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1p36 is a preferential target of chromosome 1 deletions in astrocytic tumours and homozygously deleted in a subset of glioblastomas

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Abstract

Astrocytic, oligodendroglial and mixed gliomas are the commonest gliomas in adults. They have distinct phenotypes and clinical courses, but as they exist as a continuous histological spectrum differentiating them can be difficult. Co-deletions of total 1p and 19q are found in the majority of oligodendrogliomas and considered as a diagnostic marker and a prognostic indicator. The 1p status of astrocytomas has not yet been thoroughly examined. Using a chromosome 1 tile path array, we investigated 108 adult astrocytic tumours for copy number alterations. Total 1p deletions were rare (2%), however partial deletions involving 1p36 were frequently identified in anaplastic astrocytomas (22%) and glioblastomas (34%). Multivariate analysis showed that patients with total 1p deletions had significantly longer survival (p=0.005). In 9 glioblastomas homozygous deletions at 1p36 were identified. No somatic mutations were found among the 5 genes located in the homozygously deleted region. However, the CpG island of *TNFRSF9* was hypermethylated in 19% of astrocytic tumours and 87% of glioma cell lines. *TNFRSF9* expression was upregulated after demethylation of glioma cell lines. *Akt3* amplifications were found in four glioblastomas. Our results indicate that 1p deletions are common anaplastic astrocytomas and glioblastomas but are distinct from the 1p abnormalities in oligodendrogliomas.

Keywords

Microarray; Array-CGH; Methylation; Astrocytoma; Oligodendroglioma

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Introduction

Astrocytic and oligodendroglial tumours together with the mixed oligoastrocytomas account for more than 80% of all adult human gliomas (2005-2006 Statistical Report: Primary Brain Tumors in the United States Statistical Report, http://www.cbtrus.org/reports/reports.html). They are subdivided into several malignancy grades according to the World Health Organization (WHO) classification (Kleihues & Cavenee, 2000) and prognosis varies dependent on tumour type and malignancy grade. As these three tumour types form a continuous histological spectrum, differentiating them is sometimes difficult (Kros *et al.*, 2007; Smith *et al.*, 1999).

Classical astrocytic and oligodendroglial tumours have typical genotypes. Astrocytic tumours often show gains of chromosome 7 and/or deletions of chromosome 10, mutations of *RB1* or *PTEN*, as well as amplification of *EGFR, CDK4* or *MDM2*, which are rare in oligodendroglial tumours. The great majority of oligodendrogliomas have hemizygous losses of all of 1p (total 1p deletion), almost always combined with losses of all of 19q (total 19q deletion) (Ichimura *et al.*, 2004; Reifenberger & Louis, 2003). Recent prospective control trials show that loss of one entire copy of 1p and 19q is a favourable prognostic indicator in both anaplastic oligodendrogliomas and anaplastic oligoastrocytomas (Cairncross *et al.*, 2006; van den Bent *et al.*, 2006). Attempts to find the gene(s) targeted by focusing on the smallest consistently deleted region found in a small subset of oligodendroglial tumours have been unsuccessful (Dong *et al.*, 2004; Felsberg *et al.*, 2004; Hashimoto *et al.*, 2003; Iuchi *et al.*, 2002; Nigro *et al.*, 2001). Recent reports of an unbalanced t(1;19)(q10;p10) translocation as the mechanism of co-deletions of 1p/19q in oligodendrogliomas make such an approach less likely to be rewarding (Griffin *et al.*, 2006; Jenkins *et al.*, 2006).

The status of chromosome 1 in astrocytic tumours has not received so much attention. Metaphase CGH studies report rare deletions of 1p (Koschny *et al.*, 2002), while Loss-of-Heterozygosity (LOH) analyses with limited numbers of markers indicate an overall incidence of 1p deletions of up to 20%, probably targeting 1p36 (Barbashina *et al.*, 2005; Mueller *et al.*, 2002; Schmidt *et al.*, 2002; Smith *et al.*, 1999; Ueki *et al.*, 2002; von Deimling *et al.*, 2000). However, the prognosis for patients with astrocytic tumours of any grade is much worse than that for similar grades of oligodendrogliomas or oligoastrocytomas regardless of therapy (MRC, 2001). A recent study of a series of astrocytic, oligodendrocytic and oligoastrocytic tumours of all grades suggested that the presence of a partial 1p loss was associated with poorer prognosis as compared with total 1p loss (Idbaih *et al.*, 2005). Thus the presence or absence as well as the extent of chromosome 1 deletions in these tumours and the association of such genetic abnormalities with prognosis or response to treatment requires further study (Idbaih *et al.*, 2005; Ino *et al.*, 2000; Schmidt *et al.*, 2002; Smith *et al.*, 2000).

To date the techniques used in many studies of 1p in astrocytic tumours (i.e. LOH or FISH analysis) examining a limited numbers of loci cannot accurately identify the presence or the extent of all 1p losses. Here we provide a comprehensive analysis of chromosome 1 copy number alterations in adult astrocytic tumours using a chromosome 1 tile path array. We found that deletions of chromosome 1 are present in increasing frequency among anaplastic astrocytomas and glioblastomas. Unlike oligodendrogliomas, the majority of deletions are small and involving 1p36.22-p36.23. Furthermore, we identified novel overlapping homozygous deletions in 9 glioblastomas in this region.

Results

Chromosome 1 tile path array analysis of astrocytic tumours

A total of 108 of the astrocytic tumours (9 A, 22 AA, 77 GB) were examined by a chromosome 1 tile path array. Among these were tumours with allelic imbalance at 1p detected by MSA, or copy number reduction at *SLC45A1/DNB5* detected by LightCycler analysis (qPCR) and a consecutive series of tumours collected during the year 1994 (5 A, 9 AA and 32 GB, used as an unbiased subset to estimate incidence). Twelve of the total series were examined by version 1 array alone, 65 by version 2 array alone and 31 by both. The interpretation of the data from both versions of the array gave similar findings for each of these 31 tumours (data not shown) confirming the validity of the normalization methods used. MSA and chromosome 1 array-CGH results showed good concordance, *i.e.,* array-CGH consistently indicating either loss, gain or amplification where MSA showed allelic imbalance (Fig. 1).

The chromosome 1 copy number status was determined at each clone based on the array-CGH results as either homozygous deletion, hemizygous deletion, normal copy number, copy number gain, or amplification, assuming that the tumours are near-diploid (Bigner *et al.*, 1988). The results are summarized in Table 1 and the patterns of chromosome 1 alterations in individual tumours are schematically presented in Figure 2.

Chromosome 1 deletions in astrocytic tumours

In total, copy number abnormalities on chromosome 1 were seen in 74/108 tumours (Table 1). Deletions of any part of 1p were found in 55 tumours and of 1q in 15. Deletions of one entire copy of 1p ("total 1p deletion") were only seen in 7 tumours (1 A, 3 AA, 3 GB, Table 1 and Fig. 2A). Careful re-evaluation of the histopathology of these cases revealed that two of the GBs contained small regions of oligodendroglioma-like cells. Both of these GBs also had total 19q deletions (1Mb array-CGH data, manuscript in preparation). The other five tumours, three of which also showed total 19q deletions (1 A, 2 AA), contained no significant oligodendroglial component in the tumour tissue submitted to histopathology. The commonest finding was partial deletions of 1p found in 48 tumours (1 A, 6 AA, 41 GB) and this involved 1p36 in 41 of these (1A, 6 AA, 34 GB, Table 1 and Fig. 2). The other 7 partial deletions of 1p affected different parts of 1p centromeric to 1p36. Furthermore, 9 homozygous deletions were identified in glioblastomas involving 1p36 (Figs. 1 through 3). Array-CGH analysis of patients' constitutional DNA showed normal copy number in this region, indicating that the homozygous deletions were somatic changes.

