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Copy Number Alterations in Glioma Cell Lines

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1. Introduction

Established tumor-derived cell lines are widely and routinely used as *in vitro* cancer models for various kinds of biomedical research. The easy management of these cell cultures, in contrast to the inherent difficulty in establishing and mantaining primary tumoral cultures, has contributed to the wide use of these inmortalized cell lines in order to characterize the biological significance of specific genomic aberrations identified in primary tumors. Therefore, it has been assumed that the genomic and expression aberrations of long-term established cell lines resemble, and are representative, of the primary tumor from which the cell line was derived. Indeed, the cell line-based research has been performed, not only for the definition of the molecular biology of several cancer models, but also for the investigation of new targeted therapeutic agents in a prior step to clinical practice. The use of tumor-derived cell lines has been highly relevant for the testing and development of new therapeutical agents, with several cancer cell-line panels having been developed for drug sensitivity screening and new agents' discovery (Sharma et al, 2010).

Controversial concerning the ability of tumor-derived cell lines to accurately reflect the phenotype and genotype of the parental histology has been documented. A previous report of Greshock and coworkers using array-based Comparative Genomic Hybridization (aCGH) data of seven diagnosis-specific matched tumors and cell lines showed that, on average, cell lines preserve *in vitro* the genetic aberrations that are unique to the parent histology from which they were derived, while acquiring additional locus-specific alterations in long-term cultures (Greshock et al, 2007). In contrast, a study on breast cancer cell lines and primary tumors highlight that cell lines do not always represent the genotypes of parental tumor tissues (Tsuji et al, 2010). Furthermore, a parallel genomic and expression study on glioma cell lines and primary tumors states that in this specific cancer type, cell lines are poor representative of the primary tumors (Li et al, 2008). Given the importance of the use of cell lines as models for the study of the biology and development of tumors, and for the testing of the mode of action of new therapeutical agents, the knowledge of which genomic alterations are tumor-specific or which are necessary for the maintenance of the cell line in culture, becomes essential.

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Sometimes cell line studies are interpreted in the context of artifacts introduced by selection and establishment of cell lines in vitro, given the prevalence of documented cell line-specific cytogenetic changes acquired with multiple growth passages which is associated with random genomic instability. Therefore, the ability of glioma cell-line models to accurately reflect the phenotype and genotype of the parental glioma tumors remains unstudied. The aim of this study is to compare the genomic aberrations of the most commonly used glioma cell lines for *in vitro* analysis with those alterations more prevalent in primary glioma tumors.

2. Copy number alterations in glioma cell lines

2.1 High-level DNA copy number alterations in glioma cell lines

2.1.1 Amplifications

Genomic high-level DNA copy number gains (regions of amplification, or amplicons, i.e. chromosome regions that show more than 5- to 10-fold copy number increases) were detected at 4q, 10q and 19q in two of the cell lines: SW1783 (4q12) and SF767 (10q21.2-q23.1 and 19p12) (table 1, figure 1). The MLPA analysis confirmed some of the genomic alterations observed by aCGH, such as the amplification of *PDGFRA* (4q12) which was observed in SW1783 cell line (see below table 3).

CHROMOSOME	GENES	CELL LINE (Region Size Mb)
4q12	CHIC2, GSH2, PDGFRA, KIT, KDR, SRD5A2L, TMEM165, CLOCK, PDCL2, NMU, EXOC1, CEP135, AASDH, PPAT, PAICS, SRP72, HOP, SPINK2, REST, OLR2B, IGFBP7	SW1783 (3.57)
10q21.2 - q23.1	COL13A1, H2AFY2, AIFM2, TYSND1, SAR1A, PPA1, NPFFR1, LRRC20, EIF4EBP2, NODAL, PRF1, ADAMTS14, SGPL1, PCBD1, UNC5B, SLC29A3, CDH23, PSAP, CHST3, SPOCK2, ASCC1, DNAJB12, CBARA1, CCDC109A, OIT3, PLA2G12B, P4HA1, NUDT13, ECD, DNAJC9, MRPS16, TTC18, ANXA7, ZMYND17, PPP3CB, , USP54, MYOZ1, SYNPO2L, SEC24C, FUT11, NDST2, CAMK2G, PLAU, VCL, AP3M1, ADK, MYST4, DUSP13, SAMD8, VDAC2, KCNMA1, DLG5, NAG13, POLR3A, RPS24, ZMIZ1, PPIF, SFTPD, ANXA11, MAT1A, DYDC1, DYDC2, TSPAN14, NRG3	SF767 (13.37)
19p12	ZNF43,SINE-R ,ZNF208,ZNF257	SF767 (0.28)

Table 1. Summary of high-level gains (amplifications) detected by aCGH

Amplification of the EGFR gene, located on chromosome 7, and subsequent over-expression of EGFR protein, is the most common genetic alteration found in primary glioblastoma (GBM), the most aggressive high-grade glioma. This amplification is detected in about 40% of these tumors, and is present as double-minute extrachromosomal elements (Louis et al, 2007). Amplification of the EGFR gene is often associated with structural rearrangements, resulting in tumors expressing both wild-type EGFR as well as the mutated EGFR. The most common truncated EGFR variant is the EGFRvIII one, consisting of 801-bp in-frame deletion comprising exons 2-7 of the gene.

Among the cell lines analyzed in this study, some of them derived from primary GBMs, none of them carried either amplification of the EGFR gene, nor the EGFRvIII mutant form of the receptor (Figure 2). Besides, EGFR sequence analysis of exons 18-21, coding for the tyrosine kinase domain, revealed not a mutation in this region, unlike what is found in non-small lung cancer tumors.

