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Primary Glioblastoma with Different Patterns of *EGFR* Amplification and the Relationship with Gene Expression Profile

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1. Introduction

Glioblastoma multiforme (GBM) is the most common intracranial tumor and the most aggressive of all gliomas. These tumors are very heterogeneous both histopathological and genetic. There is no consistent theory today that can explain the etiology of glioblastoma. A number of factors, both environmental and genetic, have been suggested as potential causes of the tumor. It is possibly a combination of both which initiates and triggers the pathogenesis of tumor and the sequence of genetic alterations that lead to the genesis of neoplasia. They represent 60% of all astrocytic tumors and between 12-15% of all intracranial tumors. Three new patients per 100,000 people are diagnosed every year.

Among its clinical features, age is particularly important for their development and survival. Although the tumor may arise at any age, most cases occur in adults (45-75 years). The average onset is between 53 and 62 years depending on the series (Kleihues et al., 2002, Ohgaki et al., 2004). The presence of tumor is much less frequent at earlier ages. Only 22% of cases involved patients younger than 45 years. It has been reported that cases between 0 and 20 years old only represent 3-8% of the total (Dohrmann et al., 1976). This frequency of cases in the late adulthood may indicate the need for a relatively important period of time before the accumulation of genetic damage leading to tumor formation. As for the sex of the patients there is a preference for males, about 1.5 men for every woman (Kleihues et al., 2002).

Glioblastoma is an almost exclusively supratentorial tumor, affecting both cerebral hemispheres, with a similar spatial distribution to low-grade astrocytoma and anaplastic astrocytoma, being the most affected regions the frontal, temporal and parietal lobes (Dohrmann and et al., 1976; Russell & Rubinstein, 1989). The symptoms usually develop through a short history and in most cases do not exceed 3 months. The literature reflects that 50% of cases are under the threshold of 3 months (Russell & Rubinstein, 1989). The speed of onset of symptoms can be explained by the rapid growth and by the high invasiveness and infiltration of the tumor.

The three types of treatment (surgery, radiotherapy and chemotherapy) commonly used in the treatment of other malignancies, are also used as therapeutic strategies for glioblastoma.

Metastases are rarely found, probably due to the short survival time of these patients. The removal of a tumor produces large symptomatic relief and a slight increase in survival, but practically no cure. The high infiltration capacity of the tumor and the proximity of functionally important brain regions make surgical resection unable to eliminate all tumor cells, which produce relapses in short periods of time. Radiation therapy has also shown some efficacy in improving survival time. Likewise, chemotherapy strategies active in other tumors, have shown a relative efficacy in glioblastoma (Walker et al.., 1978, Walker et al., 1980, Yung et al., 2000; Balañá et al., 2004).

The biological aggressiveness of glioblastoma multiforme makes the survival of patients short, about a year, this being significantly lower than that observed for grade II and grade III astrocytomas (5 and 3 years respectively) (Jelsma & Bucy, 1969, Sant et al., 1988, Kallio et al., 1991, Ohgaki et al., 2004).

From a histopathological point of view, glioblastoma is considered in the group of astrocytic gliomas and is the most malignant tumor in this group. According to the latest ranking of the World Health Organization (Louis et al., 2007), the tumor is classified as grade IV. It may show up as a lesion with high heterogeneity both intratumoral and intertumoral. This heterogeneity is represented by the variability of cell populations and their degree of differentiation.

Glioblastoma may be the final stage of a progression that can result from less malignant lesions (Scherer, 1940). Low-grade astrocytoma, classified as grade II, is the first step on the scale of malignancy of astrocytomas, which already makes clear the invasive nature of the tumor. This tumor shows a slight increase in cellularity and diffuse compared to normal surrounding tissue. Vascularization in these cases becomes slightly hyperplasic in any of the three variants: fibrillary, protoplasmic and gemistocytic. Microscopically, despite the invasive nature, has low destructive power and distorts little the adjacent brain structures (Russell & Rubinstein, 2006).

Although sharing some common ground with low-grade astrocytoma, anaplastic astrocytoma represents a further step in the progressive increase in cellularity, reflected in the degree of anaplasia, the cellular and nuclear pleomorphism and increased proliferative rates, and already considered as malignant astrocytoma, grade III in the WHO classification (Louis et al., 2007).

Finally, a last step in the malignant progression of the lesion, which reflects the changes with respect anaplastic astrocytoma, is morphologically expressed in a predominance of anaplastic cells and vascular proliferation with vascular hyperplasia and glomerular formations, besides the presence of necrosis

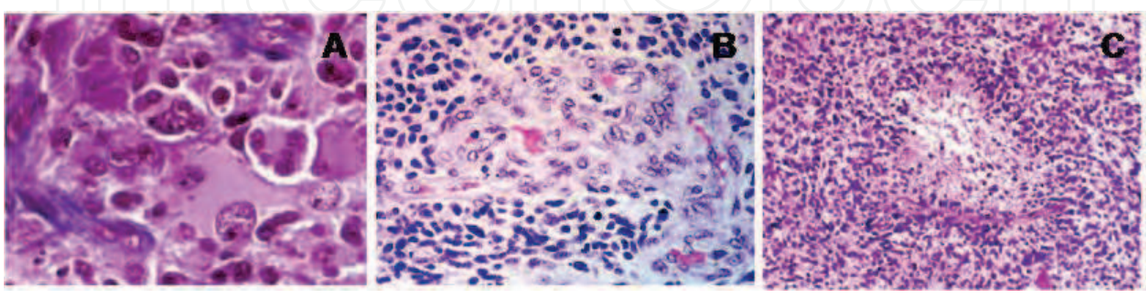

Fig. 1. Histopathological features of glioblastoma with HE techniques. A) Anaplastic histological pattern. B) Pseudoglomerular vascular hyperplasia. C) Pseudoempalizada necrosis

The glioblastoma tumor cells differentially express glial fibrillary acidic protein (GFAP). GFAP is an antigen protein that is present in astroglial cells and is part of the structure of gliofilaments. The intensity and extent of GFAP expression is highly variable (Kleihues and Cavanee, 2000). Tumor cells usually retain some astrocytic differentiation and are strongly positive, especially gemistocytic. Undifferentiated small cells stain more weakly or are clearly negative. Multinucleated giant cells are the most variable in their expression of GFAP. Within a tumor, there may also coexist regions with no GFAP expression. As the tumor progresses, there is a tendency towards the lack of GFAP expression, but this fact is not considered to have prognostic value (Schmitt, 1983).

In these tumors, Ki-67 rates are high ranging between 15-20% (Burger et al., 1986, Karamitopoulou et al., 1994). Both the number of mitosis and the proliferative rates vary depending on the tumor region studied. The spindle-like cells and the undifferentiated small cells seem to show a marked proliferative activity and may therefore have a more aggressive behavior. However, gemistocytic tumors show a lesser degree of proliferation. The determination of proliferative activity may be indicative of tumor behavior and provide additional information on the morphological diagnosis (Watanabe et al., 1997).

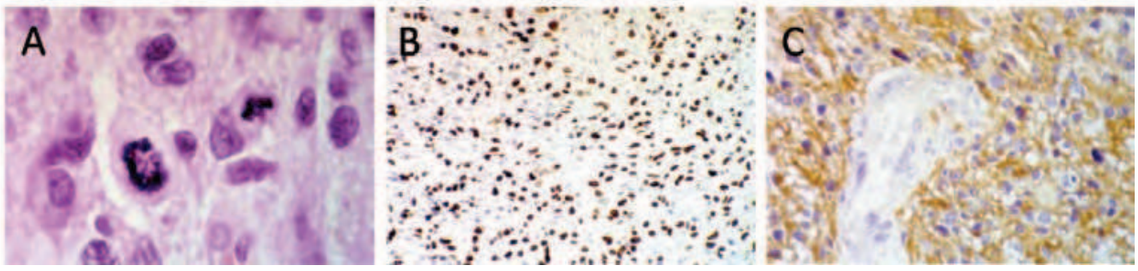

Fig. 2. Morphological and immunohistochemical features of glioblastomas. A) Mitosis in tumor cells. B) Positive expression Ki-67/MIB-1. C) GFAP positive expression.

According to the clinical concepts of Scherer (1940) with respect primary and secondary glioblastoma, most cases develop rapidly without clinical, radiological or morphological evidence of a less malignant precursor. This type of glioblastoma is considered primary or de novo glioblastoma, if also the diagnosis of glioblastoma was obtained in the first biopsy. In these cases, the tumor rapidly develops after a short history of less than 3 months in most cases. The diagnosis of secondary glioblastoma is performed only in cases with histopathological evidence of an earlier low-grade astrocytoma or anaplastic astrocytoma. In these cases, the tumor develops slowly and is considered to have arisen from the glioblastoma tumor progression of lower primary malignancy. Using these criteria, secondary glioblastomas are much less common than primary, reaching only 5% of the total (Dropcho & Soong, 1996, Ohgaki & Kleihues, 2005).

Besides the difference in the diagnosis and progression of primary and secondary glioblastoma, there are also differences in the distribution of age and sex in the two types of tumors. Primary glioblastoma affects older patients with a mean age of 62, while secondary glioblastoma affects middle-aged patients with a mean of 45 years. The distribution between the sexes is also different, dominated by men in cases of primary glioblastoma (M:F 1.5), and women in secondary (F:M 0.65) (Ohgaki & Kleihues, 2005).

