Neurogenetics
DOI 10.1007/s10048-009-0217-x

ORIGINAL ARTICLE

Intratumoral patterns of clonal evolution in gliomas

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Received: 23 April 2009 / Accepted: 21 August 2009
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Abstract Few studies have explored the patterns of clonal evolution in gliomas. Here, we investigate the cytogenetic patterns of intratumoral clonal evolution of gliomas and their impact on tumor histopathology and patient survival. Cytogenetic analysis of 90 gliomas was performed in individual tumor cells (>200 cells/tumor) using multicolor (N=16 probes) interphase—FISH. Overall, chromosome gains were more frequent than chromosome losses. Gains of chromosome 7 and/or *EGFR* amplification were detected in 91% of the cases, whereas del(9p21) (77%) and del(10q23) (78%) were the most frequent chromosome losses. Virtually, all cases (99%) showed ≥2 tumor cell clones, with higher numbers among high- versus low-grade gliomas (p=0.001). Nine different cytogenetic patterns were found in the ancestral tumor clones. In most gliomas, ancestral clones showed abnormalities of chromosome 7, 9p, and/or 10q and cytogenetic evolution consisted of acquisition of additional abnormalities followed by tetraploidization. Conversely, early tetraploidization was associated with low-grade astrocytomas—2/3 pilocytic and 3/6 grade II diffuse astrocytomas—and combined loss of 1p36/19q13 with oligodendrogliomas, respectively; both aberrations were associated with a better patient outcome (p=0.03). Overall, our results support the existence of different pathways of intratumoral evolution in gliomas

Keywords Glioma · Intratumoral heterogeneity · Clonal evolution · Cytogenetics

Electronic supplementary material The online version of this article (doi:10.1007/s10048-009-0217-x) contains supplementary material, which is available to authorized users.

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Published online: 17 September 2009

Introduction

Diffuse gliomas are the most common subtype of primary brain neoplasms in adults, accounting for around three quarters of all malignant, central nervous system tumors [1, 2]. Current classification of gliomas is based on morphological evidence of differentiation along the astrocytic (~70% of the cases) or less frequently, oligodendroglial, ependymal, or mixed cell lineages, and the histopathological degree of malignancy with >80% of all diffuse gliomas being high-grade (grade III/IV) tumors [3, 4]. Despite significant advances in standard treatment modalities, the overall prognosis of patients with gliomas remains dismal [5, 6]; however, a significant variation is observed in survival of individual patients [7–9], which cannot be explained just on the basis of tumor histopathology [10, 11]. In fact, in addition to tumor grade and the histopathological subtype, other factors such as patient's age and cytogenetics, are also recognized to be useful parameters in predicting patient outcome and survival [5, 9, 12–14].

At present it is well recognized that gliomas are genetically highly heterogeneous tumors, even within a single histopathological category [15, 16]. Many studies have been reported so far, in which cytogenetic and molecular changes associated with human gliomas have been identified using different techniques [14, 17–21]. Among other alterations, the most frequently detected molecular abnormalities include gains of chromosome 7—with or without amplification of the epidermal growth factor receptor (*EGFR*) gene— deletion and mutation of *TP53*, alteration of the retinoblastoma pathway involving the RB1 gene localized at chromosome 13q14, together with losses of the *PTEN* gene and hemizygous/homozygous deletion of the *p16* tumor suppressor gene at chromosomes 10q23 and 9p21, respectively [14, 22–26]. To date, two distinct, mutually exclusive molecular profiles have been identified, which would correspond to either de novo (primary) or secondary glioblastomas developing after progression of either a low-grade or an anaplastic astrocytoma [27, 28]. Accordingly, while primary glioblastomas show higher frequencies of *p16* deletion, *EGFR* amplification and *PTEN* mutation with a relatively low frequency of *TP53* abnormalities, secondary glioblastomas more frequently carry mutations of *TP53* in association with abnormalities of the *EGFR*, *p16*, and *PTEN* genes [28, 29]. Despite this, no study has been reported so far in which the major pathways of acquisition of genetic abnormalities are systematically analyzed in glioma patients, at the intratumoral cell level [8, 30].

In the present study we have applied multicolor interphase fluorescence in situ hybridization (iFISH) for the analysis of multiple combinations of 16 different probes specific for DNA sequences of the most frequently altered chromosome regions, to gain insight into the intratumoral cytogenetic heterogeneity of human gliomas at the single cell level. Our major goal was to define the different patterns of intratumoral clonal evolution that could contribute to the identification of those genetic profiles involved in determining the histopathological features of the tumor.

Materials and methods

Patients and samples

In the present study, tumors from 90 patients —46 males (51%) and 44 females (49%)—diagnosed with gliomas at the Neurosurgery Service of the University Hospital of Coimbra (Portugal), were analyzed. Mean age at diagnosis was 58±16 years (range, 21 to 84 years). In all tumors, histological diagnosis and classification were performed according to the WHO criteria [3] with the following distribution: 72 cases (80%) were astrocytomas (three grade I pilocytic astrocytomas, six were grade II diffuse astrocytomas, two corresponded to grade III anaplastic astrocytomas, 61 were grade IV primary or de novo glioblastomas, and two corresponded to grade IV gliosarcomas), 12 cases (13%) corresponded to oligodendrogliomas (four grade II oligodendrogliomas and eight grade III anaplastic oligodendrogliomas), four cases (4%) were oligoastrocytomas (one grade II oligoastrocytoma and three grade III anaplastic oligoastrocytomas), and, finally, two cases (2%) were diagnosed as ependymomas (one grade II ependymoma and one grade III anaplastic ependymoma).

All samples were obtained after informed consent was given by the patient and the study was approved by the Ethics Committee of the University Hospital of Coimbra. In all cases, tumor samples were obtained by conventional surgical procedures and cut into several parts. Only those tumor tissue samples shown by gross and histologic inspection to be mostly or wholly tumor were used to prepare single cell suspensions for further iFISH studies. Preparation of single cell suspensions was prospectively done by mechanical disaggregation of fresh tumor tissue samples. Tissue areas mirror-cut to those used for iFISH analysis of single cell suspensions showed always >65% of infiltrating tumor cells; the remaining cells corresponded to dead cells and eventually a few proportion of normal brain and leukocyte contamination (<5%), as assessed by microscopical analysis of hematoxylin and eosin-stained tissue specimens. Once obtained, single tumor cell suspensions were stored at −20°C until further processed. The remaining tumor sample was fixed in formalin and embedded in paraffin.

For each case, information about the histological characteristics of the tumor as well as the most relevant clinical and biological data (e.g., age, gender, and tumor localization) was recorded.

