Universidade do Minho
Escola de Ciências da Saúde

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Molecular Genetics of Paediatric *versus* Adult Brain Tumours

Genética Molecular de Tumores Cerebrais Pediátricos *versus* Adultos

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Tese de Doutoramento em Ciências da Saúde
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Trabalho efectuado sob a orientação do
Doutor Rui Manuel Vieira Reis
Professor Auxiliar da Escola de Ciências da Saúde,
Universidade do Minho, Braga, Portugal

e coorientação do
Doutor Chris Jones
Leader of the Paediatric Molecular Pathology Team,
Institute of Cancer Research, Sutton, UK

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Nome: Marta Sofia Carvalho Ribeiro Viana Pereira

Endereço electrónico: martapereira@ecsaude.uminho.pt

Telefone: (+351) 965879657

Número do Bilhete de Identidade: 12314379

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Genética Molecular de Tumores Cerebrais Pediátricos versus Adultos

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Doutor Chris Jones

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Um dia hei-de ouvi-lo em amor e saudade...

Para o meu papá, Paulino Pereira

(In memoriam)

Summary / Resumo

Summary

Brain tumours are the leading cause of cancer-related death in paediatric patients and are responsible for the greater part of the cancer-related years of life lost across all age groups. The more malignant histologies are the major contributors to the high-rates of mortality and morbidity of brain tumours. In the present thesis we focused our studies on high-grade gliomas and medulloblastomas, the most common malignant brain tumours of adults and childhood patients, respectively. Childhood and adult patients share most of the different brain tumours' histological types, despite a large variation in frequency across specific age groups. Nevertheless, there is increasing evidence that, despite being histologically similar, childhood and adult tumours have key clinical and molecular differences. The work summarized in this thesis aims to disclose molecular mechanisms particular to paediatric and adult high-grade gliomas and medulloblastomas, attempting to better understand the biology of age-specific, histologically identical, tumours.

Brain tumours are characterized by multiple genetic alterations affecting receptor tyrosine kinase (RTK) pathways. As the EGFR RTK pathway is one of the most important signalling networks in high-grade gliomas, we aimed to study EGFR molecular aberrations involved in protein activation, potentially relevant for tumour's response to EGFR-targeted therapy. The role of EGFR in adult high-grade gliomas is well-characterized, and in the present thesis we studied a unique series of Portuguese patients, aiming to understand whether the EGFR molecular alterations of these patients were in line with other populations in terms of potential biomarkers of EGFR-targeted therapy. On the other hand, EGFR is thought to be less significant in childhood tumours, although there is limited published data. Accordingly, we aimed to study the frequency and role of EGFR molecular alterations in paediatric high-grade gliomas, aiming to evaluate the presence of molecular signatures of response to existing drugs in these patients. We confirmed that EGFR represents one of the most frequently altered molecules in high-grade glioma, particularly in adult glioblastoma, and that it is also true for Portuguese patients. In addition some paediatric tumours, particularly anaplastic oligodendrogliomas, frequently presented *EGFR* aberrations and therefore are also potential candidates for EGFR-targeted therapy.

Microsatellite instability (MSI) frequency in brain tumours remains a controversial research topic, and there is a lack of clarity in the published literature. In this context, we aimed to study MSI in high-grade gliomas and medulloblastoma from adult and paediatric patients and identify MSI target genes potentially involved in MSI-related tumorigenesis. Our findings show the presence of MSI in a fraction of medulloblastoma and high-grade gliomas. Age-specific differences in MSI frequency were not present in medulloblastoma, however MSI was significantly more frequent in paediatric high-grade gliomas than in adults tumours. Moreover MSI-positivity was associated with a stable genomic profile. Overall, of the 18 MSI target-genes studied, only three were mutated, all in paediatric in MSI tumours, *MBD4* in one medulloblastoma, and *MSH6* and *DNAPKcs* in high-grade glioma As we failed to find the MMR alteration responsible for the MSI phenotype, further research is critical to clarify this topic. Nevertheless, our studies provided evidence for a potential novel molecular pathway in a proportion of medulloblastoma and paediatric high-grade gliomas, associated with the presence of MSI.

Overall, the work summarized in this thesis contributed to the knowledge of the molecular mechanisms involved in the development of childhood and adult brain tumours, and confirms that, despite being histologically indistinguishable, these tumours can be molecularly distinctive.

Resumo

Os tumores cerebrais são a principal causa de morte por cancro em crianças, sendo também os principais responsáveis na diminuição de anos de vida em doentes oncológicos de todas as faixas etárias. Os tumores de maior malignidade sãos que mais contribuem para as altas taxas de mortalidade e morbilidade características dos tumores cerebrais. Nesta tese, centramos os nossos estudos em gliomas de alto grau e meduloblastomas, os tumores cerebrais malignos mais frequentes em doentes adultos e pediátricos, respectivamente. Apesar dos diversos tipos histológicos de tumores cerebrais serem comuns a doentes de diferentes idades, existe uma significativa diferença na frequência com que ocorrem em adultos e crianças. Além disso, há cada vez mais indícios de que, tumores histologicamente semelhantes, apresentam diferenças fundamentais a nível clínico e molecular, dependendo da idade dos doentes.

Os tumores cerebrais são caracterizados por diversas alterações genéticas que afectam os receptores de tirosina cinase (RTK). Sendo a via de sinalização do RTK EGFR uma das mais importantes em gliomas de alto grau, estudámos alterações moleculares envolvidas na sua activação e potencialmente importantes na resposta tumoral à terapia dirigida. O papel desta molécula em gliomas de alto grau de doentes adultos tem sido amplamente descrito, pelo que avaliámos uma série de tumores de doentes Portugueses adultos. Neste trabalho pretendemos perceber se as alterações de EGFR nos tumores Portugueses se assemelham às descritas noutras populações, de forma a avaliar o seu potencial papel como biomarcador de terapia dirigida ao EGFR. Por outro lado, , apesar da escassez de dados publicados, pensa-se que a importância do EGFR em tumores pediátricos seja limitada. Para melhor esclarecer este assunto, estudámos a presença de alterações moleculares do EGFR em gliomas pediátricos de alto grau, com o objectivo de avaliar quais os potenciais biomarcadores na resposta a fármacos anti-EGFR. Confirmámos que o EGFR é uma das moléculas mais frequentemente alteradas em gliomas de alto grau, particularmente em tumores adultos, sendo isto também verdade para os doentes Portugueses. Igualmente os tumores pediátricos, em particular, os oligodendrogliomas anaplásicos, apresentam alterações nesta molécula e consequentemente, são também potenciais candidatos ao uso de fármacos anti-EGFR.

A ocorrência de instabilidade de microssatélites (MSI) em tumores cerebrais é um tópico de investigação controverso, sendo a literatura existente insuficiente para a esclarecer este assunto. Neste contexto, estudámos a presença de MSI em gliomas de alto grau e meduloblastomas de doentes adultos e pediátricos, assim como os genes-alvo potencialmente envolvidos na tumorigénese relacionada com MSI. Os nossos resultados demonstram a presença de MSI numa fracção de meduloblastomas e gliomas de alto grau. Em meduloblastomas não se observaram variações na presença de MSI em doentes de diferentes idades, no entanto em gliomas de alto grau, a frequência de MSI é estatisticamente mais elevada nos tumores pediátricos . Além disso, em gliomas, a presença de MSI foi associada a um perfil genómico estável. Dos mais de 18 genes-alvo analisados, foram encontradas mutações apenas em três e somente em tumores pediátricos: *MBD4* num meduloblastoma e *MSH6* e *DNAPKcs* em gliomas de alto grau. . O estudo das moléculas de "*mismatch repair*" não clarificou qual o seu papel no fenótipo de MSI, observado nestes doentes. No entanto, os nossos estudos evidenciam a presença de uma potencial nova via molecular em alguns meduloblastomas e gliomas de alto grau associada à presença de MSI.

Concluindo, o trabalho resumido nesta tese, contribuiu para o conhecimento das mecanismos molecular envolvidos no desenvolvimento de tumores cerebrais pediátricos e adultos, e confirma que apesar de histologicamente semelhantes, podem ser molecularmente distintos.

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PAPER I – Bax DA, Little SE, Gaspar N, Perryman L, Marshall L, **Viana-Pereira M,** Jones TA, Williams RD, Grigoriadis A, Vassal G, Workman P, Sheer D, Reis RM, Pearson AD, Hargrave D, Jones C. Molecular and phenotypic characterisation of paediatric glioma cell lines as models for preclinical drug development. **PLoS One**; 4(4): e5209, 2009.

PAPER II – Bax DA, Mackay A, Little SE, Carvalho D, **Viana-Pereira M**, Tamber N, Grigoriadis AE, Ashworth A, Reis RM, Ellison DW, Al-Sarraj S, Hargrave D, Jones C. A distinct spectrum of copy number aberrations in pediatric high-grade gliomas. **Clinical Cancer Research**; 16(13): 3368-77, 2010.

PAPER III – Gaspar N, Marshall L, Perryman L, Bax DA, Little SE, **Viana-Pereira M,** Sharp SY, Vassal G, Pearson A, Reis RM, Hargrave D, Workman P, Jones C. MGMT-independent temozolomide resistance in paediatric glioblastoma cells associated with a PI3-Kinase-mediated HOX / stem cell gene signature. **Cancer Research**; 70(22): 9243-52, 2010.

Abbreviations

AA – anaplastic astrocytoma

aCGH – array comparative genomic hybridization

***AKT* –** protein kinase B (PKB)

***APC* –** adenomatous polyposis coli gene

AO – anaplastic oligodendroglioma

AOA – anaplastic oligoastrocytoma

BAX – BCL2-associated X protein

BRAF – v-raf murine sarcoma viral oncogene homolog B1

***CDKN2A/CDKN2B/CDKN2C* –** cyclin-dependent kinase inhibitor 2A, 2B or 2C

cDNA – complementary deoxyribonucleic acid

CIN – chromosomal instability

CISH – chromogenic *in situ* hybridization

CNS – central nervous system

CTNNB1 – catenin beta 1

CpG – adjacent cytosine and guanine dinucleotides

CRC – colorectal cancer

DAB – 3,3'-diaminobenzidine

DNA – deoxyribonucleic acid

***EGF* –** epidermal growth factor

***EGFR* –** epidermal growth factor receptor

ERK – extracellular signal-regulated kinase

FFPE – formaline-fixed, paraffin-embedded

FISH – fluorescent in situ hybridization

FZD – frizzled

GWA – genome-wide association

HER – human epidermal receptor

HGF – hepatocyte growth factor

HGG – high-grade glioma

HNPCC – hereditary non-polyposis colorectal cancer

IDH1/IDH2 – isocitrate dehydrogenase 1 or 2

IGFR – insulin-like growth factor receptor

KRAS – v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

mAbs – monoclonal antibodies

MAPK – mitogen-activated protein kinase

***MBD4* –** methyl-CpG binding domain protein 4

MET – mesenchymal-epithelial transition factor receptor

***MGMT* –** methyl guanine methyltransferase

MLH1 – mutL homolog 1, colon cancer, nonpolyposis type 2

MLH3 – mutL homolog 3

MMR – DNA mismatch repair

MMR-D – DNA mismatch repair deficiency

***MRE11* –** meiotic recombination 11 homolog A

MSH2 – mutS homolog 2, colon cancer, nonpolyposis type 1

MSH3/MSH4/MSH5/MSH6 – mutS homolog 3, 4, 5 or 6

MSI – microsatellite instability

MSS – microsatellite stable

MS-PCR – methylation-specific PCR

mTOR – mammalian target of rapamycin

MYC – v-myc myelocytomatosis viral oncogene homolog

MYCN – v-myc myelocytomatosis viral related oncogene, neuroblastoma derived

NADPH – nicotinamide adenine dinucleotide phosphate

NF1/NF2 – Neurofibromatosis type 1/2

NF1/NF2 – neurofibromin 1/2

NSCLC – non-small cell lung cancer

O – oligodendroglioma

PCR – polymerase chain reaction

PCV – procarbazine, **CCNU** (lomustine), and vincristine

PDGFA/PDGFB – platelet-derived growth factor (alpha or beta polypeptide)

***PDGFRA/PDGFRB* –** platelet-derived growth factor receptor (alpha or beta polypeptide)

PKCs – protein kinase catalytic subunit

PI3K – phosphatidylinositol-3-kinase

PMS1/PMS2 – PMS2 postmeiotic segregation increased (1 or 2)

PTCH – patched

PTPRD – protein tyrosine phosphatase, receptor type, D

PTEN – protein tyrosine phosphatase and tensin homolog

Ras – rat sarcoma

Raf – rapidly accelerated fibrosarcoma

RB – retinoblastoma

RNA – ribonucleic acid

RT – room temperature

RT-PCR – reverse transcription-PCR

RTK – receptor tyrosine kinase

SHH – sonic hedgehog

SMO – smoothened homolog

SNP – Single-nucleotide polymorphism

STAT – signal transducers and activator of transcription

SUFU – suppressor of fused homolog

TCGA – the cancer genome atlas

TGF – transforming growth factor

TKI – tyrosine kinase inhibitor

TP53/TP73 – tumour protein 53/73

TSC – tuberous sclerosis

UTR – untranslated region

VEGF – vascular endothelial growth factor

WHO – world health organization

Wnt – wingless type mmtv integration site family

Thesis Layout

Thesis Layout

Chapter 1 presents a general introduction of the state of the art of brain tumours and genetic instability, aiming to direct attention to the work developed in the present thesis. Specifically, we review the impact, epidemiology and histology of brain tumours, as well as differential genetic instability and molecular alterations present in cancer. In particular, it is reviewed their relevance in brain tumours, focusing on high-grade gliomas and medulloblastomas and emphasising the differentiation between adult and paediatric populations.

Chapter 2 presents the rationale and aims of the thesis.

Chapter 3 comprises two pieces of work reporting EGFR molecular characterization in adult gliomas (**Sub-Chapter 3.1**) or in paediatric patients (**Sub-Chapter 3.2**). In the latter paper, the role of this molecular aberration was further explored as a potential therapeutic target in paediatric glioma cell lines. Due to the well-established importance of EGFR in the development of gliomas in contrast to medulloblastoma, in this chapter we focused only on the study of these tumours.

Chapter 4 presents two studies of MSI in brain tumours, both in medulloblastoma (**Sub-Chapter 4.1**) and high-grade glioma (**Sub-Chapter 4.2**), comprising series of adult and childhood tumours. In this chapter both medulloblastomas and high-grade gliomas were explored as in medulloblastomas literature is extremely scarce and in high-grade gliomas the frequencies reported are highly heterogeneous.

The final chapter, **Chapter 5** aims to bring together and discuss, on the basis of previous relevant literature, the major findings of the work presented throughout the thesis.

1. General Introduction

1. General Introduction

1.1 – Incidence and General Overview

Cancer represents one of the biggest health challenges of the new century. In fact, it is estimated that in the developed world, cancer will affect one in three people during their lifetime, resulting in the second leading cause of death in the United States, only preceded by heart diseases, pooling all age groups among both genders (1). Survival rates vary widely between different types of cancer, and stage of the disease at diagnosis, with later stage diagnosis and metastatic disease invariably corresponding to poorer survival. Worldwide, excluding non-melanoma skin cancer, nearly 12.7 million new cancer cases and 7.6 million cancer related deaths occurred in 2008 (2). In the paediatric population, about 10,700 new tumours were diagnosed in the United States in the same year, with almost 1,500 deaths estimated to occur in children up to 14 years of age (3). The type and aetiology of cancers that affect children and adults are different. Almost all adult cancers derive from epithelial tissue whereas paediatric cancers mostly derive from non-ectodermal embryonal tissue (4).

1.1.1 – Brain Tumours in Adult and Paediatric Populations

Primary Central Nervous System (CNS) tumours are considered to be those that originally initiate in the CNS and usually remain there. These tumours occur in people of all ages, but are more frequent in childhood and in the elderly. Instead, secondary brain tumours, which are brain metastases derived from malignant tumours elsewhere in the body, are more common in adults than in children. Primary CNS tumours, despite not leading the cancer frequency rates, rank first among cancer types for the average years of life lost, with an average of 20.1 years (5). By contrast with other malignancies, where research has lead to the establishment of more successful treatment options, the diagnosis of a CNS tumour, particularly the more malignant histological types, still has devastating effects on the patients and their relatives, not only due to the dismal prognosis of these tumours with extremely high rates of mortality, but also to the great morbidity that actual treatment options cause (6-9).

The estimated annual worldwide age-standardized incidence of malignant primary CNS tumours is 3.5 per 100,000 people, which represents more than 200,000 cases. The rate is slightly higher in males (3.9 per 100,000 people per year) than in females (3.1 per 100,000 people per year) (2). Additionally, rates appear to be higher in more developed countries (5.2 per 100,000 people per year) than in less developed countries (3.0 per 100,000 people per year) and this is thought to be a consequence of progress in diagnostic technologies and ascertainment, as well as access to adequate health care in the developed countries (2, 10). Similarly, estimated annual global age-standardized mortality of malignant primary CNS tumours is higher in males (3.0 per 100,000 people) than in females (2.2 per 100,000 people), with higher rates also in more developed regions (3.2 per 100,000 people) than in less developed regions (2.3 per 100,000 people) (2). The median age of diagnosis for all primary CNS tumours is 57 years, however the histology-specific median age ranges from 9 to 70 years (3).

In children, CNS tumours are the second most frequent malignancy after leukaemia and are therefore the most common solid tumours in childhood (1). An estimated 3,750 new cases of childhood primary non-malignant and malignant CNS tumours are expected to be diagnosed in the United States each year. This number reflects an annual incidence rate of 4.5 cases per 100,000 people. As in adults, the rate is higher in males (4.7 cases per 100,000 people) than in females (4.3 cases per 100,000 people) (3). Importantly, malignant brain tumours are the leading cause of cancer-related death in the childhood (11).

In Portugal, the annual, age-standardized, incidence (6.4 per 100,000 people) and mortality (4.3 per 100,000 people) rates of primary CNS tumours are among the highest in the world (Figure 1). Overall, it is estimated that about a thousand new cases arise per year, with about 750 people dying annually as a consequence of the disease (2).

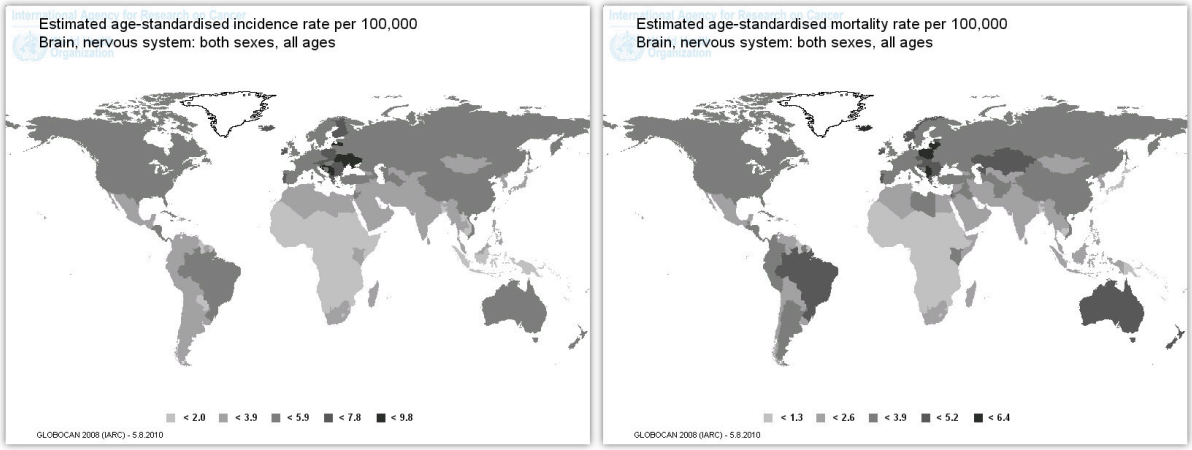

Figure 1 – Age-standardized, estimated, incidence (left map) and mortality (right map) of brain and CNS tumours in 2008. *Adapted from refernce.* (2).

1.1.2 – Histological Classification and Clinical Features

From many years now, CNS tumours have been mostly classified according to the World Health Organization (WHO) criteria (12). There are a wide range of CNS histological entities, classified according to the cell morphology and the degree of malignant behaviour. The WHO classification (12) includes a grading scheme that is a means of predicting the biological behaviour of the tumour: Grade I tumours generally have a low proliferative potential and the possibility of cure after surgical resection; Grade II tumours are generally infiltrative and, despite low level proliferative activity, often recur with some histologies tending to progress to higher grades of malignancy; Grade III tumours generally have histological evidence of malignancy, including nuclear atypia and sharp mitotic activity; Finally, grade IV tumours are histologically malignant, mitotically active and necrotic, often associated with rapid disease evolution and fatal outcome. Accordingly, CNS tumours span from benign to highly malignant, with the last mostly contributing to the high rates of mortality and morbidity among the patients. This thesis will focus only on malignant CNS tumours, more particularly high-grade gliomas (WHO grades III and IV) and medulloblastomas (grade IV), which are the most common malignant CNS tumours of adults and children, respectively.

The incidence of CNS tumours in general, but also of different histological types, varies across specific age groups (Figure 2) (13, 14).

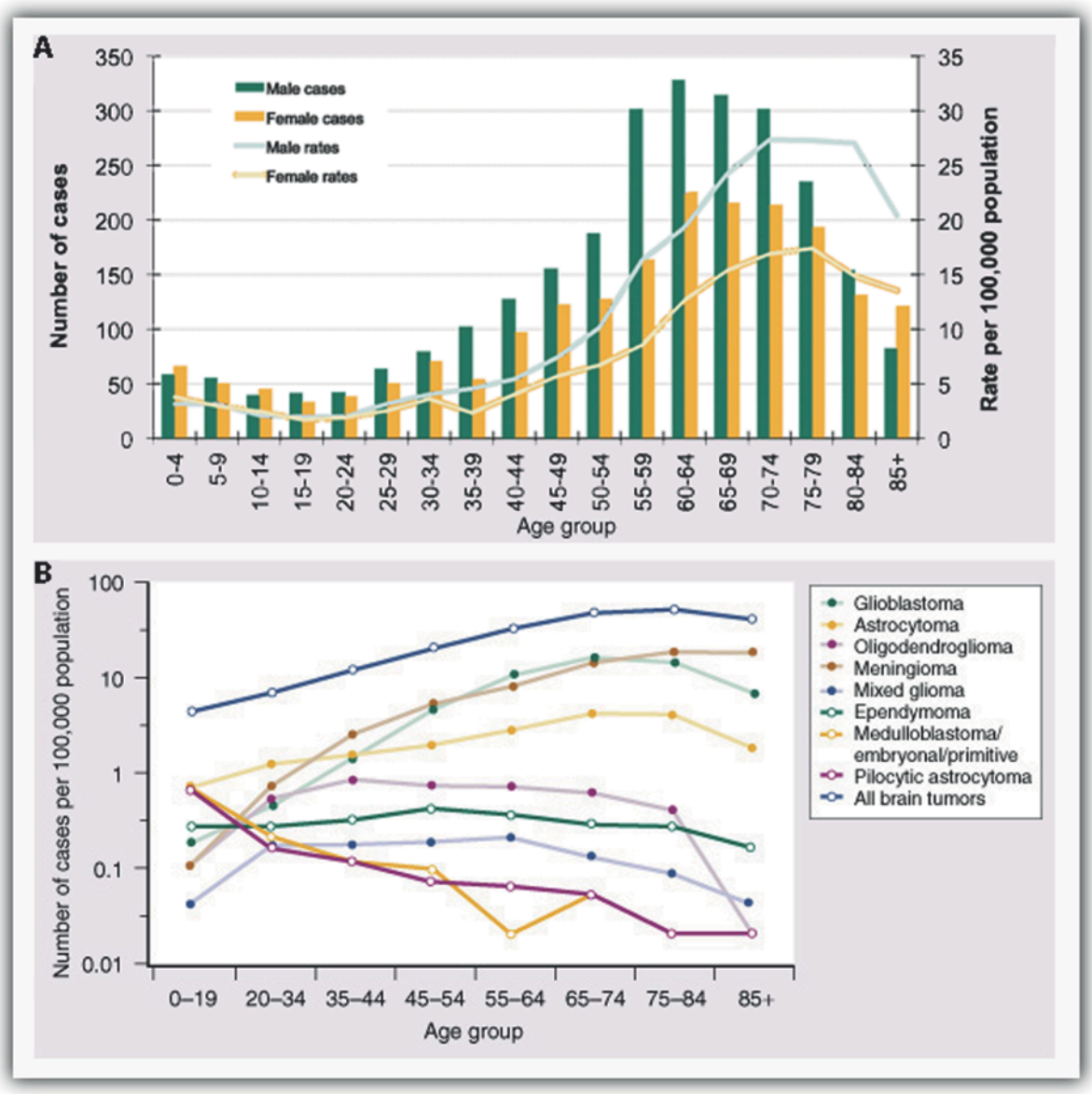

Figure 2 - Age specific incidence of primary CNS tumours. A. Numbers of new cases and age specific incidence rates, by sex, on CNS tumours. (UK incidence, 2007, data from Cancer Research UK). *Adapted from reference* (13). **B.** Age-specific incidence of primary CNS tumors by histology. "All brain tumors" includes other specific histologies not individually shown (tumors of the cranial and spinal nerves, hemangioblastomas, CNS lymphomas, germ cell tumors, tumors of the sellar region). "Astrocytoma" includes diffuse astrocytomas, anaplastic astrocytomas, unique astrocytoma variants, and astrocytomas not otherwise specified (Data from Central Brain Tumor Registry of the United States: Statistical Report: Primary Brain Tumors in the United States 1992–1997) . *Adapted from reference* (14)

Accordingly, the regions of the brain mostly affected by children and adult tumours are also different. In adults and older children, most CNS tumours are located supratentorially (cerebrum), whereas in young children they are more commonly infratentorial (arising in the cerebellum) (Figure 3) (15).

Brain tumour aetiology is thought to be multifactorial and is likely to vary by tumour type. There is a likely connection between genetics and the environment, meaning that particular genetic susceptibilities lead to increased vulnerability to environmental factors. The only proven exogenous environmental cause of brain tumours, in children or adults, is ionizing radiation, often seen in the setting of previous radiation therapy for the treatment of a former malignancy (10, 16-19).

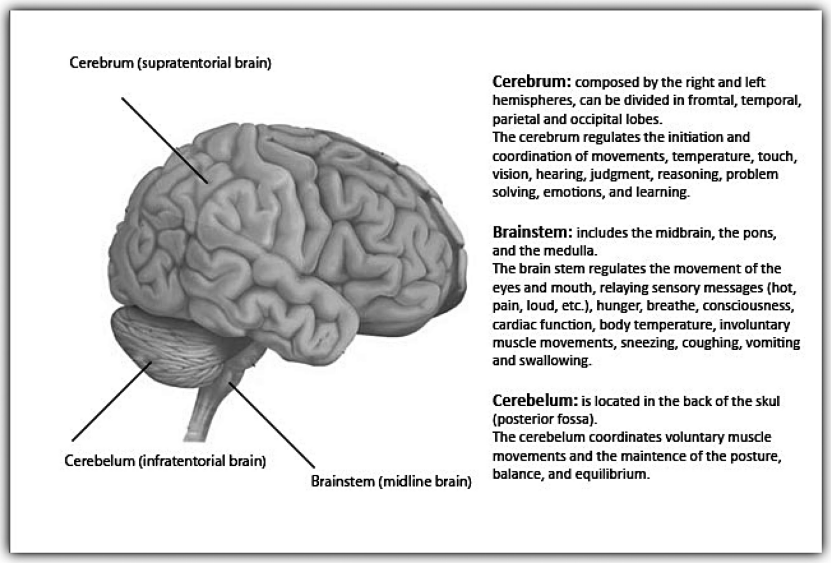

Figure 3 – Representation of brain different anatomical parts and their regulatory function. *Adapted from reference* (24).

Initial symptoms of a brain tumour vary according to the location and growth rate of the tumour (20, 21). Symptoms occur due to degradation of normal brain tissue, but mostly to the increase in intracranial pressure. Several symptoms are common among paediatric and adult patients, varying in demonstration according to the age of the patient. Infants may simply manifest distress with vague, nonspecific symptoms, such as vomiting, lack of coordination, lethargy and irritability. A peculiar symptom of babies and infants is the enlargement of the head circumference, due to the incompletely fused skull. Other common symptoms of adults and older children such as headaches, cognitive decline, personality alterations or speech changes are difficult to evaluate in infants. Eventually, symptoms are correlated with the area of the brain affected (Figure 3) (20-24). Tumours in the posterior fossa usually cause ataxia, diplopia, papilloma and nystagmus, hypotonia, gait disturbance and incoordination. Patients with supratentorial tumours develop signs and symptoms according to the area of the brain affected. Common symptoms are seizures, hemiparesia

and hemiplasia, memory loss, behaviour changes and visual field cuts. The sudden onset of symptoms is usually indicative of rapidly growing or aggressive tumours. A clinical history more likely indicates low-grade or benign neoplasias (20-23).

1.1.2.1 – High-Grade Gliomas

The brain is composed of two major cell types, neurons and glia. In the CNS, glial cells are further categorized into four specific cell types: astrocytes, oligodendrocytes, ependymal cells and microglia (25). For many years, it was believed that these normal and well-differentiated glial cells were the origin of gliomas, giving rise to the morphologically correspondent tumours, with astrocytomas, oligodendrogliomas and the mixed lineage, oligoastrocytomas, being the major subtypes. Recently, however, this theory has been called into question, as some evidence suggests that gliomas arise from stem cells or lineage-committed progenitor cells (25, 26). The majority of CNS tumours in all age groups are gliomas. The estimated annual incidence rate of gliomas in the United States is 6.0 per 100,000 people, being higher in males (7.2 per 100,000 people) than in females (5.0 per 100,000 people). Overall, the broad category of gliomas accounts for 36% of all CNS tumours and 81% of malignant ones. In young adults (20-34 years), gliomas represent 39% of all CNS tumours and 86% of the malignant tumours. In adolescents (15-19 years), 45% of all CNS tumours and 81% of the malignant ones are gliomas, whereas in children less than 15 years the frequency of gliomas is higher (56%), but they only account for 74% of malignant tumours (3). The incidence rates of gliomas by histology vary among age-specific groups; in children and adolescents grade I gliomas are the most common tumours, whereas in older patients grade IV gliomas have the highest incidence (3). The treatment of low-grade gliomas with surgical resection alone allows in most instances the possibility of cure. On the other hand, high-grade gliomas remain a difficult therapeutic challenge with a poor prognosis. Conventional treatment includes surgery, radiotherapy and chemotherapy, however despite being helpful, actual therapeutic regimens are still unable to cure high-grade gliomas. The fact that in the malignant histologies, tumour cells infiltrate into surrounding brain and are not completely removed by surgery, and that these remaining cells are often resistant to radio and chemotherapy, together with the fact that residual cells almost always allow tumour to restart growing, are the main reasons for the therapeutic failure of high-grade gliomas (22). Similarly in children, surgery plays a major role in the

treatment of gliomas, with the extent of tumour resection the most important prognostic factor in this age group. For the more malignant subtypes, focal radiation is used as first line adjuvant therapy, except in infants (22, 27). Both in adult and paediatric populations, histological type and grade of the tumour, anatomic location, extent of surgical resection, patient's age, whether radiotherapy is applied, and some chemotherapy protocols have been consistently considered prognostic factors (10, 28). As mentioned, the major subtypes of gliomas are astrocytomas, oligodendrogliomas and the mixed oligoastrocytomas, and therefore the most malignant forms of these histological types will be further discussed in this section.

Tumours arising from astrocytic lineage, **astrocytomas** represent a highly heterogeneous histological group of neoplasms. They represent about 75% of all gliomas and can occur in most parts of the brain, and are the most frequent gliomas of childhood (3). While malignant astrocytomas comprise only 2% of all adult tumours their malignant nature makes them the fourth greatest cancer-related death (29). According to the WHO classification of CNS tumours, there are several different histological types of astrocytic tumours divided into four categories based on their grade of malignancy: WHO grade I, pilocytic astrocytoma; WHO grade II, diffuse astrocytoma; WHO grade III, anaplastic astrocytoma; and WHO grade IV, glioblastoma (12). The different histological types vary in frequency, age and gender distribution, location within the brain and clinical features.

WHO grade I Pilocytic astrocytoma comprise about 6% of all gliomas (3), mostly developing without a clear gender predilection, is the most common glioma in children, in whom they mainly arise infratentorially. In the age groups 0-14 years and 15-19 years, this tumour represents about 21% and 15% of all CNS tumours, respectively. Histologically, pilocytic astrocytoma is a relatively circumscribed and slowly growing tumour having therefore, a good prognosis when treated, with surgical resection alone allowing many times the possibility of cure. These tumours, however cannot be considered benign, as when not treated they can undergo in malignant transformation and lead to patient's death (12).

WHO grade II Diffuse astrocytoma represents about 2% of all gliomas (3). There is a peak incidence for this tumour (about 60%) in young adults, between 30 and 40 years of age, with a predominance of affected males (M:F ratio, 1.18:1). It is characterized by a high

degree of cellular differentiation and slow growth occurring throughout the CNS, but being preferentially located supratentorially. Diffuse astrocytoma has an intrinsic predisposition for malignant progression to anaplastic astrocytoma and, eventually, glioblastoma (12).

Anaplastic astrocytoma (WHO grade III) accounts for about 8% of all gliomas (3, 30), there is a peak of incidence between 45 and 55 years of age, with a predominance of affected males (12, 30). Anaplastic astrocytoma is a high-grade, diffusely infiltrating glioma, preferentially located in the cerebral hemispheres. In children and young adults it can also appear in the brain stem and thalamus (30). This malignant tumour presents an increased cellularity, cytologic atypia and prominent mitotic activity, with elevated invasiveness (12, 30). Anaplastic astrocytoma may arise from diffuse astrocytoma or *de novo*, *i.e.*, without recognizable precursor lesion (12). Whereas the great majority of adults with diffuse astrocytoma (WHO grade II) experience malignant transformation, the long-term risk of malignant transformation in histologically identical neoplasms in children is less than 10% (31). In most cases, anaplastic astrocytoma will progress to the most malignant astrocytoma, glioblastoma (WHO grade IV), with an estimated mean time to progression of about 2 years. (12, 32)

Glioblastoma (WHO grade IV) is the most frequent primary brain tumour, accounting for about 51% of all gliomas and 80% of the malignant tumours (12, 22, 23). It may manifest at any age, but typically affects adults, with a peak incidence between 45 and 75 years of age, occur predominantly in males and is preferentially located in the cerebral hemispheres (12, 30). In children, it can also be found in the brain stem (30). Glioblastoma is histopathologically characterized by high cellularity, nuclear atypia, marked cellular pleomorphism, elevated mitotic activity, vascular thrombosis, microvascular proliferation and necrosis (12, 30). Similar to anaplastic astrocytoma, glioblastomas may arise *de novo*, and are designated primary glioblastomas, or can be secondary glioblastomas, slowly developing from diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). The majority (about 95%) of glioblastomas are primary tumours, develop very rapidly in elderly patients (mean 62 years) after a short clinical history and present a poorer prognosis (12). Secondary glioblastoma mostly develop in younger patients below the age of 45 (12). Due to its invasive nature, glioblastoma cannot be completely resected and despite progress in radio- and chemotherapy, less than half of the patients survive more than a year, making

glioblastoma the CNS tumour with worse prognosis, for both paediatric and adult patients (12, 30). Older age is the most significant adverse prognostic factor for glioblastoma in adults (12, 30, 33). Other prognostic factors for high-grade astrocytoma include Karnofsky Performance Scale score at diagnosis and other measures of mental and physical functionality (17, 33).

The standard therapy for adults with glioblastoma consists of concomitant radiotherapy and the chemotherapeutic agent, temozolomide, followed by adjuvant temozolomide as described by Stupp et al. (34). On the other hand, children with high-grade glioma do not seem to derive significant benefit from any single chemotherapeutic regimen (15, 21, 27). Preliminary results of studies in paediatric malignant gliomas using the Stupp protocol indicate similar PFS results in childhood glioblastoma to those in comparable adult patients, however this does not represent a significant advance when compared to paediatric historical controls (35-39). The Stupp protocol has nevertheless become a default regimen used frequently in childhood glioblastoma and will probably remain as the chemotherapy backbone for future studies, although most patients still die from their disease within 2 years (15, 21, 27). Temozolomide is also used in patients with anaplastic astrocytoma when treatment with the standard chemotherapy of nitrosurea and procarbazine is not effective.

Composed predominantly of cells morphologically resembling oligodendroglia, **oligodendrogliomas** account for a small subset of all gliomas (about 8%). Oligodendroglial tumours, regardless of the WHO grade, often are more sensitive to radio- and chemotherapeutic treatments than other glioma subtype (40-42). Other favourable prognostic factor includes younger age, frontal localization of the tumour and high Karnofsky performance status. Oligodendrogliomas are predominantly tumours of adulthood, with a peak incidence between the fourth and fifth decade of life, representing only 1-2% of all CNS tumours in children (3). Similar to most astrocytomas, oligodendrogliomas are slightly more frequent in males than females and arise preferentially in the cerebral hemispheres, mainly in the frontal lobe. Histologically, oligodendroglial tumours comprise a continuous spectrum ranging from well differentiated to highly malignant tumours; over time, oligodendrogliomas gradually become more anaplastic and evolve from low-grade into high-grade glioma with anaplastic features (12,

30, 42, 43). The WHO recognizes two grades of malignancy for oligodendrogliomas, oligodendroglioma (WHO grade II) and anaplastic oligodendrogliomas (WHO grade III), with grading a significant predictor of survival.

Oligodendroglioma (WHO grade II) is a well differentiated diffusely infiltrating tumor, characterized by uniformly round to oval cells with round nuclei and bland chromatin, with cell density usually low to moderate. Standard treatment consists of resecting the tumor as extensively as safely possible, radio or chemotherapy can also be applied (12).

Anaplastic oligodendroglioma (WHO grade III) presents high cell density, mitotic activity, nuclear atypia, microvascular proliferation and necrosis. About half of the oligodendroglial tumours are anaplastic oligodendrogliomas. Treatment includes surgery, followed by radiotherapy and/or chemotherapy, typically with PCV (procarbazine, CCNU, and vincristine) (12).

Patients with high-grade oligodendroglial or mixed gliomas harbour the best outcomes (12).

By definition, **oligoastrocytomas** have morphological characteristics of both astrocytic tumors and pure oligodendrogliomas, being frequently referred as mixed gliomas (12, 30, 42, 43). Precise data on the incidence these tumours are not available, but reported frequencies vary from 1-20% of all gliomas, with the higher percentage thought to be overestimated due to a consultation bias (12, 30, 42, 43). Oligoastrocytomas arise preferentially in the cerebral hemispheres, with males slightly more affected than females, and mainly occurring in the fifth decade of life (12, 30, 42, 43). Similar to oligodendrogliomas, these tumours can also be divided in two grades of malignancy: WHO grade II, oligodendrogliomas and WHO grade III, anaplastic oligodendrogliomas.

Oligoastrocytoma (WHO grade II) develops in middle-aged people, with median ages ranging between 35 and 45 years. Is a moderately cellular tumour with no or low mitotic activity (12).

Anaplastic oligoastrocytoma (WHO grade III) develops in individuals slightly older than the less malignant form, with age incidence peaks in the fifth decade of life. Presents histological features of malignancy, including nuclear atypia, cellular pleomorphism, high cellularity and high mitotic activity (12).

1.1.2.2 – Medulloblastomas

The histogenesis of medulloblastoma is a controversial issue. Overall, medulloblastoma is likely to be composed of biologically different subsets of tumours arising from immature neuronal precursor cells in the cerebellum (12, 44, 45). Medulloblastoma represents a highly malignant, invasive embryonic tumour, and is the most common malignant CNS tumour in children. Together with other embryonic tumours, medulloblastoma accounts for about 16% of all childhood primary CNS neoplasms (3). Globally, embryonic tumours including medulloblastoma represent 1.5% of all primary CNS tumours (3), accounting for 12-25% of all childhood brain tumours and only about 0,5-1% of all intracranial neoplasms in adults (22, 46, 47). The peak age incidence is 7 years, with 70% of medulloblastomas occurring in patients less than 16 years of age. In adulthood, 80% of medulloblastomas arise in the 21-40 years age group and rarely occur beyond the fifth decade of life. About 65% of patients are male (12, 30, 47). The annual incidence of medulloblastoma has been estimated at 0.5 per 100,000 children less than 15 years (3, 30). Medulloblastoma presents predominantly neuronal differentiation. It is composed of densely packed small round blue cells with carrot-shaped hyperchromatic nuclei, possibly presenting neuroblastic rosettes which are associated with marked nuclear pleomorphism and high mitotic activity (12, 22, 45, 47). The WHO classification of CNS tumours recognizes at least five different histological types of medulloblastoma: classic (70-85% of the cases); desmoplastic/nodular (15% in paediatric medulloblastoma compared to 30-40% in adults); anaplastic (about 10-22% of medulloblastomas); large cell (about 2-4% of cases) and medulloblastoma with extensive nodularity (about 3%) (12, 22, 47, 48). Large numbers of mitotic figures and apoptotic bodies are characteristics of the large cell and anaplastic medulloblastoma (12). Medulloblastoma histologic subtypes often are quite heterogeneous and exhibit highly variable clinical behaviour, with the anaplastic and large cell subtypes being associated with worse prognosis, whereas desmoplastic/nodular medulloblastomas portend a more favourable outcome (22, 45, 49). Medulloblastoma has an inherent tendency to metastasize via cerebrospinal fluid pathways (about 30% of patients at diagnosis), whereas spread outside the CNS is a rare event. This tendency for metastatic spread is higher in children than adults (30, 50, 51). Clinical prognostic factors include tumour size, presence of metastasis, age, and amount of tumour resected (22).

Stratification of medulloblastoma involves distinguishing high-risk and standard-risk patients, with high-risk patients those aged less than 3 years, with incomplete surgical resection of the tumour and/or with disseminated disease (12, 22, 52). Significant advances have been made in the treatment of childhood medulloblastoma, with the 5-year survival raised to 60% for high-risk disease and 80% for standard-risk tumours, however the long-term side effects of the treatment modalities applied can be severe. The amount of radiotherapy required for the disease control causes significant brain injury that may manifest as long-term neurocognitive sequelae in patients of all ages. Actual treatment includes maximal safe surgical resection and the use of combined radio- and chemotherapy for children older than 3 years. Most therapeutic approaches for high-risk patients include relatively high doses of craniospinal radiotherapy and aggressive chemotherapeutic regimens (22, 27). The treatment of infants with medulloblastoma remains highly problematic as radiation therapy is especially damaging for the developing brain of very young children. Therefore, most therapeutic approaches have focused on delaying, eliminating or reducing doses of radiotherapy by the use of increasingly aggressive chemotherapeutic approaches. A major hope for the future is that the incorporation of biological agents targeting specific signalling pathways will not only make treatment more effective, but also allow a reduction in neurotoxic therapy (12, 52).

1.1.3 – Late Effects and Quality of Life After the Treatment of a Brain Tumour

Patients of brain tumours frequently experience a combination of physical, neurocognitive and emotional deficits. The first trauma of facing a disease with a poor prognosis is potentiated by the fact that they must almost immediately make decisions about complex treatment options. After the treatment, survivors of CNS tumours generally present many complications related to the disease and treatment, summarized in Table 1. Therefore, CNS tumour patients are at increased risk of experiencing depression, perceived stress and anxiety, distress, sadness, anger and low self-esteem, which can adversely affect activity and life satisfaction (53-56).

Also for paediatric patients, brain tumours represent a difficult experience. Family of the patient have to face the reality of having a child with significant neurological, cognitive, and endocrinologic morbidity. Paediatric survivors of brain tumours face an uncertain

future. Late effects from treatment, mainly radiotherapy, may not fully manifest themselves for several years following the completion of treatment. In fact, there is a progressive deterioration until adulthood. The main factors that increase the risk for intellectual compromise are age of the child at the time of CNS radiotherapy and tumour location. Adult survivors of paediatric cancers, particularly brain tumours, have some particularities in their life when compared to normal population, such as a lower rate of employment, higher denial of insurance, less likelihood of getting married and greater difficulty in obtaining needed care (Table 1) (58).

Table 1 – Physical and Intellectual late-effects of brain tumours and their therapy, particularly radiation, on adult and paediatric survivors. *Adapted from references* (22, 57).

Effects	Adult Patients	Paediatric Patients
Physical	Second cancers	Second cancers
	Infertility	Disturbed endocrine function
	Cardiomyopathy	Cardiomyopathy
	Impaired immune system	Obesity
	Fatigue / loss of strengths	Reduction of bone mineral density
	Body image changes	Increased risk of death
	Increased risk of death	
Intellectual	Problems thinking clearly	Impaired cognition
	Problems managing easy tasks	Progressive deterioration of IQ
	Poor memory	Mental retardation
	Confusion	Learning / academic skills deficits
	Personality/behaviour changes	Problems in memory and attention
		Problems in language
		Diminished social skills
Social / economic	Loss of employment	Problems finding a job in adulthood
	Loss of insurance coverage	Denial of insurance
	Suspension driving license	Problems getting married
	Tensions in intimate relationships	

1.2 – Molecular Biology of Brain Tumours

Cancer cells usually harbour mutations in oncogenes and tumour suppressor genes. These may play a role in key cellular processes such as proliferation, apoptosis or angiogenesis. Oncogenes are genes whose deregulated activation through mutation, translocation, amplification or over-expression promotes tumorigenesis. Tumour suppressors, on the other hand, play an inhibitory role, and can be inactivated in cancer through mutation, deletion, methylation or transcriptional repression. Genomic instability present in cancer cells occurs mainly through chromosomal instability (CIN) or microsatellite instability (MSI), together with increased frequencies of molecular alterations in cancer regulatory genes (59, 60). CIN refers to the high rate by which chromosome structure and number (by gains or losses) changes over time in cancer cells compared with normal cells (61) and MSI is characterized by the expansion or contraction of the number of oligonucleotide repeats present in microsatellite sequences (61).

1.2.1 – Genetic Aetiological Factors of Brain Tumours

Brain tumours present a multifactorial aetiology, being the result of interactions between environmental exposure and genetic susceptibility. Genetic susceptibility of brain tumours comes from rare genetic syndromes and genetic polymorphisms, specifically in pathways thought to be involved in the process of brain tumour formation.

1.2.1.1 – Familial Tumour Predisposition Syndromes

A familial tumour predisposition syndrome is associated with germline mutations, typically in tumour suppressor genes, which confer an increased susceptibility of individuals to tumour formation from childhood until their adult life (62). Some of the major syndromes associated with high-grade gliomas or medulloblastomas (with genes affected by germline mutations) are neurofibromatosis type 1 (*NF1*), Li-Fraumeni syndrome (*TP53*) and Turcot syndrome (*APC* and *MLH1*/*PMS2*/*MSH2*/*MSH6*) (complete list in Table 2).

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder characterized by astrocytomas, among other tumours, together with multiple café-au-lait spots, axillary and

inguinal freckling, iris hemartomas and various osseous lesions (12). Glioblastomas are usually observed at an increased frequency in NF1 patients. *NF1* encodes neurofibromin, a protein expressed in many tissues, functioning as Ras inactivator and therefore, its loss stimulates Ras-mediated downstream signalling cascades (12, 62).

Li-Fraumeni syndrome

Li-Fraumeni syndrome is an autosomal dominant disorder characterized by several primary neoplasms in children and adults, including astrocytomas and medulloblastomas (12). About 70% of Li-Fraumeni cases present *TP53* germline mutations and approximately 50% of families with *TP53* germline mutations present Li-Fraumeni syndrome (12). Around 14% of the affected individuals' neoplasms are brain tumours, mainly astrocytomas (64%) but also medulloblastomas (12). The p53 protein is a multifunctional transcriptor factor involved in the control of cell cycle progression, DNA integrity and survival of cells exposed to DNA-damaging agents or non-genotoxic stimuli such as hypoxia (63).

Turcot syndrome

Turcot syndrome is also an autosomal dominant disorder characterized by colorectal and brain tumours, mainly glioblastomas or medulloblastomas (12). Turcot syndrome can be further divided in type 1 (glioma-polyposis) and type 2 patients (medulloblastoma-polyposis). Turcot type 1 patients present glioblastoma and have hereditary non-polyposis colorectal cancer (HNPCC) as well as germline mutations in DNA mismatch repair (MMR) genes such as *MLH1*, *PMS2*, *MSH2* and *MSH6* (12). A small fraction of the patients have biallelic mutations in the MMR genes representing an enhanced disease penetrance (64, 65). The MMR proteins are involved in the recognition and repair of base-pair mismatches on the DNA and their loss results in high mutation rates and MSI (66, 67). Turcot type 2 patients typically develop medulloblastoma, presenting familial adenomatous polyposis, together with mutations in the *APC* gene (12), which is a multifunctional tumour suppressor (68). Nevertheless, studies have described patients presenting medulloblastoma in association with homozygous germline mutations of the MMR genes, namely MSH6 (69, 70), MLH1 (71) or PMS2 (72) usually in concomitance with HNPCC.

Table 2 - Familial syndromes and genes involved causing increased risk of gliomas and medulloblastomas. *Adapted from references* (12).

Syndrome	Gene	Chromosome	CNS Tumour (e.g.)	Other Clinical Manifestations (e.g.)
Neurofibromatosis Type 1	*NF1*	17q11	Astrocytoma, neurofibroma, optic nerve glioma	Skin café-au-lait spots and axillary freckling; other tumours
Neurofibromatosis Type 2	*NF2*	22q12	Astrocytoma, schwannoma, meningioma	Posterior lens opacities, retinal hamartoma
Tuberous sclerosis	*TSC1*	9p34	Giant cell astrocytoma	Other tumours
	TSC2	16p13		
Li-Fraumeni syndrome	*TP53*	17p13	Astrocytoma, medulloblastoma	Other tumours
Turcot	*APC*	5q21	Glioblastoma, medulloblastoma	Skin café-au-lait spots, colorectal polyps
	MLH1	3p21		
	PMS2	7p22		
	MSH2	2p22-21		
	MSH6	2p16		
Gorlin	*PTCH*	9q31	Medulloblastoma	Other tumours

1.2.1.2 – Genetic Polymorphisms

Polymorphisms are genetic sequence variants that, by definition, occur in at least 1% of the general population. Compared to mutations, polymorphisms have been supposed functionally insignificant, yet a considerable number of polymorphisms affect intrinsic properties and function of the resultant protein (73-75).

Polymorphisms frequently studied in the context of brain tumours are in genes that might influence susceptibility to these tumours in concert with environmental exposures, such as genes involved in DNA repair, cell cycle regulation or inflammation. Table 3 presents a summary of representative studies reporting positive associations between polymorphisms and glioma risk (76-88).

Table 3 – Representative genetic polymorphisms associated with glioma risk. *Adapted from references* (10, 89).

Pathway	Gene	Polymorphism	Considerations/Effect
DNA Repair	*XRCC1*	W399R	Risk [76-78]
	PRKDC	G6721T	Risk [76, 82]
	PARP1	A762V	Protective [76, 89]
	ERCC1	A8092C	Risk [79, 80]
	ERCC2	Q751K	Risk [76, 79]
	MGMT	F84L	Risk [81] or Protective [78]?
Cell cycle	*MDM2*	SNP309	G/G genotype associated with higher protein expression [83]; and protection in female glioblastoma [84]
Inflammation	*IL4RA*	478T/C; 551A/G	Risk (both *IL4RA* SNPs) [85]
	IL13	1112C/T	Protection [85]
Growth signalling	*EGFR*	-216G/T	Risk [86]
	EGF	+61A/G	Risk [87, 88]

There are two glioma genome-wide association (GWA) studies reported. In one of them, five risk loci for glioma were identified at 5p15.33 (TERT rs2736100), 8q24.21 (CCDC26 rs4295627), 9p21.3 (CDKN2A-CDKN2B rs4977756), 11q23.3 (PHLDB1 rs498872), and 20q13.33 (RTEL1 rs6010620) (90). The second one, which was significantly smaller in number of samples, provided further evidence to implicate the 9p21 (CDKN2B rs1412829) and 20q13.3 (RTEL1 rs6010620) in glioma risk (91). Interestingly, the function of the five glioma susceptibility genes identified on the first GWA study (RTEL1, TERT, CCDC26, CDKN2A-CDKN2B, and PHLDB1), four (TERT, RTEL1, CCDC26, and CDKN2B) are related to telomerase. TERT is the telomerase catalytic subunit, and RTEL1 is directly involved in telomerase length, whereas the other two are related to telomerase activity. Taken together, these data provide strong evidence that common variation in telomerase-related genes might contribute to glioma predisposition (89).

1.2.2 – Patterns of Copy Number Change

CIN is characterised by an increased frequency of chromosomal alterations, resulting in gains, losses, deletions, insertions, translocations, amplifications, and rearrangements. The majority of human tumours display the CIN phenotype and the end result of CIN, aneuploidy, is observed in nearly all solid tumours (60). Tumours with the CIN phenotype usually harbour mutations in oncogenes and/or tumour suppressor genes, many of which are involved in the regulation of transcription (60). During the last decade, the use of genome-scale profiling techniques to identify the key genetic alterations underlying different tumour types allowed fundamental findings about the drivers of oncogenesis, providing the rationale for specific targeted therapies in these malignancies. The inclusion of brain tumours in such studies was delayed and it was not until 2008 that the first large-scale studies of adult glioblastoma were published (92, 93). Nevertheless, there have been an increased number of studies comprehensively mapping brain tumours genome, particularly high-grade gliomas, on the past few years, with the first studies in paediatric patients starting to emerge (94-98).

High-Grade Glioma

Array-based studies of adult glioblastoma identified common regions of chromosomal instability and gene expression signatures, allowing molecular classification of this malignancy (99-102). By comparison, paediatric high-grade glioma is an understudied disease, although several genomic studies specifically addressing childhood cancers are beginning to emerge. There is now increasing evidence that the paediatric high-grade glioma genome has certain key differences with that of histologically similar adult tumours (94, 95, 103, 104).

Paediatric glioblastomas were clearly distinguished from adult tumours by frequent gain of chromosome 1q and loss of 16q and the paucity of chromosome 7 gains and 10q losses. The most frequent focal amplifications differ, with *PDGFRA* and *EGFR* predominant in childhood and adult populations, respectively. Moreover, paediatric cases without *PDGFRA* amplification were frequently found to show overexpression of a specific *PDGFRA*-associated gene signature, which was distinct from that observed in adult cases with the 4q12 amplification (Table 4). In adults, secondary glioblastomas show overexpression or

amplification of *PDGFRA* but rarely contain *EGFR* amplification (105) suggesting that paediatric high-grade gliomas with *PDGFRA* amplification may be molecularly similar to these tumours (94). Together with *PDGFRA* amplification, deletion of *CDKN2A*/*CDKN2B*, associated with absent gene expression, is the most frequent focal event in the genome of paediatric high-grade gliomas, although this deletion event is more frequent in adult than in paediatric glioblastomas (Table 4) (92, 94, 95). In contrast, the frequencies of 13q and 14q loss are similar between paediatric and adult glioblastoma (92, 94, 95). *CDKN2A*/*CDKN2B* deletion is also frequently found in anaplastic oligodendrogliomas (106).