In the consecutive series of 46 tumours from 1994, the incidence of any deletion of 1p was 39% (18/46), the vast majority (13/46; 28%) being partial deletions involving 1p36 (0% in A, 22% in AA and 34% in GB) with one GB having a homozygous deletion of this region. Only a single GB showed total deletion of one copy of 1p. Deletions of any part of 1p were most common among GBs occurring in 16/32 GBs (50%) in this series while no deletions, or any other abnormalities of chromosome 1 were detected in the 5 As (Table 1).

Chromosome 1 status and prognosis

The potential influence of the 1p status on overall survival was studied on the 106 astrocytic tumour patients whose clinical data were available (Table 2). A univariate analysis using the log rank test showed that the tumours with total 1p deletion (1 A, 3 AA, 3 GB) had significantly longer overall survival (p=0.001, Table 2, Kaplan-Meier curve shown in Supplementary Fig. 1). Multivariate analysis using Cox' regression showed that the 1p status predicts overall survival independent of age and WHO grade (p=0.005). Similar

tendency was also observed among GB and AA patients, when analysed separately (Table 2).

Defining the commonly deleted region at 1p36

The boundaries of the 9 homozygous deletions were defined as shown in Fig. 3A. The largest homozygous deletion spanning a 2.9Mb region which included all the other 8 homozygous deletions was found in GB84 (Figs. 2B and 3A). This region was also overlapping with 36 of the tumours with hemizygous deletions with 1p36 (Fig. 2A). Five tumours with hemizygous deletions effecting 1p36 (GB63, GB152, AA14, AA29 and AA65, see Fig. 2) did not encompass this region.

Multiplex PCR at selected loci (an STS on RP11-185B14, *RERE* exons 12, 13, 14, 15 and 23, *SLC45A1/DNB5* exons 1 and 8, *TNFRSF9* exon 5) confirmed homozygous deletions at one or more of these loci in all 9 tumours (Fig 3A-D, Fig. 4A). However, a closer analysis showed that not all the homozygous deletions shared a single common region. GB219 showed homozygous deletion at exon 13 of *RERE* but not at exon 14 (Fig. 3C), defining the telomeric boundary of homozygous deletion between these loci (the region covered by clones RP11-126I6 and RP11-141M15, Fig. 3A). GB136 had homozygous deletion at exon 5 of *TNFRSF9* but not at any of the exons examined at *SLC45A1/DNB5* or *RERE* (Fig. 3B-C, Fig. 4A), defining the centromeric boundary as being telomeric to *SLC45A1/DNB5*. Thus, these results confirmed that homozygous deletions in GB219 and GB136 were not overlapping. Identical patterns of deletion were observed in xenografts of these tumours (GB219X and GB136X, Fig. 3B-D).

When these two regions were considered separately, the clones most frequently involved in the centromeric homozygous deletions were RP11-126I6, RP11-141M15, RP5-1115A15 and RP11-3J23 (8 tumours, Fig 3A). These clones contain *SLC45A1/DNB5* and a part of *RERE*. The clones most frequently involved in the telomeric region were RP5-892F13, which was deleted in 7 tumours and harbours *TNFRSF9*, as well as RP11-478I22 and RP11-431K24 (no genes mapped) and RP4-633I8 (contained a part of *RERE*). These 3 genes, as well as two other genes, *ERRFI1/MIG-6* and *PARK7*, located adjacent to *TNFRSF9*, were screened for presence of somatic mutations.

Mutation analysis of candidate genes

A total of 230 tumours (96 glioblastomas, 95 oligodendroglial tumours, 24 glioblastoma xenografts and 15 glioma cell lines) were screened for somatic mutations using either DGGE (primary tumours) or direct sequencing (xenografts and cell lines) of all coding sequences and intron sequences adjacent to the exons in *RERE* (24 exons, Accession No.NM_012102) and *SLC45A1/DNB5* (9 exons, Accession No. XM_937695). Mutations of *ERRFI1/MIG-6* (4 exons, NM_018948) were examined for 110 tumours (3 A, 12 AA, 56 GB, 24 GB xenografts and 15 glioma cell lines) and *TNFRSF9* (8 exons, NM_001561) and *PARK7* (7 exons, NM_007262) for 63 tumours (24 glioblastomas, 24 glioblastoma xenografts and 15 glioma cell lines) by DGGE. A number of nucleotide changes were identified in the coding regions or introns of *RERE, SLC45A1/DNB5* and *ERRFI1/MIG-6* (supplementary table 3), which were also observed in the patients' constitutional DNA and therefore judged to be polymorphisms. In the cell line H4, a non-synonymous homozygous nucleotide change was identified at *ERRFI1/MIG-6* (c.325G>A, D109N). Although no constitutional DNA for this cell line was available, this nucleotide change has been identified as a polymorphism in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html, refSNP ID: rs34781518) and therefore assumed as such. No nucleotide changes were identified in *PARK7* or *TNFRSF9*.

Methylation/expression analysis of candidate genes

Methylation status at the CpG islands (CGI) of the following genes in the homozygously deleted region, *CAMTA1*, *VAMP3*, *PER3*, *ERRFI1/MIG-6*, *TNFRSF9*, *SLC45A1/DNB5*, *RERE*, *ENO1*, *H6PD* and *SPSB1/SSB1*, were studied using MSP (for gene locations see Fig. 3A). No CGIs were identified for *UTS2, CA6* and *SLC2A7* (see Materials and Methods for definition of CGI used). Among them, methylation of the *TNFRSF9* CGI was found in 21/108 (19%) of primary tumours (18/77 (23%) GB, 2/22 (9%) AA, 1/9 (11%) A) and 13/15 cell lines (87%, Table 1). The methylation of *TNFRSF9* was further confirmed in two tumours (GB105 and GB152) by bisulfite-sequencing (Fig. 4C and see Materials and methods). No methylation of *TNFRSF9* was found in DNA from three normal brain specimens or one specimen of peripheral white blood cells. Methylation of *ENO1* was found in one GB and two GBs had methylation of *SPSB1/SSB1*. The other seven genes examined showed no evidence of methylation.

The expression levels of *TNFRSF9* were examined by real-time PCR using a LightCycler in 21 astrocytic tumours with methylation of *TNFRSF9* and 30 tumours without. No significant difference of expression levels between these two groups was observed (data not shown). To further investigate the association between the methylation status of *TNFRSF9* and its expression, U251, a glioblastoma cell line with methylation of *TNFRSF9*, was treated with the demethylating agent 5-aza-2′-deoxycytidine (Fig. 4B). Following treatment for 4 days, there was a 15-fold increase in *TNFRSF9* expression (Fig. 4D, top panel). MCF7, a breast cancer cell line that did not have methylation of *TNFRSF9*, showed no significant change in expression levels following the de-methylation treatment (Fig. 4D, bottom panel).