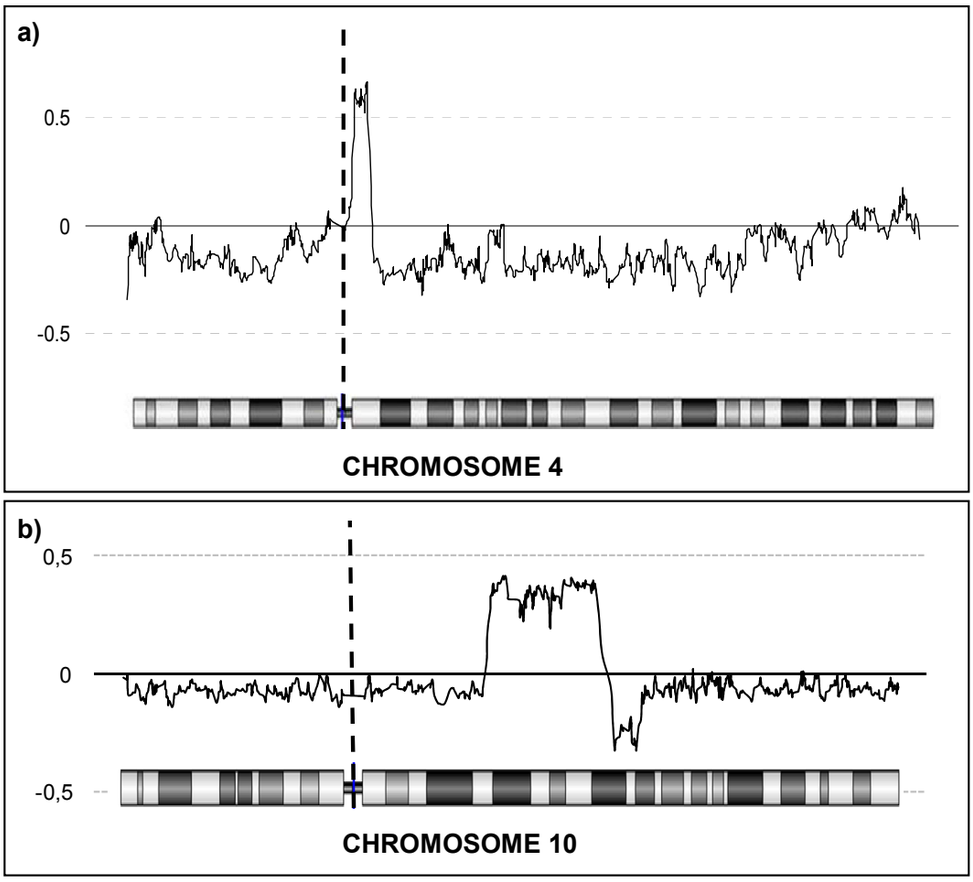


Fig. 1. aCGH results of chromosomes 4 (a) and 10 (b) in SW1783 and SF767 cell lines, respectively. Moving average of $\log_2$-genomic ratios over five neighbouring genes are plotted.

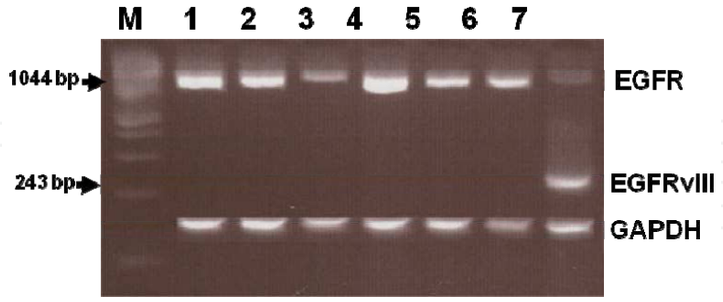


Fig. 2. RT-PCR analysis for the detection of EGFR wild-type (EGFRwt) and EGFRvIII mutant receptor. The inset shows control gene GAPDH results. Line 1: GOS3, 2: A172, 3: U118, 4: SF767, 5: T98, 6: wt EGFR control, 7: EGFRvIII control; M: molecular marker.

2.1.2 Homozygous deletions

Analysis of the high-level copy number changes detected by aCGH in the eleven glioma cell lines revealed higher frequency of genomic losses than gains. A stringent filter was applied in order to detect homozygous deletions.

Genomic homozygous losses were detected at 1p, 1q, 2q, 3p, 4q, 5q, 6q, 7p, 9p, 10p, 10q and 21p (Table 2).
Homozygous losses affecting two or more cell lines were detected at 1p33, 9p21.3-21.1, 10q23.2-23.3 and 21p11.1 (Table 2). Main target genes of these regions were: *CDKN2C* ($p18^{INK4c}$) on chromosome 1, *CDKN2A* ($p16^{INK4a}$) and CDKN2B ($p15^{INK4b}$) on chromosome 9, and *PTEN* on chromosome 10. The most frequent homozygous gene loss was the loss of *CDKN2A* ($p16^{INK4a}$) and *CDKN2B* ($p15^{INK4b}$), affecting nine (82%) and eight (73%) of 11 glioma cell lines, respectively.

2.1.2.1 Loss of *CDKN2C*

The Cancer Cell Line project (CCL) database from the Genome Cancer Project of the Sanger Institute (http://www.sanger.ac.uk/genetics/CGP/CellLines/) was used to confirm these alterations when possible. Homozygous deletion of CDKN2C (1p33) was described in this project for T98 and U87 cell lines. Homozygous deletion of CDKN2C on U373 cell line was not reported in this project. By contrast, this deletion was not reported in the study of Li and coworkers for T98 and U87 cell lines (Li et al, 2008).

2.1.2.2 Loss of *CDKN2A* and *CDKN2B*

CDKN2A (9p21.3) loss of cell lines A172, H4, SW1088, T98, U118 and U87 was reported by the CCL project. Similarly, this gene was described as not mutated in SW1783, therefore confirming our results. Data from GOS3, LN18 and U373 were not provided in this database. Deletion of the 9p21 region was also reported in A172 and U87 cell lines by Li and coworkers, again validating our findings. Strikingly, T98 cell line was not deleted in that study (Li et al, 2008). Furthermore, the MLPA analyses performed on the cell lines confirmed the homozygous deletions observed by aCGH (Table 2). Therefore homozygous deletion of the *CDKN2A* gene was present in 9 of the 11 glioma cell lines (Table 2, Figure 3).
Remarkably, there were two cell lines that lack any alteration at the *CDNK2A* locus, either by homozygous or hemizygous loss of the region.

