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Detailed Characterization of Alterations of Chromosomes 7, 9, and 10 in Glioblastomas as Assessed by Single-Nucleotide Polymorphism Arrays

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Glioblastomas are cytogenetically heterogeneous tumors that frequently display alterations of chromosomes 7, 9p, and 10q. We used high-density (500K) single-nucleotide polymorphism arrays to investigate genome-wide copy number alterations and loss of heterozygosity in 35 primary glioblastomas. We focused on the identification and detailed characterization of alterations involving the most frequently altered chromosomes (chromosomes 7, 9, and 10), the identification of distinct prognostic subgroups of glioblastomas based on the cytogenetic patterns of alteration for these chromosomes, and validation of their prognostic impact in a larger series of tumors from public databases. Gains of chromosome 7 (97%), with or without epidermal growth factor receptor (*EGFR*) amplification, and losses of chromosomes 9p (83%) and 10 (91%) were the most frequent alterations. Such alterations defined five different cytogenetic groups with a significant effect on patient survival; notably, *EGFR* amplification (29%) was associated with a better survival among older patients, as confirmed by multivariate analysis of a larger series of glioblastomas from the literature. In addition, our results provide further evidence about the relevance of other genes (eg, *EGFR*, *CDKN2A/B, MTAP*) in the pathogenesis of glioblastomas. Altogether, our results confirm the cytogenetic heterogeneity of glioblastomas and suggest that their stratification based on combined assessment of cytogenetic alterations involving chromosomes 7, 9, and 10 may contribute to the prognostic evaluation of glioblastomas. ***(J Mol Diagn 2011, 13: 634–647; DOI: 10.1016/j.jmoldx.2011.06.003)***

Gliomas are a heterogeneous group of malignant tumors that show variable localization, histopathologic features, and genetic profiles, together with a heterogeneous response to therapy but a uniformly fatal outcome.[1–11] Although no common genetic signature has been detected in all gliomas, multiple chromosomal changes have been described so far, which frequently include gains of chromosome 7 and deletions of chromosomes 9 and 10 and to a less extent also of chromosomes 1 and 19.[12–14] These genetic changes are associated with amplification of oncogenes [eg, epidermal growth factor receptor (*EGFR*)] together with deletion and/or mutation of tumor suppressor genes [eg, tumor protein p53 (*TP53*), phosphatase and tensin homolog (*PTEN*), and cyclin-dependent kinase inhibitor 2A (*p16/CDKN2A*)].[15]

Altogether, these results point out the potential involvement of different signaling pathways in gliomas, with alterations of chromosome 7, 9, and 10 participating in the most frequent tumor subtypes (eg, glioblastoma). In line with this hypothesis, we have recently shown the existence of distinct cytogenetic pathways in gliomas, by using interphase fluorescence in situ hybridization (iFISH) analysis of intratumoral patterns of chromosomal alterations, at the single-cell level.[16] Notably, specific genomic aberrations and cytogenetic profiles are associated with particular tumor histo-

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pathologic features.[17–19] Accordingly, amplification (or rearrangement) of *EGFR* is almost restricted to a fraction of all malignant gliomas, particularly glioblastomas. Among these cases, overexpression of the EGFR variant 3 mutant is most frequently detected.[20,21] Although this mutant protein is unable to bind to its ligands, it constitutively signals, conferring proliferation and survival advantages to tumor cells.[20–22] In turn, genomic deletions of chromosomes 9 and 10 at regions that harbor tumor suppressor genes are also typically found in glioblastomas, where they have been associated with the development of the tumor, its progression, and a poor prognosis.[23–26] Interestingly, monosomy 10 is associated with gain or amplification of the *EGFR* gene on chromosome 7p11.2, supporting the role of both alterations in gliomagenesis.[27,28] Other genetic abnormalities that can be frequently found in low-grade gliomas[29,30] [eg, combined del(1p)/del(19q) and *TP53* mutation] are less frequently detected in glioblastomas.[31–35]

In the past, most studies devoted to the identification and characterization of genetic/chromosomal alterations in glioblastomas have used conventional cytogenetic and molecular techniques associated with relatively low-resolution (eg, iFISH and comparative genomic hybridization). Recently, high-density single-nucleotide polymorphism (SNP) arrays have been used to characterize the most frequent genetic alterations of glioblastomas.[36–51] Newly available high-density SNP arrays allow the study of copy number (CN) changes and loss of heterozygosity (LOH) at both coding and noncoding DNA regions of the whole tumor cell genome, with high resolution; this provides a more precise map of the genetic alterations associated with CN changes in glioblastomas. Thus, SNP array studies performed in large series of patients with or without gene expression profiling have provided new insights into the potential role of new candidate genes (eg, *ERBB2*, *NF1*, and *TP53*), molecular changes (eg, *PIK3R1* and *PDGFRA/IDH1* mutations), and signaling pathways into the pathogenesis of glioblastomas.[40] In turn, based on gene expression profiles, a molecular classification of glioblastomas has been proposed that reflects the involvement of different neural lineages.[42] To the best of our knowledge, however, no classification based on the genetic changes involving the most frequently altered chromosomes (eg, 7, 9, and 10) has been proposed so far for glioblastomas.

We used high-density (500K) SNP array to investigate genome-wide CN alterations and LOH in a group of 35 primary glioblastoma patients; we focused on the identification and detailed characterization of the genetic alterations of those chromosomes more frequently altered in these tumors and the identification of groups of glioblastomas with distinct cytogenetic patterns of alteration for these 3 chromosomes, which are potentially associated with the behavior of the disease. Finally, the prognostic value of the presence of amplification of the *EGFR* gene was confirmed in a larger number of cases from four different independent series of glioblastoma patients, which have been previously reported in the literature.[41,42,45,50]

Materials and Methods

Patients and Samples

A total of 70 paired tumor ($n = 35$) and peripheral blood (PB; $n = 35$) samples from 35 patients (15 men and 20 women) diagnosed as having glioblastomas (mean ± SD age, 60 ± 14 years; age range, 30 to 84 years) who were admitted to the Neurosurgery Service of the University Hospital of Coimbra (Coimbra, Portugal) were included in this study. Before entering the study, each patient gave written informed consent to participate, and the study was approved by the Hospital's Ethics Committee. Of the 35 patients, 5 underwent complete resection of the tumor; either partial resection or just a diagnostic biopsy was performed in the other 30 cases (Table 1). Distribution according to tumor localization was as follows: 16 tumors were localized in the frontal lobe, 12 were temporal, 3 were parietal, 2 were occipital, 1 was insular, and 1 had a deep localization. Tumors were diagnosed and classified by an experienced neuropathologist according to the World Health Organization criteria.[3] At the time of closing the study, all patients had died, with a median overall survival of 11 months (range, 1 week to 67 months).