Interphase fluorescence in situ hybridization studies

Thirteen genes (*TP73*, *ANGPTL1*, *EGFR*, *ELN*, *TES*, *p16*, *ABL1*, *PTEN*, *Rb1*, *TP53*, *ZNF44*, *GLTSCR1*, and *BCR*) and three chromosome centromeres (chromosomes 7, 9, and 10) were tested by iFISH (Supplementary Table 1). In all cases, iFISH analyses were performed on freshly frozen tumor cells after fixation in 3/1 methanol/acetic (*v*/*v*; Panreac, Barcelona, Spain) with the following commercially available probes—all obtained from Vysis, Inc. (Downers Grove, IL), except the 7p12 (*EGFR*) /alphasatellite 7 DNA dual-color probe that was obtained from Q-BIOgene (Carlsbad, CA)—which were systematically used in double-stainings for the detection of numerical abnormalities of 16 different chromosome regions (please see Supplementary Table 1 for more detailed information about the probes used): (1) for chromosome 1, the LSI 1p36/LSI 1q25 dual-color probe was used; (2) for chromosome 19, the LSI 19q13/LSI 19p13 dual-color probe was employed; (3) for chromosome 7, the LSI *ELN*/LSI 7q31 dual-color probe was applied; (4) for chromosomes 9 and 22, the LSI *bcr/abl* ES dual-color probe was used; (5) in addition, also for chromosome 9, the LSI 9p21/CEP-9 dual-color probe was used; and (6) for chromosome 10, the LSI *PTEN*/CEP-10 dual-color probe, was employed. For chromosomes 13 and 17, the LSI *Rb1* and LSI *p53* probes conjugated with spectrum orange (SO) were used, respectively. In order to accurately define the exact abnormalities carried by different tumor cell clones within a tumor sample, further appropriate multicolor stainings simultaneously using up to three different probes were performed whenever required, as illustrated in Fig. 1.

Briefly, fixed tumor cells were dropped onto cleaned, poly-L-lysine-coated slides (Sigma, St. Louis, MO). The slides were then sequentially incubated for 10 min at 37°C with a solution containing 0.1 mg/ml of pepsin (Sigma), fixed in 1% acid-free formaldehyde (CH_2O; Merck, Darmstadt, Germany) in phosphate-buffered saline for 10 min at room temperature and dehydrated in increasing concentrations of ethanol (Merck) in water (70%, 90%, and 100%). Once dried, the slides containing both the cells' DNA and the probes' DNA were denaturated (6 min at 75°C) and subsequently hybridized overnight at 38°C in an Hybridizer thermocycler (Dako, Glostrup, Denmark). Once hybridized, slides were sequentially washed in 50% formamide (Merck) in a 2× saline sodium citrate (SSC) buffer (10 min at 46°C) and in 2× SSC (5 min at 46°C). Finally, tumor cell nuclei were counterstained with 75 μl of a solution containing 4,6-diamidino-2-phenylindole (0.1 μg/mL) (Sigma) and Vectashield (Vector Laboratories, Inc., Burlingame, CA), as anti-fading agent.

An Axioscope fluorescence microscope equipped with a ×100 oil objective (Zeiss, Göttingen, Germany) was used for counting the number of hybridization spots per nuclei (≥200 nuclei/slide). Only those spots with a similar size, intensity and shape in non-overlapping nuclei, were counted; doublet signals were considered as single spots. Cut-offs used to define the presence of numerical abnormalities for each individual chromosome, were based on the analysis of normal human tissue samples, as previously described in detail [31]. Briefly, gains and losses of specific chromosome regions were considered to occur when ≥5% and ≥10% of the nuclei showed an increased and decreased number of fluorescent signals (spots) with respect to normal diploid cells, respectively. Specimens were considered to carry amplification of the *EGFR* when more than 10% of the tumor cells exhibited either an *EGFR*:CEP7 ratio >2 or multiple tight clusters of hybridization signals for the *EGFR* gene probe. Definition of the exact tumor cell clones present in each sample was based on the presence of nuclei carrying identical numbers of hybridization spots for all probes analyzed in that tumor sample. In this study, only those groups of cells showing identical cytogenetic profiles which represented ≥5% of the tumor cells in a sample were considered to be a clone [31]. The ancestral tumor cell clone was identified as that tumor cell clone which carried genetic abnormalities common to all other tumor cell clones. In turn, groups of cells carrying cytogenetic abnormalities not shared by all cytogenetic altered tumor cells were considered as secondary clones.

Statistical methods

In order to assess the statistical significance of the differences observed between groups of patients, the Student *t* and the Mann–Whitney *U* tests were used for parametric and non-parametric continuous variables, respectively; for categorical variables, the X^2 test was applied (SPSS software, SPSS 12.0, SPSS Inc, Chicago, IL). Overall survival curves were plotted according to the method of Kaplan–Meier and they were compared using the log-rank test. p values ≤ 0.05 were considered to be associated with statistical significance.

Results

In this study we applied iFISH for the analysis of intratumoral cytogenetic heterogeneity at the single cell level. This approach, allowed the identification within individual samples of distinct cytogenetic profiles. In virtually every case, one or more chromosomal abnormalities were shared by all clones in the sample while others were identified in only part of all tumor cells suggesting they could correspond to earlier vs later chromosomal changes, respectively (Fig. 1).

Frequency of numerical chromosome changes

Genetic abnormalities for at least one of the 16 different chromosome probes analyzed (1p36, 1q25, 7p12, 7q11,