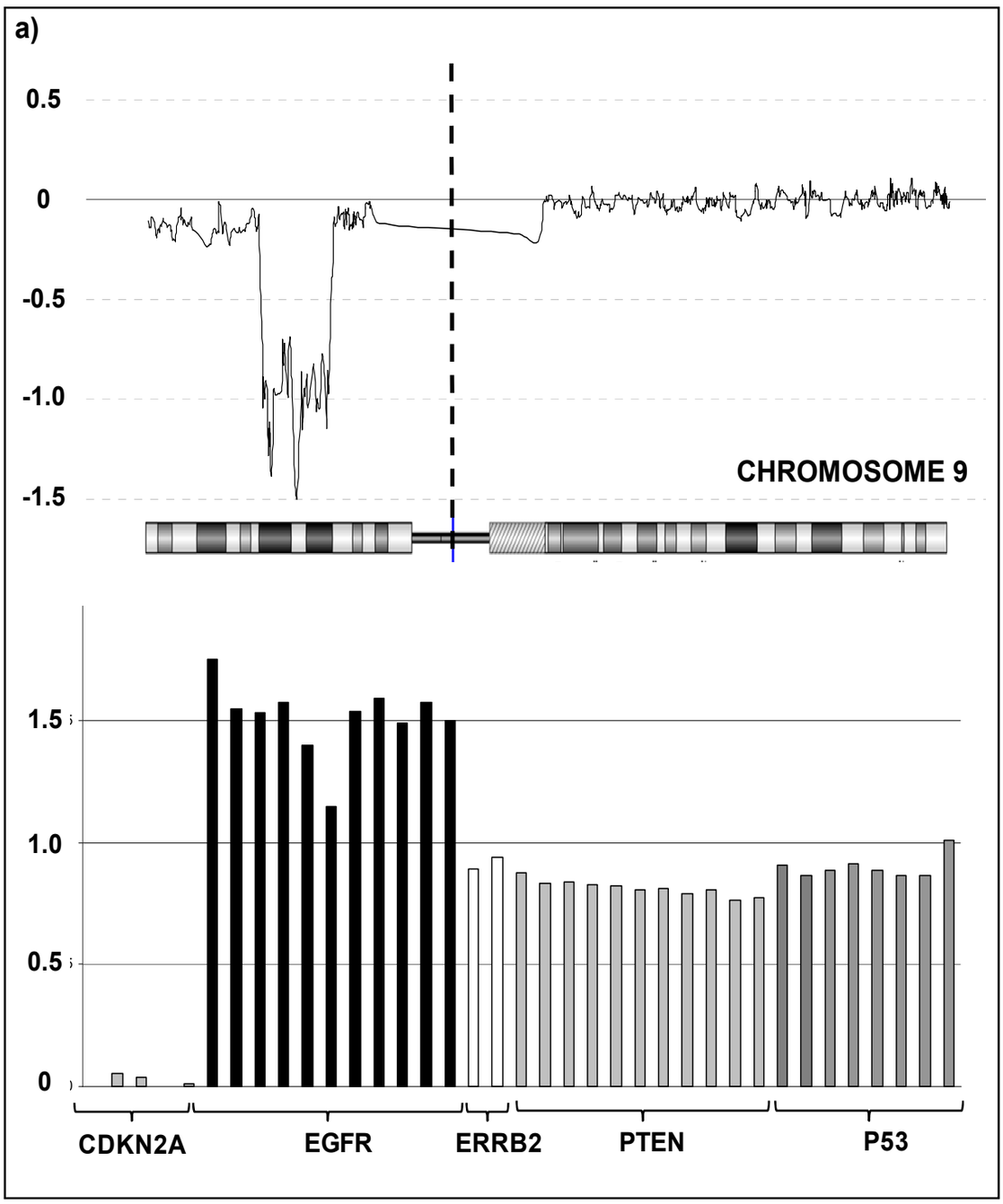
2.1.2.3 Loss of *PTEN*

The aCGH analysis revealed homozygous deletion of PTEN in SW1088 and H4 cell lines (Table 2), which was confirmed by the MLPA assay (Figure 4). In addition, homozygous deletion of PTEN in these cell lines was also reported by the CCL project. PTEN hemizygous deletion was detected in SF767 and GOS3 cell lines by aCGH and MLPA. Surprisingly, A172 cell line had homozygous deletion of all the PTEN probes of the MLPA assay except those of exons 1 and 2. This loss could not be detected by the aCGH analysis, probably because only two of the three probes included in the microarray were in the deleted part of PTEN (homozygous losses were considered as present when three consecutive clones were under the threshold 1.0) (Figure 4).
Further analyses of PTEN sequence were performed attending to PTEN expression (see Table 5 in section 3). Western-blot analysis showed PTEN expression in T98, LN18, GOS3 and SF767 (the two latter carring hemizygous deletion of the gene). Lack of protein expression was found in 7 of the eleven cell lines, three of them having homozygous deletion of PTEN. Therefore, we carried out exon-sequencing analysis of the other four PTEN deficient cell lines (U118, U87, U373 and SW1783) in order to detect putative mutations of the genomic sequence that could explain the observed suppression of protein expression. U118 and U87 presented a substitution mutation (G>T) in the splicing site of exons 8 (c.1026+1G>T) and 3 (c.209+1G>T), respectively; U373 showed an homozygous TT insertion in exon 7 causing a shift in the reading frame (c.723_724insTT); and SW1783

showed a substitution in exon 7 (c.691C>T) which results in a stop codon (CGA>TGA). The latter mutation was confirmed with the database from the Cancer Genome Project (CGP, Sanger Institute). The CGP report the same mutation that we found in cell line U373, for the U251 glioma cell line, which is derived from the same tumour as U373, and thus contains the same TT insertion mutation in PTEN.

CHROMOSOME	GENES	CELL LINE (Mb lost)
1p33	FAF1, CDKN2C	U87 (0.17), T98 (0.07), U373 (0.23)
1p31.1	LRRC44, FPGT, TNNI3K, CRYZ, TYW3	U118
1q42.2	DISC1,SIPA1L2,PCNXL2	GOS3 (1.33)
2q42.2	BAZ2B	GOS3 (0.12)
3p24.3	TBC1D5,SATB1,KCNH8, EFHB,RAB5A, SGOL1, PCAF	H4 (4.63)
3p24.1	TGFBR2	SF767 (0.23)
3p12.2-p11.2	IGSF4D,VGLL3,CHMP2B, POU1F1,HTR1F, CGGBP1	LN18 (6.32)
4q34.1	FBXO8,HPGD,GLRA3	U118 (0.40)
5q14.1	THBS4, SERINC5	SF767 (0.13)
6q22.2	ROS1,DCBLD1	U118 (0.17)
7p21.2-p21.1	ETV1, DGKB, MEOX2, OSTDC1, ANKMY2, BZW2, TSPAN13, AGR2, BCMP11, AHR, SNX13, HDAC9	SF767 (5.09)
9p22.1-p21.1	SLC24A2	LN18 (6.37)
9p21.3-p21.1	MLLT3, IFNB1	U118 (10.86), U87 (3.52), LN18
	IFNW1	U118, U87,LN18, H4 (1.22)
	KLHL9,IFNA2,IFNA8	U118, U87,LN18, H4, SW1088 (7.22)
	IFNE1,MTAP	U118, U87,LN18, H4, SW1088,A172 (0.71)
	CDKN2A	U118,U87,LN18,H4,SW1088,T98,U373,A172,GOS3 (0.18)
	CDKN2B	U118, U87,LN18, H4, SW1088, U373, A172, GOS3
	ELAVL2	U118, LN18, SW1088
	hel-N1	U118, LN18
	PLAA, IFT74, LNG01784, TEK, MOBKL2B, LRRN6C	U118, SW1088
	LINGO2	U118
10p11.21	PARD3	T98 (0.11)
10q23.2 - q23.31	MINPP1	H4 (0.73)
	PAPSS2,ATAD1,PTEN	H4,SW1088
	LIPF,ANKRD22,STAMBPL1, ACTA2, FAS,CH25H,LIPA	SW1088 (1.50)
10q25.2	TCF7L2	T98 (0.16)
12q21.2	PAWR	GOS3 (0.14)
21p11.1	BAGE4,BAGE5,BAGE3,BAGE2, BAGE	H4 A172, U118, GOS3 (0.04)

