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Characterization and Gene Expression Profiling in Glioma Cell Lines with Deletion of Chromosome 19 Before and After Microcell-Mediated Restoration of Normal Human Chromosome 19

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Abstract

Nearly 10% of human gliomas are oligodendrogliomas. Deletion of chromosome arm 19q, often in conjunction with deletion of 1p, has been observed in 65–80% of these tumors. This has suggested the presence of a tumor suppressor gene located on the 19q arm. Chromosome 19 deletion is also of interest due to the better prognosis of patients with deletion, including longer survival and better response to chemotherapy, compared with patients without deletion. Two glioma cell lines with deletion of 19q were used for chromosome 19 microcell-mediated transfer, to assess the effect of replacing the deleted segment. Complementation with chromosome 19 significantly reduced the growth rate of the hybrid cells compared with the parental cell lines. Affymetrix U133 Plus 2.0 Gene Chip analysis was performed to measure and compare the expression of the chromosome 19 genes in the chromosome 19 hybrid cell lines to the parental cell line. Probes were considered significantly different when a *P* value <0.01 was seen in all of the cell line comparisons. Of 345 probes within the commonly deleted 19q region, seven genes (*APOE*, *RCN3*, *FLJ10781*, *SAE1*, *STRN4*, *CCDC8*, and *BCL2L12*) were identified as potential candidate genes. RT-PCR analysis of primary tumor specimens showed that several genes had significant differences when stratified by tumor morphology or deletion status. This suggests that one or more of these candidates may play a role in glioma formation or progression.

INTRODUCTION

Human diffuse gliomas are generally classified into three groups: astrocytoma, oligodendroglioma, and mixed oligoastrocytoma. The distinction between the different morphological categories is based on histological differences in the tumors (Caskey et al., 2000; Louis et al., 2001). Generally, oligodendrogliomas grow more slowly, and patients survive longer than patients with astrocytomas of the same grade (Smith and Jenkins, 2000; Kitange et al., 2003). Many observed genetic alterations may underlie these growth and survival differences. One of the most recognized genetic differences is the combined deletion of chromosome arms 19q, and 1p, which is found in about 50% of

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Additional Supporting Information may be found in the online version of this article.

oligodendrogliomas (Smith et al., 1999; Smith and Jenkins, 2000; Kitange et al., 2003; Reifenberger and Louis, 2003; Barbashina et al., 2005).

The alteration of chromosome arm 19q is of particular interest for several reasons. Chromosome arm 19q deletion is seldom seen in other types of malignancies, but is a common alteration among all glioma subtypes (Smith and Jenkins, 2000). This deletion, alone and combined with the deletion of chromosome 1p, has been linked to better survival and response to chemotherapy and radiation (Smith et al., 2000; Louis et al., 2001; Perry et al., 2003; Reifenberger and Louis, 2003; Fallon et al., 2004; van den Bent, 2004; Kitange et al., 2005). Based on these observations, it has been hypothesized that chromosome arm 19q may contain a gene or genes that may be involved in the formation of gliomas. This region may also harbor a gene or genes involved in the improved response to chemotherapy and radiation. Because of these observations, many labs have examined this deletion region for potential gene targets.

Multiple methods have been used to test tumors for deletion, including fluorescence in situ hybridization (FISH), heterozygosity mapping (LOH), comparative genomic hybridization (CGH), and array CGH (CGHa) (Smith et al., 2000; Burger et al., 2001; Perry et al., 2003; Cowell et al., 2004; Kitange et al., 2005). Common regions of deletion have been mapped using various methods and can be correlated with the histological subtype of the tumor (Smith et al., 1999). It has been found that astrocytomas tend to lose a small portion of chromosome arm 19q, while oligodendrogliomas tend to lose the entire 19q arm (Smith et al., 1999; Kitange et al., 2005). We have previously screened a panel of glioma cell lines for the deletion of 1p and 19q (Law et al., 2005). In this screen two cell lines with the 19q deletion of interest, A172 and U87, were identified. Both of these cell lines have 19q deletions that encompass the minimal deletion region previously mapped and reported by our laboratory (Smith et al., 1999). Both of these cell lines were used as recipients of an intact chromosome 19 delivered by microcell-mediated transfer. Microcell-mediated transfer has been used by other groups to correct expression of genes responsible for tumorigenesis in cell lines (Astbury et al., 2001; Gagnon et al., 2006; Quaye et al., 2009). One group demonstrated that chromosome band 18q21 corrected the tumorigenic and metastatic phenotype of two PC-3 prostate cell lines (Gagnon et al., 2006). Another study by Quaye et al., lead to the identification of two candidate genes in ovarian cancer (Quaye et al., 2009). This is a report of the characterization of the resulting chromosome 19 hybrid clones, and the analysis of the gene expression profile to identify potential candidate 19q tumor suppressor genes. The candidate genes selected were also tested by Real-Time PCR (RTPCR) to measure RNA expression levels in primary gliomas.

MATERIALS AND METHODS

Cell Culture and Microcell-Mediated Chromosome Transfer

A172 and U87 glioma cell lines (provided by Dr. David James, UCSF Department of Neurosurgery, San Francisco, CA) were selected as recipient cell lines for microcell fusion based on their deletion of chromosome 19 (Law et al., 2005). A9neo19 cells (provided by Dr. Joy L. Ware, Virginia Commonwealth University, VA), a mouse sarcoma cell line with a human chromosome 19 containing a neomycin-resistance gene, were used as the donor cell line. All cells were cultured in DMEM (Gibco Invitrogen Cell Culture, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (U.S. Bio-technologies, Inc, Pottstown, PA) and 1% antibiotic-antimycotic (100×) (Gibco). The microcell-mediated fusion method has been previously described by several groups (Padalecki et al., 2001; Kost-Alimova et al., 2004). Donor A9neo19 cells were grown in DMEM supplemented with 10% FBS and 200 μg/ml G418 (Gibco). Donor cells were treated with Colcemid (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 48 hr. The resulting microcells were harvested by

centrifugation at 27,000 × *g* in the presence of 10 μg/ml cytochalasin B (Sigma-Aldrich). After centrifugation, the microcells were purified through a series of poly-carbonate filters (0.8, 0.5, and 0.3 μm), with the resulting fraction being added to the recipient A172 and U87 cells at 70–80% confluence. The microcells were added in the presence of 2 ml of 200 μg/ml phytohemaglutinin-P (PHA-P) for 10 min at 37°C (BD Biosciences, Franklin Lakes, NJ). Cells are then washed twice in serum free DMEM and treated with 1 ml of 45% polyethylene glycol (PEG) for 1 min with gentle rocking. Cells were again washed with serum free DMEM and incubated for 24 hr in DMEM with 10% FBS and 1% antibiotic-antimycotic. Cells were then split into 10 culture dishes and placed under selection with DMEM supplemented with 10% FBS and 500 μg/ml G418. After 2 weeks, the resulting colonies were isolated and propagated, after selection the cells were grown in DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic, and 100 μg/ml G418.