A difference between the two subtypes is also observed in terms of survival. Primary glioblastoma has a median survival time shorter (4.7 months) than secondary glioblastoma

(7.8 months). This difference in survival may be due to the difference in the age at which they occur. In fact, there is no significant difference after an adjustment for age (Ohgaki & Kleihues, 2005).

Primary and secondary glioblastomas are two tumor subtypes indistinguishable from a histopathological point of view.

Taking these differences and clinical presentation, the group of Kleihues et al (1997), studied the distribution of molecular features known for glioblastomas, in each of these two subtypes. These authors performed a separation of primary and secondary glioblastomas based on two different histogenetic pathways. Amplification / overexpression of EGFR and *TP53* mutation are considered the pillars of the two proposed genetic pathways for the formation of the two subtypes of glioblastomas. In principle, they were also considered mutually exclusive.

Further studies were to define more precisely the presence of these molecular alterations. Amplification in primary glioblastomas and / or overexpression of EGFR occurred in about 40% of cases. Mutations in *TP53*, however, are features of secondary glioblastomas and appeared in 65% of cases, being much less frequent in primary glioblastomas (28% of cases) (Ohgaki et al., 2004, Ohgaki & Kleihues, 2005).

This chapter is organized as follow: In the 2nd section, we have realised a description on cytogenetic and molecular characteristics of glioblastoma. We analyzed the presence of numerical chromosomal aberrations by applying cytogenetic methods in cultured cells and centromeric DNA probes specific for chromosomes 7 and 10 in material proceeding from smear preparations; and we have studied the association between these parameters. Then, in the 3rd section, characteristics of epidermal growth factor receptor gene (EGFR) and its implication in the pathways of signalization are illustrated. We have investigated the expression of activated ERK1/2 in glioblastomas using western blot analysis and immunohistochemistry, and assessed the relationship between activated ERK1/2 and genetic factors such as *EGFR* amplification, *TP53* mutation and alteration of 9p21 locus genes, as well as clinicopathological parameters. In section 4, we presented the histopathological and genetic findings of primary glioblastomas. The association between cases with *EGFR* gene amplification and cases without *EGFR* amplification with cytogenetic parameters, *TP53* mutations, *MDM2* and *CDK4* amplification, and cell cycle regulatory genes located at 9p21, was evaluated. A second aim of this study was to investigate the relationship between such genetic variations and clinical and pathological data. For last, in section 5, we investigated differences in the pattern of *EGFR* amplification in the glioblastoma. We performed FISH analysis with an *EGFR* probe in metaphases of primary cultured cells and in paraffin sections from 60 cases of primary glioblastomas. We also aimed to compare the *EGFR* copy number alterations, mRNA level and protein expression status and to correlate the FISH data with the clinical and histopathological parameters in the glioblastoma multiforme.

2. Genetic hallmarks in glioblastomas

The glioblastoma is characterized by intratumoral heterogeneity with regard to both histomorphology and genetic changes. Cytogenetically, the GBM displays highly complex karyotypes, with numerous structural and numerical aberrations, the presence of double minutes, as well as differences in ploidy level. The most common numerical chromosome aberrations are +7, +8, -9, -10, -13, -14, -22 and loss of a sex chromosome. Structural changes

involve predominantly the following chromosome arms: 1q, 2q, 6q, 7p, 7q, 9p, 14q, 17p and 18p (Jenkins et al., 1989, Bigner et al., 1990, Mitelman et al., 2004, Lopez-Gines et al., 2006). However, in vitro tissue culturing for karyotype analysis may result in the selective growth of cells with the highest proliferative activity, and these cells may not be representative of the primary tissue (Heim et al., 1989, Moertel et al., 1993).

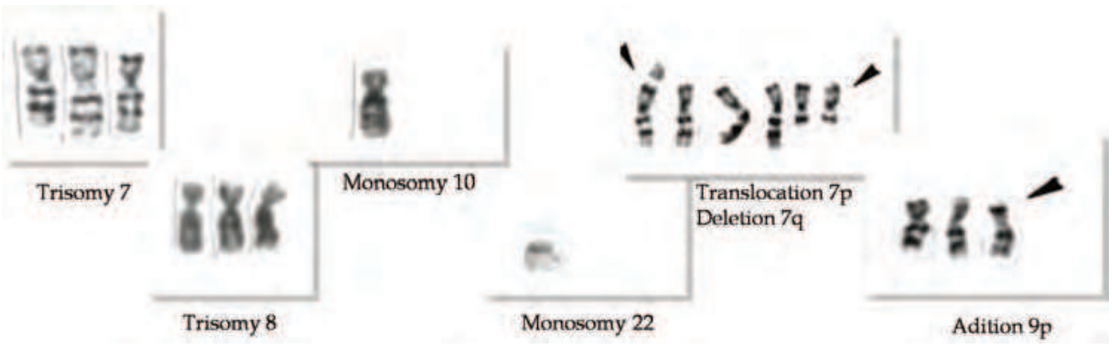

Fig. 3. Cytogenetic alterations in glioblastomas

By minimizing any in vitro culture artefact, fluorescence in situ hybridization (FISH) may more accurately depict the chromosome aberrations in tissues, like the brain, in which cellular turnover is low. These studies showed different aneuploidy for chromosomes 1, 7, 10, 17, X, and Y in astrocytomas, and in all of them the over-representation of chromosome 7 and the under-representation of chromosome 10 are the most common in GBM (Arnoldus et al., 1992, Wernicke et al., 1997, Amalfitano et al., 2000, Koschny et al., 2002, Lopez-Gines et al., 2005). FISH on interphase cells is a powerful tool for detecting different chromosome aberrations, because it can detect chromosomal changes on a cell-by-cell basis. The FISH studies of GBM have also found, that trisomy for chromosome 7 and/or monosomy for chromosome 10 are the principal anomalies, but very few of them have been carried out on about smear preparations; this is a good material and a rapid method for to detection aneuploidy, with control by hematoxilyin-eosin for the presence of tumoral cells in the preparation. According to a few publications, the association between trisomy/polisomy 7 and monosomy 10 is very different, 35-80% depending on the methods used (Steilen-Gimbel et al., 1996, Nishizaki et al., 2002, Lopez-Gines et al., 2005). The studies using karyotype analysis consider trisomy/polisomy 7 to be an early change and monosomy 10 as a progression-associated aberration (Rey et al., 1987, Bigner et al., 1990). On the contrary, other studies with FISH have suggested that in tumorigenesis, monosomy 10 may be an earlier change than the abnormalities in chromosome 7, because cases with monosomy 10 but without trisomy 7 were observed (Steilen-Gimbel et al., 1996, Amalfitano et al., 2000). It is possible that the association of both is necessary in tumorigenesis of GBM.

At the molecular level the glioblastoma multiforme has been studied extensively and the most commonly affected genes are the following:

- Loss of heterozygosity (LOH): LOH on chromosome arm 10q is the most frequent gene alteration for both primary and secondary glioblastomas; it occurs in 60-90% of cases. This mutation appears to be specific for glioblastoma multiforme and is found rarely in other tumor grades. This mutation is associated with poor survival (Fujisawa et al., 1999, Tada et al., 2001).

- Epidermal growth factor receptor (*EGFR*) gene: This oncogene is frequently amplified/overexpressed and mutated in glioblastoma. This alteration is observed in 40-50% of these tumors (Wong et al., 1992, Ekstrand et al., 1994, Wang et al., 1994).
- *TP53* tumor suppressor gene: Appears to be deleted or altered in approximately 25-40% of all glioblastoma multiformes, more commonly in secondary glioblastoma (Yoon et al., 2001, Houiller et al.., 2006).
- *PTEN: A*lso known as *MMAC* and *TEP1,* encodes a tyrosine phosphatase located at band 10q23.3. *PTEN* mutations have been found in as many as 30% of glioblastomas, more commonly in primary glioblastoma (Karlbom et al., 1993, Lin et al., 1998).
- *MDM2:* Amplification or overexpression of *MDM2* is the second most common gene amplification in glioblastoma and is observed in 10-15% of patients. Some studies show that this mutation has been associated with a poor prognosis (Reifenberger et al., 1994).
- Platelet-derived growth factor–alpha (*PDGF-alpha*) gene: The *PDGF* gene acts as a major mitogen for glial cells by binding to the *PDGF* receptor (PDGFR). Amplification or overexpression of PDGFR is typical (60%) in the pathway leading to secondary glioblastomas (Hermanson et al., 1992; Varela et al., 2004).
- Additional genetic alterations in primary glioblastomas include *INK4A* deletions (30-40%), and retinoblastoma (*RB*) gene protein alterations (Quelle et al., 1995, Stott et al., 1998, Ichimura et al., 1996).