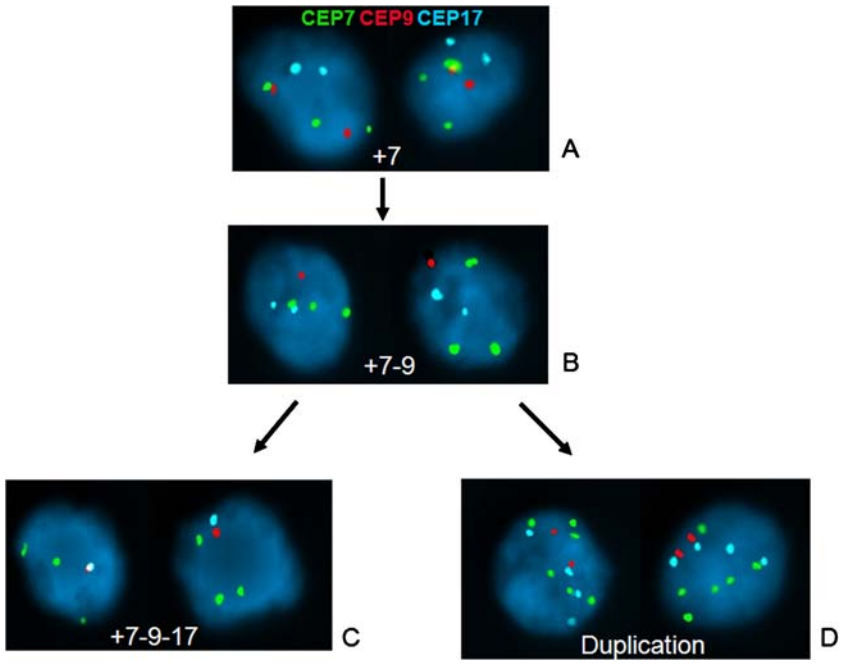

Fig. 1 Illustrating example of how different tumor cell clones were defined in a glioma using a three-color iFISH staining with probes directed against the centromeric regions of chromosomes 7, 9, and 17. As illustrated all nuclei counterstained in blue show gains of chromosome 7 (*green small dots*) while this was the only abnormality for the two nuclei displayed in **a** it was associated with loss of one copy of chromosome 9 (*red dots*) in the four nuclei shown in **b** and **c**; in this latter panel the two nuclei displayed also show loss of one blue spot (monosomy 17). In turn, the two nuclei in **d** show duplication of the fluorescence signals of all three chromosomes analyzed with six, two, and four copies of chromosomes 7, 9, and 17, respectively. *Arrows* indicate the hypothetical model of clonal evolution established for this tumor based on the abnormalities detected with the three chromosome probes

7q31, 9p21, 9q34, 10q23, 13q14, 17p13, 19p13, 19q13, and 22q11 locus and centromeres of chromosomes 7, 9, and 10) were systematically detected among the 90 gliomas studied. Overall, chromosome gains were more frequently observed than chromosome losses (68% vs 51% of all numerical chromosome abnormalities detected) and included all type of polysomies with the presence of three to nine iFISH signals/cell for individual chromosomes; gene amplification was only detected for the *EGFR* gene at chromosome 7p12 (Fig. 2a). Regarding individual chromosomes, chromosomes 7 and 9q34 were the most frequently altered, and they were gained (including amplification of the *EGFR* gene) in 91% and 79% of the tumors, respectively. Interestingly, gains of chromosome 7 were found to be associated with deletion of the 7p12 chromosome region in six cases (7%), del(7q11) in 12 (13%), del (7q31) in 16 cases (18%), and loss of centromere 7 in nine cases (10%). In contrast, gains of chromosome 9q34 were frequently associated with hemizygous/homozygous deletion of the 9p21 region. Del(9p21) together with del(10q23) corresponded to the most frequent chromosome losses and they were identified in around 80% of all cases (77% and 78%, respectively). The frequency of these and other numerical chromosomal abnormalities detected is detailed in Fig. 2a.

Intratumoral cytogenetic heterogeneity and hypothetical pathways of clonal evolution

All except one tumor (99%) showed two or more different tumor cell clones (number of tumor cells/clone of 5% to 90%) as defined by the iFISH patterns of cytogenetic abnormalities detected (Table 1). Presence of two clones was detected in five cases (6%), three clones in 17 (19%), four in 20 tumors (22%), five in 17 (19%), and ≥6 tumor cell clones were found in 30 patients (33%; Table 1). All glioma grades included tumors which displayed up to four different tumor cell clones, whereas five and six clones were only observed among high-grade tumors, with the exception of one grade II glioma (Table 1).

In 61/90 cases (68%), the cytogenetic aberrations found in common to all altered tumor cells consisted of abnormalities of either chromosome 7 (43%) or the short arm of chromosome 9 (16%) or both (9%; Fig. 2b). Accordingly, amplification of the EGFR gene was found to be present in all altered tumor cells (Fig. 3d) of 22% of the cases, while three to five copies of chromosome 7 (Fig. 3a–c) were found in all tumor cell clones of 19/90 (21%) cases (Fig. 2b). Isolated loss of either one or two copies of the 9p21 chromosome region (Fig. 3e, f) was also found to be shared by all tumor cell clones of 14/90 gliomas (16%; Fig. 2b).