Control cell lines were made by transfecting the parental cell lines with the pSVneo2 vector (BD Bioscience Clonetec, Mountain View, CA). The cell lines were selected with the same selection technique stated above, and grown under the same conditions.

Proliferation Assay

Trypan Blue exclusion was performed on all of the cell lines. Briefly, 1,000 cells were plated in six-well plates in triplicate and grown under neomycin selection, cells were counted on a hemocytometer daily for 6 days after treatment with Trypan Blue (Mediatech, Inc, Herndon, VA) as described previously (Altman et al., 1993).

Radial Migration Assay

Radial Migration Assays were performed as outlined previously (Valster et al., 2005). Briefely, 10-well Teflon®-coated slides were coated with laminin, and blocked with bovine serum albumin (Sigma-Aldrich). Medium was then applied to the wells, and cells were plated using a Cell Sedimentation Manifold (CSM, Creative Scientific Methods, Phoenix, AZ). Initial cell numbers varied by cell line: 2,000 cells for A172 lines, and 2,500 for U87 lines. Cells were allowed to adhere to the slides overnight at 37°C. The CSM was removed, and fresh medium was added to each well. The cells were then imaged at 0, 24, and 48 hr. The images were then analyzed, and diameter was used to judge the distance moved over time.

FISH and Multicolor FISH (MFISH)

FISH analysis of parental and neo19 hybrid clones was performed as described (Smith et al., 1999; Law et al., 2005). Locus-specific probes were prepared using bacterial artificial chromosome (BAC) DNA mapped to cytoband 19q13.33, labeled with dUTP Alexa 594 (Molecular Probes Invitrogen Detection Technologies, Carlsbad, CA), and BAC DNA mapped to cytoband 19p13.2, labeled with dUTP SpectrumGreen (Vysis, Downers Grove, IL). Probe solutions were pipetted into 200-μl PCR reaction tubes, vortexed, and denatured in a thermocycler at 70°C for 5 min. The denatured probe solution was placed on previously denatured slides, covered with glass coverslips, sealed with rubber cement, and hybridized in a humidified chamber at 37°C for at least 12 hr. After removal of the coverslips, slides were washed in 0.43× SSC at 70°C for 2 min, briefly rinsed in 2× SSC/0.1% NP-40 at room temperature, jet air-dried, counterstained with DAPI (100 ng 4′,6′-diamidine-2-phenylin-dole dihydrochloride per ml of Vectashield anti-fade) and covered with glass coverslips. Metaphase spreads (20–50) were analyzed and the most common copy number for each probe was recorded for each cell line. Cells hybridized with FISH probes were viewed with a fluorescent microscope equipped with a 100-W mercury lamp and filters for Texas Red, FITC, and DAPI (Vysis).

Microsatellite Analysis

PCR-based analysis was used to genotype the chromosome 19 hybrid cells. Fluorescently labeled PCR primer pairs from the ABI PRISM Linkage Mapping Set v2.5 (Applied Biosystems, Fosters City, CA) were selected to analyze micro-satellites on chromosome 19. Twenty microsatellites were selected for the 19q arm: *D19S209*, *D19S216*, *D19S220*, *D19S221*, *D19S226*, *D19S414*, *D19S418*, *D19S420*, *D19S571*, *D19S572*, *D19S865*, *D19S884*, *D19S886*, *D19S888*, *D19S894*, *D19S902*, *D19S903*, *D19S904*, *D19S921*, and *D19S931*. Two microsatellites were also selected from the 19p arm, *D19S210* and *D19S566*. DNA was extracted from the cell lines with the Puregene® DNA Purification Kit (Gentra Systems, Minneapolis, MN). PCR amplification of the samples was performed according to the protocol for the primer pairs, and the product was analyzed by Mayo Clinic College of Medicine Genotyping Shared Resource using the ABI 3100 (Applied Biosystems).

Single Nucleotide Polymorphism Assay

The GeneChip Human Mapping 10K Array *Xba* 131 (Affymetrix, Santa Clara, CA) was used to detect single nucleotide polymorphisms (SNPs). Genomic DNA from cell lines (250 ng) was digested with 10 U of *Xba* I (New England Biolabs, Ipswich, MA) for 2 hr at 37°C. Adaptor ligation, PCR, hybridization on the 10 K SNP chip, and scanning were performed according to the protocol supplied by the vendor (Affymetrix).

Affymetrix Oligonucleotide Microarray

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). Samples with clear 28S and 18S RNA bands were accepted for further analysis. Total RNA was reverse transcribed, cDNA was isolated using phenol-chloroform extraction and resuspended in RNase-free water, labeled by in vitro transcription, and fragmented by the manufacturer's guidelines (Affymetrix). The hybridization solution is made with the product, and hybridized to a test array before being hybridized to the Affymetrix U133 Plus 2.0 Chip according the manufacturer's directions (Affymetrix). Posthybridization staining and washing were processed according to the manufacturer's instructions (Affymetrix). Arrays were scanned using GeneChip Scanner 3000, and analyzed using Microarray Suite (MAS) 5.0 (Affymetrix). All chips were pooled and normalized using the FASTLO normalization algorithm (Ballman et al., 2004). Individual hybrid comparisons were made by comparing the average expression of the replicates to the average expression of the pSVneo clones. Computer modeling was used to perform a bootstrapping method in which 345 probes were selected at random, and the number of probes from that set would meet out criteria were counted. This process was duplicated 10,000 times, and a distribution of the number of probes found to meet our criteria, with up or down-regulation, were collected.