Table 2. Homozygous losses detected in glioma cell lines by aCGH


a)
0.5
0
-0.5
-1.0
-1.5
CHROMOSOME 9
1.5
1.0
0.5
0
CDKN2A
EGFR
ERRB2
PTEN
P53

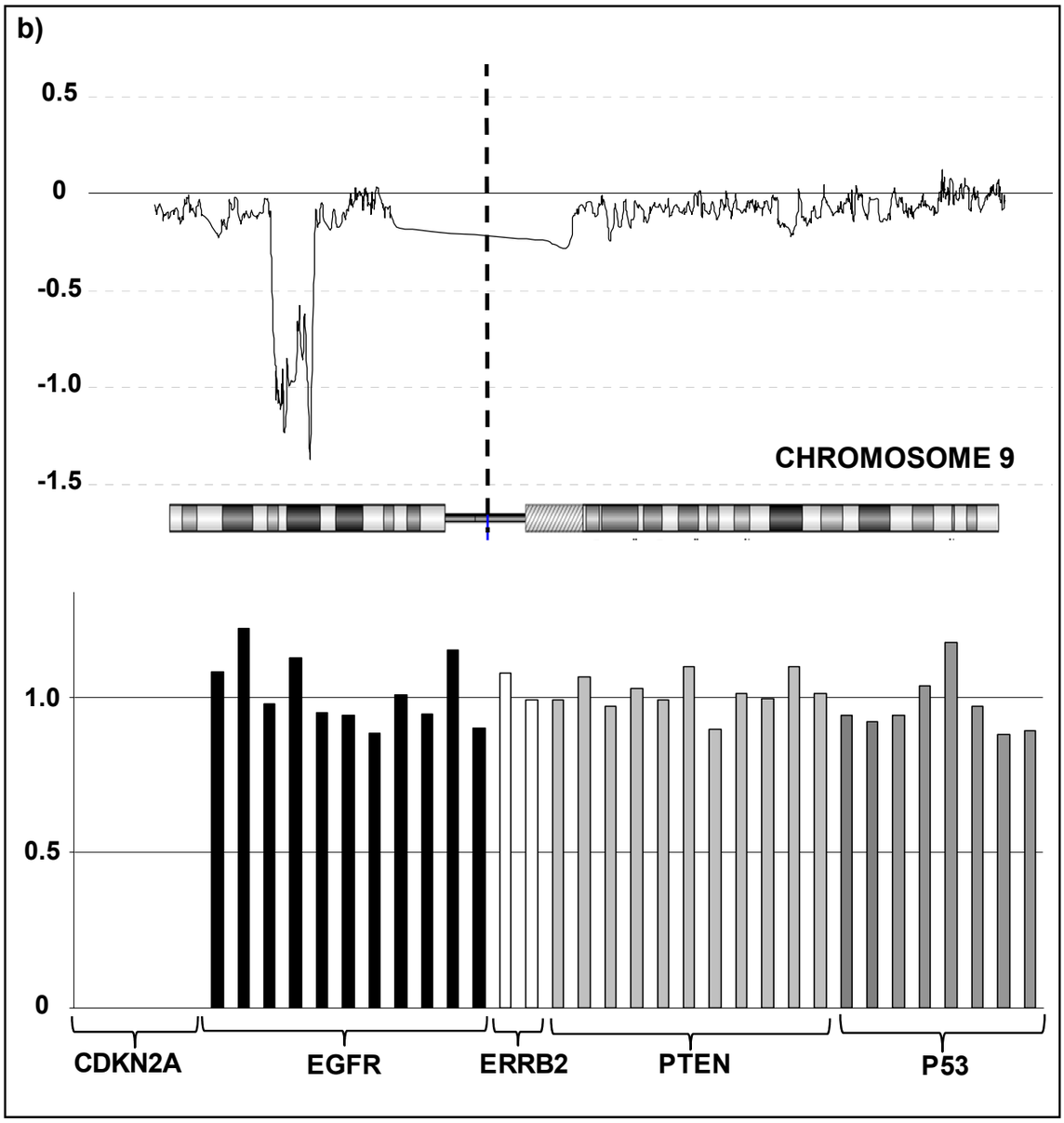


Fig. 3. Homozygous loss detected on chromosome 9 (including *CDKN2A*locus) in two representative cell lines: U118 (a) and LN18 (b). Upper panel: aCGH plot (moving average of $\log_2$-genomic ratios over five neighbouring genes); Lower panel: MLPA graph (each bar represents a probe of the MLPA assay).

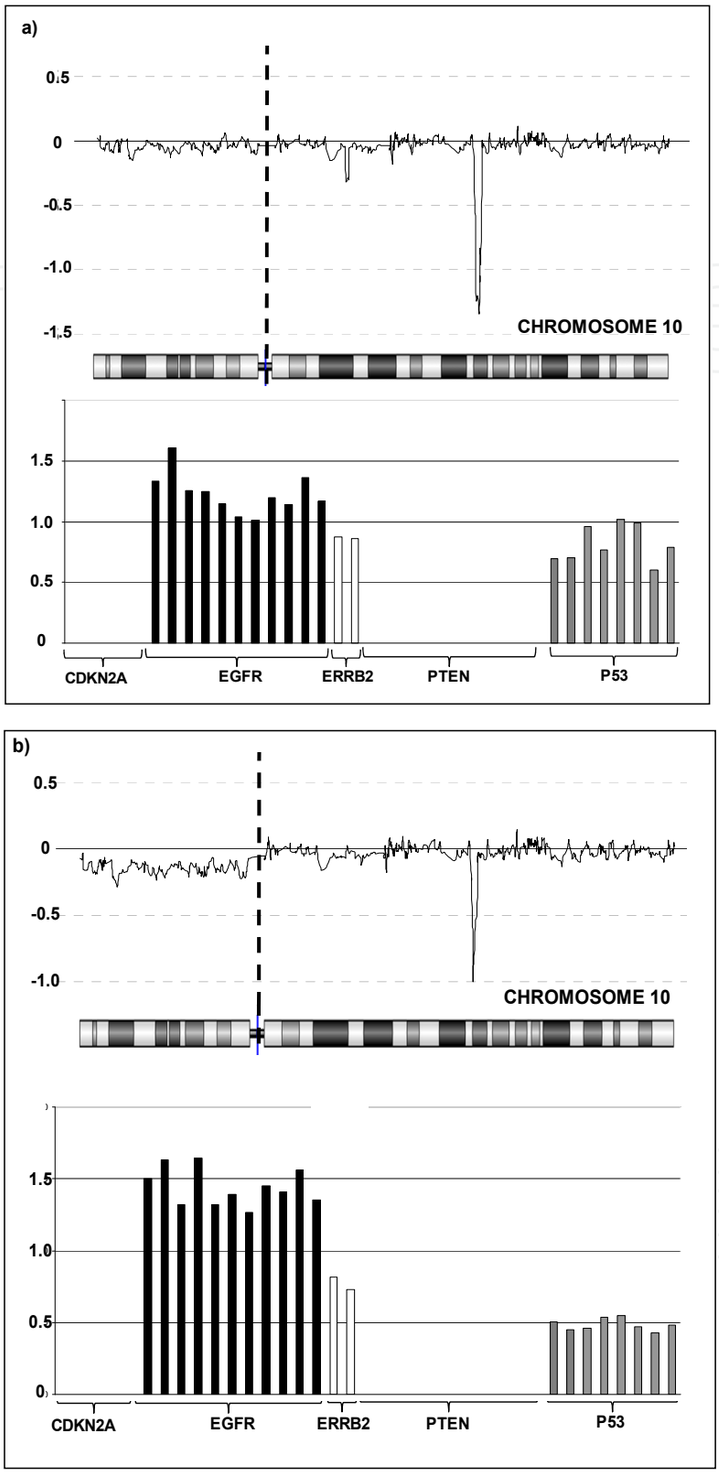


Fig. 4. Homozygous loss detected on chromosome 10 (including *PTEN*) in two representative cell lines: SW1088 (a) and H4 (b). Upper panel: aCGH plot (moving average of $\log_2$-genomic ratios over five neighbouring genes); Lower panel: MLPA graph (each bar represents a probe of the MLPA assay).

2.2 Low-level DNA copy number alterations in glioma cell lines

Analyses of the DNA copy number changes in 11 of the most commonly used glioma cell lines revealed higher frecquency of genomic losses than gains. While 22.15% of the analyzed probes were lost, only 12.35% of them presented gains. Chromosomes containing frequently gained probes among all the cell lines included chromosomes 7, 16, 17, 19 and 20. Similarly, chromosomes containing frequently lost probes included chromosomes 4, 6, 10, 13, 14 and 18 (Figure 5). Surprisingly, chromosome 9, presenting loss of the *CDKN2A/CDKN2B* locus in most of the cell lines (9 out of 11 cell lines) presented a similar percentage of gained and loss probes. This result may be explained due to this loss is relatively small in most of the cell lines, and to the low-level DNA copy number gain of most of chromosome 9 in SF767 cell line (data not shown).