Representative parts of fresh tumor tissues left after routine diagnostic histopathologic procedures had been performed were immediately snap-frozen in liquid nitrogen and stored at −80°C, until used for iFISH and DNA extraction for SNP array studies. In each case, a section cut from the tissue block used for this purpose was histologically assessed to estimate tumor cell contents. Specimens with 75% or more tumor cells in the absence of contamination by normal brain parenchyma and tumor necrosis were systematically selected for further DNA extraction and SNP array studies.

DNA Extraction and SNP Array Hybridization

DNA from both frozen tumor tissues and their paired PB leukocyte samples was purified using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA yield and purity were determined with a NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). DNA integrity was evaluated by conventional electrophoretic procedures in 1% agarose gel.

DNA samples were processed according to the Mapping 500K Array Set (Affymetrix Inc., Santa Clara, CA) protocol with two arrays, each containing 250,000 SNPs, with a mean intermarker distance of 5.8 kb (250K Nsp and Sty arrays). Briefly, total DNA (250 ng per array) from paired tumor and PB samples was separately digested with the *Nsp*I and *Sty*I restriction enzyme and ligated to the corresponding adaptors that recognize overhangs generated by the restriction enzymes. All digested DNA fragments were then used as substrates for adaptor ligation, regardless of their size. A generic primer that recognizes the adaptor sequence was used in triplicate to amplify adaptor-ligated DNA fragments through PCR. The amplified DNA was then fragmented, labeled, and hybridized to the GeneChip Human Mapping 250K Nsp

Table 1. Clinical Features of the 35 Study Patients Diagnosed as Having Glioblastoma Multiforme

Case no.	Age, years	Sex	Tumor localization	Number of relapses	Survival after surgery, months*	Karnofsky index, %	Surgical removal
G14	69	Female	Frontal	0	0	70	B
G35	50	Female	Frontal	0	2	50	ST
G54	65	Female	Parietal	0	6	60	ST
G31	71	Female	Frontal	0	7	80	ST
G43	67	Female	Temporal	0	7	70	ST
G30	71	Female	Temporal	0	9	60	B
G8	67	Female	Deep	0	9	90	ST
G45	76	Female	Temporal	0	10	60	ST
G29	49	Female	Parietal	0	12	90	B
G63	61	Female	Insular	0	13	60	ST
G41	44	Female	Frontal	0	14	60	B
G23	50	Female	Frontal	0	14	80	B
G40	45	Female	Frontal	1	15	80	ST
G10	35	Female	Temporal	0	15	80	ST
G55	54	Female	Frontal	1	17	80	ST
G62	57	Female	Occipital	1	18	90	T
G39	70	Female	Frontal	1	18	70	ST
G6	70	Female	Temporal	1	19	80	ST
G13	39	Female	Frontal	1	21	90	ST
G17	30	Female	Temporal	3	67	80	ST
G12	74	Male	Temporal	0	1	70	B
G51	60	Male	Temporal	0	2	60	B
G42	67	Male	Temporal	0	2	80	ST
G46	62	Male	Frontal	0	3	60	ST
G34	69	Male	Temporal	0	5	80	B
G15	79	Male	Parietal	0	5	80	T
G25	68	Male	Frontal	0	7	70	ST
G57	34	Male	Frontal	0	8	90	T
G64	57	Male	Occipital	0	8	60	ST
G50	84	Male	Temporal	0	11	70	ST
G56	65	Male	Frontal	0	13	80	ST
G52	56	Male	Frontal	0	21	90	B
G44	48	Male	Frontal	0	22	80	ST
G53	74	Male	Frontal	0	29	60	T
G37	70	Male	Temporal	1	32	80	T

*At the moment of closing this study, all patients had died.
B, biopsy; ST, subtotal; T, total.

or Sty arrays. After hybridization, the chips were washed and the hybridized sequences were labeled using streptavidin-phycoerythrin and assayed by fluorescence detection. Arrays were washed in an Affymetrix Fluidics Station 450 and scanned using a GeneChip Scanner 3000 (Affymetrix). The allelotype at a locus was then determined based on probe-associated fluorescence intensity data for complementary oligonucleotides to the reference sequences covering the corresponding SNP position.

Identification of CN Alterations and LOH

Identification of CN alterations and LOH was based on the analysis of a total of 500,568 SNPs for paired tumor and normal PB DNA samples. A total of 140 ".CEL" files containing data on the SNP arrays (one for each type of chip) for each type of sample (paired tumor and PB DNA) were obtained for the 35 glioblastomas using the Affymetrix GCOS software (version 1.3). The Copy Number Analysis Tool (CNAT v 4.0; Affymetrix) and the dChip 2007 software[52] (Dana Farber Institute, Harvard, MA, *http://www.dchip.org*, last accessed June 1, 2011) were used to calculate CN values and plot them according to chromosome localization. Genotypes were generated using the BRLMM algorithm included in the Genotyping Console software (version 3.0.2; Affymetrix). Normal PB samples with cutoff values of 1.30 or less and 2.50 or more (arbitrary units) were used to establish CN losses and gains. In addition, the CNAG software (version 3.3.01, The University of Tokyo, Tokyo, Japan)[53] was used to explore the state of each of the two alleles corresponding to each chromosome to distinguish between homozygous and heterozygous deletions.

LOH was defined by the presence of homozygous alleles in tumor DNA samples for alleles that were heterozygous in normal PB DNA from the same individual, and it was classified as follows: LOH by true allelic imbalance (loci at which one of the two parental copies of a chromosome is deleted) or copy neutral LOH (cnLOH) (tumor DNA showing two copies of a chromosome region from one allele in the absence of the other allele and a CN value of two).

To confirm further our findings, an additional series of 119 patients with primary glioblastomas, whose tumors had been analyzed by SNP arrays (100K, 250K, and 500K Affymetrix SNP arrays) and reported in the literature, with data on such analyses being available in an