Quantitative Real-Time Polymerase Chain Reaction (qRTPCR)

RNA was extracted from fresh frozen tumor tissue. Twenty 20-micron sections were cut from each tumor, and placed on charged slides, along with a parallel 5 micron H&E section. The H&E slides were examined by a pathologist, and areas of tumor were circled. Tumors were tested for deletion of chromosome arm 19q by FISH using standard methods for frozen sections. Frozen sections were then macrodissected and placed immediately into Trizol (Invitrogen), and frozen at −80°C. Samples were extracted as recommended by the manufacturer's instructions, and cleaned using RNeasy mini-kit RNA clean up protocol (Qiagen). Cell lines were extracted by the same method as tumors. RNA was analyzed by RT-PCR using LightCycler® RNA Amplification Kit SYBR Green I (Roche Applied Science, Indianapolis, IN). Primers, amplicon size, and $MgCl_2$ concentration are listed in Supporting Information Table 2. PCR was completed using the manufacturer's

specifications (Roche), in a volume of 20 μL containing 200 ng RNA, 2 μL forward and reverse primers at 10 μm. Reactions were run on the LightCycler® 2.0 System, an initial reverse transcription step for 10 min at 55°C, the product is then denatured for 2 min at 95°C. PCR follows, for 50 cycles, denature to 95°C, an annealing at 55°C (except for GLTSCR2 at 57°C) for 8 seconds, and an extension step at 72°C for 15 seconds with a single quantification in each cycle at during the extension step. Melting curves were then constructed by denaturing to 95°, annealing to 65°C for 5 seconds, temperature was then increased at 0.1°C/sec until a temperature of 95°C was reached, with continuous quantification of fluorescence in each sample.

RESULTS

Microcell-Mediated Chromosome Transfer

In an attempt to determine candidate genes associated with glioma on chromosome 19, micro-cell-mediated chromosome transfer was used to complement the deletion of chromosome arm 19q found in the glioma cell lines U87 and A172 (Law et al., 2005). Briefly, the A172 deletion breakpoints fall near PVRL2 and AP2A1, while those in U87 are contained within the previous region flanks by ZNF541 and RRAS (Law et al., 2005). The donor mouse cell line, A9neo19, contained one human chromosome 19 that was maintained in culture under selection with G418. A control cell line was produced by transfection of the pSVneo2 vector into both A172 and U87. Seven and 11 chromosome 19 hybrid cell lines were isolated for A172 and U87, respectively. The chromosome 19 content of the isolates were tested by FISH at the time of isolation, and periodically thereafter to monitor the continued retention of the genetic material (Supporting Information Fig. 1). MFISH was used to examine the clonal purity of the cells (data not shown), and based on these results, several cell lines were selected for further examination. The chromosome 19 status for three and four clones selected for A172 and U87, respectively, are shown in Table 1. Microsatellite analysis was completed to confirm that the donor chromosome was transferred in its entirety and retained by all of the cell lines (Supporting Information Fig. 2). The genotypes showed the successful transfer of the donor 19 alleles into the recipient cell lines. To improve resolution, the Affymetrix GeneChip Human Mapping 10K Array Xba 131 chip was used to further karyotype the chromosome 19 hybrid clones. There were a total of 150 SNPs on the chip that mapped to chromosome 19, 37 of these mapped to chromosome arm 19p. For the A172neo19 hybrids, eighteen 19p and thirty-nine 19q probes were informative. For the U87neo19 hybrids, seventeen 19p probes and thirty-six 19q probes were informative. All of the informative SNPs on chromosome 19 for each cell line were analyzed (Supporting Information Fig. 2).

The A172 hybrid cell lines maintained all five copies of chromosome 19, including two parental normal copies, the two parental deleted copies, as well as one additional transferred chromosome 19 (Table 1). FISH and microsatellite analysis showed that the transferred 19 had deleted a small portion of 19p in two of the A172 hybrid clones (A172neo19.2 and A172neo19.4, Supporting Information Fig. 2a). The U87neo19 hybrids lost one copy of chromosome 19 while the cells were maintained in culture (Table 1 and Supporting Information Fig. 1c). Microsatellite analysis and the Affymetix SNP analysis showed that the two retained chromosomes 19 were the parental deleted chromosome 19, and the transferred neo19 chromosome (Supporting Information Fig. 2b and data not shown).

Morphology, In vitro Proliferation, and Migration

After confirmation of the successful transfer of chromosome 19 into A172 and U87 glioma cell lines, the phenotype of the cells was examined. The pSVneo control cell lines and the hybrid clones had similar mophology to the parental U87 and A172 (data not shown). The

cell lines, before and after transfection, have an elongated bipolar appearance. The cells have multiple long, thin processes (data not shown). The U87 hybrid cells maintain the tendency of the parental cell line to form spheroids after becoming confluent (Golembieski et al., 1999; Bell et al., 2005), forming large spheres of cells that detach from the flask (data not shown).

The hybrid cells have a significantly slower growth rate compared with both the parental and the pSVneo control clones (Fig. 1). For both sets of cell lines, U87 and A172, the parental and the pSVneo control clones have very similar growth rates. The transfectant clones all grow more slowly, becoming significantly different by day 6 (Fig. 1).

Along with the altered growth rate, the cells also have a difference in migration rate. The chromosome 19 transfectant clones migrate more slowly than the pSVneo control cell lines (Supporting Information Fig. 3).

Gene Expression Profiling of Chromosome 19 Hybrid Glioma Cell Lines

To examine the changes in gene expression after the chromosome 19 transfer into the A172 and U87 cell lines, they were analyzed with the Affymetrix U133 Plus 2.0 Array. Three A172neo19 and four U87neo19 clones were evaluated, along with the parental and pSVneo clone for both A172 and U87 were tested. Each individual cell line was run in triplicate, and an average expression was used for the comparisons. Expression levels were normalized using the FastLo method (Ballman et al., 2004). Each cell line was compared directly with the parental pSVneo clone in a pair-wise differential analysis for a total of seven complete comparisons, each of which is an average of three replicates for each cell line (Fig. 2). Candidate genes were selected from 345 probes (Supporting Information Table 1) that are contained within the previously mapped A172 deletion region (Law et al., 2005). Altered probes were identified outside of that region of interest, but were not analyzed further.

Candidate genes were required to have a P value ≤ 0.01 in all seven cell line comparisons. In addition, all of the hybrid cell lines must have a higher probe expression level than the matching control cell lines. Ten probes met the criteria, representing seven genes: *APOE* (Apolipoprotein E, 203381_s_at, 203382_s_at, 212884_x_at), *BCL2L12* (BCL2 like 12, 233110_s_at), *CCDC8* (Coiled coil domain containing 8, 223496_s_at), *FLJ10781* (hypothetical protein FLJ10781, 218824_at), *RCN3* (Reticulocalbin-3, 61734_at, 219102_at), *SAE1* (SUMO-activating enzyme E1, 217946_s_at), and *STRN4* (zinedin or Striatin, calmodulin binding protein 4, 217903_at). More information about relative expression values, and the associated P values, are listed in Table 2. All expression differences were confirmed by RT-PCR (data not shown).