Table 4 – Summary of copy number changes in Paediatric and Adult Glioblastoma. *Adapted from references* (92, 94).

Region	Paediatric Glioblastoma (n=46)	Adult Glioblastoma (n=189)	*P*
Gains			
1q	30%	9%	0.001
7	13%	74%	<0.001
Losses			
1p	9%	2%	0.05
4q	22%	2%	<0.001
9p	17%	33%	<0.05
10q	35%	80%	<0.001
16q	24%	7%	0.003
Focal amplifications			
PDGFRA	16%	11%*	0.2
EGFR	4%	43%*	<0.001
Focal deletions			
CDKN2A	20%	55%*	<0.001

* n = 206 tumours

Even if *PDGFRA* amplification, 1q+ and 16q- events are significantly more common in paediatric patients (107-109), they are also present in a proportion of adult tumours. Similarly there is a group of paediatric high-grade gliomas containing aberrations more

commonly associated with adult disease (*EGFR* amplification, 7+, 10q-), albeit at significantly reduced frequencies (94, 95). Amplification of the *EGFR* gene on chromosome 7p is also present in 20%–30% of anaplastic oligodendrogliomas, correlating with a poor prognosis (110-113).

Further amplified genes in the childhood setting included those encoding cell cycle progression proteins (*CCND2*, *CDK4*, *MYC*, and *MYCN*), receptor tyrosine kinases (RTKs) and ligands (*EGFR*, *MET*, *IGF1R*, *PDGFB*, and *NRG1*), members of the PI3K/MAPK pathway (*PIK3C2B*, *PIK3C2G*, *PIK3R5*, *KRAS*, *AKT1*, and *S6K1*), and p53 pathway regulation (*MDM4*), some of them known oncogenes within the core signalling pathways described in adult glioblastoma (p.eg. *CDK6*, *MET* and *CCND2*) (94, 95). Additional homozygous deletions of tumour suppressor genes of known importance in glioma included *CDKN2C*, *PTEN*, *RB1*, *TP53*, and *TP73*, reflecting abrogation of common signalling pathways (92, 93) together with the deletion of *NF1* and the tyrosine phosphatase *PTPRD*, which were also deleted in adult glioblastomas (92, 114, 115). These numerous additional low-frequency amplifications and deletions identified in paediatric high-grade gliomas, such as *MYC*/*MYCN*, *CCND2*, *KRAS*, and *CDKN2C*, which seems to corroborate the hypothesis that paediatric high-grade gliomas and secondary adult glioblastomas genomes are similar (101, 116), still this premise is controversial.

IDH1 on chromosome 2q33, encodes isocitrate dehydrogenase 1 (IDH1), (117) which catalyses the oxidative carboxylation of isocitrate to α-ketoglutarate, resulting in the production of NADPH in the Krebs cycle (118). This protein forms an asymmetric homodimer (119) and is thought to play a significant role in cellular control of oxidative damage through generation of NADPH (120, 121). Recently, missense mutations in *IDH1* were found in a significant number of adult glioblastomas that tend to occur mostly in younger patients with more protracted clinical courses (93). These mutations were found exclusively on the R132 residue in the active site region of the protein (93, 122). Interestingly, a separate group of gliomas harbour mutations in the *IDH1* homologue *IDH2* at the analogous residue (R172). Further investigations have shown that mutations in *IDH1* and *IDH2* are present in high proportions of grade II and III astrocytic, oligodendroglial and mixed tumours (72 to 100%) along with secondary glioblastomas (up to 85%), but are largely absent in primary glioblastomas (5%) (93, 122-125). Additionally, *IDH* mutations are associated with other

genomic abnormalities that are typically seen in gliomas, such as *TP53* mutation low-grade astrocytoma and 1p/19q deletion in oligodendrogliomas; they are also mutually exclusive with *EGFR* amplification and chromosome 10 loss (122, 124, 126). These findings suggest that, although *IDH* mutations probably contribute to the early evolution of low-grade gliomas, remaining in the higher-grade lesions, they seem to have no role in the underlying biology of primary glioblastoma. In the childhood setting, *IDH1* hotspot mutations were not found (122, 127), distinguishing biologically paediatric high-grade gliomas from adult secondary glioblastoma. Nevertheless, *IDH1* mutations appear to be frequent (35%) in high-grade gliomas from older children (14-18 years), although less common than the reported frequency in secondary glioblastomas, these *IDH1*-mutated tumours were associated with a favourable prognosis, as observed in adults (128).

Oligodendrogliomas harbour specific copy number alterations related to response to therapy or prognosis, however a comparison between adult in paediatric populations is hard to trace, as paediatric oligodendrogliomas are extremely rare neoplasms. Still, adult tumours typically (up to 80%) show combined loss of chromosomes 1p and 19q, associated with increased chemosensitivity to treatment and favourable clinical outcome (129, 130), whereas deletions involving chromosomes 1p or 19q are rare in paediatric oligodendrogliomas and did not seem to foresee a survival advantage in paediatric high-grade gliomas (131, 132).

Another difference observed between childhood and adult high-grade gliomas is the presence of a proportion of tumours with very few, or even no detectable copy number alterations in paediatric tumours (94, 95), which is in direct contrast to data from adult glioblastoma (92, 93). This stable genomic profile is independent of histologic grade or type, and seems to convey an improved survival in patients with high-grade glioma, in contrast to those patients with an amplifier genomic profile, who do significantly worse (95).

Medulloblastoma

Similarly to glial tumours, studies of genomic copy number alterations analysing cohorts of paediatric and adult medulloblastomas are starting to emerge.

Historically, the most frequently and consistently genetic event reported in medulloblastoma is the partial or complete loss of the chromosome 17p, often in association with gain of chromosome 17q (resulting in the isochromosome 17q: i(17)q), occurring in approximately 30–50% of the medulloblastomas (133-139). Although the precise mechanism by which this genomic abnormality contributes to tumorigenesis and its prognostic importance remain unclear, the common deletion region of 17p13.2-13.3 includes several confirmed and putative tumour suppressor genes, including *TP53*, the loss of which could presumably facilitate neoplastic behaviour (45). Together with the deletion of 17p chromosomal region, *MYC* amplification is a molecular prognostic factor of medulloblastoma (22). Genomic amplifications of *MYCN* and *MYC* were described as characteristics of a subset of clinically aggressive medulloblastomas that tend to exhibit large cell/anaplastic histological features (140, 141). Additional alterations frequently fond in medulloblastomas are losses of chromosomes 1q and 10q present in approximately 20-40% of the cases (142-144).

Recently, Korshunov and colleagues (145) compared a large series of adult and paediatric subsets of tumours, using array-based comparative genomic hybridization (aCGH) (34 adult and 101 paediatric patients) and validating the results in an independent series (112 adult and 303 childhood patients) by fluorescent in situ hybridization (FISH) analysis. Despite frequencies of loss of 17p isolated or i(17)q formation were similar in both populations, isolated gains of 17q were significantly more frequent in children than adult patients, whereas monosomy of chromosome 17 was exclusively found in adults (Table 5) (145). Additionally, amplifications of MYC/MYCN prevailed in the paediatric cohort, whereas amplification of CDK6 (at 7q21.3), an alteration frequently present in adult medulloblastomas, was absent in paediatric samples (145). Of note is that both MYC/MYCN or CDK6 amplifications were associated with poor survival in paediatric and adult populations, respectively (145). Besides chromosome 17q, alterations on additional chromosome arms were shown to be significantly different between adult and paediatric medulloblastoma. Gains of chromosome 3q, 4 and 19 more frequently found in the adult tumours, while gains of chromosomes 1q, 2 and 7 and loss of 16q was more abundant in children (145). Frequency of chromosome 6 deletions was similar across adult and childhood medulloblastoma, however patterns of aberration were different, with complete

loss of the chromosome and absence of concomitant aberrations frequently more present in paediatric medulloblastoma (145). This may explain the reason why the prognostic value of chromosome 6 deletion, a well established marker for favourable outcome in medulloblastoma (146-148), was found not statistically significant for adult cases (145). A summary of the results from the validation cohort is presented on Table 5.

Table 5 – Summary of copy number changes in Paediatric and Adult Medulloblastomas (validation set of adult tumours by FISH analysis). *Adapted from reference* (145).

Region	Paediatric Medulloblastoma (n=303)	Adult Medulloblastoma (n=112)	*P*
Gains			
1q	24%	9%	<0.001
2	15%	6%	0.02
6q	11%	3%	*
7	33%	14%	<0.001
17q	17%	2%	**
Losses			
6q	12%	15%	*
17p	4%	4%	**
Focal amplifications			
CDK6	0.3%	8%	<0.001
MYC/MYCN	14%	3%	<0.001
Other Alterations			
i(17)q	39%	32%	**

* $P = 0.02$ for total Chromosome 6 aberrations; ** $P < 0.001$ for total Chromosome 17 aberrations

1.2.3 – Deregulated Molecular Pathways: Oncogenes and Tumour Suppressors

High-Grade Glioma

Comprehensive studies integrating copy number, gene expression, and mutation analyses reported that the retinoblastoma (RB), p53 and RTK/PI3K/MAPK pathways are

disrupted in adult and paediatric glioblastomas through various genetic mechanisms (92, 93).

The RB tumour suppressor pathway has been shown to be defective in a significant number of high-grade gliomas of both astrocytic and oligodendroglial lineage, either by inactivating mutations in *RB1* itself, amplification *CDK4* or *CDK6*, or deletion of the *CDKN2A* locus that encodes $p16^{INK4A}$, a positive regulator of RB (92, 93, 149-152). The study of primary adult glioblastomas showed that the overall frequency of genetic alterations in the RB signalling pathway was 78%, through $p16^{INK4A}$ homozygous deletion or mutations (52%), $p15^{INK4B}$ homozygous deletion (47%), $p18^{INK4C}$ homozygous deletion (2%), *CDK4* amplification (18%), *CCND2* amplification (2%), *CDK6* amplification (1%), *RB1* mutation or homozygous deletion (11%) (92). Alterations of this pathway are also frequent (65%) in anaplastic oligodendrogliomas (152).

Inactivation of p53 pathway has also been found in subsets of adult glioblastomas, through amplification of the p53 negative regulators *MDM2* and *MDM4*, inactivating mutations, deletions, and promoter methylation of $p14^{ARF}$, a positive regulator of p53 also encoded by the *CDKN2A* locus, together with mutations of *TP53* itself (32, 92, 93, 153, 154). Also paediatric high-grade astrocytomas frequently present *TP53* mutations, p53 protein overexpression and MDM2 overexpression (155, 156), these alterations being less frequent in younger children (<4 years) (157). The TCGA study of primary adult glioblastomas revealed that the overall frequency of genetic alterations in the p53 pathway in glioblastomas was 87%, through *TP53* mutations or homozygous deletion (35%), *MDM2* amplification (14%), *MDM4* amplification (7%), or $p14^{ARF}$ homozygous deletion or mutation (49%) (92). In previous studies, in addition to homozygous deletions, promoter methylation of $p14^{ARF}$ was frequently found in glioblastomas (153). Additionally, more than 70% of secondary glioblastomas and approximately 50% of primary glioblastomas were described to harbour, at least, one alteration in the p53 pathway (84), with *TP53* mutations found to be significantly more frequent in secondary than in primary glioblastomas (65% vs 28%) (32, 154). Approximately 50% of anaplastic oligodendrogliomas were reported to present alterations in the p53 pathway, mainly homozygous deletion or promoter methylation of the $p14^{ARF}$ gene (152).

The great majority of adult glioblastomas were found to present genetic alterations in the RTK/PI3K/MAPK pathway (92, 93). The involvement of RTKs in gliomagenesis has been repeatedly demonstrated. The presence of *EGFR* amplification and activating mutations together with protein expression has been widely associated mainly with primary glioblastomas, but also with other high-grade gliomas. EGFR amplification occurs in about 40% of adult primary glioblastomas, but rarely in secondary glioblastomas (32, 158, 159). *EGFR* amplification in adult primary glioblastomas is associated with protein overexpression and with deletion mutants, with *EGFRvIII* the most frequent type (160, 161). The EGFRvIII mutant lacks a portion of the extracellular ligand-binding domain as a result of a genomic deletion involving exons 2 to 7, leading to a mutant protein constitutively activated in a ligand-independent manner, and permanent activation of the PI3K/AKT pathway (162). About 50% of anaplastic oligodendrogliomas show EGFR overexpression, even if gene amplification is not frequent (163, 164). Besides EGFR, enhanced signalling through PDGFRA has been found to be a common feature of a significant subset of glioblastoma (165, 166). Although, in adult primary glioblastoma, activating mutations of *PDGFRA* are uncommon (167), with total gene aberrations reported in 13% of samples in TCGA (92), the frequent co-expression of both the receptor and its ligand indicates the potential for autocrine or paracrine loops boosting oncogenic signalling through the PDGF network (167). Similar findings regarding HGF and its RTK MET have also been reported for glioma (168), whereas *KIT* amplification is reported to be a common genetic mechanism underlying KIT expression in malignant gliomas (169). Therefore, enhanced RTK signalling seems to be an initial oncogenic event in the plurality of malignant gliomas, the effects of which are probably mediated in large part through oncogenic PI3K-AKT-mTOR and Ras-MAPK signalling downstream.

Underscoring this fact is the not infrequent deregulation in malignant gliomas of molecular components in these downstream networks (170, 171) the most common of which is functional loss of the tumour suppressor *PTEN*, the primary negative regulator of PI3K-AKT-mTOR signalling (40, 172). The *PTEN* gene is deleted and/or mutated in 15-40% of primary glioblastomas, but rarely altered in secondary glioblastomas (32, 92, 160, 173). In the context of core signalling pathways, Ras and AKT isoforms themselves are, respectively, mutated and amplified, in only 2% of adult glioblastomas (92), and *PIK3CA* mutations and

amplification are found in 5% and 13% of primary and secondary glioblastomas (174). Nevertheless, globally, components of the Ras-MAPK and PI3K-AKT-mTOR signalling pathways are affected in the great majority of analysed tumours (92) with about two-thirds (63%) of primary glioblastomas and one-third of secondary glioblastomas showing alterations in at least one of the *EGFR*, *PTEN*, or *PIK3CA* genes (174). Additional genetic alterations in this pathway are *NF1* mutations/homozygous deletions and *PIK3R1* mutations, present in 18% and 10% of primary glioblastomas, respectively, with an overall frequency of RTK⁄PI3K/MAPK pathway alterations of 88% in these tumours (92).

Specific genetic alterations underlying paediatric high-grade gliomas were defined primarily by directed analyses of genes that are mutated more commonly in adult tumours. Mutations in *TP53*, *CDKN2A*, and *PIK3CA* are frequent in both adult and paediatric high-grade gliomas (175-177). However, *PTEN* mutations and *CDK4* amplifications, which are frequent in adult primary glioblastoma, are less common in primary paediatric high-grade gliomas (176, 178, 179). Up to 85% of paediatric high-grade gliomas present overexpression of EGFR, however even in the presence of overexpressed protein, *EGFR* amplification and EGFRvIII mutation, aberrations commonly present in adult glioblastomas are rarely found in paediatric tumours (155, 180, 181). Considering the copy number data, there is a significantly lower frequency of pathway deregulation in paediatric high-grade gliomas, compared with that reported in adults: 25% RTK/PI3K, 19% p53 and 22% RB versus 59%, 70% and 66%, for adult glioblastoma (92, 95) (Figure 4). It is apparent that paediatric tumours show targeting of these core pathways by copy number alterations in less than half the frequency of the adult tumours. Even though isolated cases presented clear genomic events linked to activation of the sonic hedgehog (SHH) and Notch pathways activation, there are no evidences of consistently targeted pathways in paediatric high-grade gliomas.

As previously discussed, concomitant temozolomide and radiotherapy is the first-line adjuvant therapy for glioblastoma (182, 183). The improved survival benefits of temozolomide in glioblastoma are largely restricted to the subset of patients lacking expression of the DNA repair gene, *MGMT* (184). Temozolomide induces cytotoxic O6-guanine methyl adducts which are removed directly by functional MGMT, thereby producing drug resistance. Downregulation of *MGMT* usually occurs in approximately 40-57% glioblastomas by gene promoter hypermethylation, in which greater than 50%

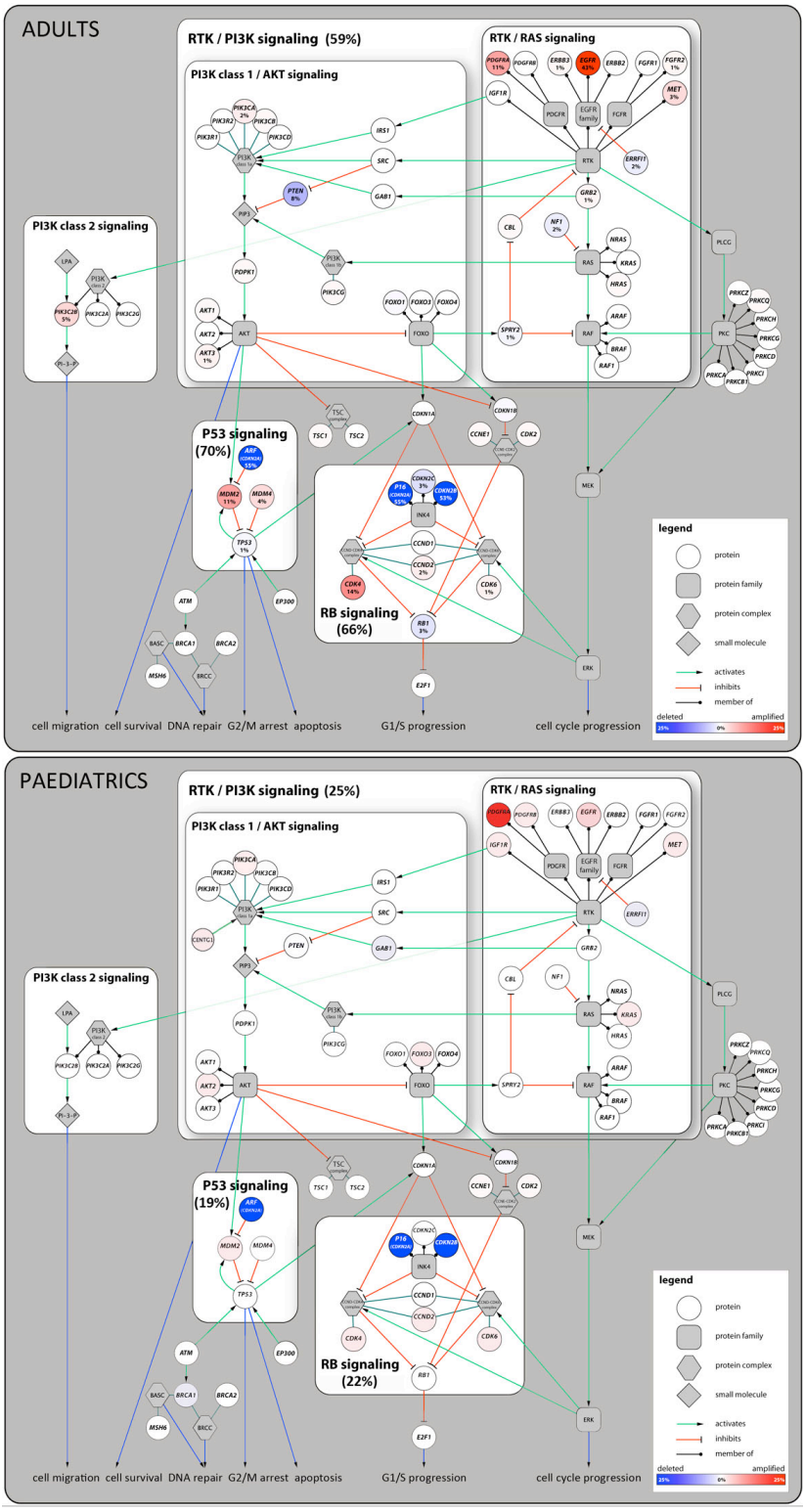

Figure 4 – Copy number alterations in core signalling Pathways in Adult and Paediatric Glioblastoma. RTK/PI3K/MAPK, p53 and RB pathways are more frequently deregulated in adult than in paediatric glioblastomas. *Adapted from references* (92, 95).

methylation has been shown to silence gene expression (184-186). Importantly, besides being associated with response to chemotherapy, *MGMT* hypermethylation has been connected with improved outcome for adult patients with glioblastoma receiving temozolomide (184, 187, 188). In the paediatric setting, *MGMT* promoter hypermethylation also predicts for response to alkylating agents (189); however, the survival of children treated with adjuvant temozolomide does not appear to be improved when compared with historical controls (35-39).

Medulloblastoma

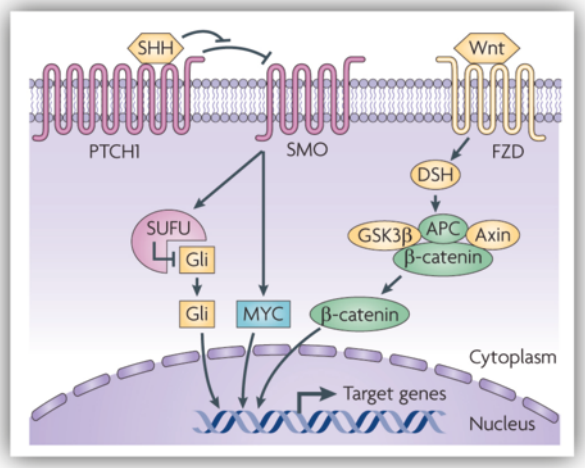

Figure 5 - Molecular Pathways Implicated in the Pathogenesis of Medulloblastoma. *Adapted from reference* (45). The binding of SHH to its receptor PTCH1 releases inhibition of SMO, releasing the Gli transcription factors from inhibitory protein complexes that typically include SuFu.
Wnt ligand binds to its receptor FZD leading to the release of its downstream effector β-catenin from an inhibitory complex that includes the APC, GSK3β and axin proteins, entering in the nucleous.

Numerous investigations have reported that components of the SHH and Wnt signalling cascades are disrupted in medulloblastomas through various genetic mechanisms (Figure 5) (190).

Genomic alterations in components of th SHH signalling pathway, specifically inactivating mutations of *PTCH1* and *SUFU* and/or activating mutations of *SMO*, have been found in approximately 15% of sporadic medulloblastomas (191-194). Additionally, allelic losses of 9q, where the *PTCH1* gene is located, have also been described in 10-18% of

medulloblastoma cases (195, 196). Overall, the SHH pathway is thought to harbour molecular alterations and play a role in the development of at least 25% of sporadic medulloblastomas (197, 198). SHH signalling is known to induce proliferation in the granule neuron precursors of the cerebellum, therefore the dysregulation of the pathway due to genomic alterations of its components probably drives medulloblastoma development through analogous downstream effects (199).

Likewise, the deregulation of the Wnt pathway has been connected to the development of medulloblastoma. Approximately 20% of sporadic medulloblastomas harbour mutations in *APC*, *AXIN1*, *AXIN2* or *CTNNB1* (β-catenin gene) (200-204), and a similarly sized fraction (18%) has separately been shown to exhibit nuclear β-catenin expression (205). However, when comparing paediatric and adult cohorts, it was found that *CTNNB1* mutations were more frequent in paediatric medulloblastomas, associated with monosomy of chromosome 6. Finally, medulloblastomas that are driven by increased Wnt signalling, as shown by nuclear β-catenin staining, may follow a relatively favourable clinical course (206). Overall, molecular disruption of this pathway is thought occur in approximately 13% of sporadic medulloblastomas (207).

Global transcriptional analyses of medulloblastoma have both emphasized and better conceptualized the molecular heterogeneity inherent to this cancer type. Initial efforts in this regard demonstrated gene expression patterns in medulloblastoma were distinct from other CNS tumours with similar histological features (208). Furthermore, transcriptional profiles were found to be predictive of clinical outcome (208, 209).

Different studies were able to classify medulloblastomas into distinct molecular subtypes (210, 211). The latest of these studies allowed the stratification of medulloblastomas into four distinct molecular sub-groups based on gene expression profiles and copy number data: WNT, SHH, Group C and Group D (211) (Figure 6A). Known targets of the Wnt pathway (*WIF1*, *DKK1* and *DKK2*) and Shh pathway (*HHIP*, *SFRP1* and *MYCN*) presented clear differential expression in their respective subgroups. Both group C and D tumours highly expressed the *OTX2* and *FOXG1B* oncogenes. Group C and WNT tumours presented high levels of *MYC* expression, while the SHH expressed *MYCN*, making group D, the only group without high levels of *MYC* family expression (Figure 6A).

Immunohistochemistry analysis of few highly expressed, sub-group specific, signature molecules (β-catenin and DKK1 for WNT; GLI1 and SFRP1 for SHH; NPR3 for Group C and KCNA1 for Group D) allowed the distinction of the four groups by this currently used technique of molecular diagnosis. SHH-driven tumours are most common in infants (≤ 3 y) and adults (≥ 16 y). Group C tumours peak in the childhood (between 3 and 10 y), and are largely absent in older patients. Group D and WNT tumours present a more distributed age, ranging from infancy to adulthood (Figure 6).

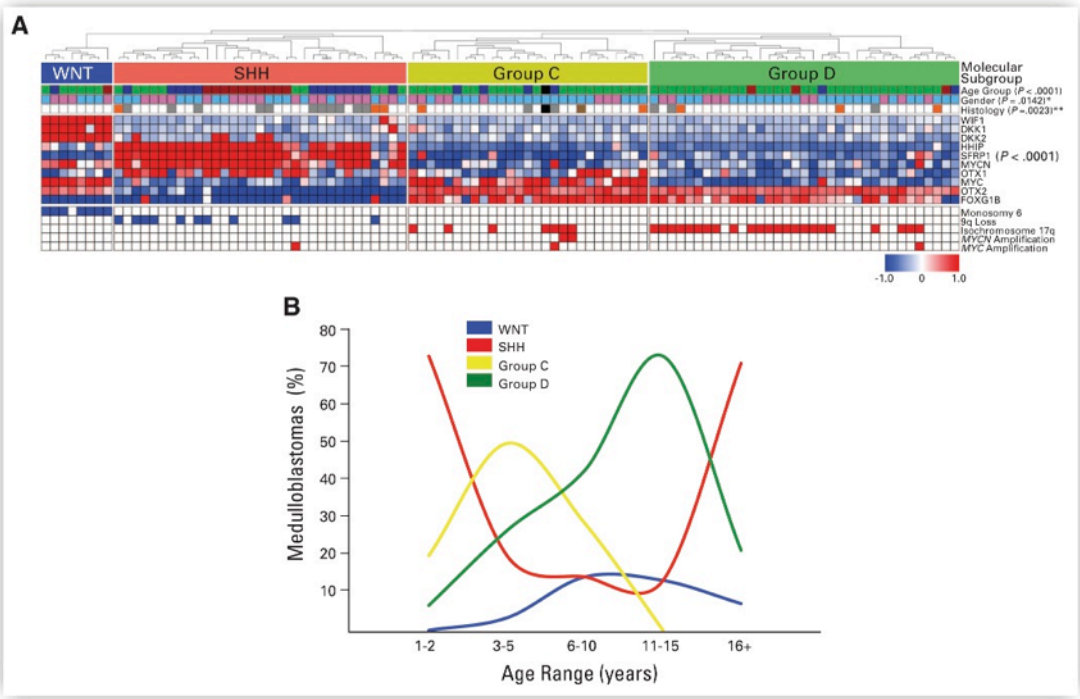

Figure 6 – A) Unsupervised hierarchical clustering from 103 primary medulloblastoma. Age groups include infants (< 3 years; blue), children (4 to 15 years; green), adults (> 16 years; red), and unknown (black). Sex includes males (blue) and females (pink). Histology includes classic (white), desmoplastic (gray), large-cell/anaplastic (orange), medulloblastoma with extensive nodularity (brown), and unknown (black). (*) *P* value determined by comparing sex prevalence in WNT/SHH versus group C/D tumours. (**) *P* value corresponds to over-representation of desmoplastic tumours in the SHH subgroup. Expression profiles for 10 genes well characterized in medulloblastoma, demonstrating their differential expression among the four subgroups in addition to common genomic aberrations known to occur in medulloblastoma are represented. Blue boxes indicate loss/deletion, red boxes indicate gain/amplification, and white boxes denote balanced copy number state for the specified genomic aberration. **B) Age at diagnosis distribution for each of the four medulloblastoma subgroups.** *Adapted from reference* (211).

1.2.4 – Microsatellite Instability (MSI)

Microsatellites are short DNA sequence repeats that are scattered throughout the human genome (212), and whose copy number varies steadily through evolution (213). Microsatellite instability (MSI) is the expansion or retraction of the number of repeats within the microsatellites and it is assumed to be generated by slippage of DNA polymerases during copying of repeats, representing a hot spot for mutagenesis (214-216).

The MMR system is the major pathway responsible for repairing base-base mispairs and short insertion/deletion loops that arise during DNA replication and as intermediates of homologous recombination (60, 217, 218). The need for such a mechanism derives from the fact that the fidelity of replicating DNA polymerases is insufficient to generate an error-free copy of genomic DNA. With single-base substitutions estimated to arise once in every 10^4-10^6 nucleotides incorporated, MMR reduces the error rate to a range of 10^{-9} to 10^{-10}, which ensures that the human genome can be duplicated without mutations (217). Left unrepaired, these structures will give rise to base-substitution and frameshift mutations, respectively (60, 217, 218). Together with the understanding of the biochemical and structural aspects of MMR, the precise biological functions of the key factors of the human MMR system, the MutS homologues MSH2, MSH3 and MSH6, and the MutL homologues MLH1 and PMS2, have been elucidated (219-221). The roles of other proteins, such as MSH4, MSH5, PMS1 and MLH3, remain largely uncharacterized. MSH6 competes for binding to MSH2 with MSH3 (222). While MLH1 can form heterodimers with PMS2 (223), PMS1 (224) or MLH3 (225), MLH1/PMS2 is the only complex with an essential role in mismatch correction (60, 226).

It was discovered in 1993 that a causal link exists between inherited mutations in the *MMR* genes and the common colon cancer predisposition syndrome HNPCC (227, 228). Since then, hundreds of studies about the frequency and characterization of MSI-positive tumours in colorectal cancer and tumours of other organs have been published. Tumours with defects in *MLH1*, *MSH2* and *PMS2* generally present MSI, whereas defects in *MSH6* do not cause the same high frequency of MSI, probably due to the ability of cells to compensate for *MSH6* absence (222). In addition, it was found that also sporadic cancers could be MMR defective, mainly due to promoter hypermethylation of *MLH1* (229-231).

Due to the widespread presence of microsatellites in DNA, mutations are expected to accumulate in the genome of tumour cells due to MMR deficiency. Some of these alterations might contribute to tumorigenesis, reflecting whether the mutation is located in a coding sequence such that it causes a frameshift mutation in the respective protein product of the gene or in an intergenic non-regulatory region (232). Genes thought to harbour mutations in microsatellites due to the MSI phenotype are commonly designated MSI target genes. There are numerous MSI target genes described, however the relevance of several of them in cancer is not clear-cut (233, 234). Some of these genes such as *TGFβRII* and *BAX* are frequently mutated in MSI-positive colorectal cancer (235-237), while other genes like *IGFRII*, *TCF4* and *AXIN*, have been reported to be altered at lower frequencies (238-240). For the MSI target genes mutations to exert a maximal effect, both alleles should be affected, yet they are often found to be mono-allelic. This implies that inactivation of a single allele might result in the production of insufficient amounts of a particular gene product (haploid-insufficiency), which is sufficient to promote carcinogenesis (67).

MSI-positive colorectal cancer appears to be related to particular clinical and histopathological features distinct from their MSI-negative counterparts. Some examples include location in the proximal colon, poor differentiation of the tumours with mucinous and signet ring cells, high tumour lymphocyte infiltration, low frequency of distant metastasis, and a comparably good prognosis (241). It has been hypothesised that, in an advanced phase of transformation, the high frequency of mutations characteristic of these tumours might make tumour cells more immunogenic, since a conspicuous intraepithelial infiltration of T lymphocytes and nodular aggregates of B cells are usually detected in MSI-positive colorectal cancers (242). Therefore, a strong immune response against MMR-deficient cells might decrease their potential for invasiveness and metastasis, and possibly explain why MSI-positive colorectal cancers have a better prognosis (67, 243). Nevertheless, while MSI-positive tumours appear to have a better prognosis than other colorectal cancers, cells with MMR deficiency were found to be resistant to certain drugs currently used in the treatment these cancers, such as 5- fluorouracil, although the evidence is somewhat contradictory in this topic (244-246). On the other hand, MSI-positive colorectal cancers are tolerant to certain drugs, in particular alkylating agents and cisplatin (247).

Due to the particular clinical characteristics and the differential response of MSI-positive colorectal cancer to therapeutic agents, MSI screening of this malignancy is of ultimate interest, and guidelines for HNPCC and MSI diagnosis have been standardized (248). Tumours should be analysed using standard panels of five microsatellite markers, and considering the number of markers presenting alterations cancers would be classified as MSI-negative or (microsatellite stable (MSS)) or MSI-positive, that are further divided into MSI-high or MSI-Low, according to the number of markers (microsatellites) altered (248).

An alternative classification of MSI relies on the size of the gains or losses within the microsatellites, rather than the number of markers with alterations. In this classification, samples presenting small length allelic shifts (≤ 6 bp) are considered Type A, whilst those presenting more drastic alterations are described as Type B MSI, as reported for colorectal cancers (249).

The presence and frequency of MSI in brain tumours is a controversial and poorly studied issue. Previous studies have evaluated the presence of MSI in brain tumours, particularly in gliomas, while in medulloblastomas MSI status has not been properly addressed. Literature has reported absence or rare incidence of MSI in adult patients, while in children results were contradictory, with reported frequencies in paediatric gliomas varying between 0 and 44% (250-260). Moreover, in contrast to colorectal cancer, which presents Type B MSI, high-grade gliomas have been described as harbouring Type A MSI (261). This appears to be a consistent difference in MSI reported in brain tumours compared with the classic MSI positive epithelial tumours such as colorectal and gastric carcinomas.

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2. Rationale and Aims

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General Aim

The general aim of this thesis was to contribute to the understanding of molecular mechanisms differentiating paediatric and adult brain tumours, with particular regard to the most common and malignant types, medulloblastoma and high-grade gliomas. The understanding of molecular alterations underlying the genesis and development of tumours is of major importance because it may allow a more reliable and objective classification of the malignancies and a selection of patients that will potentially respond to therapy. Accordingly, we intended to explore the role of receptor tyrosine kinase pathways and microsatellite instability in paediatric and adult high-grade gliomas and medulloblastomas, evaluating the presence of molecular signatures of response to existing drugs in order to identify the subsets of patients that would benefit most from different therapeutic modalities.

In view of this, the following **specific aims** were addressed:

- **Characterize the presence and frequency of EGFR molecular alterations in adult and paediatric high-grade gliomas.**

  EGFR is known to play an important role in adult high-grade gliomas, being frequently amplified and overexpressed in these tumours. Mutation by deletion of exons 2 to 7, EGFRvIII, results in a constitutively activated molecule and is thought to confer enhanced tumorigenicity in glioma models. In the paediatric setting, the role of EGFR has been less explored and the data available is limited. Nevertheless, in childhood gliomas, the receptor is known to be frequently overexpressed. Due to the well-established importance of EGFR in the development of gliomas, we focused on the study of these tumours and to address this objective we performed two independent studies, respectively, of series of adult and paediatric high-grade gliomas.

In the first study, we aimed to characterize EGFR overexpression, amplification and EGFRvIII mutation in a cohort of Portuguese adult high-grade glioma, correlating the presence of these molecular aberrations with the different histological types of tumours and survival of patients. To pursue this aim, we analysed by immunohistochemistry the expression of EGFR and the presence of the mutant EGFRvIII, and used chromogenic *in situ* hybridization (CISH) to evaluate gene amplification.

In the second study, we aimed to clarify the role of EGFR in paediatric high-grade gliomas and to assess the *in vitro* sensitivity of paediatric glioma cell line models to a selective EGFR inhibitor, erlotinib, that had shown activity in adult high-grade glioma patients. To achieve this aim, we used immunohistochemistry and CISH to evaluate expression and amplification of EGFR, RT-PCR to identify the presence of EGFRvIII and sequencing to study extracellular domain (exons 2 to 8) and tyrosine kinase domain (exons 18 to 21) mutations. We transduced glioblastoma cell lines with wild-type or mutant EGFRvIII, assessed the constitutive activation of the receptor through electrochemiluminescent immunoassay, and treated the cells with erlotinib, evaluating the effects on the expression of PI3K/MAPK pathways and on the cell cycle. Mechanisms of resistance of these cells to the inhibitor were addressed by studying up-regulation of additional phosphorylated receptor tyrosine kinases using phosphorylated RTK analysis. Based on the results, cells were submitted to combination treatment.

- **Microsatellite instability (MSI) in high-grade gliomas and medulloblastomas of adult and paediatric patients**

The presence and frequency of MSI in brain tumours is a controversial issue, with literature providing conflicting results. More specifically, in paediatric gliomas the frequencies reported are highly heterogeneous, whereas the literature in medulloblastomas is extremely scarce. In two different studies we have attempted to clarify the presence, frequency and possible relevance of MSI as a potential novel molecular pathway in series of medulloblastoma or high-grade glioma, comparing paediatric and adult tumours.

To address this objective, firstly we used a series of adult and paediatric medulloblastoma and evaluated the presence of MSI using a panel of

quasimonomorphic markers, recommended by the revised Bethesda guidelines, by multiplex PCR and genotyping. Molecular status of the mismatch repair genes (MMR) was studied by methylation specific-MLPA and immunohistochemistry. Mutation profiles of MSI-target genes were also determined by multiplex PCR followed by genotyping. Mutations of *BRAF* and *CTNNB1* were analysed by PCR single-strand conformation polymorphism.

Secondly, high-grade gliomas from adult and paediatric patients were also analysed. In this study we used the abovementioned techniques to evaluate presence and frequency of MSI and mutation profiles of MSI-target genes. MMR protein expression was determined by immunohistochemistry and *MLH1* was screened for mutations and promoter methylation by direct sequencing and MS-PCR. Mutations in MSI target genes were explored by immunohistochemistry and functional studies were performed in a mutation with unknown relevance.

3. EGFR Molecular Alterations as Potential Therapeutic Targets for Gliomas in Adult and Paediatric Populations

The results presented throughout this chapter were:

(i) Published as original articles in international peer reviewed journals:

Sub-Chapter 3.1: Marta Viana-Pereira, José Manuel Lopes, Suzie Little, Fernanda Milanezi, Diana Basto, Fernando Pardal, Chris Jones, and Rui M. Reis. Analysis of EGFR Overexpression, *EGFR* Gene Amplification and the *EGFRvIII* Mutation in Portuguese High-Grade Gliomas. *Anti-Cancer Research* 28(2A): 913-20, 2008.

Sub-Chapter 3.2: Dorine A. Bax, Nathalie Gaspar, Suzanne E. Little, Lynley Marshall, Lara Perryman, Marie Regairaz, Marta Viana-Pereira, Raisa Vuononvirta, Swee Y. Sharp, Jorge S. Reis-Filho, João N. Stávale, Safa Al-Sarraj, Rui M. Reis, Gilles Vassal, Andrew D.J. Pearson, Darren Hargrave, David W. Ellison, Paul Workman, and Chris Jones. *EGFRvIII* Deletion Mutations in Pediatric High-Grade Glioma and Response to Targeted Therapy in Pediatric Glioma Cell Lines. *Clinical Cancer Research* 15(18): 5753-61, 2009.

3.1 Analysis of EGFR Overexpression, *EGFR* Gene Amplification and the *EGFRvIII* Mutation in Portuguese High-Grade Gliomas

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Analysis of EGFR Overexpression, *EGFR* Gene Amplification and the EGFRvIII Mutation in Portuguese High-grade Gliomas

MARTA VIANA-PEREIRA[1], JOSÉ MANUEL LOPES[2,3], SUZIE LITTLE[4], FERNANDA MILANEZI[1,2], DIANA BASTO[1], FERNANDO PARDAL[5], CHRIS JONES[4] and RUI MANUEL REIS[1]

[1]*Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga;*
[2]*IPATIMUP, Porto;*
[3]*Department of Pathology, S. João Hospital, Faculty of Medicine, University of Porto, Porto;*
[5]*Department of Pathology, S. Marcos Hospital, Braga, Portugal;*
[4]*Paediatric Oncology, Institute of Cancer Research, Sutton, U.K.*

Abstract. *Background: Patients with malignant gliomas do not respond to any current therapy. Epidermal growth factor receptor (EGFR) controls several oncogenic processes, being frequently up-regulated in gliomas due to overexpression, gene amplification and gene mutation. EGFR inhibitors are being tried in gliomas, yet the molecular determinants of therapeutic response are unclear. Materials and Methods: EGFR overexpression, EGFRvIII mutation and EGFR amplification were determined by immunohistochemistry and chromogenic in situ hybridization (CISH) in 27 primary glioblastomas (GBM), 24 anaplastic oligodendrogliomas (AO) and four anaplastic oligoastrocytomas (AOA). Results: EGFR overexpression was associated with EGFR amplification, being found in 48% and 53% GBM, 33% and 40% AO and 75% and 67% AOA, respectively. EGFRvIII was found in 22% GBM, 8% AO and was absent in AOA. No association was observed between EGFR alterations and patient survival. Conclusion: We characterized, for the first time, EGFR molecular alterations in Portuguese patients with malignant glioma and identified a subpopulation of patients presenting putative biomarkers for EGFR-based therapies.*

Gliomas are the most frequent primary central nervous system (CNS) tumors (1). According to the World Health Organization (WHO), gliomas are histologically divided into astrocytic, oligodendroglial and mixed oligoastrocytic tumors, and are classified into four grades of malignancy (1, 2). Oligodendrogliomas and oligoastrocytomas are stratified into grade II and grade III (anaplastic) tumors; on the other hand astrocytomas can be subdivided into grade II, grade III and grade IV (2). The most malignant form (WHO grade IV), glioblastoma (GBM), is also the most frequent glioma subtype (1, 2). GBMs can be divided into primary glioblastomas, which arise *de novo* and are molecularly characterized by epidermal growth factor receptor (*EGFR*) overexpression/amplification, and secondary glioblastomas, which are derived from lower-grade astrocytomas and are characterized by *TP53* mutations (3). The prognosis of patients with GBM is very poor, with survival usually less than twelve months (1, 4). Recently, the introduction of temozolomide-based chemotherapy in concomitancy with radiotherapy, led to increased GBM patient survival (15 months) (4). However, these results are far from being satisfactory and there are still patients that do not respond favorably to any described therapy. Therefore, it is necessary to understand the molecular features of gliomas in order to identify novel and effective therapeutic targets.

Receptor protein tyrosine kinases (RTKs) are major regulators of cell growth signaling and their importance in tumorigenesis and progression has been extensively investigated (5). Epidermal growth factor receptor (EGFR)/HER1 is a member of the class I epidermal growth factor family, which also includes HER2, HER3 and HER4. EGFR is a transmembrane glycoprotein that is stimulated by growth factors, namely the transforming growth factor-α (TGF-α) and EGF ligands, which bind to the extracellular domain of the receptor (6). Ligand binding to EGFR activates the receptor through dimerization leading to signal transduction and activation of downstream intracellular pathways, mainly RAS/RAF/MAPK, PI3K/AKT and STATs, that regulate cellular proliferation, differentiation, migration and survival (5). Several mechanisms of aberrant EGFR activation have been reported in cancer cells, namely

Correspondence to: Rui Manuel Reis, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Tel: +351 253604825, Fax: +351 253604820, e-mail: rreis@ecsaude.uminho.pt

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overexpression of the ligand EGF and of the receptor, *EGFR* gene amplification and activating mutations. Moreover, dysregulated EGFR activation is known to act oncogenically, stimulating the growth and spread of cancer cells (5). Therefore, due to the paramount role of EGFR in tumorigenesis, several therapeutic strategies are being developed to target and inhibit signalling *via* the receptor (7-9). Monoclonal antibodies (mAbs) bind to the extracellular domain of EGFR inhibiting this region and blocking ligand binding. Cetuximab (IMC-C225, Erbitux) and Panitumumab (ABX-EGF, Vectibix) are EGFR-binding mAbs currently approved for the treatment of colorectal cancer (10). Alternatively, EGFR tyrosine kinase inhibitors (TKIs) bind to the intracellular tyrosine kinase domain, blocking kinase activity as well as the downstream signaling. Gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva), are EGFR TKIs already approved for non-small cell lung cancer (NSCLC) patients (11).

In high-grade gliomas, *EGFR* is the most frequently amplified oncogene, being present in 40% of primary GBM and poorly characterized in anaplastic astrocytomas and anaplastic oligodendrogliomas (AO) (12-14). Importantly, *EGFR* gene amplification is known to be associated with protein overexpression in gliomas (2). Additionally, in about half of GBM cases with *EGFR* amplification, the event is coupled with *EGFR* gene mutations, with EGFRvIII (also known as ΔEGFR and del2-7EGFR) the most common in GBM (2, 15, 16). This EGFR mutant oncoprotein lacks a portion of the extracellular ligand-binding domain as a result of a genomic deletion involving exons 2 to 7, resulting in a mutant protein unable to bind EGFR ligands, yet constitutively activated in a ligand-independent manner leading to overproliferation of cancer cells (6, 17). EGFR represents an attractive therapeutic target in malignant gliomas and even though there are no anti-EGFR agents approved for glioma treatment at present, there are several ongoing clinical trials evaluating the efficacy of mAbs and TKIs in gliomas (7, 18, 19).

In the present study, we aimed to characterize the most common mechanisms (*EGFR* overexpression, gene amplification and EGFRvIII mutation) involved in EGFR activation in a Portuguese cohort of high-grade (astrocytic, oligodendroglial and mixed) gliomas and to identify the subset of patients that would potentially benefit from EGFR-targeted therapies.

Materials and Methods

Patients and tumor samples. Sixty-two formalin-fixed, paraffin-embedded samples of sporadic gliomas from 55 patients were retrieved from the Department of Pathology of the S. João Hospital, Porto and S. Marcos Hospital, Braga, Portugal, as well as the available patients' clinical data, as described elsewhere (20). Tumor samples were classified according to the WHO classification of CNS tumors (2): as 31 primary GBM (WHO grade IV), 26 AO (WHO grade III), one oligodendroglioma (O) (WHO grade II) and four anaplastic oligoastrocytomas (AOA) (WHO grade III) (Tables I and II). Twenty-seven (49.1%) patients were male and 28 (50.9%) were female; the mean age was 56.1 years (range 27-79 years) (Tables I and II).

EGFR and EGFRvIII immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were used in the immunohistochemical analysis. Previously documented mouse monoclonal anti-EGFR (clone 31G7, 1:100; Zymed® Laboratories Inc., South San Francisco, CA, USA) and mouse monoclonal anti-EGFRvIII antibody (clone G100, 1:100; Zymed®Laboratories Inc.) were used as primary antibodies (18, 21). Anti-EGFRvIII antibody specifically recognizes EGFRvIII and does not cross-react with the wild-type form (18).

Immunohistochemistry for EGFR was performed as described elsewhere (22). Briefly, after deparaffinization, sections were rehydrated and washed. Antigen retrieval was achieved by 20 min incubation at 37ïC with a bacterial protease extracted from *Streptomyces griseus* (Sigma-Aldrich Co., St. Louis, MO, USA). Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 10 min and samples were then incubated with Ultra V block (Lab Vision Corporation, Fremont, CA, USA) for 10 min at room temperature (RT). After incubation with primary antibody, overnight at RT, the secondary biotinylated goat anti-polyvalent antibody (Lab Vision Corporation) was applied for 10 min followed by 10 min incubation with Streptavidine Peroxidase (Lab Vision Corporation) at RT.

For the EGFRvIII reaction, sections were deparaffinized, incubated in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity and rehydrated. Antigen retrieval was achieved by microwaving sections in 10 mM citrate buffer (pH 6.0) three times for 5 min at 700 W. Sections were then incubated with Ultra V block (Lab Vision Corporation) for 20 min at RT. After incubation with primary antibody overnight at 4ïC, the biotinylated "universal" secondary antibody (Vector Laboratories, Burlingame, CA, USA) was applied for 30 min followed by R.T.U. Vectastain®Elite ABC reagent (Vector Laboratories) incubation for 45 min at 37ïC. Both EGFR and EGFRvIII sections were incubated with the chromogen 3,3'-diaminobenzidine (DAB) (Ultravision Detection System Anti-polyvalent, HRP/DAB; DAKO Corporation, Carpentaria, CA, USA), for 10 min at RT. Haematoxylin counterstaining was performed in a Leica Auto Stainer XL (Meyer Instruments Inc., Houston, TX, USA).

A specimen of human skin and a human glioblastoma with documented expression of EGFRvIII were used as positive controls for EGFR and EGFRvIII, respectively (22,23). Neoplastic cells with membranous and/or cytoplasmic intense immunoreactivity were considered positively stained. Both the distribution and intensity of the immunoreactivity were semi-quantitatively scored as follows: – (0%), + (<10%), ++ (10-50%), and +++ (>50%). Samples with scores (–) and (+) were considered negative, and those with scores (++) and (+++) were considered positive as described elsewhere (22,23).

Chromogenic in situ hybridization (CISH). The presence of *EGFR* gene amplification was assessed with CISH using Spot-Light amplification probes for *EGFR* (Zymed®Laboratories Inc.). CISH was performed using Spot-Light CISH Polymer Detection Kit (Zymed®Laboratories Inc.) in accordance with the manufacturer's protocol, and as described elsewhere (22). Amplification was defined

Table I. *Results of EGFR analysis in glioblastomas.*

Case No.	Age (years)/ gender	IHC - EGFR	CISH - *EGFR* amplification	IHC - EGFRvIII
12	27/F	+	Not Ampl	–
13	54/F	+++	Ampl	–
14	41/M	–	Not Ampl	–
19	61/F	–	Not Ampl	–
20	77/M	+++	Ampl	–
21	67/F	–	Not Ampl	–
22	50/F	+	Ampl	++
23	73/M	+	Not Ampl	–
24	57/M	+++	Ampl	–
24†	57/M	+++	Ampl	++
26	28/M	+++	NC	–
29	51/F	+++	Ampl	–
30	69/M	–	NC	–
30†	69/M	–	ND	–
32	53/M	+++	NC	++
32†	53/M	++	ND	+
33	59/F	+++	Ampl	+
36	56/F	+++	Ampl	+++
37	66/M	–	NC	–
38	68/F	–	ND	–
40	60/F	–	NC	–
41	58/F	–	Not Ampl	–
42	60/M	+++	Ampl	–
43	73/M	+++	Ampl	–
45	66/F	++	Not Ampl	+++
48	54/M	+++	Not Ampl	–
48†	54/M	+	Not Ampl	–
94	66/F	+	NC	–
96	79/M	+	Ampl	–
97	72/M	–	Not Ampl	–
231	62/F	++	NC	+++

ND, not determined; NC, not conclusive; Ampl, amplified; Not Ampl, not amplified; †, recurrence.

Table II. *Results of EGFR analysis in anaplastic oligodendrogliomas and oligoastrocytomas.*

Case No.	Age (years)/ gender	IHC - EGFR	CISH - *EGFR* amplification	IHC - EGFRvIII
Anaplastic oligodendrogliomas				
60	65/M	++	Not Ampl	–
62	47/F	+	Not Ampl	–
64	65/F	+	NC	–
66	50/M	+	Not Ampl	–
68	36/F	–	Not Ampl	–
70	60/F	–	NC	–
71	53/M	++	Not Ampl	–
72	65/M	+++	Ampl	++
73	47/M	–	Not Ampl	–
76	70/F	–	Not Ampl	–
81	64/F	+	NC	–
83	54/M	+	Not Ampl	–
110	44/M	–	Not Ampl	–
110†	44/M	–	Not Ampl	–
112	42/F	+	Ampl	–
115	40/M	++	Not Ampl	–
116	51/M	–	Ampl	+++
118	64/F	++	Ampl	–
119	68/F	+	Ampl	–
163	51/M	+++	Ampl	–
168‡	36/M	+	ND	ND
168†	36/M	–	Not Ampl	+
170	73/M	–	Not Ampl	–
172	45/M	+++	ND	ND
172†	45/M	+++	Ampl	–
202	65/F	+++	Ampl	–
259	52/F	–	NC	–
Anaplastic oligoastrocytomas				
63	46/F	+	Not Ampl	–
203	36/F	++	NC	–
205	33/M	++	Ampl	–
206	55/F	+++	Ampl	+

ND, not determined; NC, not conclusive; Ampl, amplified; Not Ampl, not amplified; †, recurrence; ‡, oligodendroglioma grade II.

as more than 5 signals per nucleus in more than 50% of cancer cells, or when large gene copy clusters were seen. Signals were evaluated at x400 and x600 and at least 60 morphologically unequivocal neoplastic cells were counted for the presence of the gene probe signals. CISH hybridizations were evaluated in a blinded manner, by two independent observers, on a multi-headed microscope.

Statistical analysis. Statistical analysis was performed using SPSS for Windows (version 14.0; SPSS Inc., Chicago, IL, USA). The correlation between categorical variables was calculated for statistical significance using Pearson's chi-square test and the threshold for significance was $p \leq 0.05$.

Follow-up information was available for 40 out of 55 patients, with follow-up periods ranging from 1 to 93 months (median 11 months, mean 16 months). Survival duration was defined as the time between diagnosis and death. Associations among EGFR expression, amplification, EGFRvIII expression and patients' age with patients' survival were assessed using Cox proportional hazards regression analyses.

Results

EGFR protein overexpression. EGFR immunohistochemical analysis was performed on 62 glioma samples from 55 patients. Results are summarized on Table I and Table II. The EGFR neoplastic staining was membranous and/or cytoplasmic, without immunoreactivity of endothelial tumor cells (Figure 1 A and B). EGFR overexpression (2+/3+) was detected in 24 of 55 tumors (44%). A high percentage of positive cases was found in all histological types of gliomas analyzed, namely, 48% (13/27) of GBM (Table I, Figure 1A), 33% (8/24) of AO and 75% (3/4) of AOA (Table II, Figure 1B).