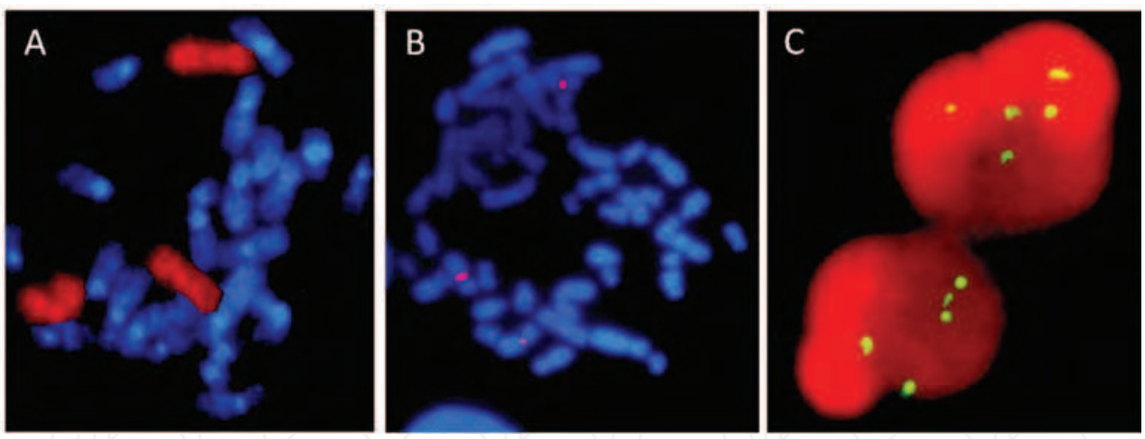

Fig. 4. FISH analysis. A) Metaphase with trisomy 7 (painting probe). B) Metaphase with trisomy 7 (probe centromere of chromosome 7). C) Interphase nuclei with polysomy of chromosome 7.

3. Epidermal growth factor receptor gene and pathways of signalization in glioblastoma multiforme

The EGFR receptor is encoded by the gene c-erbB-1 or Her-1, located on chromosome 7 in the 7p12 region (Kondo et al., 1983). EGFR was the first receptor in which the ability to phosphorylate tyrosine residues in a portion of the molecule was detected. Their activation by the ligand leads to an increase in DNA synthesis, and consequently to an increase in cell proliferation and a decrease in apoptotic activity. This stimulation of cell division begins in the actions of its tyrosine kinase activity (Carpenter & Cohen, 1990).

The oncogenic properties of EGFR receptor are associated with a constitutive and uncontrolled increase in its phosphorylation catalytic activity. The reversible phosphorylation of proteins is one of the major mechanisms for protein regulation and for control of cell physiology. The phosphorylation of certain residues of a protein can regulate its activity, its interaction with other proteins, its structure and its location. Hence, protein kinases and phosphatases are an important part in its control.

The kinases that phosphorylate tyrosine are involved in the initial steps of transmitting signals within the cell, resulting from ligand binding to the receptor (Ullrich & Schlessinger, 1990).

Phosphorylated tyrosine residues serve to the receptor as recruitment and association site for cytosolic proteins, which are activated in turn by new phosphorylations. The association of a protein to the receptor also produces the approximation of soluble proteins from the cytoplasm and thus facilitates this interaction. This will initiate the signaling pathways mediated by these molecules.

Transduction pathways that are activated in this way include the route PI3 kinase/AKT, the Ras/MAP kinase, route c-myc, protein kinase C route and route STAT. All these paths induce the cell to activate transcription of specific genes that produce cell proliferation, apoptosis resistance, invasion, metastasis and angiogenesis, ultimately leading to malignant transformation (Dai et al., 2001; Arteaga, 2002, Rao & James, 2004). Therefore, the intracellular domain of the receptor has an extracellular signal transmitted to the cell nucleus via phosphorylation cascades.

The signaling pathway RAS/RAF is altered in many tumor types, including glioblastomas. This signaling pathway through MAP kinases is involved in cell proliferation. ERK1 and ERK2 are the best studied kinases in solid tumors. ERK1/2 are activated by phosphorylation of tyrosine and threonine residues. Dual phosphorylation is necessary for full activation of ERK1/2. When ERK1/2 is activated migrates into the nucleus and phosphorylates transcription factors which affect the expression of certain genes and ultimately cell growth (Canagarajah et al., 1997, Jelinek et al., 1996). In our work we have observed that in many cases the amplification of *EGFR* is related to the dual activation of ERK1/2. Additionally, if the amplification of *EGFR* is linked to deletions or p16 gene methylation, the relationship with activation of ERK1/2 is more significant (Lopez-Gines et al., 2008).

Major intracellular signaling pathways related to aging and apoptosis involve the enzyme phosphatidylinositol 3-kinase PI3-K. This kinase phosphorylates inositol phospholipids instead of proteins, and also acts on Akt by phosphorylation. Akt itself phosphorylates MDM2, which enters the nucleus where it accumulates, causing inhibition of *TP53* (Honda et al., 1997; Roth et al., 1998).

Nuclear factor NFkB is an important mediator in the regulation of *TP53* and in the Akt signaling pathway. This regulation is achieved through an essential component of NFkB activation cascade, the protein RIP1 (death domain-containing receptor kinase interacting protein 1). RIP1 activates NFkB which causes an increase in MDM2 gene expression by inhibiting the action of *TP53* (Park et al., 2009).

Conversely, in the *P13-K/PTEN/AKT* pathway, *PTEN* tumor suppressor plays also an important role. This tumor gene encodes a phosphatase that dephosphorylates PI3-K, thus preventing the phosphorylation of Akt, and thus favoring *TP53* action. PTEN produces a negative control on Akt kinase pathway, by its action opposite to PI3-K. Therefore, alterations in this gene have a negative effect on TP53 and therefore influence the aging processes and apoptosis, and the cell migration and tumor invasion (Li et al., 1997; Knobbe et al., 2002).

The genetic pathway *TP53/MDM2* is also regulated by p14/ARF. p14 is a repressor of *MDM2*. If p14 does not exert its proper function, it will trigger an excess of free *MDM2* and consequently inactivation of TP53. The alterations of these genes are diverse. p14, which acts as a suppressor gene, typically exhibits some deletions. MDM2, on the other hand, with oncogenic activity is often amplified whereas *TP53* can show different types of mutations (Stott et al., 1998, Ichimura et al., 2000).

Finally, another pathway affected in glioblastomas is p16/Rb/*CDK4*, which controls the G1-S transition in the cell cycle and hence proliferation. In glioblastomas this pathway is impaired because p16, which normally acts as a tumor suppressor gene, has deletions. Additionally, *CDK4* amplifications are also present, giving the cells oncogenic activity. The disturbance causes Rb to be continuously phosphorylated, and as a result there is an uncontrolled cell division (Reifenberger et al., 1994, Rollbrocker et al., 1996).

All these molecules could be therapeutic targets in a tumor as aggressive as this that we are studying.

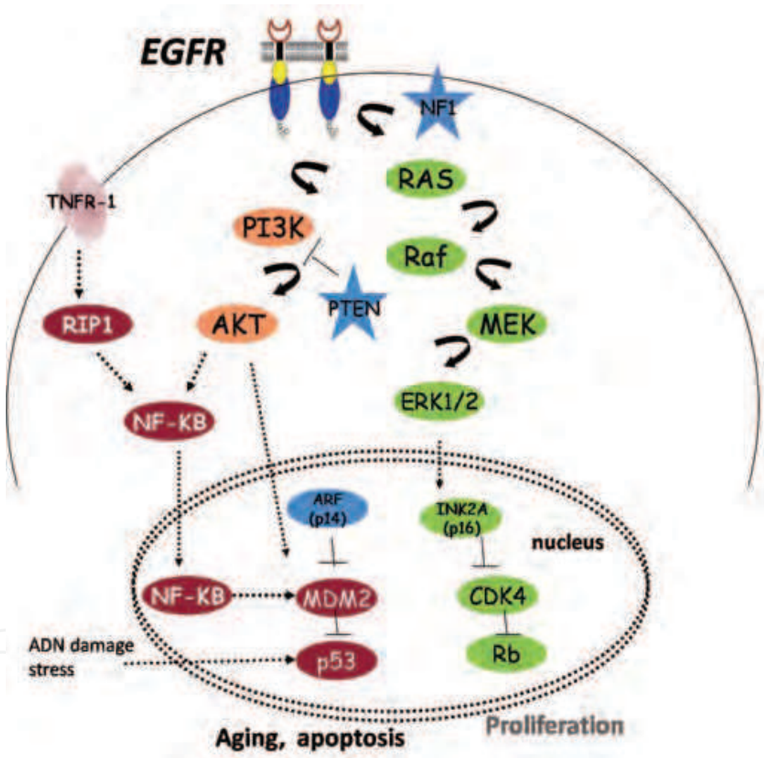

Fig. 5. Pathways of signalization in glioblastoma multiforme

Most gene amplification events in astrocytoma in general and in glioblastoma specifically, involve the EGFR gene. These gene amplification phenomena involve the duplication of certain regions of DNA that leads to the existence of multiple copies of a gene. *EGFR* amplification was an early genetic alteration involved in tumorigenesis of glioblastoma (Márquez et al., 2004).

The vast majority of authors place the percentage of glioblastomas with EGFR amplification around 40-50% (Ekstrand et al., 1992, Diedrich et al., 1995, Shinojima et al., 2003). However,

some studies extend this range up to 73% (Márquez et al., 2004). These differences have been attributed to the different methodologies used (PCR, FISH, Southern blot). *EGFR* amplification has been observed in association with the increase in the number of copies of chromosome 7, both total or partial, which is the most common karyotypic abnormality found in glioblastoma (Hurtt et al., 1992, Rao & James, 2004).

An increase in EGF receptor expression and its natural ligands has been observed using immunohistochemical techniques. This expression is not limited to the membrane, but also observed in the nucleus of tumor cells (Libermann et al., 1985, Ekstrand et al., 1991; Shinojima et al., 2003, Márquez et al., 2004).

This increase coincides with the amplification of the gene encoding the receptor (Shinojima et al., 2003, Varela et al., 2004, Márquez et al., 2004, Lopez-Gines et al., 2005). On the contrary, no expression of this marker is present in normal tissue (Márquez et al., 2004).

