., 2009; Yan et al., 2009]. Fortunately, all these mutations, except the CGT→GGT change, result in a lower T_m, rendering them theoretically detectable by our simple double COLD PCR assay. Indeed, we were able to detect some of these less frequent mutations (Supp. Fig. S1). For the detection of CGT→GGT, a full COLD PCR assay—allowing the enrichment of all possible mutations—could be performed, although the selective amplification *ratio* will probably be lower than in fast COLD PCR, because amplification, and thus enrichment in mutant sequences, begins earlier during cycling in fast COLD PCR than in full COLD PCR [Li et al., 2008; Milbury et al., 2009].

We showed that double COLD PCR HRM (followed by sequencing in case of aberrant HRM profiles) is a powerful method to evidence the presence of tumor cells with *IDH1* mutation in apparently "blank" biopsies of grade II gliomas, and is much more sensitive than immunohistochemistry for *IDH1*^{R132H}. Such procedure may be particularly useful in lesions radiologically highly suggestive of glioma, whose biopsy appears noncontributive because of the very low proportion of tumor cells. In such cases, finding an *IDH1* mutation will confirm the diagnosis of glioma, avoiding a further invasive procedure to establish diagnosis. Moreover, such approach could also be suitable for *IDH1*^{R132H} mutation detection in the cerebral spinal fluid, thus confirming glioma diagnosis without the need for surgery.

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Article 8

Houillier C, **Wang X**, Kaloshi G, Mokhtari K, Guillevin R, Laffaire J, Paris S, Boisselier B, Idbaih A, Laigle-Donadey F, Hoang-Xuan K, Sanson M, Delattre JY.

"IDH1 or IDH2 mutations predict longer survival and response to temozolomide in low-grade gliomas."

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IDH1 or *IDH2* mutations predict longer survival and response to temozolomide in low-grade gliomas

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ABSTRACT

Objectives: Recent studies have shown that *IDH1* and *IDH2* mutations occur frequently in gliomas, including low-grade gliomas. However, their impact on the prognosis and chemosensitivity of low-grade gliomas remains unclear.

Methods: Search for *IDH1* and *IDH2* mutations, loss of heterozygosity on chromosomes 1p and 19q, *MGMT* promoter methylation, and p53 expression was performed in a series of 271 low-grade gliomas and correlated with overall survival. A subgroup of 84 patients treated up-front with temozolomide was individualized. Response to temozolomide was evaluated by progression-free survival, as well as by tumor size on successive MRI scans, and then correlated with molecular alterations.

Results: *IDH* (*IDH1* or *IDH2*) mutations were found in 132/189 patients (70%). *IDH* mutation and 1p-19q codeletion were associated with prolonged overall survival in univariate ($p = 0.002$ and $p = 0.0001$) and multivariate analysis ($p = 0.003$ and $p = 0.004$). 1p-19q codeletion, *MGMT* promoter methylation, and *IDH* mutation ($p = 0.01$) were correlated with a higher rate of response to temozolomide. Further analysis of the course of the disease prior to any treatment except for surgery (untreated subgroup) showed that 1p-19q codeletion was associated with prolonged progression-free survival in univariate analysis, whereas *IDH* mutation was not.

Conclusion: *IDH* mutation appears to be a significant marker of positive prognosis and chemosensitivity in low-grade gliomas, independently of 1p-19q codeletion, whereas its impact on the course of untreated tumors seems to be limited. ***Neurology*® 2010;75:1560–1566**

GLOSSARY

GBM = glioblastoma; **KPS** = Karnofsky performance status; **LGG** = low-grade gliomas; **LOH** = loss of heterozygosity; **OS** = overall survival; **PFS** = progression-free survival; **TMZ** = temozolomide.