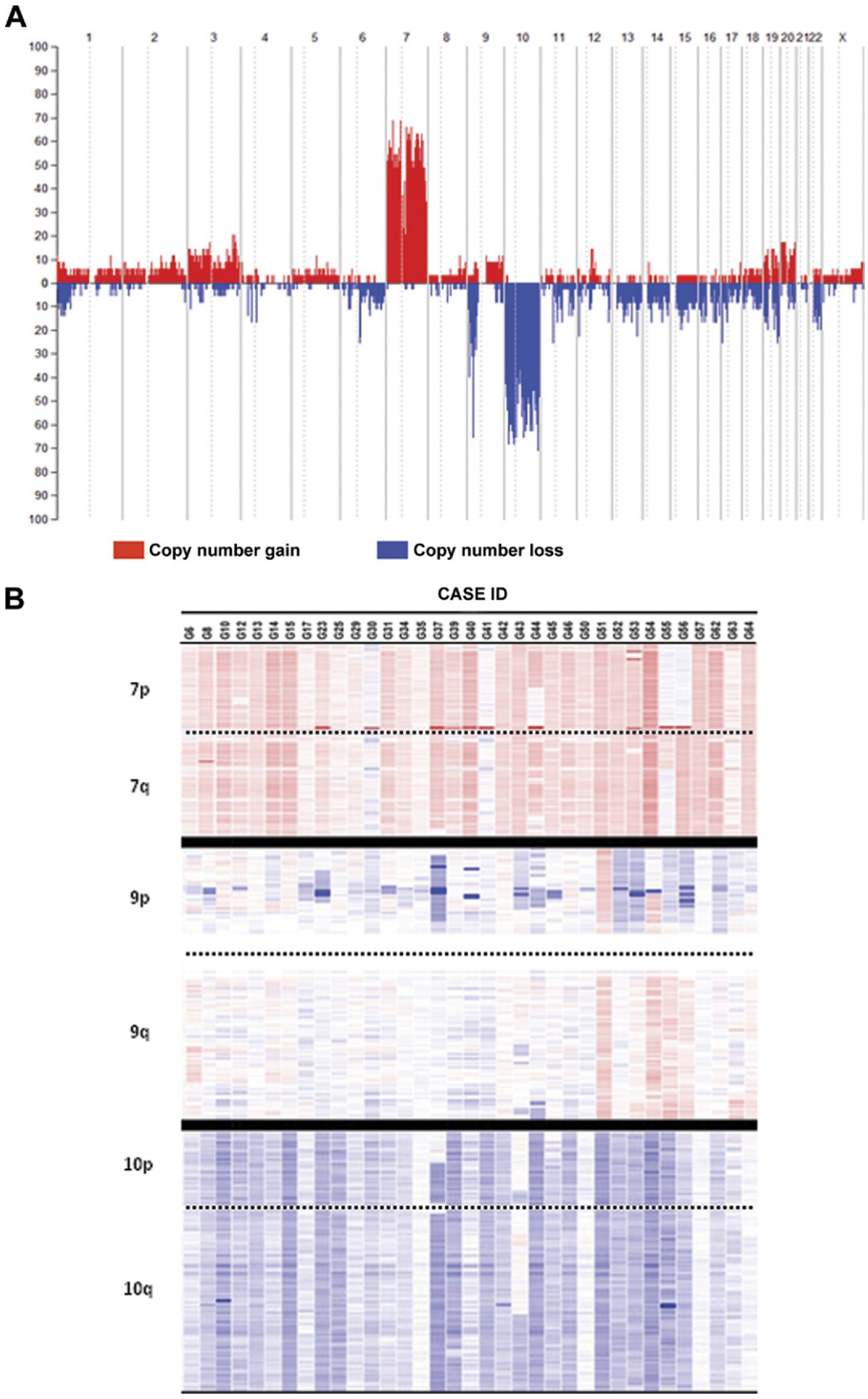


Figure 1. Frequency of CN gains (red areas) and losses (blue areas) along the tumor cell genome of 35 glioblastomas. **A:** An overview of the frequency of CN changes detected for each individual chromosome is shown. **B:** A heat map representation of the CN changes detected for chromosomes 7, 9, and 10 is displayed for each case analyzed. The intensity of colors is directly proportional to the frequency of genetic gains (red color) or losses (blue color) identified for each specific chromosomal region. Cutoff values for chromosome gains and losses were defined at CN values of 2.50 or greater and 1.30 or less, respectively.

individual patient basis, were included in this study. These additional patients corresponded to a total of five different series, with data on four of them being accessed from public databases (access codes: GSE19612,[42] E-MEXD-1330,[45] and GSE9635[50]), whereas for the other series, it was kindly provided by the authors.[41]

From these five series of glioblastomas, cases with secondary glioblastomas, tumors with simultaneously normal CN values for chromosomes 7, 9, and 10, and patients lacking survival data and/or showing low SNP call rates in the array file (<90%) were excluded from the analysis.

iFISH Studies

Confirmatory iFISH studies were performed in all cases, according to previously described methods, using dual-color probes directed against different regions of chromosomes 7, 9, and 10. Three genes (*EGFR*, *p16,* and *PTEN*) and three chromosome centromeres (7, 9, and 10) were tested with the following commercially available probes, all obtained from Vysis Inc. (Downers Grove, IL), except the 7p12 (*EGFR*)/alphasatellite 7 DNA dual-color probe, which was obtained from Q-BIOgene (Carlsbad, CA); for chromosome 9, the LSI 9p21/CEP-9 dual-color probe was used, and for chromosome 10, the LSI *PTEN*/CEP-10 dual-color probe was used.

Statistical Analyses

To establish the statistical significance of differences observed between groups, the Student's *t*-test and the Mann-Whitney *U*-test were used for parametric and non-parametric (continuous) variables, respectively; for qualitative variables, the χ^2 test was applied (SPSS software version 15.0, SPSS Inc, Chicago, IL). Survival curves were plotted according to the method of Kaplan and Meier, and the log-rank test was used to assess the statistical significance of differences observed in survival between distinct groups of patients (SPSS software). For the identification of those parameters with an independent prognostic impact on patient overall survival, the Cox regression was used; in the multivariate analysis only those variables that showed a significant impact in the univariate analysis (age and cytogenetic profile) were included. Patient overall survival was measured from the date of diagnosis until the date of death. $P < 0.05$ were considered to be associated with statistical significance.

Results

CN Changes in Glioblastomas by SNP Arrays

SNP array studies showed genetic alterations for all chromosomes in the 35 cases studied; such alterations involved either entire chromosomes or specific chromosomal regions (Figure 1). Overall, CN changes showed predominance of gains of chromosomes 7 and 20, losses of chromosomes 4, 6, 9p, 10, 15, and 17, and both gains and losses of chromosomes 1, 3, 9, 19, and 22. As could be expected, chromosomes 7, 9p, and 10 were those chromosomes more frequently altered: gains of chromosome 7 were found in all but one case (97%) and losses of chromosomes 9p and 10 were identified in 83% and 91% of all glioblastomas analyzed (Figure 1). A more detailed description of the genetic alterations found for these three chromosomes is shown in Table 2 and detailed below.

