Selective Expression of a Subset of Neuronal Genes in Oligodendroglioma with Chromosome 1p Loss

Akitake Mukasa[1, 2*]; Keisuke Ueki[4]; Xijin Ge[1]; Shumpei Ishikawa[1, 3]; Takafumi Ide[5]; Takamitsu Fujimaki[6]; Ryo Nishikawa[7]; Akio Asai[8]; Takaaki Kirino[2]; Hiroyuki Aburatani[1]

[1]Genome Science Division, Research Center for Advanced Science and Technology, [2]Department of Neurosurgery, [3]Department of Pathology, Graduate School of Medicine, University of Tokyo, Japan.
[4]Department of Neurosurgery, Dokkyo University School of Medicine, Japan.
[5]Department of Neurosurgery, Tokyo Metropolitan Bokuto Hospital, Japan.
[6]Department of Neurosurgery, School of Medicine, Teikyo University, Japan.
[7]Department of Neurosurgery, Saitama Medical School, Japan.
[8]Department of Neurosurgery, Saitama Medical Center/School, Japan.
*Current address: Ludwig Institute for Cancer Research, San Diego Branch, Calif.

Corresponding author:
Keisuke Ueki, MD, PhD, Department of Neurosurgery, Dokkyo University School of Medicine, 880 Kitakobayashi, Mibu, Tochigi 321-0293, Japan (E-mail: kuekitky@umin.ac.jp).

Gliomas are classified based mainly on microscopic resemblance to their presumed glial origin such as astrocyte and oligodendrocyte. However, more objective diagnostic criteria are indispensable for the precise treatment of patients. For instance, loss of the short arm of chromosome 1 (1p) in oligodendrogliomas is recognized as an important marker for better response to chemotherapy and longer survival of the patients. To gain insight into their molecular biological background and to identify genes characterizing each subgroup, we investigated gene expression profile of the 4 glioma subsets, oligodendroglioma with and without 1p loss, diffuse astrocytoma and glioblastoma using DNA microarray. Remarkably, most of the genes showing distinctive expression in oligodendroglioma with 1p loss were also highly expressed in normal brain tissues and had neuron-related function, which included *MYT1L*, *INA*, *RIMS2*, *SNAP91* and *SNCB*. Histological analysis also demonstrated that *MYT1L*, which were abundantly expressed in normal neuron, were certainly present in tumor cells. These results suggest that oligodendroglioma, especially with 1p loss, has more or less neuronal characteristics although oligodendroglioma is thought to originate form glial lineage cell. With further pathological studies, those neuron-related genes might be good diagnostic markers for oligodendroglioma of better prognosis as well.

Brain Pathol 2004;14:34-42.

INTRODUCTION

Gliomas are a major type of brain tumors, which constitute approximately one third of all primary brain tumors (11). Most gliomas have diffuse infiltrative trait, rendering surgical cure impossible and recurrence inevitable despite aggressive adjuvant treatment including radiotherapy and chemotherapy. Prognosis of each patient is determined primarily by the biological characteristics of tumor cells including response to treatment and rate of growth. Prediction of such biological characteristics of gliomas has been based on histological diagnosis which mainly relied on the morphological features of the tumor, and on the classification referring to the presumed origin of the tumor cells such as astrocytes, oligodendrocytes and ependymal cells. However, recent development in molecular genetic analysis have shown that even gliomas in a single histological entity can be divided into different subsets and may sometimes show different clinical features. A prominent example is that allelic loss of the short arm of chromosome 1 (1p), which is found in 60% to 80% of oligodendrogliomas, is closely associated with the chemosensitivity and longer survival (1, 8). Molecular biological background of such differences should be important information to be investigated which potentially leads to better management of gliomas, and one of powerful tools to do so is DNA microarray technology (5). Several studies have successfully demonstrated subtype specific genes in diffuse gliomas based on the expression profile analysis, and also showed that such molecular profiles could indeed help accurate prediction of clinical outcome (4, 6, 7, 17, 20, 21).

In a previous work, we have demonstrated that expression profiles of oligodendrogliomas with 1p loss are significantly different from other oligodendrogliomas, and numerous genes presumed to be related to neuronal cells are preferentially expressed in this specific subset (16). In this study, we asked whether this trait would still hold within a wider range of gliomas including astrocytic tumors. Such findings may not only be of diagnostic significance, but also would bring a new insight into glioma classification based on gene expression.

MATERIALS AND METHODS

Sample preparation. Tumor samples and paired blood samples were obtained at surgery after written informed consents. Consensus histological diagnoses were made on formalin-fixed paraffin-embedded tissues by four independent neuropathologists following the WHO classification (11). Loss of heterozygosity (LOH) assay on chromosomes 1p and 19q using microsatellite markers were performed as described previously (24). The frozen tumor sample was homogenized in Trizol (Invitrogen, Corp., Carlsbad, Calif) and total RNA was isolated following manufacturer's instructions. RNA was quantitated by ultraviolet absorbance at 260 and 280 nm and its quality was assessed by agarose gel electrophoresis.