The combined increase in the expression of ligands and receptors has led to the assumption that there is a loop autocrine/paracrine growth which stimulates neoplasic growth. Thus, by the autocrine stimulation the cell induces autoproliferation in response to their own growth factors and by the paracrine stimulation there is induction of proliferation in response to factors secreted by neighboring cells and adjacent. In any case, there is an activation of the machinery of the cell cycle and uncontrolled cell proliferation. In fact there are several studies that support the importance of the system EGF/EGFR in the tumorigenesis of glioblastoma, due to the strong coexpression of EGF receptor and its respective ligands EGF and TGF-α (Ekstrand et al , 1991; Shinojima et al., 2003).

Half of the glioblastoma cases with EGFR amplification also exhibit rearrangements of the gene, which results in a considerable variety of qualitative and structural alterations of the receptor, prompted by different mutations that can undergo the gene (Ekstrand et al., 1992, Shinojima et al., 2003, Rao & James, 2004).

The gene sequences which are amplified in glioblastoma are frequently mutated and code protein variants shorter than the native protein (Frederick et al., 2000; Shinojima et al., 2003). These mutations can affect both the 5 'region and the 3' region of the gene. Thus, the mutant protein can be affected at both the extracellular and the intracellular domain. On the other hand, the reading frame is not usually affected by these mutations (Eley et al., 1998, Frederick et al., 2000).

The most common rearrangement is a variant called EGFRvIII, a receptor with a molecular weight of 140-155 kDa that is expressed together with the normal 170 kDa protein (Sugawa et al., 1990, Sugawa et al., 1998; Wikstrand et al., 1998). This rearrangement has also been seen in other tumors where EGFR is also overexpressed (Eley et al., 1998; Arteaga, 2002). This mutation lacks a portion of the ligand binding extracellular domain as a result of genomic deletions that eliminate exons 2-7 in the EGFR mRNA (Frederick et al., 2000; Collins, 2002; Shinojima et al., 2003). This gives this form of the receptor a number of features distinct to those of the normal EGFR (Collins, 2002; Shinojima et al., 2003).

4. Primary glioblastomas with and without *EGFR* amplification

The pathogenic mechanisms leading to the development and progression of glioblastoma are still unclear. *EGFR* amplification has been identified as a genetic hallmark of primary glioblastoma and occurs in approximately 40-60% of primary glioblastomas, but rarely in secondary glioblastomas (Houiller et al., 2006, TCGA, 2008). Generally, primary glioblastomas with *EGFR* amplification show EGFR overexpression, and 70% to 90% of

those with EGFR overexpression have *EGFR* amplification (Liu et al., 2004). This oncogenetic pathway, is heavily involved in this variant of glioblastoma. However, there exist primary glioblastomas with no amplification/overexpression of the *EGFR*.

In our previous works, *EGFR* amplification was observed in 53% of the tumors, and 80% of them presented EGFR overexpression. On the other hand, 47% of our primary glioblastomas showed no *EGFR* amplification and 67% of them showed no overexpression. We studied the association between cases with *EGFR* gene amplification and cases without *EGFR* amplification with cytogenetic parameters, *TP53* mutations, *MDM2* and *CDK4* amplification, and cell cycle regulatory genes located at 9p21. Furthermore, we investigated the relationship between such genetic variations and clinical and pathological data (Benito et al., 2009).

At clinipathological level, the age distribution of cases with *EGFR* amplification closely follows that of patients with primary glioblastomas (60 years), and patients of less than 35 years of age are more infrequent. In our results, the mean age of the group with *EGFR* amplification was 57.2 years and only one patient was under 35 years of age. However, the mean age for the group of cases without *EGFR* amplification was 49.8 years and four patients were under 35 years of age. The rest of the clinicopathological findings on primary glioblastomas with and without *EGFR* amplification did not show any significant differences, although, interestingly, patients with *EGFR* amplification survived for the shortest time.

Cytogenetically, glioblastomas have shown many numerical and structural anomalies (Bigner et al., 1990, Lopez-Gines et al., 2006). In our study, the number of karyotypes with anomalies was greater in the group without *EGFR* amplification. Trisomy 7, monosomy 10 and monosomy 22 were observed in both groups, and the structural alterations of chromosomes 1, 7 and 9 were more frequent in the first group. Every case with dmin had *EGFR* amplification.

FISH on interphase cells is a powerful tool for detecting different chromosome aberrations, because it can detect chromosome changes on cell-by-cell bases. The FISH studies into primary glioblastomas have also found that trisomy for chromosome 7 and/or monosomy for chromosome 10 are the principal anomalies. In our cases, we found a high percentage (85%) of trisomy/polisomy and 70% of monosomy 10, higher in fact than those found by conventional cytogenetic analysis. Likewise, the association of both trisomy/polisomy 7 and monosomy 10 were present in 59% of the cases. In our study, both amplified and non-amplified tumors showed trisomy/polysomy of chromosome 7 and monosomy of chromosome 10. This fact suggests that these alterations could be an initial event in the tumorigenesis of glioblastoma.

At a molecular level, a close association between *EGFR* amplification and *CDKN2A* (*INK4a/ARF*) deletions is a frequent alteration in glioblastomas. Homozygous *INK4a/ARF* deletions were more frequent in primary than in secondary glioblastomas, but there was no significant difference in the overall frequency of these alterations (homozygous deletion and promoter methylation) between primary and secondary glioblastomas (Ohgaki & Kleihues, 2007). In our results on primary glioblastomas, both cases with and without *EGFR* amplification showed similar percentages of *INK4a/ARF* deletions, but the promoter methylation of *INK4a* was more frequent in the group of cases with *EGFR* amplification. As a consequence of *CDKN2A* deletions, there is a disruption of both *RB1/CDK4/P16* and *P53/MDM2/p14* pathways. *MDM2* facilitates the ubiquitin-mediated degradation of p53, which is inhibited by *p14/ARF*. This fact probably explains why both *MDM2* amplification

and *INK4a/ARF* are mutually exclusive. The amplification of *MDM2* is present in less than 10% of glioblastomas, exclusively in primary glioblastomas that lack a *TP53* mutation (Houiller et al., 2006, Ohgaki & Kleihues, 2007). In our series, *MDM2* amplification was present in 11% of the cases, all of them with *EGFR* amplification and without deletions of *INK4a/ARF*. *CDK4* promotes the phosphorylation of the Rb protein and is inhibited by p16 protein, a product of the INK4a tumor suppressor gene. Again, both alterations result in a redundant effect. In our cases, *CDK4* amplification and *INK4a/ARF* deletions were mutually exclusive, and all cases except one were present with *EGFR* amplification. We also found tightly associated *MDM2* and *CDK4* amplifications, suggesting that both genes, which map close together to the same region on 12q13, are frequently included in the same amplicon.

	mut *TP53*	del 9p21	met 9p21	- chro 10	+ chro 7	amp *CDK4*	amp *MDM2*
amp *EGFR*	4	10	18	31	42	6	9
mut *TP53*		2	8	17	18	2	0
del 9p21			4	17	16	0	0
met 9p21				24	25	2	2
- chro 10					57	3	0
+ chro 7						10	11
amp *CDK4*							7

Table 1. Co-presence of genetic alterations in glioblastomas (expressed in %).

Finally, the *TP53* pathway plays a crucial role in the development of secondary glioblastomas, *TP53* mutations being the first detectable genetic alteration. However, although this event also occurs in primary glioblastomas, it does so at a lower frequency (<30% of the cases). In our study, 18% of primary glioblastomas showed *TP53* mutations. Furthermore, we have been found two glioblastomas showing both concurrent *EGFR* amplification and *TP53* mutation.

The first case, arising *de novo* in a 51-years old woman, expressed GFAP, S100, vimentin, and had p53_LI of 27%. EGFR was overexpressed. The cytogenetic analysis showed a hypertriploid complex karyotype with rearrangements of chromosomes 1, 7, 8, and 11, and dmin. FISH studies using centromeric DNA probes for chromosomes 7 and 10 showed trisomy 7 and monosomy 10 in the majority of the cells. Molecular biology showed *EGFR* amplification, and a C176R TP53 mutation; furthermore, aberrant hypermehylation in the promoter region of p14 gene was demonstrated.

The second case arising three years later than a previous anaplastic astrocytoma in a 38-year old man, expressed GFAP, S-100, vimentin, and had p53_LI of 26%. EGFR was overexpressed. The karyotype was hypodiploid, with a t(9;18) and rearrangements of chromosomes 2, 13, and 14. Disomy 7/monosomy 10 was confirmed by FISH. At molecular level the tumor showed *EGFR* amplification and a R282W TP53 mutation.

Hypermethylation of promoter region of p16 gene was also observed. These cases showed, in common, monosomy of chromosome 10, hypermethylation in the INK4A locus, *EGFR* amplification and *TP53* mutation (Gil-Benso et al., 2007).
Our results suggest that, in primary glioblastomas, there exists a subgroup of cases closely linked with *EGFR* amplification. However, there is another subgroup of cases without *EGFR* amplification with some different clinical and genetic characteristics. This second group might include the increased activation or expression of other growth factor receptors observed in these tumors such as PDGFR or IGFR, although they are only amplified in a low percentage of cases.