Table 2. Frequency of Different Patterns of CN Alterations for Chromosomes 7, 9, and 10 in Glioblastomas as Detected by SNP Arrays (n = 35)

Chromosome	Genetic alteration	No. of cases/ total cases (%)
7	No alterations	0/35 (0)
	CN gains	34/35 (97)
	+7	23/35 (65)
	+7 and EGFR amplification	9/35 (26)
	EGFR amplification	1/35 (3)
	+7 and other amplifications	1/35 (3)
	cnLOH*	1/35 (3)
9	No alterations	4/35 (11)
	CN gains†	2/35 (6)
	CN losses	28/35 (80)
	Heterozygous del(9p)	4/35 (11)
	Heterozygous del(9p) and del(9q)	2/35 (6)
	Heterozygous del(9p) and +9q and 9p gains‡	2/35 (6)
	Heterozygous and homozygous del(9p)	12/35 (34)
	Heterozygous and homozygous del(9p) and cnLOH§	2/35 (6)
	Heterozygous and homozygous del(9p) and del(9q)	2/35 (6)
	Heterozygous and homozygous del(9p) and +9q¶	1/35 (3)
	Heterozygous and homozygous del(9p) and +9q and cnLOH	2/35 (6)
	Monosomy 9	1/35 (3)
	cnLOH*	1/35 (3)
10	No alterations	3/35 (9)
	CN gains	0/35 (0)
	CN losses	28/35 (80)
	−10	22/35 (63)
	−10 and homozygous del(10q)	4/35 (11)
	del(10p) and del(10q)	2/35 (6)
	cnLOH*	4/35 (11)

*cnLOH involving the whole chromosome.
†Chromosome 9 gains without losses of this chromosome.
‡Gain of 9p24.3 in one tumor and other gains of 9p21.1 in another case.
§cnLOH of chromosome 9p was detected in four cases; however, only two are included here (G54 and G55) because the other two cases had +9q21. In one case cnLOH involved the whole chromosome 9.
¶Two case have cnLOH (G54, G55).

CN Changes of Chromosomes 7, 9, and 10

Gains of chromosome 7 were found in all but one tumor (G41) and consisted of the gain of an entire chromosome (n = 33; 94%) and *EGFR* amplification (n = 10; 29%).

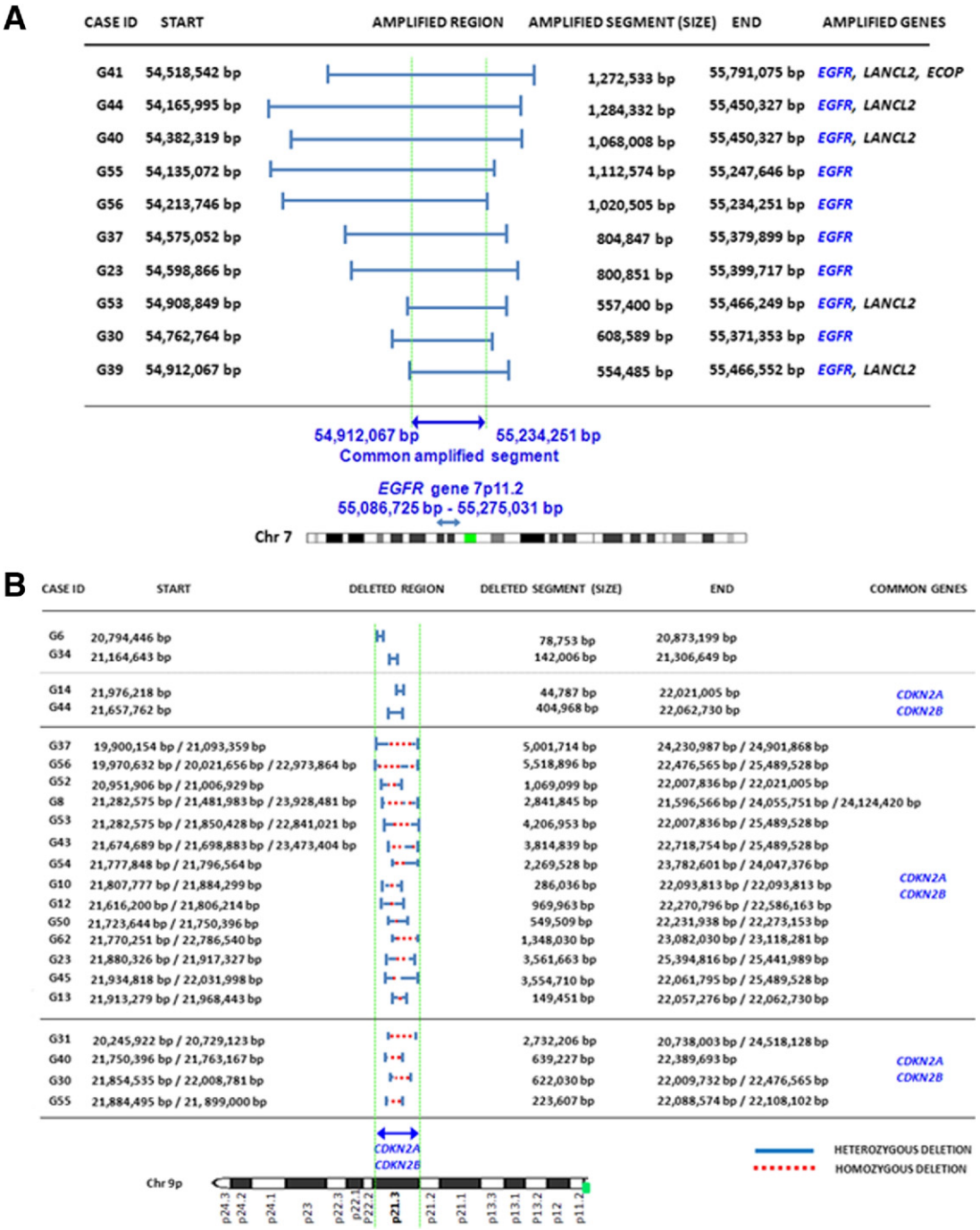


Figure 2. Detailed characterization of the amplified 7p11.2 chromosomal segments (n = 10, **A**) and the deleted chromosome 9p21.3 sequences (n = 22, **B**) in glioblastomas. Note that the *EGFR* gene extends from 55,086,725 bp to 55,275,031 bp from p*ter* positions (Entrez Gene, GeneID1956); in the 500K SNP array, the SNPs assayed in this region of chromosome 7p11.2 extended from the 55,062,691-bp to the 55,236,410-bp positions. Regarding del(9p21) no single gene was systematically deleted; however, both CDKN2A and CDKN2B were lost in all except one case.

Most of these later cases (n = 9/10) also carried gains of a whole chromosome 7. One tumor showed cnLOH of chromosome 7 (Table 2). Two cases (6%) presented amplification 7p in association with cnLOH, involving a whole chromosome 7 in one case and chromosome 7p in the other tumor. Figure 2A delineates the amplified segments at the 7p11.2 chromosomal region and their extension. As displayed, the amplified regions at chromosome 7p11.2 were variable in size, with a mean ± SD length of 908,412 ± 281,717 bp (range, 554,485 to 1284,332 bp) (Figure 2A). A more detailed analysis of the amplified 7p11.2 regions in each individual tumor showed that they typically involved a region systematically containing the *EGFR* gene, in association or not with another two genes: *LANCL2* (n = 5/10) and *ECOP* (n = 1/10) genes (Figure 2A).