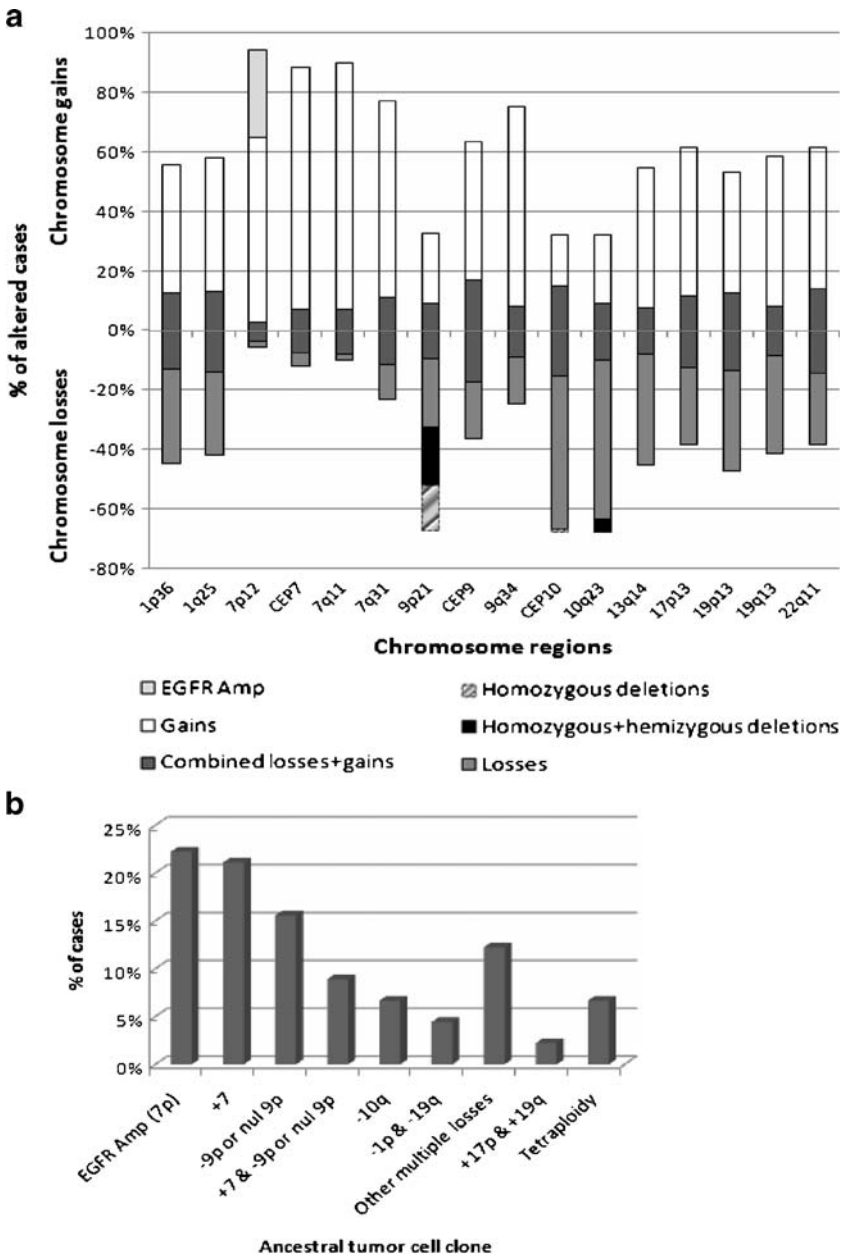

Fig. 2 Overall frequency of different gains and losses of specific chromosome regions detected in glioma tumors (**a**) and distribution of the tumors according to the cytogenetic profile of the ancestral tumor cell clone (**b**; $n=90$). Different tumors presented with distinct genetic alterations; in some cases a mixture of different abnormalities for the same chromosome was detected. In addition, it should be emphasized that low-grade astrocytomas ($n=9$) typically showed gains for all chromosomes analyzed, which were associated with a tetraploid ancestral tumor cell clone among the later cases (see Table 3); in contrast, low-grade oligodendrogliomas typically showed simultaneous l osses for chromosome 1p and 19q ($n=3/4$ cases)

Other cytogenetic aberrations found at lower frequencies in all tumor cell clones from individual cases included gains of chromosome 7 in association with hemizygous/homozygous deletion of the 9p21 chromosome region (9% of the cases), losses of chromosome 10q (7% of the cases), combined 1p36/19q13 losses (4%), coexisting deletions of several chromosome regions (12%), and coexisting gains of the 17p13 and 19q13 chromosome regions (2%); in 7% of the cases the ancestral tumor cell clones were tetraploid, with several additional changes being found in some of these tumors (Fig. 2b).

Detailed analysis of the different neoplastic cell clones present in each glioma tumor pointed out the occurrence of variable and complex patterns of intratumoral clonal evolution, as schematically illustrated in Fig. 4 and Supplementary Fig. 1. As mentioned above, in most cases (67/90; 74%), numerical chromosomal abnormalities common to all altered cells from individual tumors involved chromosomes 7, 9, and/or 10, for a total of five of nine different cytogenetic profiles (Fig. 4 and Supplementary Fig. 1). In the other tumors (23/90; 26%), common abnormalities to all genetically altered cells consisted of combined loss of the 1p36 and 19q13 chromosome regions (4/90; 4%), simultaneous losses of several chromosome regions (11/90; 12%), simultaneous gains of 17p13 and 19q13 (2/90; 2%) or a tetraploid karyotype (6/90 cases; 7%) (Fig. 4). Additional cytogenetic changes to those listed above and shared by all altered cells were highly variable

Table 1 Glioma tumors (n=90): number of cytogenetically defined tumor cell clones according to the WHO tumor grade

No. of clones/tumor	Tumor grade*				Total cases
	I (n=3)	II (n=12)	III (n=14)	IV (n=61)	
1	0/3 (0%)	1/12 (8%)	0/14 (0%)	0/61 (0%)	1/90 (1%)
2	2/3 (67%)	2/12 (17%)	1/14 (7%)	0/61 (0%)	5/90 (6%)
3	1/3 (33%)	5/12 (42%)	6/14 (43%)	5/61 (8%)	17/90 (19%)
4	0/3 (0%)	3/12 (25%)	2/14 (14%)	15/61 (25%)	20/90 (22%)
5	0/3 (0%)	0/12 (0%)	1/14 (7%)	16/61 (26%)	17/90 (19%)
≥6	0/3 (0%)	1/12 (8%)	4/14 (29%)	25/61 (41%)	30/90 (33%)

Results expressed as number of cases/total cases and percentage in brackets

WHO World Health Organization

*p<0.001

and heterogeneous. Accordingly, as illustrated in more detail in Supplementary Fig. 1, cases with amplification of the *EGFR* gene (20 cases; 22%) systematically displayed further gains of 7q (10/20 cases) and/or losses of the 10q23 (14/20 cases) in association or not with losses of one or two copies of the 9p21 region (16/20 cases) and/or loss of 1p36 chromosome region (6/20 cases). In turn, those tumors which showed gains of chromosome 7 in common to all tumor cell clones typically showed the presence of del (10q23) (5/19 cases) and/or del(9p) (2/19 cases) in association with variable patterns of other chromosome changes, except for six cases which had tetraploid cells (Fig. 4 and Supplementary Fig. 1). Cases with all tumor cell clones found to show isolated gains of +17p/+19q displayed distinct patterns of acquisition of additional chromosome gains and losses in each of the clones (Fig. 4 and Supplementary Fig. 1).

Of note, those cases which had deletions of chromosome 9p in association or not with gains of chromosome 7 in common to all altered tumor cells, displayed cytogenetic