Group	Clinical characteristics	Histophatological characteristics	Genetic alterations
1 Amplification *EGFR*	**Primary glioblastoma Mean age: 57 years Mean survival: 10.5 months**	**Mean Ki-67 : 31 Sobreexpresión EGFR p53 index: low**	**Citogenetics:+7,-10,-22 Structural: # 1, 9, dmin Amplification: *MDM2,CDK4* del 9p21; met 9p21**
2 No amplification *EGFR*	**Primary/secondary glioblastoma Mean age: 49 years Mean survival: 11 months**	**Mean Ki-67 : 24 No EGFR sobreexpresión p53 index: high**	**Citogenetics: +7, -10, -14, -15, -17, -22, Structural: # 1, 6, 11, 12 del 9p21**

Table 2. Characteristics of glioblastomas with and without *EGFR* amplification

5. Patterns of *EGFR* amplification and gene expression profile

Amplifications are mutations that result in multiple copies of genes in chromosomal regions (amplicons) and induce overexpression in cancer cells. The amount of overexpressed genes in amplified regions varies between different types of cancers, and gene expression in general was significantly regulated by DNA copy number alterations (Schwab, 1998, Myllykangas & Knuutila, 2006). Chromosomal abnormalities associated with DNA amplification can be organized as extrachromosomal copies, called double minutes (dmin); in tandem arrays as head-to-tail or inverted repeats within a chromosome, often forming a cytologically visible, homogeneously staining region (HSR); or distributed at various locations in the genome (distributed insertions) (Schwab,1998, Kuwahara et al., 2004, Albertson, 2006). *MYCN, ERBB2* and *EGFR* are the most frequently amplified genes in cancers.
According to the breakage-fusion-bridge (BFB) model of amplification, the initiating event in HSR formation is double chromatid breakage at a fragile site or telomere erosion (Coquelle et al., 1997, Murnane & Sabatier, 2004), fused sister chromatids and breaking of the anaphase bridges (Shimizu et al., 2005). BFB cycles could then result in inverted amplified structures (Toledo et al., 1992), and the mutated sister chromatids are distributed

to the daughter cells giving rise to intra-tumor heterogeneity (Gisselsson et al., 2000). Gene amplifications are also acquired by selection and unequal segregation of circular extrachromosomal chromatin (dmin and episomes). These elements are formed by looping out from the chromosomes. Dmin and episomes may also relocate in the genome after DNA double-stranded breaks to form HSR or distributed insertions (Schwab, 1999, Kuwahara et al., 2004). Instead of these above-mentioned mechanisms, N-myc duplication located at 2p24 has been described in neuroblastoma cell lines, as studied by FISH (Corvi et al., 1995). Large direct duplications may arise by unequal sister-chromatid exchange and appear to play a role during the initial stages in amplification. It is unclear whether duplication represents a prelude to amplification or an alternative pathway for activating the oncogenic potential of *MYC*. (Corvi et al., 1995, Schwab, 2004).

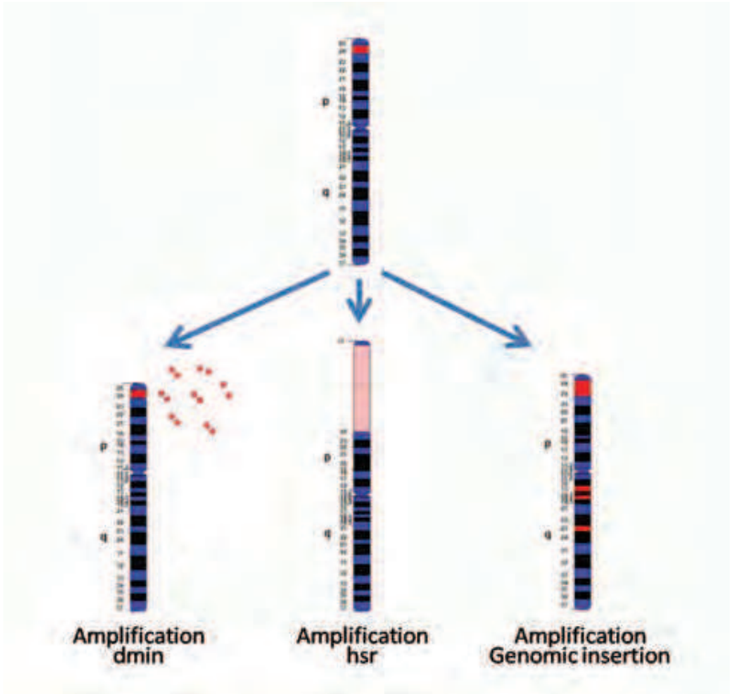

Fig. 6. Patterns of gene amplification: dmin, hsr and genomic insertion.

The first evidence of gene amplification in glioblastoma was provided by cytogenetic analyses which exhibited the presence of dmin, found in up to 50% of tumors (Bigner & Volgelstein, 1990; Thiel et al., 1992).

Molecular studies demonstrated the amplification of several genes in these tumors, especially of the EGFR gene; the amplified sequences were found located on dmin in a small number of cases, by in situ hybridization of tumor metaphases (Muleris et al., 1994, Vogt et al., 2004). However, studies by FISH in interphase nuclei are more frequent, displaying a considerable heterogeneity of *EGFR* copy number (Sauter et al., 1996, Vogt et al., 2004, Layfield et al., 2006, Mizoguchi et al., 2006). The large fraction of cases presenting a diffuse rather than a clustered pattern of amplification within single cells supports the notion that the most common pattern of *EGFR* amplification in glioblastoma biopsies is as double minutes, which ranged in size from 0.7 to 2.1 Mb and in level of amplification from 8-to-63-fold (Vogt et al., 2004). The FISH signals differed from case to case, intense and multiple,

double, faint and single. This suggests the complexity of dmin formation since both their size and the number of *EGFR* copies they contain, vary. Cell to cell variations are also likely to exist for a given tumor (Sauter et al., 1996).

Molecular screening for gene amplification revealed the frequent amplification of the *EGFR*, observed in about 35%-70% of glioblastomas (Sauter et al., 1996, Okada et al., 2003, Ohgaki and Kleihues, 2007). Differences in the frequency of *EGFR* amplification are most likely due to the different methods used, such as Southern blot, polymerase chain reaction (PCR), and fluorescent in situ hybridization.

EGFR protein overexpresssion is usually associated with gene amplification in glioblastomas and these two parameters have been studied as a potential prognostic indicator (Shinojima et al., 2003, Layfield et al., 2006). The majority of GBMs with EGFR amplification also contain the mutant EGFR gene, EGFRvIII, which is characterized by the deletion of exons 2–7, resulting in an in frame deletion variant that has a truncated extracellular domain with ligand-independent constitutive activity.

Overexpression of wild-type EGFR was not found to be an independent prognostic indicator of survival in several studies, and one study was inconclusive (Simmons et al., 2001, Shinojima et al., 2003). Four studies identified EGFR as a negative prognostic indicator of survival, one of which showed the effect only in patients younger than 45. In some of these studies, analysis was limited by small sample size, uncharacterized extent of surgical resection, and variable postoperative treatment. The prognostic impact of EGFRvIII has not been as extensively studied, but in the study that addressed this variable, the presence of EGFRvIII was found to be an independent and significant unfavorable prognosticator of survival (Ekstrand et al., 1991, Simmons et al., Smith et al., 2001, Arteaga et al., 2002, Marquez et al., 2004). *EGFR* amplification and EGFRvIII have been shown to increase glioma proliferation and invasion *in vitro*; therefore logically EGFR and/or EGFRvIII expression could exhibit a proclivity towards the development of multifocal disease, gliomatosis cerebri or ependymal seeding.

In our study we performed FISH analysis with an *EGFR* specific probe in metaphases of primary cultured cells and in paraffin sections from 60 cases of primary glioblastomas, in order to investigate differences in the pattern of *EGFR* amplification in this tumor. We compared the *EGFR* copy number alterations and gene expression with the clinical and histopathological parameters in this subset of tumors.

5.1 Methodology

5.1.1 Histopathological study

Tissue removed from the patient during the surgical resection was divided into three fragments, one for histopathological and immunohistochemical study, one for culture, and the other was frozen and stored at -80^{o}C until molecular analysis and DNA extraction were performed.

The tumoral tissue was fixed in neutral-buffered formalin during the first 48h, embedded in paraffin, sectioned and stained with HE. The samples were categorized according to the WHO classification and diagnosed as glioblastoma multiforme (Louis et al., 2007). Mitotic index values were obtained by counting the total number of mitotic figures in the tumor cells in 20 high-power fields (HPF) and in two different sections. The values are the mean number of mitoses per 10 HPF.

The immunohistochemical study was performed on paraffin-embedded sections using the avidin-biotin peroxidase method. The study was carried out with antibodies against glial

fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark), Ki-67 (MIB-1, Dako, Glostrup, Denmark), p53 (Dako, Glostrup, Denmark), and monoclonal mouse antihuman EGFR (clone H11, Dako, Glostrup, Denmark) that recognizes the wild-type EGFR and the deletion mutant form of the receptor (EGFRvIII).
Proliferation index was evaluated using MIB-1 antibody staining, and was calculated by determining the percentage of immunopositive nuclei. EGFR expression was scored according to the intensity of staining and number of staining cells, as: 0 (no staining), 1 (light or focal), 2 (moderate) and 3 (strong). Scores of 0 or 1 were defined as no overexpression; scores of 2 and 3 as overexpression (Shinojima et al., 2003).