GeneChip experiment. In addition to 2 normal brains, 6 oligodendrogliomas with 1pLOH (4 WHO grade II and 2 grade III cases), and 5 oligodendrogliomas without 1pLOH (4 grade II and 1 grade III cases) which were all reported in our previous study (16), 6 diffuse astrocytomas (grade II) and 5 glioblastomas (grade IV) were subjected to gene expression profile analysis. The high-density oligonucleotide arrays (GeneChip Human U95A array, Affymetrix, Santa Clara, Calif), which contain probe sets for approximately 12 626 human genes and ESTs, were used. Biotin-labeled cRNA was synthesized from aliquots (5 μg) of total RNA from each sample, and hybridization, washing, and detection of signals were carried out as described previously (9, 16). The Microarray Analysis Suite (MAS) 4.0 software (Affymetrix) was used to calculate the gene expression levels. The average background and noise (Raw Q) value calculated by MAS 4.0 were less than 241 (199 ± 26) and 7.13 (6.07 ± 0.78), respectively under 100% PMT setting. To allow comparison among multiple arrays, gene expression levels were normalized for each array by assigning the average of overall expression levels to be 100. The signal values of β-actin as an internal control showed <2-fold variation (4488 ± 576). The scaling factor used for all samples was 0.68 ± 0.18. The percentage of probe sets scored as detected ("Present") in each sample ranged from 48 ± 3% (42%-55%). These metrics demonstrate that the quality of each array is comparable. A value of 10 was assigned to every expression value below 10, because such low values are vulnerable to noise and artifacts.

Selection of subtype-specific genes. All glioma samples analyzed by GeneChip (N = 22) fall into 4 groups: oligodendroglial tumors with 1pLOH (n = 6), without 1pLOH (n = 5), low-grade astrocytomas (n = 6), and glioblastomas (n = 5). An ideal subtype-specific gene should have higher expression in samples of this subgroup and lower expression in samples of the other 3 types. For the selection of such genes, we used public software called Significant Analysis of Microarrays (SAM 1.21) (23), which is one of the methods to solve the statistical problem occurring in the analysis of large numbers of genes with small numbers of experiments. Basically, a score assigned by SAM is signal-to-noise (S/N) ratio called relative difference d(i), which is calculated by $\{\mu_I(i)-\mu_U(i)\}/\{s(i)+s_0\}$ when $\mu_I(i)$ and $\mu_U(i)$ denote the average levels of expression for gene(i) in group I and U, respectively, and s(i) is defined as the standard deviation of repeated expression measurements. Then, taking gene-specific fluctuations into account, SAM estimates the percentage of genes identified by chance as the false discovery rate (FDR) using permutations of the repeated measurements. SAM also identifies genes with statistically significant changes and score q-value, which is similar to the familiar "p-value."

Before SAM was applied, the control probes and genes called absent (not detected) by the expression algorithm in MAS 4.0 software or less than 100 in all 24 samples were excluded because of low confidence of scarcely expressed genes. Then by the pre-filtering, the 2756 probe sets whose maximum and minimum expression levels among 22 tumor samples differed by more than 100, and had more than 5-fold difference, were selected for the following statistical analysis.

Comparison with normal brain tissue data. To see expressions of the selected genes in normal brain tissues, we used Affymetrix U95A array expression data in the Gene Expression Atlas on the website of Genomics Institute of the Novartis Research Foundation (22), in addition to the data obtained from our two normal whole brain samples. This database contains 2 whole brain, a cerebral cortex, 2 cerebellum, 2 caudate nucleus, 2 amygdala, 2 thalamus, 3 corpus callosum and 2 spinal cord. These data were linearly scaled to the same target signal (100) as in our own expression data. After this conversion, the expression levels of internal control genes such as β-actin (4425 ± 1122) in these normal tissues were similar to our data. The average gene expression levels in our normal brains and those in downloaded samples (whole brain) were also well correlated (coefficient r = 0.88) among pre-filtered genes. Hierarchical clustering was carried out by the programs Cluster and TreeView using selected 80 subtype-specific genes (3).

Quantitative real-time PCR. Quantitative real-time PCR (qPCR) was performed using iCycler (Bio-Rad, Hercules, Calif). cDNA was synthesized with oligo-dT primer from 2 μg total RNA using SuperScript Preamplification System (Invitrogen). The aliquot of cDNA were amplified by Taq polymerase for 40 cycles, consisted of 15 seconds of denaturing at 94°C, 15 seconds of annealing at 63-70°C, and 30 seconds of extension at 72°C with monitoring of the SYBR Green I dye intercalation signal. Each PCR reaction was done in triplicate. For each sample, relative expression of a gene to the expression in reference cDNA mixture of several cell lines and tissues was calculated, and the expression of each gene was then normalized using β-*actin* expression of the same sample as an internal control. The following primer sets and annealing temperature (Tm) were used: forward (F) 5′-AGAAG GAGAT CACTG CCCTG GCACC-3′, reverse (R) 5′-CCTGC TTGCT GATCC ACATC TGCTG-3′ and Tm 65°C for β-*actin*; F 5′-AATTA TTTCG GGGCT CTGCG GAACC-3′, R 5′-GCACC TTGCT TCAGC TCTCA AAACG-3′ and Tm 67°C for *MYT1L*; F 5′-TACCA CCCGG TCCCC ACTTT ATTGC-3′, R 5′-TTCGG GCCAC CCCTA CTTCT TCTCC-3′ and Tm 63°C for *L1CAM*; F 5′-CAGAC TCTGG CAACA CCTGC AATGG-3′, R 5′-CACGG GCCCG GATGA TTTCT ACCTC-3′ and Tm 70°C for *RIMS2*; F 5′-GAAGT GGCCC AGGAA GCTGC TGAAG-3′, R 5′-CAGGG ACAGA ATTGT GCTGC TGGTG-3′ and Tm 70°C for *SNCB*; F 5′-GAGGA CCGTC ATCAG GCCGA CATTG-3′, R 5′-GGCCA TCTCC CACTT GGTGT TCCTC-3′ and Tm 70°C for *NEFH*; F 5′-CCCTT TCCCC AAAAG TAGCG TAACC-3′, R 5′-TTGAC AGGAC GGCGA CTGTG AGAC-3′ and Tm 68°C for *OLIG1*. The specificity of the amplification products was validated using post-amplification melt curve analysis. Differences of gene expression in oligodendroglioma with 1pLOH and the other gliomas were tested by Kruskal-Wallis analysis.