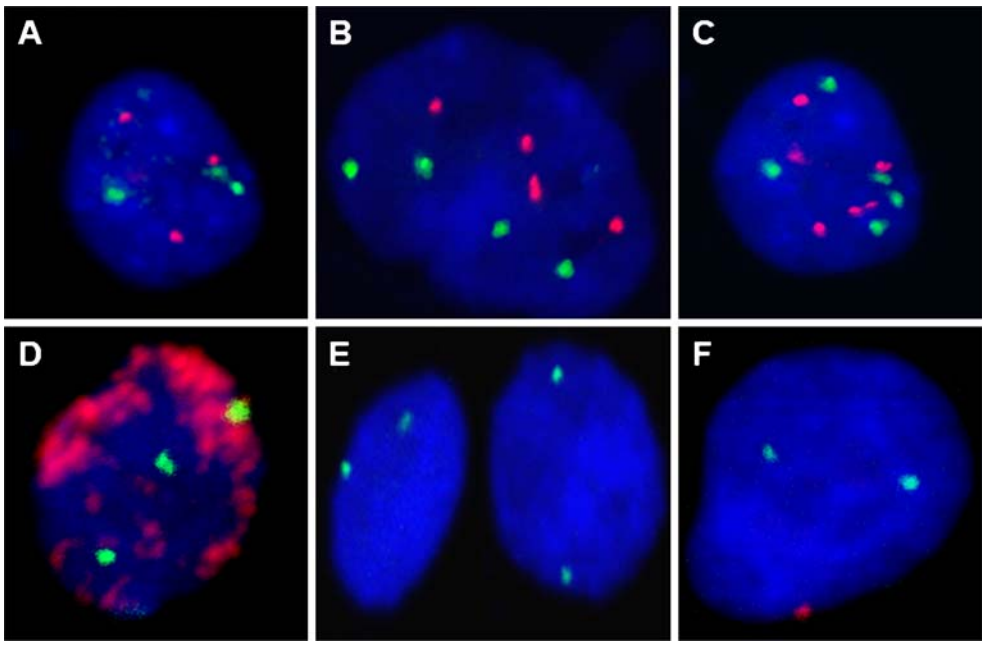

Fig. 3 Illustrating examples of some of the most frequently detected chromosomal abnormalities identified in glioma tumor cells by interphase fluorescence in situ hybridization (iFISH); original magnification ×1,000. In **a**–**c**, nuclei (*blue background signal*) corresponding to tumors showing gains—trisomy (**a**), tetrasomy (**b**), and pentasomy (**c**)—of chromosome 7 (*red spots*) are displayed; in these panels (**a** to **c**) green spots correspond to a chromosome 7 centromeric probe. Amplification of the *EGFR* locus (7p12) was found in 20 gliomas (*red spots*) and it is illustrated in (**d**) where green signals also correspond to the fluorescence emissions associated with a centromeric probe for chromosome 7. **e** and **f** show illustrating examples of two and one tumor cell carrying nul(9p21) and del(9p21), as revealed by the absence of *red spots* or the presence of a *single red spot*, respectively; these three cells also show *two green spots* each, corresponding to a probe directed to the centromeric region of chromosome 9

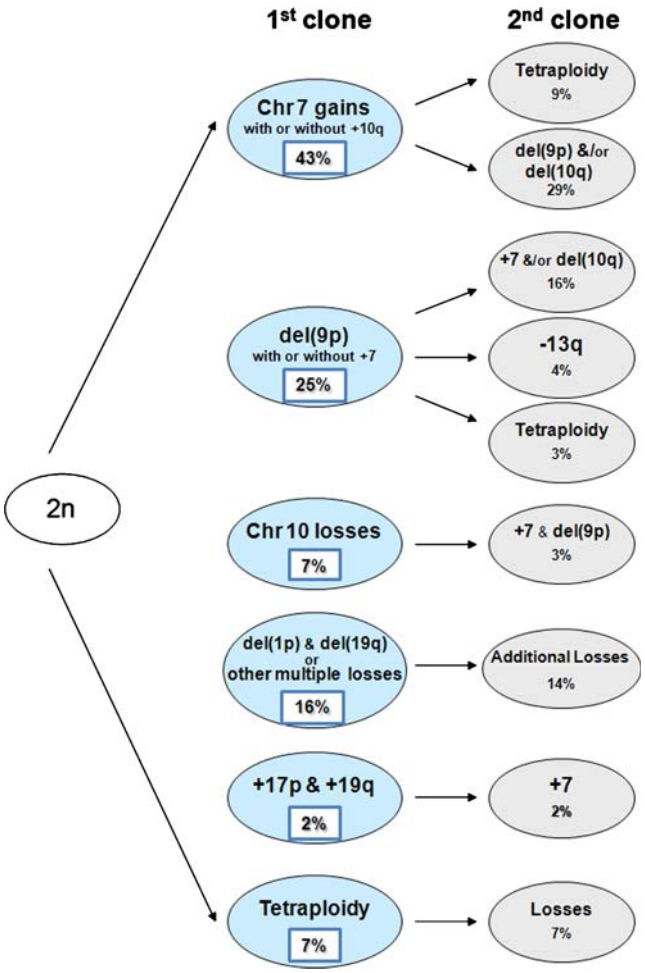

Fig. 4 Hypothetical pathways of intratumoral clonal evolution of gliomas (n=90) defined on the basis of the most frequent cytogenetic patterns detected for the 16 different chromosome probes analyzed. In this figure, only the most frequently detected ancestral tumor cell clones and their corresponding subsequent second clones are displayed. Noteworthy, most (five of six tumors) tetraploid cases corresponded to low-grade astrocytomas and around half of all tumors showing del(1p)/del(19q) and/or other multiple losses in their ancestral tumor cell clone were low-grade gliomas (two diffuse astrocytomas, four oligodendrogliomas, and one ependimoma out of 15 cases); in contrast, high-grade gliomas predominated among the other cytogenetic groups. For more detailed information on the exact cytogenetics profiles of each clone please see Supplementary Fig. 1

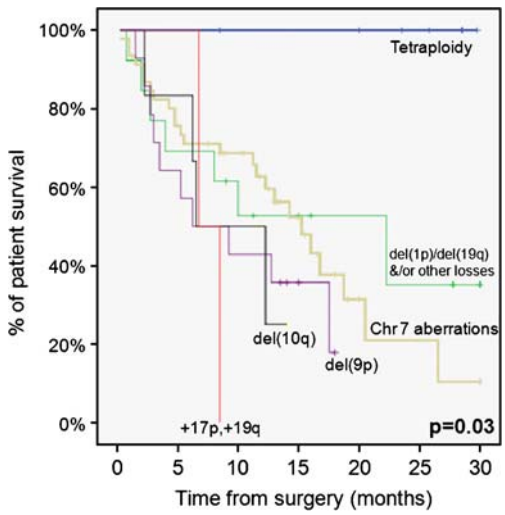

Fig. 5 Overall survival curves of gliomas grouped according to the cytogenetic abnormalities found to be common to all altered tumor cells. Of note, most (five of six tumors) tetraploid cases corresponded to low-grade astrocytomas and around half of all tumors showing del (1p)/del(19q) and/or other multiple losses in their ancestral tumor cell clone were low-grade gliomas (two diffuse astrocytomas, four oligodendrogliomas, and one ependimoma out of 15 cases); in contrast, high-grade gliomas predominated among the other cytogenetic groups

patterns with between one and four additional abnormalities of chromosomes 1p, 7, 10q, 13q, and 17p; in addition, 3% of these cases with del(9p21) with or without gains of chromosome 7 in common to all tumor cell clones, showed tetraploidization (Fig. 4 and Supplementary Fig. 1). As it may be inferred from what is describe above, tetraploidization with variable losses and gains of one or more chromosomes was a relatively common finding and it was detected in secondary clones of around half of the cases (47/90; 52%), in addition to those cases (7%) that presented tetraploidy in common to all tumor cell clones (Fig. 4 and Supplementary Fig. 1). However, in some subgroups such as cases with combined del(1p) and del(19q), secondary clones showed multiple additional chromosome losses in the absence of a tetraploid karyotype (Fig. 4 and Supplementary Fig. 1)

Interestingly, once gliomas were grouped according to the cytogenetic profile found to be common to all altered tumor cells, a longer overall survival was observed (p=0.03) for cases that displayed either tetraploidy or del(1p) associated with del(19q) compared to patients who had abnormalities of chromosome 7, del(9p), del(10q) and simultaneous gains of chromosome 17p and 19q (Fig. 5).