5.1.2 Cytogenetic analysis and fluorescence in situ hybridization

Cytogenetic analyses were performed by short-term culture of the tumors. Fresh tumor samples were disaggregated with 2mg/mL of collagenase II. The cells were seeded in flasks using RPMI-1640 medium supplemented with 20% foetal bovine serum, L- glutamine, and antibiotics. The cells were processed after 72 h of culture by a standard technique. Air dried slides were banded by trypsin-Giemsa. Karyotypic analyses were performed according to ISCN (1995).

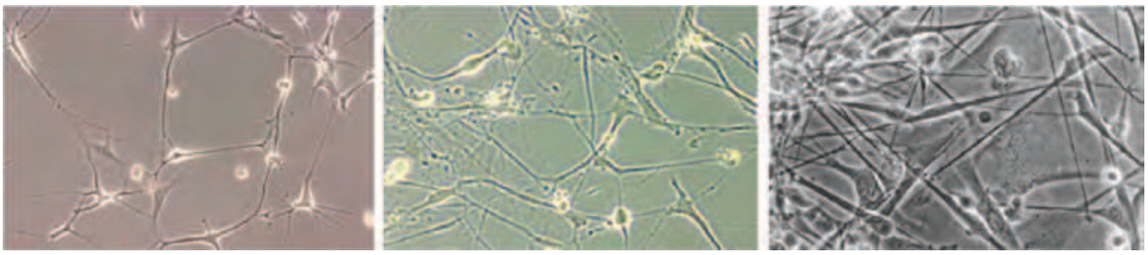

Fig. 7. Cultured cells of glioblastoma

To evaluate EGFR gene status, dual-color FISH was performed on cultured cells and on paraffin sections. FISH was carried out using the LSR EGFR Spectrum Orange/CEP 7 Spectrum Green Probe from Vysis (Abbott Laboratories, Downers Grove, IL, USA. Cat. No. 32-191053).

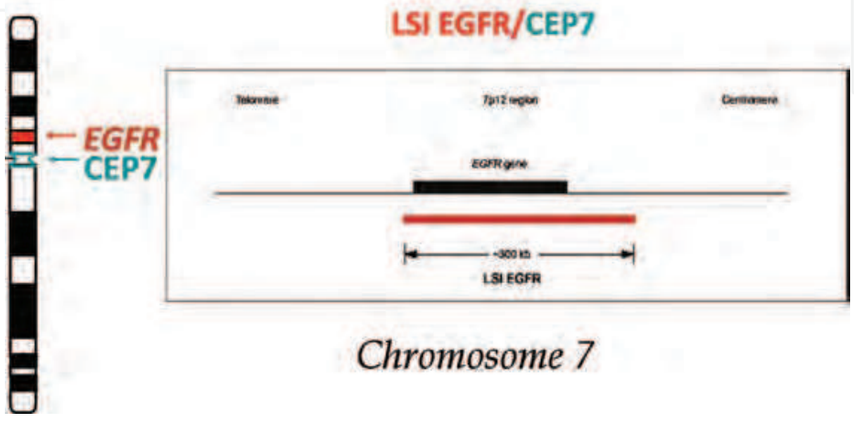

Fig. 8. LSR EGFR Spectrum Orange/CEP 7 Spectrum Green Probe (Vysis).

Cultured cells were treated with Colcemid (0.02 µg/mL) (Gibco BRL, Grand Island, NY, USA) for 80 minutes, and chromosomes were prepared by conventional fixation. The paraffin embedded tissues were cut at 5-µm and mounted on Superfrost/Plus microscope slides. Hybridizations were performed according to the instructions that accompany the probe. Counterstaining of nuclei was carried out using DAPI. The fluorescent signal was detected using a photomicroscope Axioplan 2 and Axiophot 2 (Zeiss) equipped with a set of the appropriate filters. Signals were counted in 100-200 cultured cell nuclei, in all the possible metaphases and in 100 non-overlapping tumor cell nuclei in the paraffin sections. The mean signal number for the EGFR gene and CEP 7 was calculated for each case, as well as the EGFR gene/CEP 7 ratio. The EGFR gene was scored as amplified in individual cells when the EGFR/control signal ratio was greater than 2 (Layfield et al., 2006).

5.1.3 Real-time quantitative PCR

Tumor DNA was extracted from 10-15 mg using DNeasy kit extraction (Qiagen, Valencia, CA). Quantitative PCR was performed using an ABI Prism 7900 HT Fast Real-Time PCR System (Applied Biosystem, Foster City, CA) to analyze *EGFR* copy number. Primer sequences for *EGFR* were: forward GTGCAGATCGCAAAGGTAATCAG; reverse GCAGACCGCATGT GAGGAT; and probe FAM-CCCCTCCCCGTATCTC-MGB. Primer sequences for *RNase P* and *GAPDH* used as references genes were purchased to TaqMan RNase P and GAPDH Detection Reagents (P/N: 4316831).

Target and reference genes were amplified in separate wells. Each 20 µl assay contained 20 ng of genomic DNA, 900 nM each of forward and reverse primers for the gene (*RNasa P*, and *GAPDH* as reference gene, and *EGFR* as target gene), and 250 nM of labelled gene specific probe in 1x TaqMan Universal PCR Master Mix. The TaqMan universal PCR Master Mix (Applied Biosystems) contained AmpliTaq Gold DNA polymerase, AmpErase uracyl-N-Glycosylase, deoxynucleotide triphospates with dUTP that replace dTTP, and optimized buffers.

Individual samples were run in triplicate. PCR conditions were: 2 min at 50ºC (initial incubation for activate AmpErase), 10 min at 95ºC (Activation of AmpliTaq Gold DNA Polymerase) followed by 40 cycles of 15 sec at 95ºC (melt) and 1 min at 60ºC (anneal/extend). Real Time data was collected by the SDS 2.1 software. Each replicate was normalized to reference gene (*RNasa P* and *GAPDH*) to obtain ΔCt, and average ΔCt for each sample (from the 3 replicates) was calculated. All samples were normalized to a calibrator sample to determine ΔΔCt. Relative quantity is $2^{-\Delta\Delta Ct}$, and copy number is 2x (Relative quantity) (Livak & Schmittgen, 2001).

Theoretically, a normal sample has two copies for each gene. Such an assumption is right in cell lines, but taking into consideration that human tumor tissue samples may be contaminated by normal cells, we use a copy number range approach and consider: Homozigous Deletion (HD) 0 - 0.4; Borderline HD-Loss of Heterozigosity (LOH) > 0.4 - 0.6; LOH > 0.6 – 1.4; Borderline LOH-Normal > 1.4 – 1.6; Normal > 1.6 - 2.4; Borderline Amplification > 2.4 – 2.6; and Amplification > 2.6.

5.1.4 Single nucleotide polymorphism (SNPs) array analysis for assessment of EGFR copy number status

Nucleic Acid Isolation and microarray experiment: Fresh frozen tumor tissues (10-20 mg) from patients with glioblastoma were used to extract high molecular weight, genomic DNA using DNeasy kit extraction (Qiagen, Valencia, CA). DNA quality was assessed by

electrophoresis in 1% of agarose gel. Two hundred and fifty DNA nanograms were used for hybridization on Genechip Human Mapping 100K as recommended by the manufacturer (Affymetrix, Santa Clara, CA). The mapping 100K set provides a broad coverage of the human genome (92%), with an average distance between SNPs of 23.6 Kb. This set includes two arrays, each with more than 50,000 SNPs. The processing was performed following the manufacturer's guidelines. Arrays were scanned by means of a GeneChip Scanner 3000, and GeneChip Operating software was used to define Absent or Present Call and generate CEL files.

Data Analysis: CEL files were imported into dChipSNP arrays analysis software (Lin et al., 2004). Arrays were normalized against the array with median overall intensity (Baseline array) employing the *Invariant set* normalization method (Li & Wong 2001a). Probe set signal intensity was obtained by using a model based on expression index (PM/MM) method (Li & Wong, 2001b). Average array call rate was > 95 %. We employ a Hidden-Markov model to identify LOH regions from unpaired tumor samples, taking into account SNP intermarker distance, SNP heterozigosity rates, and the haplotype structure of the genome (Beroukhim et al., 2006). We used 60 CEPH parents as normal reference genotype and we removed inferred LOH regions consistent with 95% of homozygous markers in normal reference samples. Copy number alterations at each SNP locus were determined using the probe level signal intensity data. Reference signal distribution was obtained using a trimmed analysis with 80 % of the samples.

5.1.5 EGFR mRNA expression

Nucleic acid isolation: RNA was extracted from 19 glioblastoma samples. Five pilocytic astrocytomas without *EGFR* amplification were used as reference. For total RNA extraction, 10-20 mg of frozen tumor samples were homogenized with Ultra Turrax and total RNA was isolated using mirVANA kit (Ambion Inc, Austin, TX) following the manufacturer's instructions. Purified RNA was quantified by UV absorbance at 260 and 280 nm and RNA quality was assessed using a spectrophotometer and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples with 28S/18S ratio of ≥ 1.1 and no evidence of ribosomal peak degradation were included.

Microarray experiments: Total RNA (1-15μg) was used to generate double-stranded cDNA. cDNA synthesis and cRNA labelling were performed using the protocol for one-cycle cDNA synthesis. Biotin-labeled cRNA (20 μg) was fragmented and hybridized overnight to Affymetrix HU133plus2.0 genechip. Protocols were performed as recommended by Affymetrix. Arrays were washed, stained with streptavidin phycoerythrin and scanned to generate an image file. Scan quality was assessed by the inspection of visible microarray artifacts, grid placement, background intensity, and housekeeping gene expression. GeneChip operating software (GCOS) was used to define Absent or Present Call and generate CEL files. Arrays with < 30% "Present" call for the 47,000 probe sets and signal 3´/5´ratio of GDAPH control 0.5 ≤ ratio ≤ 4.5 were omitted.