Chromosome 1 amplification in astrocytic tumours

Amplification was noted in 10 tumours (1 AA, 9 GB, see Table 1 and Figs. 2 and 5). Four glioblastomas, GB291, GB225, GB153 and GB263 had amplicons of various sizes, all including a common region at 1q44 between RP11-150L22 and RP11-350M10 (Fig. 5). This region contained four genes, *SDCCAG8, AKT3, ZNF238* and *NP_001013732* (Fig. 5B, lower panel). Further amplicons were found in GB197 at 1p36.21 between RP11-219C24 and RP5-1108H3, a region included in a larger amplicon in GB251 (between RP11-474O21 and RP11-227B14 with interruptions). The commonly targeted region in these two cases includes 5 genes (*PRDM2, PRAMEF8, PRAMEF9, LRRC38* and *PDPN*). Yet another amplicon on 1p31.3-p32.1 in AA99 (between RP11-351G1 and RP11-776H12) was entirely included in a larger amplicon in GB168 (between RP5-994L8 and RP4-537K17). This region does not contain any known genes. Other amplified regions were found in GB149 (1p35.1; RP11-131M11 - RP1-149P10) and GB126 (1p34.2-p34.3**;** RP3-423B22 - RP4-562N20).

Discussion

This study focused on profiling chromosome 1 alterations and precisely defining the minimum common regions of alterations in astrocytic tumours. Our results show that deletions at 1p36 are common abnormalities among astrocytic tumours. They are generally small and overlap with the 9 homozygous deletions we identified in glioblastomas. It occurred in increasing incidence with malignancy grade, suggesting that this region is involved in progression. In contrast, total deletions of 1p were rare in all grades of astrocytoma. Thus, deletions affecting 1p are common abnormalities in both astrocytic and oligodendroglial tumours, however the patterns of deletions are different. Moreover, total 1p deletions in this astrocytoma series had a significantly longer overall survival independent of age and malignancy grade as compared to the tumours with normal 1p status or other patterns of deletions (p=0.005 in Cox’s regression).

The association between 1p status and prognosis of astrocytic tumours has been controversial (He *et al.*, 2001; Homma *et al.*, 2006; Schmidt *et al.*, 2002). Some of these studies examined a limited number of loci on 1p36, in which case the different types of 1p deletion cannot be distinguished. In line with our study, Idbaih *et al* compared total and partial 1p deletions in gliomas and found that total deletions of 1p, but not partial deletions, were associated with longer survival (Idbaih *et al.*, 2005). Our results suggest that tumours with total 1p deletions and those with partial 1p36 deletions belong to biologically distinct classes of tumours, and must be clearly distinguished. This is particularly important when an assessment of 1p and 19q status is used in stratification or selection for clinical trials or recommended as a supplement to morphological diagnosis. Great care must thus be taken when choosing loci for conventional microsatellite or FISH analysis of 1p.

We identified 9 homozygous deletions at 1p36 in GBs. Detailed mapping revealed that homozygous deletions of GB219 and GB136 did not overlap, indicating that two separate regions were being targeted (Fig. 3). In the telomeric region defined by the homozygous deletion of GB136, RP5-892F13, RP11-478I22 and RP11-431K24 were most frequently deleted. *TNFRSF9* located on RP5-892F13 was deleted in 7 tumours including GB263, which had an isolated homozygous deletion at this clone. No somatic mutations were found in *TNFRSF9*. However hypermethylation of the CGI of *TNFRSF9* was identified in approximately 20% of primary astrocytic tumours and 87% of glioblastoma cell lines.

TNFRSF9 encodes the CD137 (ILA / 4-1BB) protein, a member of tumour necrosis factor receptor family (reviewed in (Watts, 2005)). TNFRSF9/CD137 is expressed on the surface of activated T-cells. Antigen presenting cells express its ligand as a cell surface trans-membrane protein (TNFSF9/4-1BBL). TNFRSF9/CD137 mediated signals promote cell survival and the secretion of various chemokines by T-cells. The role of TNFRSF9/CD137 in non-immune cells is not clear. It is interesting that *TNFRSF9* is homozygously deleted, or hemizygously deleted with methylation of the retained allele in a subset of astrocytic tumours and glioma cell lines, while no such methylation was found in normal brain tissues. Although no significant difference of *TNFRSF9* expression levels was observed between primary tumours with *TNFRSF9* methylation and those without, a strong association between the methylation status of *TNFRSF9* and its mRNA expression was demonstrated by demethylating cultured glioblastoma cells with 5′-aza-2′-deoxycytidine (Fig. 4D). The lack of correlation between expression levels and methylation status in primary tumour tissue may be due the presence of non-neoplastic cells such as macrophages that express high levels of this receptor (Melero *et al.*, 1997) and that are commonly present in high numbers in glioblastomas. *TNFRSF9* was the only gene found frequently altered among the genes recognised so far in the homozygously deleted region. *TNFRSF9* was not methylated in GB219 or GB176, in which *TNFRSF9* was not involved in the homozygous deletions, excluding the possibility that *TNFRSF9* is the sole target of all 1p36 deletion cases (Fig. 3A).

RERE was identified as a gene coding for a protein homologous to the atrophin-1 protein, in which abnormal glutamine expansion causes dentatorubral-pallidoluysian atrophy (DRPLA), a rare hereditary neurodegenerative disorder (Yanagisawa *et al.*, 2000). Ectopically expressed RERE has been shown to induce apoptosis in a caspase-3-dependent manner (Waerner *et al.*, 2001), suggesting that *RERE* may be involved in promoting apoptosis and is therefore a potential TSG. All but one homozygous deletion (GB136) involved at least a part of *RERE*, however no somatic mutations or promoter hypermethylation were identified in *RERE* after screening a large number of gliomas. Thus the significance of *RERE* homozygous deletions in glioblastomas remains obscure.

The possibility still exists that other gene(s) or regulatory sequences in this region may be being targeted. Several non-coding RNA genes have been mapped in this region. The presence of novel, as yet unrecognised gene(s) cannot be excluded. The lack of recurrent breakpoints for gains/deletions (see Fig. 2) indicates that a recurrent translocation with a resultant a novel fusion gene is unlikely.

Hemizygous 1p36 deletions also occurred outside the homozygously deleted region defined above (e.g., GB63, GB152 and AA14, see Fig. 2B). These deletions overlap with minimally deleted regions reported previously (Barbashina *et al.*, 2005; Dong *et al.*, 2004). Barbashina *et al.* defined the minimal region of deletion around *CAMTA1*. A limited analysis of this gene identified no mutations (Barbashina *et al.*, 2005). *CAMTA1* was also included in the homozygously deleted regions in 5GBs of our series (Fig. 3A). *TP73*, a homologue of *TP53* involved in regulation of cell proliferation and apoptosis, is located on 1p36.32, telomeric to the homozygously deleted region but was included in a number of hemizygous deletions (Fig. 2B). No somatic mutations of *TP73* have been identified so far (Alonso *et al.*, 2001), however hypermethylation of the promoter region has been reported in a subset of gliomas (Watanabe *et al.*, 2002). Bagchi *et al* identified *CHD5* (Chromodomain helicase DNA binding domain 5) as a candidate tumour suppressor gene using mice genetically engineered to harbour alterations in the chromosomal region corresponding to human 1p36 (Bagchi *et al.*, 2007). Mouse *Chd5* was shown to positively regulate the p19Arf/p53 pathways and mouse embryonic fibroblasts deficient in *Chd5* formed tumours in nude mice following transplantation. Human gliomas with *CHD5* deletions showed low expression levels. *CHD5* maps telomeric to but not within the homozygously deleted region we identified (Fig. 2B). So far no somatic mutation of *CHD5* has been reported in human tumours but this requires further investigation. Interestingly, among the other genes mapped to this region, neither *Camta1* nor *Tp73* showed the ability to modulate proliferation in the study of Bagchi et al, while *Tnfrsf9* was not examined.