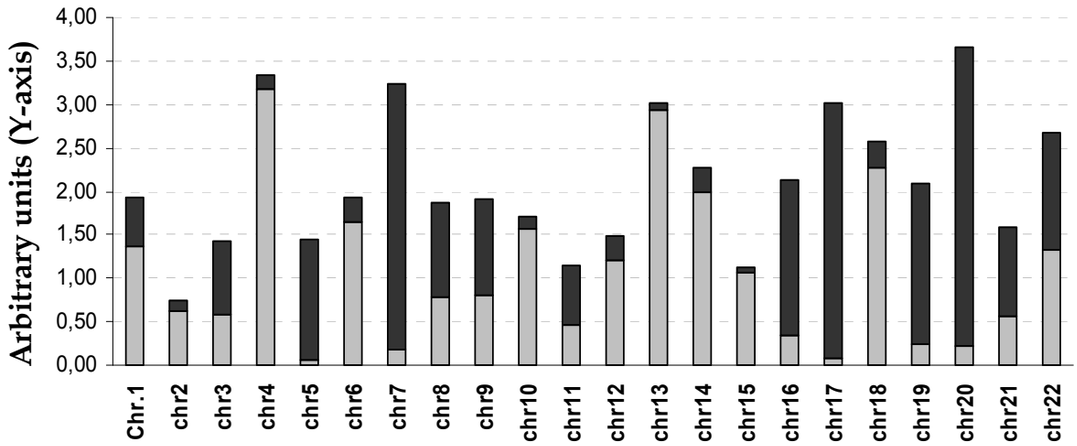


Fig. 5. Percentage of low-level DNA copy number gains (black) and losses (grey) relative to the analyzed probes in the microarray per chromosome.

Chromosome 7 was one of the most gained chromosomes, with complete or almost complete chromosome 7 gain in SW1088 and GOS3 cell lines, or with relative wide regions of gain in H4, U373, U118 or A172 cell lines. Gain of the *EGFR* gene (located at 7p12) was evaluated by MLPA assays, showing EGFR low-level copy number gain in 8 out of the 11 cell lines (table 3).

Other gains detected by MLPA analysis were *PI3KCA*, *BRAF* and *BIRC5*. Three of the cell lines presented a *PI3KCA* gain (3q). *PIK3CA* is one of the three genes encoding components of PI3K which is involved in activation of AKT signaling. Amplification of *PIK3CA* has been observed in various types of cancer, including gliomas (Karakas, 2006; Kita, 2007; Vogt, 2006). *BRAF* oncogene (7q34) was gained in five of the cell lines. BRAF is a serine/threonine kinase that is frequently activated in many types of cancer by a specific mutation (V600E). In pilocytic astrocytomas, BRAF is frequently activated by tandem duplication and rearrangement of part of the gene, resulting in fusion proteins containing the kinase domain (exons 9-18). Activation of BRAF through these mechanisms of duplication or fusion is infrequent in diffusely infiltrating astrocytic gliomas (Bar et al, 2008; Riemenscheneider et al, 2010). All the cell lines analyzed in this study were obtained from adult patients with high grade gliomas.

BIRC5 or survivin (17q) was gained in five of the cell lines. Survivin, which promotes cell proliferation, angiogenes and inhibits apoptosis, is frequently overexpressed in proliferating tissues and tumors (Zhen et al, 2005). In gliomas, survivin overexpression is significantly associated with tumorigenesis and progression, and with a worse prognosis of patients (Shirai et al, 2009). Previous studies revealed, as well, BIRC5 gain and overexpression in oligodendroglial tumors (Blesa et al, 2009). High expression of BIRC5 in nervous system tumors have been already reported (Das, 2002; Hogdson, 2009; Sasaki, 2002).
As a summary, at the gene-level, the most represented gains and losses in the 11 analyzed cell lines are shown in table 4.

	CHROMOSOME	GENE NAME	CELL LINE
HOM LOSS	9p21	*CDKN2A*	U373, U118, SW1088, GOS3, A172, H4, T98, U87, LN18
	10q23	*PTEN*	A172, SW1088, H4
HEMI LOSS	1p13.2	*NRAS*	A172, H4
	10q23	*PTEN*	SF767, GOS3
GAIN	1p13.2	*NRAS*	U373
	1q32	*PI3KC2B*	A172
	2q35	*IGFBP2*	SW1088
	3q26.3	*PIK3CA*	A172, SW1783, H4
	7p12	*EGFR*	U373, U118, SW1088, GOS3, A172, H4, T98, SF767
	7q34	*BRAF*	U87, U373, SW1088, GOS3, T98
	17p11.2	*TOM1L2*	LN18
	17q25	*BIRC5*	H4, LN18, T98, U373, SW1783
	21q22.3	*RUNX1*	H4, A172, T98
A	4q11	*PDGFRA*	SW1783

Table 3. Summary of gene-specific MLPA-validated copy number alterations (HOM LOSS: homozygous loss; HEMI LOSS: one copy loss; GAIN: low-level copy number gains; A: amplifications).

GAIN		HOMOZYGOUS DELETION	
Gene (location)	**Total**	**Gene (location)**	**Total**
EGFR (7p12)	8	CDKN2A (9p21)	9
BRAF (7q34)	5	CDKN2B (9p21)	8
BIRC5 (17q25)	5	MTAP (& others; 9p21)	6
PI3KCA (3q26.3)	3	BAGE (21p11.1)	4
		PTEN (10q23)	3
		CDKN2C (1p33)	3

Table 4. Summary of the alterations most represented on the eleven glioma cell lines studied. (Total: number of cell lines presenting the alteration described)