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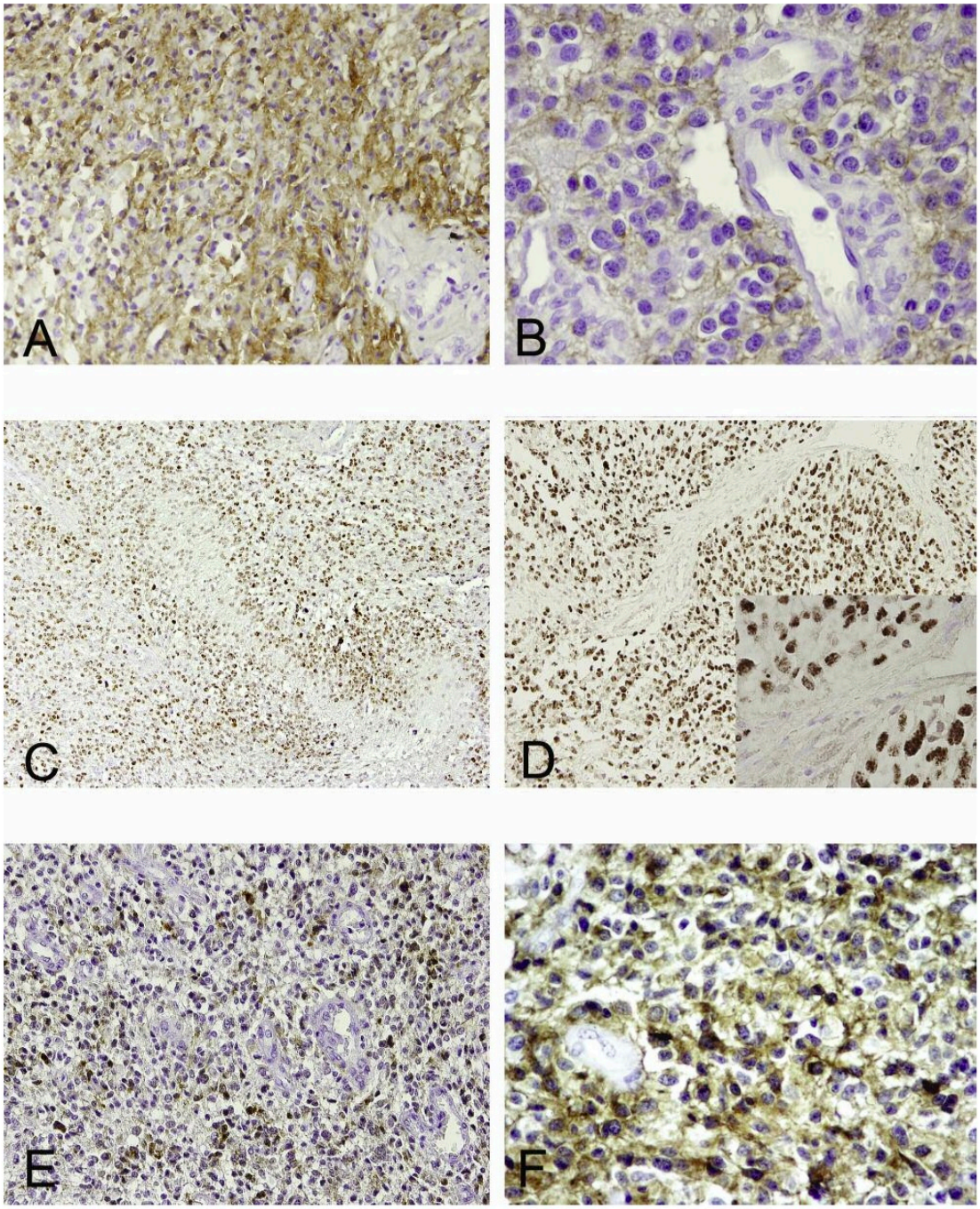

Figure 1. *EGFR cytoplasmic/membranous immunohistochemistry expression in GBM (A, x200) and AO (B, x400). EGFR amplification revealed by high nuclear signaling in GBM (C, x100) and AO (D, x100; inset, x600). EGFRvIII cytoplasmic/membranous immunohistochemistry expression in GBM (E, x200) and AO (F, x400).*

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EGFR amplification. CISH analysis was possible in 54 tumors and was conclusive in 42 (78%) of the cases (Tables I and II). The pattern of EGFR signals observed using CISH was compatible with double minute amplification (Figure 1C and D). Overall, *EGFR* gene amplification was detected in 20 (48%) high-grade gliomas. Specifically, *EGFR* gene amplification was observed in 53% of GBM (10/19) (Table I, Figure 1C), 40% of AO (8/20) (Table II, Figure 1D) and 67% of AOA (2/3) (Table II). Of the 20 gliomas with *EGFR* amplification, 15 (75%) exhibited EGFR overexpression ($p=0.001$). All recurrent cases with available CISH analysis for both primary and recurrent samples, namely two GBM (cases 24 and 48) and one AO (case 110), showed the same *EGFR* amplification status.

EGFRvIII protein overexpression. EGFRvIII expression analysis was performed in 60 samples of 55 gliomas and results are summarized in Table I and Table II. EGFRvIII staining was predominantly cytoplasmic and was observed only in neoplastic cells, not in endothelial tumor cells (Figure 1E and F).

EGFRvIII overexpression was observed in 22% of GBM (6/27) (Table I, Figure 1E), 8% of AO (2/24) (Table II, Figure 1F) and was absent in AOA (0/4) (Table II). In GBM, all but one sample with EGFRvIII overexpression also exhibited EGFR overexpression (Table I, $p=0.114$). Similarly, with the exception of one case, all informative samples with EGFRvIII overexpression also showed *EGFR* gene amplification (Table I, $p=0.596$). Both AO cases with EGFRvIII overexpression depicted *EGFR* gene amplification, (Table II, $p=0.068$) one of them also with EGFR overexpression (Table II, $p=0.602$).

Clinical significance of EGFR overexpression, EGFR amplification and EGFRvIII overexpression. Association between patient age and EGFR overexpression was assessed for glioma, GBM and AO patients (data not shown). EGFR overexpression in GBM patients tended ($p=0.082$) to occur in younger patients. Multivariate analysis (Cox proportional hazards) was used to determine the association between patient age, EGFR overexpression, *EGFR* amplification or EGFRvIII overexpression and overall survival for GBM and AO. The median age of patients was calculated for GBM (60 years) and AO (51 years) and these values were used to split patients into younger and older groups. Survival time was calculated after one year for GBM patients and after five years for AO patients. No correlation was found between EGFR overexpression, *EGFR* amplification, or EGFRvIII overexpression and overall survival of GBM and AO patients (Table III). EGFR overexpression showed a tendency ($p=0.054$, Table III) for being associated with shorter survival in AO patients. There was a significant correlation ($p=0.014$, Table III) between being older and better survival in AO patients.

Table III. *Multivariate analysis for the effect of EGFR overexpression, EGFR amplification, EGFRvIII overexpression and age on survival of GBM and AO patients.*

Variable	Hazard ratio (95% confidence interval)	*p*-value
GBM (1-year survival)		
EGFR overexpression	3.655 (0.731-18.277)	0.114
EGFR amplification	1.073 (0.167-6.885)	0.941
EGFRvIII overexpression	0.732 (0.131-4.095)	0.723
Median age (<60 or ≥60 years)	1.222 (0.204-7.333)	0.826
AO (5-year survival)		
EGFR overexpression	36.927 (0.942-1447.576)	0.054
EGFR amplification	0.112 (0.007-1.710)	0.116
EGFRvIII overexpression	NS	
Median age (<51 or ≥51 years)	0.065 (0.007-0.570)	0.014

NS: Insufficient data for statistical evaluation.

Discussion

In this study, we characterized, for the first time, the presence of EGFR alterations in a series of high grade gliomas from Portuguese patients. We studied a total of 31 GBM and observed that the frequency of GBM presenting EGFR overexpression (48%), *EGFR* amplification (53%) and EGFRvIII overexpression (22%) were in line with the published literature (24-26). Similarly to several studies a positive correlation ($p=0.012$) was found between EGFR overexpression and gene amplification in GBM (14, 18, 24, 27). Additionally, all but one GBM with EGFRvIII overexpression presented gene amplification, which is also in agreement with previous studies (24, 25). It was previously reported that older patients with GBM have higher rates of EGFR overexpression and amplification (24, 28). We found no statistical differences between patient age and EGFR overexpression, *EGFR* gene amplification, or EGFRvIII overexpression in the same cases.

EGFR activation status in anaplastic oligodendrogliomas has been less frequently reported (13, 27, 29, 30). We analyzed 24 cases and observed EGFR overexpression in 33%, *EGFR* amplification in 40% and EGFRvIII mutation in 8% of anaplastic oligodendrogliomas. EGFR overexpression tended to correlate with gene amplification ($p=0.094$). Other authors described *EGFR* amplification in anaplastic oligodendrogliomas and frequencies varied from 0-42.5% (13, 27, 31). A possible reason for this discrepancy is the distinct methodologies used. Regarding EGFRvIII expression, results are few and contradictory. Wikstrand and colleagues analyzed 5 anaplastic oligodendrogliomas and described a frequency of 20% of EGFRvIII expression (30); however, in another study from the same group EGFRvIII was not found to be expressed in 25 anaplastic oligodendrogliomas (29).

The influence of EGFR overexpression, *EGFR* gene amplification and EGFRvIII overexpression in patient prognosis has been highly controversial for gliomas (24, 25, 28, 32-37). To clarify this issue, we performed a multivariate analysis and found no association between these EGFR alterations and patients' overall survival in our cohort.

EGFR is becoming an important therapeutic target, with some anti-EGFR drugs already being used in clinical practice and several novel EGFR inhibitors under development (38). Among the two major approaches, using EGFR-TKIs and mAbs, the former seems to be more suitable for gliomas due to their low molecular weight potentially being better at overcoming the blood–brain barrier (39). In NSCLC, the presence of activating mutations in the EGFR kinase domain was associated with selective EGFR-TKI sensitivity, allowing the selection of patients with a higher probability of clinical response to gefitinib and erlotinib (11). However, these mutations have never been found in glioma cell lines (11), or in glioma patients (11, 40, 41). Recently, Lee and colleagues reported EGFR activation in GBM due to missense mutations in the EGFR extracellular domain (42). They reported that transformed cells with the EGFR ectodomain mutations had increased sensitivity to erlotinib; however studying DNA samples from a previous clinical trial, these authors were unable to associate EGFR ectodomain mutations with clinical responses to EGFR TKI inhibitors (42).

The few clinical trials with gefitinib and erlotinib in gliomas included a small number of patients and variable molecular markers, insufficient for conclusive results regarding patients' response and *EGFR* molecular status. Rich and colleagues, in a phase II trial, described a monotherapy study with gefitinib in GBM patients and found neither objective tumor response nor association between EGFR expression, *EGFR* amplification, or EGFRvIII expression and gefitinib response (43). Franceschi and colleagues' recent phase II trial investigated the role of gefitinib in patients with high-grade gliomas and reported an 18% stable disease rate without any correlation of EGFR expression or gene status and tumor response (44). Vogelbaum *et al.* described a monotherapy with erlotinib in patients with recurrent GBM and found 25% with stable disease and 25% with partial response rates; although *EGFR* amplification was observed in about 50% of tumors, it was not associated with erlotinib response (45). In a phase II study with erlotinib in recurrent GBM, Cloughesy *et al.* described a 33% stable disease rate and EGFR expression associated with a slight tendency for better patient outcome (46). Haas-Kogan *et al.* reported that the presence of EGFR overexpression and gene amplification, associated with low levels of activated Akt, were associated with response to erlotinib, suggesting that these molecular alterations could be predictive markers for EGFR-TKI sensitivity in gliomas (18). Mellinghoff *et al.* described that coexpression of EGFRvIII and phosphatase and tensin homolog (PTEN) (RTK downstream negative regulator) was associated with a better response to gefitinib and erlotinib in recurrent GBM (19). In general, results with erlotinib seem to be more promising than with gefitinib, possibly due to its targeting of both EGFRvIII mutant and wild-type EGFR (39).

There have also been clinical trials involving EGFR-targeted mAbs in gliomas (9, 47). A phase I/II clinical trial using the EGFR mAb h-R3 in malignant glioma enrolled 29 patients with an objective response in 37.9% and stable disease in 41.4% of the cases (47). Another phase I/II trial using cetuximab in GBM is ongoing (9). There are several *in vitro* and *in vivo* studies reporting EGFR-targeted mAbs effects in glioma. Recently, Johns *et al.* studied the efficacy of two EGFR-specific mAbs (mAbs 806 and 528) against glioma cell line-derived xenografts expressing EGFR and EGFRvIII and reported that their efficacy was dependent on EGFR overexpression and receptor activation status (48). Currently, considerable efforts are being made to design anti-EGFRvIII strategies, such as mAbs and vaccines, since this mutated form constitutes a tumor-specific target that is not present in normal cells.

In conclusion, in the present study, we found that a high percentage of GBM and AO exhibited EGFR overexpression and amplification, as did a significant proportion of GBM and a small proportion of AO expressing EGFRvIII. Our results represent the first step for the identification of Portuguese glioma patients who could respond to specific therapies targeting EGFR.

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3.2 EGFRvIII deletion mutations in paediatric HGG and response to targeted therapy in paediatric glioma cell line

Human Cancer Biology

EGFRvIII Deletion Mutations in Pediatric High-Grade Glioma and Response to Targeted Therapy in Pediatric Glioma Cell Lines

Dorine A. Bax,[1] Nathalie Gaspar,[1,2,4] Suzanne E. Little,[1] Lynley Marshall,[1,2,3] Lara Perryman,[1] Marie Regairaz,[4] Marta Viana-Pereira,[1,5] Raisa Vuononvirta,[1] Swee Y. Sharp,[2] Jorge S. Reis-Filho,[6] João N. Stávale,[8] Safa Al-Sarraj,[7] Rui M. Reis,[5] Gilles Vassal,[4] Andrew D.J. Pearson,[1,3] Darren Hargrave,[3] David W. Ellison,[9] Paul Workman,[2] and Chris Jones[1]

Abstract

Purpose: The epidermal growth factor receptor (EGFR) is amplified and overexpressed in adult glioblastoma, with response to targeted inhibition dependent on the underlying biology of the disease. EGFR has thus far been considered to play a less important role in pediatric glioma, although extensive data are lacking. We have sought to clarify the role of EGFR in pediatric high-grade glioma (HGG).

Experimental Design: We retrospectively studied a total of 90 archival pediatric HGG specimens for EGFR protein overexpression, gene amplification, and mutation and assessed the *in vitro* sensitivity of pediatric glioma cell line models to the small-molecule EGFR inhibitor erlotinib.

Results: Amplification was detected in 11% of cases, with corresponding overexpression of the receptor. No kinase or extracellular domain mutations were observed; however, 6 of 35 (17%) cases harbored the EGFRvIII deletion, including two anaplastic oligodendrogliomas and a gliosarcoma overexpressing EGFRvIII in the absence of gene amplification and coexpressing platelet-derived growth factor receptor α. Pediatric glioblastoma cells transduced with wild-type or deletion mutant EGFRvIII were not rendered more sensitive to erlotinib despite expressing wild-type PTEN. Phosphorylated receptor tyrosine kinase profiling showed a specific activation of platelet-derived growth factor receptor α/β in EGFRvIII-transduced pediatric glioblastoma cells, and targeted coinhibition with erlotinib and imatinib leads to enhanced efficacy in this model.

Conclusions: These data identify an elevated frequency of *EGFR* gene amplification and *EGFRvIII* mutation in pediatric HGG than previously recognized and show the likely necessity of targeting multiple genetic alterations in the tumors of these children. (Clin Cancer Res 2009;15(18):5753–61)

Amplification, overexpression, and/or mutation of the epidermal growth factor receptor (EGFR) represent a compelling set of molecular genetic indicators for targeted therapy in adult glioblastoma. About 40% of glioblastomas show amplification of the *EGFR* gene locus, and about half of these tumors express a mutant receptor (EGFRvIII) that is constitutively active due to an in-frame truncation within the extracellular ligand-binding domain (1). In addition, novel missense mutations have been reported in the extracellular domain of tumors and cell lines (2), and recently, additional mutations have been described outside of these regions (e.g., in the transmembrane domains), although the significance of these is not yet clear (3).

EGFRvIII is caused by deletion of exons 2 to 7, resulting in a protein that lacks a ligand-binding domain and is constitutively activated and is further resistant to down-regulation due to a low rate of receptor endocytosis (4). EGFRvIII has been shown

Authors' Affiliations: [1]Paediatric Oncology and [2]Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research; [3]Paediatric Oncology, The Royal Marsden NHS Foundation Trust, Sutton, United Kingdom; [4]Pharamacology and New Treatments of Cancer, Institut de Cancérologie, Gustave Roussy, Villejuif, France; [5]Life and Health Science Research Institute, Universidade do Minho, Braga, Portugal; [6]Breakthrough Breast Cancer, The Institute of Cancer Research; [7]Neuropathology, Kings College Hospital, London, United Kingdom; [8]Department of Pathology, Universidade Federal de Sao Paulo, Sao Paulo, Brazil; and [9]Neuropathology, St Jude Children's Research Hospital, Memphis, Tennessee

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D.A. Bax and N. Gaspar contributed equally to this work.

Requests for reprints: Chris Jones, Paediatric Oncology, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, United Kingdom. Phone: 44-20-8722-4416; Fax: 44-20-8722-4321; E-mail. chris.jones@icr.ac.uk.

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Translational Relevance

Dysregulation of epidermal growth factor receptor (EGFR) is common in adult glioblastoma but has been considered less so in the pediatric setting, although unequivocal data have been lacking. This article details the largest and most comprehensive study of EGFR gene alterations in high-grade glioma of childhood yet undertaken. In addition to an elevated frequency of gene amplification in comparison with previous smaller series, we have identified for the first time the presence of EGFRvIII deletions in pediatric cases. By generating stably EGFRvIII-overexpressing pediatric glioblastoma cell lines, we show that the deletion is not sufficient to confer increased sensitivity to the EGFR inhibitor erlotinib due to the specific coactivation of platelet-derived growth factor receptor α/β in these cells. Thus, we provide evidence for a novel, pediatric-specific mechanism for erlotinib resistance in glioblastoma.

to confer enhanced tumorigenicity both in human glioma cells *in vitro* and in xenografts in immunodeprived mice (5, 6), and in a xenograft model, tumorigenicity is directly proportional to EGFRvIII receptor load (7). EGFRvIII has also been shown to confer resistance to both radiotherapy and chemotherapy (8, 9). Constitutive activation by EGFRvIII stimulates proliferation and inhibits apoptosis predominantly through stimulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway but also via the Ras/mitogen-activated protein kinase (MAPK) signal transduction pathway (10, 11). These data may explain the observation that patients with EGFRvIII-expressing tumors have a shorter interval to clinical relapse and poorer survival than patients with EGFRvIII-negative tumors (12).

Erlotinib (Tarceva, OSI-774) is a selective inhibitor of the EGFR tyrosine kinase and has shown activity in adult high-grade glioma (HGG) patients (13). A differential therapeutic response has been reported in these cases, with a variety of factors reported to be predictive for treatment efficacy, including activating mutations of *EGFR* and a wild-type *PTEN* (14, 15). Published preclinical data show equivocal results in adult glioblastoma model systems engineered to overexpress the deletion mutant. Erlotinib has been shown to inhibit EGFRvIII, blocking constitutive EGFRvIII kinase activity and the growth of EGFRvIII-transformed cells (15, 16). Furthermore, long-term exposure to erlotinib decreased EGFRvIII expression in transformed cells and selectively down-regulated the EGFRvIII-mediated induction of oncogenes that drive invasion in transformed glioblastoma cells (17). By contrast, other reports have shown that *EGFR* amplification/mutation even in the presence of functional PTEN did not render sensitivity to EGFR inhibitors (18, 19). This combination of alterations may therefore be necessary but not sufficient for conferring glioblastoma sensitivity to EGFR kinase inhibition (20). Consequently, one could infer the existence of additional determinants of glioblastoma sensitivity to EGFR kinase inhibition yet to be identified. For instance, activating mutations of *PIK3CA*, as recently shown in adult and pediatric glioblastomas, could account for tumor insensitivity to EGFR inhibition.

HGGs in children seem to be clinically and biologically distinct from their adult counterparts. Glioblastomas in adults are classified as primary or secondary based on progression from preexisting low-grade lesions and on distinct patterns of molecular abnormalities (21). Although some molecular abnormalities encountered in pediatric cases are reminiscent of secondary adult glioblastoma [*TP53* mutation (22) and platelet-derived growth factor receptor α (PDGFRα) overexpression (23)], these neoplasms rarely originate from preexisting low-grade lesions (24). Unfortunately, our understanding of these differences is restricted by the relative paucity of molecular data derived from pediatric tumors.

EGFR has been considered to play a less important role in pediatric HGG, although this issue is compounded by the limited available data. The receptor seems to be frequently overexpressed, usually in the absence of gene amplification, with reports varying from 10% to 80% of pediatric nonbrain stem HGGs (25–29). Interestingly, the primary site of the tumor may affect the type of genetic changes involved, with data indicating that *TP53* mutation and *EGFR* overexpression are more frequent in diffuse intrinsic pontine glioma than in supratentorial sites in children (25).

We have sought to clarify the role of EGFR in pediatric HGG and to assess the *in vitro* sensitivity of pediatric glioma cell line models to erlotinib. These data identify a higher prevalence of *EGFR* gene amplification and *EGFRvIII* mutation in pediatric HGG than previously recognized. We further investigated the potential of treatments targeting the receptor in deletion mutant-positive cases.

Materials and Methods

Tumor samples. HGG samples from 90 patients were obtained after approval by Local and Multicenter Ethical Review Committees. The collection consisted of 53 glioblastoma multiforme, 16 anaplastic astrocytomas, 3 anaplastic oligodendrogliomas, 3 brainstem gliomas, and 25 other WHO grade 3 or 4 lesions. All cases were archival formalin-fixed, paraffin-embedded (FFPE) tissues. The presence of tumor tissue in these samples and the tumor type was verified on a H&E-stained section independently by two neuropathologists (D.W.E. and S.A-S.).

Immunohistochemistry and chromogenic* in situ *hybridization. Immunohistochemistry was done on representative 4- or 5-μm FFPE sections. EGFR overexpression was assessed using the mouse monoclonal antibody 31G7 (Zymed) at a dilution of 1:50 using the Envision-HRP system (K4006, Dako) as previously described (30). EGFRvIII was assessed using a 1:100 dilution of the mouse monoclonal antibody G100 (Zymed) as a primary and antigen retrieval by microwaving in 10 mmol/L citrate buffer (pH 6.0) for 3 × 5 min (31). PDGFRα was assessed using the polyclonal rabbit primary antibody RB-1691 (LabVision) at 1:150 dilution with 15 min antigen retrieval in 10 mmol/L citrate buffer (pH 6.0; ref. 32). Chromogenic *in situ* hybridization (CISH) was done using Spot-Light amplification probes for *EGFR* (Zymed) according to the manufacturer's protocol and as reported earlier (30). Cases were considered to be amplified for EGFR when >50% of the neoplastic cells harbored (*a*) more than five signals per nuclei or (*b*) large gene copy clusters. Immunohistochemical and CISH analyses were done with observers blinded to the results of *EGFR* and sequencing data.

Mutation analysis. Genomic DNA was isolated from representative 10-μm-thick unstained tissue sections containing >85% tumor cells, as determined from a serial H&E-stained section, using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. Exons 2 to 8, coding for the extracellular domain of EGFR, and exons 18 to 21, coding for the receptor tyrosine kinase (RTK) domain of EGFR, were

amplified by PCR (33). Products were purified using the QIAquick PCR Purification kit and subjected to bidirectional sequencing using BigDye Terminator Mix 3.1 (Applied Biosystems) according to the manufacturer's instructions. Capillary sequencing was done in duplicate on an ABI 3100 genetic analyzer (Applied Biosystems), and sequences were evaluated for the presence of mutations using Mutation Surveyor Software (SoftGenetics LLC). A reverse transcription-PCR (RT-PCR) was developed for detection of the EGFRvIII mutation from FFPE sections. RNA was extracted from representative 10-μm-thick unstained tissue sections containing >85% tumor cells, as determined from a serial H&E-stained section, using the Recover-All kit (Ambion). cDNA was synthesized from 200 ng RNA using SuperScript II reverse transcriptase (Invitrogen Ltd.). The deletion region was amplified with primers in exon 1 (5′-GGGCTCTGGAGGAAAAGAAA-3′) and exon 9 (5′-CCTCCATCTCATAGCTGTCG-3′) amplifying a region of 91 bp when the deletion is present and 892 bp in the wild-type. The presence of the EGFRvIII fragment was confirmed in duplicate by direct sequencing.

Cell culture and generation of isogenic cell lines. Glioblastoma cell lines U87MG and KNS42 were obtained from the American Type Culture Collection (LGC Promochem) and Japan Cancer Research Resources !s] cell banks, respectively. SF188 was kindly provided by Dr. Daphne Haas-Kogan (University of California at San Francisco, San Francisco, CA), whereas UW479, Res259, and Res186 were kindly provided by Dr. Michael Bobola (University of Washington, Seattle, WA). Cells were grown as monolayers in DMEM/F12 Ham's medium + 10% FCS in 5% CO_2. *EGFR* constructs for isogenic cell lines were created by PCRs done using Phusion DNA polymerase enzyme (Finnzymes) to minimize the risk of error. Full-length wild-type *EGFR* was amplified from A431 cell line cDNA using the forward primer 5′-TCTTCGGCTAGCAACGATGCGACCCTC-3′ and the reverse primer 5′-ATGCGGCCGCTCATACTATCCTCCGTGGT-3′, which contained *Nhe*I and *Not*I enzyme restriction sites, respectively. The *EGFRvIII* mutant was created using splice overlapping extension PCR. Two PCRs were done to generate two fragments with overlapping ends surrounding the deleted region (exon 1: forward, 5′-TCTTCGGCTAGCAACGATGCGACCCTC-3′; reverse, 5′-TGTCACCACATAATTACCTTTCTTTTCCTCCAGAGCC-3′; exon 8: forward, 5′-GGAAAAGAAAGGTAATTATGTGGTGACAGATCACGGC-3′; reverse, 5′-GCCGCGTATGATTTCTAGATTCTCAAAGGC-3′). These fragments were then combined in a subsequent "fusion" PCR in which the overlapping ends annealed, allowing 3′ overlap of each strand to serve as a primer for the 3′ extension of the complementary strand. The result was a fragment of 499 bp with *EGFRvIII* deletion and *Nhe*I and *Xba*I restriction sites at the 3′ and 5′ ends. The remaining EGFR cDNA sequence was amplified using forward primer 5′-GAATCTAGAAATCATACGCGGCAGGACC-3′ and reverse primer 5′-ATGCGGCCGCTCATACTATCCTCCGTGGT-3′, which contained *Xba*I and *Not*I enzyme restriction sites. The vector F527 (34) was digested by *Nhe*I and *Not*I and the PCR fragments by the enzyme corresponding to their restriction sites. The cDNA fragment was then extracted from an agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen). The ligation of inserts and vector F527 was done using T4 DNA ligase (Invitrogen). Electrocompetent XL1 blue *Escherichia coli* (kindly provided by Betsy Julienne, Institut de Cancérologie Gustave Roussy, Villejuif, France) was transformed by electroporation in the presence of the ligation products, and selection was done by bacterial culture on ampicillin LB agar Petri dishes. Colonies showing the correct orientation by restriction digestion were further designated to sequencing. The DNA from plasmid with the correct sequence was then efficiently isolated from *E. coli* using Maxiprep using the PureLink HiPure Plasmid Filter Purification kit (Invitrogen). U87MG and SF188 were then transfected with either the F527 empty vector, or F527-EGFR wild-type or F527-EGFRvIII plasmids using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Stable clones were obtained after 4 wk of antibiotic selection by 1 μg/mL puromycin.

Western blot analysis. Cells at 80% confluence were trypsinized, washed with PBS, and lysed for 1 h at 4°C in lysis buffer (Cell Signaling) and a complete mini protease inhibitor cocktail (Roche Diagnostics). Cells were then centrifuged at 11,000 rpm at 4°C for 15 min, and protein concentration was determined (bicinchoninic acid assay, Pierce). Total protein extracts (30 μg/lane) were separated electrophoretically in 4% to 20% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Invitrogen). Immunodetection was done using antibodies directed against EGFR, phosphorylated and total extracellular signal-regulated kinase 1/2, phosphorylated/total Akt, and PTEN (all 1:1,000; Cell Signaling) as well as glyceraldehyde-3-phosphate dehydrogenase (1:2,000; Chemicon). Blots were revealed with peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (GE Healthcare) followed by enhanced chemiluminescence solution (GE Healthcare).

Detection of phosphorylated RTKs. The levels of phosphorylated and total wild-type EGFR were determined from 2.5 μg of protein lysate by an electrochemiluminescent immunoassay (MesoScale Discovery) according to the manufacturer's instructions. A phosphorylated RTK assay (R&D Systems) was used to screen multiple RTKs. Cells were lysed in radioimmunoprecipitation assay lysis buffer (Cell Signaling) and a complete mini protease inhibitor cocktail (Roche Diagnostics), as described for Western blot analysis. Phosphorylated PDGFRα/β was measured in a sandwich ELISA assay (Cell Signaling) according to the manufacturer's instructions.

Growth inhibition studies. Erlotinib (Euroasian Chemicals PVT Ltd.), imatinib (LC Laboratories), the dual PI3K and mammalian target of rapamycin inhibitor PI-103 (provided by Piramed Ltd.), and the MAPK/extracellular signal-regulated kinase kinase (MEK) inhibitor PD325901 (provided by Dundee University, Dundee, United Kingdom) were obtained in powder form and diluted in either DMSO or water at 10 mmol/L stock solutions. Growth inhibition was determined using the sulforhodamine B (SRB) assay as described previously (35). Briefly, 10^3 cells were seeded into 96-well microtiter plates and allowed to attach for 36 h. Compounds at a range of concentrations were added in quadruplicate wells for 6 d (at least three doubling times) in a volume of 200 μL/well. Cells were then fixed with ice-cold 10% trichloroacetic acid for 30 min, then washed with tap water, and dried. The plates were subsequently stained with 0.4% SRB in 1% acetic acid for 15 min, and excess stain was washed off with 1% acetic acid. After the plates were dried, the stain was solubilized with 10 mmol/L Tris base, and the absorbance was determined at 570 nm. The IC_{50} was calculated as the drug concentration that inhibits cell growth by 50% compared with control growth using GraphPad Prism 4 program (GraphPad Software). All values are given as mean ± SD of at least three independent experiments.

Statistical analysis. All statistical tests were done in R2.6.1.[10] Correlations between categorical values were done using the χ^2 and Fisher's exact tests. Correlations between continuous variables were done using Student's *t* test or the Mann-Whitney *U* test. Cumulative survival probabilities were calculated using the Kaplan-Meier method, with differences between survival rates analyzed with the log-rank test. All tests were two tailed, with a confidence interval of 95%. *P* values of <0.05 were considered statistically significant.

Results

EGFR is amplified and overexpressed in pediatric HGGs. We collected a series of 90 HGGs from patients under the age of 19 years (median, 11.3 years) for molecular analysis. All samples were archival FFPE pathology specimens, with variable quantity and quality of material available as sections for immunohistochemistry or DNA/RNA extraction. To evaluate gene dosage and receptor expression of EGFR, we used CISH and immunohistochemistry on tissue sections.

We observed gene amplification in 8 of 74 (11%) evaluable cases. These consisted of 6 glioblastomas (6 of 43, 14%) and 2 anaplastic astrocytomas (2 of 11, 18%). No amplifications were

[10] http://www.r-project.org/

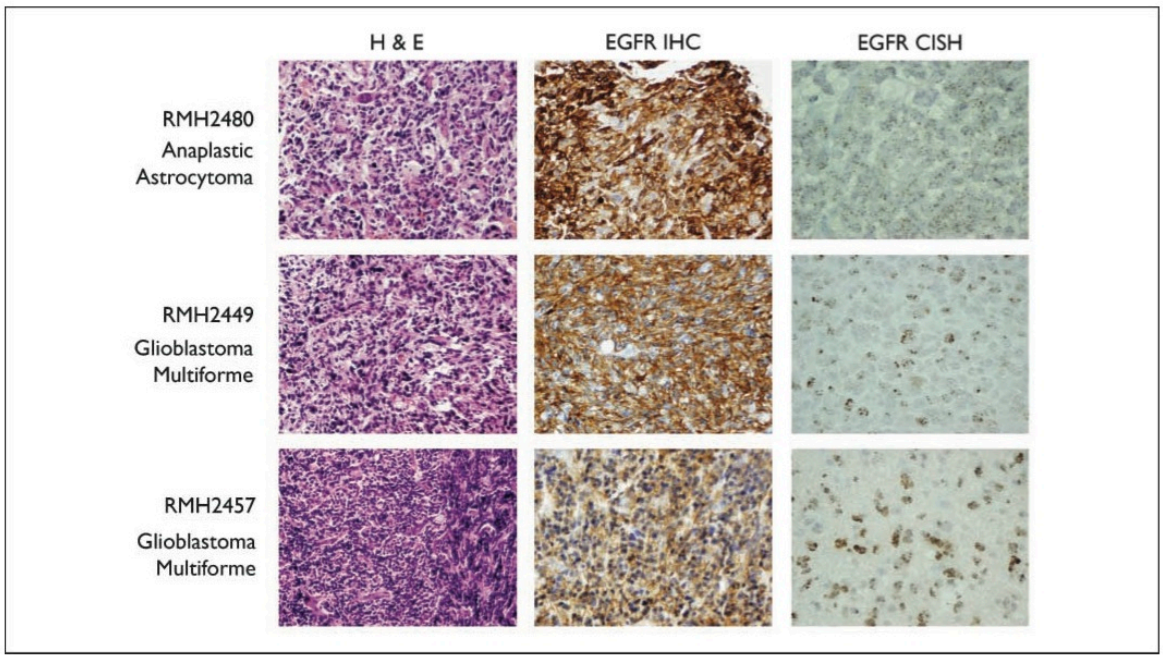

Fig. 1. EGFR expression and amplification in pediatric HGG. H&E-stained sections, immunohistochemistry for wild-type EGFR, and EGFR CISH for three representative cases of pediatric HGG. Amplifications were present either as discrete signals at a copy number of 9 to 10 per nucleus (RMH2480), in scattered cells throughout the tumor (RMH2449), or in dense clusters in the vast majority of the tumor cells (RMH2457).

seen in any other high-grade lesion. Polysomy of chromosome 7 was noted in an additional 14 of 74 (19%) cases and included 10 cases of glioblastoma, 3 anaplastic astrocytoma, and a gliosarcoma.

These amplifications were mostly present as dense clusters of nuclear signals representing a high-level DNA copy number gain in the vast majority of the tumor cells (Fig. 1, RMH2457); however, additional patterns were also observed. These included discrete signals at a copy number of 9 to 10 per nucleus, observed in a case of anaplastic astrocytoma (Fig. 1, RMH2480), or high-density clusters, present only in scattered cells throughout the tumor, in a glioblastoma (Fig. 1, RMH2449).

All cases of pediatric HGG harboring *EGFR* amplification were found to express the protein at high levels (Fig. 1). There was no association between *EGFR* amplification and clinical outcome ($P = 0.457$, log-rank test for overall survival) or age at diagnosis ($P = 0.591$, Mann-Whitney *U* test).

Pediatric HGGs harbor vIII deletions but no activating mutations in the extracellular or kinase domains. We further screened our pediatric HGG cases for mutations in the *EGFR* gene. Previously reported activating mutations in the kinase domain (in non–small cell lung cancer and others) and the extracellular domain (adult glioblastoma) were screened by PCR amplification of exonic sequences and direct sequencing. We observed no mutations in 60 (kinase domain) and 66 (extracellular domain) pediatric cases. Although the lack of kinase domain mutations is consistent with data from adult glioblastoma, the absence of extracellular domain mutations in our pediatric series is significantly discordant with the published frequency of 18 of 132 (13.6%; ref. 2) in adult glioblastoma ($P = 0.0019$, Fisher's exact test).

In addition, we developed a RT-PCR assay applicable to FFPE samples to screen for the presence of the EGFRvIII deletion mutation. A product of 91 bp spanning exons 1 and 8 was generated from extracted mRNA in cases harboring deletion of exons 2 to 7, which was verified by direct sequencing (Fig. 2). We detected six cases of EGFRvIII in 35 assessable cases (17%). These included 1 of 20 (5%) glioblastoma, a single case of gliosarcoma, and an increased frequency in anaplastic astrocytoma (2 of 6, 33%) and anaplastic oligodendroglioma (2 of 3, 67%). Despite the small numbers, these latter cases had a statistically significant increased frequency compared with other pediatric HGG ($P = 0.036$, Fisher's exact test). Interestingly, the glioblastoma and anaplastic astrocytoma cases were *EGFR* amplified, whereas the anaplastic oligodendroglioma and gliosarcomas were not. A summary of mutation data is given in Table 1.

Pediatric glioma cell lines and response to erlotinib. In addition to the primary tumors, we also screened our panel of pediatric glioma cell lines for *EGFR* mutations as described above. None of five lines harbored any point mutations, deletions, or amplifications nor significant levels of protein or mRNA expression of the receptor (data not shown). Treatment with the small-molecule EGFR inhibitor erlotinib revealed a general lack of sensitivity, with an IC_{50} of >50 μmol/L for most of the pediatric cell lines. The most sensitive cell line was the glioblastoma SF188 line ($IC_{50} = 8.3 \pm 0.7$ μmol/L) derived from an 8-year-old male patient, which is *PTEN* wild-type and harbors mutant *TP53*.[11]

[11] Bax DA*, Little SE*, Gaspar N, et al. Molecular and phenotypic characterisation of paediatric high grade glioma cell lines as models for preclinical drug development PLoS ONE 4: e5209.

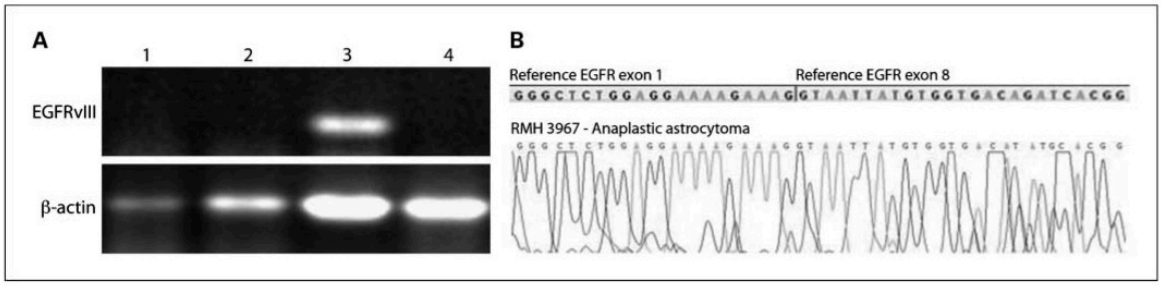

Fig. 2. Detection of EGFRvIII deletions in pediatric HGG. *A,* lane 3, RT-PCR amplifying a product of 91 bp in EGFRvIII-positive cases. *B,* sequencing of the RT-PCR product established the presence of sequence spanning exons 1 and 8 of the *EGFR* gene.

Therefore, to study the effects of EGFR overexpression and vIII deletion on pediatric glioma cells, we constructed isogenic cell line models using the *PTEN* wild-type pediatric glioblastoma cell line SF188 as well as the *PTEN*-deleted adult glioblastoma line U87MG. We successfully transduced either EGFR wild-type or vIII into both cell lines, resulting in stable protein overexpression (Fig. 3A). Treatment of these cell line models with erlotinib inhibited both PI3K and MAPK pathways and induced a G_1 arrest in all cells (Supplementary Fig. S1) but revealed no statistically significant differences in efficacy between EGFR-transduced (wild-type and vIII) and parental/empty vector for both U87MG and SF188 (Fig. 3B). Isolated clones showed a modest (2-fold) increase in sensitivity when overexpressing the vIII deletion in both cell lines; however, this was not generalizable. This was despite showing that vIII-transduced cells harbored constitutive activation of the receptor, as determined by an electrochemiluminescent immunoassay (detecting transactivation of the endogenous EGFR rather than the mutant) and also phosphorylated RTK array analysis (detecting both wild-type and deletion mutant phosphorylation directly).

Mechanisms of resistance to erlotinib in SF188 cells. As EGFR signals through both PI3K and MAPK, we sought to determine whether the presence of either wild-type or mutant *EGFR* would render glioblastoma cells more sensitive to agents inhibiting these pathways. Regardless of *PTEN* status, there was no consistent change in efficacy of EGFR-transduced U87MG or SF188 cells with the dual PI3K/mammalian target of rapamycin inhibitor PI-103 (Fig. 4A; refs. 36, 37) or the MEK inhibitor PD325901 (Fig. 4B).

We next focused on the pediatric SF188 line to investigate the possible biological basis for the lack of increased efficacy of erlotinib in EGFR-overexpressing clones despite these cells harboring wild-type *PTEN*. Selecting the most erlotinib-sensitive clones transduced by EGFR, we first showed that there was no difference in response to small-molecule inhibition due to either mutant or wild-type EGFR expression over a period of 72 hours despite a modest diminution of downstream signaling in the EGFR mutant SF188 cells (data not shown).

In U87MG cells, there is coactivation of EGFR and MET, which allows for continued oncogenic RTK signaling in the presence of small-molecule inhibition of EGFR (38, 39). To look for similar association in our pediatric glioma cells, we assayed the SF188 clones with phosphorylated RTK antibody arrays (Fig. 5). There was only very low levels of constitutive EGFR activation in the control cells, reflecting the data from the electrochemiluminescent immunoassay (Fig. 3C and D), and no additional RTK activation. There was an increase in phosphorylated EGFR on transduction with the wild-type receptor and a further significant increase on constitutive activation by the EGFRvIII deletion mutant. In concert with this, we observed a significant up-regulation of phosphorylated PDGFRα and PDGFRβ as well as a modest increase in activated MER, Tie-2, and Flt-3 (Fig. 5A). The increased levels of phosphorylated PDGFRAα/β were confirmed by a sandwich ELISA assay in our SF188 model (Supplementary Fig. S2) and were not seen in U87MG:EGFR wild-type or vIII mutant-overexpressing cells (data not shown). Four of the six (50%) pediatric HGG samples positive for EGFRvIII also showed strong expression of

Table 1. Summary of EGFR mutations in pediatric HGGs

	WHO grade	Amplification (CISH)	vIII deletion (RT-PCR)	Extracellular domain	Kinase domain
All pediatric HGG	—	8/74 (11%)	6/35 (17%)	0/66	0/60
Glioblastoma multiforme	IV	6/43 (14%)	1/20 (5%)	0/34	0/30
Brain stem glioma	IV	0/3	0/1	0/3	0/2
Anaplastic astrocytoma	III	2/11 (18%)	2/6 (33%)	0/12	0/12
Anaplastic oligodendroglioma	III	0/4	2/3 (67%)*	0/4	0/4
Other pediatric HGG	—	0/13	1/5 (Gliosarcoma)	0/13	0/12

*Significantly higher than that seen in other tumors (P = 0.036, Fisher's exact test).

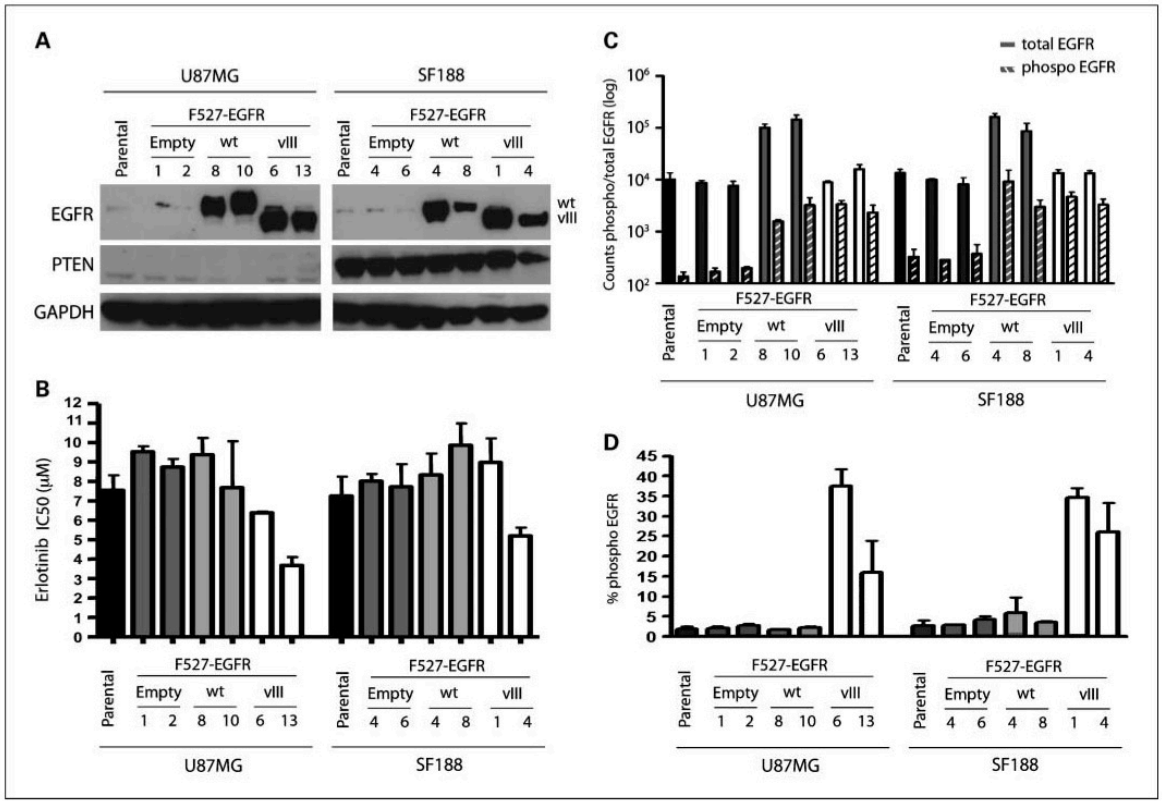

Fig. 3. Glioblastoma cell lines stably overexpressing the EGFRvIII deletion mutant have constitutive activation but not increased sensitivity to erlotinib. *A,* expression of EGFR and PTEN by Western blot in adult (U87MG) and pediatric (SF188) glioblastoma cell lines stably expressing EGFR wild-type (185 kDa) and vIII deletion mutation (145 kDa). U87MG cells are *PTEN* deleted, and SF188 cells express the wild-type protein. *B,* sensitivity of the isogenic cell lines to erlotinib, as determined by the SRB assay. There were no statistically significant differences in the IC_{50} values of any of the cell lines. *C,* constitutive activation of the receptor, in EGFRvIII-overexpressing cell lines. Absolute counts for total and phosphorylated EGFR are plotted on a $\log_{10}$ scale. *D,* percentage of phosphorylated EGFR to total protein, as determined by an electrochemiluminescent immunoassay, in the isogenic cell line models.

PDGFRα protein by immunohistochemistry (Fig. 5B), a greater frequency than the population as a whole (36%). Intriguingly, these four cases included all those EGFRvIII-positive cases in the absence of gene amplification (both anaplastic oligodendrogliomas and a gliosarcoma) as well as an anaplastic astrocytoma. The number of positive cases was too small to assign a statistical association ($P = 0.14$, Fisher's exact test) but shows the cosegregation of these abnormalities in the clinical setting.

Finally, we tested whether inhibition of PDGFR would enhance the sensitivity of these cells to erlotinib treatment. We measured cell viability of the SF188:EGFRvIII cells treated with a fixed dose of 10 μmol/L erlotinib alone and in combination with 10 μmol/L imatinib. Treatment with a combination of both small molecules showed a significant decrease in cell viability compared with either compound alone ($P < 0.01$, *t* test; Fig. 5C).

Discussion

Although the oncogenic significance of activated EGFR signaling is well established in primary adult glioblastoma, in the pediatric setting the picture is less clear. We have carried out the largest molecular analysis to date of EGFR in the HGGs of childhood and identified the presence of the EGFRvIII deletion mutation in pediatric cases.

EGFRvIII was noted in WHO grade 4 glioblastoma and WHO grade 3 anaplastic astrocytoma cases in association with gene amplification and receptor overexpression. The few previous reports on EGFR in pediatric gliomas suggested that amplification was less common in pediatric glioblastomas than in adults, although the frequency in these small series ranged from 0% to 25%, measured using a variety of different assays on differing collections of tumor histologies (25–29). We observed an overall frequency of 11% amplification, with up to 14% in glioblastoma and 18% in anaplastic astrocytoma. Of particular interest was the observation that in some cases the high-density clusters of amplified probe signal were seen only in scattered cells throughout the tumor. This may explain the underestimation reported in earlier studies and highlights the benefit of using a technique such as CISH to determine gene amplification, as it allows histologic evaluation concurrent with the molecular analysis.

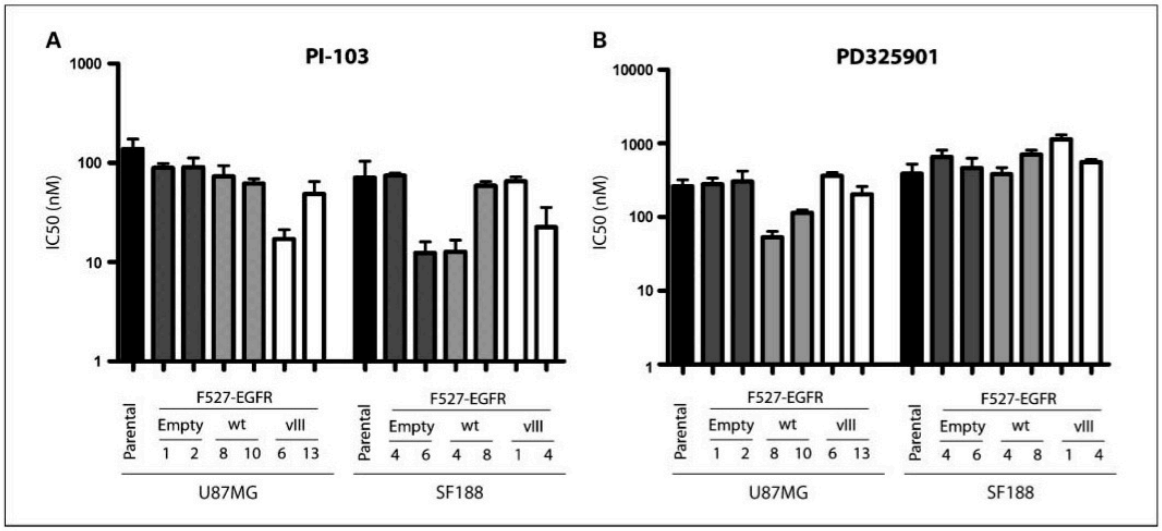

Fig. 4. Pediatric glioblastoma cell lines transduced with wild-type or vIII deletion mutant EGFR were not rendered more sensitive to inhibitors of the PI3K or MAPK pathways. Response of transduced U87MG and SF188 cells to inhibitors of PI3K/mammalian target of rapamycin (PI-103; *A*) and MEK (PD325901; *B*), as determined by the SRB assay. There were no statistically significant differences in the IC_{50} values of any of the cell lines.

We further observed EGFRvIII expression, in the absence of gene amplification, in two of three WHO grade 3 anaplastic oligodendrogliomas and a single case of WHO grade 4 gliosarcoma. Although there are no reports to our knowledge on the presence of EGFRvIII in pediatric anaplastic oligodendrogliomas, the deletion has previously been identified in an adult case (40), and more recently, we have observed 2 of 24 adult cases to harbor the deletion (31). Both of these studies further reported that

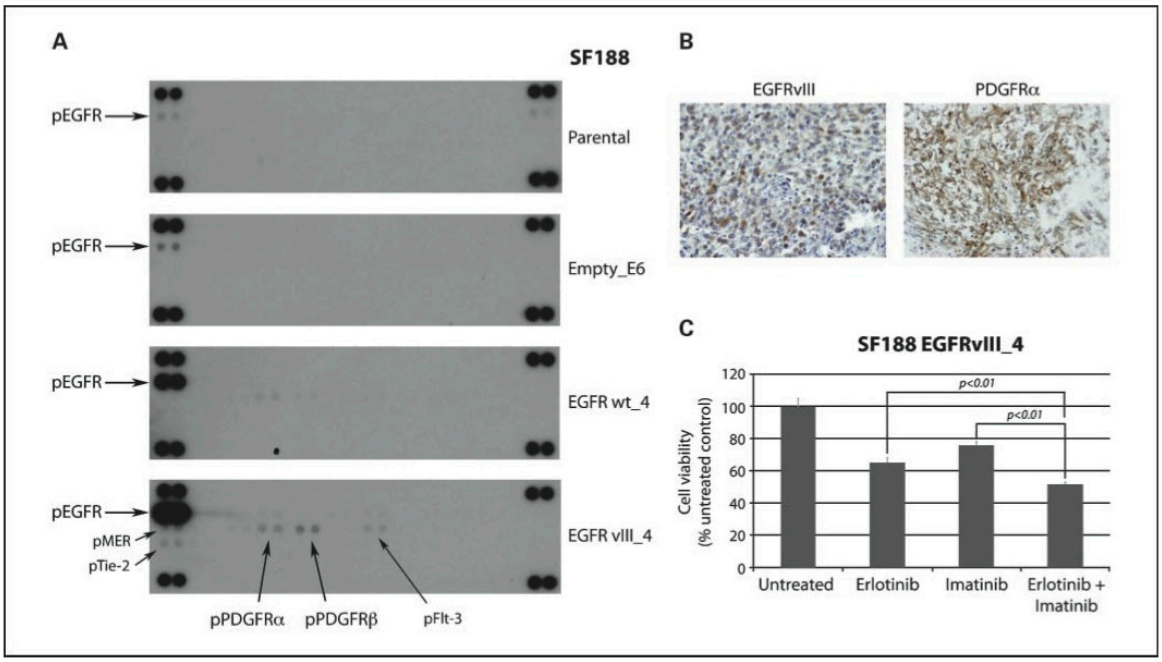

Fig. 5. Coactivation of PDGFRα/β in SF188 cells stably expressing EGFRvIII. *A*, phosphorylated RTK array analysis of isogenic SF188 cells shows low levels of constitutive EGFR activation in the control cells, and no additional RTK activation, in contrast to a large increase on constitutive activation by the EGFRvIII deletion mutant in association with a significant up-regulation of phosphorylated PDGFRα and PDGFRβ (also seen to a lesser extent with the wild-type receptor) as well as MER, Tie-2, and Flt-3. *B*, immunohistochemistry for EGFRvIII and PDGFRα in a case of pediatric gliosarcoma (RMH2473) showing coexpression of the receptors in the clinical setting. *C*, coinhibition of EGFR and PDGFR by erlotinib and imatinib showed a significant decrease in SF188: EGFRvIII cell viability than either compound alone ($P < 0.01$, *t* test).

the mutant receptor was amplified, and the significance of our findings of EGFRvIII expression in the absence of amplification is not clear; however, it may be significant that these anaplastic oligodendroglioma cases also had strong PDGFRα expression, as genomic alterations in *EGFR* and *PDGFRA* tend to be mutually exclusive in pediatric glioblastomas (41). Intriguingly, in a mouse model genetically engineered to express both EGFRvIII and Ras under a GFAP promoter, GFAP-V(12)Ha-ras;GFAP-EGFRvIII, but not GFAP-V(12)Ha-ras;GFAP-EGFR(wt) double transgenic mice, had decreased survival and developed oligodendrogliomas and mixed oligoastrocytoma tumors, instead of the fibrillary astrocytomas observed in GFAP-V(12)Ha-ras mice (6). The role of EGFR, and specifically the vIII deletion mutant, remains to be clarified in anaplastic oligodendroglioma, and assessing the translational significance in children is hampered by a lack of cell line models.