We identified 10 novel amplifications on chromosome 1. Most notably, 4 glioblastomas had amplifications encompassing *AKT3*. Akt3 (PKBγ) is one of three isoforms of phosphoinositide-dependent serine-threonine protein kinases, Akt (Protein Kinase B). Akt plays a central role in the PI3K-Akt-mTOR signalling pathway, which is involved in regulating many cellular functions including cell proliferation, growth survival, cell motility and angiogenesis (Bellacosa *et al.*, 2005). Deregulation of this pathway such as mutations of *PTEN* is common in glioblastomas. Components of this pathway are frequently altered in diverse tumour types. Amplification involving the *AKT3* locus has been particularly observed in hepatitis C virus-associated hepatocellular carcinomas (Hashimoto *et al.*, 2004), although previous reports on glioblastomas have not found this (Knobbe & Reifenberger, 2003). As our tumour series was not selected for alterations of this region (see Results), the frequency of amplification would be about 5% (4/77). Interestingly, two of the four GBs in our series with *AKT3* amplification also had *PTEN* mutations ((Schmidt *et al.*, 1999) and unpublished data). *PTEN* mutation and *AKT3* amplification/overexpression may have a synergetic effect. A precise amplicon mapping with correlation to mRNA expression is underway.

The data presented here revealed that chromosome 1 status in gliomas, particularly in astrocytic tumours, is much more complex than previously thought. A thorough assessment of 1p status across different glioma types, correlation of the findings with clinical features, the identification of what is being targeted as well as an understanding of the biological consequences of the abnormalities will establish their clinical significance and improve our understanding of the molecular mechanisms underlying the progression of the astrocytic gliomas.

Materials and methods

All chromosome 1 array clones, positions of copy number variations and log2-ratio are shown in supplementary table 1. All primers, PCR and gel conditions are shown in supplementary table 2.

Tumour materials and nucleic acid extraction

A total of 265 primary astrocytic tumours including 22 astrocytoma malignancy grade II (prefixed as A), 61 anaplastic astrocytoma malignancy grade III (AA) and 182 glioblastoma malignancy grade IV (GB), as well as 24 glioblastoma xenografts (established from 24 glioblastomas included in the above series) and 15 glioma cell lines were included in the study. In addition, 95 oligodendroglial tumours were included in the mutation analysis of selected genes. The histopathological diagnosis of the astrocytic tumours was reviewed by one neuropathologist (V.P.C) and made according to the WHO classification (Kleihues & Cavenee, 2000). Collection, handling and DNA/RNA extraction of tumour tissues and the patients' blood samples were as described previously (Ichimura *et al.*, 2000). cDNA was generated using total RNA that had been treated with RNase-free DNaseI as published (Liu *et al.*, 2005). The study was approved by the Ethical Committee of the Karolinska Hospital (No. 91:16) and Cambridge Local Research Ethics Committee, Cambridge, UK (Ref. LREC 03/115).

Microsatellite analysis

The following chromosome 1 microsatellite markers were used; (1p) *D1S468*, *D1S2667*, *D1S228*, *D1S247*, *D1S1598*, *D1S220*, *D1S464*, *D1S418*, (1q) *D1S2655*, *D1S2703*. Microsatellite analysis (MSA) was performed as previously described (Ichimura *et al.*, 1998).

Chromosome 1 tile path array

Two versions of chromosome 1 tile path array were used. The first version has been previously described (Buckley *et al.*, 2005). The second version consisted of the same set of clones with an additional 113 chromosome 1 clones added to fill some gaps in the first version. In total, 2222 clones (1437 BACs, 778 PACs, 7 cosmids) covering 97.9% of the sequenced regions of chromosome 1 were included for the analysis (NCBI build 35, (Gregory *et al.*, 2006)). The second version also included a set of 538 BACs that were distributed over all autosomes with an average midpoint interval of 5.2Mb (5Mb clone set). The majority of the tumours (96 tumours) were studied using the second version, of which 31 were examined using both to confirm reproducibility. The construction, hybridization and analysis of the chromosome 1 tile path array versions 1 and 2 were carried out according to the published protocol with minor modifications (Fiegler *et al.*, 2003; Ichimura *et al.*, 2006). For normalization, the median of the ratio of test/reference spot intensity of all clones on either 1p or 1q was used as the normalization factor in the first version of array. The chromosomal arm on which most microsatellite markers showed allelic balance was chosen for normalization. In the second version, the median of test/reference spot intensity ratios of all autosomal clones in the 5-Mb clone set was used as a normalization factor independent of the prior knowledge of chromosome 1 allelic status.

Multiplex PCR, DGGE and sequencing

Multiplex PCR was performed and the copy number (normal value = 2) at each target locus was calculated as described (Schmidt *et al.*, 1999). PCR products on an agarose gel were quantified using a Labworks software and GelDoc-It Imaging System (UVP, Cambridge, UK). Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing were optimized and

performed as described (Ichimura *et al.*, 2000). Primer design and sequencing analysis were carried out using MacVector v8.1 (MacVector, Inc. Cambridge, UK) or Accelrys Gene 2.0 (Accelrys, Cambridge, UK).

Methylation analysis

Bisulfite-modification of genomic DNA and methylation-specific PCR (MSP) was performed according to the published protocol with minor modifications (Herman *et al.*, 1996). CpG islands (CGI) for the genes at 1p36.22-p36.23 (*CAMTA1*, *VAMP3*, *PER3*, *ERRFI1/MIG-6*, *TNFRSF9*, *SLC45A1/DNB5*, *RERE*, *ENO1*, *H6PD* and *SPSB1/SSB1*) were identified using the Methprimer software (http://www.urogene.org/methprimer/index.html) with the following criteria: Island size > 100bp, G+C content > 50.0%, Observed to Expected ratio of CpG dinucleotides > 0.60. Primers for methylation-specific PCR (MSP) were designed within the defined CGIs to specifically amplify bisulfite-modified methylated sequences. Bisulfite-modified DNA was amplified using a primer pair for *ACTB* that amplified bisulfite-modified sequences regardless of their original methylation status to confirm the efficiency of modification (Harden *et al.*, 2003). Unmodified genomic DNA, bisulfite-modified normal blood DNA, *Sss*I methylase-treated and bisulfite-modified normal blood DNA, and no template control were included in every PCR run as a control.

Primers for bisulfite-sequencing of *TNFRSF9* were designed to amplify bisulfite-modified sequences of the entire CGI regardless of the methylation status. The PCR products were then subcloned into a pCR4-TOPO vector according to the manufacturer's recommendations (Invitrogen, Paisley, UK) and a minimum of 10 clones from each construct were sequenced.