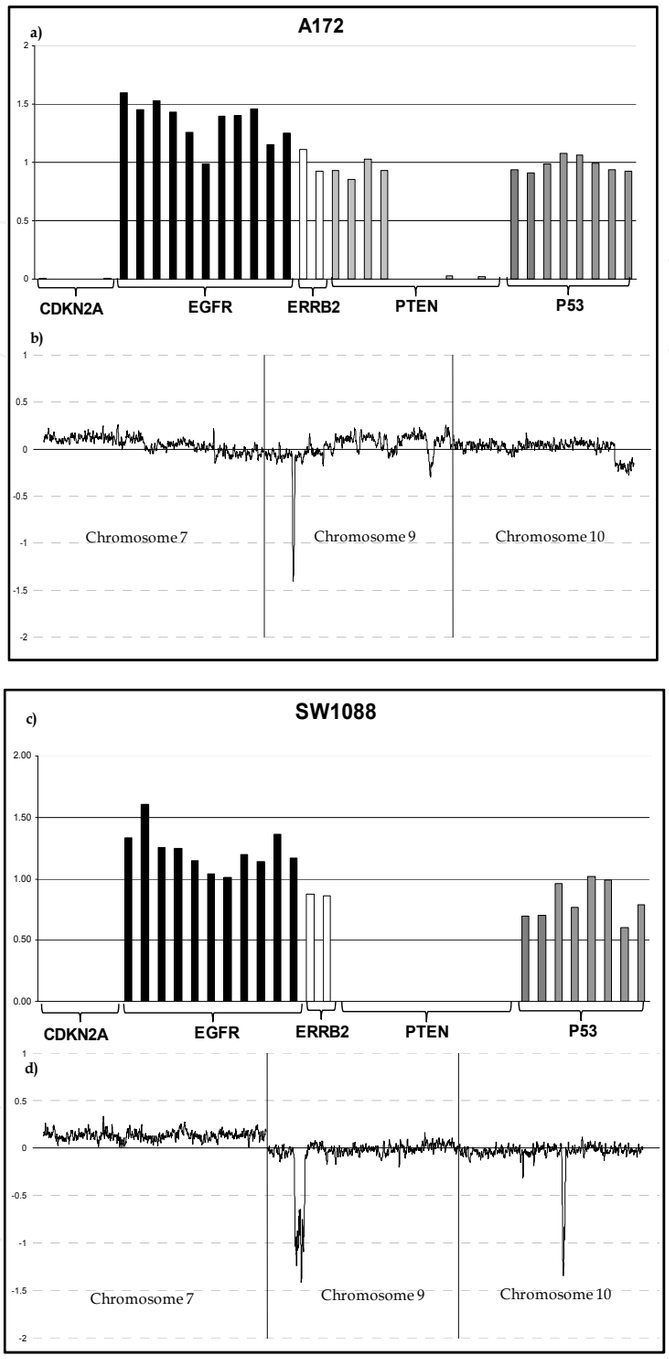


Fig. 6. Genomic analysis of A172 (a, b) and SW1088 cell lines (c, d). a) MLPA analysis (each bar represents a probe of the MLPA assay) showing *EGFR* gain, *CDKN2A* homozygous deletion, and *PTEN* homozygous deletions except for exons 1 and 2. b) aCGH analysis (moving average of $\log_2$-genomic ratios over five neighbouring genes) of chromosomes 7, 9 and 10. c) MLPA analysis showing *EGFR* gain, and homozygous deletions of *CDKN2A* and *PTEN* d) aCGH analysis of chromosomes 7, 9 and 10. Observe that no *PTEN* deletions (10q23.2) were detected in A172 cell line compared to SW1088.

3. Comparison between copy number alterations in glioma cell lines and primary tumors

Gliomas are the most frequent primary brain tumors, and include a variety of different histological tumor types and malignancy grades. High-grade gliomas are those graded as III or IV according to the criteria of the World Health Organization (WHO) classification system (Louis et al, 2007), including anaplasic astrocytoma (WHO grade III) and GBM (WHO grade IV). High-grade gliomas may arise from diffuse astrocytoma WHO grade II or III, or *de novo*, i.e. without evidence of a less malignant precursor lesion. GBM is the most frequent primary brain tumor. Primary GBM manifest rapidly de novo, while secondary GBM develops slowly from diffuse or anaplastic astrocytomas.

It is important to note that most of the cell lines used in this study derived from astrocytoma tumors of high-grade (8 cell lines: T98, LN18, U373, SW1088, H4, SW1783, U118, and A172), with the exception of GOS3 cell line that was derived from a high-grade mixed tumor with oligodendroglial component.

From a genetic point of view, progression to malignancy in gliomas is a multistep process, driven by the sequential acquisition and accumulation of genetic alterations. Distinctions between the genetic alterations identified in primary and secondary GBM have been made, with *TP53* mutations occurring more commonly in secondary GBMs and *EGFR* amplifications, and *PTEN* mutations occurring more frequently in primary GBMs. However, none of these alterations sufficiently distinguishes between primary and secondary GBM.

Recently, a comprehensive sequencing and genomic copy number analysis of GBM tumors showed that the majority of the tumors analyzed had alterations in genes encoding components of each of the *TP53*, *RB1*, and *PI3K* pathways, previously known to be altered in GBMs (Parsons et al, 2008). In these tumors, all but one of the cancers with mutations in members of a pathway did not have alterations in other members of the same family, suggesting that such alterations are functionally equivalent in tumorigenesis. Opposite to what is found in primary and secondary GBMs, glioma cell lines usually harbor functional alterations of the three pathways simultaneously (e.g. SW1088, SW1783 or U118, table 5).

Alteration mutations of the tumor suppressor gene *TP53* (located at 17p13.1) and loss of heterozygosity on chromosome arm 17p are frequent in secondary GBM. While *TP53* copy number analysis showed nor gains or losses in the cell lines tested, neither by CGH nor by MLPA, point mutations have been reported by the Sanger database in some of the analyzed cell lines (Table 5).

Primary GBM, on another hand, characterises by *EGFR* amplification or overexpression, *PTEN* mutation, trisomy of chromosome 7, monosomy of 10 and genomic gains of 12p, 19q and 20q (Riemenschneider et al, 2010).

Regarding alterations of *PTEN* gen (*PI3K* pathway), loss of chromosome 10 is one of the most frequent alteration in primary GBM tumors (60-80% of cases). While many tumors show loss of one entire copy of chromosome 10, loss of heterozygosity (LOH) studies have reported the involvement of several regions of LOH, suggesting several potential tumor suppressor genes in addition to *PTEN*. The cell lines analyzed in our study frequently presented alteration of *PTEN* gene (nine out of 11 cell lines), either by mutation or genomic loss. Absence of PTEN protein expression in these cell lines was confirmed in seven of these cell lines by western blot (data not shown).

Concerning amplifications, EGFR high-level copy number gain is the most frequent alteration found in primary GBM. As mentioned before, this alteration is present as double-

minutes, i.e. small and circular fragments of extrachromosomal DNA that are replicated in the nucleus of the cell during cell division but that, unlike actual chromosomes, lack centromere or telomere. This EGFR amplification has not been detected in any of the analyzed glioma cell lines, probably due to the difficulty in maintaining a highly unstable extrachromosomal fragment that lacks centromere, in long-term cultures. A recent report, however, describes another type of EGFR gain in which extra copies (in small numbers) of EGFR are inserted in different loci of chromosome 7 (Lopez-Gines et al, 2010). The presence of this type of gain in glioma cell lines remains to be studied.

	RB pathway		*PI3K* pathway			*TP53* pathway	
	CDKN2A	***PTEN***	***PTEN seq***	***EGFR***	***EGFRvIII***	***Tp53***	***p53 mut***
T98G	del HOMO	N	-	G	No	N	p.M237I
LN18	del HOMO	N	-	N	No	N*	nd
SF767	N	del HEMI	-	G	No	N*	nd
U373	del HOMO	N*	c.723_724insTT	G	No	N	nd
U87MG	del HOMO	N*	c.209+1G>T	N	No	N*	nd
SW1088	del HOMO	del HOMO*	-	G	No	N	p.R273C
H4	del HOMO	del HOMO*	-	G	No	N*	nd
SW1783	N	N*	c.691C>T	N	No	N	p.R273C
U118	del HOMO	N*	c.1026+1G>T	G	No	N	p.R213Q
GOS3	del HOMO	del HEMI	-	G	No	N	nd
A172	del HOMO	del HOMO*,#	-	G	No	N*	nd

*Protein expression not detected (Western-blot or Immunohistochemistry, data not shown) #deletion except for exons 1 and 2. del HOMO: homozygous deletion; del Hemi: hemizygous deletion; G: Gain; N: No copy number change; No: EGFRvIII mutation not detected; p53 mut: data from the Sanger database; nd: no data from available.

Table 5. Alterations of the RB, TP53 and PI3K pathways.