In situ hybridization. Tumor samples and adjacent normal brain tissue stored at -80°C were embedded into Tissue-Tek OCT compound (Sakura Finetek, Torrance, Calif) and cryosectioned (7-μm thick), and then fixed in 4% paraformaldehyde. For the detection of *MYT1L* mRNA, the sections were treated with proteinase K

GeneChip probe number	Genbank accession number	Gene	Symbol	Expression level†					Locus
				Oligodendroglioma		Astrocy-toma	GBM	Normal whole brain	
				1pLOH (+)	1pLOH (-)				
32712_at	AB029029	Myelin transcription factor 1-like *‡	MYT1L	125 ± 70	13 ± 5	20 ± 19	10 ± 0	203 ± 102	2p
37210_at	S78296	Internexin neuronal intermediate filament protein α	INA	622 ± 182	177 ± 54	235 ± 102	136 ± 18	816 ± 85	10q
34526_s_at	AF052108	Hypothetical protein LOC157627	LOC157627	154 ± 77	30 ± 15	39 ± 50	11 ± 2	272 ± 120	8p
38551_at	U52112	L1 cell adhesion molecule *	L1CAM	479 ± 273	58 ± 62	44 ± 46	73 ± 43	620 ± 167	Xq
41589_at	Y15065	Potassium voltage-gated channel, KQT-like	KCNQ2	119 ± 86	32 ± 25	10 ± 0	10 ± 0	325 ± 146	20q
40234_at	X96484	DiGeorge syndrome critical region gene 6	DGCR6	443 ± 209	258 ± 167	19 ± 21	59 ± 60	439 ± 175	22q
33426_at	Y00064	Chromogranin B (secretogranin 1)	CHGB	233 ± 139	85 ± 44	49 ± 44	16 ± 9	282 ± 148	20p
32709_at	L39833	Potassium voltage-gated channel, shaker-related	KCNAB1	81 ± 36	23 ± 10	27 ± 15	26 ± 20	180 ± 33	3q
37568_at	U79242	Clone 24816 mRNA sequence		97 ± 33	20 ± 10	44 ± 15	30 ± 11	140 ± 22	
38163_at	AB018294	Regulating synaptic membrane exocytosis 2 *	RIMS2	133 ± 58	29 ± 14	39 ± 33	11 ± 2	140 ± 58	8q
34520_at	AJ012582	Potassium voltage-gated channel, brain 2	HCN2	79 ± 28	39 ± 16	25 ± 13	11 ± 2	144 ± 26	19p
36965_at	U13616	Ankyrin 3, node of Ranvier (ankyrin G)	ANK3	196 ± 83	78 ± 70	63 ± 35	44 ± 19	490 ± 215	10q
38699_at	X00734	Tubulin beta 5	TUBB5	500 ± 152	217 ± 115	138 ± 76	94 ± 90	1785 ± 282	19p
37580_at	AF036271	SH3-domain GRB2-like 3	SH3GL3	127 ± 59	50 ± 25	45 ± 37	12 ± 4	377 ± 132	15q
39095_at	M58018	Myosin, heavy polypeptide 7, cardiac muscle, β	MYH7	95 ± 79	20 ± 13	14 ± 8	18 ± 10	10 ± 0	14q
40733_f_at	D89377	Msh homeo box homolog 2	MSX2	79 ± 33	32 ± 5	30 ± 5	36 ± 6	31 ± 24	5q
41675_at	AB014556	Synaptosomal-associated protein, 91kDa homolog	SNAP91	341 ± 151	167 ± 118	74 ± 85	10 ± 0	950 ± 127	6q
38174_at	X99688	Pleckstrin and Sec7 domain protein	PSD	374 ± 198	164 ± 45	133 ± 70	65 ± 16	1068 ± 259	10q
33947_at	U18550	G protein-coupled receptor 3	GPR3	112 ± 20	50 ± 24	32 ± 23	38 ± 15	133 ± 39	1p
34788_at	AL049365	cDNA DKFZp586A0618		194 ± 72	82 ± 80	42 ± 27	58 ± 60	120 ± 8	
37060_at	U79289	Clone 23695 mRNA sequence		72 ± 35	22 ± 2	32 ± 16	27 ± 8	215 ± 30	
38855_s_at	D82343	Olfactomedin 1	OLFM1	762 ± 599	236 ± 56	222 ± 215	143 ± 114	2059 ± 155	9q
180_at	S82470	Leukocyte receptor cluster (LRC) member 4	LENG4	74 ± 33	29 ± 17	23 ± 15	22 ± 8	121 ± 74	19q
40653_at	U32439	Regulator of G-protein signaling 7	RGS7	133 ± 32	43 ± 15	59 ± 32	61 ± 37	371 ± 61	1q
34527_r_at	AF052108	Hypothetical protein LOC157627	LOC157627	154 ± 30	61 ± 22	88 ± 42	45 ± 11	213 ± 59	
41792_at	L78207	Sulfonylurea receptor	ABCC8	417 ± 233	172 ± 78	148 ± 91	88 ± 16	205 ± 30	11p
37857_at	AL080188	MT-protocadherin	KIAA1775	141 ± 61	68 ± 22	48 ± 33	17 ± 9	48 ± 15	10q
1998_i_at	U19599	BCL2-associated X protein	BAX	92 ± 14	61 ± 7	28 ± 10	34 ± 6	160 ± 108	19q
40753_at	AF053136	Synuclein β*	SNCB	146 ± 146	11 ± 2	26 ± 25	41 ± 17	953 ± 263	5q

Table 1. Highly expressed genes in oligodendroglioma with 1pLOH.
† Expression level of each gene was demonstrated as Mean ± S.E.M in each subgroup.
‡ The genes examined by qPCR are indicated by *.