Activating mutations in the kinase domain of *EGFR* predict for sensitivity to small molecular inhibitors in tumors such as non–small cell lung cancer; however, these have not been reported in adult glioblastomas, and their absence from our pediatric series was not unexpected. By contrast, the identification of oncogenic point mutations in the extracellular domains in adult glioblastoma, and the resultant efficacy of receptor-targeting strategies, led to the expectation that we may also observe these base changes in childhood cases. The lack of such mutations once again serves to highlight the potential biological differences between gliomas arising at different ages.

Expression of the EGFRvIII mutant in association with wild-type PTEN has been reported to predict for sensitivity to small-molecule EGFR inhibitors, such as erlotinib, in the clinic (15), although in some model systems constitutive EGFR activation did not render the glioblastoma cells sensitive. We sought to clarify the situation in the childhood disease by screening a series of well-characterised[11] glioma cell lines derived from pediatric patients for *in vitro* sensitivity to erlotinib. These lines, consisting of both *PTEN* null and wild-type, all expressed EGFR at only low levels and were relatively insensitive to small-molecule inhibition by erlotinib. Transduction of the *PTEN*-expressing pediatric glioblastoma cell line SF188 with EGFRvIII did not greatly enhance the effects of erlotinib on cell viability nor downstream signaling.

EGFRvIII and the wild-type receptor have recently been shown to differentially activate downstream pathways, suggesting that therapeutic approaches toward tumors expressing these two distinct receptors should be fundamentally different (38). The expectation that targeting the MAPK pathway in wild-type EGFR, and the PI3K pathway in EGFRvIII-expressing cells, was not borne out by our data with engineered SF188 or U87MG cells, although in part this may be explained by the significant transactivation of the wild-type receptor in cells transduced with the mutant.

Previous reports have shown that coexpression of EGFRvIII and PTEN in U87MG cells rendered them highly susceptible to growth arrest by erlotinib compared with controls and U87 cells transfected with *EGFR* wild-type and *EGFRvIII* alone (15). In common with a recent study using a panel of serially propagated glioblastoma xenografts (20), it seems from our experiments with pediatric SF188 cells that the combination of PTEN expression with *EGFRvIII* mutation may be insufficient to confer obligate sensitivity to the small-molecule inhibitor.

The above data strongly suggest the presence of additional determinants of sensitivity to EGFR inhibitors in SF188 cells and possibly to pediatric HGGs in the clinic. The recent discovery of activating mutations in PI3K family member genes, such as *PIK3CA* and *PIK3R1*, in malignant glioma reiterates the importance of multiple mechanisms to increase signaling through this pathway (3, 42). SF188 harbors amplification at chromosome 12q14 of several genes, including *CDK4* and *CENTG1*, also known as the PI3K enhancer *PIKE-A* (43), a ubiquitously expressed GTPase, which binds to and enhances Akt kinase activity in a guanine nucleotide–dependent manner (44). Additional genomic aberrations in SF188 include amplification of *MYC* at 8q24,[11] which can be stabilized by PI3K (45) and provides an additional attenuation of this pathway in SF188 cells. Both amplification events have been reported in pediatric glioblastoma patient samples (43, 46).

Coactivation of RTKs, and the concept of "kinase switching," is an emerging concept that may additionally explain the lack of efficacy of agents targeting activated RTKs, such as EGFR. In adult glioblastoma, EGFR and MET seem frequently to be coactivated, with *in vitro* data supporting cotargeting both receptors (39). Furthermore, in adult glioblastoma models, the MET receptor seems strongly phosphorylated as a function of EGFRvIII levels. This has been hypothesized to be a result of an intermediary signaling component such as Src, as has been postulated for human bladder carcinoma (38). In the pediatric SF188 cells, we observed a significant activation of the closely related PDGFRs PDGFRα and PDGFRβ on transduction with EGFRvIII (and to a lesser extent with the wild-type receptor). We have previously reported high levels of Src family kinase expression in pediatric glioma cell lines, and SF188 in particular,[11] and the role of these key regulators of signal transduction in the transactivation and/or switching between constitutively activated EGFRvIII and PDGFRα/β warrants further investigation.

The identification of *EGFRvIII* deletions in pediatric HGGs and the possible resistance mechanisms to EGFR inhibitors conferred by known oncogenic amplifications have significant translational potential. In particular, PDGFRs are overexpressed at high frequency in pediatric glioblastomas, and specifically with three of six EGFRvIII-positive cases in this study, and this may be due to a differential, amplification-independent mechanism of action in anaplastic oligodendrogliomas. Coupled with evidence of enhanced efficacy from our *in vitro* model systems, these data provide a strong rationale for cotargeting with small-molecule inhibitors of both RTKs, potentially overcoming resistance to single agents in appropriate patient cohorts (47).

Disclosure of Potential Conflicts of Interest

P. Workman is employed by, has received a commercial research grant from, has an ownership interest in, and is a consultant for Piramed Pharma.

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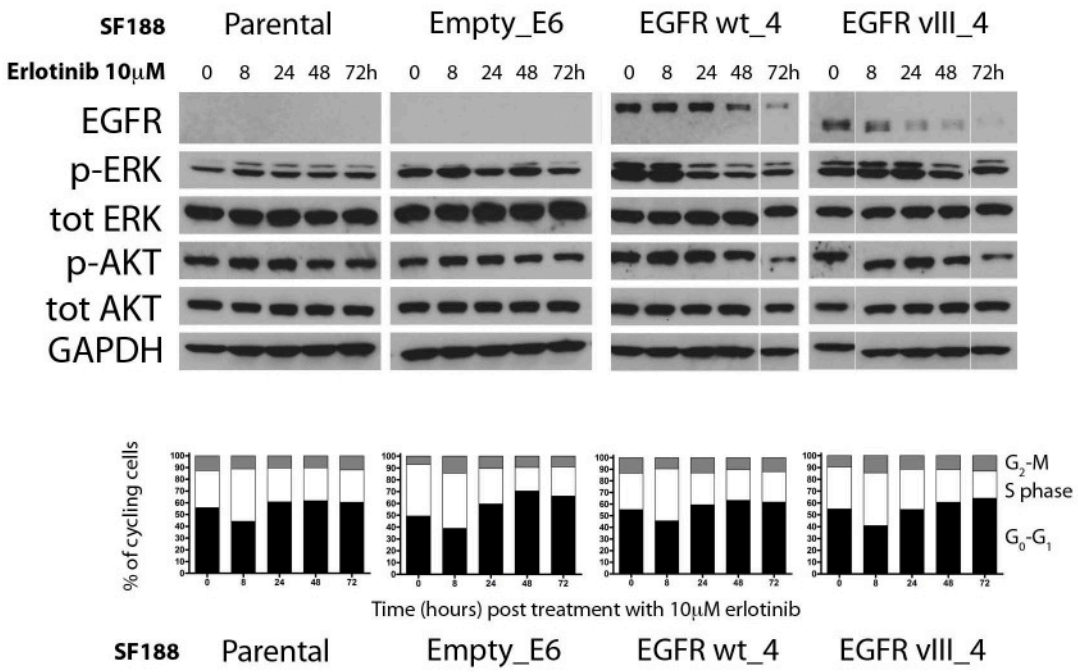
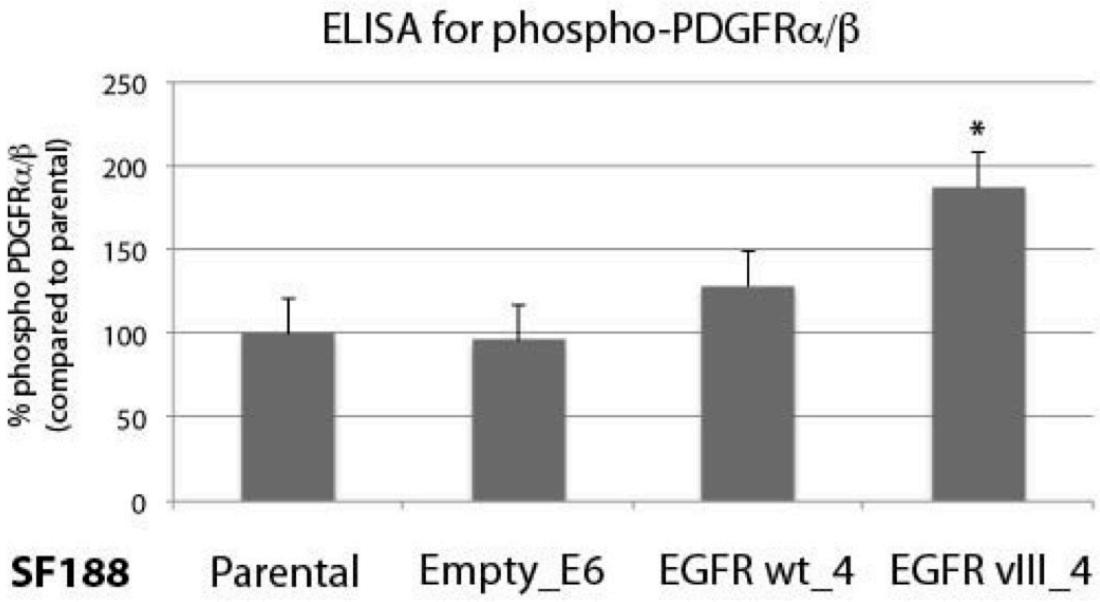
Supplementary Figure S1



Supplementary Figure S2



4. Microsatellite Instability in High-grade Gliomas and Medulloblastomas of Adult and Paediatric Patients

The results presented throughout this chapter were:

(i) Published as original articles in international peer reviewed journals:

Sub-Chapter 4.1: Marta Viana-Pereira, Inês Almeida, Sónia Sousa, Bethânia Mahler-Araújo, Raquel Seruca, José Pimentel and Rui M Reis. Analysis of Microsatellite Instability in Medulloblastoma. *Neuro-oncology* 11(5):458-67, 2009.

(ii) Submitted for publication as an original article to an international peer reviewed journal:

Sub-Chapter 4.2: Marta Viana-Pereira, Alicia Lee, Sergey Popov, Dorine A Bax, Safa Al-Sarraj, Leslie R Bridges, João N Stávale, Darren Hargrave, Chris Jones and Rui M Reis. Microsatellite Instability in Pediatric High Grade Glioma is Associated with Genomic Profile and Differential Target Gene Inactivation, 2010.

4.1 Analysis of Microsatellite Instability in Medulloblastoma

NEURO-ONCOLOGY

Analysis of microsatellite instability in medulloblastoma

Marta Viana-Pereira, Inês Almeida, Sónia Sousa, Bethânia Mahler-Araújo, Raquel Seruca, José Pimentel, and Rui Manuel Reis

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal (M.V.-P., I.A., R.M.R.); Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal (S.S., R.S.); Histopathology Department, Addenbrooke's Hospital, University of Cambridge, Cambridge, United Kingdom (B.M.-A.); Laboratory of Neuropathology, Hospital de Santa Maria, Institute of Molecular Medicine, Lisbon, Portugal (J.P.)

Medulloblastoma is the most common malignant brain tumor in children. The presence of microsatellite instability (MSI) in brain tumors, particularly medulloblastomas, has not been properly addressed. The aim of the present study was to evaluate the role of MSI in medulloblastoma carcinogenesis. MSI status was determined in 36 patients using a pentaplex PCR of quasimonomorphic markers (NR27, NR21, NR24, BAT25, and BAT26). Methylation status of mismatch repair (MMR) genes was achieved by methylation-specific multiplex ligation-dependent probe amplification (MLPA). In addition, MutS homolog 6 (MSH6) expression was determined by immunohistochemistry. Mutations of 10 MSI target genes (*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*) were studied by pentaplex PCR followed by analysis with GeneScan 3.7 software. Mutation analysis of hotspot regions of *β-catenin* (*CTNNB1*) and *BRAF* (*v-raf murine sarcoma viral oncogene homolog B1*) oncogenes was performed by PCR single-strand conformation polymorphism analysis followed by direct sequencing. Among the 36 tumors, we found four (11%) cases with instability, one with high MSI and three with low MSI. Methylation analysis of MMR genes in cases presenting shifts on the MSI markers revealed mild hypermethylation of *MSH6* in 75% of cases, yet MSH6 was expressed in all the tumors. The MSI target genes *MBD4* (*methyl-CpG binding domain protein 4*) and *MRE11* (*meiotic recombination 11 homolog A*) were mutated in two different tumors. No *CTNNB1* or *BRAF* mutations were found. This study is the most comprehensive analysis of MSI in medulloblastomas to date. We observed the presence of MSI together with mutations of MSI target genes in a small fraction of cases, suggesting a new genetic pathway for a role in medulloblastoma development. *Neuro-Oncology 11, 458–467, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00196, January 29, 2009. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-115)*

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Brain tumors are the second most common malignancy among children and the leading cause of cancer-related death in pediatric patients. The overall incidence rate for pediatric brain tumors (occurring from birth through 19 years) is 4.3 per 100,000 person-years.[1] Among the different histological entities, medulloblastoma is the most common childhood malignant brain tumor, accounting for approximately 20% of all pediatric intracranial tumors, with a peak incidence between 3 and 4 years of age.[1] Medulloblastomas are less common in adults, with a peak incidence between 20 and 35 years.[2] Current therapy for this malignancy is very aggressive, including maximum surgical resection, craniospinal radiotherapy, and adjuvant chemo-

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Address correspondence to Rui Manuel Reis, Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal (rreis@ecsaude.uminho.pt).

therapy, yet the medulloblastoma 5-year survival rate is only 50%–60%,[3,4] and these aggressive procedures frequently have serious neurocognitive and endocrine sequelae in survivors, particularly in younger patients.[5]

Genetic instability is a paramount feature of cancer, which leads to accumulation of genetic alterations that varies from subtle changes in DNA sequence to chromosomal abnormalities.[6] Microsatellite instability (MSI) is a particular type of genetic instability affecting short sequences of DNA repeats (microsatellites) found throughout the genome.[6] MSI was first described in hereditary nonpolyposis colorectal cancer (HNPCC) and is present in the majority of these patients; currently, MSI analysis of this malignancy is standardized by the Bethesda guidelines.[7] In colorectal cancer (CRC), the MSI phenotype appears to be related to particular clinical and histopathological features, including location in the proximal colon, tumors poorly differentiated with mucinous and signet ring cells, high tumor lymphocyte infiltration, low frequency of distant metastasis, and a comparably good prognosis.[8] The MSI phenotype is a consequence of deficient DNA mismatch repair (MMR), which fails to recognize errors introduced in microsatellite regions during DNA replication. The loss of function of MMR family genes (*MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, *PMS1*, and *PMS2*) is caused by germline mutations in hereditary malignancies, whereas in sporadic cancers, *MLH1* (*mutL homolog 1, colon cancer, nonpolyposis type 2*) promoter methylation has been shown to be the main cause of gene silencing.[9] As naturally occurring replication errors are not efficiently repaired, tumors with MMR deficiencies have a higher number of nucleotide insertions/deletions in genes harboring microsatellites.[6,9] The accumulation of activating or inactivating frameshift mutations in genes that regulate cell functioning, such as *TGFBR2* (*transforming growth factor β type II receptor*) and *BAX* (*BCL2-associated X protein*), is thought to be responsible for the tumorigenic process of MSI in MMR-deficient cells.[9] Particularly important to oncological research is the evidence that many of these mutated genes, already identified in different tumors, also appear to have a role in the therapeutic response of different anticancer drugs.[10–12]

Previous studies have evaluated the presence of MSI in brain tumors, mainly gliomas. An absence or a rare incidence of MSI in adults and contradictory results in pediatric patients have been reported.[13–21] In medulloblastoma, MSI status has not been properly characterized.

The aim of the present study was to evaluate the presence of MSI in medulloblastomas, using a panel of markers recommended by the revised Bethesda guidelines.[7] In addition, in tumors presenting MSI, we assessed the molecular status of MMR genes and the mutation profiles of 10 potential MSI target genes (*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*). Furthermore, we analyzed mutations of *BRAF* (*v-raf murine sarcoma viral oncogene homolog B1*) and *β-catenin* (*CTNNB1*).

Material and Methods

Patients and Tumor Samples

Formalin-fixed, paraffin-embedded samples from 36 cases of medulloblastoma were retrieved from the Pathology Department of Santa Maria Hospital, Lisbon, Portugal. Tumor samples were classified according to WHO criteria.[22] Thirty-four of the 36 cases were classic medulloblastomas, and the remaining two were classified as desmoplastic medulloblastomas. Of the patients, 22 (61.1%) were male and 14 (38.9%) were female; the mean age was 19.5 years (range, 1.5–70 years; Table 1).

DNA Extraction

DNA was extracted from 10-μm-thick formalin-fixed, paraffin-embedded tumor samples as previously described.[23] Briefly, tissues were deparaffinized by a serial extraction with xylene and ethanol (100%/70%/50%), and separately selected areas of tumor and normal tissue, when available, were microdissected using a sterile needle and carefully collected into a 0.2-mL PCR tube. DNA was extracted using QIAamp DNA Micro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

MSI Analysis

The MSI evaluation was performed using a multiplex PCR comprising five quasimonomorphic mononucleotide repeat markers (NR27, NR21, NR24, BAT25, and BAT26).[24] Primer sequences were described previously.[24] Each antisense primer was end-labeled with 6-carboxyfluorescein (FAM), hexachloro-6-carboxyfluorescein (HEX), or tetrachloro-6-carboxyfluorescein (TET) fluorescent marker. PCR was performed using the Qiagen Multiplex PCR Kit, and then products were separated using an ABI Prism 310 single capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA). The MSI status of the tumor was analyzed using GeneScan analysis software (version 3.7; Applied Biosystems). Cases exhibiting instability at two or more markers were considered to have high MSI (MSI-H), those with instability at one marker were defined as having low MSI (MSI-L), and those showing no instability were defined as microsatellite stable (MSS), as previously described.[25] DNA from the cell lines HCT15 (MSI) and DAOY (MSS) were used as positive and negative controls, respectively. The quasimonomorphic variation range of each marker (described by Buhard et al.[26]) was established in our analysis using a series of DNA from six healthy people.

Mutation Analysis of MSI Target Genes

Selected genes containing repeated sequences previously described as frequent targets for instability were chosen for frameshift mutation study by fragment analysis and further genomic sequencing confirmation. The selected

Table 1. Detection of microsatellite instability (MSI) in medulloblastomas using five mononucleotide repeat markers

Case	Gender/Age (Years)	Histological Type	MSI Detection					
			NR27	NR21	NR24	BAT25	BAT26	MSI State
M1	M/11	Classic	86	106	123	148	179	MSS
M2	F/16	Classic	86	107	123	148	180	MSS
M3	M/35	Classic	87	107	122	148	180	MSS
M4	M/33	Classic	86	107	123	148	180	MSS
M5	M/1.5	Classic	86	**102–107**	122	148	179	MSS
M6	M/10	Classic	86	107	123	147	180	MSS
M7	F/70	Classic	86	**102–106**	123	147	180	MSS
M8	M/20	Classic	87	106	122	148	180	MSS
M9	F/26	Classic	86	**102–107**	123	147	180	MSS
M10	F/10	Classic	86	106	123	147	180	MSS
M11	F/1.5	Classic	87	106	123	148	180	MSS
M12	F/1.5	Classic	87	106	123	148	180	MSS
M13	M/21	Desmoplastic	**84**	105	122	146	**168**	**MSI-H**
M14	M/11	Classic	86	107	122	148	179	MSS
M15	M/28	Classic	87	107	123	146	179	MSS
M16	M/34	Classic	86	107	122	148	179	MSS
M17	M/6	Classic	86	106	123	148	180	MSS
M18	F/24	Classic	86	106	122	147	180	MSS
M19	F/34	Classic	87	106	123	148	179	MSS
M20	M/12	Classic	86	106	123	147	180	MSS
M21	M/10	Classic	86	105	122	**147–150**	179	**MSI-L**
M22	F/16	Classic	86	106	123	148	180	MSS
M23	M/8	Classic	86	107	123	148	NA	MSS
M24	M/7	Classic	87	106	123	148	180	MSS
M25	M/35	Classic	87	107	123	**144–148**	179	**MSI-L**
M26	M/2	Classic	86	106	123	147	180	MSS
M27	M/65	Classic	86	107	122	148	180	MSS
M28	M/39	Classic	86	107	123	147	180	MSS
M29	F/8	Classic	87	107	122	147	179	MSS
M30	F/26	Classic	86	106	122	148	179	MSS
M31	F/2	Classic	86	107	122	146	180	MSS
M32	M/33	Classic	86	106	122	147	180	MSS
M33	M/4	Classic	86	107	123	148	180	MSS
M34	F/9	Classic	86	107	123	147	180	MSS
M35	M/13	Classic	**84**	106	122	146	179	**MSI-L**
M36	F/18	Desmoplastic	86	106	123	148	180	MSS

Abbreviations: M, male; MSS, microsatellite stability; F, female; MSI-H, high microsatellite instability; MSI-L, low microsatellite instability; NA, not amplified. Boldface indicates MSI markers presenting alterations.

genes were *transcription factor-4* (*TCF4*; poly[A]9), *X-ray repair cross-complementing protein 2* (*XRCC2*; T8), *methyl-CpG binding domain protein 4* (*MBD4*; A10), *meiotic recombination 11 homolog A* (*MRE11*; T11), *ataxia telangiectasia and Rad3 related checkpoint kinase 1* (*ATR*; A10), *MSH3* (A8), *TGFBR2* (A10), *RAD50 homolog* (*RAD50*; A9), *MSH6* (C8), and *BAX* (G8).[27,28] PCR was performed with primers, end-labeled with FAM, HEX, or TET fluorescent markers, specific for each selected candidate gene, as previously described.[27,28] PCR products were separated using an ABI Prism 310 single capillary genetic analyzer (Applied Biosystems), and the PCR products profiles were analyzed using GeneScan 3.7 software (Applied Biosystems). Several normal DNA samples were used to establish profile patterns for each gene, and mutation analysis was performed comparing the peak pattern alterations with the reference peak size and pattern.[27,28] Analyses of samples presenting abnormal profiles were repeated three times by multiplex and monoplex PCR. In addition, PCR followed by direct sequencing was performed to confirm the presence of a frameshift mutation.

Mutation Analysis of CTNNB1 and BRAF Oncogenes

Screening of hotspot mutations on *CTNNB1* exon 3 and *BRAF* exon 15 was carried out by PCR single-strand conformation polymorphism as previously described.[29–31]

Methylation Analysis of the MMR System Genes

The study of *MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, and *PMS2* (*PMS1 postmeiotic segregation increased 2*) MMR gene methylation was performed by methylation-specific MLPA kit ME011 according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands).[32] Briefly, 100 ng tumoral DNA was denaturated in 5 µl ultrapure water at 98°C for 5 min and then incubated with the probe mix for 20 h at 60°C. After probe hybridization, each sample was divided into two tubes. Half of the sample was ligated using a ligase enzyme, and in the other half ligation was combined with HhaI digestion, resulting in ligation of the methylated sequences only. The resulting products were amplified by PCR using a FAM-labeled primer following manufacturer's instructions. PCR products were analyzed on an ABI Prism 310 single capillary genetic analyzer (Applied Biosystems) using GeneScan 3.7 software (Applied Biosystems). Duplicate experiments were performed for methylation analysis, and average ratios were calculated. Additionally, the overall average of the different probes of the same gene was calculated. Data analysis was performed as described by the manufacturer. We interpreted (average) ratios as absence of hypermethylation (0.00–0.24), mild hypermethylation (0.25–0.49), moderate hypermethylation (0.50–0.74), and extensive hypermethylation (≥0.75), as previously described.[33]

Immunohistochemistry to the MSH6 MMR System Protein

Immunohistochemistry analysis of MSH6 protein was performed using 3-µm paraffin-embedded tissue sections. Tissue sections were deparaffined, rehydrated in graded ethanol, and washed. Antigen retrieval was achieved by microwave treatment in 1 mM EDTA (pH 8.0) for 15 min. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2. To block nonspecific protein binding, sections were incubated with R.T.U. normal horse serum (R.T.U. vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). A primary antibody to MSH6 (clone 44, purified mouse anti-MSH6; BD Transduction Laboratories, BD Biosciences, Erembodegem, Belgium) was applied at a concentration of 1:100 and incubated overnight at room temperature. Antigen–antibody complexes were revealed by a 10-min incubation with R.T.U. biotinylated universal antibody antirabbit/mouse IgG (H + L) (R.T.U. vectastain Elite ABC kit; Vector Laboratories) followed by incubation with R.T.U. vectastain Elite ABC reagent (R.T.U. vectastain Elite ABC kit; Vector Laboratories) for 10 min 3,3/-Diaminobenzidine (Dako Liquid DAB, DakoCytomation, VitaReal, Carpinteria, CA, USA) was used as a chromogen. Slides were counterstained with hematoxylin. Normal colon tissue was used as a positive control. A negative control was also used (DakoCytomation N-Universal Negative Control Mouse, DakoCytomation).

Results

MSI Analysis

MSI analysis was performed for 36 tumors using a pentaplex PCR of quasimonomorphic markers recommended by the revised Bethesda guidelines.[7] Among all samples, we found four (11.1%) cases with instability—one with MSI-H and three with MSI-L—and 32 MSS (88.9%) (Table 1, Fig. 1). Regarding adult and pediatric patients, we found 13% (2 of 15) MSI tumors (M13, M25) in the adult set, one of which was MSI-H (M13). In pediatric samples, two cases (9.5%) were MSI-L (M21, M35). Case M13 presented mutations in NR27 and BAT26 markers, cases M21 and M25 presented alterations in BAT25, and M35 presented alterations in NR27. Three cases (M5, M7, and M9) presented allele variants in NR21. Case M13 had adjacent normal DNA available, which did not exhibit the alterations present in the tumor DNA (Fig. 1). In addition, the MSI status of both tumor and normal DNA was confirmed by direct sequencing.

MMR Gene Methylation

Promoter abnormal methylation of MMR genes is the main mechanism underlying MSI phenotype in sporadic tumors. Therefore, we analyzed methylation of the main

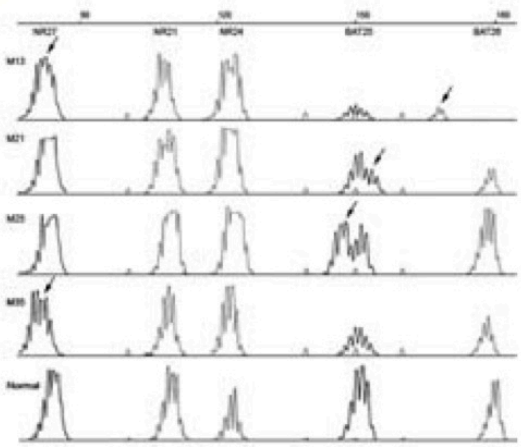

Fig. 1. Altered markers in the microsatellite instability cases: pentaplex PCR with the microsatellite quasimonomorphic markers (NR27, NR21, NR24, BAT25, and BAT26). Case M13 presented mutation in NR27 and BAT26 microsatellite markers, cases M21 and M25 were mutated in the BAT25 marker, and M35 in NR27 (altered markers indicated with arrows).

Table 2. Methylation status of mismatch repair (MMR) genes in medulloblastomas

		MMR Gene Methylation (Average)					
Case	MSI Status	MLH1	MLH3	MSH2	MSH3	MSH6	PMS2
M5	MSS	U (0.15)	U (0.05)	U (0.08)	U (0.17)	U (0.19)	U (0.10)
M7	MSS	U (0.05)	U (0.02)	U (0.07)	U (0.19)	U (0.16)	U (0.11)
M9	MSS	U (0.11)	U (0.00)	U (0.05)	U (0.17)	**M (0.27)**	U (0.09)
M13	MSI	U (0.15)	U (0.06)	U (0.11)	U (0.20)	**M (0.29)**	U (0.13)
M21	MSI	U (0.11)	U (0.18)	U (0.06)	U (0.23)	**M (0.26)**	U (0.11)
M25	MSI	U (0.10)	U (0.00)	U (0.05)	U (0.15)	U (0.23)	U (0.07)
M35	MSI	U (0.02)	U (0.08)	U (0.08)	U (0.00)	U (0.12)	U (0.07)

Abbreviations: MSI, microsatellite instability; *MLH1 and MLH3, mutL homolog 1, colon cancer, nonpolyposis, types 2* and *3*; *MSH2*, *MSH3*, and *MSH6*, *MutS homologs 2*, *3*, and *6*; *PMS2*, *PMS1 postmeiotic segregation increased 2*; MSS, microsatellite stability; U, unmethylated; M, methylated. Boldface indicates the presence of gene methylation.

MMR genes (*MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, and *PMS2*) in the MSI-H tumor (M13), in three tumors presenting MSI-L (M21, M25, and M35), and in the three additional cases presenting an allele variant in the NR21 marker (Table 2). Except for *MSH6*, none of the other MMR genes showed promoter gene hypermethylation. *MSH6* presented mild hypermethylation in 43% (three of seven) of the cases analyzed: in two MSI (M13 and M21) and one MSS (M9).

Immunohistochemistry of the MSH6 MMR System Protein

The immunohistochemical assay was performed to complement the methylation study of *MSH6* and to understand the effects of mild gene hypermethylation on protein expression levels. All cases exhibited MSH6 positivity, but cases M5 and M25 showed weaker staining (Fig. 2).

Mutation Analysis of MSI Target Genes

Selected MSI target genes (*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*) were analyzed for frameshift mutations in the MSI tumors and in the cases presenting an allele variant in the NR21 marker (Table 3). Among the genes studied, *MBD4* and *MRE11* have been shown to be mutated in one MSI sample each (1 of 4 = 25%) in M13 (MSI-H) and M21 (MSI-L). Both *MBD4* and *MRE11* had a heterozygous insertion of one base pair. Therefore, two of the four tumors with instability presented mutation in one MSI target gene. The presence of frameshift mutations was confirmed by direct sequencing and demonstrated to be heterozygous by both techniques (Figs. 3 and 4).

Mutation Analysis of CTNNB1 *and* BRAF *Oncogenes*

Because the Wingless/Wnt signal transduction pathway is involved in medulloblastoma development, we searched for mutations in its critical downstream effector *CTNNB1*. All cases were analyzed for mutations in exon 3. No medulloblastoma showed any *CTNNB1* genetic alteration.

BRAF mutations, particularly the V600E hotspot mutation, have been described to be involved in colorectal carcinomas exhibiting MSI. None of the MSI medulloblastomas exhibited *BRAF* exon 15 mutations.

Discussion

MSI was first identified in and is present in about 90% of HNPCC cases.[34] This phenotype has also been described in many sporadic human malignancies and is present in approximately 10%–15% of colorectal, endometrial, and gastric cancers.[34] The few studies reporting MSI status in brain tumors showed that this phenotype is a rare event (0%–8%) in adult sporadic CNS tumors.[13–17,19] Regarding pediatric data, the results are contradictory: MSI was found in 0%–27% of CNS tumors studied.[13–16,18–21] In the present study, we screened 21 pediatric and 15 adult medulloblastomas for MSI. The overall incidence of instability was 11% (4 of 36 cases), with three cases showing MSI-L and a single case with MSI-H, from two adults and two pediatric patients. According to these results, the presence of MSI in medulloblastomas appears not to be age-related, in contrast to data from other CNS tumors. The few studies that analyzed MSI status in medulloblastomas reported the absence of genetic instability.[15,21,35] However, these studies evaluated a very small number of cases, some not differentiating medulloblastomas from other primitive neuroectodermal tumors,[15,21] using panels of microsatellite markers that were limited for MSI status assessment.[16,17,21] In this work, we used a gold standard panel of microsatellite markers recommended by the revised Bethesda guidelines for CRC.[7] This panel of mononucleotide markers provides high specificity and sensitivity for MSI detection, and their quasimonomorphic nature allows the analysis of MSI status without the need to evaluate corresponding normal tissue.[24]

The main mechanism driving MSI in sporadic tumors has been shown to be methylation of MMR genes. We analyzed the methylation of the *MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, and *PMS2* MMR genes in tumors with MSI and in those presenting an allelic variant of the NR21 marker but considered MSS, and found mild

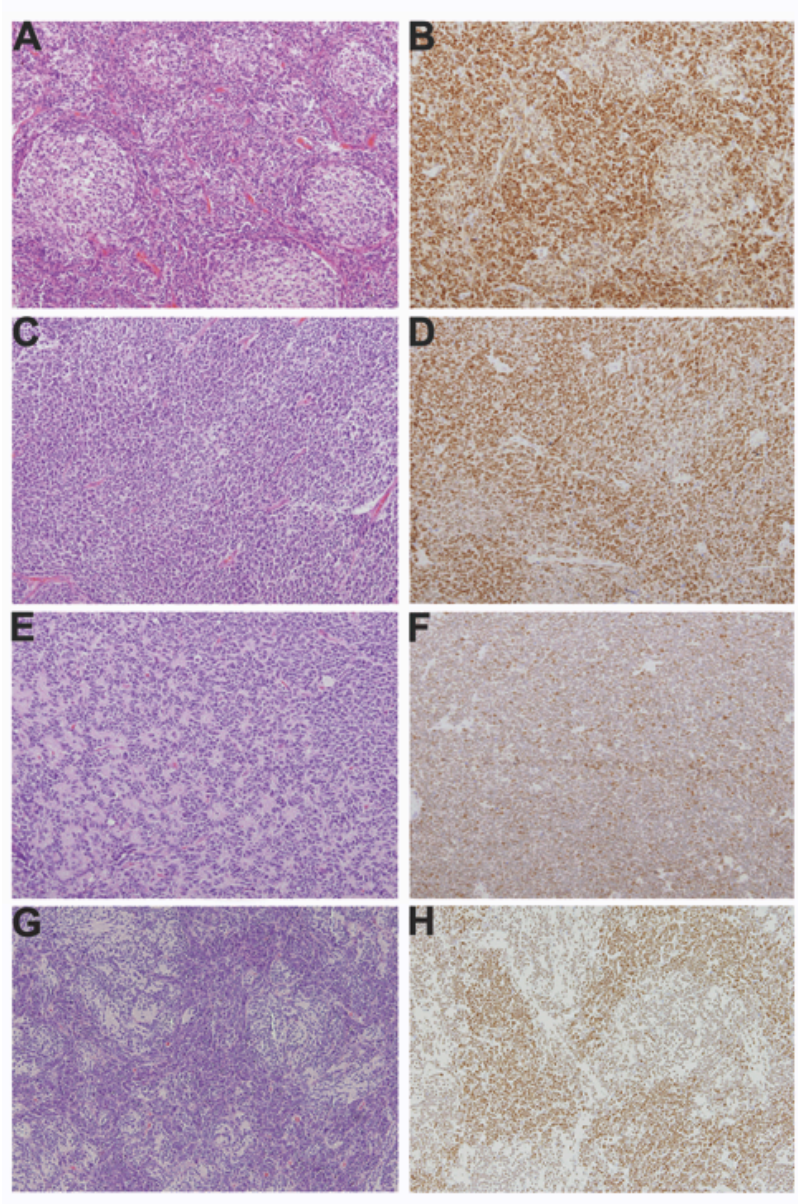

Fig. 2. Representative histology and immunohistochemistry results: hematoxylin and eosin (A, C, E, and G) and MutS homolog 6 (MSH6) immunohistochemistry (B, D, F, and H) for the microsatellite-instability–positive medulloblastomas M13 (A and B), M21 (C and D), M25 (E and F), and M35 (G and H). Original magnification: ×100.

hypermethylation levels of the *MSH6* gene in two cases of MSI (M13 and M21) and one case of MSS (M9). Nevertheless, the presence of mild hypermethylation was not transduced in a lack of protein expression and might not be the cause of the MSI phenotype. It remains to be determined in MSI-positive medulloblastomas which MMR protein is affected and by which mechanism. The presence of MMR deficiencies is well correlated with the MSI status in several tumors, such as CRC,[36] ovarian carcinoma,[37,38] and endometrial carcinoma,[38,39] where MSI-H tumors present inactivation of MMR proteins whereas the genes leading to MSI-L are unclear.[7,40] However, in other tumor entities such as Ewing tumors, such associations were not reported.[41] This suggests that MMR protein deficiencies in MSI-positive tumors depend not only on MSI levels but also on the tumor

Table 3. Candidate microsatellite instability target gene frameshift mutations detected in medulloblastomas

Case	TCF4 (A9)	XRCC2 (T8)	MBD4 (A10)	MRE11 (T11)	ATR (A10)	MSH3 (A8)	TGFBR2 (A10)	RAD50 (A9)	MSH6 (C8)	BAX (G8)	Mutation Frequency
M5	A	A	A	A	A	A	A	A	A	A	0 of 10
M7	A	A	A	A	A	A	A	A	A	A	0 of 10
M9	A	A	A	A	A	A	A	A	A	A	0 of 10
M13	A	A	A	**P** (T11–12)	A	A	A	A	A	A	1 of 10
M21	A	A	**P** (A10–11)	A	A	A	A	A	A	A	1 of 10
M25	A	A	A	A	A	A	A	A	A	A	0 of 10
M35	A	A	A	A	A	A	A	A	A	A	0 of 10
Tumors with mutation	0 of 7	0 of 7	1 of 7	1 of 7	0 of 7	0 of 7	0 of 7	0 of 7	0 of 7	0 of 7	

Abbreviations: *TCF4, transcription factor-4*; *XRCC2, x-ray repair cross-complementing protein 2*; *MBD4, methyl-CpG binding domain protein 4*; *MRE11, meiotic recombination 11 homolog A*; *ATR, ataxia telangiectasia and Rad3 related checkpoint kinase 1*; *MSH3, MutS homolog 3*; *TGFBR2, transforming growth factor β type II receptor*; *RAD50, RAD50 homolog*; *MSH6, MutS homolog 6*; *BAX, BCL2-associated X protein*; A, absent; P, present.

type. Data on MMR gene alterations are scarce in CNS tumors. In medulloblastoma, only one study has examined MMR protein expression.[42] The authors reported the absence of any deficiency in MLH1, MSH2, and PMS2 proteins in a series of 22 medulloblastomas.[42]

In order to evaluate the mutagenic effect of the MSI phenotype in medulloblastomas, we performed a mutation analysis of candidate MSI target genes. We studied 10 candidate genes—*TCF4*, *XRCC2*, *MBD4*, *MRE11*, *ATR*, *MSH3*, *TGFBR2*, *RAD50*, *MSH6*, and *BAX*—previously described to be frequently mutated in MSI tumors such as colorectal, urothelial, or endometrium cancers.[27,28,43] Most of the candidate target gene mutations were primarily found and mainly analyzed in MSI-H CRC. Although several of these mutations have already been reported in different MSI-H tumors, this is not true for all different MSI cancers. We found alterations in *MBD4* and *MRE11* genes in two of the four MSI

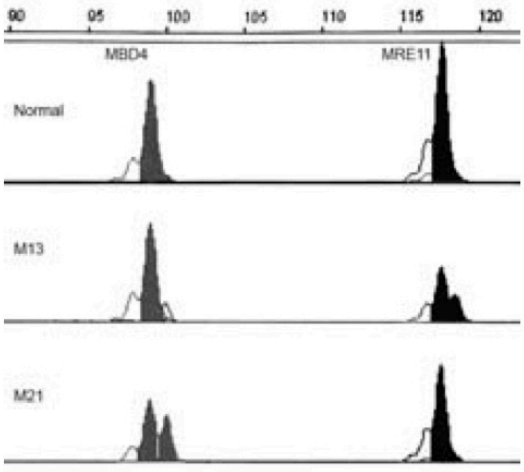

Fig. 3. Microsatellite instability target gene mutations: fragment analysis of *methyl-CpG binding domain protein 4* (*MBD4*) and *meiotic recombination 11 homolog A* (*MRE11*) for normal DNA from a healthy person, and mutated fragments by insertion of one nucleotide in MRE11 in case M13 and in MBD4 in case M21.

medulloblastomas, one with MSI-H and one with MSI-L. Overall, among the 10 candidate target genes studied, this represents 20% of genes mutated. This rate of mutation in candidate target genes, although smaller than what is regularly stated for CRC, is comparable to frequencies reported for other tumor entities such as pancreatic ductal adenocarcinomas, which presents 25% mutated genes,[44] and it is higher than the incidence reported in other studies,[45] indicating that different tumors can present mutations in different targets for instability.[46,47] In addition, as mentioned above, mutations in candidate target genes were mainly reported only in MSI-H tumors, in different tumor entities.[44,48] Among these different cancers, MSI-L malignancies generally do not present mutations in the candidate target genes frequently mutated in MSI-H tumors, but our results raise the question of whether this is true for all MSI-L tumors.

MBD4 is a member of the methyl-CpG binding protein family, which possesses a methyl-CpG binding domain (MBD) and a glycosylase repair domain, repairing mismatched G-T residues at methylated CpG sites.[49] Previous studies in colorectal, endometrial, and gastric tumors reported that truncating mutations of *MBD4*, due to the deletion of one nucleotide in the A10 tract of exon 3, result in proteins without the glycosylase repair domain and therefore with defective glycosylase activity.[49–51] In addition, MBD4 truncated protein had the capacity to compete with wild-type protein in a dominant negative manner, causing the accumulation of errors in the DNA.[51] In our study we observed not a deletion but a nucleotide insertion at the A10 tract of the *MBD4* gene. Similar to deletion, insertion of a nucleotide in the A10 microsatellite region is also suggested to result in a truncated and defective protein.[50]

MRE11 is a member of the MRE11/NBS1/RAD50 (MNR) complex, which is essential for the maintenance of DNA integrity. This complex plays a central role in recognizing and repairing double-strand breaks through homologous recombination or nonhomologous end-joining repair pathways. It was previously suggested that homo- or heterozygous deletions in the poly(T)11 within *MRE11* intron 4 cause aberrant splicing, with skipping of exon 5, leading to a premature stop codon and generation of a truncated protein.[52] In this study,

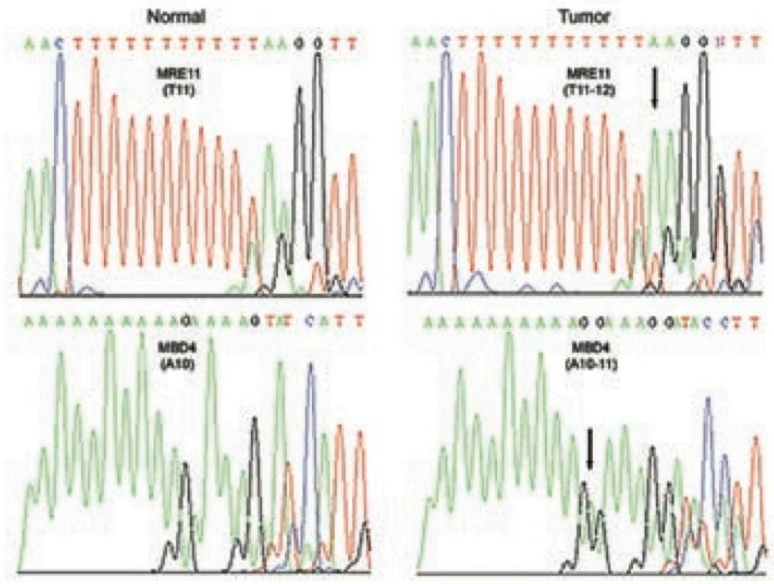

Fig. 4. DNA sequencing of the microsatellite instability target genes *meiotic recombination 11 homolog A* (*MRE11*; T11) and *methyl-CpG binding domain protein 4* (*MBD4*; A10): normal DNA from a healthy person for the *MRE11* and *MBD4* genes (left) and the mutated cases M13 for *MRE11* and M21 for *MBD4* (right). Arrows indicate the insertion of one nucleotide.

we observed a heterozygous insertion in *MRE11* T11 in case M13. This allele expansion was also identified in a CRC tumor sample of a Lynch syndrome patient, and can also result in aberrant splicing signals and premature stop codons of *MRE11*.[52] Nevertheless, the exact functional role of the poly(T)11–12 *MRE11* mutation is not clear, and the same mutation was also reported in a lymphoma cell line.[53]

Although the majority of medulloblastomas occur sporadically, they can occur associated with Turcot syndrome type 2,[22] and it also has been reported in young members of families with Lynch syndrome.[54–56] Interestingly, biallelic germline mutations in *MSH6*[54,55] or *MLH1*[56] were described in three different patients that developed medulloblastoma. Tumors presenting *MSH6* mutations were also found to lack protein expression,[54,55] whereas in medulloblastoma with *MLH1* mutation, protein expression was not reported.[56] Despite due diligence, it was not possible to obtain the family history of the four patients with MSI medulloblastoma to assess their potential inherited nature. Therefore, we cannot exclude the possibility that these MSI-presenting tumors arose in a familial cancer context. Aiming to unravel this question, somatic *BRAF* mutations associated with sporadic CRC MSI were screened, and no mutation was detected. However, at variance with CRC, *BRAF* mutations have never been detected in medulloblastomas, which thus does not exclude the sporadic nature of our MSI-positive medulloblastomas.

β-Catenin is a key player in the Wingless/Wnt signal transduction pathway that is involved in medulloblastomas, and mutations were reported previously in only 5%–9% of the cases.[57,58] Aiming to better characterize our samples and to determine if *CTNNB1* could be related to MSI status in medulloblastomas, we searched for mutations in its hotspot region but found no evidence. Although no *BRAF* or *CTNNB1* mutations were found in the hotspot region screens in these medulloblastomas, we cannot exclude the potential existence of mutations in other regions of the genes.

In conclusion, this study is the most comprehensive analysis of MSI in medulloblastomas to date. We found a total of four cases (11%) with instability, three with MSI-L, and one with MSI-H, two of which presented mutations in *MBD4* and *MRE11* MSI target genes, which have never before been reported in medulloblastomas. While further studies analyzing a larger series of both pediatric and adult medulloblastomas are warranted to assess the frequency of MSI, the present work suggests the existence of a potential novel molecular pathway in a fraction of medulloblastomas associated with the presence of MSI.

Acknowledgments

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4.2 Microsatellite Instability in Paediatric High Grade Glioma is Associated with Genomic Profile and Differential Target Gene Inactivation

Microsatellite Instability in Pediatric High Grade Glioma is Associated with Genomic Profile and Differential Target Gene Inactivation

Marta Viana-Pereira[1,2], Alicia Lee[2], Sergey Popov[2,3], Dorine A Bax[2], Safa Al-Sarraj[4], Leslie R Bridges[5], João N Stávale[6], Darren Hargrave[3], Chris Jones[2,3]* and Rui M Reis[1]*

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; [2]Section of Paediatric Oncology, Institute of Cancer Research, Sutton, UK; [3]Paediatric Oncology, Royal Marsden Hospital, Sutton, UK; [4]Department of Clinical Neuropathology, Kings College Hospital, London, UK; [5]Neuropathology, St George's Hospital, London, UK; [6]Department of Pathology, Federal University of São Paulo, S. Paulo, Brazil

ABSTRACT

High grade gliomas (HGG) are one of the leading causes of cancer-related deaths in children, and there is increasing evidence that pediatric HGG may harbor distinct molecular characteristics compared to adult tumors. We have sought to clarify the role of microsatellite instability (MSI) in pediatric *versus* adult HGG. MSI status was determined in 144 patients (71 pediatric and 73 adults) using a well established panel of five quasimonomorphic mononucleotide repeat markers. Expression of MLH1, MSH2, MSH6 and PMS2 was determined by immunohistochemistry, *MLH1* was assessed for mutations by direct sequencing and promoter methylation using MS-PCR. DNA copy number profiles were derived using array CGH, and mutations in eighteen MSI target genes studied by multiplex PCR and genotyping. MSI was found in 14/71 (19.7%) pediatric cases, significantly more than observed in adults (5/73, 6.8%; p=0.02, Chi-square test). MLH1 expression was downregulated in 10/13 cases, however no mutations or promoter methylation were found. MSH6 was absent in one pediatric MSI-High tumor, consistent with an inherited mismatch repair deficiency associated with germline *MSH6* mutation. MSI was classed as Type A, and associated with a remarkably stable genomic profile. Of the eighteen classic MSI target genes, we identified mutations only in *MSH6* and *DNAPKcs* and described a polymorphism in *MRE11* without apparent functional consequences in DNA double strand break detection and repair. This study thus provides evidence for a potential novel molecular pathway in a proportion of gliomas associated with the presence of MSI.

Keywords: glioblastoma; microsatellite instability; mismatch repair; MRE11; MLH1

***Corresponding authors:** Rui M Reis, School of Health Sciences, University of Minho, Campus de Gualtar, 4710-029, Braga, Portugal; Email: rreis@ecsaude.uminho.pt; Tel: (+351)253604825; Fax: (+351)253604820
Chris Jones, Section of Paediatric Oncology, Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey, SM2 5NG, UK; Email: chris.jones@icr.ac.uk; Tel: (+44)(0)2087224416; Fax: (+44)(0)2087224321

INTRODUCTION

Pediatric gliomas comprise a diverse group of lesions which account for more than half of all childhood CNS tumors. In contrast to adults, where WHO grade IV glioblastomas predominate, in children the most common form are WHO grade I pilocytic astrocytomas [1]. Despite the rarity of high grade gliomas (HGG) in children, they are one of the leading causes of cancer-related deaths in this age group, with a current two year survival rate of 10-15% [2]. Although histologically similar to those which arise in adults, HGG in children may have distinct clinical features, including anatomical site of presentation [2] and response to chemotherapy [3].

New evidence also demonstrates that pediatric HGG harbor distinct genetic characteristics compared to adult tumors. Recent large-scale genomic studies on adult glioblastomas defined the key genetic aberrations, and proposed the 'core signaling pathways' driving gliomagenesis [4,5]. Similar studies on pediatric tumors demonstrated fewer genomic events targeting these pathways, and identified alterations in PDGF-driven signaling to be prevalent in the majority of pediatric tumors, in contrast to adults, where EGFR is the predominant target [6]. The finding of a significant proportion of childhood HGG to harbor few chromosomal imbalances is one key difference to those seen in adults [6], and raises questions regarding the underlying biological basis for these highly aggressive tumors.

The presence of microsatellite instability (MSI), is another feature that has been described to be more frequent in pediatric than in adult brain tumors [7-11]. Nevertheless, the results are conflicting, with reported frequencies in pediatric gliomas varying between 0-44%, possibly due to different sensitivities of the methods used to detect MSI status [7-17]. MSI was first described and is better characterized in hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) [18], where it is thought to arise due to germline mutations in mismatch repair (MMR) genes, mainly in *MLH1*, *MSH2*, *MSH6* and *PMS2* [19]. Germline mutations in these genes have also been described in Turcot's syndrome, which predispose to gliomas [1].

Both in familial and sporadic colorectal cancer (CRC), the presence of MSI is associated with mutations in genes harboring microsatellites in their coding or regulatory regions. Genes involved in DNA repair, cell growth inhibition and apoptosis are targeted, although the frequency varies between cancers [20], and an extensive analysis has not been previously performed in MSI gliomas. We have sought to clarify the role of MSI in pediatric *versus* adult HGG, and report a higher prevalence of MSI in childhood cases. We have further investigated the role

of MMR system proteins, genomic instability and 18 known target genes in pediatric MSI HGG.

MATERIALS AND METHODS

Cases and DNA isolation

Formalin-fixed paraffin-embedded (FFPE) HGG samples from 71 children and young people (<23 years) and 73 adults (32-79 years) were obtained after approval by Local and Multicenter Ethical Review Committees from King's College Hospital and St George's Hospital, UK and Federal University of São Paulo, Brazil (Supporting Information 1). All the samples enrolled in the present study were unlinked and unidentified from their donors. Due the retrospective nature of the study, no informed consent from participants was obtained. DNA was isolated using the QIAamp DNAMini kit (Qiagen) according to the manufacturer's instructions.

MSI analysis

MSI was assessed using a multiplex PCR comprising five quasimonomorphic mononucleotide repeat markers (NR27, NR21, NR24, BAT25 and BAT26), as previously described [21]. Products were separated using an ABI Prism 3100 genetic analyzer (Applied Biosystems) and results analyzed with GeneScan Analysis software, version 3.7 (Applied Biosystems). In the absence of matched normal DNA, MSI was defined as MSI-High (MSI-H, instability at three or more markers) MSI-Low (MSI-L, instability at one or two markers) or microsatellite stable (MSS, absence of instability) [22]. The quasimonomorphic variation range (QMVR) of each marker, previously described [23] was established in our analysis using a series of DNA samples from 30 controls which were randomly selected from cancer-free blood donors at the ICR, UK and São Marcos Hospital, Portugal.

Mutation analysis of MSI-targeted genes

Selected genes containing repeat sequences, previously described as frequent targets for MSI in other cancers [24,25,26], were chosen for mutation screening (primers available upon request): *ATM* (poly(T)13), *ATR* (poly(A)10), *AXIN2* (poly(G)7; poly(C)5; poly(C)6), *BAX* (poly(G)8), *BLM* (poly(A)9), *BRCA1* (poly(A)8), *BRCA2* (poly(A)8), *DNAPKcs* (poly(A)10), *MBD4* (poly(A)10), *MRE11* (poly(T)11), *MSH3* (poly(A)8), *MSH6* (poly(C)8), *PTEN* exon 7 (poly(A)6), *PTEN* exon 8 (poly(A)6), *RAD50* (poly(A)9), *TCF4* (poly(A)9), *TGFβRII* (poly(A)10), *WISP3* (poly(A)9) and *XRCC2* (poly(T)8). PCR and genotype analysis were performed as previously described [21], except *AXIN2,* which was directly sequenced after PCR [25]. Samples presenting abnormal profiles were

direct sequenced to confirm the presence of frameshift mutations.