Thus, at least for what concerns to the EGFR amplification, glioma cell lines seem not to resemble primary tumors. This result contrast to what is found in breast cancer cell lines, where amplification of *ERBB2* (17q12) is detected indeed more frequently in cell lines that in primary tumors (Tsuji et al, 2010). Of note, amplification of *ERBB2* takes place within homogeneously staining regions, where the extra copies of the gene are integrated within the chromosome, thus allowing its maintenance in established cell lines.

Similarly, other amplifications reported in primary GBM tumors have not been found in these cell lines, such as those of 1q (*MDM4*, *PIK3C2B*), 7q (*MET*, *PEX1*, *CDK6*), 12p (*CCDN2*) 12q (*MDM2*, *GLI*, *CDK4*) or 13q (Rao et al, 2010; Ruano et al, 2006). The only common amplification detected in glioma cell lines and tumors was that of 4q (*PDGFRA*) which was detected in SW1783 cell line. *PDGFRA* encodes for a cell surface tyrosine kinase receptor of the members of the platelet-derived growth factor family. These growth factors are mitogens for cells of mesenchymal origin and activate intracellular signaling through the MAPK, PI3K and PKCgamma pathways with roles in the regulation of many biological processes including embryonic development, angiogenesis, cell proliferation and differentiation. On the other hand, to our knowledge, amplifications of 10q and 19p detected in SF767 cell line have not been reported before in glioma tumors.

Digital karyotyping for eight tumor-derived cultured samples and one bulk tumor was used by Rao and coworkers (2010) to describe genomic alterations in GBM. This group described

amplifications in 1q, 7p, 8q and 12q, and homozygous deletions in 1p, 9p and 9q. However 7p11.2-12.1 (*EGFR*), 8q24.21 (*MYC*) and 12q15 (*MDM2*) amplifications were found just in case of the tumor sample, consistent with previous observations that adherent GBM cells tend to lose EGFR amplification during culturing. The most frequent amplifications found by this group was 12q13.3-q14.1, which targeted *GLI1* and *CDK4* oncogenes, affecting 3 samples. Two of the samples showed amplification of *PI3KC2B* and *MDM4* in 1q32.1. Table 6 shows comparison of our results with those published by Rao and coworkers (2010). Low-level copy number gains (e.g. *PI3KC2B*: A172 cell lines; *EGFR:* 8/11 cell lines) but not amplifications were detected in the cell lines.

The p16ink4a/CDK4/RB1 pathway is important for the control of progression through G1 into the S phase of the cell cycle. In GBM tumors, alterations affecting this pathway are found at an overall frequency of 40-50% (Louis et al, 2007). Homozygous deletions affecting *CDKN2A* locus (9p21) were described by digital karyotyping in 44% of cultured samples (four out of nine) (Rao et al, 2010). Our study reveals 82% (9/11) and 73% (8/11) of cell lines carrying homozygous deletions for *CDKN2A* and *CDKN2B* genes, respectively (Table 6).

Chromosome band	**Target oncogene**	**Rao % (n=9)**	**Our group % (n=11)**
Amplifications/Gains (G)			
1q32.1	PIK3C2B	22	9 (G)
1q32.1	MDM4	22	0
7p11.2-12.1	EGFR	11	73 (G)
8q24.21	MYC	11	45 (G)
12q13.3-q14.1	GLI1,CD4	33	18 (G)
12q14.1	Unknown	22	9 (G)
12q15	MDM2	11	0
Homozygous deletions			
1p36.31-p36.23	TP73, LRRC47, DFFB	33	18
9p21.3-22.3	CDKN2A, CDKN2B	44	82, 73
9q34.3	CACNA1B	44	0

Table 6. Comparison of results obtained by Digital Karyotyping (Rao et al, 2010) with aCGH alterations observed in glioma cell lines. Only amplification data from Rao´s study was available.

Finally, regarding the number of DNA copy number alterations in cell lines, the lost probes almost doubled the gained ones, with an average of losses and gains per cell line of 9,908 and 5,072 probes, respectively. This result contrast to what is observed in primary GBM tumors, having similar numbers of gains and losses (Ruano et al, 2006). Accordingly, similar results were obtained in tumor-derived cell lines from other histologies (Greshock et al, 2007) and specifically in breast cancer cell lines (Tsuji et al, 2010; Naylor et al, 2005), with more alterations found in cell lines than in tissue specimens, as a general trend. In fact, genomic losses in breast cancer cell lines almost doubled the gains (Tsuji et al, 2010). These observations may suggest the accumulation of genomic alterations in long-term cultures that are not present in primary tissues.

4. Cell culture specific aberrations

Several of the frequent genomic alterations detected in glioma cell lines are not found in primary tumors, suggesting that some of the commonly seen alterations *in vitro* could be artifacts secondary to the selection pressure for optimal cell growth *in vitro* following years of passage. This observation has been reported previously in gliomas (Li et al, 2008), but the presence of acquired locus-specific alterations in culture has also been recognized in tumors and cell lines of other histologies (Greshock et al, 2007). For example, genome-specific copy number alterations of chromosomes 5 (gained), 8, 11 and 18 (lost) in glioma cell lines have been attributed exclusively to the phenotype of established cell lines. Furthermore, other copy number alterations not commonly found in cell lines, such as those of specific areas of chromosomes 2, 3, 6 and 8 have been rarely observed in primary tumors.

Our findings (Figure 5) have identified areas of low-level gain on chromosomes 5, 16 and 17 affecting between 5 and 7 cell lines, which do not feature GBM tumors. In addition, areas of loss of chromosomes 6, 8, 11, and, most importantly, loss of chromosome 18 have been identified in most of cell lines. These alterations seem to be culture-associated changes present in cell lines and suggest a genomic instability phenotype in established cell lines that is not present in primary tumor tissues.

Absence of chromosome 13 deletions in glioma cell lines, which were commonly found in primary GBMs, was reported by Li and coworkers (2008) as a striking discrepancy between cell lines and tumors. Our study, however, did detected chromosome 13 losses (Figure 5). In the present study, complete loss of chromosome 13 was identified by aCGH in H4, LN18, U373, SW1088 and U118 cell lines, while partial loss was detected in U87, SF767, SW1783 and A172 cell lines. No loss was observed in T98 and GOS3 cell lines. Curiously, cell lines analyzed in common by our study and that of Li, had partial chromosome 13 loss in our study and partial chromosome 13 LOH in the study of Li and coworkers (U87 and A172), or no chosmosome 13 loss in both studies (T98).

5. Material and methods

5.1 Cell lines and cell culture

The human glioma cell lines GOS3, U87MG (U87), A172, SW1783, U118 MG (U118), T98G (T98), SW1088, H4, LN18, U373MG (U373) and SF767WL (SF767) were kindly provided by Dr. Velasco (Complutense University of Madrid, Spain) or Dr. Setién (Catalan Institute of Oncology, Spain). These cell lines were maintained in RPMI medium containing 10% FBS (Gibco, Grand Island, NY) in standard culture conditions. Total DNA and RNA were extracted from cell cultures according to standard phenol-chloroform and Trizol (Invitrogen, Carlsbad, CA) techniques, respectively. Nucleic acids obtained were quantified using NanoDrop-1000 (NanoDrop Technologies, Inc., Wilmington, DE).