Cytogenetic profiles and tumor grade

Upon analyzing the frequency of the distinct cytogenetic abnormalities detected in gliomas according to tumor histopathology, several remarkable differences were noted between astrocytic and oligodendroglial, as well as between grade I/II and grade III/IV cases (Table 2). Accordingly, astrocytic grade I/II tumors (9/72 cases; 13%) were mainly characterized by the presence of gains for all chromosome regions analyzed (including all three astrocytic grade I tumors) consistent with tetraploidy in five of nine cases. In contrast, high-grade astrocytic tumors (63/72 tumors; 88%) showed more complex and heterogeneous genetic alterations, with a significantly higher frequency of chromosome abnormalities (Table 2).

Table 2 Frequency of different cytogenetic abnormalities detected in glioma tumors classified according to tumor histopathology

	Astrocytic			Oligodendroglial		Oligoastrocytic		Ependymoma	
	Grade I	Grade II	Grades III/IV	Grade II	Grade III	Grade II	Grade III	Grade II	Grade III
	Pilocytic	Diffuse	Anaplastic/GBM[a]		Anaplastic		Anaplastic		Anaplastic
Chromosomal abnormalities	(*n*=3)	(*n*=6)	(*n*=63)	(*n*=4)	(*n*=8)	(*n*=1)	(*n*=3)	(*n*=1)	(*n*=1)
Chr 1p losses	0	1/6 (17%)	17/63 (27%)	3/4 (75%)	5/8 (62%)	0	0	0	1/1 (100%)
Chr 1p gains	3/3 (100%)	4/6 (67%)	24/63 (38%)	0	3/8 (38%)	0	2/3 (67%)	0	0
Chr 1p gains and losses	0	1/6 (17%)	18/63 (29%)	1/4 (25%)	0	1/1 (100%)	1/3 (33%)	0	0
Chr 7 losses	0	0	0	1/4 (25%)	1/8 (13%)	0	0	1/1 (100%)	0
Chr 7 gains/amplification	3/3 (100%)	4/6 (67%)	43/63 (68%)[b]	0	5/8 (62%)	0	3/3 (100%)	0	1/1 (100%)
Chr 7 gains/amp and losses	0	2/6 (33%)	20/63 (32%)[c]	2/4 (50%)	2/8 (25%)	1/1 (100%)	0	0	0
Chr 9p losses	0	1/6 (17%)	39/63 (62%)	1/4 (25%)	6/8 (75%)	1/1 (100%)	2/3 (67%)	0	1/1 (100%)
Chr 9p gains	3/3 (100%)	4/6 (67%)	11/63 (17%)	0	2/8 (25%)	0	1/3 (33%)	0	0
Chr 9p gains and losses	0	1/6 (17%)	13/63 (21%)	1/4 (25%)	0	0	0	0	0
Chr 9q losses	0	0	11/63 (17%)	1/4 (25%)	2/8 (25%)	0	0	0	0
Chr 9q gains	3/3 (100%)	4/6 (67%)	39/63 (62%)	1/4 (25%)	6/8 (75%)	0	3/3 (100%)	0	1/1 (100%)
Chr 9q gains and losses	0	2/6 (33%)	10/63 (16%)	0	0	1/1 (100%)	0	0	0
Chr 10q losses	0	1/6 (17%)	42/63 (67%)	2/4 (50%)	3/8 (38%)	0	1/3 (33%)	0	1/1 (100%)
Chr 10q gains	3/3 (100%)	4/6 (67%)	10/63 (16%)	0	2/8 (25%)	0	1/3 (33%)	0	0
Chr 10q gains and losses	0	1/6 (17%)	9/63 (14%)	1/4 (25%)	2/8 (25%)	1/1 (100%)	1/3 (33%)	0	0
Chr 13q losses	0	2/6 (33%)	21/63 (33%)	2/4 (50%)	3/8 (38%)	1/1 (100%)	1/3 (33%)	0	0
Chr 13q gains	3/3 (100%)	4/6 (67%)	25/63 (40%)	1/4 (25%)	3/8 (38%)	0	2/3 (67%)	0	0
Chr 13q gains and losses	0	0	11/63 (17%)	0	1/8 (13%)	0	0	0	0
Chr 17p losses	0	1/6 (17%)	17/63 (27%)	1/4 (25%)	3/8 (38%)	0	0	0	0
Chr 17p gains	3/3 (100%)	3/6 (50%)	28/63 (44%)	1/4 (25%)	2/8 (25%)	0	3/3 (100%)	0	1/1 (100%)
Chr 17p gains and losses	0	2/6 (33%)	13/63 (21%)	1/4 (25%)	3/8 (38%)	1/1 (100%)	0	0	0
Chr 19q losses	0	0	19/63 (30%)	4/4 (100%)	4/8 (50%)	1/1 (100%)	0	0	0
Chr 19q gains	3/3 (100%)	3/6 (50%)	31/63 (49%)	0	3/8 (38%)	0	2/3 (67%)	0	1/1 (100%)
Chr 19q gains and losses	0	3/6 (50%)	10/63 (16%)	0	0	0	1/3 (33%)	0	0
Chr 22q losses	0	1/6 (17%)	14/63 (22%)	2/4 (50%)	2/8 (25%)	0	1/3 (33%)	0	0
Chr 22q gains	3/3 (100%)	3/6 (50%)	25/63 (40%)	1/4 (25%)	5/8 (62%)	0	2/3 (67%)	0	1/1 (100%)
Chr 22q gains and losses	0	2/6 (33%)	19/63 (30%)	0	1/8 (13%)	1/1 (100%)	0	0	0

The chromosomal abnormalities were divided in three groups: losses, gains (including several polysomies—trisomy, tetrasomy, pentasomy, etc.—and amplification detected only in chromosome 7) and finally combined gains and losses