A recent genome-wide mutational analysis of glioblastomas (GBM) has led to the identification of somatic mutations on codon 132 in the isocitrate dehydrogenase 1 (*IDH1*) gene.[1] IDH1 is an enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate.[2] This reaction leads to NADPH production and is thought to play a role in cellular protection from oxidative stress.[3]

Additional studies have demonstrated that *IDH1* mutation is restricted to gliomas, with few exceptions, including leukemia.[4–7] The mutation is rare in primary GBM (3% to 7%) but frequent in secondary GBM and grade III and grade II gliomas (50% to 90%).[6,8–11] IDH2 has functions similar to those of IDH1 but *IDH2* mutations are much less common (1% to 6% of mutations in a series of grade II and grade III gliomas).[9]

Preliminary studies demonstrate that *IDH1* mutations are associated with prolonged survival, at least on univariate analysis.[6,8,11] However, few studies have focused on low-grade gliomas (LGG, grade II), a subtype of glial tumors with great prognostic variability. To date, the only molecular biomarker predictive of good prognosis in LGG is the loss of chromosomes 1p-19q.[12–14] In this

Supplemental data at www.neurology.org

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study, we analyzed *IDH* mutations in a large cohort of LGG to determine their impact on prognosis and sensitivity to treatment and their relationship to loss of 1p-19q.

METHODS **Selection of patients.** This work is based on the analysis of a database created in January 1997 which collects clinical information on patients treated in our department for primary brain tumor.[15] The following inclusion criteria were selected: 1) age 18 years or above at time of surgery; 2) histologic diagnosis of a cerebral LGG according to WHO classification (WHO grade II, 2007), including astrocytomas, oligodendrogliomas, and oligoastrocytomas; 3) detailed clinical information at diagnosis and during follow-up; and 4) availability of paired blood and tumor samples, obtained after informed consent, for molecular analysis. Patients with anaplastic pathology features, dysembryoplastic neuroepithelial tumors, pilocytic astrocytomas, or gangliogliomas were excluded.

Standard protocol approvals, registrations, and patient consents. The study was approved by the ethical committee of the Pitié-Salpêtrière Hospital. All patients gave their written informed consent.

Molecular analysis. DNA from both blood and tumor tissue was extracted using a commercial kit (Qiagen, QIAmp DNA Mini-Kit) according to the manufacturer's instructions.

Only frozen samples were used for *IDH1* and *IDH2* analysis. The genomic region spanning wild-type R132 of *IDH1* was analyzed by direct sequencing using the following primers: 5′ TGTGTTGAGATGGACGCCTATTTG and 3′ TGCCACCAACGACCAAGTC, as previously described.[1]

The genomic region spanning wild-type R172 of *IDH2* was analyzed by direct sequencing using the following primers: IDH2f 5-GCCCGGTCTGCCACAAAGTC and IDH2r 5-TTGGCAGACTCCAGAGCCCA, as previously described.[9]

Loss of heterozygosity (LOH) on chromosomes 1p, 19q, 9p, and 10q was detected by microsatellite analysis on blood and tumor DNA, as previously reported.[16] We considered only "true" 1-19q codeletion implying a complete loss of chromosomes 1p and 19q, which corresponds to the translocation t(1;19)(q10;p10)[17] and is correlated with good prognosis in oligodendrogliomas.[18]

DNA methylation status of the *MGMT* promoter was determined by bisulfite modification and subsequent nested MSP, a 2-stage PCR approach, as previously described.[19]

The expression of p53 was detected on 5-μm sections of formalin-fixed and paraffin-embedded tissues, as reported previously.[15] Expression of p53 was defined as moderate to strong (++ to +++) staining of more than 10% of nuclei.

Subgroup analysis. Since analysis of the whole series allowed us to identify molecular alterations associated with a better prognosis (see below), we attempted to see if these molecular changes were predictive of a slower spontaneous natural history of the disease or of a better response to treatment. Two subgroups were specifically reviewed: 1) a subgroup of patients who had no adjuvant treatment after surgery until first progression, allowing study of the "natural history" of the disease; 2) a subgroup of patients with evaluable residual tumor who received temozolomide (TMZ) as first-line postoperative treatment, allowing analysis of molecular predictors of treatment response. Analysis of the subgroup who received radiotherapy was not performed because MRI follow-up of LGG after radiotherapy is hampered by the frequent development of radiation-induced white matter changes around the initial tumor which does not allow a clear distinction between the effects of treatment on white matter and the tumor itself. In this setting, evaluating PFS on MRI after radiotherapy was found unreliable, particularly because of our focus on the stages preceding obvious anaplastic transformation of the tumor.