Validation of Microarray Data and Expression of Candidate Genes in Glioma Cell Lines

To estimate the false discovery rate (FDR) bootstrapping methodology was used. In a bootstrapping simulation using a sample size of 345 probes, up to five would be expected to be significantly different by random chance (P value <0.01, Supporting Information Fig. 4a). In our experiment, we observed 10 probes that were up-regulated, twice that expected by the bootstrapping method, compared with parental in all of the cell line comparisons.

To estimate independently the FDR, a genome-wide count of probes was performed. In all seven samples across the whole genome, 63 probes were significantly up-regulated, and 42 probes were significantly down-regulated, (Supporting Information Fig. 4b). Based on the total number of probes on the array, and the selected P value of 0.01, 78 probes would be expected to achieve significance by random chance (versus the 105 found). Of the total up-regulated genes, 13 were found on 19q, and 8 were found on 19p. This clustering on

chromosome 19 supports the hypothesis that some of the expression changes were not due to chance alone.

RNA Expression of Candidate Genes in Glioma by RT-PCR

RNA expression analysis in primary gliomas was used to assess the relevance of candidate genes detected by the chromosome 19 complementation experiments. An initial set of 52 tumors was used to screen eight genes of interest (*APOE, BCL2L12, CCDC8, FLJ10781, GLTSCR2, RCN3, SAE1*, and *STRN4*) along with three control genes (*GAPDH, KDELR1*, and *AP2S1*). Primers used for RT-PCR are listed in Supporting Information Table 2. The test group of tumors contained 24 astrocytomas, 9 mixed oligoastrocytomas, and 18 oligodendrogliomas. All of the tumors were examined by a pathologist (C.G.) to confirm initial diagnosis, and tested for deletions on chromosome arms 1p and 19q by FISH. Of the 52 tumors, 36 were found to be not deleted for 19q, and 16 deleted for 19q (Table 3).

All of the tumors from the test set were stratified by morphology to look for differences in expression (Supporting Information Fig. 5a). Significant differences were seen in the astrocytoma and mixed oligoastrocytoma groups for *APOE* ($P \leq 0.00011$), *BCL2L12* ($P \leq 0.019$), and *FLJ10781* ($P \leq 0.041$, Supporting Information Fig. 5a). A comparison of the astrocytomas and oligodendrogliomas showed a significant difference for *APOE* ($P \leq 0.019$) and *BCL2L12* ($P \leq 0.01$, Supporting Information Fig. 5a). Only one gene was significantly different when the mixed oligoastrocytomas were compared with the oligodendroglioma, *APOE* ($P \leq 0.042$, Supporting Information Fig. 5a). When the astrocytoma group was compared to the pooled mixed oligoastrocytomas and oligodendrogliomas groups, both *APOE* ($P \leq 0.00042$) and *BCL2L12* ($P \leq 0.0031$, Supporting Information Fig. 5a) are significant.

APOE was found to have significantly different expression between the deleted and not deleted groups ($P \leq 0.0091$, Fig. 3a). *BCL2L12* also showed a significant difference between the deleted and not deleted tumors ($P \leq 0.00066$, Fig. 3a). Because *SAE1* comparisons gave *P* values close to the cutoff at 0.05, it was selected along with *BCL2L12* for further validation. *APOE* was not included for further validation, as it showed higher expression in tumors with deletion (Fig. 3a). A complete listing of the *P* values from the different comparisons is found in Supporting Information Table 3.

A validation set ($n = 26$) of tumors was selected for the further testing of selected genes. Most comparisons within the validation set did not reach statistical significance, but this is likely due to the size and composition of the set of tumors tested. *BCL2L12* showed a significant reduction in expression in the oligodendrogliomas compared to the astrocytomas ($P \leq 0.05$, Supporting Information Fig. 5b). *RCN3* also exhibited a significant reduction in expression between the same two groups of tumors ($P \leq 0.048$, Supporting Information Fig. 5b). The validation was then combined with the test set (n = 77). The difference in *BCL2L12* expression between the deleted and not deleted groups was highly significant ($P \leq 0.000095$, Fig. 3c). *SAE1* also showed a significant difference between the deleted and not deleted groups ($P \leq 0.05$, Fig. 3c).

DISCUSSION

This report demonstrates the use of microcell-mediated transfer to restore human chromosome 19 into deleted glioma cell lines A172 and U87. Deletion of chromosome arm 19q, alone and in combination with 1p, is a frequent alteration observed in human oligodendrogliomas (Smith et al., 1999; Smith and Jenkins, 2000; Reifenberger and Louis, 2003), suggesting that chromosome arm 19q may contain a gene, or genes, involved in the development of these tumors. In addition, evidence from retrospective studies has shown

that patients with 19q and/or 1p deletion have a better response to chemotherapy and radiation, and exhibit longer survival than those without these alterations (Smith et al., 2000; Fallon et al., 2004; Felsberg et al., 2004). Therefore chromosome 19 has been an area of investigation in patients with oligodendrogliomas. Bone fide oligodendroglioma cell lines with combined 1p and 19q deletion are not available. Thus we hypothesized that astrocytic cell lines with 19q deletion may be useful cellular reagents for identifying chromosome 19q genes associated with gliomas. Two glioma cell lines have been identified, A172 and U87, which contained chromosome 19q deletions encompassing the minimal deletion region in oligodendrogliomas and astrocytomas (Smith et al., 1999; Law et al., 2005).

Previous literature has shown that A172 and U87 may have some characteristics of oligodendroglioma, besides the presence of the 1p and 19q deletions. U87 xenografts have been shown to stain positively for galactocerebroside, which has been shown to be a marker of oligodendrogliomal lineage (Joshi and Mishra, 1992; Ecsedy et al., 1999). A172 and U87 have also been seen to have deletions in *PTEN* and *CDKN2A*, similar to anaplastic oligodendroglioma which may have homozygous deletions of *CDKN2A* and deletions of *PTEN* (Cairncross et al., 1998; Adachi et al., 1999; Reifenberger and Louis, 2003; Law et al., 2005). We do not claim that these cell lines are oligodendroglial in nature, but that they may have some similar characteristics.