Immunohistochemistry

Immunohistochemistry for MLH1, MSH2, MSH6, PMS2 and MRE11 was performed using the Vectastain ABC system (Vector), according to the manufacturer's instructions. Antigen retrieval was achieved in boiling waterbath in Tris-EDTA pH 9.0 for 20 min and primary antibodies MLH1 (G168-15, 1:25, BD Biosciences), MSH2 (FE11, 1:150, Calbiochem), MSH6 (44, 1:100, BD Biosciences), PMS2 (A16-4, 1:200, BD Biosciences) and MRE11 (12D7, 1:500, Abcam) were incubated overnight at 4ºC.

For DNAPKcs staining, the Ultravision Plus Detection System (LabVision) was used according to the manufacturer's instructions. Antigen retrieval was performed with microwave treatment in citrate buffer pH 6.0 for 15 min and primary antibody (Ab-4 cocktail, 1:100, NeoMarkers) incubated 2h at RT.

Microscopic analysis was done by a blinded pathologist (S. P.). Sections without staining in the tumor cells were considered to have a lost expression (-). Samples without nuclear staining but positivity in the cytoplasm or with < 5% tumor cells with nuclear staining were considered with diminished expression (+). Samples with the scores ++ (5-50% nuclear staining in tumor cells) or +++ (> 50% nuclear staining in tumor cells) were considered positive.

Mutation analysis and methylation-specific (MS) - PCR of *MLH1*

Mutation analysis of *MLH1*, exons 1 to 19, was performed by PCR, using primers previously described [27], followed by direct sequencing.

For *MLH1* promoter methylation detection, DNA was treated with sodium bisulphite using the Epitect kit (Qiagen) according to the manufacturer's instructions. MS-PCR was performed using primers previously described [28].

Cell culture and ionizing irradiation

The cell lines Daudi, Raji and Jurkat were kindly provided by Dr Sue Colman / Prof Mel Greaves (ICR, UK). Cells were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Cells were seeded in 35 mm Petri dishes and treated with 1.5 Gy ionizing irradiation (IR) using an X-ray source irradiator (HS-MP1, AGO) operating at 250 kv and 10 mA. Cells were allowed to recover for 1h, 4h or 24h and then processed for immunofluorescence staining or western-blot analysis.

Immunofluorescence

Cells were spun at 200 rpm for 5 min on a Shandon Cytospin 3 centrifuge (Thermo

Fisher Scientific), fixed according to a modified Strecks pre-extraction protocol, permeabilized and blocked, as described [29]. Primary antibodies directed against MRE11 (Sheep polyclonal, 1:500, previously described [30]), 53BP1 (NB100-304, rabbit polyclonal, 1:100, Novus Biologicals) and phospho-Histone H2A.X (Ser139) (JBW301, mouse monoclonal, 1:2000, Millipore) were applied overnight at 4ºC and secondary antibodies, Alexa Fluor 594 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen) 1h at RT. Cell nuclei were stained with DAPI (Invitrogen). Images were taken using a Zeiss Axioplan 2 microscope and Smartcapture 2 software (Digital Scientific).

Western blot analysis

Protein extracts were separated in a 3-8% Tris-Acetate gel (Invitrogen) and transferred to PVDF membranes (GE Healthcare). Immunodetection was performed using antibodies directed against phospho-ATM (Ser1981) (10H11.E12, 1:500, Cell Signaling), ATM (MAT3-4G10/8, 1:1000, Sigma-Aldrich), and MRE11 (12D7, 3μg/mL, Abcam).

Quantitative Real-Time PCR

cDNA was prepared from 1 μg of RNA by random primed reverse transcription using Superscript III (Invitrogen). qRT-PCR was performed using SYBR green master mix (Applied Biosystems) on an ABI 7900HT loaded with the SDS2.1 software. Primers used were as follows: total MRE11 mRNA (exons 2-4), forward: CCAGGGGTTCTTGGAGAAG and reverse: TTTCCTTGAGGGCTTATTTTCA; MRE11 distinguishing the aberrant and wild-type transcript (exons 2-6) included the same forward primer and reverse: CCAGCACAACTTAAAATGTC; GAPDH, forward: GCCACCCAGAAGACTGTGGATGGC and reverse: CATGATGGCCATGAGGTCCACCAC. GAPDH mRNA levels were measured as an internal control. The number of amplification cycles to half maximal saturation of the PCR product was determined by measuring the integration of the fluorescent dye into the PCR products. The ratio of the level of MRE11 expression relative to the GAPDH control was calculated. Samples were analyzed on a 4% agarose gel loaded after saturation of the PCR reaction.

Statistical Analysis

All statistical tests were done in SPSSv16.0 (SPSS Inc.). Correlations between categorical values were done using the two-tailed Chi-square and Fisher's exact tests. A p value of < 0.05 was considered significant.

RESULTS

MSI is more common in pediatric HGG than in adults

MSI analysis was performed in 144 HGG, 71 pediatric and 73 adults, using a pentaplex PCR of quasimonomorphic markers as recommended by the revised Bethesda guidelines [18]. A total of 19 samples (13.2%) presented instability, 1 MSI-H (<1%) and 18 MSI-L (12.5%) cases, with the remaining 125 tumors stable (86.8%). The MSI-H case, RMH2452, was a three years old girl with glioblastoma, presenting instability at four markers (NR27, NR21, BAT25 and BAT26) (Figure 1).

Overall, there were 14/71 (19.7%) MSI-positive pediatric cases, significantly more than observed in adults (5/73, 6.8%; p=0.02, Chi-square test). The pediatric MSI cases comprised 11 glioblastomas, two anaplastic astrocytomas and one anaplastic oligodendroglioma, with an age range of between 4 months and 20 years. The adult MSI-L cases were glioblastomas of ages 62-75 years (Table1). Individual microsatellite data for each case is provided in full in Supporting Information 1.

Inactivation of mismatch repair proteins in familial and sporadic MSI pediatric HGG

Aiming to determine the underlying nature of the MSI observed we sought to investigate whether key components of the MMR system were intact, screening the MSI-positive samples for expression of MLH1, MSH2, MSH6 and PMS2 by immunohistochemistry (Table 1, Supporting Information 2). 10/13 (76.9%) pediatric samples with MSI showed an absent or diminished expression of MLH1 in the tumor cells, often in concert with reduced MSH2

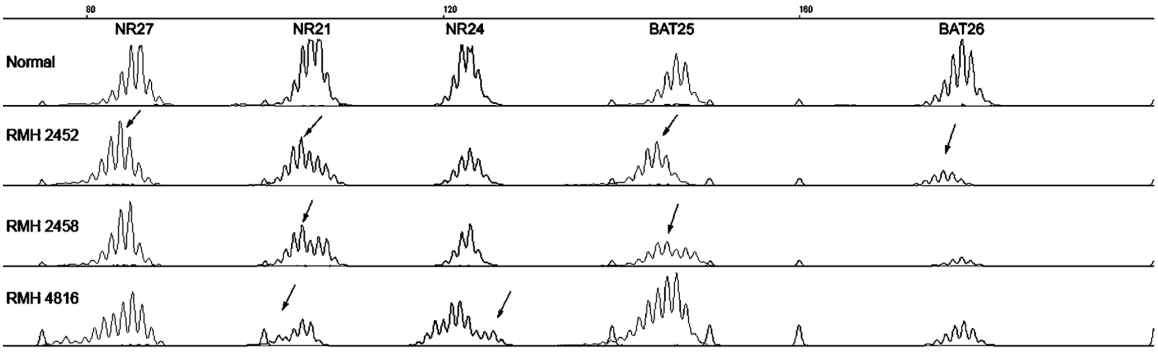

Figure 1 - Microsatellite instability in pediatric HGG. Representative electropherogram traces for three MSI-positive pediatric HGG, presenting sequence alterations in more than one quasimonomorphic marker. Case RMH2452 (MSI-H) presented alterations in NR27, NR21, BAT25 and BAT26; RMH2458 (MSI-L) in NR21 and BAT25; and RMH4816 (MSI-L) in NR21 and NR24. Alterations in relation to a control trace are indicated with arrows.

Table 1 – Clinical characterization and MMR status of MSI cases

Case	*Gender / Age*	*WHO grade*	*Diagnosis*	*MSI status*	*Immunohistochemistry*				*MLH1 mutation screening*	*MLH1 methylation status*
					MLH1	*MSH2*	*MSH6*	*PMS2*		
Pediatric										
2452	*F / 3*	*IV*	*GBM*	*MSI-H*	*+++*	*+++*	*-*	*++*	*ND*	*ND*
2458	F / 9	IV	GBM	MSI-L	+++	+++	+++	++	A	A
4816	M / 14	IV	GBM	MSI-L	+	++	++	++	A	NA
2444	M / 19	IV	GBM	MSI-L	-/+	+++	+/++	++	A	A
2457	F / 13	IV	GBM	MSI-L	-/+	++	+++	-/+	A	A
2470	F / 14	IV	GBM	MSI-L	MC	MC	+/++	MC	A	A
3952	F / 14	IV	GBM	MSI-L	-	NS	NA	++	NA	NA
3954	M / 20	IV	GBM	MSI-L	+++	++	+++	+++	A	A
3962	F / 0.3	III	AA	MSI-L	+	-	++/+++	-/+	A	A
3967	F / 8	III	AO	MSI-L	+	++	++/+++	NS	A	A
3969	F / 14	IV	GBM	MSI-L	MC	MC	+/++	MC	A	A
4820	M / 16	IV	GBM	MSI-L	+	+	+	++	NA	NA
4823	M / 15	IV	GBM	MSI-L	+	++	-	++	NA	NA
4839	M / 16	IV	GBM	MSI-L	NS	++	++	++	NA	NA
Adult										
3248	M / 67	IV	GBM	MSI-L	+++	+++	+++	++	A	NA
3283	M / 63	IV	GBM	MSI-L	+++	++	++/+++	+++	A	NA
3415	M / 70	IV	GBM	MSI-L	NA	NA	NA	NA	NA	NA
3416	F / 75	IV	GBM	MSI-L	-	+++	+++	+++	A	NA
3465	F / 62	IV	GBM	MSI-L	NS	+++	+++	+++	A	NA

Abbreviations: M, male; F, female; GBM, glioblastoma; AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; MSI-H, microsatellite instability-High; MSI-L, microsatellite instability-Low; MC, mostly cytoplasmatic; NS, non-specific; A, absent; ND, not done; NA, not available

(4/10, 40%) or PMS2 expression (4/10, 40%). There was, however, no evidence of mutation (assessed by direct sequencing) or hypermethylation (by MS-PCR) of *MLH1* in these cases. Due to the FFPE nature of the samples, it was not possible to screen for *MLH1* mutations and promoter methylation in all MSI samples (Table 1). Moreover, on samples screened for *MLH1* mutations, the rate of successful exons sequenced ranged from 58% (11/19 exons) to 100% (19/19 exons), according sample DNA quality. In contrast to pediatric, the four adult MSI-L cases presented positive immunoreactivity for MMR proteins.

A single case was negative only for MSH6. This patient (RMH2452, MSI-H) presented multiple café-au-lait spots in the absence of other clinical features of neurofibromatosis-1 (NF1). There was no family history of NF1, although the maternal great grandmother had endometrial cancer in her early forties. Although constitutional DNA was not available for testing, this patient's clinical history is consistent with an inherited MMR deficiency such as Turcot's syndrome, associated with germline *MSH6* mutation.

MSI-positive pediatric HGG have distinct genomic profiles and differential target genes compared with other tumor types

As the presence of MSI reflects a nucleotide-level form of genetic instability, work in CRC suggests that large scale chromosomal instability is reduced or absent in MSI-positive cases [31,32]. In order to determine whether this may also be true for pediatric HGG, we examined copy number profiles on 9/14 MSI-positive cases for which sufficient quantity and quality of DNA was available, and compared this to a similarly profiled cohort of 26 pediatric MSS cases (Array Express accession number E-TABM-857) [33]. A total of four MSI-positive cases harbored a 'flat' or 'stable' profile, with minimal or no copy number alterations detectable on the 32K tiling-path BAC array platform used (Figure 2, Table 2).

This is a pattern of genomic stability present in approximately 20% of pediatric HGG, but almost entirely absent from similar adult tumors [6,34]. Four MSI-L cases harbored a small number of whole chromosome arm gains or losses, and fell into the 'aneuploid' category of genomic profile. There was a single, MSI-L, case (RMH3954) that had a highly rearranged genome, with 19 distinct alterations. No MSI-positive cases contained any high-level amplifications or homozygous deletions.

Table 2 – Genomic alterations and Candidate MSI target genes frameshift mutations in MSI samples

Case	*MSI status*	Genomic subtype	Copy number changes	Altered MSI Target Genes
Pediatric				
2452	*MSI-H*	*Stable*	*5q31+, 21q21-*	*MSH6 (C)9*
2458	MSI-L	Stable	None	-
4816	MSI-L	NA	NA	-
2444	MSI-L	Aneuploid	1q+, 9q+	MRE11 (T)11/12
2457	MSI-L	Aneuploid	4q-, 5q-,7p12+, 9p21-, 13q-, 19+	-
2470	MSI-L	Aneuploid	5q-, 10q-, 14q-, 15q-, 19+, 20p-, Xp-	-
3952	MSI-L	Aneuploid	1p-, 6p-, 12q- 13q-, 19-, 22q-	-
3954	MSI-L	Rearranged	1p-, 1q+, 2q+, 3q-, 4p+, 4q+, 4q-, 5q-, 6p-, 6q+, 7+, 8p+, 8q-, 11p-, 11q+, 12q-, 13q-, 18-, 19q-	-
3962	MSI-L	Stable	None	-
3967	MSI-L	Stable	None	-
3969	MSI-L	NA	NA	DNAPKcs (A)9/10
4820	MSI-L	NA	NA	-
4823	MSI-L	NA	NA	-
4839	MSI-L	NA	NA	MRE11 (T)11/12
Adult				
3248	MSI-L	NA	NA	MRE11 (T)11/12
3283	MSI-L	NA	NA	-
3415	MSI-L	NA	NA	-
3416	MSI-L	NA	NA	-
3465	MSI-L	NA	NA	-

Abbreviations: MSI-H, microsatellite instability-High; MSI-L, microsatellite instability-Low; NA, not available

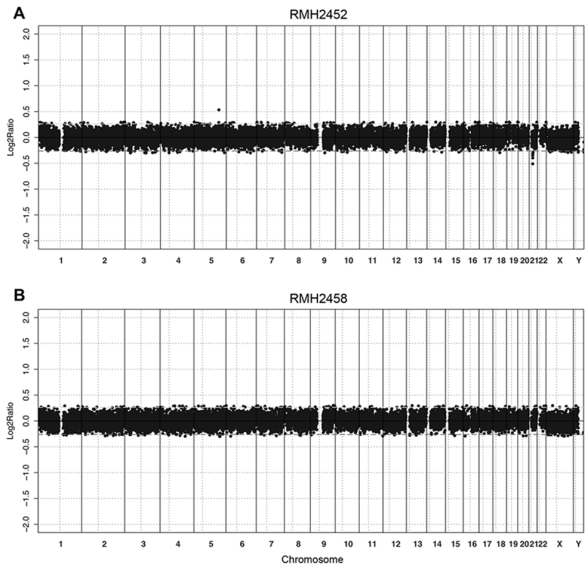

Figure 2 - Genomic stability in MSI pediatric HGG. Array CGH genome plots are shown for two MSI-positive pediatric HGG, the MSI-H RMH2452 (A) and the MSI-L RMH2458 (B), demonstrating lack of large scale alterations detectable on the 32K BAC platform. Log_2 ratios for each clone (y axis) are plotted according to chromosomal location (x axis). The centromeres are represented by vertical lines.

Overall there were less copy number changes in MSI (mean 5.78, range 0-19) than MSS cases (mean 8.35, range 0-25), although there was no statistical difference in the groups (p=0.37, t test), reflecting the highly rearranged MSI-L case, and the presence of 4/26 (15.4%) genomically stable cases by array CGH that were also MSS.

The presence of MSI confers an increased susceptibility for acquiring mutations in various target genes containing single nucleotide repeat sequences [20]. Selected CRC target genes involved in apoptosis (*BAX*), tumor growth (*TGFβRII*), WNT pathway (*AXIN2, TCF4, WISP3),* DNA repair (*ATM, ATR, BLM, BRCA1, BRCA2, DNAPKcs, MBD4, MRE11, MSH3, MSH6, RAD50, XRCC2*) and PI3-kinase signaling (*PTEN*) were analyzed for mutations in MSI-positive HGG samples (Table 2).

The pediatric MSI-H sample was found to contain a homozygous single base insertion in the poly(C)8 tract of *MSH6* poly(C)9, a frameshift which results in a truncated protein, confirmed in duplicate by genotyping and direct sequencing (Figure 3A). Somatic mutations on the poly(C)8 sequence of *MSH6*, are strongly associated with initiating *MSH6* germline mutations [35], adding further evidence to this patient having an inherited MMR deficiency syndrome.

A further MSI-L sample (RMH3969) presented a heterozygous single base deletion in the poly(A)10 sequence on the exon 5 of *DNAPKcs* (poly(A)9/10), however this mutation did not influence protein expression, as observed by immunohistochemistry (Figure 3B). Two other MSI-L cases, RMH2444 (Figure 3C) and RMH3952 harbored a heterozygous single base insertion in the poly(T)11 tract on the intron 4 of *MRE11 (*poly(T)11/12). Immunohistochemistry again revealed positivity of the protein (Figure 3C). This insertion was also observed in 1/5 adult cases with MSI-L (RMH3248). No additional target gene mutations were observed in our series (Table 2).

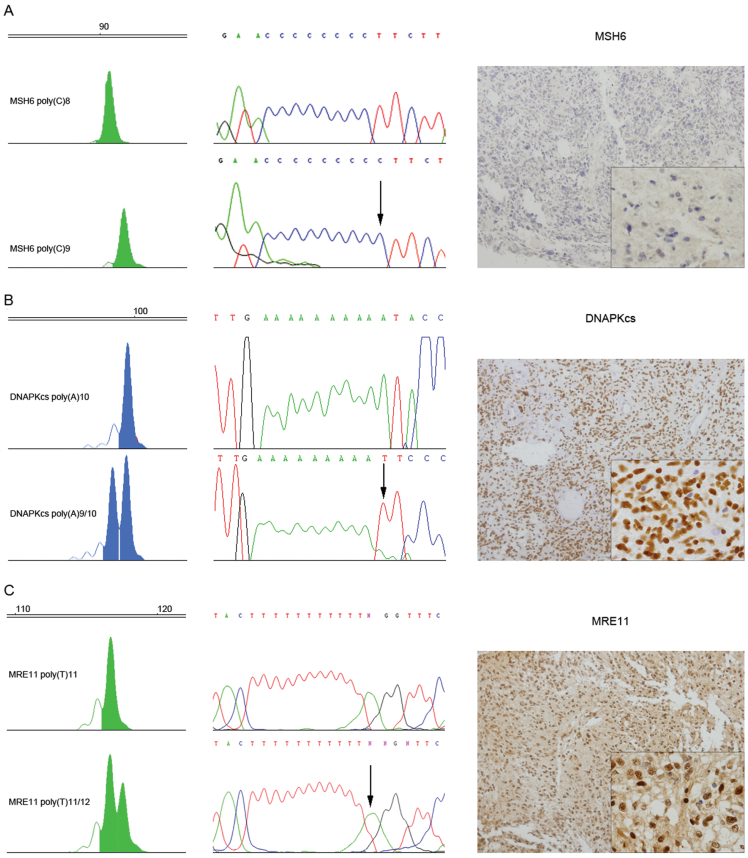

Figure 3 - Target gene screening in MSI pediatric HGG. Electropherogram traces, direct sequencing, and immunohistochemistry for MSI target genes presenting frameshift mutations in pediatric HGG (DNA alterations indicated with arrows). (A) RMH2452 (MSI-H) presenting a homozygous insertion of one bp in the MSH6 poly(C)8 tract and loss of protein expression. (B) RMH3969 (MSI-L) presenting a heterozygous deletion of one bp in the DNAPKcs poly(A)10 tract, and retention of protein expression. (C) RMH2444 (MSI-L) presenting a heterozygous insertion of one bp in the MRE11 poly(T)11 tract and retention of protein expression.

MRE11 poly(T)11/12 is a previously unrecognized polymorphism with no apparent functional consequence in DNA double strand break detection and repair

As MRE11 represented a potentially unknown contributor to the pathogenesis of HGG associated with MSI, we determined whether the specific *MRE11* poly(T)11/12 mutation affects the normal function of the protein, a member of the MRE11-RAD50-NBS1 (MRN) complex involved in DNA repair. As models we used three leukemia/lymphoma cell lines (Figure 4A): Daudi, *MRE11* poly(T)11/12; Raji, *MRE11* poly(T)11/11, wild type, and DNA repair proficient; and Jurkat, *MRE11* poly(T)10/11 known to generate alternative splicing in exon 5 of *MRE11* (Figure 4B) and DNA repair deficient [26]. The presence of an aberrant transcript was detected by RT-PCR of exons 2 to 6 with a truncated product detected in Jurkat cells (Figure 4C). Levels of total MRE11 mRNA were quantified by qRT-PCR amplification of exons 2 to 4 which generates a single size product, for both wild-type and the aberrant transcript. The ratios of the mRNA expression levels of MRE11, relative to GAPDH, were similar across all cell lines (Figure 4B) while protein levels were lower in Jurkat (Figure 4C).

To assess the proficiency of MRE11-associated DNA repair in *MRE11* poly(T)11/12 Daudi, we monitored the

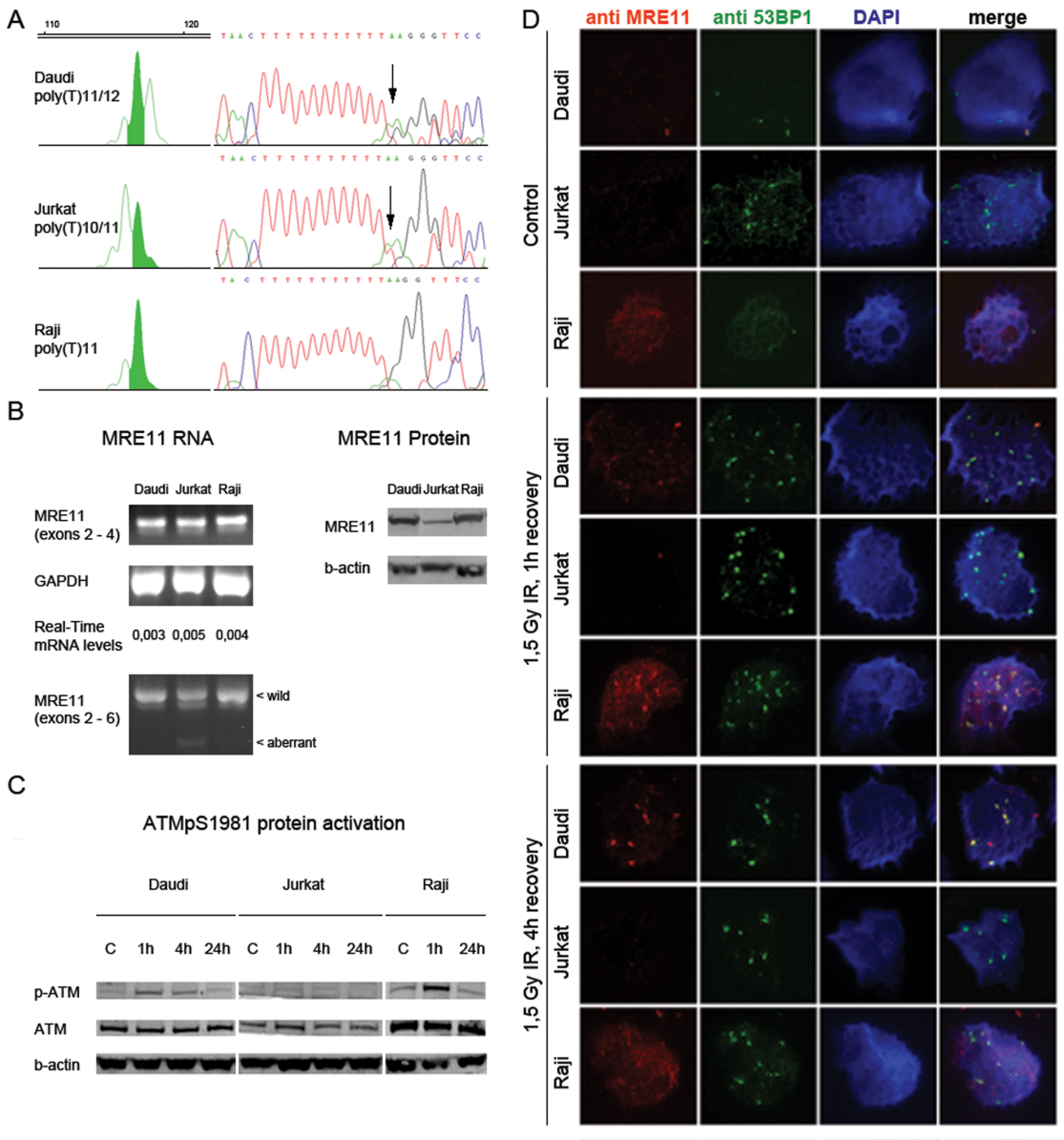

Figure 4 - *MRE11 poly(T)11/12 sequence variant has no apparent functional* consequence on DNA damage detection and repair (A) Electropherograms and sequencing traces of the MRE11 poly(T)11 tract in leukemia/lymphoma cells: Daudi (heterozygous insertion of one bp, poly(T)11/12); Jurkat (heterozygous deletion of one bp, poly(T)10/11); and Raji (wild-type, poly(T)11). (B) MRE11 expression in Daudi, Jurkat and Raji cells. qRT-PCR was used to calculate the ratio of MRE11 expression (amplification of exons 2 to 4) relative to the GAPDH control, independently of the presence of the aberrant transcript observed in Jurkat. PCR products were loaded on the gel at saturation of the reaction, being not quantitative. MRE11 protein levels were assessed by Western blot, and were considerably lower in Jurkat. (C) ATM-pS1981 levels were assessed by Western blot in Daudi, Jurkat and Raji after 1.5Gy irradiation of cells and 1h, 4h or 24h recovery. There was an increase of ATM phosphorylation 1h after irradiation in Daudi and Raji cells that decreased at the later time points. Jurkat showed no ATM phosphorylation after exposure to irradiation. (D) DNA repair foci was observed by immunofluorescence after 1.5Gy IR followed by 1h and 4h recovery. Irradiation resulted in the formation of both MRE11 and 53BP1 foci in Daudi and Raji cells, whereas Jurkat failed to form MRE11 foci. Nuclei were counterstained with DAPI. Merged figure presents co-localization of MRE11 and 53BP1 foci visualized in yellow.

repair response following DNA damage induced by ionizing radiation. After exposure to irradiation, auto-phosphorylation of ATM on serine-1981 takes place in response to the formation of DNA double stranded breaks (DSB) [36]. 1.5 Gy IR resulted in no ATM phosphorylation in Jurkat, while Raji and Daudi, presented an increase of ATM phosphorylation 1h after IR followed by a slight decrease in longer time points (Figure 4C). Another response to ionizing radiation is the accumulation of DNA repair proteins after exposure to irradiation that can be visualized in the form of foci associated with recruitment of essential repair complexes to the sites of DNA damage [37]. After 1.5 Gy IR, 53BP1 foci formation after IR was proficient in all cell lines as these can form even in the absence of a functional MRN complex (Figure 4D). In Daudi and Raji, the co-localization between MRE11 and 53BP1 foci observed was suggestive of the formation of functional repair complexes, in contrast to Jurkat, where no MRE11 foci were present. Compared to Jurkat and Daudi, Raji non-irradiated cells showed higher background levels of DNA damage as detected by formation of gH2AX foci (Supporting Information 3). In summary, these results suggest that the *MRE11* poly(T)11/12 mutation does not result in a compromised DNA damage repair response in Daudi cells.

Although *MRE11* poly(T)11/12 alteration could not be found in the dbSNP database, this specific base change was recently identified in the germline of a Caucasian male [38]. We subsequently screened a series of 72 control DNA samples, and discovered *MRE11* poly(T)11/12 to be present in 5/72 (6.9%) healthy volunteers. Although this frequency is less than that observed in the MSI-positive cases (3/19, 15.8%), the difference is not statistically significant (p=0.23, Chi-square test). Thus we conclude that *MRE11* poly(T)11/12 is a previously unrecognized polymorphism with no functional consequences on the DNA repair response.

DISCUSSION

Published studies on MSI in HGG have produced contradictory results. These differences have variously been attributed to the number of samples studied, the heterogeneity and accuracy of methods used to determine MSI status. We sought to address this by carrying out a large study of HGG using the most robust and sensitive screens available, and report a significantly elevated frequency of MSI in pediatric (19.7%) *versus* adult (6.8%) tumors.

One of the key considerations in the assessment of MSI is in the use of mononucleotides *versus* polynucleotides in the panel of markers used. The quasimonomorphic nature of the mononucleotides we used makes these most

suitable for MSI detection, particularly when matching germline DNA is not available, and display a higher sensitivity [23,39]. Many of the early studies used polynucleotide markers only and found a frequency of MSI ranging from of 0 to 37% in series of 7 to 56 gliomas from pediatric or age not discriminated series [12-15]. Including both mononucleotide and polynucleotides did little to improve consistency, with contrasting results of 0 to 44% in series of 1 to 9 pediatric gliomas [8-10]. Recent studies using mononucleotides only were also contradictory. Alonso *et al*. 2001 [11] reported a frequency of 27% MSI in 45 pediatric HGG using BAT25 and BAT26 as markers, while Eckert *et al*. 2007 [16] found no evidence of MSI in 41 cases using CAT25, BAT25 and BAT26. Vladimirova *et al*. 2007 [17] used the same panel of five mononucleotides as in the present study, and yet found a reduced frequency of 3.2% MSI in 126 pediatric HGG in comparison to our 19.7%. One key difference between our studies that possibly accounts for the diversity of frequencies is the use of 30 controls to establish the QMVR in our work. This approach allowed us to assess smaller allelic shifts of the markers as any variation outside the QMVR was considered as instability. Although the data presented by previous studies do not allow for re-analysis using this technique, we speculate that our increased frequency of MSI may be related to enhanced detection sensitivity [39].

Another important analytical difference may be found in the use of an alternative classification of MSI, which takes into account the size of the allelic shifts in the markers, rather than the number of markers with alterations. In this classification, samples presenting small length change (≤ 6 bp) are considered Type A, whilst those presenting more drastic alterations are described as Type B MSI, as reported for CRC [40]. Pediatric HGG with constitutive MMR deficiency have been previously described as possessing Type A MSI [41] and we also observed Type A MSI in our sporadic, and likely syndromic cases. This is also consistent with our previous observations in medulloblastoma [21], and appears to be a consistent difference in MSI reported in CNS tumors compared with the classic MSI positive epithelial tumors such as colorectal and gastric carcinomas. The observation that brain tumors present smaller changes in the MSI markers may represent a cause of the inconsistency found on MSI frequencies reported in literature.

Having established that a proportion of our pediatric HGG cases harbored MSI, we were keen to determine how this related to other genomic abnormalities. Microsatellite and chromosomal instability have been considered to be mutually exclusive [31], and although recent reports have demonstrated

that a minority of tumors can present both MSI-H and chromosomal instability, the frequency and degree of chromosomal alterations in colorectal and gastric MSS cancers is much higher than in the MSI-H cases [32]. Pediatric HGG differ from those found in adults by comprising a proportion of tumors with few or no detectable copy number changes by microarray analysis [6]. The MSI-H sample in our current cohort fell into that category. The MSI-L cases also had relatively fewer alterations than the population as a whole, while the presence of MSI and chromosomal instability were not mutually exclusive. In particular, there were several cases with 'stable' genomes which did not present MSI, suggesting that although this phenotype was associated with a proportion of cases with no gross alterations, it was not a general explanation for this key difference between the childhood and adult disease.

MSI is a molecular feature resulting mainly from inactivating alterations of the MMR system [19]. We studied MMR inactivation by immunohistochemistry on the 11 MSI tumors and observed that MLH1 expression was absent or reduced in 10 samples, 5 of which also presented reduced PMS2 and/or MSH2 expression. Considering the high percentage of pediatric HGG lacking MLH1 expression, molecular deficiencies in this gene appear to be the origin of the MSI phenotype in most of the cases, although we did not observe point mutations or hypermethylation in these samples.

MSH6 expression was absent in a single sample that also presented a homozygous insertion of a single nucleotide in the poly(C)8 track of *MSH6*, which is a target of MSI. This is the first report of this mutation in brain tumors. *MSH6* poly(C)9 leads to a truncated protein and is probably the cause of the total absence of protein assessed by immunohistochemistry. *MSH6* mutations in the poly(C)8 microsatellite are thought to arise due to germline mutations of the MMR genes, making of *MSH6* a target for somatic mutations in the presence of MMR germline mutations [35]. These germline mutations usually occur in the context of familial tumors and accordingly this patient had a clinical history consistent with a MMR deficiency syndrome such as Turcot, in which Type A MSI has been reported in affected children with glioblastoma [42]. Recently, *MSH6* mutations have been demonstrated to arise in gliomas as a consequence of treatment with temozolomide, and have been implicated in drug resistance and the presence of a hypermutated phenotype [4,43]. Apart from the sample RMH2458, which did not harbor any *MSH6* mutation, the tumors analyzed in this study, including the *MSH6* mutated RMH2452, were primary tumors, and have not been previously exposed to radio or chemotherapy treatment. Therefore MSH6 findings are not

related with a previous exposure to temozolomide.

A major consequence of MSI is the accumulation of additional mutations in key oncogenic target genes. To our knowledge, only five known MSI target genes have been screened, with mutations reported in a single case each of *IGFIIR* [7] and *PTEN* [9], and of *TGFβRII* in 71% cases [14], although this latter observation was not seen elsewhere [7,9]. We investigated the mutational status of 18 classical target genes in MSI HGG and, apart from *MSH6*, found alterations only in two other target genes, *DNAPKcs* and *MRE11,* which are involved in DNA DSB repair. The *DNAPKcs* poly(A)9/10 sequence variant has been described in samples of gastric and endometrial tumors [44,45], but has not been previously reported in gliomas. In subclones of the CRC cell line HCT-8 it failed to confer additional deficiency to DNA DSB repair compared to the parental line [46], and we found no alterations in protein expression in the MSI glioma sample presenting this mutation. *MRE11* poly(T)11/12 has previously been reported in a single case of MSI CRC [47], a single case of MSI medulloblastoma [21], and in the Daudi lymphoma cells [26]. Our findings here demonstrate that it appears to be an undocumented polymorphism with no functional consequences on DNA damage detection and repair. Thus it seems that the classical target genes for MSI in other tumor types are not frequently mutated in gliomas, and the field would benefit from a bioinformatic approach focusing on specific repeat sequences in coding regions for identifying novel, possibly glioma-specific MSI target genes as has been carried out in other tumor types [48].

The presence of MSI in pediatric HGG may have important translational implications. Specifically, in CRC, MSI-positive patients appear to show a differential response to 5-fluorouracil alone [49] as well as adjuvant therapy with irinotecan, fluorouracil, and leucovorin [50]. Furthermore, the majority of MSI-positive tumor cell lines of different tissue origins (endometrial, ovarian, prostate, and colorectal carcinomas) appear hypersensitive to drugs that produce DNA DSBs such as bleomycin [46]. Given the considerable impact of abrogated DNA repair capacity on gliomagenesis, identification of even a subset of cases with a differential response to existing chemotherapeutics would be of immense clinical benefit in these extraordinarily treatment refractory tumors.

In conclusion, we identified a subset of glioma patients presenting MSI with molecular alterations distinctive of this phenotype suggesting an association of MSI with the development of gliomas.

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SUPPORTING INFORMATION

1. Clinicopathological characteristics and microsatellite screening data of all HGG samples. Full QMVR range is provided.

2. Immunohistochemistry of MMR proteins in MSI-positive samples. H&E staining as well as expression of MLH1, MSH2, MSH6 and PMS2 are shown for cases RMH2452, RMH2458 and RMH4816. Original magnification x200 (inset x600).

3. Immunofluorescence for γH2AX foci in Daudi, Jurkat and Raji cells. Cells were treated with 1.5Gy IR and allowed to recover for 1h and 4h. Higher background levels of DNA damage were observed in Raji cells as seen by the formation of foci in the non-irradiated cells.

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Supporting Information 1

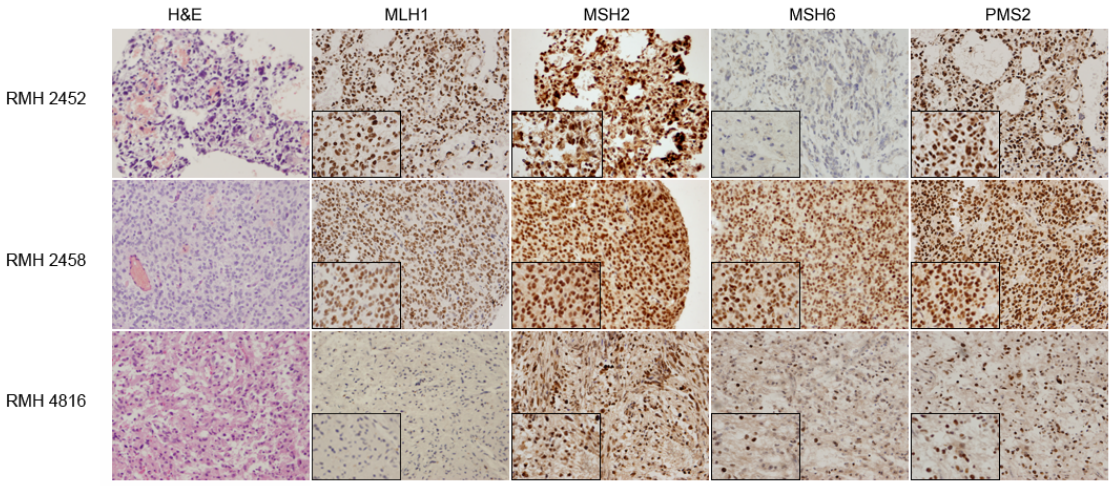
Pediatric Samples

Pediatric case	*Gender / Age (y)*	*Histological type*	*MSI detection*					
			NR27	*NR21*	*NR24*	*BAT25*	*BAT26*	*MSI status*
OMVR			85-86	105-106	122-123	145-148	177-179	
RMH2444	M / 19	OA (3)	85	106	**122-124**	146	178	MSI-L
RMH2446	F / 17	GBM	85	105	123	145	178	MSS
RMH2447	F / 13	AO	85	106	123	147	178	MSS
RMH2448	F / 5	GBM	85	105	123	147	178	MSS
RMH2449	F / 11	GBM	85	106	123	146	178	MSS
RMH2450	M / 0.5	GBM	85	106	123	146	178	MSS
RMH2452	F / 3	GBM	**84**	**104-106**	123	**144**	**176**	MSI-H
RMH2457	F / 13	GBM	85	105	123	**146-149**	178	MSI-L
RMH2458	F / 9	GBM (Secondary)	85	**104-106**	123	**145-147**	178	MSI-L
RMH2460	M / 16	AA	86	106	122	147	177	MSS
RMH2463	F / 12	GBM	86	106	122	147	177	MSS
RMH2465	M / 13	GBM	85	106	123	146	178	MSS
RMH2466	M / 9	GBM	85	106	123	146	177	MSS
RMH2467	F / 10	GBM	85	105	122	147	178	MSS
RMH2469	F / 4	Gliomatosis cerebri	85	105	123	146	178	MSS
RMH2470	F / 14	GBM	85	105	123	**143-148**	178	MSI-L
RMH2472	F / 12	GBM	85	106	122	146	178	MSS
RMH2474	M / 11	Gliosarcoma	85	106	123	146	178	MSS
RMH2475	M / 11	GBM	86	106	122	148	178	MSS
RMH2476	M / 8	AA	85	106	123	146	178	MSS
RMH2478	F / 11	GBM	86	106	123	146	178	MSS
RMH2480	M / 11	AA	85	106	122	146	178	MSS
RMH3547	M / 12	AA	85	106	123	147	178	MSS
RMH3548	F / 9	AA	85	105	122	147	178	MSS
RMH3549	M / 6	GBM	85	106	122	147	178	MSS
RMH3550	F / 10	GBM	86	106	122	148	178	MSS
RMH3551	M / 20	GBM	85	105	122	146	179	MSS
RMH3552	F / 5	AA	85	106	122	146	178	MSS
RMH3554	F / 8	GBM	85	106	122	146	NA	MSS
RMH3555	F / 9	GBM	85	106	122	147	177	MSS
RMH3556	M / 0.8	GBM	86	105	123	147	179	MSS
RMH3557	F / 5	Giant cell GBM	85	106	123	146	177	MSS
RMH3558	F / 10	AA	85	106	122	147	177	MSS
RMH3559	F / 16	GBM	85	106	123	147	177	MSS
RMH3561	F / 10	GBM	85	106	122	146	177	MSS
RMH3564	M / 12	GBM	85	105	122	147	178	MSS
RMH3565	M / 8	AA	85	106	122	146	179	MSS
RMH3566	F / 10	AA	86	105	122	146	178	MSS
RMH3567	F / 10	AA	86	105	122	146	178	MSS
RMH3568	M / 17	AA	85	105	123	147	178	MSS
RMH3942	M / 11	AO	85	104	122	145	177	MSS
RMH3945	F / 4	AA	85	105	122	147	178	MSS
RMH3947	M / 6	GBM	85	106	123	148	178	MSS
RMH3952	F / 14	GBM	85	**101-106**	122	146	178	MSI-L
RMH3954	M / 20	GBM	85	106	122	**146-148**	178	MSI-L
RMH3955	F / 19	GBM	86	106	122	146	178	MSS
RMH3957	M / 4	GBM	85	106	123	146	178	MSS
RMH3959	M / 22	AA	85	106	123	146	178	MSS
RMH3960	M / 11	GBM	85	105	122	147	178	MSS
RMH3962	F / 0.3	AA	**82**	105	122	145	179	MSI-L
RMH3965	M / 12	GBM	85	105	123	146	178	MSS
RMH3966	M / 11	GBM	85	106	123	146	177	MSS
RMH3967	F / 8	AA	**84**	105	122	147	178	MSI-L
RMH3968	F / 21	AA	85	106	122	147	178	MSS
RMH3969	F / 14	GBM	**84**	105	122	146	177	MSI-L
RMH3490	M / 11	GBM	85	105	122	147	178	MSS
RMH3492	M / 21	AO	85	106	122	146	178	MSS
RMH4811	M / 17	GBM	85	105	122	146	178	MSS
RMH4816	M / 14	GBM	85	**101-104**	**121-130**	146	179	MSI-L
RMH4818	M / 18	GBM	85	106	122	147	178	MSS
RMH4820	M / 16	GBM	85	**101-104**	122	145	178	MSI-L
RMH4822	F / 16	GBM	85	105	122	146	177	MSS
RMH4823	M / 11	GBM	85	105	**121-124**	146	179	MSI-L
RMH4825	F / 1	GBM	85	105	122	145	NA	MSS
RMH4826	M / 18	GBM	85	106	123	146	NA	MSS
RMH4831	F / 13	GBM	85	105	123	147	178	MSS
RMH4833	F / 18	GBM	85	106	122	146	177	MSS
RMH4835	F / 6	GBM	85	105	123	146	178	MSS
RMH4836	M / 14	GBM	85	106	122	146	177	MSS
RMH4839	F / 16	GBM	85	**101-105**	122	146	178	MSI-L
RMH4840	M / 7	GBM	85	104	123	147	177	MSS

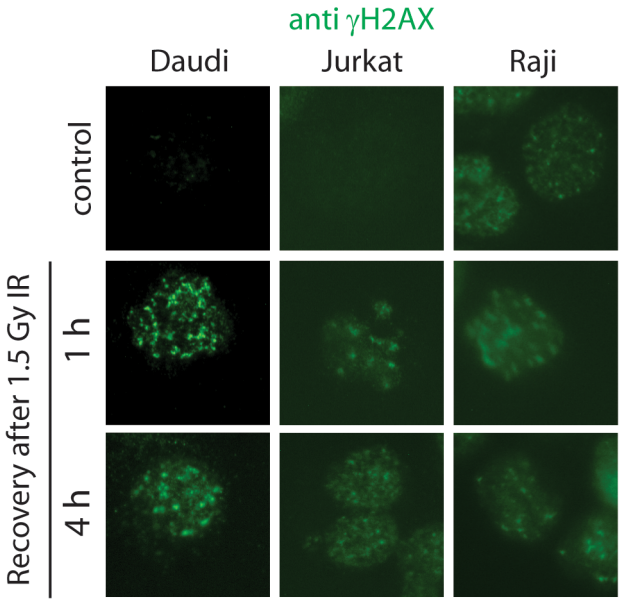
Adults

Adult case	*Gender/Age(y)*	*Histological type*	*MSI detection*					
			NR27	*NR21*	*NR24*	*BAT25*	*BAT26*	*MSI status*
RMH3243	M / 60	GBM	85	106	123	147	179	MSS
RMH3244	M / 68	GBM	86	106	122	146	178	MSS
RMH3246	M / 42	GBM	85	106	123	147	178	MSS
RMH3247	M / 63	GBM	85	106	123	146	178	MSS
RMH3248	M / 67	GBM	**82-86**	105	122	145	178	MSI-L
RMH3249	M / 48	GBM	85	105	123	147	179	MSS
RMH3250	M / 48	GBM	86	106	122	146	178	MSS
RMH3251	M / 65	GBM	NA	106	122	146	177	MSS
RMH3252	M / 66	GBM	85	105	122	145	178	MSS
RMH3253	F / 63	GBM	86	106	122	147	178	MSS
RMH3255	M / 70	GBM	85	106	123	146	178	MSS
RMH3256	M / 65	GBM	85	106	123	146	179	MSS
RMH3257	F / 35	GBM	85	106	123	146	178	MSS
RMH3258	M / 44	Gliosarcoma	85	106	123	146	178	MSS
RMH3260	M / 69	GBM	85	106	123	146	179	MSS
RMH3262	M / 70	GBM	85	106	123	146	178	MSS
RMH3263	F / 60	GBM	86	106	123	146	177	MSS
RMH3265	F / 59	GBM	85	106	123	146	177	MSS
RMH3266	F / 56	GBM	85	105	122	146	178	MSS
RMH3267	M / 61	GBM	85	106	122	146	178	MSS
RMH3269	M / 58	GBM	86	106	122	147	178	MSS
RMH3273	M / 71	GBM	85	105	123	147	178	MSS
RMH3274	F / 61	GBM	85	106	122	146	178	MSS
RMH3279	F / 69	GBM	85	106	123	145	178	MSS
RMH3281	M / 53	GBM	85	105	123	146	178	MSS
RMH3282	F / 62	GBM	85	106	123	146	178	MSS
RMH3283	M / 63	GBM	85	106	123	147	**176-179**	MSI-L
RMH3284	F / 61	Gliosarcoma	85	106	123	146	178	MSS
RMH3287	M / 41	GBM	86	106	123	147	177	MSS
RMH3288	F / 65	GBM	85	105	123	147	177	MSS
RMH3292	F / 68	GBM	86	105	123	147	178	MSS
RMH3293	M / 65	GBM	86	105	122	147	179	MSS
RMH3294	M / 69	GBM	86	106	122	147	177	MSS
RMH3295	M / 55	GBM	85	105	123	147	178	MSS
RMH3296	M / 55	GBM	85	105	122	146	179	MSS
RMH3297	F / 46	GBM	85	105	123	146	178	MSS
RMH3300	F / 66	GBM	85	107	123	147	178	MSS
RMH3301	M / 54	GBM	85	105	123	146	178	MSS
RMH3302	M / 45	GBM	86	106	123	147	178	MSS
RMH3332	M / 56	GBM	85	105	122	147	178	MSS
RMH3362	F / 56	GBM	85	105	123	146	177	MSS
RMH3363	F / 70	GBM	85	106	122	146	178	MSS
RMH3364	M / 70	GBM	85	106	123	146	178	MSS
RMH3366	M / 32	AA	85	106	123	146	178	MSS
RMH3368	M / 65	GBM	86	106	123	147	179	MSS
RMH3379	F / 55	GBM	85	105	123	147	179	MSS
RMH3383	M / 51	GBM	85	106	123	147	178	MSS
RMH3384	M / 54	GBM	85	105	122	146	178	MSS
RMH3392	M / 73	GBM	85	106	122	146	178	MSS
RMH3405	M / 56	GBM	85	106	123	147	177	MSS
RMH3409	M / 68	GBM	85	105	123	145	177	MSS
RMH3410	F / 68	GBM	85	105	123	146	178	MSS
RMH3411	F / 59	GBM	85	105	123	147	178	MSS
RMH3412	M / 52	GBM	85	106	122	146	177	MSS
RMH3414	M / 49	GBM	85	105	123	146	177	MSS
RMH3415	M / 70	GBM	85	**101-106**	122	146	178	MSI-L
RMH3416	F / 75	GBM	85	106	123	**146-148**	178	MSI-L
RMH3441	M / 56	Gliosarcoma	85	105	123	147	178	MSS
RMH3442	F / 68	GBM	85	105	123	147	179	MSS
RMH3444	F / 76	GBM	86	106	123	146	178	MSS
RMH3445	M / 62	GBM	85	105	123	146	178	MSS
RMH3446	M / 79	GBM	85	105	123	146	178	MSS
RMH3447	M / 66	GBM	86	106	123	146	178	MSS
RMH3449	F / 76	GBM	86	105	123	146	178	MSS
RMH3451	F / 56	GBM	85	106	123	146	178	MSS
RMH3456	M / 31	GBM	85	106	123	146	178	MSS
RMH3458	F / 49	GBM	85	106	123	146	179	MSS
RMH3459	M / 40	GBM	85	106	123	146	178	MSS
RMH3462	F / 55	GBM	85	105	122	146	178	MSS
RMH3465	F / 62	GBM	85	105	122	**146-148**	179	MSI-L
RMH3466	F / 69	GBM	85	106	123	147	178	MSS
RMH3467	M / 79	GBM	86	106	123	147	178	MSS
RMH3471	M / 69	GBM	85	106	122	147	178	MSS

Supporting Information 2



Supporting Information 3



5. General Discussion

5. General Discussion

A growing body of evidence has started to bring to light genetic differences between paediatric and adult brain tumours. The work described throughout this thesis aimed to give a further input on this major topic in neuro-oncobiology research. Hence, we highlight the relevance of EGFR and the presence and frequency of MSI in paediatric and adult malignant brain tumours.

5.1 – EGFR Molecular Alterations as Potential Therapeutic Targets for Gliomas in Adult and Paediatric Populations

The tyrosine kinase receptor EGFR induces proliferation and differentiation of multiple cell types, particularly of epithelial origin (1). Both overexpression and structural alterations of EGFR are frequent in various types of cancer, suggesting a role in the pathogenesis of multiple malignancies (2). Likewise, in high-grade gliomas, there are multiple mechanisms through which EGFR mediates tumour initiation and progression (Figure 1). *EGFR* gene amplification and overexpression are striking features of adult primary glioblastoma but are rare in low-grade gliomas or secondary glioblastoma (Figure 1B) (3-5). The most common and best-studied *EGFR* mutant in gliomas, the *EGFRvIII* (also known as *EGFR* type III, del2-7 or *ΔEGFR*), is generated from a deletion of exons 2 to 7 of *EGFR*, resulting in an in-frame deletion of 267 amino acids from the extracellular domain of the receptor (6, 7). Despite being unable to bind any ligand, this mutant signals constitutively, and is usually co-expressed with the wild type receptor in glioblastoma and present when *EGFR* is amplified (Figure 1D) (6, 8-10). EGFRvIII phosphorylation levels are only 10% of the activated EGFR wild type, yet it is thought to play a key role in gliomagenesis (11). Co-expression of EGFR ligands has also been detected, suggesting that autocrine loops contribute to malignant progression (Figure 1C) (12, 13). In our first study (summarized in Sub-Chapter 3.1) we evaluated these well-known EGFR molecular alterations in a series of adult Portuguese high-grade glioma patients, a population which had not previously been studied. The results were in agreement with earlier reports for adult high-grade gliomas from other countries (6, 14-19), yet added further contribution to the characterization of *EGFR* status, particularly

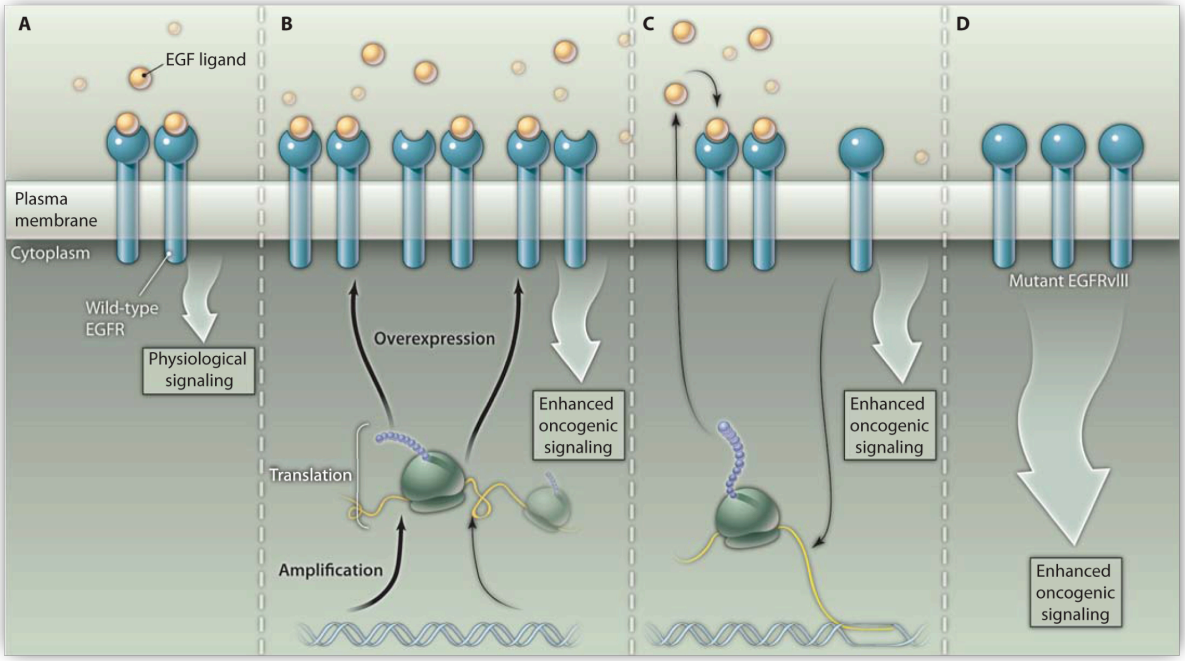


Figure 1 – Mechanisms of EGFR activation in high-grade gliomas. A) Normal EGFR and EGF signalling. B) EGFR overexpression (protein level)/amplification (DNA level) resulting in an increase of competent receptor signalling complexes at cell surface and enhanced oncogenic signalling. C) Increased matrix protease activity or the EGFRvIII mutant stimulates the secretion of EGF-family ligands, activating autocrine loops and leading to stimulation of wild-type EGFR and oncogenic signalling. D) EGFRvIII mutant results in constitutive receptor activation and diminished turnover, enhancing oncogenic signalling. *Adapted from reference* (9).

regarding the *EGFRvIII* mutation in anaplastic oligodendrogliomas, which was poorly described in literature (6, 20-23). In the paediatric group, the relevance of the EGFR signalling pathway is less well understood. In this context we aimed to clarify the role of EGFR in a large series of paediatric high-grade glioma (summarized in Sub-Chapter 3.2). A summary of the directly comparable main results of our adult and paediatric works is presented in Table 1.