Evaluation of response to upfront temozolomide. The decision to treat patients after surgery was made at "brain tumor conferences." Criteria for immediate postoperative treatment included the following: focal deficit, uncontrolled seizures, age above 45 years, biopsy only, or documented radiologic progression.

Patients who were initially treated with TMZ, prior to any other treatment except for surgery, were selected. This first-line regimen has been increasingly used over the last decade to delay radiotherapy and its feared side effects on cognitive functions.[20–22] Patients whose tumors harbored no contrast enhancement at diagnosis, but did show contrast enhancement at the beginning of TMZ, were excluded because secondary contrast enhancement raised the question of a malignant transformation of the tumor.

TMZ was administered orally from day 1 to day 5 at a starting dose of 200 mg/m^2, repeated every 28 days after the first daily dose of TMZ. In the absence of unacceptable toxicity (repeated grade IV blood toxicity despite 25% dose reduction) or of disease progression, patients continued to receive TMZ for at least 12 cycles and up to 30 cycles. Follow-up was based on clinical examination and brain MRI with gadolinium infusion repeated every 2 months.

Radiographic response to TMZ was evaluated on the measurable change of the product of the 2 largest perpendicular diameters of the tumor on the axial planes of the MRI (T2/fluid-attenuated inversion recovery–weighted images), as reported previously.[23] In brief, partial response was defined as ≥50% reduction in the size of the T2/fluid-attenuated inversion recovery tumor; minor response was defined as ≥25% to 50% reduction in the tumor size. Patients must be on stable or reduced doses of corticosteroids and show stable or improved neurologic status. Progressive disease was defined as greater than 25% increase in T2 hypersignal or contrast enhancement, or tumor-related neurologic deterioration. Stable disease was defined as any other clinical status not meeting the criteria for partial response, minor response, and progressive disease lasting for at least 6 months.

Statistical methods. Frequency distribution and summary statistics were calculated for all clinical, histologic, and molecular variables. The χ^2 test was used to test the association between molecular alterations and between radiologic response to chemotherapy and molecular alterations. Progression-free survival (PFS) and overall survival (OS) were both used to study the prognostic impact of the analyzed variables. PFS was defined as the time from the beginning of the treatment (surgery or chemotherapy) until the first unequivocal clinical or radiologic sign of progressive disease. Probability estimates for PFS and OS were calculated using the Kaplan-Meier method. The log-rank test was used to test for equality of the PFS and OS distributions. Multivariate analysis was performed with the multivariate Cox proportional hazard regression model analysis. The significant variables in univariate analysis were included in the multivariate model: age (>55 vs <55 years), gender, Karnofsky performance status (KPS) (>80 vs ≤80), type of surgery, 1p-19q and *IDH* status, as well as histologic subtype and *MGMT* promoter status. Two-sided p values <0.05 were considered significant.

RESULTS **Analysis of the whole series.** ***Clinical data.*** A total of 271 patients fulfilled the inclusion criteria.

Table 1 Main clinical characteristics

	Full population	TMZ as adjuvant treatment	No adjuvant treatment
No.	271	84	171
Median age at diagnosis, y (range)	39 (18-78)	39 (18-78)	38 (18-62)
M/F	1.4	1.3	1.4
Median preoperative KPS (range)	90 (70-100)	90 (70-100)	90 (70-100)
Biopsy, % (n)	23 (62/265)	49 (40/81)	7 (11/167)
Partial resection, % (n)	36 (95/265)	27 (22/81)	36 (60/167)
Gross total resection, % (n)	41 (108/265)	24 (19/81)	57 (96/167)
Astrocytomas, % (n)	17 (47/271)	13 (11/84)	18 (30/171)
Oligoastrocytomas, % (n)	24 (66/271)	21 (18/84)	26 (45/171)
Oligodendrogliomas, % (n)	58 (158/271)	66 (55/84)	56 (96/171)
Early radiotherapy	50	0	0
Delayed radiotherapy	81	36	59
Early chemotherapy	58	50	0
Delayed chemotherapy	126	34	95
Median follow-up, mo (95% confidence interval)	69.2 (60.3-78.7)	63.4 (55.5-78.7)	76 (68.6-89.6)
Median PFS, mo	41.3	34.6	41.3
Median survival, mo	133.3	103.9	136.5

Abbreviations: KPS = Karnofsky performance status; PFS = progression-free survival; TMZ = temozolomide.