Using the astrocytic cell lines may not identify oligodendroglioma-specific genes, but genes that are involved in glioma in general. Oligodendrogliomas are characterized by a high frequency (65–80%) of deletions of 1p and 19q (Smith et al., 1999; Perry et al., 2003; Fallon et al., 2004). In contrast, these alterations are only seen in 20–40% of mixed oligoastrocytomas, and 5–10% of astrocytomas (Smith et al., 1999; Perry et al., 2003; Fallon et al., 2004). Using FISH mapping and LOH, Smith et al., showed that a panel of astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas with 19q deletion contain a common deletion region that maps to 19q13.33 (Smith et al., 1999). It has been demonstrated by CGHa that oligodendrogliomas commonly lose the entire 19q arm, while astrocytomas lose only a portion of the 19q arm (Cowell et al., 2004; Kitange et al., 2005). The whole arm deletion has recently been shown to be mediated by a translocation involving chromosomes 1 and 19 (Jenkins et al., 2006).

The frequent deletion of chromosome arm 19q in glioma suggests that this chromosome arm harbors a tumor suppressor gene related to the tumor formation. We expected that complementation of human chromosome 19 in glioma cell lines A172 and U87 should affect not only the genotype, but also the phenotype of these cell lines. This was confirmed by the reduction in proliferation seen in the hybrid clone cell lines (Fig. 1). The cells were also seen to migrate less after the replacement of chromosome 19 (Supporting Information Fig. 3). It is possible that the changes may be a result of more than one gene on the transfected chromosome 19, inside and/or outside of the deleted region in 19q. The artificial elevation of the copy number of many genes might also contribute to the phenotypic changes observed. However this cannot be the case for the U87 clones. All U87 clones maintained two copies of chromosome 19, one deleted and one not deleted chromosome, the same number as the parental cell line.

Combining complementation with gene expression analysis yielded seven candidate genes within the 19q region commonly deleted in glioma. It was expected that an increase in expression whould be seen in a larger number of genes. Other genes have increase expression, but the expression difference was not significant in all the cell lines compared. The change in gene copy number from four copies to five copies in the A172 cell lines may not cause that much of a change in gene expression, where as a change from two copies to three copies in the deletion region was expected to cause a larger change in expression.

Those genes were than examined in primary gliomas to judge there potential involvement in the primary tumors.

BCL2L12 expression levels vary widely among the gliomas tested. The astrocytoma group (mostly GBMs) expressed *BCL2L12* at a significantly higher level than the group of mixed oligoastrocytomas or oligodendrogliomas. It is important to note that gain of chromosome 19 was not observed in the group of astrocytomas, an alteration that is sometimes seen in GBM (Vranova et al., 2007). Previous work has demonstrated an increased expression of *BCL2L12* in GBM compared with the surrounding normal brain by RT-PCR (Stegh et al., 2007). Stegh et al. also demonstrated that an increased expression of *BCL2L12* caused a resistance to apoptosis in glioma cell lines, and that RNAi caused an increased susceptibility to cell death. The authors hypothesized that the intrinsic apoptotic pathway was involved in this cell death (Stegh et al., 2007). We found a significant difference in the expression of *BCL2L12* when the tumors were divided based on deletion status, even after the removal of the astrocytomas (see Fig. 3 and Supporting Information Table 3).

Over-expression of *BCL2L12* has been fount to be a good prognostic factor in breast cancer (Thomadaki et al., 2007). Patients that tested positive by RT-PCR for *BCL2L12* were found to be at a decreased risk for relapse or death. Both multivariate analysis and Kaplan-Meier survival curves showed an increased disease-free survival and overall survival time in *BCL2L2*-positive patients. Similar studies have been performed in colon cancers, showing an overexpression of *BCL2L12* in colon tumors when compared with adjacent normal mucosa (Mathioudaki et al., 2004). Expression of *BCL2L12* was generally associated with larger tumors, and a statistically significant shortening of time to relapse in patients with follow-up (Mathioudaki et al., 2004).

RCN3 is also a potentially important candidate gene. There is a significant difference seen between the expression in astrocytomas and oligodendrogliomas in the combined group (Supporting Information Figs. 5b and 5c). Little is known about the function of *RCN3*, also known as reticulocalbin-3. *RCN3* is an EF-hand calcium-binding protein. It functions in the secretory pathway, and has been shown to bind to a PACE4 precursor protein (Tsuji et al., 2006). It is not currently clear how this protein could affect tumorigenesis or if there is any prognostic significance to the expression of *RCN3*.

SAE1 was also found to have some significant differences in expression in the deleted versus not deleted tumors, and thus is also a candidate gene. *SAE1* was seen to be differentially expressed in the deleted and not deleted groups (Fig. 3c). *SAE1* functions as an activating enzyme in the sumolyation of proteins. This sumolyation has been seen to affect multiple cell functions, including apoptosis and cell-cycle regulation.

APOE exhibited a large difference in expression among the morphology groups, and between the deleted and not deleted tumors in the test set. Interestingly, the deleted tumors had a higher expression than the not deleted tumors (Fig. 3a). The expression of *APOE* was lower in the astrocytomas than in the other two groups of tumors. In contrast to our RNA data, previous studies had shown a higher protein expression by immunohistochemistry in astrocytoma compared with oligodendroglioma (Rousseau et al., 2006).

Tews et al., demonstrated a difference in two of the candidate genes shown here, *STRN4* (aka. *ZIN*) and *FLJ10781*, in a microarray comparison of 1p/19q deleted tumors (Tews et al., 2006). We did not reproduce either of these findings. Tews et al. showed that STRN4 was significantly different in a comparison between 14 tumors with LOH 1p/19q, and 18 tumors without LOH. We found no significant difference with regard to deletion status (*P* value ≤0.71), or with regard to any of the morphology groupings (see Supporting Information Table 3). Tews et al. also showed a significant difference in *FLJ10781* expression during a

comparison of strictly Grade 3 oligodendrogliomas when divided by LOH 1p/19q. Our group of tumors contained seven Grade 3 oligodendrogliomas (four deleted, three not deleted). Doing the same comparison done by Tews et al., we did not find a difference ($P \leq 0.62$). A comparison of our MOA and O, Grades 2 and 3 we also did not find a difference when the tumors were compared by deletion status of chromosome 19 (P value ≤ 0.65) unlike the study completed by Tews et al.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Adachi J, Ohbayashi K, Suzuki T, Sasaki T. Cell cycle arrest and astrocytic differentiation resulting from PTEN expression in glioma cells. J Neurosurg. 1999; 91:822–830. [PubMed: 10541240]

Altman SA, Randers L, Rao G. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. Biotechnol Prog. 1993; 9:671–674. [PubMed: 7764357]