Real-time quantitative PCR

Gene copy number change at exon 6 of *SLC45A1/DNB5* and mRNA expression of *TNFRSF9* and *RERE* were assessed by real-time PCR using a FastStart DNA Master SYBR Green I® with LightCycler® (Roche Diagnostics, Mannheim, Germany). *WI-3306* (STS at 2q21) and the 18S ribosomal RNA gene were used as an internal reference for copy number and expression analysis respectively. Samples were amplified in duplicate using a LightCycler version 3.5 software according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). A consistent calibrator (mixture of various genomic DNA or cDNA) was included in each run for normalization. The PCR products of the target or reference genes, directly or subcloned into a pCR4-TOPO vector, were used in a dilution series to determine relative standard curves for efficiency correction. After the amplification, the Crossing Points for individual samples were automatically determined using the Second Derivative Maximum Method using LightCycler version 3.5. The PCR efficiency-corrected and normalized target/reference ratio was then determined using a RelQuant software (Roche Diagnostics, Mannheim, Germany). For *SLC45A1/DNB5*, a gene dosage was calculated as a relative target/reference ratio of each tumour DNA to that of control DNA (average of five normal tissue DNA).

5-aza-2′-deoxycytidine treatment

For each treatment group, 2×10^5 cells (U251 or MCF7) were plated in a T25 flask in triplicate. After 24 hours, cells were cultivated with the medium containing 1 μM of 5-aza-2′-deoxycytidine for either 48 hours or 96 hours. The medium containing the reagent was changed every 24 hours. After the treatment, cells were cultivated for further 48 hours without 5-aza-2′-deoxycytidine, RNA extracted and expression levels quantified using real-time PCR as described above.

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Statistical analysis

The log rank test was used for survival analysis, assessing the potential prognostic value of the 1p status. Cox' regression analysis was performed to adjust for age and WHO grade. All statistical analysis were performed using Statistical Package for the Social Sciences ver 14.0 (SPSS).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CGH	comparative genomic hybridisation
TSG	tumour suppressor gene
MSA	microsatellite analysis
PAC	P1-derived artificial chromosome
BAC	bacterial artificial chromosome
DGGE	denaturing gradient gel electrophoresis
CGI	CpG island
MSP	methylation-specific PCR

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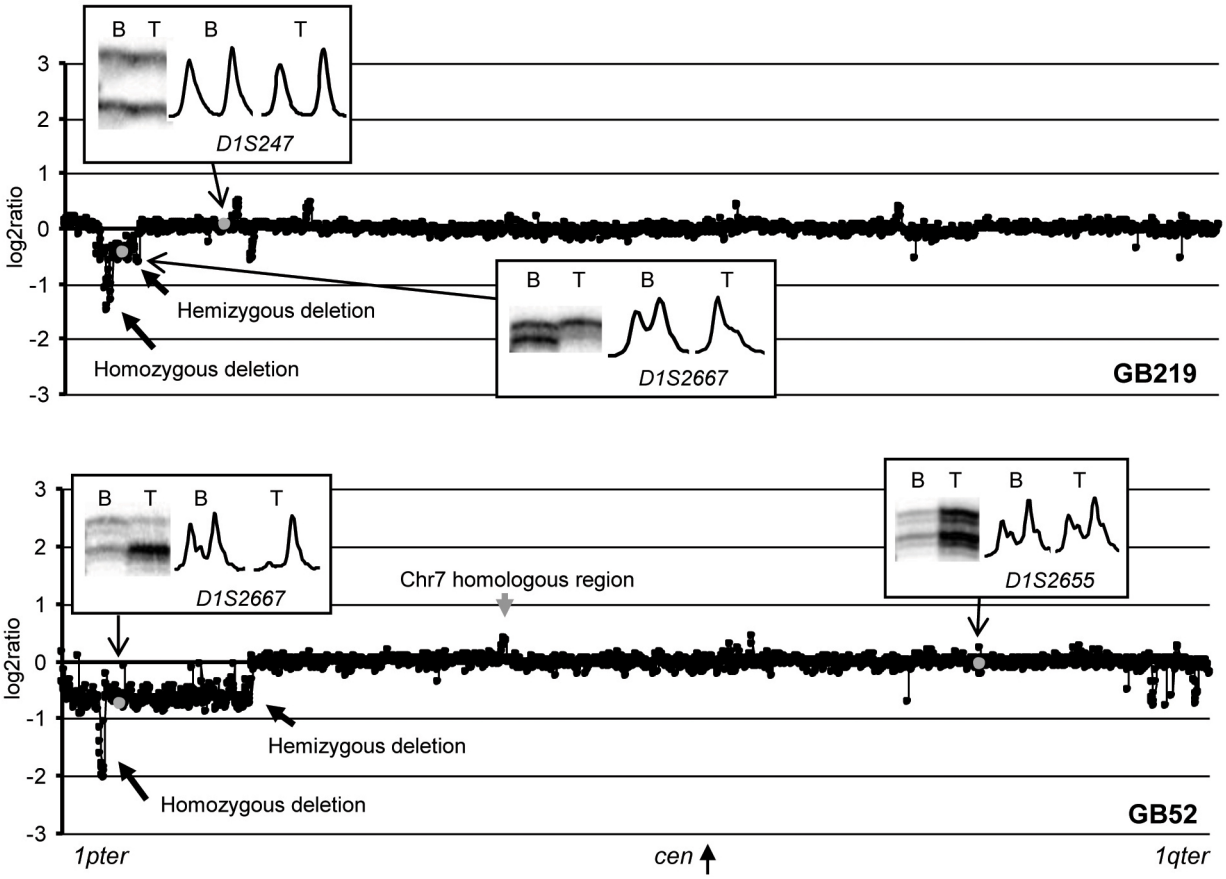

Fig. 1.

Log2 ratio plots of chromosome 1 in two glioblastomas, GB219 and GB52. Insets show microsatellite data displaying allelic balance (*D1S247* in GB219 and *D1S2655* in GB52) and imbalance (*D1S2667* in GB219 and GB52). Positions of clones that harbour the corresponding markers are indicated by grey spots. Hemizygous and homozygous deletions are indicated by arrows (see also Fig. 2). A grey arrow in GB52 indicates the region that appeared as copy number gain due to homology to a part of chromosome 7 which showed trisomy in this tumour (see Fig. 2 legend).