Their main clinical characteristics are summarized in table 1.

Molecular analysis. LOH on 1p-19q, *IDH1* mutation, *IDH2* mutation, *MGMT* promoter methylation, and p53 expression were found in 32% (84/263), 66% (125/189), 6% (7/121), 81% (150/185), and 48% (102/213) of the patients. (Variation in denominator is explained by the fact that all tests could not be performed in all patients.) As previously described,[24–26] 1p-19q codeletion and p53 expression were strongly mutually exclusive ($p = 4.10^{-12}$).

IDH1 and *IDH2* mutations were always mutually exclusive. *IDH* mutation (*IDH1* or *IDH2*) was strongly associated with 1p-19q codeletion ($p = 0.0009$). Only 8 patients harboring *IDH* mutation were not 1-19q codeleted. *IDH* mutation was not correlated with p53 expression ($p = 0.11$) or *MGMT* promoter methylation ($p = 0.41$). *MGMT* promoter methylation was associated with LOH on 1p-19q ($p = 0.003$).

Correlations between histology, clinical course, and molecular findings. There was no association between histopathologic subtype and *IDH* mutation but the classic correlations between an oligodendroglioma phenotype and 1p-19q codeletion ($p = 7.10^{-12}$) or between an astrocytoma phenotype and p53 expression ($p = 4.10^{-8}$) were also present in this series.

There was no association between age and *IDH* status.

Progression-free survival. As indicated in tables 2 and 3, univariate and multivariate analysis revealed that 1p-19q codeletion and *MGMT* promoter methylation were associated with increased PFS, whereas *IDH* mutation was not.

Overall survival. In univariate analysis, several clinical and pathologic factors were associated with increased OS: age at diagnosis <55 years, female gender, KPS >80, absence of neurologic deficit at diagnosis, and gross total resection (vs biopsy or partial resection). Among molecular markers, LOH on 1p-19q and *IDH* mutation were strongly correlated with increased OS ($p = 0.0001$ and $p = 0.002$) (table 2) whereas MGMT promoter methylation status was not. The population could therefore be divided into 3 main prognostic groups (figure 1): patients with 1p-19q codeletion and *IDH* mutation (n = 50) had a median OS of 151 months, patients with only *IDH* mutation (n = 77) had an OS of 110 months, and patients with neither of these alterations (n = 46) had an OS of 68 months ($p = 0.0002$).

In a multivariate analysis including the main clinical and molecular variables, KPS >80, LOH on 1p-19q, and *IDH* mutation ($p = 0.003$) remained strongly associated with prolonged survival (table 3).

Subgroup analysis. ***Patients with no initial adjuvant treatment.*** In order to analyze the impact of molec-

Table 2 Correlations between clinical or molecular factors and PFS or OS: Univariate analysis

	No. of observations	Median PFS, mo	p	Median OS (months)	p
Male	158	41.4	0.4	110	0.01
Female	113	39.9		161.7	
Age at diagnosis <55 y	245	41.4	0.4	136.5	0.001
Age at diagnosis >55 y	26	27.6		62.4	
Preoperative KPS ≤80	39	25.7	0.01	78	<0.0001
Preoperative KPS >80	208	42.8		150.9	
No neurologic deficit	141	40.4	0.008	136.5	0.002
Neurologic deficit	18	19.5		53.9	
Biopsy	62	36	0.09	91.7	0.0004
Partial resection	95	35.6		150.9	
Gross total resection	108	48		151.6	
Astrocytoma	47	34.3	0.11	103.9	0.98
Oligodendroglioma	158	42.6		133.3	
Oligoastrocytoma	66	39.6		136.5	
Postoperative radiotherapy	50	56.8	<0.0001	84.4	0.9
Delayed radiotherapy	81	23.9		103.9	
Postoperative chemotherapy	58	38.4	0.08	Not reached	0.1
Delayed chemotherapy	126	35.4		119.2	
No LOH 1p-19q	179	35.9	0.002	103.9	0.0001
LOH 1p-19q	84	49.8		161.7	
No *IDH* mutation	57	43.3	0.47	83.9	0.002
IDH1 or *IDH2* mutation	132	43.9		136.5	
No p53 expression	111	48	0.02	133.3	0.2
p53 expression	102	36.8		111.4	
MGMT methylated	150	41.3	0.001	119	0.3
MGMT unmethylated	35	23		Not reached	

Abbreviations: KPS = Karnofsky performance status; LOH = loss of heterozygosity; OS = overall survival; PFS = progression-free survival.

ular factors on the spontaneous course of the disease, we evaluated PFS in 171 patients who had no adjuvant treatment after surgery until first progression. Their main clinical characteristics are summarized in table 1.