Astbury C, Jackson-Cook CK, Culp SH, Paisley TE, Ware JL. Suppression of tumorigenicity in the human prostate cancer cell line M12 via microcell-mediated restoration of chromosome 19. Genes Chromosomes Cancer. 2001; 31:143–155. [PubMed: 11319802]

Ballman KV, Grill DE, Oberg AL, Therneau TM. Faster cyclic loess: Normalizing RNA arrays via linear models. Bioinformatics. 2004; 20:2778–2786. [PubMed: 15166021]

Barbashina V, Salazar P, Holland EC, Rosenblum MK, Ladanyi M. Allelic losses at 1p36 and 19q13 in gliomas: Correlation with histologic classification, definition of a 150-kb minimal deleted region on 1p36, and evaluation of CAMTA1 as a candidate tumor suppressor gene. Clin Cancer Res. 2005; 11:1119–1128. [PubMed: 15709179]

Bell HS, Whittle IR, Bader SA, Wharton SB. Discovery of a perinecrotic 60 kDa MDM2 isoform within glioma spheroids and glioblastoma biopsy material. Neuropathol Appl Neurobiol. 2005; 31:191–202. [PubMed: 15771712]

Burger PC, Minn AY, Smith JS, Borell TJ, Jedlicka AE, Huntley BK, Goldthwaite PT, Jenkins RB, Feuerstein BG. Losses of chromosomal arms 1p and 19q in the diagnosis of oligodendroglioma. A study of paraffin-embedded sections. Mod Pathol. 2001; 14:842–853. [PubMed: 11557779]

Cairncross JG, Ueki K, Zlatescu MC, Lisle DK, Finkelstein DM, Hammond RR, Silver JS, Stark PC, Macdonald DR, Ino Y, Ramsay DA, Louis DN. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. J Natl Cancer Inst. 1998; 90:1473–1479. [PubMed: 9776413]

Caskey LS, Fuller GN, Bruner JM, Yung WK, Sawaya RE, Holland EC, Zhang W. Toward a molecular classification of the gliomas: Histopathology, molecular genetics, and gene expression profiling. Histol Histopathol. 2000; 15:971–981. [PubMed: 10963139]

Cowell JK, Barnett GH, Nowak NJ. Characterization of the 1p/19q chromosomal loss in oligodendrogliomas using comparative genomic hybridization arrays (CGHa). J Neuropathol Exp Neurol. 2004; 63:151–158. [PubMed: 14989601]

Ecsedy JA, Holthaus KA, Yohe HC, Seyfried TN. Expression of mouse sialic acid on gangliosides of a human glioma grown as a xenograft in SCID mice. J Neurochem. 1999; 73:254–259. [PubMed: 10386978]

Fallon KB, Palmer CA, Roth KA, Nabors LB, Wang W, Carpenter M, Banerjee R, Forsyth P, Rich K, Perry A. Prognostic value of 1p, 19q, 9p, 10q, and EGFR-FISH analyses in recurrent oligodendrogliomas. J Neuropathol Exp Neurol. 2004; 63:314–322. [PubMed: 15099021]

Felsberg J, Erkwoh A, Sabel MC, Kirsch L, Fimmers R, Blaschke B, Schlegel U, Schramm J, Wiestler OD, Reifenberger G. Oligodendroglial tumors: Refinement of candidate regions on chromosome arm 1p and correlation of 1p/19q status with survival. Brain Pathol. 2004; 14:121–130. [PubMed: 15193024]

Gagnon A, Ripeau JS, Zvieriev V, Chevrette M. Chromosome 18 suppresses tumorigenic properties of human prostate cancer cells. Genes Chromosomes Cancer. 2006; 45:220–230. [PubMed: 16281261]

Golembieski WA, Ge S, Nelson K, Mikkelsen T, Rempel SA. Increased SPARC expression promotes U87 glioblastoma invasion in vitro. Int J Dev Neurosci. 1999; 17:463–472. [PubMed: 10571408]

Jenkins RB, Blair H, Ballman KV, Giannini C, Arusell RM, Law M, Flynn H, Passe S, Felten S, Brown PD, Shaw EG, Buckner JC. A t(1;19)(q10;p10) mediates the combined deletions of 1p and 19q and predicts a better prognosis of patients with oligodendroglioma. Cancer Res. 2006; 66:9852–9861. [PubMed: 17047046]

Joshi PG, Mishra S. Galactocerebroside mediates Ca2+ signaling in cultured glioma cells. Brain Res. 1992; 597:108–113. [PubMed: 1335816]

Kitange G, Misra A, Law M, Passe S, Kollmeyer TM, Maurer M, Ballman K, Feuerstein BG, Jenkins RB. Chromosomal imbalances detected by array comparative genomic hybridization in human oligodendrogliomas and mixed oligoastrocytomas. Genes Chromosomes Cancer. 2005; 42:68–77. [PubMed: 15472895]

Kitange GJ, Templeton KL, Jenkins RB. Recent advances in the molecular genetics of primary gliomas. Curr Opin Oncol. 2003; 15:197–203. [PubMed: 12778011]

Kost-Alimova M, Fedorova L, Yang Y, Klein G, Imreh S. Microcell-mediated chromosome transfer provides evidence that polysomy promotes structural instability in tumor cell chromosomes through asynchronous replication and breakage within late-replicating regions. Genes Chromosomes Cancer. 2004; 40:316–324. [PubMed: 15188454]

Law ME, Templeton KL, Kitange G, Smith J, Misra A, Feuerstein BG, Jenkins RB. Molecular cytogenetic analysis of chromosomes 1 and 19 in glioma cell lines. Cancer Genet Cytogenet. 2005; 160:1–14. [PubMed: 15949564]

Louis DN, Holland EC, Cairncross JG. Glioma classification: A molecular reappraisal. Am J Pathol. 2001; 159:779–786. [PubMed: 11549567]

Mathioudaki K, Scorilas A, Papadokostopoulou A, Xynopoulos D, Arnogianaki N, Agnanti N, Talieri M. Expression analysis of BCL2L12, a new member of apoptosis-related genes, in colon cancer. Biol Chem. 2004; 385:779–783. [PubMed: 15493871]

Padalecki SS, Johnson-Pais TL, Killary AM, Leach RJ. Chromosome 18 suppresses the tumorigenicity of prostate cancer cells. Genes Chromosomes Cancer. 2001; 30:221–229. [PubMed: 11170278]