5.2 Comparative genomic hybridization

Copy number analyses of the 11 glioma cell lines were screened by array-based Comparative Genomic Hybridization (aCGH) in the Microarrays Analysis Service of the CIPF (Centro de Investigación Principe Felipe, Valencia). "Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis" protocol Version 4.0 (Agilent Technologies, Palo Alto, California USA. p/n G4410-90010) was followed to obtain labeled DNA. 2000 ng of DNA from samples and reference DNA (pool of sex-matched normal brain DNA) was

fragmented and labeled (Cyanine 3-dUTP for the cell lines DNA and cyanine 5-dUTP for the reference DNA) according to the "Agilent Genomic DNA labeling kit plus" protocol. Labeled DNA was hybridized with Human Genome CGH Microarray 44 k (Agilent p/n G4426B-014950) containing 45,214 probes with 42,494 distinct biological features. Arrays were scanned in an Agilent Microarray Scanner (Agilent G2565BA). Data was analyzed using DNA Analytics 4.0 CGH Module (Agilent Technologies). Genomic alterations were detected using an ADM-2 algorithm with two different filters: one, used to detect low level alterations, , detects those alterations affecting to three consecutive probes with a ratio above or below to 0.25; the other, used to obtain amplifications or homozygous deletion, included in 2.1 section, detects only three consecutive probes above or below a ratio of 1.0.

5.3 MLPA analysis

Specific gene alterations were validated by Multiple ligation probe assay experiments (MLPA®, Mrc-Holland, The Netherlands) with SALSA MLPA kit P105 Glioma-2 for EGFR, PTEN, CDKN2A and p53. Besides, SALSA® MLPA® kit P173 was used to detect copy number alteration of several genes which are frequently altered in several tumors, such as: BCL2L11, BIRC5, BRAF, ERBB4, JAK2, NRAS, PDGFRA, PIK3C2B, PIK3CA. MLPA assays were carried in total DNA from the eleven cell lines, obtained by standard methods, following manufacturers' conditions. Polymerase chain reaction products were separated and quantified on an ABI PRISM 310 DNA sequencer (Applied Biosystems), and electropherograms were analyzed using GeneMapper v.3.7 software (Applied Biosystems). Three nontumor reference samples were included in each run.

5.4 EGFRvIII analysis

Presence of EGFR vIII variant was determined by RT-PCR from total RNA of the cell cultures. cDNA was obtained from lμg of total RNA using the Superscript System (Gibco®). Primers and PCR conditions used were previously described (Lee et al, 2006). Amplifications products were visualized in bromure ethydium 2% agarose gel.

5.5 EGFR and PTEN sequence analysis

Mutations in exons 1 to 9 of PTEN gene and 18 to 21 of the EGFR gene were screened by direct sequencing in an ABI PRISM 310 DNA Analyser (Applied Biosystems) according to the manufacturer's instructions. PCR primers and conditions for EGFR amplification were previously described (Hsieh et al, 2006).

Exon	Upstream primer 5'-3'	Downstream primer 5'-3'	Annealing T (°C)
1	TCCTCCTTTTTCTTCAGCCAC	GAAAGGTAAAGAGGAGCAGCC	56
2	GCTGCATATTTCAATCAAACTAA	ACATCAATATTTGAAATAGAAAATC	54
3	TGTTAATGGTGGCTTTTTG	GCAAGCATACAAATAAGAAAAC	56
4	TTCCTAAGTGCAAAAGATAAC	TACAGTCTATCGGGTTTAAGT	56
5	TTTTTTTTTCTTATTCTGAGGTTAT	GAAGAGGAAAGGAAAAACATC	51
6	AGTGAAATAACTATAATGGAACA	GAAGGATGAGAATTTCAAGC	54
7	AATACTGGTATGTATTTAACCAT	TCTCCCAATGAAAGTAAAGTA	56
8	TTTTTAGGACAAAATGTTTCAC	CCCACAAAATGTTTAATTTAAC	54
9	GTTTTCATTTTAAATTTTCTTTC	TGGTGTTTTATCCCTCTTG	54

Table 7. PTEN sequence and annealing temperature used for PCR reactions of nine exon primers

6. Conclusion

High-level copy number alterations have been observed in cell lines of different sources such as breast, melanoma or lung tumors. Some authors suggest that some of the commonly seen alterations in the glioma cell lines can be due to the *in vitro* cell growth process following long term passage cultures. These observations are based on (i) the comparison of the genomic alterations of glioma and other non glioma cancer cell lines: some of these alterations are common between established cancer cell lines from different origin and uncommon in glioma tumors (Li et al, 2008). ii) Differential expression analyses suggest that established cancer cell lines share an underlying molecular similarity more closely related to their *in vitro* culture conditions than to their original tumor type of origin. Although some functional signalling pathways are up-regulated both in glioma tumors and glioma cell lines (epidermal growth factor receptor, vascular endothelial growth factor receptor, p53, PI3K pathway), there are some others gene expression sets whose up-regulation is just seen in cancer cell lines (cell cycle, proteasome activity, purine metabolism, mitochondrial activity).
Our findings show that established glioma cell lines and glioma tumours have differences in genomic alterations, concluding that glioma cell lines may not be such an accurate representation or model system for primary gliomas as would be desirable. As opposed to primary tumors, glioma cell lines did not present either *EGFR* amplification, or presence of EGFRvIII variant, events that are frequent in high-grade gliomas. Homozygous *CDKN2A* deletion was frequently observed in glioma cell lines, as occur in cell lines derived from other histologies and in glioma tumors. Chromosome 7 gain and *PTEN* deletions represent the most specific glioma alterations present in these cell lines.
The easy of management of glioma cell lines make these cell lines as good candidate models for exploring basic glioma biology and for the use and discovery of therapeutic agents in preclinical screens. However, it is of interest that cell clycle-related alterations of gene expression are importantly affected in these cell lines, and that most drugs have been tested for cytotoxicity against rapidly dividing cells. Therefore, selection bias toward the identification of therapeutic agents involved in molecular functions more related to the long term culture than to glioma biology could occur.
On the other hand, many efforts are being done to create adequate culture conditions that allow the maintenance of the genomic profiles of the original tumor, such as glioma stem-like cell cultures, which may be more representative of their parent tumors. Several reports have demonstrated that glioma cultures under serum free conditions and stimulated with mitogens, epidermal growth factor and fibroblast growth factor, grow as neurospheres and maintain a phenotype and genotype closer to that typical of primary tumours compared to traditional serum-derived cell lines and culture techniques (Fael Al-Mayhani et al, 2009; Ernst et al, 2009). Perhaps, the standardization of this culture method could enhance and improve the research with cell lines in brain tumors.

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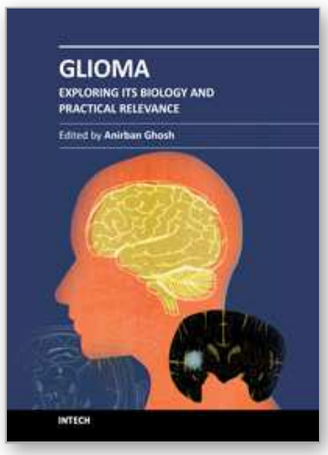
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The tittle 'Glioma - Exploring Its Biology and Practical Relevance' is indicative of its content. This volume contains 21 chapters basically intended to explore glioma biology and discussing the experimental model systems for the purpose. It is hoped that the present volume will provide supportive and relevant awareness and understanding on the fundamental advances of the subject to the professionals from any sphere interested about glioma.

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