Table 3 Correlations between clinical or molecular factors and PFS or OS: Multivariate analysis

	PFS		OS	
Covariate	p	Hazard ratio	p	Hazard ratio
Age>55 y	0.8	1.13	0.2	2.17
Female	0.5	1.18	0.6	0.82
KPS >80	0.07	0.56	0.0003	0.21
Gross total resection vs partial resection and biopsy	0.08	0.75	0.2	0.7
Histology	0.5	0.89	0.7	1.1
LOH 1p-19q	0.04	0.6	0.004	0.3
IDH mutation	0.7	0.92	0.003	0.32
MGMT promoter unmethylation	0.02	2.3	0.8	1.15

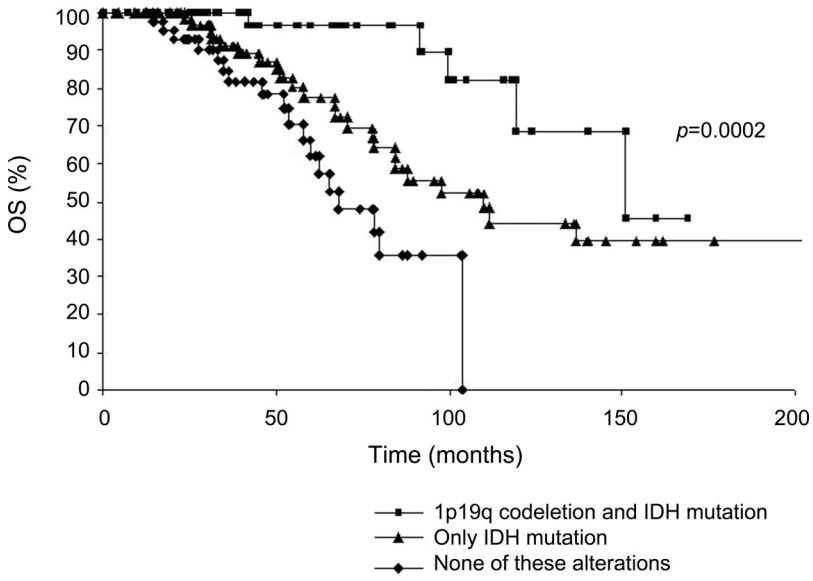
Abbreviations: KPS = Karnofsky performance status; LOH = loss of heterozygosity; OS = overall survival; PFS = progression-free survival.

In univariate analysis, KPS >80 ($p = 0.01$), absence of neurologic deficit at diagnosis ($p = 0.02$), gross total resection (vs biopsy or partial resection) ($p = 0.0002$), absence of p53 expression ($p = 0.04$), and LOH on 1p-19q ($p = 0.05$) were associated with prolonged PFS, whereas *IDH* mutation ($p = 0.43$) and MGMT promoter methylation ($p = 0.07$) were not correlated with PFS (figure 2). Only KPS and resection remained significant on multivariate analysis.

Patients initially treated with TMZ. Eighty-four patients were initially treated with TMZ, prior to any other treatment except for surgery. Their main characteristics are summarized in table 1.

IDH mutation was detected in 33/49 (67%) tumors and LOH on 1p-19q in 31/83 (37%) tumors. All but one patient with 1p-19q codeletion also had *IDH* mutation ($p = 0.003$).