Perry A, Fuller CE, Banerjee R, Brat DJ, Scheithauer BW. Ancillary FISH analysis for 1p and 19q status: Preliminary observations in 287 gliomas and oligodendroglioma mimics. Front Biosci. 2003; 8:a1–a9. [PubMed: 12456321]

Quaye L, Dafou D, Ramus SJ, Song H, Gentry Maharaj A, Notaridou M, Hogdall E, Kruger Kjaer S, Christensen L, Hogdall C, Easton DF, Jacobs I, Menon U, Pharoah PD, Gayther SA. Functional complementation studies identify candidate genes and common genetic variants associated with ovarian cancer survival. Hum Mol Genet. 2009; 18:1869–1878. [PubMed: 19270026]

Reifenberger G, Louis DN. Oligodendroglioma: Toward molecular definitions in diagnostic neuro-oncology. J Neuropathol Exp Neurol. 2003; 62:111–126. [PubMed: 12578221]

Rousseau A, Nutt CL, Betensky RA, Iafrate AJ, Han M, Ligon KL, Rowitch DH, Louis DN. Expression of oligodendroglial and astrocytic lineage markers in diffuse gliomas: Use of YKL-40, ApoE, ASCL1, and NKX2–2. J Neuropathol Exp Neurol. 2006; 65:1149–1156. [PubMed: 17146289]

Smith JS, Jenkins RB. Genetic alterations in adult diffuse glioma: Occurrence, significance, and prognostic implications. Front Biosci. 2000; 5:D213–D231. [PubMed: 10702383]

Smith JS, Perry A, Borell TJ, Lee HK, O'Fallon J, Hosek SM, Kimmel D, Yates A, Burger PC, Scheithauer BW, Jenkins RB. Alterations of chromosome arms 1p and 19q as predictors of survival in oligodendrogliomas, astrocytomas, and mixed oligoastrocytomas. J Clin Oncol. 2000; 18:636–645. [PubMed: 10653879]

Smith JS, Alderete B, Minn Y, Borell TJ, Perry A, Mohapatra G, Hosek SM, Kimmel D, O'Fallon J, Yates A, Feuerstein BG, Burger PC, Scheithauer BW, Jenkins RB. Localization of common deletion regions on 1p and 19q in human gliomas and their association with histological subtype. Oncogene. 1999; 18:4144–4152. [PubMed: 10435596]

Stegh AH, Kim H, Bachoo RM, Forloney KL, Zhang J, Schulze H, Park K, Hannon GJ, Yuan J, Louis DN, DePinho RA, Chin L. Bcl2L12 inhibits post-mitochondrial apoptosis signaling in glioblastoma. Genes Dev. 2007; 21:98–111. [PubMed: 17210792]

Tews B, Felsberg J, Hartmann C, Kunitz A, Hahn M, Toedt G, Neben K, Hummerich L, von Deimling A, Reifenberger G, Lichter P. Identification of novel oligodendroglioma-associated candidate tumor suppressor genes in 1p36 and 19q13 using microarray-based expression profiling. Int J Cancer. 2006; 119:792–800. [PubMed: 16550607]

Thomadaki H, Talieri M, Scorilas A. Prognostic value of the apoptosis related genes BCL2 and BCL2L12 in breast cancer. Cancer Lett. 2007; 247:48–55. [PubMed: 16647810]

Tsuji A, Kikuchi Y, Sato Y, Koide S, Yuasa K, Nagahama M, Matsuda Y. A proteomic approach reveals transient association of reticulocalbin-3, a novel member of the CREC family, with the precursor of subtilisin-like proprotein convertase, PACE4. Biochem J. 2006; 396:51–59. [PubMed: 16433634]

Valster A, Tran NL, Nakada M, Berens ME, Chan AY, Symons M. Cell migration and invasion assays. Methods. 2005; 37:208–215. [PubMed: 16288884]

van den Bent MJ. Advances in the biology and treatment of oligodendrogliomas. Curr Opin Neurol. 2004; 17:675–680. [PubMed: 15542975]

Vranova V, Necesalova E, Kuglik P, Cejpek P, Pesakova M, Budinska E, Relichova J, Veselska R. Screening of genomic imbalances in glioblastoma multiforme using high-resolution comparative genomic hybridization. Oncol Rep. 2007; 17:457–464. [PubMed: 17203188]

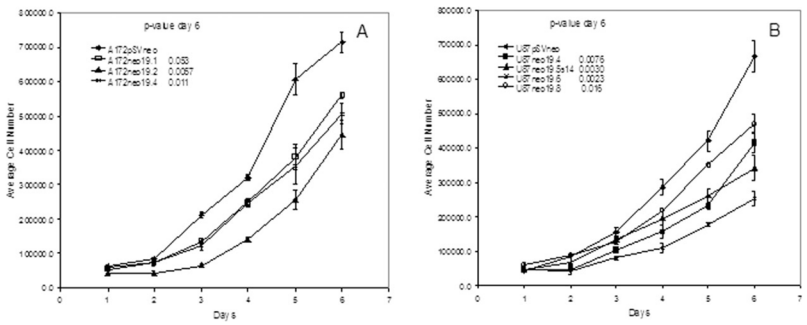

Figure 1.

Proliferation of cell lines, assessed by Trypan Blue Exclusion assay. Cells were plated on day 0 on tissue culture treated plates, with 50,000 cells per well. Values listed in the legends are the *P* value when compared with the appropriated parental pSV control cell line on day 6. (A) A172 cell lines and (B) U87 cell lines; 227 × 97 mm (72 × 72 DPI).

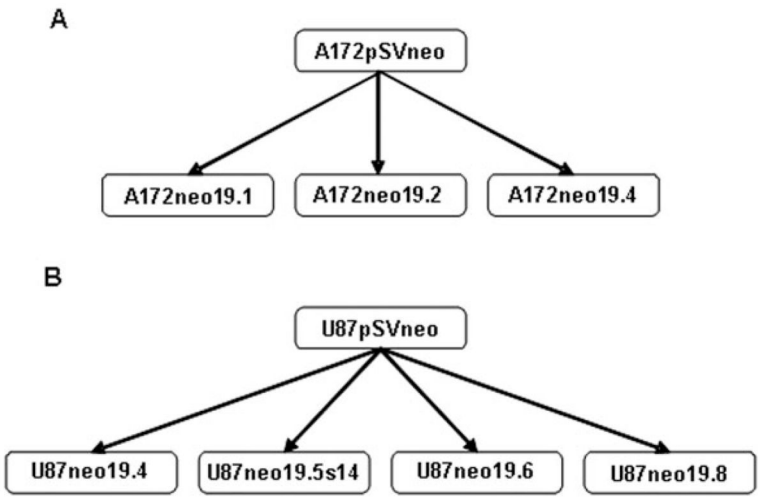

Figure 2.
Schematic of the cell line comparisons used for the Affymetrix U133 Plus 2.0 Array. Data from each of the hybrid cell lines was compared with the data collected for the parental control cell line containing the pSVneo vector; 254 × 190 mm (96 × 96 DPI).