Data analysis: CEL files were imported into dCHIP program. Arrays were normalized against the array with median overall intensity (Baseline array) employing the *Invariant set* normalization method (Li & Wong 2001a). Probeset signal intensity was obtained by using a model based on expression index (PM/MM) method (Li & Wong, 2001b). EGFR probesets with Present Call and expression level ≥10 in ≥ 45 % samples were selected. Probesets that pass established criteria were: 1565484_x_at; 201983_s_at; 201984_s_at; 211551_at; 211607_x_at.

5.1.6 Statistical analysis

In this study we used no parametric test for Ki67 levels, *EGFR* copy number, EGFR transcript expression, and percentage of cells found to be *EGFR* amplification by FISH. First, the Kruskal-Wallis test was used to assess significant differences among established groups, and then the comparisons among the pairs of groups were assessed by means of the Mann-Whitney U test. We used the Bonferroni method to correct the p-values in order to avoid type I error.

Pearson´s coefficient was used to assess the significant correlation between: *EGFR* copy number measured by qPCR, EGFR transcript expression and percentage of *EGFR* positive cells measured by FISH. The significance of the differential protein expression was contrasted by comparing the *EGFR* amplification type using Fischer´s exact test (one-tailed). Survival curves for amplification types, KPS groups (≤80, >80), and age (<55, ≥55 years) were evaluated by the Kaplan-Meier method and were compared with the log-rank test. The statistical tests significance was determined at p-value ≤ 0.05. The analysis was performed with SPSS 15.0 software (SPSS Inc., Chicago, IL).

5.2 Levels of *EGFR* amplification in glioblastoma

Sixty glioblastomas were analyzed successfully by FISH in cultured cells and paraffin sections. We have analyzed the type of *EGFR* amplification, the fraction of amplified cells, and the number of signals of *EGFR* in each tumor.

On basis of the *EGFR* status, the gene copy number and the type of amplification, the cases were categorized into three groups: GBM-h: high level EGFR gene amplification and dmin, **GBM-l:** low level EGFR gene amplification and insertions, and GBM-n: no EGFR gene amplification (Lopez-Gines et al., 2010).

GBM-h: The 47% of the cases was included in this group. The 50% of patients were male and 50% female. Patient age ranged from 38 to 72, with a mean age of 56 years. Survival ranged from 1 to 24 months, with a mean of 9 months.

Histologically, all tumors demonstrated features of glioblastoma with pleomorphic, astrocytic tumor cells, prominent microvascular proliferation, and necrosis. In every case, the expression of GFAP was confirmed in the neoplastic cells. The number of mitoses ranged between 1 and 20, with a mean of 7. The mean Ki-67 of the tumors was 38%; range: 10-80%.

The type of amplification was dmin with a variable number of copies affecting over 25% of the cells in most cases. Only two cases contained less than 10% of amplified cells in paraffin sections.

By using SNP arrays, we inferred the presence of frequent copy number gains in the region of the *EGFR* locus. In tumor samples, an excess of EGFR gene copies was identified by copy number variation from the reference set and from the copy number of the control samples. Gene copy numbers at 7p12.1 were validated by quantitative PCR. Every case showed a strong correlation of these values with the copies demonstrated by FISH (Pearson's correlation coefficient= 0.77; p-value≤ 0.01).

Gene expression have a significant, positive correlation between the *EGFR* copy number and the transcript gene expression (Pearson's correlation coefficient = 0.75; p-value≤ 0.01). The higher percentage of cases of amplified *EGFR* detected by FISH was correlated with a higher gene expression (Pearson's correlation coefficient = 0.85; p-value≤ 0.01).

The EGFR protein expression was evaluated by immunohistochemistry. Cases with a high level of amplification, manifested EGFR overexpression (except in two cases).

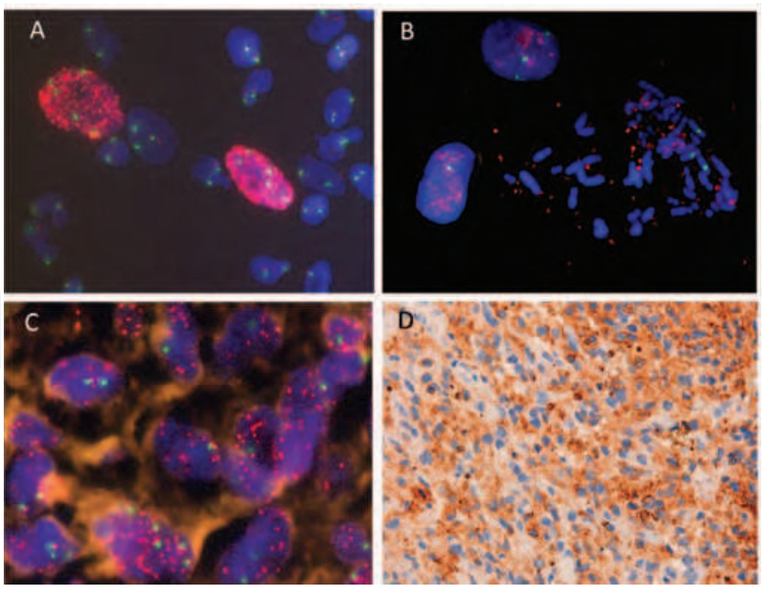

Fig. 9. High-level of amplification of the *EGFR* present in GBM-h group. Probe for EGFR and centromere 7 were labelled with red and green respectively. A) Interphase nuclei of cultured cells. B) Metaphase showing trisomy 7 and *EGFR* amplification as dmin C) Nuclei in paraffin sections showing high level of amplification. Probe for EGFR and centromere 7 were labelled with red and green respectively. D) Immunoreactivity for EGFR: Strong staining.

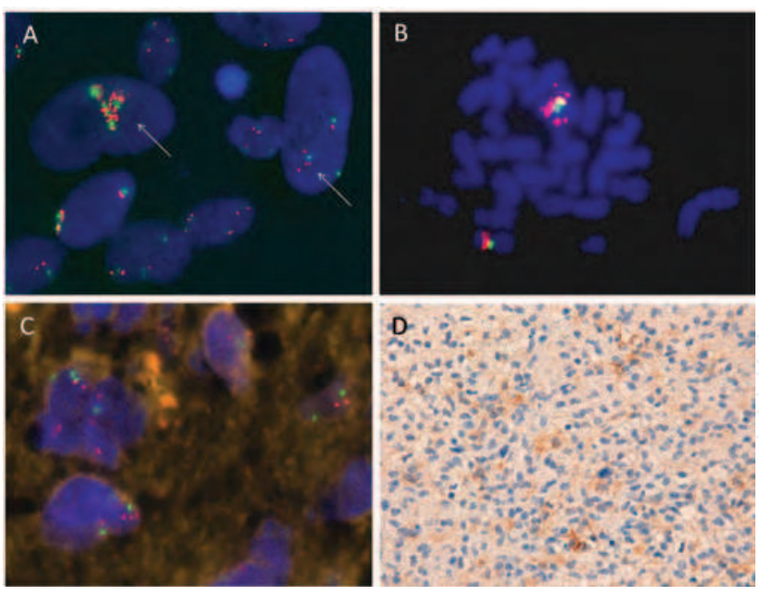

Fig. 10. Low-level of amplification of the *EGFR* present in GBM-l group. A) Different interphase nuclei of cultured cells showing low-level (arrow) of amplification. B) Metaphase spread with extra copies of *EGFR* inserted in p and q arms of chromosomes 7. C) Tumoral cells in paraffin section with low-level amplification/copy number gains with other cells exhibiting a normal copy number of the EGFR gene. D) Immunoreactivity for EGFR with focal staining.

GBM-l: The 21% of the cases was included in this group. The 69% of patients were male and 31% female. Patient age ranged from 24 to 73, with a mean age of 48 years. Survival ranged from 3 to 23 months, with a mean of 12 months.
Histologically, all tumors demonstrated features of glioblastoma with pleomorphic, astrocytic tumor cells, prominent microvascular proliferation, and necrosis. In every case, the expression of GFAP was confirmed in the neoplastic cells. The number of mitoses ranged between 0 and 20, with a mean of 4. The mean Ki-67 of the tumors was 24%; range: 5-50%. The type of amplification showed extra copies of *EGFR* inserted in different locus of chromosome 7, both in p and q arms. In this form of amplification, the number of copies was small, and the percentage of cells with *EGFR* amplification, rarely affects more than 15% of the cells. These cases presented low values validated by quantitative PCR. The increase in mRNA caused by amplification is not always proportional to the number of gene copies. Non increase of RNA expression was found in this group. The EGFR protein expression was variable, only one third of the cases scored EGFR overexpression.
GBM-n: The 32% of the cases was included in this group. The 63% of patients were male and 37% female. Patient age ranged from 22 to 76, with a mean age of 49 years. Survival ranged from 3 to 24 months, with a mean of 11 months.
Histologically, all tumors demonstrated features of glioblastoma with pleomorphic, astrocytic tumor cells, prominent microvascular proliferation, and necrosis. In every case, the expression of GFAP was confirmed in the neoplastic cells. The number of mitoses ranged between 0 and 10, with a mean of 3.The mean Ki-67 of the tumors was 23%; range: 5-50%.
These cases without *EGFR* amplification presented the values of quantitave PCR nearly 2, and non increase of RNA expression was found. The immunohistochemistry analysis showed no overexpression in 79% of cases and overexpression in 21% of cases.