Allelic loss of chromosome 9p21 was the most common alteration found for chromosome 9 (27/35 cases;

Table 3. Detailed Characterization of the Localization and Deletion Size of 9p and Commonly Lost Chromosomal Segments at 9p21.3 Detected in Glioblastomas

Type of deletion	Case ID	Deleted segment		No. of deleted genes	Deleted genes	
		Start (bp)	End (bp)		*MLLT3*	*KIAA1797*
Heterozygous (n = 4)	G6	20,794,446	20,873,199	10		○
	G34	21,164,643	21,306,649	6		
	G14	21,976,218	22,021,005	3		
	G44	21,657,762	22,062,730	16		
Heterozygous and homozygous (n = 14)	G37	19,649,652	24,901,868	128	○	○
	G56	19,970,632	26,131,011	10	●	●
	G52	20,951,906	22,021,005	53		○
	G8	21,282,575	24,124,420	7		
	G53	21,282,575	22,021,005	146		
	G43	21,674,689	26,027,837	32		
	G54	21,777,848	24,047,376	4		
	G10	21,807,777	22,093,813	3		
	G12	21,616,200	22,586,163	6		
	G50	21,723,644	22,273,153	3		
	G62	21,770,251	23,118,281	10		
	G23	21,880,326	25,441,989	33		
	G45	21,934,818	26,741,666	5		
	G13	21,913,279	22,062,040	2		
cnLOH plus heterozygous and homozygous (n = 4)	G31	20,245,922	24,518,128	10	○	●
	G40	21,750,396	22,389,693	3		
	G30	21,854,535	22,476,565	30		
	G55	21,884,495	22,108,102	3		

(table continues)

Deleted genes are noted with a circle; genes for which homozygous deletions were observed are noted with a solid circle. Case G17 presented monosomy 9 and homozygous losses from 9p22.1 to p21.3. (n = 22/35 cases).

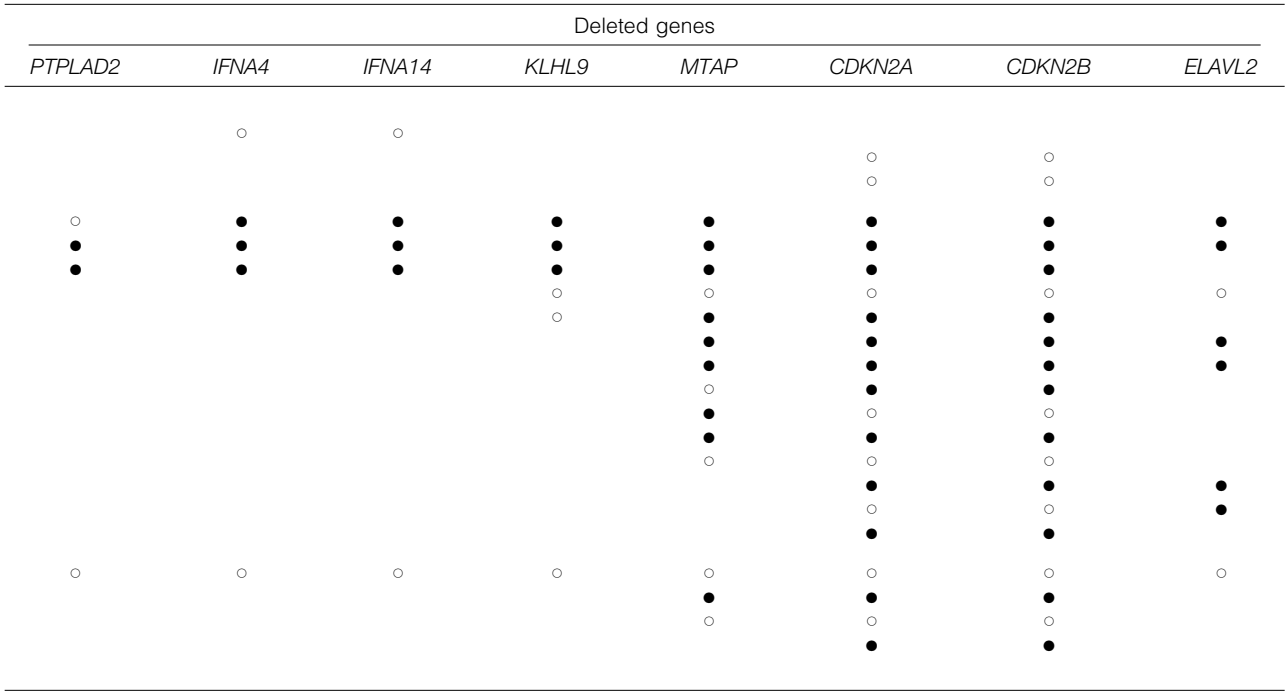
77%); in addition, monosomy 9 in association with homozygous del(9p21) was detected in one case (case G17; 3%), and cnLOH of an entire chromosome 9 was found in another case (case G57, 3%) (Table 2; see also Supplemental Table S1 at *http://jmd.amjpathol.org*). Seven tumors showed gains of chromosome 9q, consisting of partial gains (n = 5; cases G6, G14, G54, G55, G56, and G63) or gain of an entire chromosome 9 (n = 1; case G51); some of these cases (n = 5/7) showed additional coexisting losses of chromosome 9p (cases G6, G14, G54, G55, and G56). A more detailed analysis of chromosome 9 sequences in cases with del(9p) revealed a wide spectrum of allelic losses regarding the size of the deleted regions, ranging from 44,787 to 5518,896 bp. Overall, deletions within the short arm occurred much more frequently than in the long arm of chromosome 9 (n = 27 versus 4 cases), with several different patterns: i) heterozygous del(9p) (n = 8) (cases G41, G39, G6, G14, G29, G34, G64, and G35); ii) combined heterozygous and homozygous del(9p) (n = 15) (cases G23, G53, G44, G56, G30, G37, G13, G52, G62, G12, G45, G8, G10, G43, and G50); and iii) cnLOH combined with heterozygous and homozygous del(9p) (n = 4) (cases G55, G40, G31, and G54). From those cases showing cnLOH with or without del(9p) (n = 5), complete loss of chromosome 9p was found in 3 cases (9%); the other two glioblastomas had cnLOH involving the whole chromosome 9, in association with heterozygous and homozygous del(9p21) in one tumor (case G40) (Table 2; see also Supplemental Table S1 at *http://jmd.amjpathol.org*). Despite all these patterns, cases with del(9p21.3) (n = 22) or monosomy 9 (n = 1) almost systematically displayed in common loss of the *CDKN2B* and the *CDKN2A* genes (21 of 22 cases), in association with loss of the *MTAP* gene in 15 cases (Table 3). Other frequently deleted genes included the *MLLT3* (4/35 cases), *KIAA1797* (6/35 tumors), *PTPLAD2* (5/35 cases), *IFNA4* (6/35 patients), *IFNA14* (6/35 cases), *KLHL9* (7/35 tumors), and *ELAVL2* (9/35 cases) genes (Table 3). One additional tumor (G17) presented loss of an entire chromosome 9 in association with homozygous del(9p21) also involving the *CDKN2A/CDKN2B* genes.