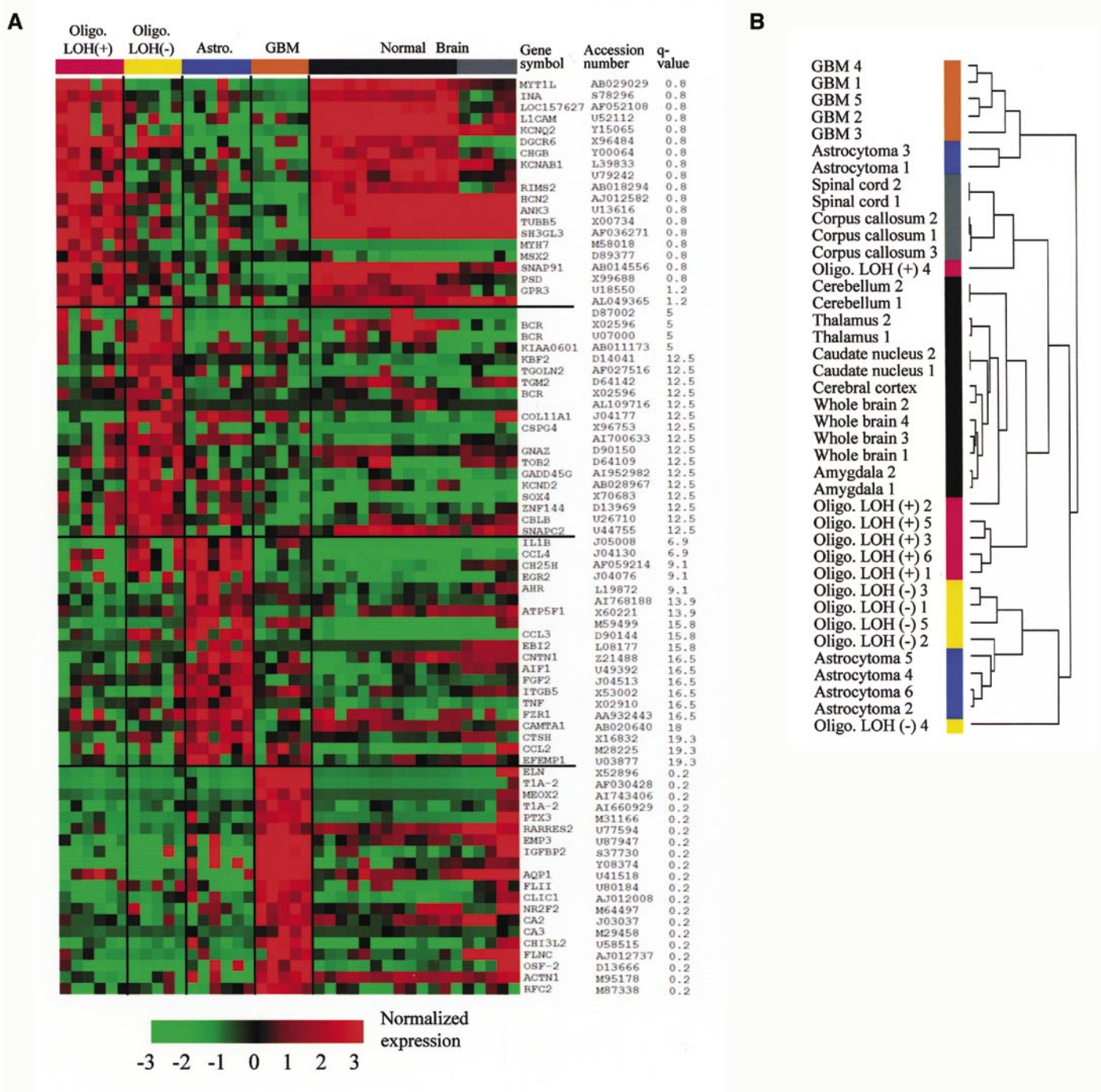

Figure 1. A. The genes characterizing 4 subgroups of diffuse gliomas. Each column represents a sample and each row represents a gene. Expression of each gene in 22 gliomas, together with 18 normal brain tissues was demonstrated in color gradation after normalization. Red indicates increased expression, and green indicates decreased gene expression. The order of samples are oligodendroglioma with 1pLOH (1-6), without 1pLOH (1-5), astrocytoma (1-6), glioblastoma (1-5), our whole brain (1, 2), downloaded whole brain (3, 4), cerebral cortex (1), cerebellum (1, 2), caudate nucleus (1, 2), amygdala (1, 2), thalamus (1, 2), corpus callosum (1-3) and spinal cord (1, 2). Note that the genes showing higher expression in oligodendroglioma with 1pLOH were also highly expressed in normal brain, except for corpus callosum and spinal cord. **B.** The result of hierarchical clustering using selected 80 subtype-specific genes. The oligodendrogliomas with 1pLOH were clustered into the same group with the normal brains, and were more similar to whole brain, cerebral cortex, cerebellum, caudate nucleus, amygdala and thalamus than corpus callosum and spinal cord. Two astrocytomas were clustered together with glioblastomas.