Figure 1 Overall survival (OS) according to the 1p19q and *IDH* status

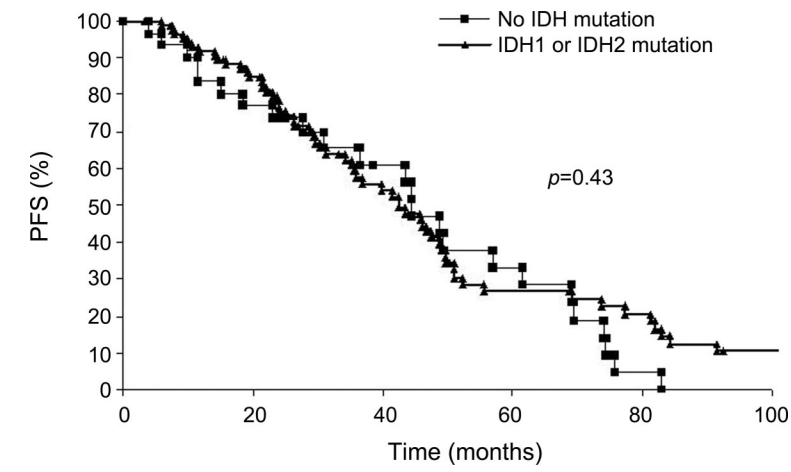


Response to TMZ was assessable in 74 of these patients. Patients received a median number of 18 cycles of TMZ (range 2–28 cycles). Eleven patients (15%) achieved a partial response, 23 patients (31%) achieved a minor response, 22 patients (30%) were stable, and 18 (24%) patients had progressive disease. None of the patients developed myelodysplastic syndrome or myelogenous leukemia during the entire follow-up period.

The objective rate of response was higher in the *IDH* mutated group (17/28 patients: 61%) than in the *IDH* wild-type group (2/12 patients: 17%) ($p = 0.01$). The patients with 1p-19q codeletion ($p = 0.002$) and the patients with *MGMT* methylation ($p = 0.02$) also had a higher rate of objective response (figure e-1 on the *Neurology*® Web site at www.neurology.org). Three subgroups of different chemosensitivity were identified (figure e-2): patients with both 1p-19q codeletion and *IDH* mutation had the best response rate to TMZ (80%), as compared to patients with *IDH* mutation alone (33%) and to patients with none of these alterations, who had the worst response rate to TMZ (17%) ($p = 0.004$).

Median PFS was increased in patients with *MGMT* methylation ($p = 0.01$), 1p-19q codeletion ($p = 0.06$), and *IDH* mutation ($p = 0.07$). PFS was 37.9 months in 1p-19q/*IDH* mutated patients, 32.9 months in the *IDH* mutated alone subgroup, and 18.7 months in patients with neither alteration. However, these differences did not reach significance. The number of events was too low to evaluate survival.

Figure 2 Progression-free survival (PFS) according to *IDH* status in the untreated group



DISCUSSION This study indicates that *IDH* mutation in LGG constitutes a major independent favorable prognostic factor for survival, whose importance seems comparable to the 1p-19q codeletion. We also found some evidence that *IDH* mutation is a molecular predictor of response to TMZ, but that its role on the spontaneous growth of LGG before anaplastic transformation is more questionable.

Our data confirm that *IDH1* mutations are frequent in LGG, occurring in two-thirds of patients, whereas *IDH2* mutations are much rarer (only 6%). As previously reported,[9] these 2 mutations are mutually exclusive (100% of cases in our series), suggesting that they are involved in similar tumorigenesis pathways. *IDH1* and *IDH2* mutations were therefore grouped together in our prognostic analysis.

As expected, the 2 classic groups of LGG comprising 1p-19q codeleted tumors (mainly oligodendrogliomas) and tumors with p53 expression (mainly astrocytomas) were clearly identified in this series,[24–26] although astrocytomas were less represented in our series than in the literature.[24,27] Of note was the close association between 1p-19q codeletion and *IDH* mutations, as also noted by others.[6,8] Most of the patients with 1p-19q codeletion also had *IDH* mutations. The reverse was not true, since many patients had *IDH* mutations without 1p-19q codeletion.

In contrast with another study,[10] we did not identify a relationship between age and *IDH* status in astrocytomas; however, these data should be viewed with caution because there were only 34 astrocytomas with a known *IDH* status in our series.