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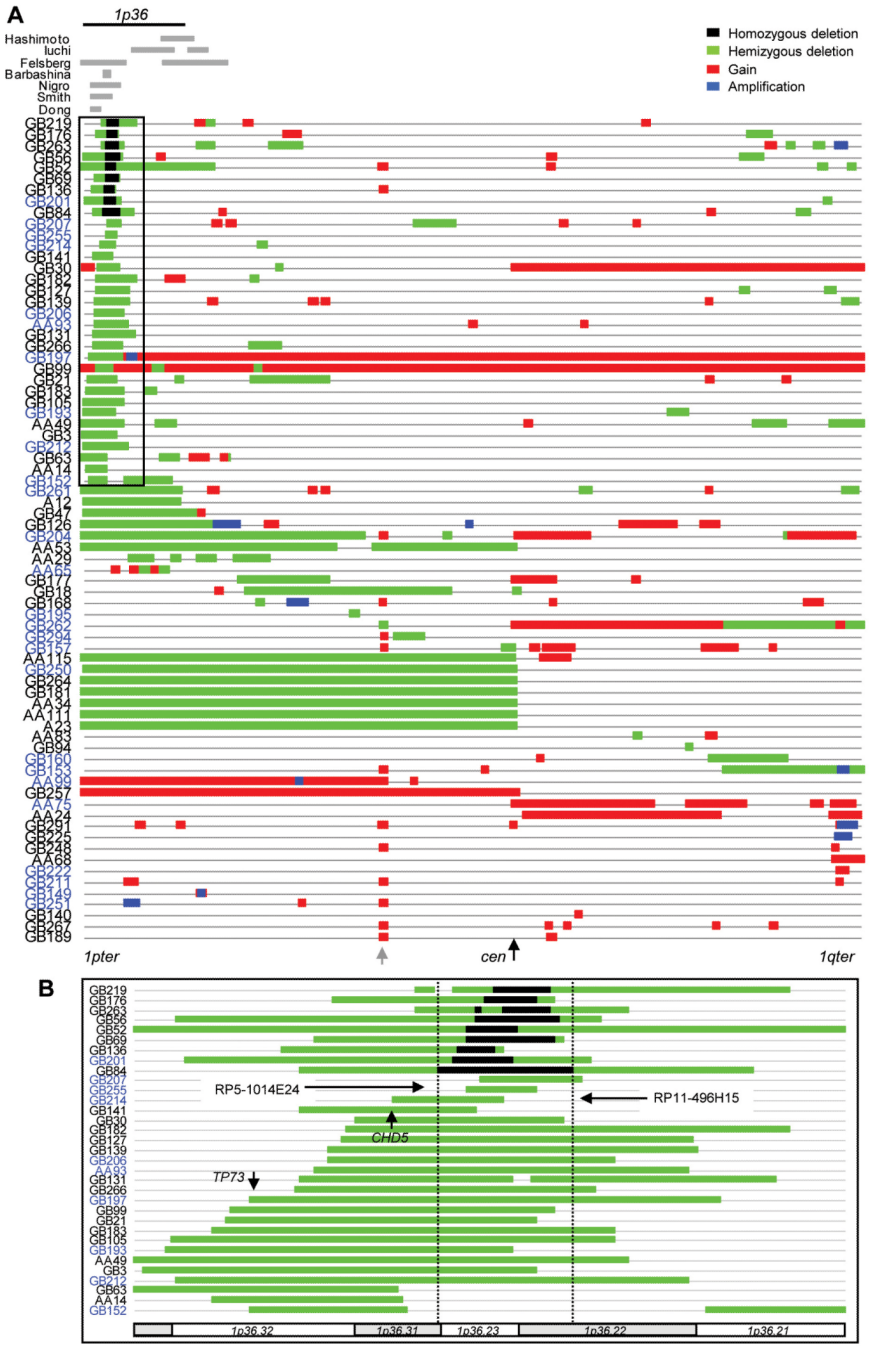

Fig. 2.

A. A diagram mapping all copy number abnormalities on chromosome 1. Copy number status is schematically represented along chromosome 1 (*1pter* on the left to *1qter* on the right) by colour-coded bars as indicated. Chromosomal band 1p36 is indicated at the top. Previously reported minimum regions of deletion by LOH analysis in various gliomas are schematically shown by grey bars (references, Hashimoto (Hashimoto *et al.*, 2003), Iuchi (Iuchi *et al.*, 2002), Felsberg (Felsberg *et al.*, 2004), Barbashina (Barbashina *et al.*, 2005), Nigro (Nigro *et al.*, 2001), Smith (Smith *et al.*, 1999), Dong (Dong *et al.*, 2004)). The 1p36 region shown in more detail in Fig. 2B is boxed. A region homologous to chromosome 7 is indicated by a grey arrow all tumours with chromosome 7 gain (as judged by the 5Mb clone

results) showed copy number gain in this region, most likely because of sequence homology. Codes for the 1994 tumours are in blue. *B*. A detailed view of copy number abnormalities at 1p36.21-33 for cases that had deletions within the region. Only deletions are shown. The boundaries of the largest homozygous deletion (GB84), telomeric break point at RP3-438L4 and centromeric at RP11-807G9, are indicated by dotted lines. For more precise borders of homozygous deletions see Fig. 3. Chromosomal bands are indicated below.

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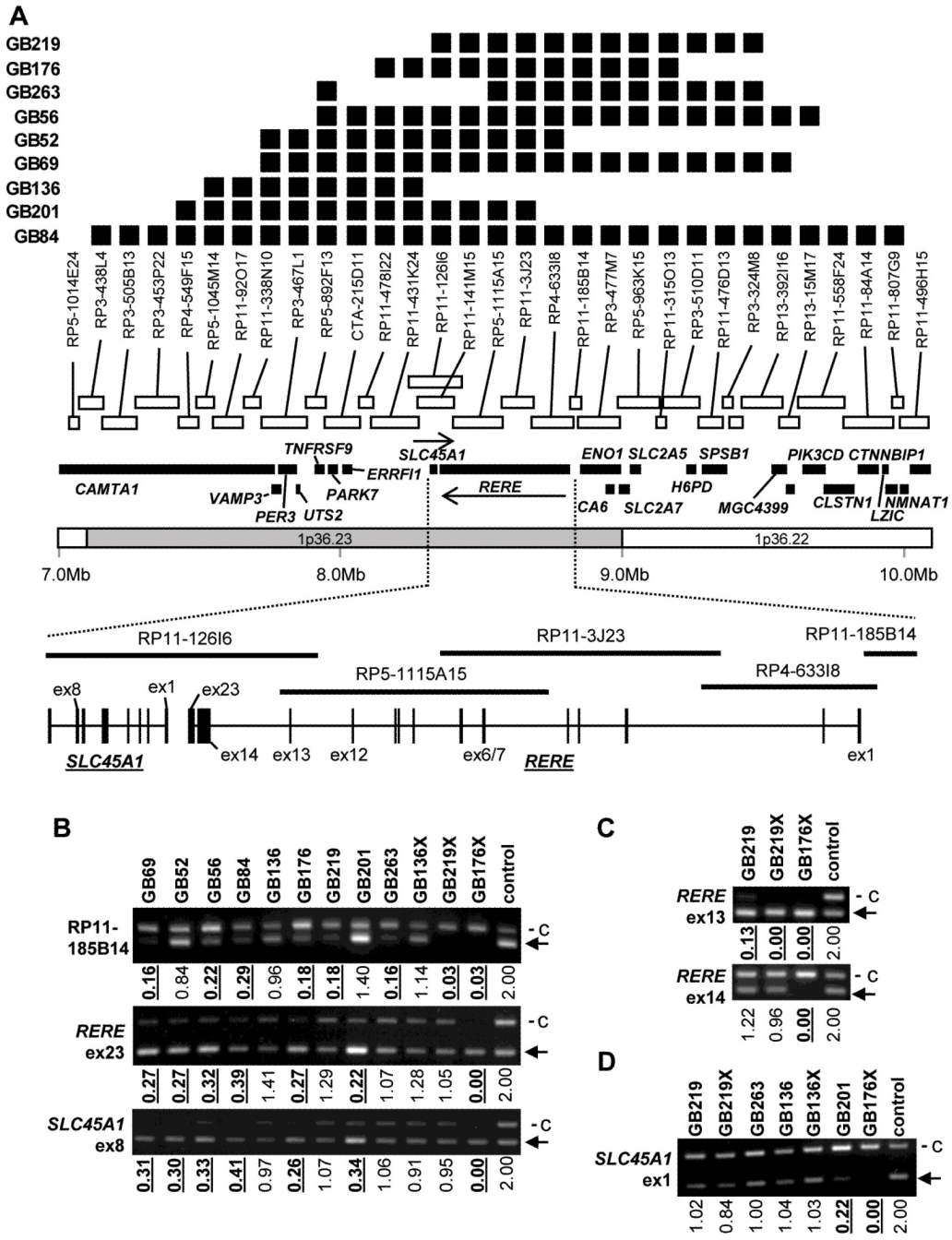

Fig. 3.