Genetic losses of chromosome 10 consisted of monosomy 10 in 26 of 35 glioblastomas (74%) in association with homozygous del(10q) in 4 cases (11%), isolated del(10p) coexisting with del(10q) in two cases (6%), and cnLOH for the entire chromosome 10 in four cases (11%); three tumors (9%) did not show any CN change for chromosome 10, and gains of chromosome 10 were systematically absent (Table 2; see also Supplemental Table S1 at *http://jmd.amjpathol.org*).

Cytogenetic CN Profiles of Glioblastomas According to the Alterations of Chromosomes 7, 9, and 10

On the basis of the pattern of CN alterations observed for chromosomes 7, 9, and 10, glioblastomas were grouped into five distinct cytogenetic profiles (Table 4; see also Supplemental Table S1 at *http://jmd.amjpathol.org*): i) tumors exhibiting amplification of the *EGFR* gene (n = 10; 29%); ii) glioblastomas with gains of chromosome 7, losses along chromosome 10, and del(9p) or cnLOH 9 (n = 17; 48%); iii) tumors displaying gains of chromo-

Table 3. *Continued*



some 7 without monosomy 10 (n = 3; 9%); iv) tumors that had gains of chromosome 7 and monosomy 10, in the absence of del(9p21.3) (n = 4; 11%); and v) tumors with gains of an entire chromosome 9 (n = 1; 3%).

Overall, no clear association was found between these cytogenetic profiles and other clinical variables, including tumor localization, except for patient survival. Accordingly, despite the dismal outcome observed in all cases, the cytogenetic profile of the tumor (as defined by the cytogenetic pattern of CN alterations observed for chromosomes 7, 9, and 10 by SNP arrays) had a significant effect on overall survival ($P < 0.0001$) (Figure 3A): tumors with *EGFR* gene amplification exhibited the longest (P = 0.005) survival rates versus all other cases (Figure 3B and Table 4), specifically among older (>60-year-old) patients (P = 0.01). This also holds true when patients undergoing complete tumor resection and those not undergoing resection were separately considered (data not shown).

The presence and frequency of these five cytogenetic groups were then confirmed among the 119 glioblastoma cases collected from other series in the literature: pattern 1, 61 of 119 cases (51%); pattern 2, 57 of 119 (45%); pattern 3, 0 of 119 (0%); pattern 4, 4 of 119 (3%); and pattern 5, 2 of 119 cases (2%) (see Supplemental Table S2 at *http://jmd.amjpathol.org*). In addition, the impact of the cytogenetic profiles on overall survival was also confirmed when the whole series of glioblastoma patients (n = 154) was evaluated, both when the five different cytogenetic subgroups were considered (P = 0.0001; Figure 3D) and when cases with *EGFR* amplification were compared with all other cases in the whole series (P =

Table 4. Cytogenetic Subgroups of Glioblastomas as Defined by the CN Alterations Detected by SNP Arrays for Chromosomes 7, 9, and 10 and Association With Patient Overall Survival (n = 35)

Pattern	Chromosomal abnormalities			No. of cases (%)*	Overall survival (range)†
	Chr7	Chr9	Chr10		
I	EGFR AMP	del(9p21)	−10 or del(10p) and del(10q) or cnLOH	10/35 (29)	16 (9–32)
II	+7	del(9p) or cnLOH	−10 or del(10p) and del(10q) or cnLOH	17/35 (48)	8 (0–21)
III	+7	del(9p21) or +9q	No monosomy 10	3/35 (9)	13 (11–67)
IV	+7	Normal 9p21.3	−10	4/35 (11)	4 (2–7)
V	+7	+9	−10	1/35 (3)	2

*Results are expressed as number of cases/total cases analyzed with percentages.
†Median (range) overall survival in months.
B, biopsy; ST, subtotal; T, total.

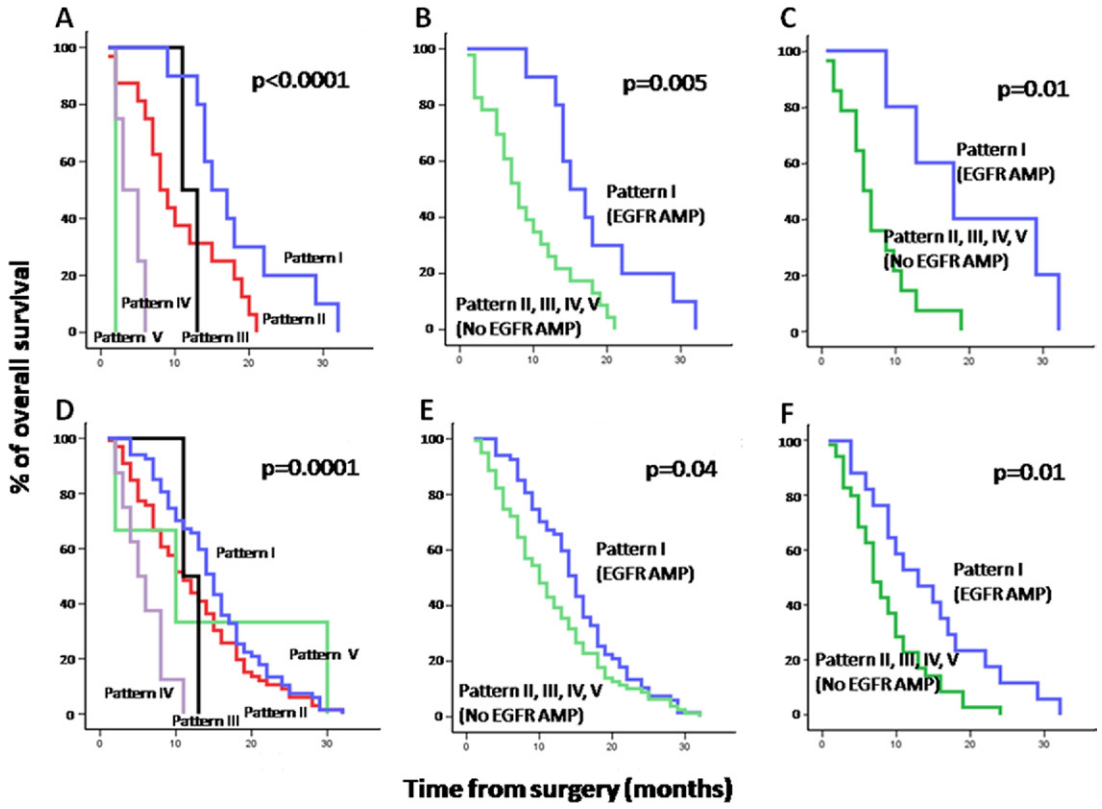


Figure 3. Overall survival curves of glioblastoma patients (n = 35) from our patients (**A–C**) and our patients plus cases from five other series (154 total cases) from the literature (**D–F**), according to the different cytogenetic patterns of alteration detected for chromosomes 7, 9, and 10 (**A** and **D**) and the presence of *EGFR* amplification in the whole series (**B** and **E**) and among patients older than 60 years (**C** and **F**). Cytogenetic patterns by SNP array studies corresponded to the following profiles: pattern 1, *EGFR* amplification; pattern 2, +7/del(9p) or cnLOH 9/−10 or del(10p) with del(10q); pattern 3, +7/without monosomy 10; pattern 4, +7/−10/absence of del(9p21.3); and pattern 5, +7 and + 9.