(code S3004; Dako, Glostrup, Denmark) diluted to 1:5000 at room temperature for 10 minutes, and 2 ng/μl of biotin-labeled oligonucleotide probe (antisense of 5′-ACATG GCTGT CACTG GATTT AGGCT TTCTG TCCTC C-3′ and sense of 5′-GGAGG ACAGA AAGCC TAAAT CCAGT GACAG CCATG T-3′) was hybridized at 37°C overnight. A GenPoint catalyzed signal amplification system (Dako) was used following manufacturer's instructions, and DAB substrate (Dako) was used to visualize amplified signal. The tissues were counterstained with hematoxylin. Furthermore, to test the quality of the mRNA in samples, positive and negative fluorescein-conjugated peptide nucleic acid (PNA) probes against glyceraldehyde

3-phosphate dehydrogenase, and PNA in situ hybridization detection kit (code K5201; Dako) were used according to the manufacturer's instruction.

Immunohistochemistry. Normal brain slides prepared simultaneously for in situ hybridization were immunolabeled. The slides were pretreated with microwave for total 20 minutes in citrate buffer pH 6.0, and then incubated with mouse anti-neuronal nuclei (NeuN) monoclonal antibody (Chemicon, Temecula, Calif) at 1:100 dilution for one hour at room temperature. A LSAB kit and a DAB substrate (DAKO) were used to visualize the antibody biding, and tissues were counterstained with hematoxylin.

RESULTS

The subtype-specific genes. SAM identified 29, 0, 0, and 247 subtype-specific genes with statistical significance of q-value <1.25% for oligodendroglioma with 1pLOH, without 1pLOH, astrocytoma and glioblastoma, respectively (highly expressed genes in oligodendroglioma with 1pLOH were listed in Table 1). Since SAM is applied 4 times to each subtype, the overall statistical significance for these genes is 5% after Benjamini correction for multiple testing. Then, we tried to select the same number of genes from each subtype of glioma for the subsequent clustering analysis, though SAM identified different number of genes as statistically significant. In this manuscript, main focus of our analysis was oligodendrogliomas with 1pLOH, in which 29 genes were identified as significantly highly expressed by SAM. On the other hand, genes list more than 20 in oligodendrogliomas without 1pLOH and astrocytoma had higher q-value and FDR. Therefore, we decided to select each 20 genes, which were sufficiently specific for oligodendroglioma with 1pLOH and glioblastoma, and were still acceptable for oligodendroglioma without 1pLOH and astrocytoma. Accordingly, we selected each 20 probe sets which showed lower q-value in each subgroup, as subtype-specific genes of oligodendroglioma with 1pLOH, without 1pLOH, astrocytoma and glioblastoma, of which median FDR were within 1.2, 12.5, 19 and 1.9 %, respectively (Figure 1A). Some of those genes showed consistency with other studies, such as insulin-like growth factor binding protein 2 (*IGFBP2*) whose higher expressions in glioblastoma were reported in the previous microarray studies (4, 20, 21).

Most of the genes that showed distinctively higher expression in oligodendroglioma with 1pLOH also showed similarly high expression in the normal brain, while the genes showing higher expression in other glioma subgroups did not have such a trend (Table 1, Figure 1A). Notably, many of those genes were considered to have neuron-related function. For example, myelin transcription factor 1-like (MYT1L) is thought to be a neuron specific transcription factor (10); internexin neuronal intermediate filament protein α (INA) may act as a neuron-specific intermediate filament protein (15); regulating synaptic membrane exocytosis 2 (RIMS2) and synaptosomal-associated protein 91kDa homolog (SNAP91) are supposed to be synapse related molecules; β-synuclein (SNCB) may play a role in neuronal plasticity and abundant in neurofibrillary lesions (2). L1 cell adhesion molecule (L1CAM), chromogranin B (CHGB), ankyrin 3 (ANK3), tubulin β5 (TUBB5), SH3-domain GRB2-like 3 (SH3GL3), pleckstrin and Sec7 domein protein (PSD), olfactomedin 1 (OLFM1), regulator of G-protein signaling 7 (RGS7), potassium voltage-gated channels such as KCNQ2, KCNAB1 and HCN2 are all thought to be expressed in neuronal cells. Besides those known genes, ESTs such as hypothetical protein LOC157627 and clone 23695 also seem to be abundantly expressed in the brain and neural tissue according to the public database such as UniGene, though their functions in the nervous system are not yet proven.

Using these 80 subtype-specific genes in total, clustering analysis was performed on the 22 tumors and 18 normal brain and spinal cord tissues (Figure 1B). The oligodendroglial tumors with 1pLOH were clustered into the same group with the normal brains, indicating their similarity in the expression pattern of the selected genes. Furthermore, oligodendroglioma with 1pLOH were more similar to whole brain, cerebral cortex, cerebellum, caudate nucleus, amygdala and thalamus than corpus callosum and spinal cord, possibly because corpus callosum and spinal cord consist mostly of glial cells than neurons. Two astrocytomas were clustered together with glioblastomas.