The small amount of previous studies on EGFR in paediatric high-grade gliomas suggested that gene amplification is less common in paediatric than in adult primary glioblastomas, however the reported frequencies were highly heterogeneous (24-28). The variety of techniques used is postulated as the main cause of the heterogeneity of the results. Of note, in both works (Sub-Chapters 3.1 and 3.2), we used CISH for evaluation of *EGFR* amplification, which allows a histological evaluation and consideration of focal high-density clusters of amplification, unlike other molecular techniques, such as quantitative PCR. Comparing adult and childhood tumours, *EGFR* amplification was found at significantly

higher frequencies in glioblastomas or when pooling all high-grade gliomas of adult patients (P<0.01, Two tailed Fisher's exact test). Statistical significance was not achieved in oligodendroglial tumours, probably due to the reduced number of paediatric samples and homogeneity of results (0/4, 0% amplification).

Table 1 – Summary of the findings from the studies on EGFR in adult and paediatric high-grade gliomas

	Overexpression (IHC)		***Amplification (CISH)***		***EGFRvIII (IHC or RT-PCR)***	
All high-grade gliomas*						
Adult (n=55)	24/55 (44%)	-	20/42 (48%)	P<0.0001**	9/55 (16%)	-
Paediatric (n=74)	15/32 (47%)		8/74 (11%)		6/35 (17%)	
Glioblastoma						
Adult (n=27)	13/27 (48%)	-	10/19 (53%)	P<0.01**	6/27 (22%)	-
Paediatric (n=53)	7/18 (39%)		6/43 (14%)		1/20 (5%)	
Anaplastic oligodendroglioma						
Adult (n=24)	8/24 (33%)	-	8/20 (40%)	-	2/24 (8%)	P<0.05**
Paediatric (n=3)	1/1 (100%)		0/3 (0%)		2/3 (67%)	

*Includes 27 primary glioblastomas, 24 anaplastic oligodendrogliomas and 4 anaplastic oligoastrocytomas in the adult setting and 53 glioblastomas, 16 anaplastic astrocytomas, 3 anaplastic oligodendrogliomas, 3 brainstem gliomas and 25 other WHO grade 3 or 4 lesions in the paediatric series. ** Two tailed Fisher's exact test.

Two different approaches were used to assess *EGFRvIII* presence in our works and both proved to be reliable. In the first article, we performed immunohistochemistry with a described EGFRvIII specific antibody (15). In the second study, it was developed an RT-PCR assay suitable for use in FFPE samples and the consistency of the technique was confirmed by direct sequencing. The presence of *EGFRvIII* in high-grade astrocytomas was associated with gene amplification and wild-type receptor overexpression, either in adult or in paediatric tumours, and overall frequencies were in line with the literature (27).

Frequencies of EGFRvIII were only significantly different among anaplastic oligodendroglioma (Table 1), with a higher incidence reported in paediatric tumours, yet the numbers of paediatric cases were very small. In the adult anaplastic oligodendroglioma,

EGFRvIII expression was accompanied by amplification of the receptor, whereas in paediatric tumours the absence of *EGFR* amplification in the presence of the mutant makes the significance of the mutation less clear. As the transforming ability of EGFRvIII has shown to only manifest itself in the context of other genetic alterations (29-32), it may be of note that these paediatric EGFRvIII-positive cases without gene amplification, presented strong PDGFRA expression, even if genomic alterations in *EGFR* and *PDGFRA* tend to be mutually exclusive in paediatric glioblastomas (33). Importantly, it has been described for adult glioblastomas, that EGFRvIII expression results in the cross-activation of other RTKs, such as MET, AXL or EphA2 (34). The PDGFRA co-expression with EGFRvIII, was not observed in our adult anaplastic oligodendrogliomas cases (35).

Anti-EGFR treatment seems to be effective in patients with EGFR tyrosine kinase mutations in lung cancer (36-39), however these mutations have not been found in glioma cell lines (37) or in tumour tissue from adult patients (37, 40, 41). By contrast, mutations in the EGFR extracellular domain were identified in glioblastoma samples, yet not associated with better responses to EGFR tyrosine kinase inhibitors (42). Recently, TCGA data have confirmed the presence of C-terminal deletions (5) and extracellular domain point mutations in a significant percentage of glioblastoma tumours (43). Biological material limitations in the adult high-grade glioma series, together with the scientific knowledge at that time, did not allow us to perform mutation studies on these tumours. Nevertheless such analysis was done in the paediatric samples, though we have not found mutations either in the tyrosine kinase or in the extracellular domains of *EGFR*, emphasising the potential biological differences between high-grade gliomas of different age-groups.

Initial studies have demonstrated that a subset of patients with co-expression of EGFRvIII and PTEN appeared to be more sensitive to anti-EGFR therapy with erlotinib (Tarceva), a selective EGFR and EGFRvIII tyrosine kinase inhibitor (TKI), in glioblastoma, enhancing this drug as a promising EGFR TKI for clinical use (44, 45). Also, it has been shown that erlotinib was more efficient in the adult glioblastoma U87MG cells co-expressing PTEN and EGFRvIII, compared to controls or cells transfected with EGFR wild-type or EGFRvIII alone (44). Nevertheless, a subsequent study reported that the concomitant expression of EGFRvIII with PTEN was not predictive of improved survival in patients treated with erlotinib (46). In the paediatric study, this topic was further explored, using paediatric glioma cell

lines as models to study the *in vitro* sensitivity of these cells to erlotinib. The rationale for using these cells for preclinical and mechanistic studies has been provided in a previous study (see appendix, paper I). Our data shows that co-expression of PTEN and EGFRvIII does not confer enhanced sensitivity to the inhibitor, comparing the paediatric glioblastoma, PTEN wild-type, cell line SF188 and the adult glioblastoma, PTEN deleted, cell line U87MG. In addition, the presence of wild type or mutant *EGFR* did not alter the efficiency of PI3K or MAPK pathways inhibitors in these cells. These findings suggest that more complex molecular signatures associated with individual tumours may need to be identified for clinically effective targeting of the EGFR in high-grade gliomas. As it was previously referred, EGFRvIII seems to activate MET and combined treatment of MET and EGFR kinase inhibitors conferred higher efficacy than single treatment of either compound (34). In agreement with the observations in high-grade glioma tumours, the presence of EGFRvIII in the paediatric glioblastoma cell line induced activation of PDGF receptors, whereas this was not observed in the adult cell line. Treating the paediatric cells with erlotinib in combination with imatinib, a TKI of PDGFR, revealed an enhanced efficacy of the combined treatment compared with the use of either compound alone.

Overall, the fact that EGFR represents one of the most frequently altered molecules in high-grade glioma renders it an outstanding therapeutic target. This is particularly true for adult patients, mainly those with glioblastoma, and was confirmed during the work developed in this thesis that it is also true for Portuguese high-grade glioma patients. Likewise, paediatric tumours, particularly anaplastic oligodendrogliomas, seem to frequently harbour *EGFRvIII* deletions and therefore are also potential candidates for EGFR targeted therapy. In spite of this, it has been shown in preclinical and clinical studies that the presence of EGFR molecular alterations is, *per se*, not sufficient to assure a response to treatment with EGFR TKI in high-grade gliomas (44, 47-49). Instead, a multifactorial molecular predisposition might dictate the susceptibility of individual tumours to targeted therapies. In this context, it is important to further study and understand the underlying molecular mechanisms responsible for resistance or sensitivity to targeted therapies and define subgroups of resistant / responsive tumours. So far, the combined therapy of different drugs targeting EGFR signalling pathway at different levels or the combined

inhibition of multiple RTKs co-activated in high-grade gliomas seem to be most promising to overcome resistance toward EGFR-targeted monotherapy (50-52).

5.2 – Microsatellite Instability in High-grade Gliomas and Medulloblastomas of Adult and Paediatric Patients

DNA is continuously exposed to numerous different insults, endogenous and exogenous, that can ultimately result in DNA mutation and alteration of cell behaviour. DNA repair mechanisms are therefore essential for keeping DNA integrity and prevent tumourigenesis (53, 54). Consequences of failure of these molecular pathways are well illustrated in colorectal cancer, mainly HNPCC where it is present in about 90% of the tumours. In these cancers, MMR-deficient cells adopt a mutator phenotype in which there is a significant increase in cellular mutation rates (55, 56). The most obvious molecular signature of this mutator phenotype is the presence of MSI (57).

The classification of MSI more commonly adopted takes into account the number of markers presenting frameshift alterations. MSI tumours are therefore classified as MSI-H when, comparing to germline DNA, two or more markers present allelic shifts or, in the absence of constitutive DNA, at least three markers are altered; when, respectively, one or two markers present alterations, tumours are classified as MSI-L; and when all markers are normal tumours are MSS (58-60). Accordingly, in Sub-Chapter 4.1 the presence of normal adjacent tissue allowed the comparison between tumour and germline DNA and so MSI-H was considered when two or more markers were mutated. In the high-grade glioma study, Sub-Chapter 4.2, germline DNA was not available and so MSI-H was defined when 3 or more markers presented alterations. In parallel with this classification, an alternative qualitative distinction of MSI, which considers the size of the allelic shifts in the markers, has been proposed (61, 62). Samples presenting small length changes (≤6 bp) are designed Type A MSI whereas those with more extreme variations are defined as Type B MSI. We consistently observed type A MSI in the brain tumours studied. Both MSI-positive medulloblastoma and high-grade glioma presented small length alterations within the microsatellites and we hypothesise that this might be one of the primary reasons for the high variation frequency of MSI in brain tumours reported in the literature (63-73).

In our series of high-grade gliomas, the frequency of MSI was significantly higher in paediatric than adult tumours. Even if it is not clear-cut, taking into account data previously published, this could be anticipated (63-67). Our data represents a step forward into this controversial topic of discussion and contributes to the identification of a further molecular distinction between paediatric and adult high-grade gliomas. Moreover, the MSI-positive paediatric tumours mostly presented a stable genomic profile at the chromosomal level, even if microsatellite and chromosomal instability were not found to be mutually exclusive. Importantly, the presence of a proportion of tumours with few or absent copy number alterations distinguishes paediatric high-grade gliomas from their adult counterparts (see appendix, paper II) and therefore we hypothesise that MSI might represent an alternative form of genetic instability, at least in a proportion of these paediatric tumours with no gross chromosome number alterations. In medulloblastoma, we found no difference in MSI frequency between adult and paediatric tumours, suggesting that the presence of MSI in these tumours is not age-related. Overall, reviewing literature (see Chapter 1), it seems that there are less molecular differences between adult and paediatric medulloblastomas than those reported in high-grade gliomas, in concordance with our data.

Mismatch repair deficiencies have been associated with paediatric brain tumours in a hereditary context. Case reports have described MMR germline mutations combined with NF1-like clinical features in children presenting medulloblastoma or high-grade glioma, described as a “mismatch repair-deficiency (MMR-D) syndrome” (62, 74-85). A summary of these cases is presented in Table 2. In the medulloblastoma series (Sub-Chapter 4.1), only after the publication of the study was it possible to retrieve clinical data from some of the MSI-positive patients, and therefore this issue was not further explored therein. Data was available for two MSI-L cases (M21 and M25) and it was possible to conclude that M21 presented a familial history of cancer (Figure 2A). There is specific clinical and molecular information missing, such as presence or otherwise of NF1-like clinical features or which MMR molecule is abnormal, however it is likely that patient M21 presents a MMR-D syndrome. In our series of high-grade glioma (Sub-Chapter 4.2), the paediatric patient presenting MSI-H had clinical characteristics of NF1 (multiple *café-au-lait* spots), with a family history unremarkable, except for a maternal great grandmother, who had endometrial cancer in her early forties (Figure 2B). The clinical history of the paternal family

is largely unknown. Even though there was no constitutional DNA available for evaluation of MMR germline mutations, data imply that this patient probably has a *MSH6* germline mutation, again presenting a MMR-D syndrome.

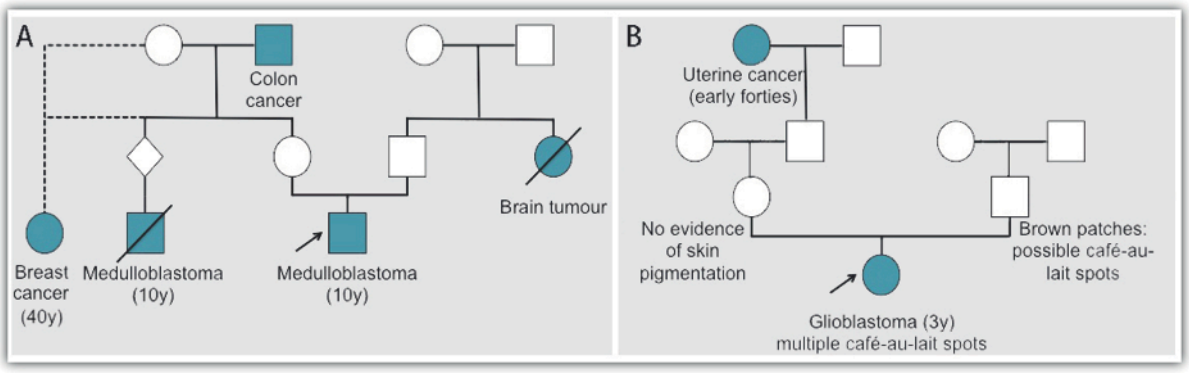


Figure 2 – Pedigree trees from likely MMR-D syndrome cases of our series. A) Pedigree tree of medulloblastoma sample M21, showing a cancer-family history. B) Pedigree tree of glioblastoma sample RMH2452 and clinical features. Mother had no evidence of skin pigmentation, whereas father presented brown patches, possible *café-au-lait* spots. Clinical history form the remaining paternal family is scarce. Family members to whom cancer was diagnosed are presented in green.

Independently of the hereditary or sporadic nature of the MSI-positive brain tumours, we were not able to identify the MMR molecular alteration underlying the MSI phenotype. In the medulloblastoma study we analysed only methylation of the MMR genes (*MLH1, MLH3, MSH2, MSH3, MSH6* and *PMS2*) on the assumption that the majority of our samples were sporadic. MSH6 immunohistochemistry was performed on the basis of the methylation results and expression was found in all MSI-positive samples and therefore it is unlikely that MSH6 is responsible for the MSI phenotype observed. The inclusion of the other MMR proteins in the analysis possibly could have been helpful in identifying the causes of MSI in medulloblastoma. On the other hand, in the high-grade glioma study, we analysed the core MMR protein (MLH1, MSH2, MSH6 and PMS2) inactivation by immunohistochemistry and observed a frequent absence of MLH1 expression. Nevertheless, mutations or promoter methylation of the gene were not observed. It is important to mention that due to the poor quality of the FFPE samples and the limited amount of tissue, successful sequencing of the entire coding region of the gene was not possible for the majority of the samples, and an absence of hotspot mutations in MLH1 related to MSI-positivity means that we cannot completely rule out this mechanism of inactivation. We can

Table 2 – Cases reported in the literature with MMR-D associated with brain tumours, including the likely MMR-D cases found in our studies.

Family	*Malignancy*	*Age at diagnosis*	*Café-au-lait spots*	*Affected gene*	*Reference*
1	AML Medulloblastoma	6 7	Yes	*MLH1*	Wang et al. (1999)[74]
7	Anaplastic oligodendroglioma Colorectal cancer	14 and 17 18	NS	*PMS2*	De Rosa et al. (2000)[75]
	Neuroblastoma	13	NS		
2	Oligodendroglioma Rectosigmoid carcinoma	10 12	Yes	*MSH6*	Menko et al. (2004)[76]
3	Glioblastoma	8	Yes	*MSH6*	Hegde et al. (2005)[77]
4	Duodenal adenocarcinoma Colonic adenoma Glioblastoma	16 16 17	Yes	*PMS2*	Agostini et al. (2005)[78]
5	Pilocytic astrocytoma Lymphoma	9 10	Yes	*MSH6*	Ostergaard et al. (2005)[79]
	Spinal glioblastoma	2	Yes		
6	Glioblastoma Wilms tumour	4 4	Yes	*MLH1*	Wagner et al. (2003)[80] Poley et al. (2007)[81]
7	Lymphoma Anaplastic oligodendroglioma	4 6	Yes	*MSH6?*	Poley et al. (2007)[81]
	Medulloblastoma	6	Yes		
8	Medulloblastoma AML Colon carcinomas	7 10 13	Yes	*MSH6*	Scott et al. (2007)[82]
9	Glioblastoma	6	Yes		
	Glioblastoma Colorectal cancer Small bowel cancer Urotel carcinoma	6 15 15 15	Yes	*PMS2*	Kruger et al. (2008)[83]
	Glioblastoma	9	Yes		
10	Glioblastoma	10	Yes	*PMS2*	Giunti et al. (2009)[62]
11	Glioblastoma	4	NS	*MLH1*	Giunti et al. (2009)[62]
	Glioblastoma	12	NS	*NS*	
12	Colorectal cancer Astrocytoma WHOII	14 14	Yes	*MSH2*	Toledano et al. (2009)[84]
	Anaplastic astrocytoma	13	Yes	*NS*	
13	Medulloblastoma Duodenal adenocarcinoma	 16	Yes	*PMS2*	Roy et al. (2009)[85]
14	Medulloblastoma	10	NS	*?*	Viana-Pereira et al. (2009)
15	Glioblastoma	3	Yes	*MLH6?*	Viana-Pereira et al. (2010)

speculate that mutations in other MMR genes can be the cause of MLH1 lack of expression, as absence of more than one protein was frequently observed (86). An alternative indirect mechanism of somatically silencing the MMR proteins has been recently described (87). Overall, it was reported that overexpression of the microRNA (miR) miR-155 significantly down-regulates MSH2, MSH6 and MLH1, explaining the loss of expression of MMR proteins in samples lacking a genetic or epigenetic cause of MMR inactivation (87). It is therefore possible that the down-regulation of MLH1 in our samples is happening through an indirect approach and this should be further evaluated.

Several genes affected by MSI have been identified in different cancers. A positive selective pressure should lead to the frequent detection of frameshift mutations at microsatellite loci in MSI-positive tumours. It is, however, not always clear which of these are “driver mutations”, that truly contribute to tumourigenesis, and which are “passenger mutations” that indicate the presence of a MMR defect but have little impact on disease progression (88, 89). In theory, for these frameshift mutations to exert a maximal effect both alleles should be affected. However, mutations in the coding sequences of MSI target genes are often found to be mono-allelic, implying that inactivation of a single allele results in the production of insufficient amounts of the gene product, ultimately affecting the phenotype of the cells and promoting carcinogenesis (56). We found only few of the “classical” MSI target genes mutated on the MSI-positive brain tumours, indicating that the MSI-target genes can be tissue-specific, as previously hypothesised (90). All MSI target genes mutated in our samples are involved in different DNA repair pathways: *MBD4*, *DNAPKcs*, *MSH6* and *MRE11*. The mutations described in our studies have never been reported in brain tumours.

MBD4 was mutated in a paediatric medulloblastoma sample. A heterozygous insertion of one nucleotide in the poly(A)10 tract of exon 3 is thought to produce a truncated protein. MBD4 is member of the methyl-CpG binding protein family, involved in the repair of mismatched G-T residues at methylated CpG sites (91). *MBD4* mutations were previously described in colorectal, endometrial, pancreatic and urothelial MSI-positive tumours (92-95), but never in brain tumours. Importantly, *in vitro* studies demonstrated that truncated MBD4 has the capacity to bind to methylated DNA sites and compete with wild-type protein in a dominant negative manner, causing the accumulation of errors in the DNA (96).

Furthermore, *MBD4* mutations may also play a role in tumour treatment, where overexpression of truncated *MBD4* in heterozygous cell lines results in an increased sensitivity to cisplatin and the resistance to etoposide *in vitro* (97). These drugs have already been used in clinical trials with medulloblastomas presenting positive results (98).

The *DNAPKcs* poly(A)9/10 mutation was previously described in gastric, endometrial and colorectal tumours (90, 99, 100). Cells use two main mechanisms to repair double strand breaks (DSB), non-homologous end-joining (NHEJ) and homologous recombination (HR), depending on the phase of the cycle and the nature of the DSB ends (101). DNAPKcs plays a central role in non-homologous end joining repair leading to the recruitment and activation of end-processing enzymes (102). In subclones of the CRC cell line HCT-8 the heterozygous single nucleotide deletion in the poly(A)10 tract failed to confer additional deficiency to DNA DSB repair compared to the parental line (103). Accordingly, we found no alterations in the levels of protein in the MSI sample presenting this mutation, however additional experiments would be necessary to rule out the functional role of this mutation in gliomas.

A paediatric glioblastoma presented a homozygous single nucleotide insertion in the poly(C)8 tract of *MSH6* exon 5. This mutation results in a truncated protein and accordingly, we saw absence of protein expression in the mutated sample. Also, it is thought that *MSH6* represents a preferential target for somatic mutations in *MSH6* germline mutation carriers (104), a topic previously discussed.

MRE11 is a member of the MRE11/NBS1/RAD50 (MNR) complex, which is involved in the recognition and repair of DSB through HR or NHEJ, and so is essential for the maintenance of DNA integrity. Large mono- or biallelic deletions in the poly(T)11 of the *MRE11* intron 4 cause aberrant splicing, skipping of exon 5, introduction of a premature stop codon and generation of a truncated protein with a strong reduction or absence of MRE11 expression (105). Absence of *MRE11* leads to a functional impairment of the MRN complex and DSB repair, ultimately resulting in the development of cancer. We found a heterozygous single base insertion in the poly(T)11 tract of *MRE11* in one medulloblastoma and three glioblastoma with MSI-positivity. This alteration had been previously found in the constitutional DNA of a patient with MSI-positive, MRE11 mutated CRC (105). As the role of

MRE11 poly(T)11/12 was unclear in MSI tumours, this alteration was further studied for functional consequences. We used Daudi, a described lymphoma cell line harbouring this genotype, as a model and observed that this alteration seems to be of no consequence for DNA damage repair response. Moreover, the screen of *MRE11* poly(T)11/12 in 72 healthy blood donors revealed an incidence of 6.9%, proving to be a previously unrecognised polymorphism. Therefore, *MRE11* poly(T)11/12 rather than a "passenger mutation", represents a polymorphism, without apparent functional consequences on DSB repair, but of unknown significance in brain tumour risk.

We believe that our studies provided a major contribution to this subject, as mutations in these genes have not been extensively studied in brain tumours before, and all the mutations that we found have not been previously described in these cancers. Meanwhile, new MSI target genes in alternative pathways are being described. Recently, Melo et al. (106) identified inactivating mutations by insertion of a nucleotide in the poly(A)7 coding microsatellite repeat on exon 32 of *XPO5* in the context of MSI-positivity in colorectal, gastric and endometrial cancers. XPO5 mediates precursor miRNA (pre-miRNAs) nuclear export and consequently, inactivation of this molecule traps pre-miRNAs in the nucleus, down-regulating mature miRNA levels in the cells and promoting tumourigenesis. Moreover, genes classically involved in brain tumour development, such as EGFR, are frequently and differentially mutated in MSI tumours (107-109). We hypothesise that, in MSI-positive brain tumours, a differential target gene inactivation from that which is classically found is present. In this perspective, a bioinformatic approach for the identification of glioma-specific MSI target genes would be of major importance to the research area.

It is widely accepted that MSI-positive colorectal cancers have a better prognosis that the MSS ones, but they are still life-threatening. It is hypothesised that the high-frequency of mutations might turn MMR-deficient cells more immunogenic (110) and that a consequent robust immune reaction might decrease tumour cells' potential for invasiveness and metastasis resulting in a better prognosis of these cancers (111). On the other hand, the MMR-deficient cells were found to be resistant to certain drugs currently used in the treatment of cancer. MMR function includes the mediation of response to certain forms of DNA damage-inducing agents, including alkylating agents (112). This is of high importance

for brain tumours as temozolomide is an alkylating agent and MMR deficiencies have been linked to resistance to this drug (113).

Overall, we identified the presence of MSI in a subset of medulloblastomas and paediatric high-grade gliomas associated with molecular alterations distinctive of this phenotype, suggesting a potentially new genetic pathway correlated with these brain tumours development. As MSI is associated in other tumour types to a differential response to chemotherapy, the presence of MSI even in a small subset of brain tumours may have important translational implications in these extraordinarily treatment refractory malignancies.

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6. Appendix

Paper I

Dorine A. Bax, Suzanne E. Little, Nathalie Gaspar, Lara Perryman, Lynley Marshall, Marta Viana-Pereira, Tania A. Jones, Richard D. Williams, Anita Grigoriadis, Gilles Vassal, Paul Workman, Denise Sheer, Rui M. Reis, Andrew D. J. Pearson, Darren Hargrave, Chris Jones. Molecular and Phenotypic Characterization of Paediatric Glioma Cell Lines as Models for Preclinical Drug Development. *PLoS ONE* 4(4): e5209, 2009.

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Molecular and Phenotypic Characterisation of Paediatric Glioma Cell Lines as Models for Preclinical Drug Development

Dorine A. Bax[1☯], Suzanne E. Little[1☯], Nathalie Gaspar[1,2,3], Lara Perryman[1], Lynley Marshall[1,2,4], Marta Viana-Pereira[1,5], Tania A. Jones[6], Richard D. Williams[1], Anita Grigoriadis[7], Gilles Vassal[3], Paul Workman[3], Denise Sheer[6], Rui M. Reis[5], Andrew D. J. Pearson[1,4], Darren Hargrave[4], Chris Jones[1]*

1 Paediatric Oncology, The Institute of Cancer Research, Sutton, United Kingdom, **2** Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Sutton, United Kingdom, **3** Pharmacology and New Treatments of Cancer, Institut de Cancérologie Gustave Roussy, Villejuif, France, **4** Paediatric Oncology, The Royal Marsden NHS Foundation Trust, Sutton, United Kingdom, **5** Life and Health Science Research Institute (ICVS), Universidade do Minho, Braga, Portugal, **6** Neuroscience Centre, Institute of Cell and Molecular Science, Bart's and The London School of Medicine & Dentistry, London, United Kingdom, **7** Breakthrough Breast Cancer Unit, Guy's Hospital, London, United Kingdom

Abstract

Background: Although paediatric high grade gliomas resemble their adult counterparts in many ways, there appear to be distinct clinical and biological differences. One important factor hampering the development of new targeted therapies is the relative lack of cell lines derived from childhood glioma patients, as it is unclear whether the well-established adult lines commonly used are representative of the underlying molecular genetics of childhood tumours. We have carried out a detailed molecular and phenotypic characterisation of a series of paediatric high grade glioma cell lines in comparison to routinely used adult lines.

Principal Findings: All lines proliferate as adherent monolayers and express glial markers. Copy number profiling revealed complex genomes including amplification and deletions of genes known to be pivotal in core glioblastoma signalling pathways. Expression profiling identified 93 differentially expressed genes which were able to distinguish between the adult and paediatric high grade cell lines, including a number of kinases and co-ordinated sets of genes associated with DNA integrity and the immune response.

Significance: These data demonstrate that glioma cell lines derived from paediatric patients show key molecular differences to those from adults, some of which are well known, whilst others may provide novel targets for evaluation in primary tumours. We thus provide the rationale and demonstrate the practicability of using paediatric glioma cell lines for preclinical and mechanistic studies.

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* E-mail: chris.jones@icr.ac.uk

☯ These authors contributed equally to this work.

Introduction

Paediatric high grade glioma is a clinically devastating and biologically under-studied cancer of the central nervous system. The poor clinical outcome of these children is largely due to the intrinsic drug resistance of these tumours coupled with a lack of understanding of the basic molecular pathology of the disease [1].

Unlike adults, little is known about the mechanisms of tumorigenesis of glioblastoma in children. *TP53* mutation and PDGFRα overexpression, alterations associated with secondary glioblastoma in adults, have been reported at high frequencies in paediatric cases, [2], however these tumours rarely originate from pre-existing low grade lesions [3]. Furthermore, although there are clear morphological similarities between high grade gliomas of all ages, the WHO classification system is based upon adult cases, and may not be truly representative of the histopathological diversity of the childhood disease [4]. Firm conclusions are hampered by the limited number of studies performed on small sample sizes.

Drug treatment efficacy also differs. In adult glioblastoma, introduction of combined chemoradiotherapy of concomitant and adjuvant temozolomide (TMZ) and radiotherapy has provided a modest survival benefit [5], particularly in patients with an epigenetically silenced O^6-methylguanine-DNA-methyltransferase (*MGMT*) gene [6]. In children, hypermethylated *MGMT* promoter predicts for response to alkylating agents [7], however the survival of children treated with adjuvant temozolomide does not appear to

be improved when compared with historical controls [8–11]. Similarly, the disappointing results obtained in early phase clinical trials by the use of inhibitors directed against EGFR [12] and PDGFR [13] are likely in part due to inadequate patient selection, although as yet there are no validated markers predictive of response in high grade gliomas, and it is unclear whether there are differences in the paediatric setting.

The mechanisms of drug resistance in paediatric high grade glioma are poorly understood, in part due to the limited availability of suitable models of the disease. Nearly all preclinical and functional work is carried out in a relatively small panel of adult glioblastoma cell lines *in vitro*, and it is currently unclear how representative these are of the underlying biology of paediatric lesions.

In order to begin to address these questions, we have carried out a detailed molecular and phenotypic characterisation of a series of paediatric high grade glioma cell lines in comparison to routinely used adult lines. Through an integrated profiling approach, we demonstrate that glioma cell lines derived from paediatric patients show key molecular differences to those from adults, some of which are well known, whilst others may provide novel targets for evaluation in primary tumours. We thus provide the rationale for using paediatric high grade glioma cell lines for preclinical and mechanistic studies.

Materials and Methods

Cell culture

Adult glioblastoma cell lines, A172, LN229, SF268, U87MG, U118MG and U138MG were obtained from ATCC (LGC Promochem, Middlesex, UK). Paediatric glioblastoma KNS42 cells were obtained from the JCRB (Japan Cancer Research Resources) cell bank. Paediatric SF188 cells were kindly provided by Dr Daphne Haas-Kogan (UCSF, San Francisco, CA, USA), whilst UW479, Res259 and Res186 were kindly provided by Dr Michael Bobola (University of Washington, Seattle, WA, USA). They were grown as monolayers in DMEM/F12 Ham's medium+10% FCS in 5% CO_2.

Immunohistochemistry

Cells were grown to 70–80% confluency in chambered slides (Nunc, Rochester, NY, USA) fixed in either 4% paraformaldehyde:0.5% TritonX-100 in PBS (GFAP, synaptophysin, vimentin, nestin and CD133) or ice cold methanol (S100). The cells underwent a peroxidase block (Dako, Ely, UK) followed by washes in 0.05%Tween in TBS prior to incubation with primary antibody – GFAP (clone 6F2, 1:50 dilution, Dako); synaptophysin (SY38, 1:10, Dako); vimentin (V9, 1:100, Dako), S100 (B32.1, 1:20, Abcam, Cambridge, UK), nestin (10C2, 1:400, Miltenyi Biotech, Bergisch Gladbach, Germany), or CD133 (W6B3C1), 1:40, Miltenyi Biotech). Staining was visualised by the Envision horseradish peroxidase system (Dako) according to the manufacturer's instructions.

Genomic analysis

500 ng of DNA was analysed on the Affymetrix GeneChip Human Mapping 250K Nsp and Sty arrays by the UCL Institute of Child Health Gene Microarray Centre, according to the manufacturer's protocol. The raw data were processed and genotypes called using the BRLMM method of the Affymetrix GTYPE package. Copy number ratios were calculated by the Affymetrix CNAT 4 algorithm, using quantile normalisation and a Gaussian smoothing bandwidth of 0.1 Mb. Normal references were provided by public data from an unmatched panel of 30 Caucasian female DNA samples, the Centre d'Etude du Polymorphisme Humain (CEPH) trio mothers, analysed on the same platform by The International HapMap Consortium (International HapMap Consortium 2007, http://www.hapmap.org). The copy number data set was compared to the positions of known copy number variations (CNVs) in the HapMap populations displayed on the UCSC genome browser and any copy number changes in the current data that overlapped with these CNVs were noted. LOH was inferred using the linkage disequilibrium Hidden Markov Model as implemented in dChip (http://www.dchip.org). False positives were reduced by excluding apparent LOH regions present in more than 10% of all 60 CEPH trio parents. LOH was taken as copy neutral if the copy number measured by dChip was close to diploid (1.7–2.3 copies). All data have been submitted according to MIAME guidelines [14] to the public data repository ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-TABM-579.

Multiplex fluorescence *in situ* hybridisation (M-FISH) analysis was performed on metaphase spreads prepared from Res259 cells using a Vysis SpectraVysion probe (Abbot Molecular, Abbott Park, IL, USA) following the manufacturer's instructions as previously described [15]. Images were captured using a Zeiss Axiophot microscope equipped for epifluorescence using cooled CCD-camera (Hamamatsu, Welwyn Garden City, UK). Image analysis was performed using SmartCapture X software (Digital Scientific, Cambridge, UK).

Fluorescent in situ hybridisation (FISH)

FISH analysis was carried out on metaphase chromosome preparations as previously described [16]. Probes directed against CDK4 (RP11-66N19,RP11-571M6), PDGFRA (RP11-231C18, RP11-626H4, RP11-117E8), and PTEN (RP11-105A10) were labelled with Cy5 (GE Healthcare, Amersham, UK), whilst commercial probes against RB1 (Abbot Molecular) and CDKN2A/B (Abbott Molecular) were SpectrumOrange-labelled. Appropriate centromeric probes were labelled with either fluorescein or SpectrumGreen. Hybridised preparations counterstained with DAPI in antifade (Vector Laboratories Inc., Burlingame, CA, USA). Images were captured using a cooled charge-coupled device camera (Photometrics, Tuscon, AZ, USA).

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)

Promoter methylation was assessed by kits ME001, ME002 and ME011 according to manufacturer's instructions (MRC-Holland, Amsterdam, Netherlands) [17]. Briefly, 100 ng of tumour DNA was denatured in 5 μL of Ultra-Pure Water at 98°C for 5 min and then incubated with the probe mix for 20 h at 60°C. After probe hybridization, each sample was divided into two different tubes. Half of the sample was ligated using a ligase enzyme and in the other half ligation was combined with *Hha*I digestion resulting in ligation of the methylated sequences only. The resulting products were amplified by PCR using a FAM-labeled primer, following manufacturer's instructions. PCR products were analyzed on ABI Prism 310 single capillary genetic analyzer (Applied Biosystems) using the GeneScan 3.7 software (Applied Biosystems). Duplicate experiments were performed for methylation analysis and average ratios were calculated. We interpreted ratios as absence of hypermethylation (0.00–0.24), mild hypermethylation (0.25–0.49), moderate hypermethylation (0.50–0.74), and extensive hypermethylation (≥0.75), as previously described [18].

Western blot analysis

Cells at 80% confluence were trypsinised, washed with PBS and lysed for 1 hr at 4°C in lysis buffer (Cell Signaling, Hertfordshire,

UK) and a complete mini protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cells were then centrifuged at 11,000 rpm at 4°C for 15 min, and protein concentration determined (Pierce BCA assay, Rockford, IL, USA). Total protein extracts (30 μg/lane) were separated electrophoretically in 4–20% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Invitrogen Ltd, Paisley, UK). Immunodetection was performed using antibodies against cRAF (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), phosphorylated and total Erk1/2, Akt, GSK3β and S6 (all 1:1000, Cell Signaling), and GAPDH (1:2000, Chemicon, Hampshire, UK). Blots were revealed with peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (GE Healthcare, Amersham, UK) followed by ECL chemiluminescence solution (GE Healthcare).

mRNA expression profiling

10 μg of total RNA was used to generate labelled cRNA and hybridised to U133Plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA) containing more than 40,000 transcripts by the UCL Institute of Child Health Gene Microarray Centre according to standard manufacturer protocols. Probe intensity data for each array was normalized to a baseline array with median signal intensity using the "invariant set" model. Model-based expression was performed using the perfect-match/mismatch (PM/MM) model to summarize signal intensities for each probe set. All data have been submitted according to MIAME guidelines [14] to the public data repository ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-TABM-579. For the supervised identification of genes exhibiting differential expression between paediatric and adult high grade cell lines, expression values were subjected to a variation filter to exclude probe sets with minimal variation across the samples, and an absolute signal to noise metric of greater than 1.5 was subsequently used to identify marker probe sets using GenePattern software (http://www.broad.mit.edu/cancer/software/genepattern/);. Differentially expressed co-ordinate sets of genes were identified using Gene Set Enrichment Analysis (GSEA, www.broad.mit.edu/gsea/) with a nominal p value cut-off of 0.001.

Real-time quantitative RT-PCR

cDNA was prepared from 1 μg of tumour or reference RNA by random primed reverse transcription using Superscript II (Invitrogen, Paisley, UK). TaqMan® Gene Expression Assays were obtained from Applied Biosystems (Warrington, UK) for LYN (Hs00176719), CRKL (Hs00178304), AXL (Hs00242357) and EPHA6 (Hs00297133). PCRs were performed in a 10 μl reaction volume containing 5 μl 2× buffer/enzyme mix, 0.5 μl 20× assay mix, 0.5 μl 20× GAPDH endogenous control assay mix (Hs99999905) and 1 μl input cDNA. Assays were run on an Applied Biosystems 7900 Sequence Detection System and results analysed by the standard curve method. Data were normalised to Universal Human Reference RNA (Stratagene, La Jolla, California, USA).

Results

Paediatric glioma cell lines grown as monolayers in vitro express astrocytic and stem cell markers

All paediatric glioma cell lines were derived from astrocytomas of differing grades arising in patients aged 3–16 years old. They were established *in vitro* as monolayer cultures, with doubling times of between 24–48 hours, and demonstrated a mixture of stellate and bipolar morphologies, with some cells of polygonal, cuboidal or flattened appearance (Table 1). The astrocytic nature of the cells was confirmed in culture by immunohistochemistry with a range of glial markers (Figure 1). All cells showed some degree of GFAP-positivity, although SF188 and Res259 were only weakly expressing. All cells were strongly positive for S100 and vimentin, with the exception of UW479, which was vimentin negative. There was little synaptophysin expression, save for the occasional isolated cell in SF188 and KNS42 cultures. There was a surprisingly high level of expression of stem cell markers in the paediatric glioblastoma lines, with both SF188 and KNS42 strongly positive for nestin, and containing 7% and 4% CD133-positive cells respectively.

Genomic profiling reveals numerous chromosomal abnormalities and focal amplifications/deletions

Affymetrix 500K SNP array analysis revealed all paediatric glioma cell lines to display complex genomes with numerous gross chromosomal copy number abnormalities and rearrangements. After exclusion of known copy number variants, this high-resolution copy number profiling additionally highlighted a number of focal copy number aberrations, which were confirmed by FISH on metaphase preparations (Figure 2). The glioblastoma cell line SF188 harboured high-level amplifications of several oncogenes known to be present in paediatric glioblastoma tumour samples. These included *MYC* at 8q24, *CCND1* at 11q13, *CDK4* at 12q14, all of which were due to extrachromosomal double minute formation, and overexpression of the corresponding proteins was

Table 1. Characteristics and immunophenotype of paediatric glioma cells grown as monolayers.

Cell line	WHO grade	Diagnosis	Age	Sex	Morphology	Doubling time	Immunohistochemistry					
							GFAP	S100	Vimentin	Synapto-physin	Nestin	CD133
SF188	IV	Glioblastoma multiforme	8 yrs	male	stellate, polygonal, bipolar	26 hrs	++	++	+++	++		7%
KNS42	IV	Glioblastoma multiforme	16 yrs	male	cuboidal, polygonal, bipolar	48 hrs	+++	+++	+++	++		4%
UW479	III	Anaplastic astrocytoma	13 yrs	female	polygonal, stellate	28 hrs	+++	+++	−	+		1%
Res259	II	Diffuse astrocytoma	4 yrs	female	polygonal, cuboidal, bipolar	24 hrs	+	++	+++	−		0%
Res186	I	Pilocytic astrocytoma	3 yrs	female	stellate, flattened, bipolar	46 hrs	+++	++	+++	−		0.1%

The astrocytic nature of the cells was confirmed in culture by immunohistochemistry with a range of glial and stem cell markers. (+++) strongly positive; (++) moderately positive; (+) weakly positive; (−) negative.

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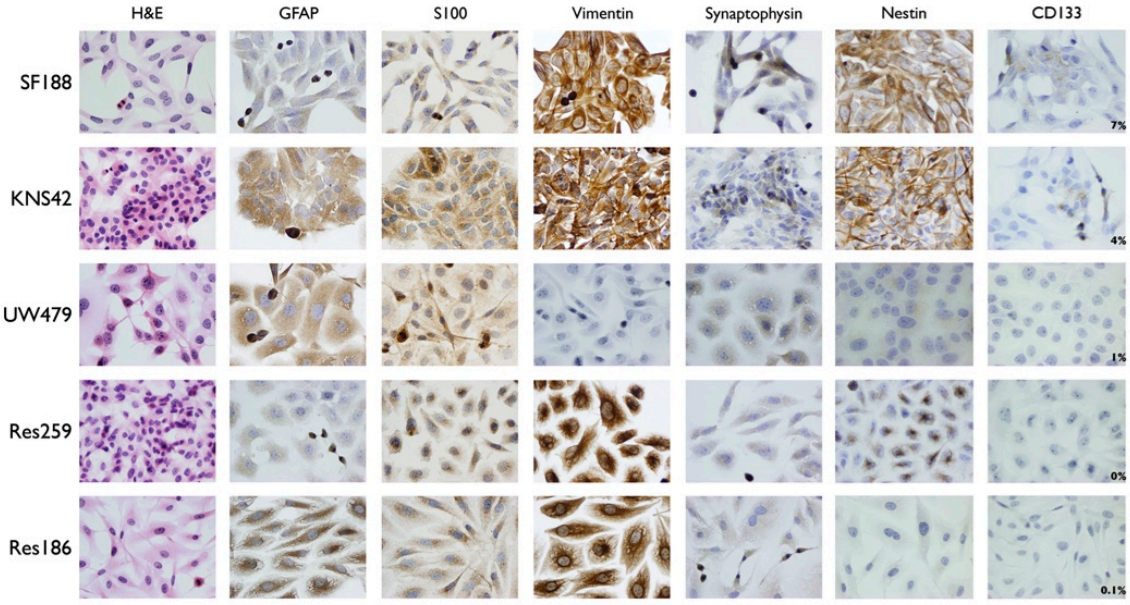

Figure 1. Immunophenotyping of paediatric glioma cell lines. All lines were grown as monolayers and stained with a variety of glial and stem cell markers including glial fibrillary acidic protein (GFAP), S100, vimentin, synaptophysin, nestin and CD133. H&E – haematoxylin and eosin. All images original magnification ×400.
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confirmed by Western blotting (data not shown). There was furthermore a focal deletion of *NF1* at 17q11.2. KNS42 glioblastoma cells, by contrast, contained no genuine amplifications, but instead its highly rearranged genome harboured low-level copy number gains at loci such as 3q26 (*PIK3CA*), and hemizygous deletions at known tumour suppressor loci such as 13q14 (*RB1*). FISH analysis confirmed that this was due to the loss of a single copy from an otherwise triploid genome. Homozygous deletion at the *CDKN2A/B* locus at 9p12 was observed in the anaplastic astrocytoma UW479 line, which additionally harboured numerous high level copy number changes including 3p11-p12, 6p21, 18p11 and 19q12. *CDKN2A/B* deletion was also observed in Res259 astrocytoma cells, which also contained the well-described 4q12 amplicon, resulting in high level gains of the oncogenes *PDGFRA* and *KIT*, and low-level gain of a region including *KDR/VEGFR2*. This was found from FISH and M-FISH analysis to be due to an unbalanced translocation between chromosomes 4 and 19, resulting in a der(19)t(4;19). Finally, Res186, originally derived from a pilocytic astrocytoma, harboured the least complex genomic profile, although was found to have an intragenic homozygous deletion of *PTEN* at 10q23.

Copy neutral and epigenetic events in paediatric high grade glioma cells

In addition to the copy number changes, we also observed several copy neutral loss of heterozygosity (LOH) events (Figure 2). KNS42 cells exhibited focal copy neutral LOH at 19p13 (*KLF2*) and 21q21 (*HEMK2*), whilst larger regions were observed in UW479 cells at chromosome 2q11-qter and in Res186 at 7p11-pter and 17p13 (*TP53*). Although the significance of these events is not clear, it is expected that such loci may harbour genes in which the remaining allele may harbour a point mutation, as is the case for *TP53*.

In order to explore epigenetic events in these lines we carried out methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) on 39 genes known to be hypermethylated at CpG islands in human cancer. Certain genes such as *RASSF1A* and *ASC* were found to be methylated in all cell lines tested (Table 2). There was specific promoter hypermethylation in *FHIT* in SF188 and Res259, *HIC1* in Res259 and *TP73* in Res186 cells. Interestingly, UW479 additionally harboured extensive promoter hypermethylation at a host of genes assayed including *APC*, *CASP8*, *CD44*, *CDH13*, *CHFR*, *ESR1*, *GSTP1*, *IGSF4*, *MGMT*, *PAX5A*, *PAX6*, and *RARB*. Of direct relevance to clinical resistance to alkylating agents were the observations of methylation in the mismatch repair gene *MLH3* in Res259 cells, which also harboured high levels of promoter hypermethylation of *MGMT*, along with KNS42 cells. Low to moderate *MGMT* methylation was also observed in UW479 and Res186 cells.

Differential constitutive activation of PI3K, MAPK and downstream pathways

Protein expression was measured by Western blot to examine constitutive pathway activation of several key signalling transduction networks of importance in gliomagenesis (Figure 3). There was a modest activation of the MAPK pathway as determined by levels of phosphorylated Erk 1/2 in the paediatric high grade lines SF188 and UW479 as compared to KNS42 and the low grade lines (Res259, Res186). Although Akt pathway activation was seen in all paediatric cells, this was considerably lower than that observed in our panel of adult glioblastoma lines, except in the *PTEN* null line Res186, which showed high levels of phospho-Akt. Phosphorylated GSK3β levels were low in UW479, Res259 and Res186, and almost entirely absent in the glioblastoma lines SF188 and KNS42. Although there was some degree of ribosomal protein

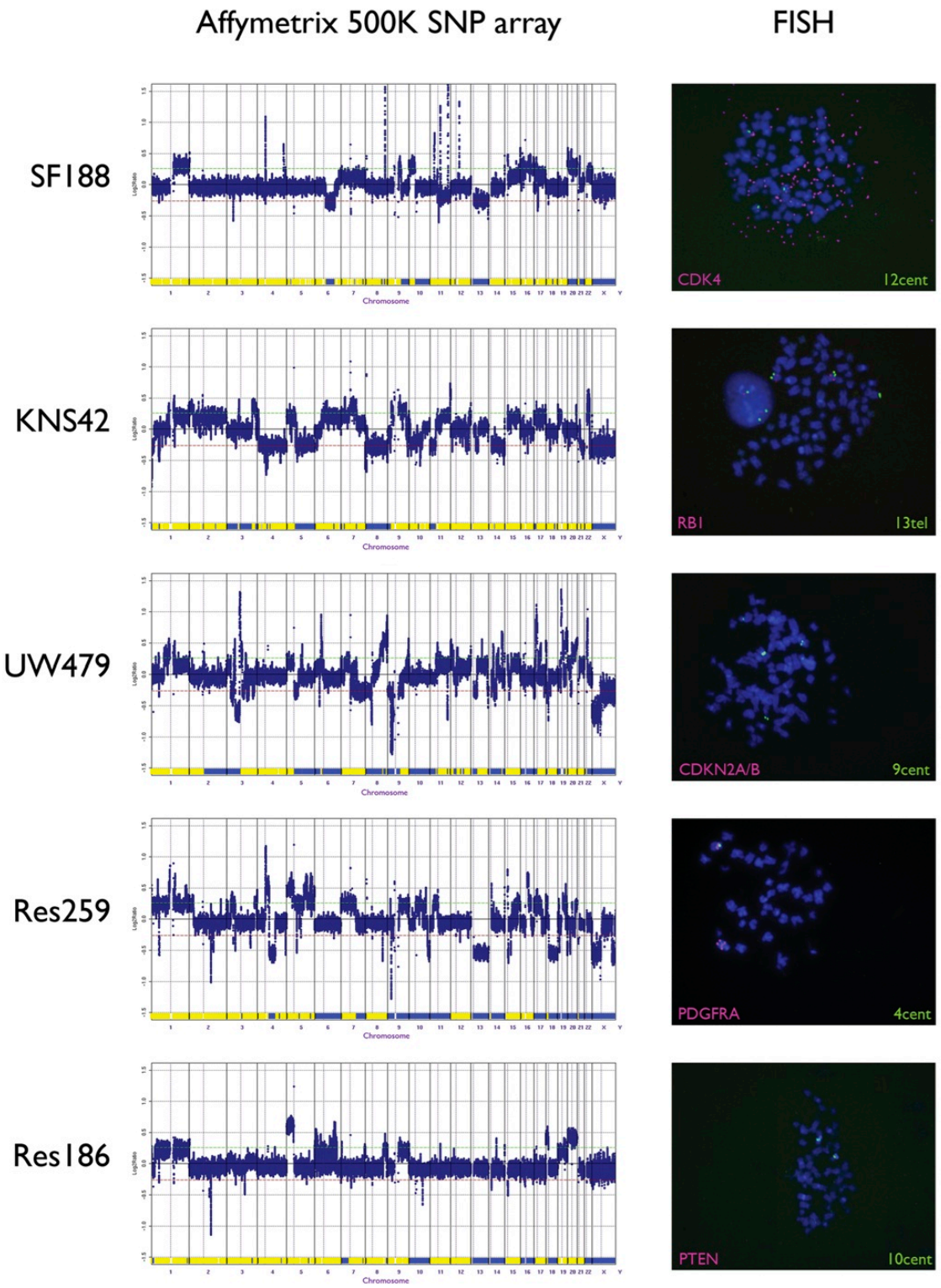
Affymetrix 500K SNP array
FISH
SF188
KNS42
UW479
Res259
Res186
Chromosome
CDK4
12cent
RB1
13tel
CDKN2A/B
9cent
PDGFRA
4cent
PTEN
10cent

Figure 2. Genomic profiling of paediatric glioma cell lines. Copy number and loss of heterozygosity (LOH) profiles were generated by Affymetrix 500K SNP arrays. Log_2 ratios are plotted (y axis) for each probeset according to chromosomal location (x axis). Loss (blue) and retention (yellow) of heterozygosity is depicted in the lower portion of the plots. Fluorescent *in situ* hybridisation validation of selected copy number changes is represented with clones for *CDK4*, *RB1*, *CDKN2A/B*, *PDGFRA* and *PTEN* (ICy5/SpectrumOrange) and appropriate centromeres (fluorescein/SpectrumGreen).
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Table 2. Methylation-specific multiplex ligation-dependent probe amplification of 39 genes in paediatric glioma cell lines.

Gene	SF188	KNS42	UW479	Res259	Res186
ASC	52%	66%	52%	81%	100%
APC	2%	2%	100%	1%	2%
ATM	0%	2%	1%	1%	0%
BRCA1	1%	1%	2%	3%	3%
BRCA2	0%	0%	0%	0%	0%
CASP8	1%	3%	71%	1%	3%
CD44	0%	2%	48%	1%	0%
CDH13	2%	24%	94%	4%	0%
CDKN1B	0%	0%	0%	0%	0%
CDKN2A	2%	1%	**DEL**	**DEL**	3%
CDKN2B	3%	4%	**DEL**	**DEL**	4%
CHFR	0%	1%	81%	0%	0%
DAPK1	5%	0%	0%	0%	0%
ESR1	25%	51%	31%	74%	55%
FHIT	62%	0%	0%	56%	2%
GATA5	10%	0%	14%	86%	94%
GSTP1	3%	5%	75%	0%	70%
HIC1	2%	2%	7%	92%	0%
IGSF4	0%	0%	44%	0%	0%
MGMT	11%	79%	26%	63%	57%
MLH1	1%	1%	1%	1%	2%
MLH3	0%	0%	0%	70%	0%
MSH2	0%	0%	0%	0%	0%
MSH3	0%	0%	3%	5%	7%
MSH6	0%	0%	0%	7%	0%
PAX5A	43%	46%	99%	53%	100%
PAX6	0%	0%	100%	0%	0%
PMS2	0%	0%	3%	3%	0%
PTEN	4%	1%	1%	2%	**DEL**
RARB	5%	4%	56%	2%	0%
RB1	5%	0%	0%	0%	23%
RASSF1A	95%	90%	100%	100%	100%
STK11	0%	2%	0%	0%	0%
THBS1	5%	9%	9%	10%	0%
TIMP3	0%	16%	0%	1%	2%
TP53	1%	4%	3%	1%	22%
TP73	0%	0%	0%	0%	43%
VHL	0%	0%	0%	0%	0%
WT1	0%	88%	7%	69%	21%

Values give percentage methylation at CpG islands in the genes promoter.
DEL = gene deletion. n.d = not done.
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S6 activation in all lines, this was particularly pronounced in KNS42 and Res259, with very high levels of phospho-S6. Taken together, although variable, we observed constitutive activation of one or more of these signal transduction pathways in all the paediatric glioma lines.