0.04; Figure 3E) and among older (>60-year-old) patients (P = 0.01; Figure 1F). Noteworthy, amplification of the *EGFR* gene (P = 0.01) together with age (P = 0.04) emerged as the best combination of independent variables to predict overall survival in the multivariate analysis.

CN Alterations by SNP Arrays Versus iFISH Analyses

CN changes identified by SNP arrays for chromosomes 7, 9, and 10 were confirmed in most cases by iFISH studies. However, discrepancies were observed by iFISH in two tumors (see Supplemental Table S3 and Supplemental Figure S1 at *http://jmd.amjpathol.org*). One of these cases showed tetrasomy and trisomy for chromosome 9p (n = 1) but a diploid profile by SNP arrays, whereas the other displayed coexistence of nulisomy 9p plus monosomy 9p and monosomy 10 by iFISH but an SNP array profile with both gain and loss of small regions of chromosome 9 and a diploid 10q23 profile.

Discussion

In recent years, increasingly heterogeneous genotypic profiles have been identified in glioblastomas. Accumulating evidence indicates that such variability reflects progressive acquisition and accumulation of multiple combined genetic events in single cells, accounting for gliomagenesis and potentially also for the behavior of the disease. Detailed characterization of common genetic changes in single chromosomes and shared genetic profiles in individual tumors will contribute to the identification of commonly altered genes and molecular profiles for a better understanding of the molecular mechanisms of the disease and its variable biological, histopathologic, and clinical features. Overall, chromosomes 7, 9, and 10 have been reported as those more frequently altered in glioblastomas.[46,50,51,54] Despite this, to the best of our knowledge, no study has attempted to classify glioblastomas on the basis of the distinct patterns of combined alterations of these three chromosomes.

We used high-density SNP arrays for detailed characterization of those CN changes and genotypic profiles involving the three most frequently altered chromosomes in a group of 35 cases of glioblastomas. Overall, our results show that with very few exceptions, SNP arrays allow detection of underlying genetic changes of chromosomes 7, 9, and/or 10 whenever specimens contain 75% or more tumor cells, as confirmed by iFISH studies. Through this approach, we confirmed the existence of previously reported genomic abnormalities for these three chromosomes.[13–15] In addition, use of high-resolution SNP arrays allowed accurate and detailed delineation of those sequences affected by CN changes (eg, gains, amplifications, and homozygous or heterozygous

deletions) and allelic imbalances (eg, LOH and cnLOH) and identification of the specific genes involved; overall, five different patterns of combined alterations for these three chromosomes were observed.

Noteworthy, gains of chromosome 7 were identified in virtually every case. This highlights the relevance of complete gains of this chromosome in the development of glioblastomas and the potential pathogenic contribution of multiple oncogenes coded in it. In addition, we also confirm and extend on previous observations that have suggested that *EGFR* is the most frequently amplified oncogene in glioblastomas[5,11,55,56] because *EGFR* was the only oncogene found to be amplified in common in cases with multiple copies of the 7p11.2 chromosomal region. Interestingly, precise localization of the *EGFR* amplicons revealed amplification of other adjacent genes in many cases, particularly the *LANCL2* gene. Noteworthy, from those genes involved in 7p11.2 amplification, only *EGFR* showed increased expression in gene expression profiling,[7,57] further supporting the unique and relevant role of this oncogene in glioblastomas versus the other genes (eg, *LANCL2*).

Regarding chromosome 9, more heterogeneous patterns of CN changes were observed, from which heterozygous and/or homozygous del(9p) was the most common alteration. Interestingly, although homozygous deletions were restricted to relatively small sequences of chromosome 9p21, heterozygous del(9p) extended to larger chromosomal regions. Notably, common deleted segments at 9p21 almost systematically involved the *CDKN2B/p15* tumor suppressor gene in association with *CDKN2A/p16* and the *MTAP* housekeeping genes. Del(9p21) is known to play an important role in the development and progression of many different types of cancer through deregulation of cell cycle and/or apoptosis.[58] The *CDKN2A* locus has been claimed to play a crucial role in this regard. *CDKN2A* codes for two gene products, p16 and p14, that control both the Rb and the p53 pathways; p16 binds to CDK4 and CDK6 and inhibits the catalytic activity of CDK/cyclin D complexes to activate cell cycle through RB phosphorylation. In turn, p14 blocks MDM2 inhibition of p53 activity, thereby leading to stabilization of p53.[11] Because deletion of the *CDKN2A/B* locus causes deregulation of two crucial pathways involved in many types of cancer, loss of the *MTAP* gene activity could be viewed as potentially irrelevant. However, deficiency of the MTAP protein (an enzyme involved in the metabolism of methionine and purines) has also been detected in multiple types of malignant neoplasms in association with deletion of the *CDKN2A* and *CDKN2B* loci,[59] as also found in our glioblastoma cases. Most interestingly, it has been shown that *MTAP* can be lost independently of *CDKN2A/p16*, which suggests that loss of *MTAP* may indeed play a role in tumor biology.[60–62]

Taken together, these results raise the question about which of these three genes is/are critical target genes in glioblastomas. On the basis of our results, *CDKN2A/p16* and *CDKN2B/p15* are the most frequently altered in cases with heterozygous and homozygous deletions in line with previous large-scale multidimensional analyses performed by The Cancer Genome Atlas Research Network.[40] The *CDKN2B* gene (*p15*; *INK4b*) is located adjacent to *p16* (*INK4a*) on 9p21 and is co-deleted in a high proportion of human cancers. p15 (INK4b) is a member of the family of cyclin-dependent protein kinases that inhibits CDK4B. Because expression of CDKN2B is induced by transforming growth factor β, p15 may act as an effector of the transforming growth factor β–mediated cell cycle arrest pathway. In line with our results, data from both mutational and functional studies indicate that *CDKN2B/p15* deletion could likely be the target of del(9p21).[63]