The validation studies using quantitative real-time PCR. Of the 29 genes that showed significantly higher expression in oligodendroglioma with 1pLOH, we selected four known genes for further validation study using qPCR. We also analyzed a gene for neurofilament heavy polypeptide (NEFH) which is known to be expressed in normal brain, and OLIG1 gene that is reported to be expressed specifically in oligodendrogliomas (12, 14). Forty-seven samples including 24 samples used in the microarray experiment and 23 additional gliomas were analyzed. The relative expression levels in qPCR of each six gene, *MYT1L*, *L1CAM*, *RIMS2*, *SNCB*, *NEFH* and *OLIG1* were shown in Figure 2. *MYT1L*, *L1CAM*, *RIMS2* and *SNCB* showed significantly higher expression in oligodendrogliomas with 1pLOH than other gliomas ($p<0.0001$, <0.005, <0.0001 and <0.001, respectively), and normal brains also had higher expression as expected from GeneChip data (Note that Y-axis in Figure 2 represents the relative gene expression level to the average expression in normal brain). We recognized, however, some exceptional cases (one of them was indicated by circle in Figure 2) that had higher expression of these genes in other glioma subgroups. Including such cases, gliomas showing higher expression in one of the 4 genes usually had similarly higher expression in other 3 genes as well. The expression levels of *NEFH* in gliomas were much lower than normal brain. *OLIG1* were highly expressed in gliomas comparing to normal brain, though it was not specific to oligodendrogliomas.

The results of qPCR corresponded well to the GeneChip data, and the correlation between the data from the qPCR and the GeneChip were 0.88 for *MYT1L*, 0.77 for *L1CAM*, 0.87 for *RIMS2*, and 0.98 for *SNCB* respectively, using Pearson correlation coefficient.

The expression of neuron-related molecules in oligodendroglioma with 1pLOH. To exclude the possibility of contaminated normal neurons as the source of the higher expression of neuronal genes in oligodendroglioma with 1pLOH, we performed in

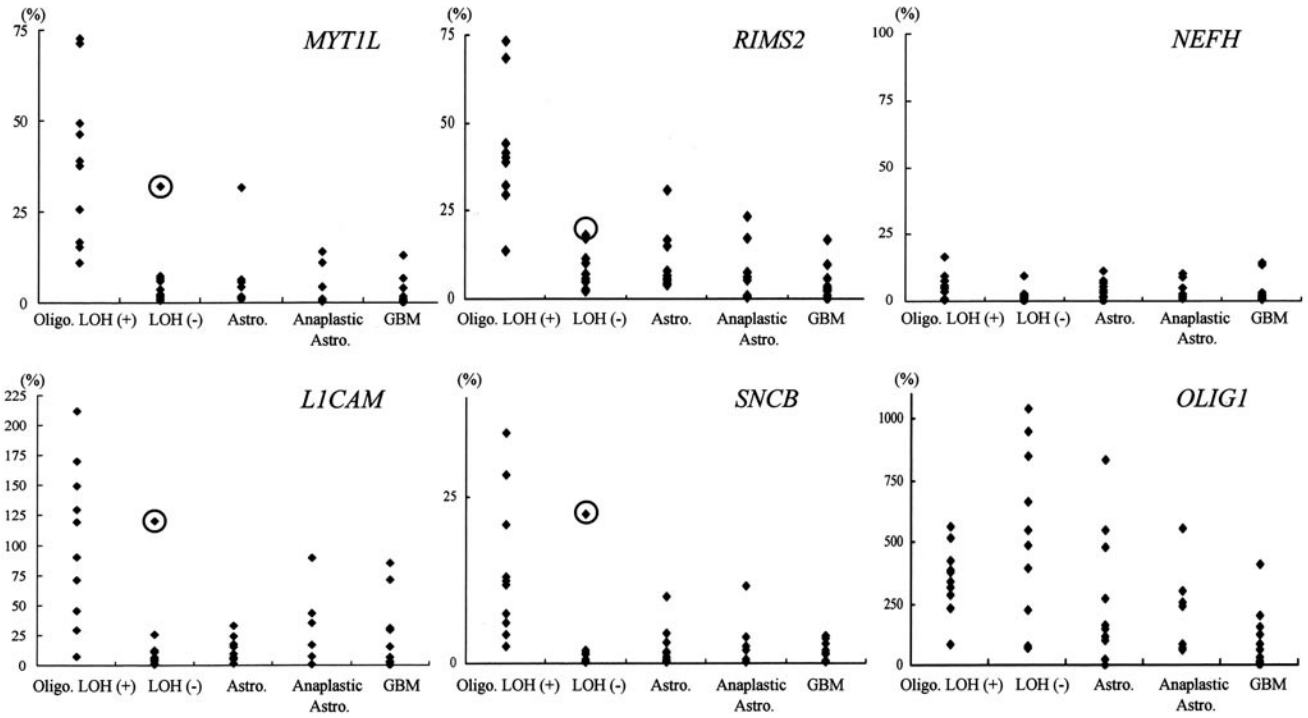

Figure 2. Quantitative real-time PCR analysis on oligodendrogliomas with 1pLOH (n = 10), without 1pLOH (n = 10), low-grade diffuse astrocytomas (n = 9), anaplastic astrocytomas (n = 7), glioblastomas (n = 9) and normal brains (n =2). Note that Y-axis represents the relative gene expression level to the average expression in normal brains. *MYT1L*, *L1CAM*, *RIMS2* and *SNCB* showed significantly higher expression in oligodendrogliomas with 1pLOH than other gliomas ($p<0.0001$, <0.005, <0.0001 and <0.001, respectively) using the Kruskal-Wallis test. There were two exceptional cases; one oligodendrogliomas without 1p loss (indicated by circle) and one astrocytoma showed higher expression in those four genes. *NEFH* showed consistent lower expression in all glioma samples comparing with normal brain (<20%). Most of glioma samples had higher *OLIG1* expressions.