[a] Two gliosarcomas included in this group. Cases without genetic abnormalities for some chromosome regions were not considered in this table

[b] Fourteen of these cases presented *EGFR* amplification

[c] Eleven of these cases presented *EGFR* amplification

Interestingly, a significant association was also found between tumor grade and the iFISH patterns of cytogenetic abnormalities detected in common to all tumor cell clones identified in individual cases (Table 3). Accordingly, while grade I/II astrocytic tumors showed either gains of chromosome 7 or tetraploidy in common to all altered tumor cells, high-grade astrocytic tumors (grades III/IV) revealed more complex cytogenetic patterns, with a considerable lower frequency of tetraploid tumors (Table 3). Although the number of cases analyzed for other histopathological subtypes was rather limited, combined loss of the 1p36 and 19q13 regions was most frequently found in common to all tumor cell clones in oligodendrogliomas (4/12 cases; 33%, particularly in low-grade cases, three of four cases). Despite this, as found in high-grade oligoastrocytic tumors, anaplastic oligodendrogliomas, in addition to 1p36/19q13 losses, showed more heterogeneous cytogenetic patterns in common to all tumor cell clones with respect to oligodendroglial grade II tumors; similarly to what was observed among high-grade astrocytomas, these included gains of chromosome 7, losses of the 9p21 chromosome region and other multiple chromosome losses (Table 3).

Discussion

Until now, few studies have been reported in which the cytogenetic heterogeneity of glioma tumors is assessed at the intratumoral cell level [32, 33], and only Lopez-Gines et al. [34] and Wemmert et al. [33] have used iFISH analyses based on a limited number of chromosome probes, to assess clonal evolution in glioblastomas and pilocytic astrocytomas, respectively. Through the study of 25 glioblastomas Lopez-Gines et al. [34] suggested that trisomy/polysomy 7 and monosomy 10 were frequently associated in glioblastomas, whereas *EGFR* amplification could represent a later event not associated with the former profile (gains of chromosome 7/chromosome 10 losses). In turn, Wemmert et al. [33] confirmed the occurrence of intratumoral cytogenetic heterogeneity among 14 pilocytic astrocytomas, through iFISH analysis of numerical abnormalities for chromosomes 7, 8, 17, and the *TP53* gene.

In this study, we used a multicolor iFISH approach aimed at the investigation of a large number (n=16) of different chromosome regions, including those more frequently altered in gliomas, to assess intratumoral genetic heterogeneity in a series of 90 patients studied at diagnosis. In order to identify the exact sequence of accumulation of chromosomal abnormalities in individual tumors carrying multiple subclones, we assumed that karyotypic abnormalities shared by all subclones should represent relatively early changes; in contrast, later cytogenetic changes would only be present in some of the

Table 3 Gliomas (n=90): relationship between the cytogenetic profile of ancestral tumor cell clones and tumor histopathology

	Astrocytic			Oligodendroglial		Oligoastrocytic		Ependymoma	
	Grade I	Grade II	Grades III/IV	Grade II	Grade III	Grade II	Grade III	Grade II	Grade III
	Pilocytic	Diffuse	Anaplastic/GBM[a]		Anaplastic		Anaplastic		Anaplastic
Ancestral tumor cell clone	(n=3)	(n=6)	(n=63)	(n=4)	(n=8)	(n=1)	(n=3)	(n=1)	(n=1)
Chr 1p and chr 19q losses	0/3 (0%)	0/6 (0%)	0/63 (0%)[b]	3/4 (75%)	1/8 (12%)	0/1 (0%)	0/3 (0%)	0/1 (0%)	0/1 (0%)
Chr 7 gains/amplifications	1/3 (33%)	1/6 (17%)	33/63 (52%)	0/4 (0%)	1/8 (12%)	1/1 (100%)	1/3 (33%)	0/1 (0%)	1/1 (100%)
Chr 9p losses and chr 7 gains	0/3 (0%)	0/6 (0%)	16/63 (25%)	0/4 (0%)	4/8 (50%)	0/1 (0%)	2/3 (67%)	0/1 (0%)	0/1 (0%)
Chr 10q losses	0/3 (0%)	0/6 (0%)	6/63 (10%)	0/4 (0%)	0/8 (0%)	0/1 (0%)	0/3 (0%)	0/1 (0%)	0/1 (0%)
Chr 17p and chr 19q gains	0/3 (0%)	0/6 (0%)	2/63 (3%)	0/4 (0%)	0/8 (0%)	0/1 (0%)	0/3 (0%)	0/1 (0%)	0/1 (0%)
Several losses	0/3 (0%)	2/6 (33%)	5/63 (8%)	1/4 (25%)	2/8 (25%)	0/1 (0%)	0/3 (0%)	1/1 (100%)	0/1 (0%)
Tetraploidy	2/3 (67%)	3/6 (50%)	1/63 (2%)	0/4 (0%)	0/8 (0%)	0/1 (0%)	0/3 (0%)	0/1 (0%)	0/1 (0%)

[a] Two gliosarcomas included in this group

[b] 20 of these cases presented *EGFR* amplification as ancestral tumor cell clone

other tumor cell clones. In line with this concept of clonal evolution, our results show that in virtually all gliomas, ≥2 cytogenetically related tumor cell clones which carry different chromosomal abnormalities, are present.

Overall, nine different cytogenetic profiles were identified in the ancestral tumor cell clones of the 90 cases analyzed. These most commonly involved partial or complete gains of chromosome 7—including amplification of the *EGFR* gene—hemizygous or homozygous deletion of 9p21 and del(10q); interestingly, combinations of such alterations were either already detected in the ancestral tumor cell clone or acquired in subsequent, highly represented clones. Of note, these complex cytogenetic patterns typically occurred prior to tetraploidization—a cytogenetic profile detected during evolution of a great proportion of all tumors analyzed—in the context of a relatively high number of tumor cell clones/sample and they were characteristic of high-grade astrocytic tumors.