Expression signatures of functionally relevant genes distinguishes between high grade paediatric and adult glioma cell lines

In order to investigate the differences between the paediatric- and adult-derived cell lines at the gene expression level, we carried out expression profiling using Affymetrix U133 Plus2.0 arrays. For the comparison, we excluded the lines Res259 and Res186, derived from low-grade tumours, in order to avoid the possible

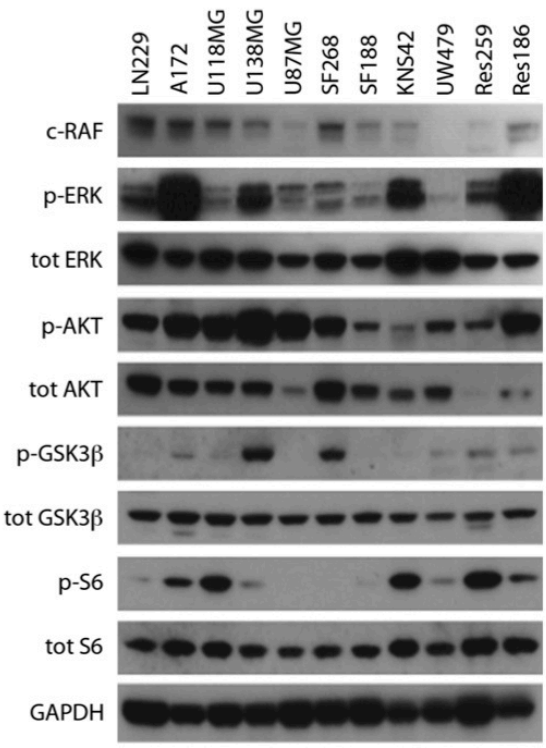

Figure 3. Constitutive activation of key signalling pathways in glioma cell lines. Western blots analysis of c-Raf, phospho/total Erk1/2, phospho/total Akt, phospho/total GSK3β, phospho/total S6 and GAPDH as loading control in adult (LN229, A172, U118MG, U138MG, U87MG, SF268) and paediatric (SF188, KNS42, UW479, Res259, Res186) glioma cell lines.
doi:10.1371/journal.pone.0005209.g003

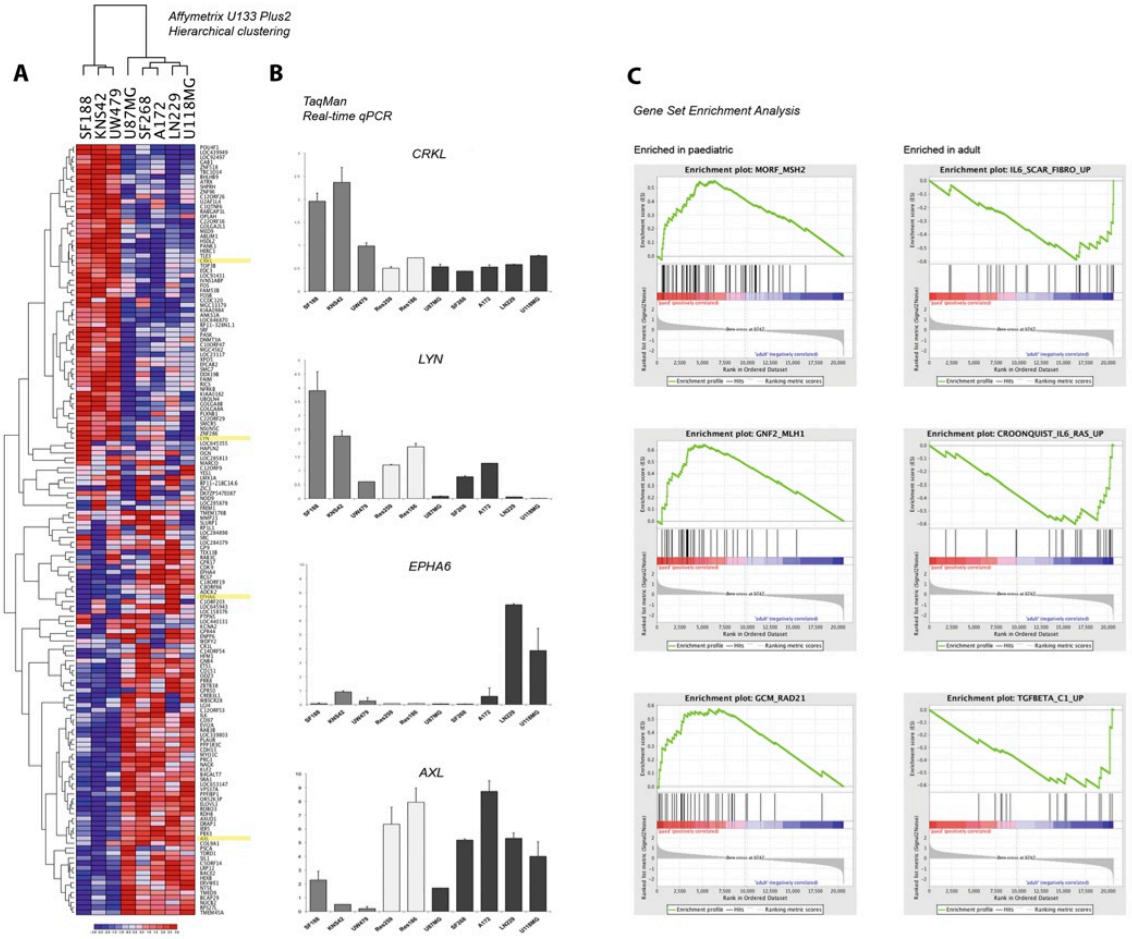

Figure 4. Expression profiling of paediatric and adult glioblastoma cell lines. (A) Heatmap demonstrating hierarchical clustering of 93 differentially expressed genes between paediatric (SF188, KNS42, UW479) and adult (LN229, A172, U118MG, U87MG, SF268) high grade glioma cell lines. (B) Quantitative real-time (TaqMan) RT-PCR confirming differential expression of *CRKL*, *LYN*, *EPHA6* and *AXL*. Expression values are plotted relative to Universal Human Reference RNA. (C) Gene Set Enrichment Analysis highlighting co-ordinated differential expression of gene sets defined *a priori*. Enriched in paediatric high grade glioma cell lines - MORF_MSH2, GNF2_MLH1, GCM_RAD21, DNA_replication_reactome; enriched in adult lines IL6_SCAR_FIBRO_UP, CROONQUIST_IL6_RAS_UP, TGFBETA_C1_UP. Nominal p value<0.001.
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confounding factors of grade. Despite the small number of lines profiled, we were able to identify 93 genes significantly differentially expressed between paediatric and adult high grade glioma cell lines (Figure 4A). The differentially expressed genes included several kinases which were validated by quantitative real-time RT-PCR (Figure 4B). These included members of the Src family kinases - *LYN* and the adaptor molecule *CRKL*, highly expressed in paediatric lines, along with *SRC* itself and also *YES1*. Also upregulated in the paediatric *vs* adult lines were several early response genes including *FOS* and *FOSB*. Highly expressed in the adult lines were the receptor tyrosine kinases *EPHA6* and *AXL*, and also *CDK9* and *ILK*.

In addition, gene set enrichment analysis (GSEA) highlighted co-ordinated differentially high expression of several gene lists associated with maintenance of DNA replication and repair in paediatric *vs* adult cell lines (MORF_MSH2, GNF2_MLH1, GCM_RAD21, DNA_replication_reactome) (Figure 4C). Furthermore, gene sets representing co-ordinated regulation of extracellular matrix protein in response to cytokines were found enriched in the adult compared to the paediatric lines (IL6_SCAR_FIBRO_UP, CROONQUIST_IL6_RAS_UP, TGFBETA_C1_UP). Although the nominal p values of these analyses was less than 0.001, q values for estimates of the false discovery rate were all greater than 0.9, presumably due to the small sample size.

Discussion

Understanding and exploiting the similarities and differences between adult and paediatric gliomas is an important strategy for the development of new targeted therapies. The intrinsic drug resistance of high grade gliomas represents a major challenge in the clinical management of children with the disease, and efforts to

understand and overcome this are hampered by a lack of mechanistic data in paediatric glioma models. We have sought to address this by collecting and carrying out a detailed molecular and phenotypic characterisation of the few available paediatric glioma cell lines, with a view to incorporating them into ongoing drug development programmes.

There is an acute clinical need to develop better treatment strategies for the refractory high grade gliomas in childhood, and having lines representative of paediatric glioblastoma (WHO grade IV – SF188 and KNS42) and anaplastic astrocytoma (WHO grade III – UW479), would be extremely useful in preclinical and mechanistic studies of the disease. These lines all grew as either stellate or bipolar cells, with some polygonal features, in monolayers, and the immunophenotypes were representative of high grade glial tumours, including expression GFAP, S100 and vimentin. There were slight discrepancies with a previous publication referring to some of these lines [19], with a greater degree of S100 staining in UW479, and less GFAP in Res259 in our study, however it unclear whether this is reflective of methodological differences, culture conditions or changes in gene expression over time.

It is of considerable interest to note the relative high levels of CD133 positive cells in both SF188 (7%) and KNS42 (4%). Intrinsic drug resistance in the proportion of clinical glioblastomas comprised of tumour stem cells has been suggested to play a key role in drug resistance in this disease, and the high CD133-positive content of these cells grown as monolayers may make them particularly relevant in preclinical drug screening programmes.

All three high grade lines had highly complex genomic profiles and harboured amplifications and deletions at several known cancer genes dysregulated in paediatric glioma, including *CCND1*, *MYC*, *CDK4*, *PIK3CA*, *CDKN2A/B*, and *RB1* [2,20–23]. Although there are no molecular alterations yet described that are diagnostic for paediatric high grade glioma, these genes cover a broad spectrum of reported genomic aberrations. In addition, large scale chromosomal imbalances such as gain of chromosomes 1q and 7, and losses of 10q and 13q, reported at high frequencies in primary tumours [24], are present within these cell lines.

There were contrasting genomic profiles in the two paediatric glioblastoma lines. SF188 harboured an amplifier phenotype, with coordinated amplification of *CCND1* and *CDK4* evidence of a lack of cell cycle control by virtue of dysregulation of the RB1 pathway. By contrast, KNS42 cells achieved a similar end through direct deletion of *RB1* itself. No *RB1* hypermethylation was detected. Simultaneous abrogation of core signalling pathways in glioblastomas, as recently reported for primary tumours [25,26], are in evidence in both these cell lines, with further disruption to the p53 pathway (through point mutations of *TP53* in both lines), and the RTK/PI3K/AKT pathway via *NF1* deletion in SF188 and *PIK3CA* copy number gain in KNS42.

Interestingly, UW479 cells, as well as harbouring numerous high level amplifications, also had extensive promoter hypermethylation at a variety of epigenetically regulated genes, including *APC*, *CASP8*, *CD44*, *CDH13*, *CHFR*, *ESR1*, *GSTP1*, *IGSF4*, *MGMT*, *PAX5A*, *PAX6*, and *RARB*. They also had, in common with all the lines tested, methylated *RASSF1A* and *TMS1* genes. *RASSF1A* promoter methylation is one of the most common molecular changes in cancer, and may modulate multiple apoptotic and cell cycle checkpoint pathways [27]. *TMS1/ASC* is an intracellular signaling molecule with proposed roles in the regulation of apoptosis, nuclear factor-κB activation, and cytokine maturation, and has been found to be hypermethylated in approximately 40% of adult glioblastoma cell lines and tumours [28].

Of potential significance in preclinical drug screening was the observation that UW479 and Res186 cells to harbour methylated GSTP1. Glutathione-S-transferase π acts to enzymatically conjugate glutathione to the reactive metabolites of alkylating agents, and up-regulation in glioblastoma tumour specimens has been correlated with resistance to BCNU [29]. Of further direct translational relevance was the observation that KNS42 cells were found to have methylation in the *MGMT* promoter, but counter to the expectation in glioblastoma cells, this did not confer a sensitivity to *in vitro* treatment with the alkylating agent temozolomide [30].

Two of the cell lines (Res259 and Res186) were reported to be diffuse astrocytoma (WHO grade II) and pilocytic astrocytoma (WHO grade I), which would make them extremely valuable *in vitro* models. Due to the difficulty in culturing these lesions successfully, and the relatively stable genomes reported in the human disease [31–33], it is likely that these lines may have acquired genetic alterations in culture. Interestingly, both lines harboured genetic alterations reminiscent of high grade gliomas in humans - including the archetypal 4q12 amplification spanning *PDGFRA* and *KIT* in Res259, and *PTEN* deletion in Res186. Despite this, their immunophenotypes more closely resembled low grade lesions, with an absence of synaptophysin expression, in contrast to the occasional positive cells seen in the high grade lines, and higher grade tumours in humans [34]. Whether or not they are truly reflective of the lesion from which they were derived, they are likely to make excellent models for the study of specific pathway modulation, *i.e.* that of the receptor tyrosine kinase and PI3K/PTEN systems. This is of particular importance, as the paediatric high grade lines were all *PTEN* wild-type, and there was significantly lower constitutive levels of phosphorylated Akt in these cells than a selection of the commonly used adult glioblastoma lines.

Similarly, constitutive activation of additional key signalling pathways would further increase the usefulness of the cell line panel for drug development. The MAPK pathway is represented in the KNS42 glioblastoma cells, which otherwise has little constitutive activation of Akt. High levels of phosphorylated S6 in both KNS42 and Res259 cells shows a significant activation of the mTOR pathway, potentially independent of upstream PI3K/Akt signalling. mTOR is emerging as a therapeutic target in human gliomas [35], and extending this to the paediatric setting will be facilitated by the use of these models for screening mTOR inhibitors, alone and in combination strategies.

Global measures of gene expression also served to highlight the differing genetic background of the paediatric lines. The differential expression of diverse kinases including *LYN*, *CRKL*, *EPHA6* and *AXL* between the high grade glioma cells derived from patients of different ages suggests a number of potential new drug targets, and provides impetus for molecular pathology investigations to confirm their presence *in vivo*. Two such observations are the overrepresentation of genes associated with DNA integrity in the paediatric cells, and underrepresentation of those associated with the immune response. As both of these areas are under intense research for the development of novel therapeutic agents in gliomas [36–40], measurement of the specific elements responsible for their modulation is needed in order to ensure the most appropriate model is utilised for mechanistic and preclinical assessment.

The paediatric lines have already proven their usefulness *in vitro* with studies identifying differential drug sensitivity and mechanisms of action/resistance [19,30,41,42]. As an adjunct to this, we are currently establishing and profiling them as *in vitro* neurosphere cultures, and *in vivo* as xenografts in immunodeprived mice. It is

clear, however, that to fully represent the heterogeneity present in human glioma [25], and particularly those derived from children, additional well-characterised models will be required if we are to reliably test novel agents aimed at treating these diverse, clinically refractory tumours.

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Author Contributions

Conceived and designed the experiments: PW DH CJ. Performed the experiments: DB SL NG LP LM MVP TJ RW. Analyzed the data: DB SL NG LP LM MVP TJ RW AG GV PW DS RR AP CJ. Contributed reagents/materials/analysis tools: GV PW DS RR AP DH. Wrote the paper: CJ.

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Paper II

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Human Cancer Biology

Clinical Cancer Research

A Distinct Spectrum of Copy Number Aberrations in Pediatric High-Grade Gliomas

Dorine A. Bax[1], Alan Mackay[3], Suzanne E. Little[1], Diana Carvalho[1,6,7], Marta Viana-Pereira[1,6], Narinder Tamber[3], Anita E. Grigoriadis[4], Alan Ashworth[3], Rui M. Reis[6], David W. Ellison[8], Safa Al-Sarraj[5], Darren Hargrave[2], and Chris Jones[1,2]

Abstract

Purpose: As genome-scale technologies begin to unravel the complexity of the equivalent tumors in adults, we can attempt detailed characterization of high-grade gliomas in children, that have until recently been lacking. Toward this end, we sought to validate and extend investigations of the differences between pediatric and adult tumors.

Experimental Design: We carried out copy number profiling by array comparative genomic hybridization using a 32K bacterial artificial chromosome platform on 63 formalin-fixed paraffin-embedded cases of high-grade glioma arising in children and young people (<23 years).

Results: The genomic profiles of these tumors could be subclassified into four categories: those with stable genomes, which were associated with a better prognosis; those with aneuploid and those with highly rearranged genomes; and those with an amplifier genotype, which had a significantly worse clinical outcome. Independent of this was a clear segregation of cases with 1q gain (more common in children) from those with concurrent 7 gain/10q loss (a defining feature of adults). Detailed mapping of all the amplification and deletion events revealed numerous low-frequency amplifications, including *IGF1R*, *PDGFRB*, *PIK3CA*, *CDK6*, *CCND1*, and *CCNE1*, and novel homozygous deletions encompassing unknown genes, including those at 5q35, 10q25, and 22q13. Despite this, aberrations targeting the "core signaling pathways" in adult glioblastomas are significantly underrepresented in the pediatric setting.

Conclusions: These data highlight that although there are overlaps in the genomic events driving gliomagenesis of all ages, the pediatric disease harbors a distinct spectrum of copy number aberrations compared with adults. *Clin Cancer Res; 16(13); 3368–77. ©2010 AACR.*

The use of genome-scale profiling techniques to identify the key genetic aberrations underlying various tumor types has led to fundamental discoveries about the drivers of oncogenesis, and provides the rationale for specific targeted therapies in these lesions. Until recently, the application of such studies to the fields of high-grade glioma specifically, and childhood tumors in general, have lagged behind the adult epithelial cancers. This is now rapidly changing, with large screens of adult glioblastoma through collaborative networks (1) or single institutions (2) joining an increasing number of smaller independent studies (3–7) in comprehensively mapping the glioblastoma genome.

There are also beginning to emerge genomic studies specifically addressing childhood cancers, and there is mounting evidence that the pediatric high-grade glioma genome has certain key differences with that of histologically similar adult tumors. An early study using metaphase comparative genomic hybridization (CGH; ref. 8) highlighted distinct chromosomal changes in 23 childhood cases, a result borne out in a later 10K single nucleotide polymorphism (SNP) array study of a further 14 high-grade tumors (9), and more recent studies of 18 pediatric glioblastoma on Illumina 100K arrays (10), and 20 high-grade tumors using molecular inversion probes (11). Most recently, we participated in a collaborative effort to carry out molecular profiling of 78 pediatric high-grade gliomas by Affymetrix 500K SNP and U133 Plus2.0 expression arrays (12). From all these studies, it seems clear that although there are many large-scale chromosomal and

Authors' Affiliations: [1]Section of Paediatric Oncology, The Institute of Cancer Research, and [2]Paediatric Oncology, Royal Marsden Hospital, Sutton, United Kingdom; [3]Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, and [4]Breakthrough Breast Cancer Unit and [5]Clinical Neuropathology, Kings College Hospital, London, United Kingdom; [6]Life and Health Sciences Research Institute, University do Minho, Braga, Portugal; [7]Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal; and [8]St Jude Children's Research Hospital, Memphis, Tennessee

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D.A. Bax and A. Mackay contributed equally to this work.

Corresponding Author: Chris Jones, Section of Paediatric Oncology, Institute of Cancer Research, Sutton, Surrey, SM2 5NG, United Kingdom. Phone: 44-0-20-8722-4416; Fax: 44-0-20-8722-4321; E-mail: chris.jones@icr.ac.uk.

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Translational Relevance

Pediatric high-grade gliomas represent clinically devastating and biologically understudied tumors of the central nervous system. Little is known about the key genomic alterations that arise in childhood cases, nor of the specific differences between these and the adult disease. We present the copy number profiling of a large series of these rare tumors, and identify numerous low-frequency events previously unreported in pediatric high-grade glioma, including the potential therapeutic target *IGF1R*. Tumors could be classified into distinct genomic subtypes, with marked differences in clinical outcome, and an idealized $PDGFRA^{amp}$, 1q+, 16q-genotype was considerably enriched in pediatric cases, in contrast to the $EGFR^{amp}$, 7+, 10q- cases more commonly associated with adults. We further highlight the importance of platelet-derived growth factor (PDGF) signaling in this context, through the most commonly observed genomic amplification of *PDGFRA*, as well as a unique amplification of *PDGFRB*, providing strong rationale for clinically targeting this pathway in children with this disease.

specific genetic amplification/deletion events common to tumors from patients of all ages, there are certain events found at significantly different frequencies in pediatric versus adult lesions.

One of the most immediately apparent differences was the high frequency of chromosome 1q gains and 16q losses, and the lower frequency of (often concurrent) gain of chromosome 7 and loss of 10q in childhood cases compared with adults. Although there were numerous low-frequency amplifications and deletions such as *MYC/MYCN*, *CCND2*, *KRAS*, and *CDKN2C*, which seemed to show the pediatric high-grade glioma genome to be similar to clinical secondary adult glioblastomas (13, 14), a lack of *IDH1* mutations in the childhood setting showed the distinct biological pathways active during pathogenesis (12).

The most common amplification in the pediatric cases was at 4q12, with shortest region of overlap (SRO) and expression analyses identifying the amplicon driver to be *PDGFRA* (12). This was present in up to 17% of primary pediatric glioblastoma, and 29% of diffuse intrinsic pontine glioma, and was also found in 50% of cases of high-grade glioma arising as a secondary malignancy after cranio-spinal radiation (post-IR). Many cases without *PDGFRA* amplification were still found to show overexpression of a specific *PDGFRA*-associated gene signature, which was itself distinct from that observed in adult cases with the 4q12 amplification. Taken together, platelet-derived growth factor (PDGF)-driven signaling seems to be preferentially activated in the majority of pediatric tumors, in contrast to adults, where epidermal growth factor receptor (EGFR) is implicated as the predominant target (12).

Although these studies are beginning to unravel the key features of the pediatric high-grade glioma genome, the total number of cases studied remains considerably smaller than for adult tumors. This is of particular importance given the lower frequency of the majority of genetic aberrations detected in childhood cases. Validating these low-frequency events in independent cohorts as being recurrent abnormalities, as well as the likely identification of novel isolated copy number changes will aid our understanding of the key pathways underlying the diversity of high-grade gliomas in children. To this end we carried out an array CGH study of 63 cases of pediatric high-grade glioma from formalin-fixed, paraffin-embedded (FFPE) archival pathology specimens on a 32K tiling-path bacterial artificial chromosome (BAC) platform.

Materials and Methods

Samples and DNA extraction

High-grade glioma samples from 63 patients (<23 years old) treated at the Royal Marsden Hospital (RMH), Sutton, and the Newcastle Royal Infirmary, United Kingdom, were obtained after approval by local and multicenter ethical review committees. The collection consisted of 37 glioblastoma multiforme, 14 anaplastic astrocytomas, 4 anaplastic oligodendrogliomas, 4 diffuse intrinsic (brain stem) gliomas, 2 astroblastoma, 1 oligoastrocytoma, and 1 gliosarcoma. All cases were archival FFPE tissues. The presence of tumor tissue in these samples and the tumor type were verified on a H&E-stained section independently by two neuropathologists (DWE and SA-S). Nine of the cases were previously profiled from a frozen tumor specimen in the collaborative SNP study (12). DNA was extracted using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol and quantitated on a NanoDrop spectrophotometer (Thermo Scientific).

Array CGH

All raw and processed data have been deposited in Array Express (http://www.ebi.ac.uk/microarray-as/ae/; E-TABM-857). The array CGH platform used in this study was constructed at the Breakthrough Breast Cancer Research Centre and comprises 31,619 overlapping BAC probes covering the human genome at an approximate resolution of 50 kb (A-MEXP-1734). Hybridizations were carried out as previously described (15) and slides were scanned using an Axon 4000B scanner (Axon Instruments) with images analyzed using Genepix Pro 4.1 software (Axon Instruments). The median localized background slide signal for each clone was subtracted and each clone Cy5/Cy3 ratio was normalized by local regression (loess) against fluorescence intensity and spatial location. Clones overlapping known copy number variants were removed for statistical and visualization purposes, but not for mapping of specific amplifications

and deletions, which was done according to the March 2006 build of the human genome sequence (hg18).

Data analysis

All data transformation and statistical analysis were carried out in R 2.9.0 (http://www.r-project.org/) and BioConductor 2.4 (http://www.bioconductor.org/), making extensive use of modified versions of the package aCGH in particular (15). For identification of DNA copy number alterations, data were smoothed using a local polynomial adaptive weights procedure for regression problems with additive errors, with thresholds for assigning "gain" and "loss" set at 0.1 (3 × SD of control hybridizations). For visualization purposes, the processed $\log_2$ ratios were colored green (gain) or red (loss) after segmentation and copy number determination.

To assess the significance of the genomic alterations, we applied an algorithm similar to those previously described, namely, Genomic Identification of Significant Targets in Cancer (GISTIC; ref. 13) and Genome Topography Scanning (GTS; ref. 16), taking into account the frequency, amplitude, and focality of the observed amplifications ($\log_2$ ratio >1.0) and deletions ($\log_2$ ratio <–0.75). This was calculated as the product of the absolute $\log_2$ ratio, the number of clones in each segment, and the frequency within the entire cohort, scaled to the absolute maximum for amplifications/deletions separately, and overplotted on the frequency histogram for gains and losses described above.

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization (FISH) analysis was carried out on FFPE sections as previously described (17). Probes directed against *MYCN* (pool of clones RP11-1183P10, RP11-674F13 and RP11-754G14), *PIK3CA* (RP11-4B14, RP11-642A13, RP11-379M20), *PDGFRA* (RP11-819D11, RP11-58C6), *SKP2* (RP11-749P08, CTD-2010F22), *PDGFRB* (RP11-211F05, RP11-21I20), *MYC* (RP11-440N18, RP11-237F24, CTD-2034C18), *CDK4* (RP11-66N19, RP11-277A02, RP11-672O16), *MDM2* (RP11-611O02, RP13-618A08, CTD-2067J14), and *IGF1R* (CTD-2015I17, RP11-203H14, RP11-189B22) were labeled with Cy3 (GE Healthcare), whereas chromosome-specific control probes at loci of no copy number change were labeled with fluorescein (GE Healthcare). Hybridized preparations were counterstained with 4′, 6-diamidino-2-phenylindole in antifade (Vector Laboratories Inc.). Images were captured using a cooled charge-coupled device camera (Photometrics).

Statistics

All statistical tests were done in R2.9.0. Correlations between categorical values were done using the χ^2 and Fisher's exact tests. Correlations between continuous variables were done using Student's *t* test or the Mann-Whitney *U* test. Cumulative survival probabilities were calculated using the Kaplan-Meier method on uniformly treated patients within our cohort from the same institution (RMH), with differences between survival rates analyzed with the log-rank test. Important prognostic information (including extent of resection, Karnofsky performance score) was not available for all cases in this retrospective study, so multivariate analysis could not be done. All tests were two-tailed, with a confidence interval of 95%. *P* values of <0.05 were considered statistically significant.

Results

Distinct patterns of copy number change in the pediatric high-grade glioma genome

Previously we utilized whole genome amplification strategies for array CGH studies of tumors extracted from FFPE specimens (18). In this study, however, we were able to utilize a cohort of samples for which sufficient material was available to avoid the previous approach. We were able to generate high-quality copy number profiles from an unselected series of 63 pediatric high-grade gliomas using 32K tiling-path BAC arrays from which the tumor cell purity could be verified as >90% without the need for additional steps.

We observed a mean number of large-scale (whole chromosome or chromosomal arms) gains and losses of 5.8 per sample (median, 4; range, 0-22), with more losses (mean, 3.5; median, 3; range, 0-14) than gains (mean, 2.3; median, 2; range, 0-11). There was a further mean of 1.8 focal amplifications/deletions per sample (median, 1; range, 0-11), again with a slightly increased number of deletions (mean, 1.0; median, 0; range, 0-8) compared with amplifications (mean, 0.8; median, 0; range, 0-4). The list of observed alterations is given for the full dataset in Supplementary Table S1.

We were able to subtype the samples into four groups based upon the pattern of their genomic profiles. First was a group of tumors that had a very stable genome, with few (<3), low-level, focal changes. This subtype comprised 13 of 63 (20.6%) cases, and included 8 tumors (12.7%) that harbored no detectable copy number alterations on our 32K BAC platform (Fig. 1A). The second type contained only large, single copy alterations involving whole chromosomes or chromosomal arms, resulting in aneuploidy in the absence of any high-level amplifications in 22 of 63 (34.9%) cases, the largest subgroup we observed (Fig. 1B). The third type harbored numerous, low-level, intrachromosomal breaks resulting in multiple gains and losses and a highly rearranged genome. This group was also defined for this purpose by exclusion of cases with bona fide amplicons, and comprised 11 of 63 (17.5%) of the cohort (Fig. 1C). Finally, we considered those tumors with single or multiple high-level ($\log_2$ ratio >1.0) amplifications, regardless of the genomic background, as belonging to the fourth, "amplifier" subtype. This group consisted of 17 of 63 (27.0%) of cases (Fig. 1D).

There were no significant correlations between genomic subtype and WHO grade or histology ($P > 0.05$, Fisher's exact test), with glioblastomas, anaplastic astrocytomas,

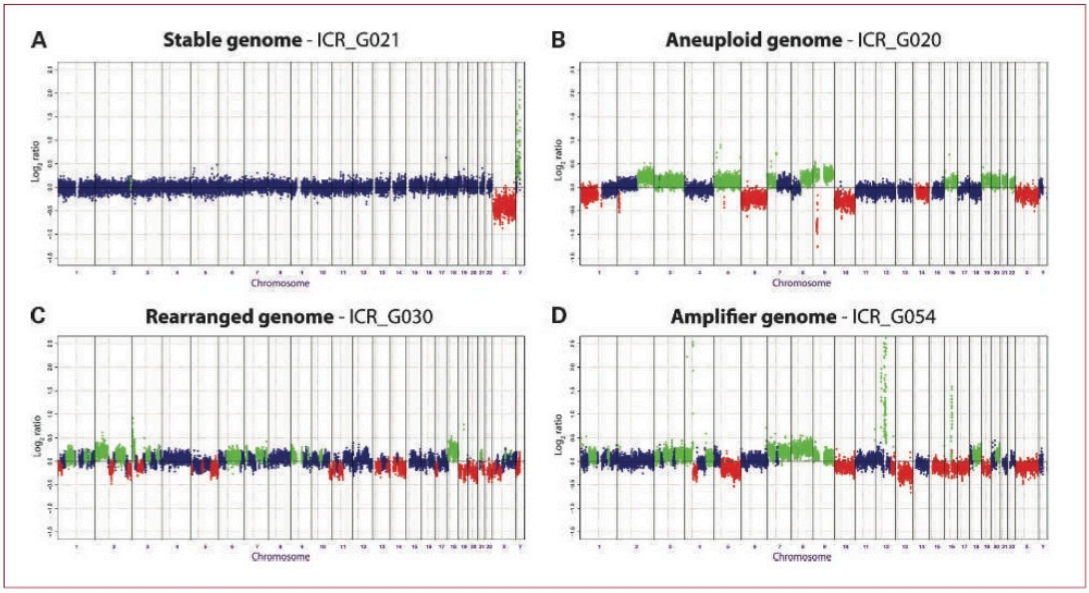

Fig. 1. Pediatric high-grade gliomas comprise different subtypes of copy number profiles. Sample genome plots are given for stable (A), aneuploid (B), rearranged (C), and amplifier (D) genomes within our sample cohort. Log_2 ratios for each clone (*Y*-axis) are plotted according to chromosomal location (*X*-axis). Vertical lines, centromeres; green points, gains; red points, losses.

and anaplastic oligodendrogliomas spread across all subtypes. Of note, there were no "stable" genomic cases among the series of five patients that were treated for a previous malignancy by cranio-spinal radiation (post-IR; Supplementary Table S1). There was also no association of copy number profiles with age at diagnosis ($P > 0.05$, Mann-Whitney *U* test), although the amplifier group did not include any infant tumors (<3 years). However, when we investigated the overall survival of the patients treated at a single institution (RMH), we detected significant differences by retrospective univariate analysis in the clinical outcome of cases according to the genomic profile of the tumor. The stable genome cases showed a trend towards better prognosis when compared with all other cases ($P = 0.0755$, log-rank test), whereas the samples with an amplifier genome had a significantly shorter time to death

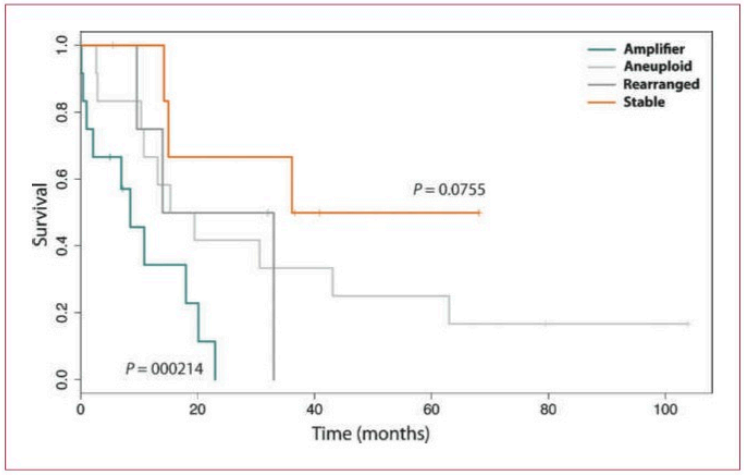

Fig. 2. Genomic subtypes of pediatric high-grade glioma have prognostic relevance. Kaplan-Meier plot for overall survival of pediatric high-grade gliomas treated at a single institution stratified according to genomic subtype. The stable genome cases showed a trend towards better prognosis when compared with all other cases ($P = 0.0755$, log-rank test), whereas the samples with an amplifier genome had a significantly shorter time to death ($P = 0.00214$, log-rank test).

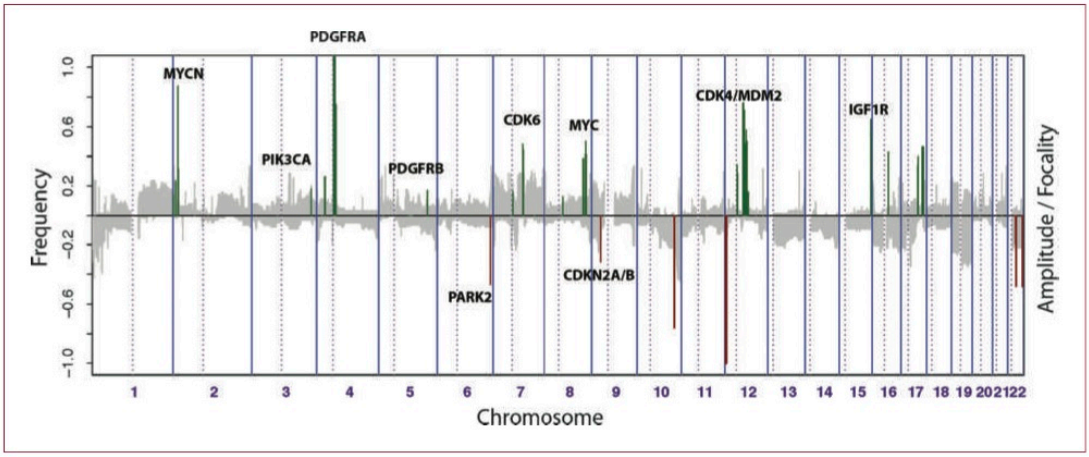

Fig. 3. Summary and significance of genomic aberrations in pediatric high-grade glioma. The proportion of tumors in which each clone is gained or lost is plotted in grey (*Y*-axis) for each BAC clone according to genomic location (*X*-axis). A measure of the frequency, amplitude, and focality of high-level events was calculated for each affected clone and was overplotted for amplifications (green) and deletions (red), scaled to the absolute maximum for each.

(P = 0.00214, log-rank test; Fig. 2). The aneuploid and rearranged cases fell in between, and were representative of the survival characteristics of the cohort as a whole, suggesting that they may need to be considered together as falling between the extremes of the other two groups.

One of the defining features of pediatric high-grade glioma is the frequent gain of chromosome 1q (12 of 63, or 19.0%, versus 17 of 189, or 9.0%, of adult cases; ref. 1; P = 0.039, Fisher's exact test) and loss of 16q (11 of 63, or 17.5%, versus 14 of 189, of 7.4%; P = 0.028, Fisher's exact test); in contrast to adult glioblastoma cases, in which gains of chromosome 7 (12 of 63, or 19.0%, versus 140 of 189, or 74.1%; $P < 0.0001$, Fisher's exact test) and losses of 10q (10 of 63, or 15.9%, versus 152 of 189, or 80.4%; $P < 0.0001$, Fisher's exact test) predominate. In our FFPE cohort, we noticed a clear distinction of 1q gain cases from those with concurrent 7 gain/10q loss (7+/10q-, 8 of 63, or 12.7%), with only a single case harboring both abnormalities. Neither event was significantly associated with any clinicopathologic parameters, although there was a trend towards shorter survival in the 1q+ cases (P = 0.0865, log-rank test). Neither abnormality was seen in any infant cases.

Mapping of focal amplifications and deletions to known oncogenes and novel loci

As we had with large-scale alterations, we observed numerous focal amplifications and deletions. In summary, we identified 47 unique amplification and 32 unique deletions. All these events are detailed in full in Supplementary Table S2 (amplifications) and Supplementary Table S3 (deletions).

The most common amplicon was at 4q12 (10 of 63, or 15.9%), and deletion at 9p21 (10 of 63, or 15.9%, consisting of 8 homozygous, 2 hemizygous). Mapping the SRO in these cases narrowed these regions specifically to *PDGFRA* and *CDKN2A*, respectively, confirming the initial observations that these are by far the most common amplifications/deletions in pediatric high-grade glioma (12). Other common events included amplification of *MYCN* at 2p24 (3 of 63, or 4.7%) or *MYC* at 8q24 (2 of 63, or 3.2%), together giving a frequency of 7.9% (5 of 63) of cases with genomic MYC family dysregulation; and 3 of 63 (4.7%) *EGFR* amplification at 7p12 – a lower frequency than observed in our recent chromogenic *in situ* hybridization study of a larger cohort of which this series is a subset, reflecting the focal nature of the amplification event in a small number of tumors identified by molecular pathology (19).

For the remaining aberrations, we highlighted the SROs where they were found to be recurrent. However, as most were present only in a single case, and we were unable to narrow down gained/lost regions, the result was that we identified a total of 1,026 amplified and 1,243 deleted genes across our series. To facilitate the identification of key oncogenic events in pediatric high-grade glioma, we sought to assign significance to the genomic aberrations we observed. Inspired by algorithms such as GISTIC (13) and GTS (16), we developed a simple measure based upon three key features of our data for each clone on the array: (*a*) frequency of high-level amplification/homozygous deletion, (*b*) absolute magnitude of the change, and (*c*) focality of the segmented copy number change. This amplitude/focality measure was then scaled to the maximum and minimum for amplifications/deletions, respectively, and plotted over the frequency of low-level gains and losses on the same histogram (Fig. 3).

As well as *PDGFRA* (the highest scoring gene) and *CDKN2A*, this analysis highlighted the importance of several known oncogenes, amplified at low frequency in our series, but at high magnitude, and in a focally restricted

manner. These included *PIK3CA* (3q26), *CDK6* (7q21), and *CDK4* (12q14), the first two previously reported in adult glioblastoma, but not in pediatric cases, and present here in a single case. We also identified amplifications of two additional receptor tyrosine kinases: *IGF1R* at 15q26 (Fig. 4A) and *PDGFRB* at 5q33 (Fig. 4B). Such an approach further highlighted the potential significance of known deletions targeting *PARK2* at 6q6 and *MGMT*, *PTPRE*, and others at 10q26, as well as unique events for which the candidate gene is unknown at 10q25 (Fig. 4C) and 11q14 (Fig. 4D).

We were able to validate nine of these lower-frequency amplification events by carrying out FISH on our FFPE sections using specific probes against *MYCN*, *PIK3CA*, *PDGFRA*, *SKP2*, *PDGFRB*, *MYC*, *CDK4*, *MDM2*, and *IGF1R* (Fig. 5).

Glioblastoma core signaling pathways are not commonly activated by copy number changes in pediatric patients

One of the most important findings from recent large-scale genomic profiling studies of adult glioblastoma was the identification of three core signaling pathways that were abrogated by amplification, deletion, and/or mutations of key genes in the vast majority of cases. Considering only the copy number data from these studies, 59%, 70%, and 66% of cases were found to have at least one genetic event targeting the receptor tyrosine kinase/phosphoinositide 3-kinase (RTK/PI3K), p53, or RB pathways, respectively (1, 2).

We mapped the copy number changes in our pediatric cases to the same pathways, which included many of the genes described above, as well as others described in adult

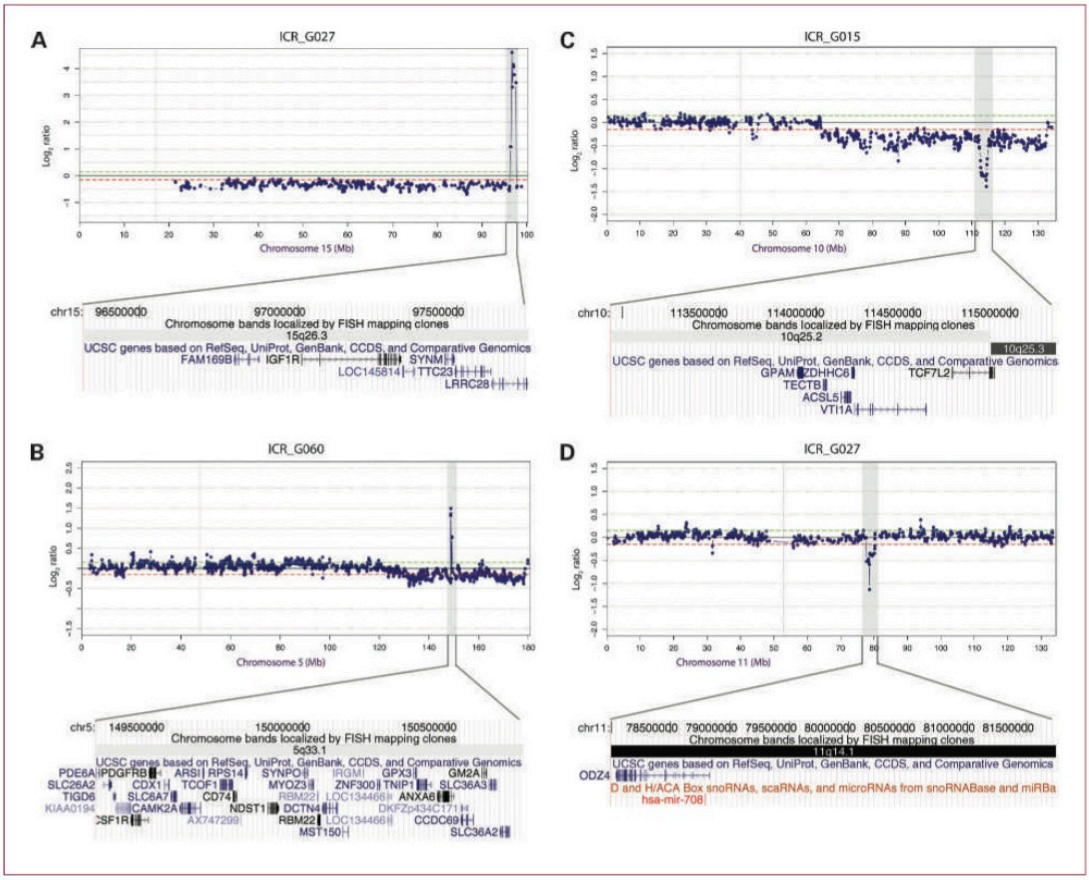

Fig. 4. Novel low-frequency amplifications and fine-mapping focal deletions in pediatric high-grade glioma. Chromosome plots for chromosome 15, targeting *IGF1R* (A); chromosome 5, targeting *PDGFRB*/*CSF1R* (B); chromosome 10, mapping a deletion at 10q25.2-q25.3 (C); and chromosome 11, resolving a deletion at 11q14 to *ODZ4* and hsa-mir-708 (D). Log_2 ratios for each clone are plotted (*Y*-axis) for each BAC clone according to location (*X*-axis) along the length of the chromosome, with genes and microRNA within the minimal regions plotted underneath according to positional information from the UCSC Genome Browser (hg18).

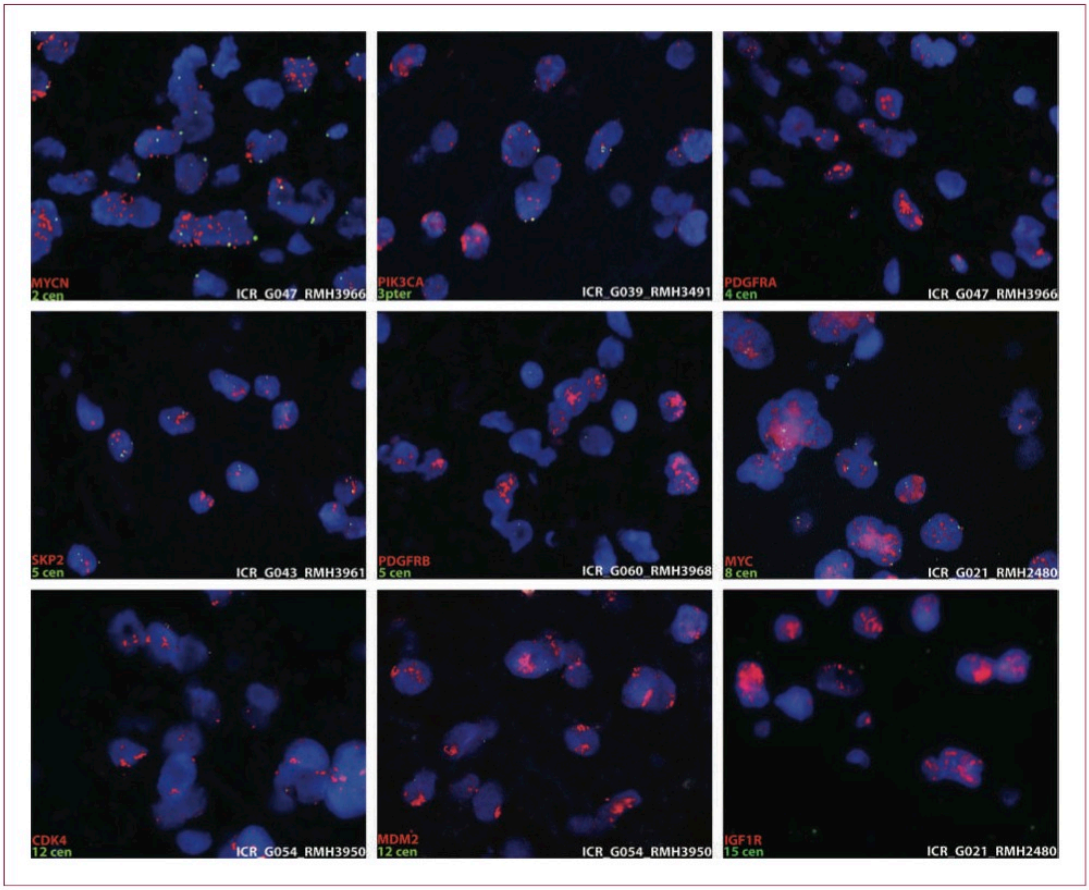

Fig. 5. FISH validation of low-frequency amplifications in pediatric high-grade glioma. Specific probes for *MYCN*, *PIK3CA*, *PDGFRA*, *SKP2*, *PDGFRB*, *MYC*, *CDK4*, *MDM2*, and *IGF1R* were labeled with Cy3 (red) and cohybridized to interphase nuclei on FFPE specimens with chromosome-specific control probes labeled with fluoroscein.

glioblastoma, including *MET*, *KRAS*, and *AKT2* (RTK/PI3K), *MDM2* (p53), and *CCND2* (RB). Despite this, we observed a significantly lower frequency of pathway dysregulation compared with that reported in adults: 16 of 63 (25%) RTK/PI3K, 12 of 63 (19%) p53, and 14 of 63 (22%) RB (all $P < 0.0001$, Fisher's exact test; Fig. 6). Even after removing the stable genome subtype from this analysis, it is apparent that pediatric tumors show targeting of these core pathways by copy number alterations in less than half as many instances than in adults.

To explore whether other canonical pathways may be activated by this mechanism preferentially in childhood tumors, we mapped amplified/deleted genes in those tumors without core pathway targeting via GenMAPP. Although there were isolated cases with clear genomic events linked to activation of the Sonic Hedgehog (*GLI2* amplification, *HHIP* deletion) and Notch (*DLL3* amplification, *DLK1* deletion) pathway activation, there was no consistently targeted pathway in these cases, nor was there specific enrichment of any additional pathway across the entire cohort.

Discussion

We were previously part of a collaborative study setting out to comprehensively map the copy number alterations present in the pediatric high-grade glioma genome, in which we used Affymetrix 500K SNP arrays on a series of 78 cases available as frozen tumor samples (12). Those data revealed an overlapping, but distinct, underlying molecular genetics of the childhood disease when compared with recent large-scale genomic analyses of adult high-grade glioma (1, 2). Along with the common amplification/deletion targets of *PDGFRA* and *CDKN2A/B*, there were numerous low-frequency events targeting both well-recognized

oncogenes and novel loci. The present study had three purposes: (*a*) to validate the high-frequency events in an independent set of samples, analyzed on an independent microarray platform; (*b*) to extend the sample set to provide evidence of recurrence of the low-frequency events previously reported; and (*c*) to identify novel low-frequency events, which by their nature may have been missed in the earlier study.

The most frequent focal events were *PDGFRA* amplification and *CDKN2A/B* deletion, and the most common large-scale gains and losses included chromosomes 1q and 16q, respectively. The $PDGFRA^{amp}$, 1q+, 16q- events were significantly more common in the childhood setting (10, 11, 20), although it is important to note that they are present in a proportion of adult tumors. Similarly, we observed a group of tumors in our cohort containing aberrations more commonly associated with the adult disease, namely $EGFR^{amp}$, 7+, 10q-, albeit at significantly reduced frequencies. That they tended towards exclusivity suggests they represent archetypes for different ends of the spectrum of the disease.

One of the most intriguing differences observed in the pediatric setting was the presence of a proportion of cases of high-grade tumors with very few, or even no detectable copy number alterations. This was true on both BAC

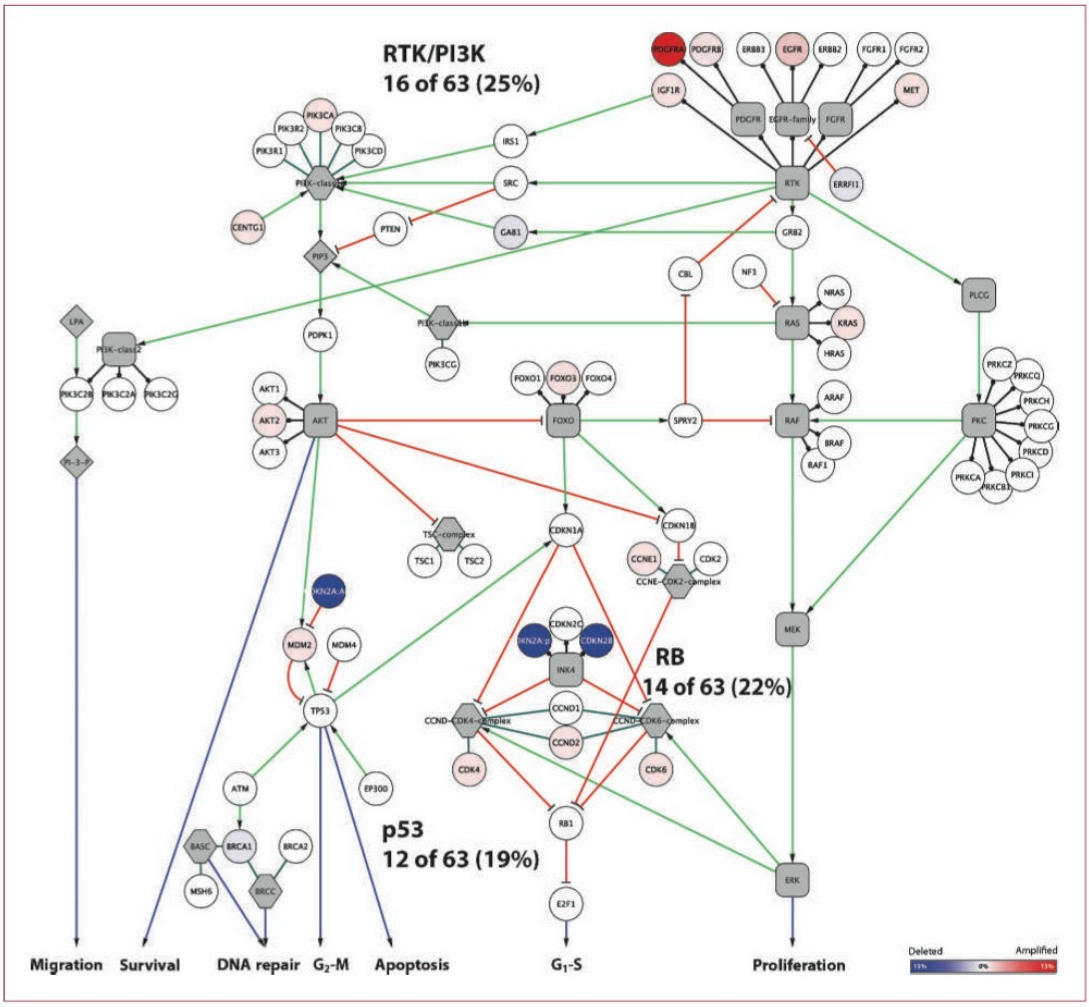

Fig. 6. Glioblastoma core signaling pathways are dysregulated by copy number changes less frequently in pediatric than in adult tumors. Signaling pathway heatmap of interactions defined by the cancer genome atlas (1). Red, genes with amplifications; blue, genes with focal deletion. The overall frequency of copy number alteration in pediatric high-grade glioma for each pathway is listed, and is significantly lower than in adults (25% RTK/PI3K, 19% p53, and 22% RB versus 59%, 70%, and 66% for adult glioblastoma, respectively).

(approximately 32,000 probes, 100 kb resolution) and SNP (approximately 500,000 probes, 6 kb resolution) platforms (12), and is in direct contrast to data from adult tumors (1, 2). This stable genomic profile is independent of histologic grade or type, and seems to convey an improved survival in patients with high-grade glioma, in contrast to those patients with an amplifier genomic profile, who do significantly worse.

Another of the defining features of the pediatric high-grade glioma genome is the numerous low-frequency amplifications and deletions present only in isolated cases in any given study. By nearly doubling the number of these rare tumors for which we have genomic data, we have been able to ascertain aberrations as recurrent across 132 cases. These include amplifications of known oncogenes within the core signaling pathways described in adult glioblastoma, such as *CDK6* (10), *MET*, and *CCND2*, as well as novel targets. These include *ID2* at 2p25, previously found in association with the *MYCN* amplicon at 2p24, possibly part of a single event, identified here as an independent target in its own right. ID2 is a helix-loop-helix transcription factor that has previously been shown to be widely expressed in astrocytic tumors (21, 22), and may play a role in negatively regulating cell differentiation and promoting cell survival (23, 24). Another amplicon at 17q22 was also confirmed in the FFPE series, with a SRO analysis identifying *RNF43* as the most likely target. RNF43 is a ubiquitin ligase that promotes cell growth and is upregulated in colon cancer (25, 26), but has not previously been implicated in gliomagenesis.