situ hybridization for *MYT1L* transcripts. Eleven samples containing good quality of mRNA confirmed by in situ hybridization using PNA probe as a positive control were evaluated; 2 normal brain tissues, 3 oligodendrogliomas with 1pLOH, 3 oligodendrogliomas without 1pLOH, 2 astrocytomas and 1 glioblastoma (Figure 3). *MYT1L* expressions were detected in 2 normal brain tissues and were highly expressed in cells containing large nuclei (Figure 3A, C). The cells containing large nuclei were also immunostained with anti-neuronal nuclei (NeuN) antibody and were assumed to be neurons (Figure 3A, inset). Expression of *MYT1L* transcripts was clearly demonstrated in 2 of 3 oligodendrogliomas with 1pLOH (Figure 3B, D), but not in oligodendrogliomas without LOH (Figure 3E), astrocytomas (Figure 3F) nor glioblastoma (Figure 3G).

DISCUSSION

In this study, we demonstrated that some of the genes showing higher expression in oligodendroglioma with 1p loss compared to the other major subtypes of gliomas were functionally neuron-related genes, with the expression at the similar levels in normal neurons. Although it was rather unexpected that neuron-related genes were expressed in gliomas, contamination of normal neurons in the samples of oligodendroglioma with 1p loss was not likely, because *i)* allelic losses observed on the microsatellite analysis were almost complete in all cases, indicating that the examined tissues consisted mostly of tumor cells, and *ii)* our in situ hybridization for *MYT1L* transcripts demonstrated that these genes were indeed expressed in the tumor cells. Furthermore, *iii)* expressions of other neuron specific genes expressed in normal brain tissues, such as gene encoding neurofilament subunit (*NEFH*, *NEFM* and *NEFL*), were much lower in oligodendrogliomas with 1p loss than those in normal brain. Therefore, the microarray analysis represented expression profile of the tumor cells, not normal neurons.

The qPCR analysis on several genes confirmed the microarray analysis results, and validated them on additional 23 gliomas samples. The results were mostly consistent, showing similar levels of higher expression in oligodendrogliomas with 1p loss but not in other gliomas. However, there were 2 exceptional cases; one oligodendroglioma without 1p loss and one astrocytoma showed higher expression in those genes. On re-reviewing the histology of those 2 tumors, we noticed that the oligodendroglioma case had occasional ependymoma-like portion, but the astrocytoma case was typical astrocytoma without any unusual morphology. We consider that these exceptional cases may reflect the heterogeneity of yet unknown background. To be noted was that the changes of expression levels of the genes were always to the same trend in all gliomas including the exceptional cases, suggesting a possible functional link among those genes.

We also compared WHO grade II (n = 8) and grade III (n = 3) oligodendrogliomas using Mann-Whitney test with cut-off p-values of 0.05, and 368 genes were detected as differentially expressed by grade (whole list of the selected genes would be available on request). Downregulated genes in grade III tumors included genes for CD44, alpha 1 syntrophin, connexin43 gap junction protein, CCAAT/enhancer binding protein delta, and chemokine receptor 4. Genes upregulated in grade III tumors included