A. Top; A schematic diagram of homozygous deletions (indicated by closed boxes). Middle; A schematic diagram of physical locations of the clones on the array, the genes in the region, the corresponding chromosomal bands and nucleotide position ruler (modified after Ensembl 33.35e, September 2005). Orientation of transcription is indicated by an arrow above corresponding genes. Bottom; A schematic diagram of exon positions of *RERE* and *SLC45A1/DNB5* as well as the BAC clones. *B-D*. Multiplex PCR at an STS in RP11-185B14, *RERE* exon 23 and *SLC45A1/DNB5* exon 8 (*B*), *RERE* exon 13 and 14 (*C*) and *SLC45A1/DNB5* exon 1 (*D*). Xenografts are indicated by suffix X after the code corresponding to their parental primary tumours. The densitometrically calculated copy

numbers are shown below the gel photographs (normal = 2, homozygous deletions (< 0.6) are indicated by bold text and underlining). c, control locus (*WI-3306*, an STS at 2q21). Arrows indicate the bands representing the target locus.

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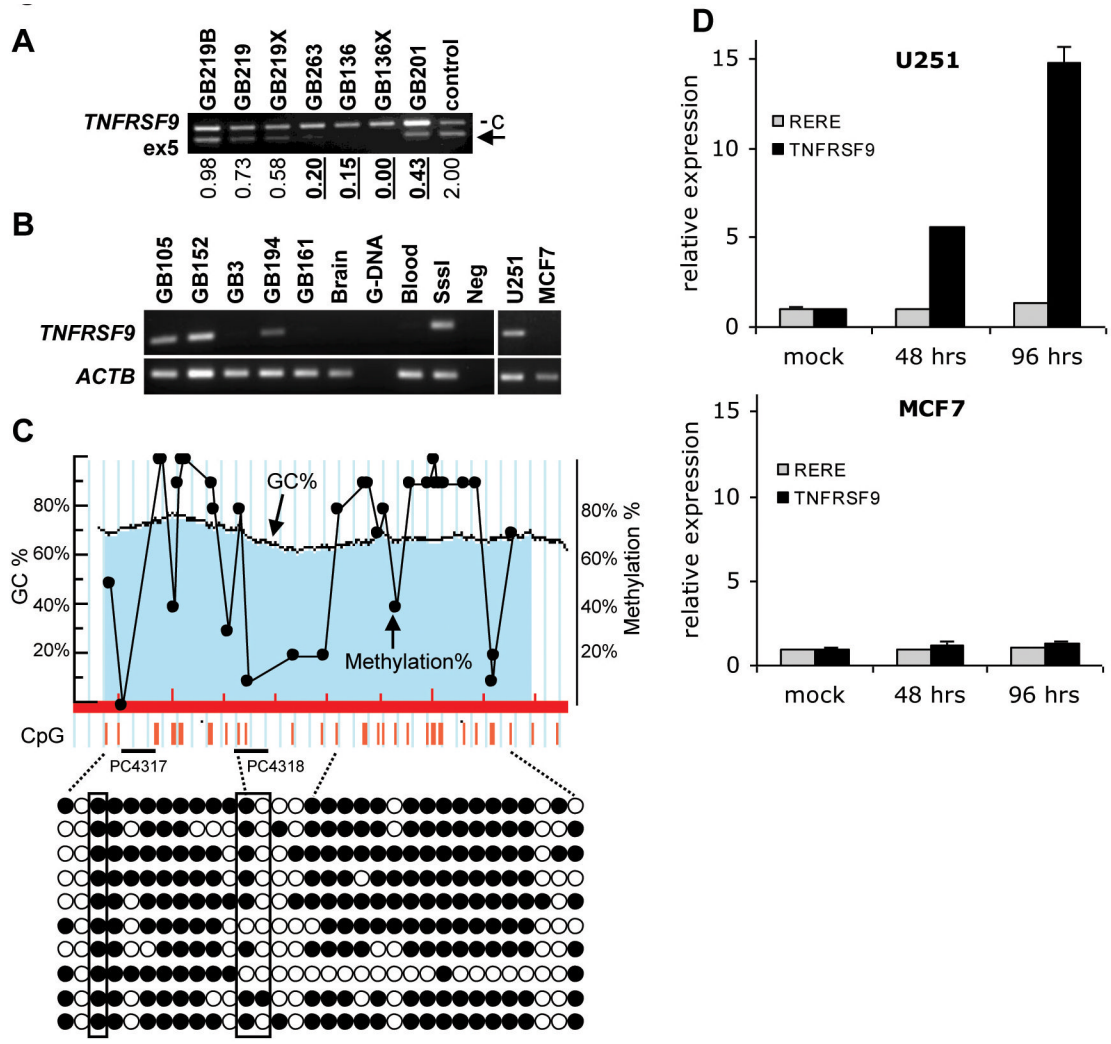

Fig. 4.

Analysis of *TNFRSF9* alterations. *A.* Multiplex PCR at *TNFRSF9* exon 5. The densitometrically calculated copy numbers are shown below the gel photographs (normal = 2, homozygous deletions (< 0.6) are indicated by bold text and underlining). c= control locus (*WI-3306*, an STS at 2q21). An arrow indicates the bands representing the target locus. *B.* MSP at the CpG island of *TNFRSF9*. The primer pair used for *TNFRSF9* specifically amplifies bisulfite-modified methylated sequences. PCR products for *ACTB* using the primer pair that amplifies bisulfite-modified sequences regardless of methylation status are shown to indicate successful bisulfite modification. G-DNA, unmodified genomic DNA; Blood, modified DNA from WBC; SssI, *SssI* methylase treated DNA as positive control; Neg, no template. *C.* Bisulfite sequencing of *TNFRSF9* in GB105. Upper panel is a diagram of the CpG island of *TNFRSF9* (abscissa, nucleotide position; left ordinate, GC%; shaded area, CpG island). The positions of CpG dinucleotides in the island are indicated by vertical red bars. Frequency of methylation at each CpG dinucleotides in the 10 sequenced clones, each of which is presented in diagram form in the lower panel (closed circle, methylated; open circle, unmethylated), is superimposed in the upper panel (left ordinate, methylation%). The position of MSP primer pair (PC4317/PC4318) is indicated on the bottom of upper panel and the CpG dinucleotides recognized by the primers are indicated in boxes in the lower panel. *D.* mRNA expression levels of *TNFRSF9* and *RERE* in glioma

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cell line U251 and breast carcinoma cell line MCF7 after treatment with 5′-Aza-2′-deoxycytidine for either 48 hours or 96 hours. Expression levels relative to that of the mock experiment are shown.