Altogether, these results clearly show that complex karyotypes in which combined gains of chromosome 7—including amplification of the *EGFR* gene—del(9p21) and/or del(10q23) coexist, are crucial events for the development of a great majority of gliomas with an astrocytic phenotype; in addition, they support previous observations about the role of combined alterations of the *EGFR*, $p16/p14^{ARF}$, and/or *PTEN* genes in the malignant (high-grade) transformation of these tumors [10, 24, 35, 36]. In line with this, it has been previously shown that most gliomas coexpress the *EGFR* and *EGF* molecules, which are involved in an autocrine loop of growth stimulation [37]. Of note, *EGFR* mediates its cellular effects through the PI3K-Akt pathway [38], which is repressed by the expression of *PTEN* [39, 40]; loss/mutation of this later gene results in a direct increase in cell growth together with a blockade of apoptosis and up-regulation of vascular endothelial growth factor production [37, 40, 41]. Similarly, deletion of *p16* and $p14^{ARF}$ would also favor cell proliferation in these tumors, particularly when associated with mutations or epigenetic silencing of the remaining copy of these genes [40, 42]. Such a cytogenetic profile consisting of disruption of the *p16–pRb–*$p14^{ARF}$*–TP53* cell cycle control pathways, inactivation of tumor suppressor genes on chromosome 10 (e.g. *PTEN*) and amplification of the *EGFR* gene, has been associated with high-grade gliomas with an astrocytic phenotype [40] as also found in our series. In turn, the higher number of tumor cell clones observed among high-grade gliomas suggests a high genetic/chromosomal instability, which would facilitate the emergence and sequential selection of more aggressive subclones that would lead to tumor progression and more aggressive histopathological features. Of note, this high genetic/chromosomal instability cannot be therapy-induced since the studied glioblastoma multiforme are primary (or *de novo*) GBM, without any previous treatment.

Other less frequent cytogenetic profiles recurrently observed in the ancestral tumor cell clones included tetraploidy, combined loss of 1p36/19q13 and combined gains of chromosomes 17 and 19; while the later pattern was only found in a few high-grade glioblastomas, the former were characteristic of low-grade tumors with a better outcome and an astrocytic or oligodendroglial/mixed phenotype, respectively. In contrast to high-grade gliomas, few cytogenetic profiles were detected in common to all altered tumor cells in grade I/II astrocytic tumors. Such profiles were consistent with tetraploidy and less frequently, with chromosome 7 gains in the absence of losses of chromosomes 9p21 and 10q23, immediately followed by tetraploidization in subsequent tumor cell clones. Based on these observations, it could be hypothesized that early occurrence of tetraploidization in association with duplication of the *BRAF* gene in pylocytic astrocytomas [43, 44] could prevent histopathological grade I/II astrocytomas from evolving into an invasive phenotype and a highly progressive clinical behavior tied to aggressive histopathological features; this could be due to the fact that $p16/p14^{ARF}$ and chromosome 10q losses that occur after tetraploidization would have a more limited impact in the aggressiveness of the tumor because at least one copy of each allele of these genes would still be retained. In line with this, a tetraploidy checkpoint has been associated with prevention of tumor progression among gliomas [45, 46] and these cytogenetic profiles were also much less frequently observed among high-grade gliomas, where acquisition of multiple cytogenetic abnormalities leading to relatively complex cytogenetic profiles, typically preceded tetraploidization. Although early tetraploidization was associated with a significantly better clinical outcome, this could be due to its association with low-grade astrocytomas and deserves further multivariate analyses in larges series of patients to determine its potential independent prediction value.

Combined losses of 1p36 and 19q13, were detected in common to all tumor cells in 3/4 (75%) grade II oligodendrogliomas, while only 1/8 (12%) high-grade oligodendrogliomas showed this cytogenetic profile in its ancestral tumor cell clone. At present, the molecular mechanisms involved in the ontogeny of oligodendroglial tumors remains largely unclear. However, recent reports point out the potential involvement of *p21* [47], and other recently identified oligodendroglioma-associated candidate tumor suppressor genes localized at the 1p36 and 19q13 chromosome regions, in the development of oligodendrogliomas [48, 49]. In fact, it has been shown that, del(1p) and del(19q), as well as translocations involving these chromosome regions are the genetic hallmark of oligodendroglial tumors [50–53] as also found in our series; however, it should be noted that in line with our findings, losses of chromosomes 1p and 19q could be less commonly

detected in anaplastic/grade III versus grade II oligodendrogliomas [3, 49].

Altogether, these results may suggest that while astrocytic and oligodendroglial tumors may progress [27, 28, 54] from histologically low- to high-grade tumors, frequently, different genetic pathways could be involved in histologically low- versus high-grade gliomas [55]. In line with this hypothesis, previous studies aimed at dissecting the genetic pathways of tumor progression through the comparison of the karyotypes of different histological subgroups of gliomas [4, 9–11] have pointed out the existence of two clearly distinct cytogenetic patterns. One corresponds to the majority of cases representing primary or *de novo* glioblastomas, which are genetically characterized by loss/mutation of 10q/*PTEN*, *EGFR* amplification and/or *p16* deletion with a low frequency of *TP53* mutations. The second cytogenetic pattern is characteristic of secondary glioblastomas, which develop through progression from low-grade diffuse astrocytomas or anaplastic astrocytomas where *TP53* mutation is the most frequent and earliest detectable genetic alteration [24, 30]. Our results support and extend on this hypothesis by showing that, at the intratumoral cell level, early tetraploidization together with combined 1p36/19q13 losses are associated with low-grade astrocytomas and oligodendroglial tumors, respectively; of note, tetraploidy was similarly found in pilocytic grade I as well as grade II, diffuse astrocytomas as also reported by others [56, 57]. In contrast, the ancestral tumor cell clones of high-grade gliomas are more likely associated with early occurrence of combined gains/amplification of the *EGFR* gene in chromosome 7, hemizygous or homozygous del (9p21) and/or deletion of the *PTEN* gene in chromosome 10q, followed by acquisition of complex karyotypes prior to tetraploidization. Based on these observations, it could be hypothesized that early tetraploidization in gliomas with an astrocytic phenotype could prevent these tumors from having an aggressive behavior, even in the presence of *TP53* mutation/deletion [40, 58, 59]; in contrast, occurrence of tetraploidization at relatively advanced stages of the disease, after gains of chromosome 7/*EGFR* amplification, del(9p21) and/or del(10q23) have already occurred, would not have such an impact. These observations together with the limited number of low-grade tumors included in our study point out the need to confirm our observations in larger series of low-grade gliomas, in which comparative analysis of the cytogenetic profiles of primary versus secondary high-grade tumors are also analyzed.

Acknowledgments This work has been partially supported by grants from FCT (Portuguese Foundation for Science and Technology, PhD fellowships SFRH/BD/11820/2003 and SFRH/BD/23086/2005), FCG (Portuguese Calouste Gulbenkian Foundation, Project Ref. 68708) and Spanish Network of Cancer Research Centers (RD06/0020/0035; RTICC; Instituto de Salud Carlos III, Ministerio of Science and Innovation, Madrid, Spain).

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