Homozygous deletions now apparent as recurrent lesions include those at 14q32, encompassing a large number of microRNAs, as well as the gene *DLK1*. DLK1 is a δ-like homolog that acts to inhibit Notch signaling through specific binding interactions with the receptor (27), and may play diverse roles in cellular transformation and differentiation (28). Although we have now observed two cases of homozygous deletion, other mechanisms of downregulation may be active, as *DLK1* is present at an imprinted locus, with increased methylation upstream of *GTL2* leading to reduced expression in other tumor types (29). Other deletions may have a more complicated role in gliomagenesis such as those on chromosome 16q. The SNP study identified a large deletion in a single tumor that is present as two separate events at 16q12 and 16q21 in two independent cases here, targeting numerous candidates including clusters of Iroquois homeobox genes, metallothioneins, and coiled-coil domain containing genes. By contrast, a homozygous deletion observed in the present study overlaps two independent loci previously reported at 11q14 to target a single microRNA, hsa-mir-708, and a single gene, *ODZ4*. Although little seems known about mir-708, the odd Oz/ten-m homolog 4 is expressed in the developing and adult central nervous system, and seems to act as an important transcriptional regulator associated with neurodevelopment (30, 31).

Finally, we were also able to identify several novel amplifications and deletions, the significance of many of which is not yet clear. There were some genes identified that were also present in adult glioblastoma studies which had not previously been reported in pediatric high-grade glioma, such as *AKT2*, *CCNE1*, *GLI2*, *MDM2*, *PARK2*, and *PIK3CA*. There were other previously unreported genes that may be associated with specific glioblastoma- related signaling pathways such as AKTIP (16q12), an Akt-interacting protein that acts as an activator of the PI3K pathway (32), and *PIK3C3* (18q12), also known as Vps34, a member of the PI3K family associated with autophagy (33). There were numerous others with potential functional relevance unknown.

We also noted rare amplifications at receptor tyrosine kinases considered less likely to be driven by copy number gain. Firstly was a very high level gain of *IGF1R* at 15q26 (11). Insulin-like growth factor (IGF) signaling has previously been implicated in gliomagenesis, primarily on the basis of high levels of the ligand *IGF2* in glioblastoma specimens (34). The growth-promoting effects of IGF2 that were shown were mediated via IGF1R and the PI3K regulatory subunit PIK3R3. Of particular relevance to the childhood setting was the observation of a mutual exclusivity between *IGF2*-associated tumors and *EGFR*-driven cases, suggesting that the IGF pathway may play a prominent role in pediatric tumors, possibly in concert with PDGF receptor (PDGFR)-related signaling.

Secondly was an amplicon at 5q33 which included *PDGFRB* (and another receptor tyrosine kinase *CSF1R*). Given the clear importance of PDGFR signaling on pediatric high-grade gliomas, it is perhaps unsurprising that there may be multiple mechanisms active in driving tumorigenesis through a common pathway. To this end, we also observed recurrent amplification of the ligand *PDGFB* (22q13) in the previous SNP study (12), and here further observed focal copy number gain at 7p22 encompassing *PDGFA*. That these unique genomic events have thus far been found to be restricted to pediatric tumors adds further evidence to a distinct underlying genetics driving archetypal high-grade gliomas in children, one that is largely PDGF-driven, and forms a discrete pole within the diversity of glioma biology. Understanding the most appropriate ways of efficaciously targeting these pathways in the most appropriate patient populations will hopefully overcome the disappointing early-phase clinical trials observed thus far with PDGFR inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Paper III

Dorine A. Bax, Suzanne E. Little, Nathalie Gaspar, Lara Perryman, Lynley Marshall, Marta Viana-Pereira, Tania A. Jones, Richard D. Williams, Anita Grigoriadis, Gilles Vassal, Paul Workman, Denise Sheer, Rui M. Reis, Andrew D. J. Pearson, Darren Hargrave, Chris Jones. MGMT-Independent Temozolomide Resistance in Pediatric Glioblastoma Cells Associated with a PI3-Kinase-Mediated HOX/Stem Cell Gene Signature. *Cancer Research* 70(22): 9243-52, 2010.

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Therapeutics, Targets, and Chemical Biology

Cancer Research

MGMT-Independent Temozolomide Resistance in Pediatric Glioblastoma Cells Associated with a PI3-Kinase–Mediated *HOX*/Stem Cell Gene Signature

Nathalie Gaspar[1,2,3], Lynley Marshall[1,2,4], Lara Perryman[1], Dorine A. Bax[1], Suzanne E. Little[1], Marta Viana-Pereira[1,5], Swee Y. Sharp[2], Gilles Vassal[4], Andrew D.J. Pearson[1,3], Rui M. Reis[5], Darren Hargrave[3], Paul Workman[2], and Chris Jones[1]

Abstract

Sensitivity to temozolomide is restricted to a subset of glioblastoma patients, with the major determinant of resistance being a lack of promoter methylation of the gene encoding the repair protein DNA methyltransferase MGMT, although other mechanisms are thought to be active. There are, however, limited preclinical data in model systems derived from pediatric glioma patients. We screened a series of cell lines for temozolomide efficacy *in vitro*, and investigated the differential mechanisms of resistance involved. In the majority of cell lines, a lack of *MGMT* promoter methylation and subsequent protein overexpression were linked to temozolomide resistance. An exception was the pediatric glioblastoma line KNS42. Expression profiling data revealed a coordinated upregulation of *HOX* gene expression in resistant lines, especially KNS42, which was reversed by phosphoinositide 3-kinase pathway inhibition. High levels of *HOXA9/HOXA10* gene expression were associated with a shorter survival in pediatric high-grade glioma patient samples. Combination treatment *in vitro* of pathway inhibition and temozolomide resulted in a highly synergistic interaction in KNS42 cells. The resistance gene signature further included contiguous genes within the 12q13-q14 amplicon, including the Akt enhancer PIKE, significantly overexpressed in the KNS42 line. These cells were also highly enriched for CD133 and other stem cell markers. We have thus shown an *in vitro* link between phosphoinositide 3-kinase–mediated *HOXA9/HOXA10* expression, and a drug-resistant, progenitor cell phenotype in MGMT-independent pediatric glioblastoma. *Cancer Res; 70(22); OF1–10. ©2010 AACR.*

Introduction

Glioblastoma is the most common tumor of the central nervous system, affecting patients of all ages, and being essentially refractory to treatment; the clinical outcome remains dismal regardless of age at diagnosis. The median survival of a patient with glioblastoma is 15 months, and this has improved little in the last four decades (1). The mainstays of treatment during this time have been surgical resection and radiotherapy, often with nitrosurea-based chemotherapy. The use of adjuvant temozolomide has more recently emerged as a new standard of care in glioblastoma, with concurrent and sequential treatment in the initial therapy of patients resulting in a modest improvement in median survival (2).

Authors' Affiliations: [1]Paediatric Oncology, and [2]Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, and [3]Paediatric Oncology, The Royal Marsden NHS Foundation Trust, Sutton, United Kingdom; [4]Pharmacology and New Treatments of Cancer, Institut de Cancérologie Gustav Roussy, Villejuif, France; and [5]Life and Health Science Research Institute (ICVS), Universidade do Minho, Braga, Portugal

Corresponding Author: Chris Jones, Paediatric Oncology, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey, SM2 5NG, United Kingdom. Phone: 44-0-20-8722-4416; Fax: 44-0-20-8722-4321; E-mail: chris.jones@icr.ac.uk.

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Temozolomide is a DNA methylating agent that is orally bioavailable, crosses the blood:brain barrier, and exhibits schedule-dependent antitumor activity (3). The improved survival benefits in glioblastoma are largely restricted to the subset of patients lacking expression of the DNA repair enzyme O^6-methylguanine-DNA-methyl-transferase (MGMT; ref. 4). Temozolomide induces cytotoxic O^6-guanine methyl adducts that are removed directly by functional MGMT, thereby producing drug resistance. Downregulation of MGMT usually occurs in tumors by gene promoter hypermethylation, in which >50% methylation has been shown to silence gene expression (5).

Deficiencies in DNA mismatch repair (MMR) are also linked to resistance to alkylating agents such as temozolomide (6), as are elevated levels of Ape1/Ref-1, a major component of the base excision repair (BER; ref. 7) system, with attempts to enhance temozolomide-induced cytotoxicity by disrupting BER by means of inhibition of poly-(ADP-ribose)-polymerase (PARP) proving effective *in vitro* and *in vivo* (8).

The vast majority of the above work has taken place in adult glioblastoma and preclinical models derived from adult patients. In the pediatric setting, *MGMT* promoter hypermethylation predicts for response to alkylating agents (9); however, the survival of children treated with adjuvant temozolomide does not seem to be improved when compared with historical controls (10–14). The mechanisms of drug resistance in

pediatric high-grade glioma are poorly understood, in part due to the lack of availability of suitable models of the disease. We screened a series of pediatric and adult glioma cell lines for temozolomide efficacy *in vitro*, and investigated the differential mechanisms of resistance involved, highlighting the involvement in pediatric cells of processes outside of the usual MGMT/MMR/BER axis.

Materials and Methods

Cell culture

Adult glioblastoma cell lines A172, LN229, SF268, U87MG, U118MG, and U138MG, and pediatric glioma cell lines SF188, KNS42, UW479, Res259, and Res186 were obtained and cultured as previously described (15). For the spheroid formation assay, cells were grown in neurosphere medium, which consisted of NDiff RHB-A medium (Stem Cell Sciences) supplemented with epidermal growth factor and fibroblast growth factor 2, each at 20 ng/mL.

Growth inhibition studies

Temozolomide was obtained from Apin Chemicals, O^6-benzylguanine from Calbiochem, and PI-103 from Piramed Pharma or synthesized in-house. Growth inhibition was determined using the sulforhodamine B (16) or MTS (17) assay as previously described. To attempt reversion of resistance to temozolomide, O^6-benzylguanine was added at the highest nontoxic concentration (10–15% of cell growth inhibition,

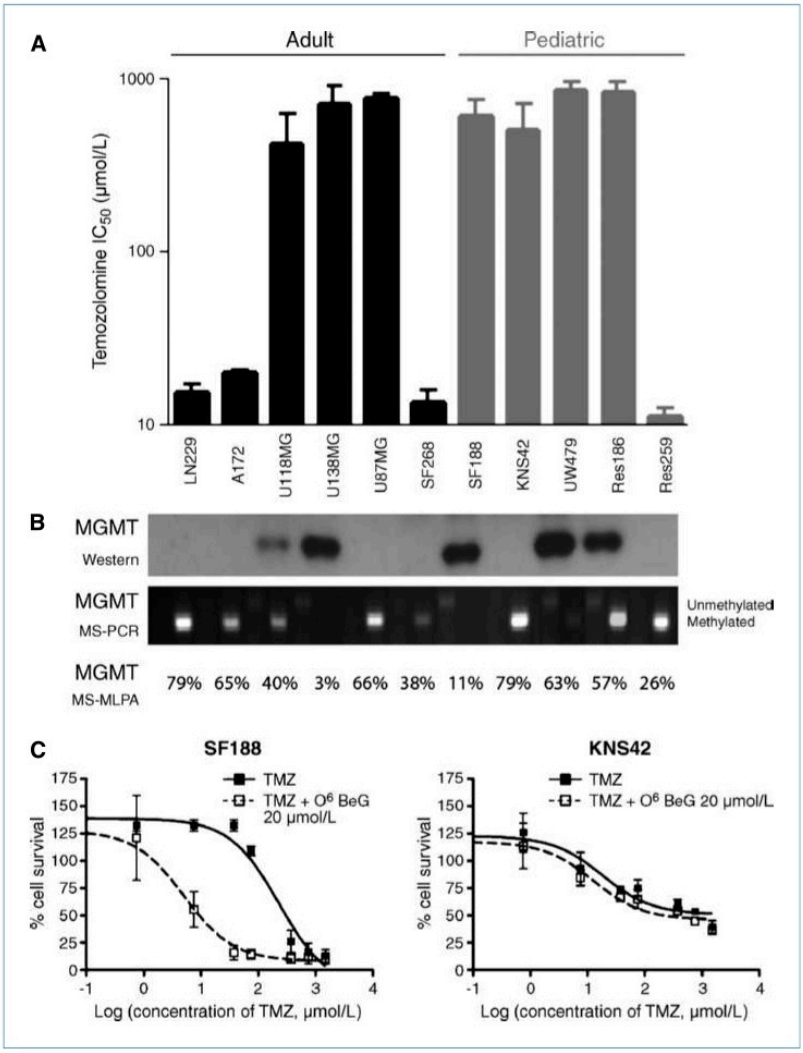


Figure 1. Sensitivity of pediatric and adult glioma cell lines to temozolomide and relationship to MGMT status. A, adult (LN229, A172, U118MG, U138MG, U87MG, SF268) and pediatric (SF188, KNS42, UW479, Res186, Res259) glioma cells were treated with temozolomide, and cytotoxicity was assessed by the sulforhodamine B assay. IC_{50} values are plotted on a $\log_{10}$ scale. B, Western blot for MGMT protein expression correlated with extent of promoter methylation as assessed by MS-PCR and MS-MLPA. In most cases expression correlates with temozolomide resistance, with the exception of U87MG and KNS42 cells, which are hypermethylated, do not express the protein, and are resistant to temozolomide. C, SF188 and KNS42 cells were treated with MGMT substrate analogue O^6-benzylguanine, showing the MGMT-dependent nature of temozolomide (TMZ) resistance in SF188, but not KNS42 cells. Growth inhibition was determined by the sulforhodamine B assay. Concentration of temozolomide is on a $\log_{10}$ scale.

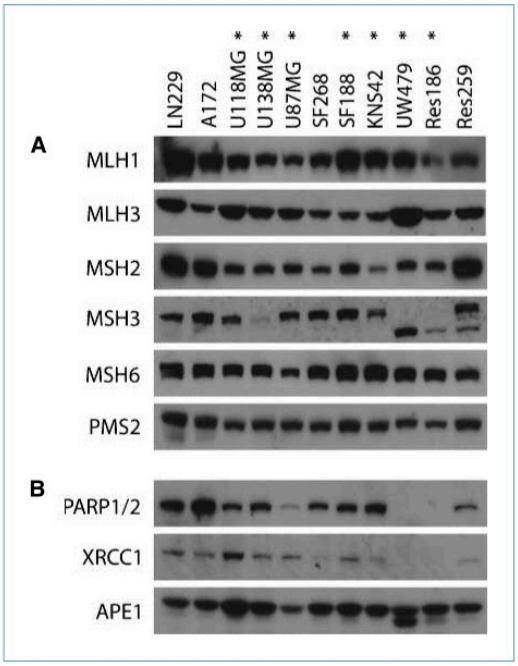


Figure 2. Assessment of DNA MMR and BER pathways in pediatric and adult glioma cell lines. Western blot for proteins involved in MMR (A) and BER (B). Although deficiencies in MSH3 are noted in U138MG, UW479, and Res186, and there is some variability in PARP and XRCC1 expression, U87MG and KNS42 have normal expression of proteins involved in both pathways. *, temozolomide-resistant cell lines.

20 μmol/L). For the assessment of combination effects, cells were treated with increasing concentrations of drugs either alone or concurrently at their equipotent molar ratio and combination indices were calculated by the method of Chou and Talalay (18). All values are given as mean ± SD of at least three independent experiments.

Promoter methylation analysis

Cell line DNA was treated with sodium bisulphite using the Epitect kit (Qiagen) according to the manufacturer's instructions. Methylation-specific (MS) PCR for the *MGMT* promoter was performed as described previously (19). MS–multiplex ligation-dependent probe amplification (MLPA) was carried out as previously reported (15) according to the manufacturer's instructions (MRC-Holland; ref. 20). *HOXA9/HOXA10* methylation was assessed by comparing expression profiles of 5-Aza-2′-deoxycytidine–treated cells with vehicle-treated controls on Illumina Human-6 v2 Expression BeadChips (Illumina Inc.), ArrayExpress accession number E-TABM-858.

Western blot analysis

Immunodetection was performed as previously described (15) using antibodies against MGMT (1:500; Zymed), MLH1 (1:500; Pharmingen), MLH3 (1:500; Santa Cruz Biotechnologies), MSH2 (1:500; Calbiochem), MSH3 (1:250; BD Bioscience), MSH6, PMS2 (both 1:500; BD Bioscience), PARP1/2 (1:1,000; Cell Signaling), XRCC1 (1:500; Cell Signaling), APE1 (Novus Biochemicals), p85, p110α (Cell Signaling), p110β, p110δ (Santa Cruz), PIKE-A/PIKE-L (all 1:1,000; Abcam), phospho-Akt^{Ser473}, Akt (both 1:1,000; Cell Signaling), and GAPDH (1:2,000; Chemicon).

mRNA expression profiling analysis

Cell line expression profiling by Affymetrix U133 oligonucleotide arrays has been previously described (ref. 15; ArrayExpress accession number E-TABM-579). Supervised analysis was performed using an absolute signal-to-noise metric of >1.5 in GenePattern software (http://www.broad.mit.edu/cancer/software/genepattern/). Coordinate gene regulation was identified using gene set enrichment analysis (GSEA; www.broad.mit.edu/gsea/), with a nominal *P* value cutoff of 0.001. "Core enriched" genes are defined as belonging to the leading-edge subset within the gene set, and thus contribute the most to the enrichment result. Assessment of *HOX* gene expression after 24-hour treatment with PI-103 at 5 × IC_{50} was carried out using Illumina HT-12 BeadChips (ArrayExpress accession number E-TABM-890). Affymetrix U133 expression data from The Cancer Genome Atlas (TCGA) glioblastoma study (21) was assessed for cross-correlations of probesets corresponding to *HOXA9* by calculating Pearson's correlation coefficients in R. GSEA and clinical correlations were further carried out on a published dataset (22) of Affymetrix U133 expression array profiling of 78 pediatric high-grade gliomas (Gene Expression Omnibus accession number GSE19578; http://www.ncbi.nlm.nih.gov/geo/).

Immunofluorescence and flow cytometry

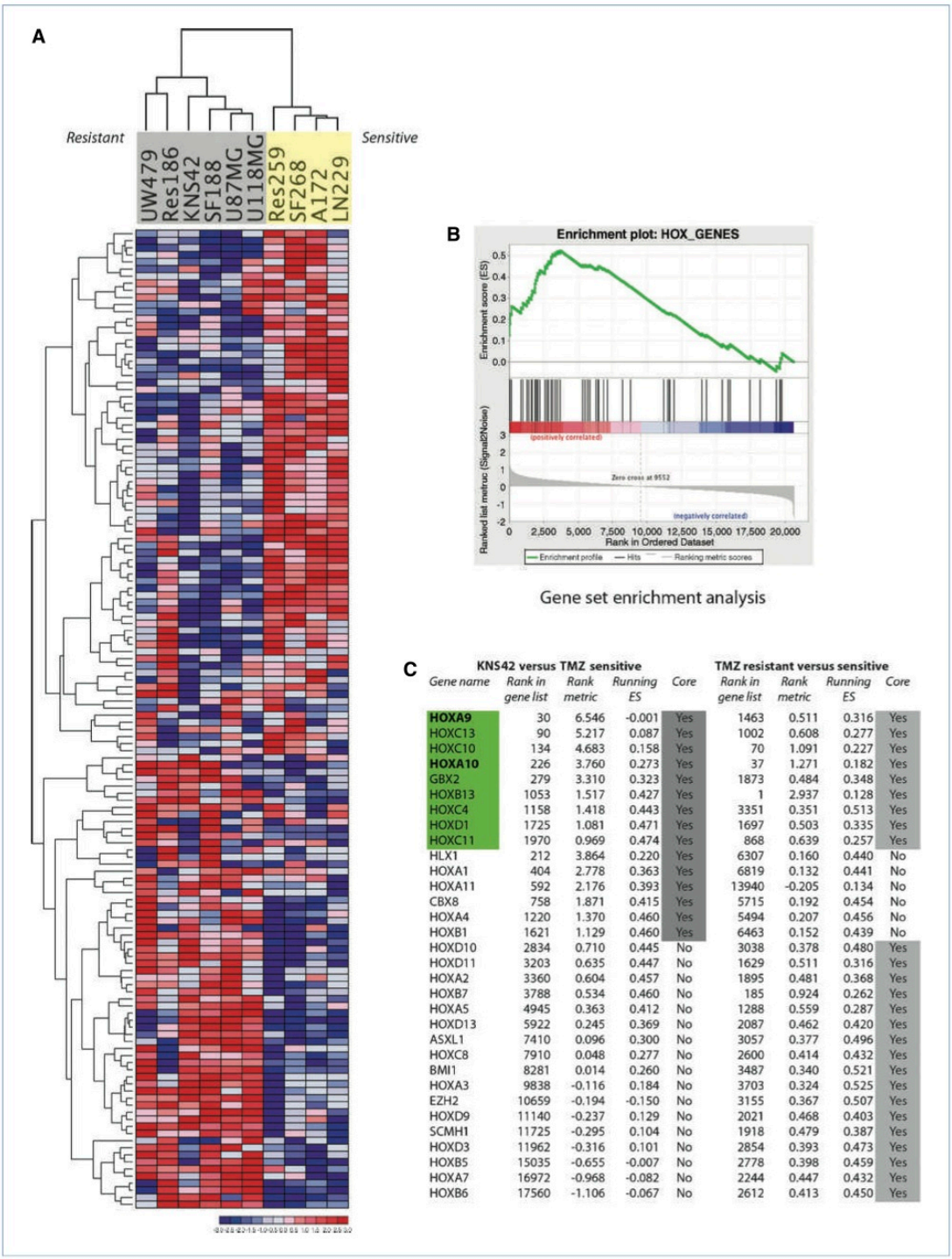
CD133 protein expression was measured by both flow cytometry using a BD fluorescence-activated cell sorting Vantage SEDiVa system (BD Biosciences) and immunofluorescence on cytospin preparations, using anti-CD133 antibody (AC133/1, Miltenyi Biotec) at 1:50 and 1:100 dilution, respectively. Cells were costained with nestin (196908, R & D Systems) and visualized with 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.). Cell cycle analysis was also carried out by flow cytometry.

Results

Sensitivity of glioma cell lines to temozolomide *in vitro* is largely but not exclusively dependent on *MGMT* promoter methylation and lack of protein expression

We first determined the response to temozolomide *in vitro* of our panel of five pediatric (SF188, KNS42, UW479, Res259, Res186) and six adult (A172, LN229, SF268, U87MG, U118MG, U138MG) glioma cell lines. Four lines (A172, LN229, SF268, Res259) were classed as temozolomide sensitive, with IC_{50} values of between 10 and 20 μmol/L (Fig. 1A). The remaining cells were resistant to treatment with the alkylating agent, with IC_{50} values of >500 μmol/L.

We assessed *MGMT* promoter methylation by methylation-specific PCR and MLPA, and protein expression by Western



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	KNS42 versus TMZ sensitive				TMZ resistant versus sensitive			
Gene name	*Rank in gene list*	*Rank metric*	*Running ES*	*Core*	*Rank in gene list*	*Rank metric*	*Running ES*	*Core*
HOXA9	30	6.546	-0.001	Yes	1463	0.511	0.316	Yes
HOXC13	90	5.217	0.087	Yes	1002	0.608	0.277	Yes
HOXC10	134	4.683	0.158	Yes	70	1.091	0.227	Yes
HOXA10	226	3.760	0.273	Yes	37	1.271	0.182	Yes
GBX2	279	3.310	0.323	Yes	1873	0.484	0.348	Yes
HOXB13	1053	1.517	0.427	Yes	1	2.937	0.128	Yes
HOXC4	1158	1.418	0.443	Yes	3351	0.351	0.513	Yes
HOXD1	1725	1.081	0.471	Yes	1697	0.503	0.335	Yes
HOXC11	1970	0.969	0.474	Yes	868	0.639	0.257	Yes
HLX1	212	3.864	0.220	Yes	6307	0.160	0.440	No
HOXA1	404	2.778	0.363	Yes	6819	0.132	0.441	No
HOXA11	592	2.176	0.393	Yes	13940	-0.205	0.134	No
CBX8	758	1.871	0.415	Yes	5715	0.192	0.454	No
HOXA4	1220	1.370	0.460	Yes	5494	0.207	0.456	No
HOXB1	1621	1.129	0.460	Yes	6463	0.152	0.439	No
HOXD10	2834	0.710	0.445	No	3038	0.378	0.480	Yes
HOXD11	3203	0.635	0.447	No	1629	0.511	0.316	Yes
HOXA2	3360	0.604	0.457	No	1895	0.481	0.368	Yes
HOXB7	3788	0.534	0.460	No	185	0.924	0.262	Yes
HOXA5	4945	0.363	0.412	No	1288	0.559	0.287	Yes
HOXD13	5922	0.245	0.369	No	2087	0.462	0.420	Yes
ASXL1	7410	0.096	0.300	No	3057	0.377	0.496	Yes
HOXC8	7910	0.048	0.277	No	2600	0.414	0.432	Yes
BMI1	8281	0.014	0.260	No	3487	0.340	0.521	Yes
HOXA3	9838	-0.116	0.184	No	3703	0.324	0.525	Yes
EZH2	10659	-0.194	-0.150	No	3155	0.367	0.507	Yes
HOXD9	11140	-0.237	0.129	No	2021	0.468	0.403	Yes
SCMH1	11725	-0.295	0.104	No	1918	0.479	0.387	Yes
HOXD3	11962	-0.316	0.101	No	2854	0.393	0.473	Yes
HOXB5	15035	-0.655	-0.007	No	2778	0.398	0.459	Yes
HOXA7	16972	-0.968	-0.082	No	2244	0.447	0.432	Yes
HOXB6	17560	-1.106	-0.067	No	2612	0.413	0.450	Yes

blot (Fig. 1B). Extensive methylation resulted in an absence of MGMT protein expression in LN229, A172, U87MG, SF268, Res259, and KNS42 cells. There was, for the most part, a direct correlation between MGMT methylation/lack of expression and temozolomide sensitivity. An exception to this was the pediatric glioblastoma KNS42 cell line, which displayed insensitivity in the absence of MGMT protein, implying that alternate mechanisms of resistance must be operative. To confirm this, we treated pediatric glioblastoma SF188 and KNS42 cells with temozolomide in the presence of the substrate analogue O^6-benzyl guanine (O^6BeG), which depletes the enzyme and increases cytotoxicity (ref. 23; Fig. 1C). Treatment with 20 μmol/L O^6BeG increased the efficacy of temozolomide in SF188 cells nearly 40-fold (IC_{50} without O^6BeG = 194 μmol/L; IC_{50} in the presence of O^6BeG = 5 μmol/L), thus confirming the dependence of these cells on MGMT in conferring temozolomide resistance. By contrast, no such effect was seen in KNS42 cells, showing the MGMT-independent nature of the insensitivity.

Dysregulation of MMR and BER proteins do not explain resistance to temozolomide in MGMT-deficient KNS42 cells

Enzymes involved in DNA MMR (MLH1, MLH3, MSH2, MSH3, MSH6, PMS2) were evaluated by Western blot (Fig. 2A). Although we identified three temozolomide-resistant lines with abrogated expression of MSH3 (U138MG, UW479, and Res186), there was no deficiency in KNS42 or U87MG cells. These data correlated well with levels of promoter methylation assessed by MS-MLPA (15). We further investigated components of the BER pathway, including PARP1/2, XRCC1, and APE1. Although temozolomide-resistant UW479 and Res186 seemed to lack PARP1/2 and XRCC1 expression, there were no apparent alterations in other glioma lines, including KNS42 or U87MG (Fig. 2B).

Identification of a *HOX*/stem cell gene expression signature associated with temozolomide resistance in pediatric glioblastoma cells

Using expression microarrays, we identified 135 genes differentially expressed between sensitive and resistant glioma cell lines (Fig. 3A). Included in this list were *MGMT* and *PARP2*, despite these enzymes not explaining the resistance in all cell lines. Also included were several kinases, including *MAPK9* and *CDK6*, which may prove suitable targets for pharmacologic modulation; *PIK3C3* (Vps34), suggesting a possible link to the autophagic response (24); and genes encoding elements of the immune response such as *IL10* and *IL16*.

When we applied GSEA to our data, we observed coordinated differential expression of the HOX_GENES set (MSigDB C2:curated gene sets) in resistant versus sensitive cell lines (Fig. 3B), with an enrichment score of 0.54 [nominal $P = 0.01$; false discovery rate (FDR) $q = 0.403$]. Furthermore, the HOX_GENES list was also identified as significant using a GSEA "Preranked" analysis based on differentially expressed genes between KNS42 alone and temozolomide-sensitive cell lines (Fig. 3C). In these analyses, the genes in both core-enriched lists, which contribute to the leading-edge subset within the gene set (25), included *HOXA9, HOXA10, HOXB13, HOXC4, HOXC10, HOXC11, HOXC13, HOXD1*, and *GBX2*.

As coordinated expression of *HOX* genes had recently been noted in glioblastoma clinical samples (26), and was reported as evidence of a "self-renewal" signature as it included the stem cell marker *PROM1* (CD133), we sought further evidence for this in a published glioblastoma dataset and our cell line models. When we investigated TCGA expression profiles of 163 glioblastomas for genes that correlated with the top-ranking *HOX* gene in our KNS42 GSEA list, *HOXA9*, we noted a remarkable parallel expression of numerous other homeobox genes (Fig. 4A). Of the top 47 genes by this analysis, 18 were homeobox genes found at 9 distinct genomic loci, and 7 were included in the self-renewal signature of Murat and colleagues (26). These latter genes included *PROM1*. Intriguingly, the vast majority of the non–homeobox genes identified by this analysis are contiguous genes found commonly amplified in glioblastoma at the genomic locus 12q13-q14.

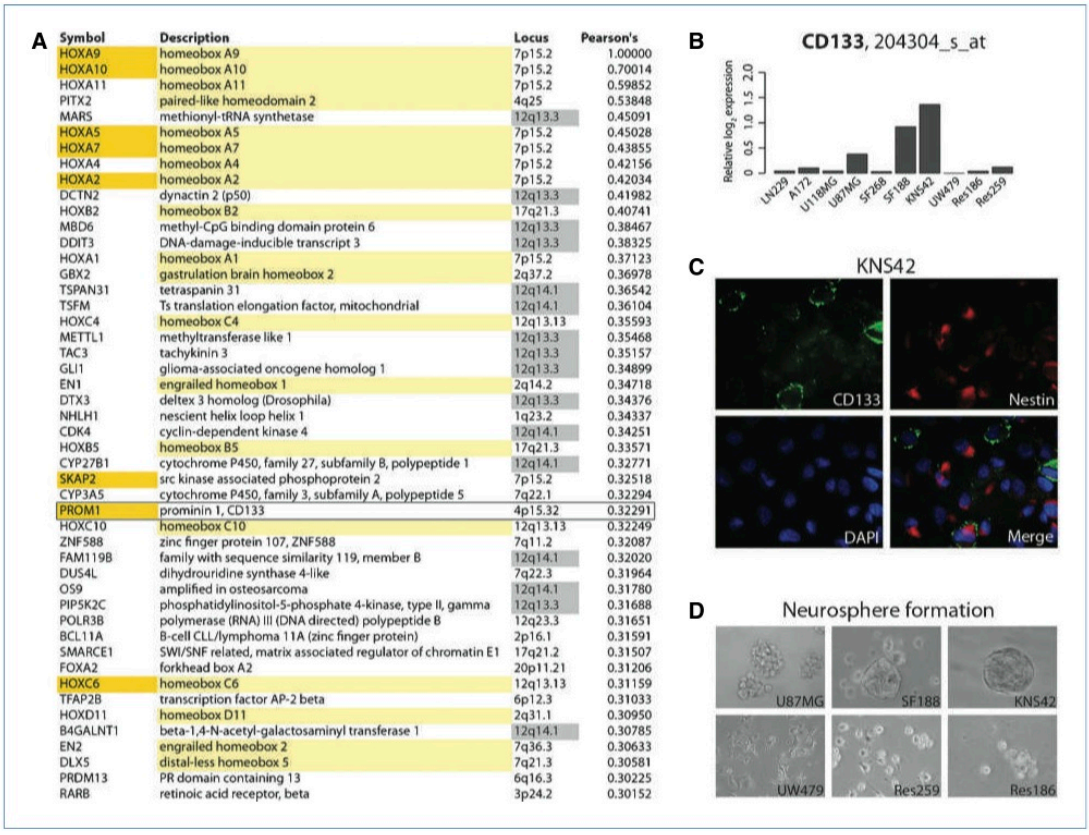
To determine whether an enrichment of the stem cell marker CD133 may be playing a role in the resistance of KNS42 cells to temozolomide, we assessed the levels of mRNA expression relative to the other cell lines (Fig. 4B), and noted considerably higher levels of *PROM1* in KNS42 cells than in any other line in our panel. Of note, the only other two cell lines to express *PROM1* at above background levels were the similarly temozolomide-resistant U87MG and SF188. This was visualized by immunofluorescent staining for CD133, colabeled with nestin (Fig. 4C). We had previously reported the relatively high levels of stem cell markers in SF188 and KNS42 by immunocytochemistry (15), and to more accurately quantify this, we used the more sensitive flow cytometry analysis to reveal an usually high degree of expression in KNS42, with 17.0% of cells positive for CD133. There were also high levels of CD133-positive cells in the SF188 line (4.8%) compared with 0.0% to 0.02% in other cell lines. KNS42 cells grown as monolayers also expressed by far the greatest levels of other stem cell markers such as nestin, SOX2, and musashi-1 (Affymetrix U133, data

Figure 3. Expression profiling reveals a HOX/stem cell signature–associated temozolomide resistance in glioma cell lines. A, heatmap showing hierarchical clustering of 135 differentially expressed genes between resistant (UW479, Res186, KNS42, SF188, U87MG, U118MG; gray highlight) and sensitive (Res259, SF268, A172, LN229; yellow highlight) high-grade glioma cell lines. B, GSEA highlighting coordinated differential expression of gene sets defined *a priori*. Enriched in temozolomide-resistant cell lines were the HOX_GENES set (enrichment score = 0.54, nominal $P = 0.01$; FDR $q = 0.403$). C, ranked genes derived from GSEA of KNS42 versus temozolomide-sensitive cell lines, and all temozolomide-resistant versus sensitive lines. Genes are provided along with their rank in the total gene list and rank enrichment metric, and running enrichment scores are provided for both analyses. Gray highlight, genes present within the core enrichment signature; green highlight, genes present in both analyses; bold, genes also present in the *HOX* gene signature in Murat and colleagues (26), *HOXA9* and *HOXA10*.

not shown). Consistent with the cancer stem/progenitor cell–associated gene expression profile, neurosphere formation assays showed that KNS42 cells form tight three-dimensional spheroids, which may be serially passaged and undergo self-renewal. In contrast, SF188 cells form smaller, more loosely packed spheres, whereas U87MG grow as cell aggregates rather than neurospheres per se. Neither UW479, Res259, nor Res186 formed spheres under these conditions. Taken together, KNS42 cells seem to have a significant cancer stem/progenitor cell–associated gene expression signature and biological phenotype.

Expression of *HOXA9/HOXA10* is a result of phosphoinositide 3-kinase–mediated demethylation, inhibition of which synergistically interacts with temozolomide in KNS42 cells

Treatment with the demethylating agent 5-aza-2′-deoxycytidine resulted in highly differential levels of expression of *HOXA9* and *HOXA10* in all pediatric cell lines with the exception of KNS42, in which no changes were observed, indicative of a lack of methylation in the untreated cells (Fig. 5A). As a recent study has proposed a mechanism for this observation whereby transcriptional activation of the *HOXA* cluster is reversible by a phosphoinositide 3-kinase (PI3K) inhibitor through an epigenetic mechanism involving histone H3K27 trimethylation (27), we sought to investigate whether this mechanism was active in our system. Treatment for 1, 8, and 24 hours with the dual PI3K/mTOR inhibitor PI-103 (28–30) at 5 × IC_{50} resulted in significantly reduced expression of both *HOXA9* and *HOXA10* in KNS42 cells in a time-dependent manner (Fig. 5B). These effects were not due to fluctuations in *HOX* gene expression with the cell cycle, as diminished *HOXA9/HOXA10* was observed as early as one hour posttreatment, at which time there was no evidence of G_1 arrest (Fig. 5C), despite inhibition of PI3K as seen by reduced phospho-Akt levels (Fig. 5D).



Symbol	Description	Locus	Pearson's
HOXA9	homeobox A9	7p15.2	1.00000
HOXA10	homeobox A10	7p15.2	0.70014
HOXA11	homeobox A11	7p15.2	0.59852
PITX2	paired-like homeodomain 2	4q25	0.53848
MARS	methionyl-tRNA synthetase	12q13.3	0.45091
HOXA5	homeobox A5	7p15.2	0.45028
HOXA7	homeobox A7	7p15.2	0.43855
HOXA4	homeobox A4	7p15.2	0.42156
HOXA2	homeobox A2	7p15.2	0.42034
DCTN2	dynactin 2 (p50)	12q13.3	0.41982
HOXB2	homeobox B2	17q21.3	0.40741
MBD6	methyl-CpG binding domain protein 6	12q13.3	0.38467
DDIT3	DNA-damage-inducible transcript 3	12q13.3	0.38325
HOXA1	homeobox A1	7p15.2	0.37123
GBX2	gastrulation brain homeobox 2	2q37.2	0.36978
TSPAN31	tetraspanin 31	12q14.1	0.36542
TSFM	Ts translation elongation factor, mitochondrial	12q14.1	0.36104
HOXC4	homeobox C4	12q13.13	0.35593
METTL1	methyltransferase like 1	12q13.3	0.35468
TAC3	tachykinin 3	12q13.3	0.35157
GLI1	glioma-associated oncogene homolog 1	12q13.3	0.34899
EN1	engrailed homeobox 1	2q14.2	0.34718
DTX3	deltex 3 homolog (Drosophila)	12q13.3	0.34376
NHLH1	nescient helix loop helix 1	1q23.2	0.34337
CDK4	cyclin-dependent kinase 4	12q14.1	0.34251
HOXB5	homeobox B5	17q21.3	0.33571
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1	12q14.1	0.32771
SKAP2	src kinase associated phosphoprotein 2	7p15.2	0.32518
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	7q22.1	0.32294
PROM1	prominin 1, CD133	4p15.32	0.32291
HOXC10	homeobox C10	12q13.13	0.32249
ZNF588	zinc finger protein 107, ZNF588	7q11.2	0.32087
FAM119B	family with sequence similarity 119, member B	12q14.1	0.32020
DUS4L	dihydrouridine synthase 4-like	7q22.3	0.31964
OS9	amplified in osteosarcoma	12q14.1	0.31780
PIP5K2C	phosphatidylinositol-5-phosphate 4-kinase, type II, gamma	12q13.3	0.31688
POLR3B	polymerase (RNA) III (DNA directed) polypeptide B	12q23.3	0.31651
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	2p16.1	0.31591
SMARCE1	SWI/SNF related, matrix associated regulator of chromatin E1	17q21.2	0.31507
FOXA2	forkhead box A2	20p11.21	0.31206
HOXC6	homeobox C6	12q13.13	0.31159
TFAP2B	transcription factor AP-2 beta	6p12.3	0.31033
HOXD11	homeobox D11	2q31.1	0.30950
B4GALNT1	beta-1,4-N-acetyl-galactosaminyl transferase 1	12q14.1	0.30785
EN2	engrailed homeobox 2	7q36.3	0.30633
DLX5	distal-less homeobox 5	7q21.3	0.30581
PRDM13	PR domain containing 13	6q16.3	0.30225
RARB	retinoic acid receptor, beta	3p24.2	0.30152

Figure 4. Coordinated upregulation of *HOX* genes in primary glioblastomas and a striking degree of CD133 positivity on KNS42 cells. A, genes coordinately expressed with *HOXA9* from the TCGA dataset (21) were determined by calculating Pearson's correlation coefficients. All genes with values >0.3 are listed, in rank order. Yellow highlight, homeobox genes; orange highlight, genes also present in the *HOX* gene/self-renewal signature of Murat and colleagues (26), including *PROM1* (CD133; box); gray highlight, genes found within the 12q13-q14 amplicon. B, Affymetrix expression analysis of *PROM1* (CD133) mRNA levels in the panel of glioma cell lines, plotted as relative $\log_2$ expression. C, immunofluorescence assay showing extensive expression of stem cell markers CD133 (green) and nestin (red) in KNS42 cells grown as a monolayer. D, neurosphere formation assay highlighting the tight three-dimensional spheroids formed by KNS42 cells in contrast to the other cell lines studied.

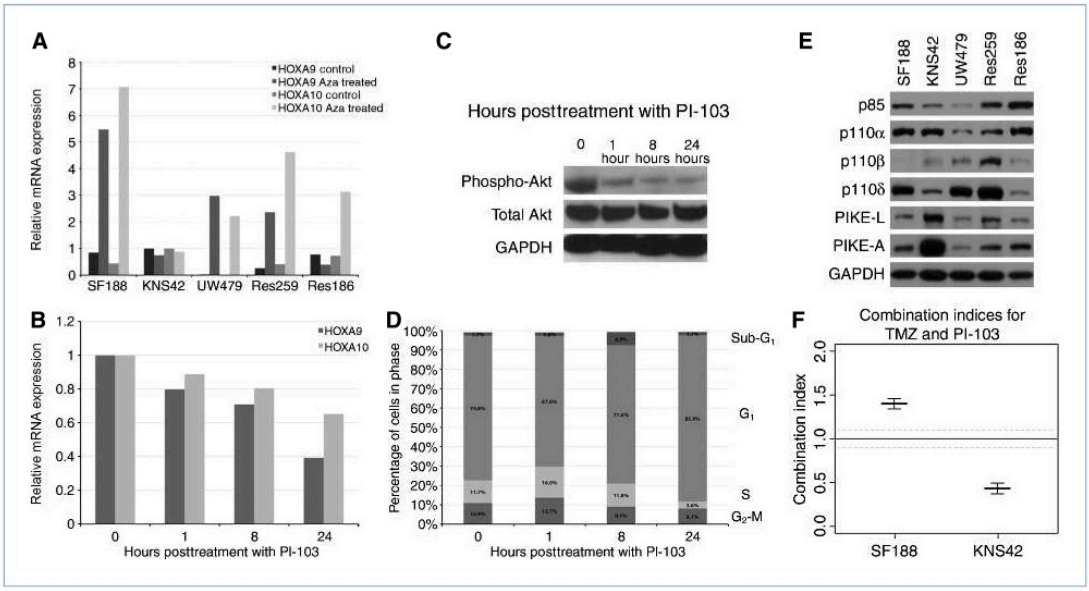


Figure 5. *HOXA9/HOXA10* expression in KNS42 cells is driven by a lack of promoter methylation in a PI3K-dependent manner. A, relative mRNA expression levels of *HOXA9* and *HOXA10* before and after treatment with 5-Aza-2′-deoxycytidine in pediatric glioma cell lines. An absence of expression changes after 5-Aza-2′-deoxycytidine treatment in KNS42 cells is indicative of an absence of constitutive promoter methylation. B, *HOXA9/HOXA10* expression is reduced by treatment with PI-103 in KNS42 cells in a time-dependent manner. Treatment with the dual PI3K/mTOR inhibitor PI-103 at 5 × IC_{50} for 1, 8, and 24 hours led to a reduction in expression of both *HOXA9* and *HOXA10* in KNS42 cells as early as 1 hour posttreatment. C, Western blot analysis of phospho- and total Akt after treatment of KNS42 cells with PI-103 at 5 × IC_{50} for 1, 8, and 24 hours. Inhibition of PI3K signaling is observed at the earliest time point. D, cell cycle analysis of KNS42 cells after treatment with PI-103 at 5 × IC_{50} for 1, 8, and 24 hours. There was no G_1 arrest evident at the early time points at which reduced *HOXA9/HOXA10* expression was observed. E, Western blot analysis of PI3K regulatory and catalytic subunits and enhancers in pediatric glioma cells. Expression of the specific Akt enhancer proteins PIKE-A and PIKE-L were considerably elevated in KNS42 cells compared with other pediatric glioma cells. F, synergistic interaction of PI-103 and temozolomide (TMZ). Cotreatment with PI-103 and temozolomide resulted in a high degree of synergy in MGMT-independent KNS42 cells (combination index = 0.43) as calculated by the median effect analysis. By contrast, SF188 cells showed an antagonistic interaction (combination index = 1.401).

Next we sought to determine whether there was any specific dysregulation of the PI3K/PTEN system in KNS42 cells that may be responsible for the *HOX* gene overexpression. Mutation screening for *PTEN, PIK3CA, PIK3R1*, and *PIK3R3* did not identify any sequence variations (data not shown), and Western blot analysis confirmed a lack of overexpression of PI3K regulatory and catalytic subunits (Fig. 5E). By contrast, there were significantly elevated levels of the enhancer proteins PIKE-A and especially PIKE-L in KNS42 cells in comparison with the other lines. Both PIKE proteins are encoded by the *CENTG1* (*AGAP2*) gene found within the 12q13-q14 amplicon coordinately upregulated in association with the *HOX* cluster, and likely represent a significant target for this genomic event in human glioblastoma.

Finally, we investigated the efficacy of targeting PI3K as a strategy for overcoming temozolomide resistance in our pediatric glioma cells. Combination treatment *in vitro* of temozolomide with the dual PI3K/mTOR inhibitor PI-103 resulted in a highly synergistic interaction in KNS42 as measured by median effect analysis (combination index = 0.43; Fig. 5F). By contrast, in SF188 cells an antagonistic response was observed with the same combination (combination index = 1.40).

HOXA9/HOXA10 expression is associated with shorter survival in pediatric high-grade glioma patients

To assess the translational relevance of *HOX* gene expression in pediatric high-grade glioma patient samples, we examined published data detailing expression profiles of 78 tumors arising in childhood (22). Although the number of long-term (>3 years) survivors is small, we identified 49 genes that were differentially expressed between patients with long and short (<1 year) overall survival (Fig. 6A). Included in this list were *HOXA2, HOXA5, HOX7*, and *HOXA9*. By applying GSEA to the dataset, we identified coordinated upregulation in the short-term survivors of genes at the chromosome 7p15 cytoband, with an enrichment score of 0.68 (nominal $P < 0.001$) albeit with a high false discovery rate value (FDR $q = 0.930$). With the *HOXA* cluster found at this locus, running GSEA on the *HOXA* gene list itself gave a highly significant enrichment score of 0.90 (nominal $P < 0.001$, FDR $q < 0.001$; Fig. 6B). There was considerable correlation between gene expression of all members of the *HOXA* family in the pediatric samples (Pearson's correlation coefficients 0.03–0.90). Taking the values of *HOXA9* and *HOXA10*, and segregating samples into "high" and "low" expressers (combined expression greater or less than the 75th percentile of all values, respectively), we showed a

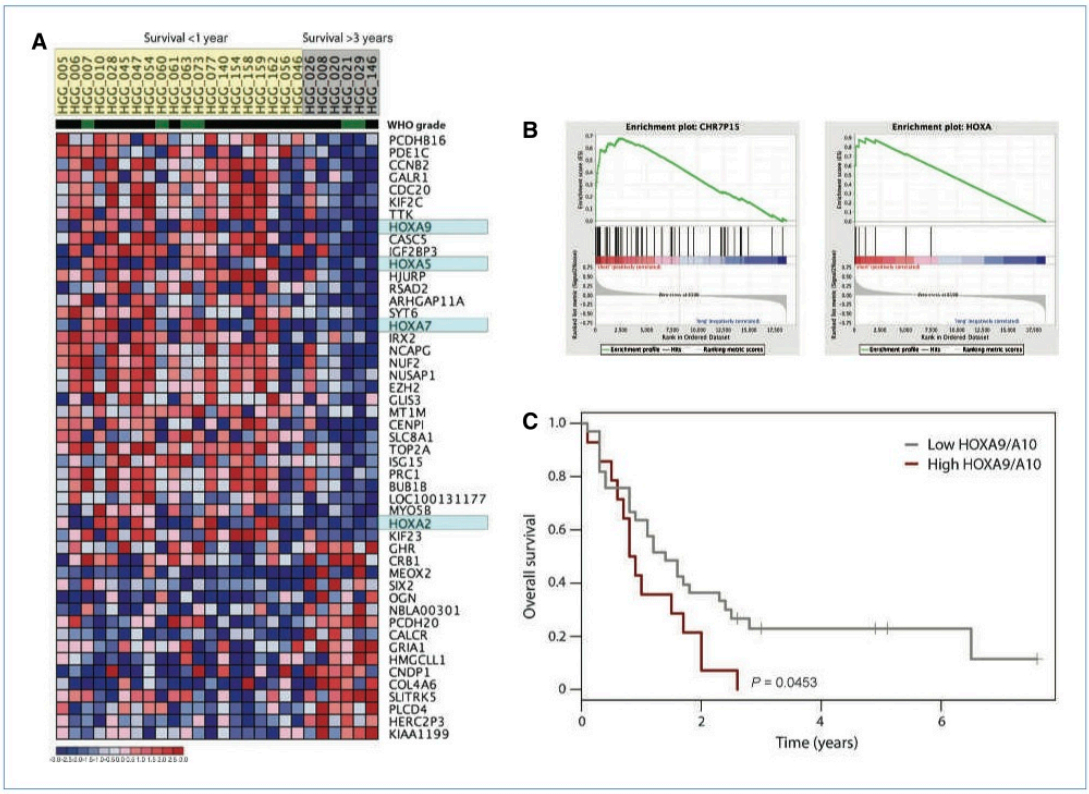


Figure 6. High levels of *HOXA* gene expression are associated with shorter survival in pediatric high-grade glioma patients. A, heatmap representing differentially expressed genes between short (<1 year; yellow highlight) and long-term (>3 years; gray highlight) survivors. WHO grade IV (black) and III (green) tumors are indicated. Light blue, *HOXA* genes. B, GSEA analysis showing significant enrichment of genes at the chromosome 7p15 locus (enrichment score = 0.68, nominal $P < 0.001$, FDR $q = 0.930$), and of *HOXA* genes in particular (enrichment score = 0.90, nominal $P < 0.001$, FDR $q < 0.001$), upregulated in short-term survivors. C, Kaplan-Meier plot showing a significantly shorter survival of patients with high levels of *HOXA9/HOXA10* gene expression ($P = 0.0453$, log-rank test).

significantly reduced overall survival of pediatric high-grade glioma patients with high *HOXA9/HOXA10* expression (log-rank test, $P = 0.0453$; Fig. 6C) independent of the WHO grade of the tumor ($P = 0.635$, Fisher's exact test).

Discussion

Promoter methylation of the *MGMT* gene is generally accepted as the major determinant of sensitivity to the alkylating agent temozolomide in glioblastoma cells, and as such has major significance in the treatment of these patients. We identified the pediatric glioblastoma cell line KNS42 to be resistant to temozolomide *in vitro* despite an absence of MGMT expression, a competent MMR system, and an intact double-strand break repair pathway. Clues as to the mechanism of resistance in these cells may help in identifying factors that contribute to childhood glioblastoma patients who remain refractory to temozolomide treatment.

Gene expression profiling of a panel of pediatric and adult glioma cell lines highlighted coordinated expression of numerous *HOX* genes in the resistant cell lines, most especially KNS42, and provided *in vitro* model system evidence in support of data from temozolomide-treated adult glioblastoma patients (26). Using a similar expression profiling and GSEA approach, Murat and coworkers identified a *HOX*-dominated gene cluster as an independent predictive factor of resistance. Integrating the core gene lists of the present study to that dataset highlights *HOXA9* and *HOXA10* as the key effectors in both systems. This converges with a recent study identifying the *HOXA* cluster, and *HOXA9* in particular, to be independent negative prognostic markers in adult glioblastoma (27). Herein we provide evidence for an additional prognostic role in pediatric high-grade glioma.

HOX genes are essential in axis determination during embryonic development and are known to be involved in cancer, including glioblastomas (31, 32); it is not immediately apparent, however, what role they may play in resistance to

alkylating agents. Costa and colleagues propose that *HOXA9* exerts antiapoptotic and proproliferative effects after upregulation via an epigenetic mechanism controlled by PI3K and independent of mTOR (27). Our findings confirm this observation, and add to previous evidence showing the synergistic interactions of temozolomide and PI3K pathway inhibitors in *in vitro* and *in vivo* models of adult glioblastoma (33); this combination may therefore also be beneficial in glioblastoma patients with a MGMT-independent *HOX* gene signature-associated mechanism of resistance to temozolomide.

Murat and colleagues suggested strong HOXA10 expression in glioblastoma-derived neurospheres to be in line with a role of *HOX* genes in the glioma stem-like cell compartment, and showed that the resistance signature as a whole is evocative of self-renewal (26). Of note is the presence of a high proportion (17.0%) of CD133-positive cells present in our KNS42 cell monolayer cultures, with additional expression of other stem cell markers in coordination with the *HOX* gene signature. Such a *HOX*/stem cell signature was also found to be tightly regulated in an analysis of TCGA glioblastoma expression data (21). The pediatric glioblastoma cell line KNS42 may be an excellent experimental model for investigating such interactions with MGMT-independent treatment failure.

We also found a remarkable link between the *HOX*/stem cell signature and coordinated overexpression of genes within the CDK4 amplicon at 12q13-q14 in glioblastoma patient samples. This association was also present, and correlated with poor response to temozolomide chemoradiotherapy, in the Murat and colleagues dataset (26). Here we provide a possible mechanism for this coexpression of *HOX* and 12q13-q14 genes in the form of overexpression of the Akt enhancer PIKE (*CENTG1, AGAP2*), also present at very high levels in the unamplified KNS42 cells, which may drive the changes in H3K27 methylation of the *HOXA* cluster mediated via PI3K pathway signaling (27).

It is apparent that a variety of processes, not all involving repair or tolerance of alkyl lesions, may promote alkylator resistance (23). Along with providing therapeutic guidance for those patients whose tumors are intrinsically resistant to treatment, characterization of additional determinants of resistance is necessary to develop new targets for therapy in tumors that acquire resistance to temozolomide *in vivo* in the presence of hypermethylated MGMT.

Disclosure of Potential Conflicts of Interest

N. Gaspar, L. Marshall, L. Perryman, D.A. Bax, S.E. Little, M. Viana-Pereira, S.Y. Sharp, A.D.J. Pearson, P. Workman, and C. Jones are or were employees of The Institute of Cancer Research, which has a commercial interest in the development of PI3K inhibitors and operates a rewards-to-inventors scheme. P. Workman and his team have been involved in a commercial collaboration with Yamanouchi (now Astellas Pharma) and with Piramed Pharma, and intellectual property arising from the program has been licensed to Genentech. P. Workman was a founder of, consultant to, Scientific Advisory Board member of, and stockholder in Piramed Pharma, which was acquired by Roche.

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