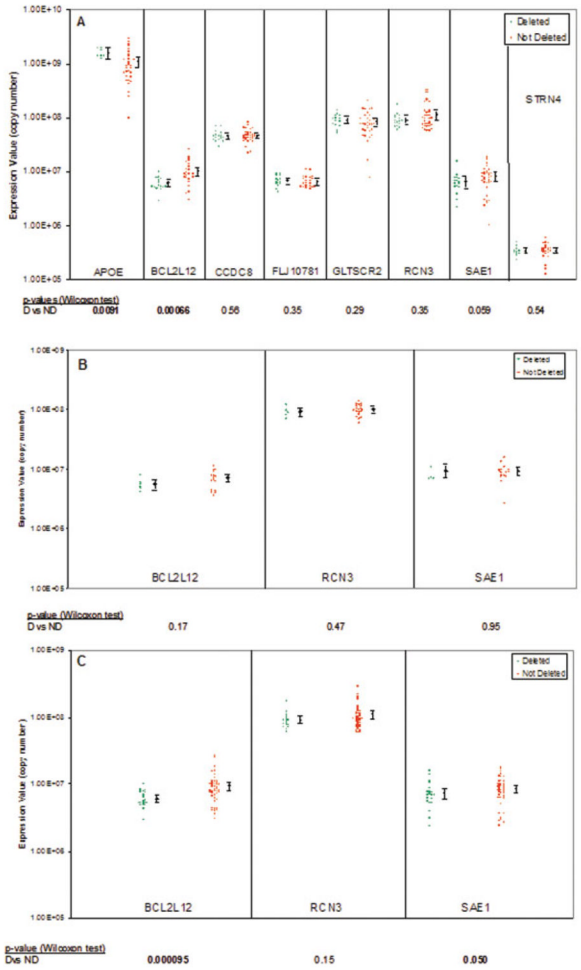

Figure 3.

RTPCR examining RNA copy number variation in primary tumor specimens for candidate genes. Individual tumors are represented by the individual spots. The black bars indicated the mean ± 2 SEM. (A) Test set of tumors (n = 51) stratified by deletion status of chromosome 19q. (B) Validation set of tumors (n = 26) stratified by tumor deletion status of chromosome 19q. (C) Combined set of tumors (n = 77) stratified by deletion status of chromosome 19q. The table beneath the graph show the P values (Wilcoxon test); 213 × 324 mm (72 × 72 DPI).

TABLE 1

Chromosome 19 Content of A172neo19 and U87neo19 Clones

	A172 clones					U87 clones			
	# Chromosome 19					# Chromosome 19			
	Initial isolates		Later passages			Initial isolates		Later passages	
Clone ID	Not deleted 19q	Deleted 19q	Not deleted 19q	Deleted 19q	Clone ID	Not deleted 19q	Deleted 19q	Not deleted 19q	Deleted 19q
A172	2	2	2	2	U87	1	1	1	1
A172pSVneo	2	2	2	2	U87pSVneo	1	1	1	1
A172neo19.1	3	2	3	2	U87neo19.4	2	1	1	1
A172neo19.2	3	2	3	2	U87neo19.5s14	2	1	1	1
A172neo19.4	3	2	3	2	U87neo19.6	2	1	1	1
					U87neo19.8	2	1	1	1

Genes Chromosomes Cancer. Author manuscript; available in PMC 2011 October 11.

TABLE 2

Affymetrix Microarray Data for the Selected Candidate Genes

Gene Symbol	Affymetrix Id	Location	A172 hybrid mean expression value ± SD	U87 hybrid mean expression value ± SD	A172 mean fold change	U87 mean fold change	A172 average *P* value	U87 average *P* value
APOE	203381_s_at	19q13.31	726 ± 176	164 ± 202	3.93	3.59	1.3×10^{-5}	6.4×10^{-6}
APOE	203382_s_at	19q13.31	677 ± 154	461 ± 149	3.23	2.96	3.8×10^{-5}	1.8×10^{-7}
APOE	212884_x_at	19q13.31	690 ± 94	442 ± 97	2.29	1.97	6.1×10^{-6}	8.8×10^{-5}
BCL2L12	233110_s_at	19q13.33	624 ± 42	404 ± 34	1.18	1.53	4.0×10^{-3}	9.6×10^{-5}
CCDC8	223496_s_at	19q13.32	196 ± 14	166 ± 29	1.30	1.54	1.4×10^{-3}	1.5×10^{-4}
FLJ10781	218824_at	19q13.32	253 ± 20	221 ± 98	1.89	2.40	7.4×10^{-6}	4.9×10^{-5}
RCN3	61734_at	19q13.33	392 ± 53	422. ± 50	1.80	2.29	4.0×10^{-5}	4.4×10^{-7}
RCN3	219102_at	19q13.33	308 ± 26	275 ± 22	1.34	1.58	2.6×10^{-4}	1.8×10^{-4}
SAE1	217946_s_at	19q13.32	1659 ±159	1328 ± 206	1.30	1.86	208×10^{-3}	2.0×10^{-3}
STRN4	217903_at	19q13.32	483 ±49	382 × 30	1.19	1.43	5.9×10^{-3}	1.7×10^{-3}

TABLE 3

Tumors Analyzed by RT-PCR

	Test set				Validation set				Complete set			
(N)	**A (24)**	**MOA (9)**	**O (18)**	**Total (51)**	**A (9)**	**MOA (8)**	**O (9)**	**Total (26)**	**A (33)**	**MOA (17)**	**O (27)**	**Total (77)**
Deleted	1	6	8	15	0	2	4	6	1	8	12	23
Not deleted	23	3	10	36	9	6	5	20	32	9	15	56
Grade 1	0	0	1	1	0	0	0	0	0	0	1	1
Grade 2	1	3	9	13	0	3	9	12	1	6	18	25
Grade 3	1	6	7	14	0	3	0	3	1	9	7	17
Grade 4	22	0	1	23	9	2	0	11	31	2	1	34

A, astrocytoma; MOA, mixed oligoastrocytoma; O, oligodendroglioma.