A primary result to arise from this study is that *IDH* mutations are strongly associated with prolonged survival of LGG in univariate as well as in multivariate analysis. Several studies found that *IDH* mutations were correlated with prolonged survival in gliomas of various grades.[6,8] In a recent work,[11] we

found that 1p-19q codeletion and *IDH1* mutation were independent markers of good prognosis in a multivariate analysis of a series of gliomas (where all grade tumors were pooled). More recently, a correlation between OS and *IDH1* was found in a small cohort of 49 low-grade astrocytomas.[28] However, the relationship between *IDH* mutation and 1p-19q codeletion in LGG remained unclear. This point is important to clarify because the close link between these 2 alterations raises the question that prolonged survival in patients exhibiting *IDH* mutation merely reflects the major prognostic impact of the frequently associated 1p-19q codeletion. In fact, in this large LGG series, *IDH* mutation was a favorable marker of survival, independently of the 1p-19q status. Thus, the population could be divided into 3 main prognostic groups according to their 1p-19q/*IDH* status: first, the patients with 1p-19q codeletion and *IDH* mutation (OS of 151 months), second, the patients with only *IDH* mutation (OS of 110 months), and third, the patients with neither alteration (OS of 68 months).

A second finding of this study is the association between *IDH* mutation and response to TMZ.

In contrast with a previous study[28] that focused on previously irradiated LGG treated with TMZ after malignant transformation, we studied a group of LGG who received up-front temozolomide before any evidence of anaplastic transformation. Previous studies have identified 2 molecular markers of chemosensitivity in LGG: 1p-19q codeletion and *MGMT* promoter methylation,[19,29] but there are no available data on the role of *IDH* mutation. Again, we found 3 subgroups of decreasing chemosensitivity. The best response rate was observed in patients harboring both 1p-19q deletion and *IDH* mutation with a 80%/13%/7% rate of objective response/stable/progressive disease, whereas these rates were 33%/59%/8% in patients with only *IDH* mutation and 16%/25%/59% in patients with none of these alterations. These data suggest that both 1p-19q codeletion and *IDH* mutations are favorable predictors of response to TMZ.

Although PFS was about twice as long in *IDH*-mutated patients (37.9 and 32.9 months in patients with and without concomitant 1p-19q codeletion) as compared to *IDH*-intact patients (18.7 months), this difference did not reach significance, possibly because of insufficient power due to the limited number of patients.

The mechanisms underlying the increased rate of response of *IDH* mutated tumor are unknown, but *IDH* mutation could promote treatment-induced apoptosis by inhibiting cellular protection against oxidative stress. The relationship between *IDH* mutation, *MGMT* promoter methylation, and chemosensitivity could not be analyzed because of the very low incidence of unmethylated *MGMT* in the TMZ-treated subgroup.

In order to study the role of *IDH* mutation on the spontaneous growth (natural history) of LGG, we analyzed PFS in a series of 171 patients who had no adjuvant treatment after surgery until first progression. Except for a much higher rate of tumor resection (93% vs 51% of partial or total resection), those patients in whom a wait-and-see attitude was selected did not differ from the TMZ-treated group in term of age, KPS, or molecular prognostic factors (1p-19q, *IDH*, *MGMT*). As previously reported,[30] we found that the 1p-19q codeletion was associated with increased spontaneous PFS in the absence of treatment. Conversely, spontaneous PFS did not differ in *IDH*-mutated and *IDH*-intact patients. This finding could account for the lack of effect of *IDH* mutation on PFS in the whole series (n = 271), since this untreated subgroup (n = 171) constitutes a vast majority of the entire population. This result suggests that *IDH* mutation status does not profoundly influence the natural course of the disease when additional treatments are not administered. In contrast with the 1p-19q deletion, which seems to be both a marker of slower spontaneous growth and a marker of response to treatment in LGG, *IDH* mutation could primarily act as a predictor of response, at least in the phase preceding anaplastic progression. However, this result should be interpreted with caution, because determining PFS precisely in LGG is difficult.

Although additional studies are needed to better delineate the role of *IDH* mutation in the natural course of LGG and response to treatment, the prognostic importance of this alteration already appears clear, deserving a systematic analysis in future trials and possibly in the prognostic workup of newly diagnosed LGG.

AUTHOR CONTRIBUTIONS

Statistical analysis was conducted by Dr. J. Laffaire and Dr. C. Houillier.

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