Regarding the specific mechanism by which these genes are inhibited, LOH at 9p21 was a relatively rare event, whereas combined homozygous and heterozygous deletions (associated or not with cnLOH events) were relatively common in our and other studies[64]; this finding suggests that all three genes (*CDKN2A/p16*, *CDKN2B/p15*, and *MTAP*) may be inactivated in glioblastomas by a large deletion event. In line with this hypothesis, a large mapping study of 545 primary tumors[65] showed that tumors containing homozygous del(9p21) minimally have a 170-kb region deleted that includes both the *MTAP* and *p16* loci, as also found here. However, homozygous deletion does not seem to be the only mechanism leading to inactivation of these tumor suppressor genes in glioblastomas because cases with heterozygous deletions were also found at higher frequency in our study. In another study on 85 brain tumor samples of different histologic features and grade, *CDKN2B/p15* and *CDKN2A/p16* genes were found to be methylated in only 4% and 7% of the cases, respectively; interestingly, *CDKN2A* was methylated only in glioblastoma samples (6% of the cases), and none of the samples showed simultaneous methylation of both the *p15* and *p16* genes[66]; this finding suggests that methylation of these genes does not play a major role in the development of glioblastomas. Interestingly, however, gene expression profiling of glioblastomas shows a significant impact on the expression of CDKN2A in cases with not only homozygous but also heterozygous del(9p21), whereas this does not affect the expression of the other two genes (data not shown). In any case, point mutations of these genes should be investigated in parallel in these cases. Because emerging CN analyses of glioblastoma samples confirmed the *CDKN2A/CDKN2B* locus to be the most common homozygous deletion at 9p21, detailed characterization of the deletion at chromosome 9p21 and the lost genes becomes particularly relevant. In this study, detailed mapping of the 9p21.3 region shows distinct patterns and extents of del(9p21) among the tumors analyzed. In addition, our results also show that the deleted locus encompassed not only genes with well-established tumor suppressor functions in glioblastomas but also multiple other less known genes (eg, the *ELAVL2*, *MLLT3*, *KIAA1797*, *PTPLAD2*, and *KLHL9* genes). These findings strengthen the hypothesis that suggests the presence of additional candidate tumor suppressor genes mapped to this region.[67]

Overall, approximately three-quarters of all glioblastomas analyzed showed chromosome 10 losses, which most frequently consisted of monosomy 10 and cnLOH of

an entire chromosome 10. These findings point to the loss of more than one tumor suppressor gene, localized both in the short and the long arms of this chromosome. In this regard, extensive losses of chromosome 10 sequences have been associated with progression of astrocytoma,[68] and several regions along this chromosome (eg, 10q23, 10q24, 10q25-26, 10p13, and 10p14-p15) have been consistently proposed to harbor tumor suppressor genes (eg, the *PTEN/MMAC1*, *DMBT1,* and *LGI1* genes).[69] Although it has been previously suggested that the *PTEN* gene could be a preferential target of del(10q),[70] in our series, losses of chromosome 10 mainly involved the entire chromosome. Despite this, our results highlight the fact that other regions at 10q11.21, 10q21.3, and 10q.23.33 (with loss of the *HNRPF*, *PAKDB,* and *CUL2* genes, the *CXXCC*, *CCPRL1*, *STOX1,* and *DDX50* genes; and the *IRE* gene, respectively) were more frequently lost and could act as potential preferential targets of deletion in glioblastomas. Likewise, those genes encompassed within these deleted loci could also represent novel candidate tumor suppressor genes involved in glioblastoma tumorigenesis, in addition to *PTEN*.

In this study, as in other larger series of glioblastomas,[14,18,19,27,71] gains of chromosome 7 and losses of chromosomes 9 and 10 frequently coexisted in the same tumor, but different patterns were observed for these abnormalities. Accordingly, glioblastomas that exhibited *EGFR* gene amplification also showed extensive losses of chromosome 10, del(9p21), and trisomy 7 in all but one case. Conversely, in more than half of the cases, monosomy 10 coexisted with trisomy 7 in the absence of *EGFR* gene amplification with or without del(9p21). Altogether, these findings suggest that these alterations may occur independently from each other, with *EGFR* amplification appearing to be a later event in the development of glioblastomas versus trisomy 7 and monosomy 10. Nevertheless, their combination could be crucial in the malignant transformation process for which the underlying mechanism is still poorly understood. In this regard, several candidate genes in chromosome 10 with putative reciprocal relationship to *EGFR* have been identified, with great emphasis on the *PTEN* gene. Complementary deregulation of the EGFR and PTEN pathways often results in constitutional signaling through PI3-kinase and Akt, leading to altered cell proliferation and survival.[72] A recent study by Yadav et al[28] also suggests a tumorigenic synergism between loss of the annexin A7 (*ANXA7*) gene at 10q21.1-q21.2 and *EGFR* amplification, with ANXA7 haploinsufficiency acting as a positive regulator of EGFR signaling in glioblastomas. This study also demonstrates a cross-talk among the *ANXA7*, *PTEN*, and *EGFR* genes, which leads to constitutive activation of the PI3K-AKT signaling pathway and, ultimately, to malignant transformation. Taken together, these findings suggest that cytogenetic profiles, more than isolated chromosomal alterations, should be considered in evaluating the impact of CN alterations in disease behavior.

On the basis of CN alterations of chromosomes 7, 9, and 10, five different genetic profiles were identified in our series and confirmed to be present in other series from the literature[41,42,45,50] from which cases with amplification of the *EGFR* gene, in association with monosomy 10 and del(9p21), clearly showed a better outcome in our 35 cases and when data on 119 additional glioblastoma patients from four previously reported series[41,42,45,50] were considered. Controversial results have been reported about the prognostic value of EGFR amplification/overexpression in glioblastomas. Although some authors claim there is no association with survival,[73,74] others state that this aberration is a negative prognostic factor.[75,76] In turn, an association between EGFR overexpression and a better prognosis in older glioblastoma patients has also been reported,[25,26,33,77] in line with our observations. Noteworthy, we did not find an association between tumor cytogenetics and other disease characteristics, such as patient age[76] and tumor localization, among other features.[55,78]

Simmons et al[78] and Batchelor et al[5] have previously found that EGFR overexpression is associated with a trend toward a worse prognosis in young patients and a better outcome in older cases; likewise, in a series of 220 primary glioblastomas Houillier et al[55] also documented an association between *EGFR* amplification and increased survival in older patients, which could be associated with the existence of additional as-yet-unidentified specific molecular alterations in older patients. In the present study, we confirm the prognostic value of *EGFR* amplification in patients older than 60 years in our small patient series and in a larger series of patients from four independent studies previously reported in the literature.[5,55,78,79]

In summary, our high-density analysis of the CN alterations of chromosomes 7, 9, and 10 disclosed five subgroups of patients defined by unique cytogenetic profiles, which are associated with patient outcome, with tumors with *EGFR* amplification showing a longer overall survival among older patients. In addition, our results provide further evidence about the relevance of the *EGFR*, *CDKN2A/B,* and *MTAP* genes, together with other genes coded in chromosome 10, in the malignant transformation of glioblastomas. Further studies in larger series of glioblastoma patients are necessary to investigate the functional interaction between these genes and more precisely delineate their pathogenetic role and clinical impact in glioblastomas.

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