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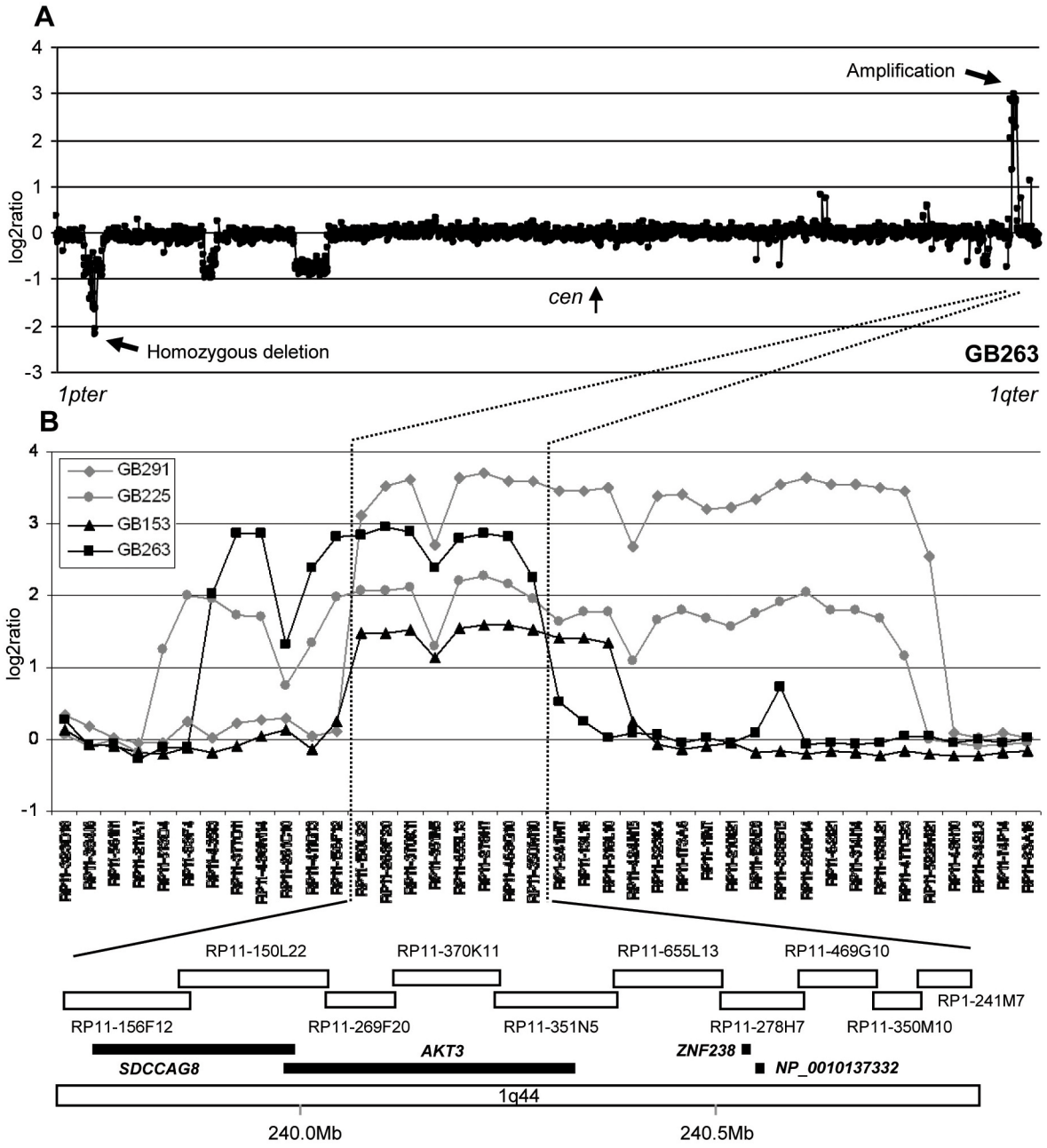

Fig. 5.

A. A log2 ratio plot of chromosome 1 in GB263. Homozygous deletion at 1p36.22-p36.23 (see also Fig. 3) and amplification at 1q44 are indicated by arrows. *B.* Superimposed log2 ratio plots of GB291, GB225, GB153 and GB263 around the amplicons at 1q44. A schematic diagram of physical locations of clones and genes in the minimal region of overlap, corresponding chromosomal bands and nucleotide position ruler are shown below the plot (modified after Ensembl 33.35e, September 2005).

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Table 1

Summary of chromosome 1 status in 108 astrocytic tumours

[1]Tumor	Total no.	1p status										1q status						Amplification		TNFRSF9 methylation		Any chr1 abnormality	
		[2]Total 1p deletion		[3]Partial 1p36 deletion		Homozygous deletion		Any 1p deletion		[4]1p gain		[4]Any 1p abnormality		Any 1q deletion		1q gain		Any 1q abnormality							
All cases																									
A	9	1	11%	1	11%	0	0%	2	22%	0	0%	2	22%	0	0%	0	0%	0	0%	0	0%	1	11%	2	22%
AA	22	3	14%	6	27%	0	0%	9	41%	3	14%	10	45%	1	5%	7	32%	7	32%	1	5%	2	9%	14	64%
GB	77	3	4%	34	44%	9	12%	44	57%	21	27%	50	65%	14	18%	26	34%	33	43%	9	12%	18	23%	58	75%
total	108	7	6%	41	38%	9	8%	55	51%	24	22%	62	57%	15	14%	33	31%	40	37%	10	9%	21	19%	74	69%
1994 cases																									
A	5	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
AA	9	0	0%	2	22%	0	0%	2	22%	3	33%	3	33%	0	0%	1	11%	2	22%	1	11%	1	11%	4	44%
GB	32	1	3%	11	34%	1	3%	16	50%	7	22%	20	63%	7	22%	9	28%	12	38%	4	13%	8	25%	22	69%
total	46	1	2%	13	28%	1	2%	18	39%	10	22%	23	50%	7	15%	10	22%	14	30%	5	11%	9	20%	26	57%

[1] A, diffuse astrocytomas malignancy grade II; AA, anaplastic astrocytomas; GB, glioblastomas; 1994 cases, tumors operated within the calendar year of 1994 (see text)

[2] the number of cases with hemizygous deletion of all 1p clones with or without other abnormalities

[3] tumors that have deletion at 1p36 but not for the whole 1p

[4] Gains limited to the homologous region to chromosome 7 were not included

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Table 2

Median post-operative survival in astrocytic tumours

	All tumours		Glioblastomas		Anaplastic astrocytomas	
1p status	***(n=)***	**Survival in days**	***(n=)***	**Survival in days**	***(n=)***	**Survival in days**
Normal 1p	(41)	292	(23)	156	(11)	590
Total 1p deletion	(7)	4091	(3)	3321	(3)	>5119
1p36 deletion	(40)	301	(33)	299	(6)	406
Other 1p alteration	(18)	240	(17)	279	(1)	60
[1]Univariate analysis		*p=0.001*		*p=0.002*		*p=0.021*
[2]Multivariate analysis		*p=0.005*		*p=0.017*		*p=0.06*

[1]Univariate analysis using log rank test.

[2]Multivariate analysis including age and WHO grade using Cox' Regression