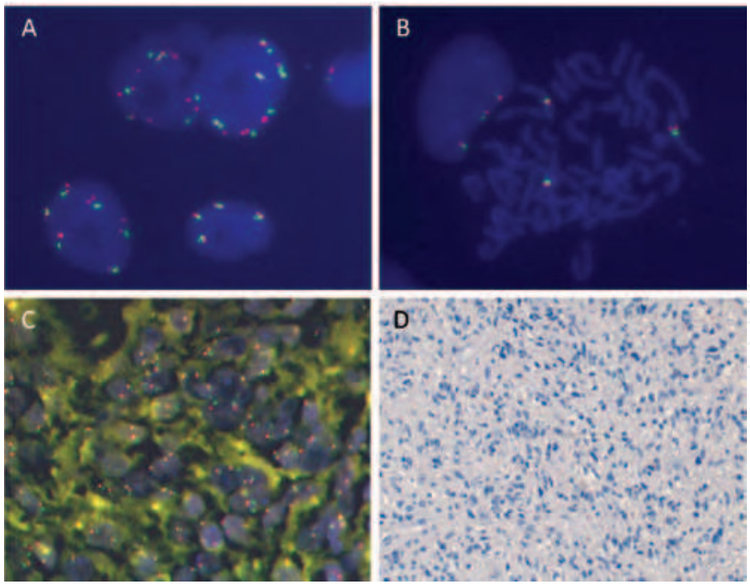

Fig. 11. No amplification of the *EGFR* present in GBM-n group. A) Different interphase nuclei of cultured cells showing no amplification. B) Metaphases spread with three centromeres of chromosome 7 and three copies of *EGFR*. C) Non-amplified tumoral cells of paraffin sections. D) No immunoexpression for EGFR

On the basis of these results, the model of amplification of group GBM-l could correspond to a variant of the distributed insertion mechanism, or a consequence of a process of duplication like the one demonstrated in neuroblastoma cell lines (Corvi et al., 1995), and we suggest that this mechanism could be considered as an early stage of amplification in glioblastoma. This hypothesis is corroborated by the findings of both status of amplification, dmin, and extra copies located in chromosome 7, in two cases. In glioblastomas, the cut-points that define amplification are controversial (Smith et al., 2001, Layfield et al., 2006), although the most commonly accepted criteria is that *EGFR* amplification exists when the EGFR gene/chromosome per cell ratio was ≥2, or ≥15 copies of *EGFR* per cell in ≥10% of analyzed cells (Capuzzo et al., 2005, Nagasaka et al., 2007).

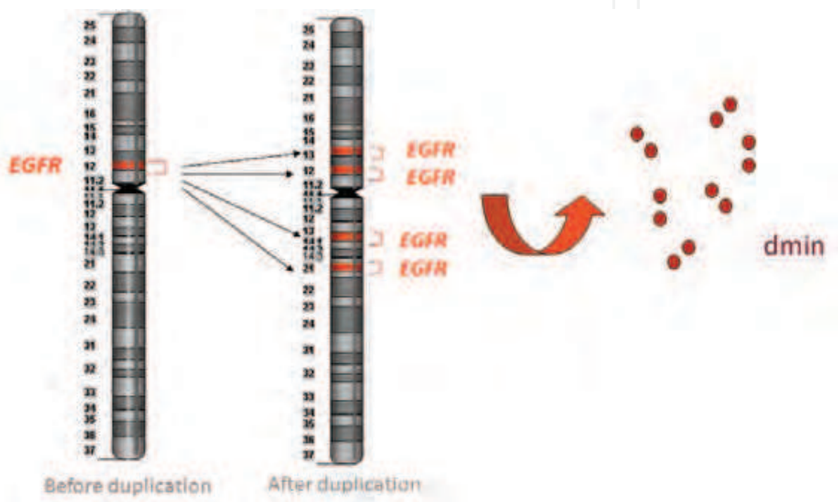

Fig. 12. Model of amplification of *EGFR* correspond to a variant of the distributed insertion mechanism.

In addition, there was considerable heterogeneity in the centromere 7 (CEP7) copy number in these tumors. The relationship between trisomy/polysomy of chromosome 7 and EGFR gene amplification has been analyzed. In our study, both amplified and non-amplified tumors showed trisomy/polysomy of chromosome 7. This fact suggests that this alteration could be an initial event in the tumorigenesis of glioblastoma.

Cases	Status *EGFR*	Copies *EGFR* (% cases)			CEP 7 (% cases)				% of cells		q-PCR	EGFR expression		
		2-10	6-25	25->100	dis	dis/tri	tri	tri/pol	1-20	25-95		0-1	2-3	(%cases)
46%	Amplification dmin	0	0	100	6	6	50	38	12	88	19-227.8	3	97	
22%	Amplification insertion	0	100	0	10	30	30	30	80	20	3.3 – 7.2	69	31	
32%	No amplification	100	0	0	8	0	46	46	-	-	2.1 -3.1	79	21	

Table 3. Characterization of the three types of *EGFR* amplification found in glioblastoma

5.3 Statistical correlation of *EGFR* copy alterations with clinicopathological parameters

The Kruskal Wallis test was used to assess the differences among the three amplification groups and it showed significant differences in the percentage of cells with positive *EGFR* amplification detected by FISH, the *EGFR* copy number quantified by PCR, and the EGFR transcript expression assessed by microarrays (p-value ≤ 0.01). In the percentage of *EGFR* positive cells and *EGFR* copy number, all bilateral comparisons of the three amplification groups showed statistically significant differences, after the Bonferroni correction (GBM-h vs GBM-l, GBM-h vs GBM-n, GBM-l vs GBM-n) (p-value ≤ 0.01). The EGFR expression values of amplification types GBM-h and GBM-l were statistically significant after the Bonferroni correction (p-value ≤ 0.01). No significant differences were found between types GBM-l and GBM-n. The mean Ki-67 value of group GBM-h exhibited statistically significant differences with respect the rest (p-value < 0.05). Ki-67 mean values for the different groups were 38% for the GBM-h group, 24% for the GBM-l group and 23% for the GBM-n group.

None of the survival curves showed any significant differences in established groups according to *EGFR* amplification type, and age (<55, ≥55 years). As we expected, younger patients (<55 years) showed a slightly higher survival rate. Interestingly, patients with *EGFR* amplification type GBM-h survived for the shortest time, a mean of 9 months.

Amplification in general and amplicons in particular are important for both prognosis and targeted therapies. Specific amplicons, such as *MYCN* in neuroblastomas, and *MYC* and *ERBB2* in breast cancer, also have prognostic significance (Albertson, 2006). *EGFR* amplification status has been studied as a potential prognostic indicator. The majority of the early reports showed that amplification of the *EGFR* was associated with a poorer prognosis than that associated with non-amplified glioblastomas (Simmons et al., 2001, Shinojima et al., 2003). Other studies failed to confirm this relationship. Our study did not show that the type of *EGFR* amplification led to significant differences in survival rates; although, interestingly, patients of group GBM-h, with *EGFR* amplification type dmin survived for the shortest time.

6. Conclusion

Glioblastoma multiforme is characterized by intratumoral heterogeneity in both histomorphological and genetic changes, displaying a wide variety of numerical chromosome aberrations, the most common of which are trisomy 7 and monosomy 10. The amplification of the epidermal growth factor receptor (EGFR) gene is the most frequently reported genetic abnormality.

Our studies about histopathological and genetical characterization of glioblastoma multiforme suggest that, in primary glioblastomas, there exists a subgroup of cases closely linked with *EGFR* amplification. However, there is another subgroup of cases without *EGFR* amplification with some different clinical and genetic characteristics. This second group might include the increased activation or expression of other growth factor receptors observed in these tumors such as PDGFR or IGFR, although they are only amplified in a low percentage of cases.

Furthermore, we proposed that *EGFR* amplification is an important and frequent pathway in glioblastomas. This amplification is expressed as dmin in a subset of glioblastoma, but we have observed another type of amplification of the EGFR gene. This is seen at chromosome level as distributed insertions of this gene in chromosome 7 itself. Detection of this pattern

of amplification of *EGFR* may further improve the prognostic value of genomic diagnosis of this disease.

Since amplification is often associated with poor prognosis and is a mechanism of resistance to therapies, it will be important to identify the genes or pathways (or both) that promote amplification in tumors, so that they might be targeted as part of a combination therapy to prevent the evolution of resistance to drugs designed to arrest or eradicate the tumor.

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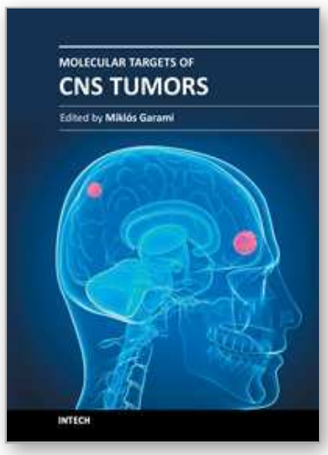

Molecular Targets of CNS Tumors

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Molecular Targets of CNS Tumors is a selected review of Central Nervous System (CNS) tumors with particular emphasis on signaling pathway of the most common CNS tumor types. To develop drugs which specifically attack the cancer cells requires an understanding of the distinct characteristics of those cells. Additional detailed information is provided on selected signal pathways in CNS tumors.

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