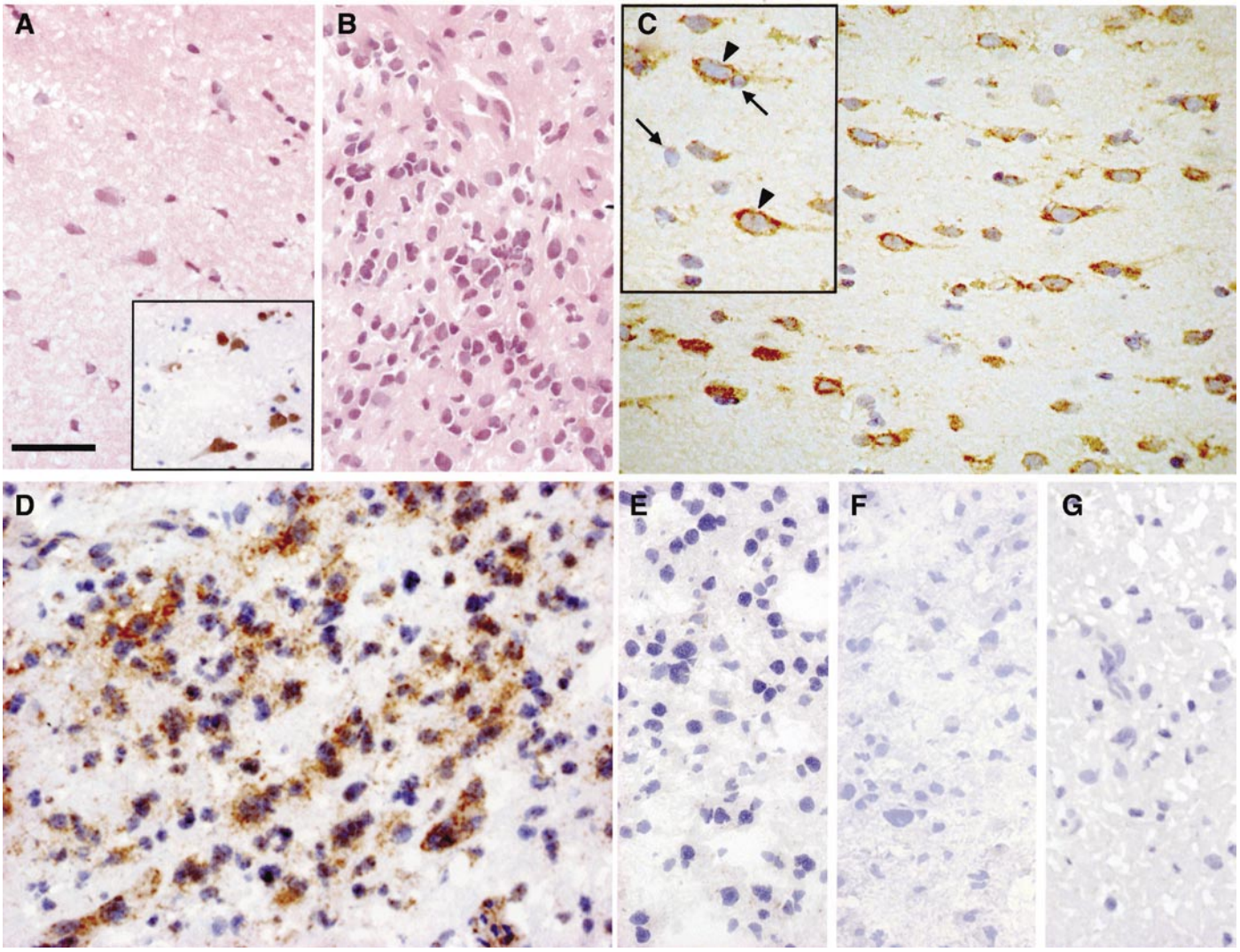

Figure 3. In situ hybridization for *MYT1L* transcripts, with corresponding H&E staining of normal brain (**A**) and oligodendroglioma with 1pLOH (**B**) on frozen section. *MYT1L* expressions were strongly observed in neurons (arrowheads), which contain large nuclei, comparing with the surrounding cells with small, round nuclei which were putatively considered as oligodendrocytes (arrows) (**C**). The cells containing large nuclei were also immunostained with anti-neuronal nuclei (NeuN) antibody (inset, **A**). Oligodendroglioma with 1pLOH actually expressed MYT1L transcripts (**D**), though oligodendroglioma without 1pLOH (**E**), astrocytoma (**F**) and glioblastoma (**G**) did not. (Bar = 50 μm [A, B]; 30 μm [C, D, E, F, G]).

genes for myosin and interferon induced proteins. These genes listed above were concordant with previous report (25), suggesting the consistency of this DNA array analysis. However, further analysis of differentially expressed genes by tumor grade was not performed in this study, because statistical confidence for this data might be limited by small number of our grade III tumors.

Both Myt1 and its homologue Myt1l are zinc finger proteins of CCHC class that are expressed in neurons at early stages of differentiation. While Myt1 is expressed in cells of glial lineage, Myt1l is not detected in glial cells but co-expressed with Tuj1 in neurons around terminal mitosis (10). Therefore, Myt1l is supposed to play a role in the development of neurons. In our study, *MYT1L* was also expressed in neuron of normal adult human brain. The fact that oligodendroglioma with 1p loss express a subset of neuron specific genes like *MYT1L* would raise a question whether these tumor originate from the same glial progenitor cells as the other gliomas. Neurocytic differentiation and variable degrees of neuronal marker expression have been reported in oligodendrogliomas (19, 26, 27), and neuron-like physiological properties of oligodendroglioma cells have been observed as well (18). On the other hand, *OLIG1* and *OLIG2* genes, which are crucial in maturation of oligodendrocyte and its progenitor, were strongly expressed in oligodendrogliomas as previously reported 12, 14), although the expression of those 2 genes were not specific to oligodendrogliomas in our data. Therefore, these results together may suggest that oligodendroglioma with 1p loss have both neuronal and glial differentiation patterns at least on a certain group of genes. In line with such a still hypothetical proposition, recent studies indicated that some oligodendrocytes might share the same progenitor cells with neurons (13, 28).

Since the era of Baily and Cushing's inaugural works, classification of gliomas has been based upon hypothetical origins of the tumor, which were assigned to each tumor type according to their morphological features. With the rapid advancement in developmental biology of nervous system at the molecular level, such classifications could be reorganized using molecular markers related to neural development. Our

observations suggest that genetic subsets in gliomas may well be one of the subjects for such possible redefinition in the future. A clinically important question is whether these neuronal genes such as *MYT1L* could be used to identify more favorable subset of gliomas as diagnostic markers, independently to 1p loss. A recent report showed that in certain malignant gliomas, the expression profiling using microarray was successful in identifying a set of genes more accurate in predicting prognosis of patients than histological diagnosis (17). Notably, 2 patients of astrocytoma grade II which were clustered together with glioblastomas in our series (Figure 1B), indeed showed the clinical course equivalent to glioblastomas, suggesting usefulness of microarray for predicting patients' prognosis. However, almost all oligodendroglioma patients in the current study are still alive and therefore we do not have sufficient prognostic data for analysis at this point. Further investigation with more data, both in number of cases and length of follow-up, would certainly answer such question in the future.

ACKNOWLEDGMENTS

This work was supported by a Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (No. 11671357 and No. 13470184). This study was carried out as a part of The Technology Development for Analysis of Protein Expression and Interaction in Bioconsortia on R&D of New Industrial Science and Technology Frontiers which was performed by Industrial Science, Technology and Environmental Policy Bureau, Ministry of Economy, Trade and Industry, and entrusted by New Energy Development Organization (NEDO).

We thank Drs Yoichi Nakazato, Takanori Hirose, Nobuaki Funata, and Junko Hirato for reviewing the histology. We also thank Drs Masato Nakafuku and Tetsuya Taga for helpful discussion of the manuscript. We thank expert help of Dr Hirokazu Taniguchi, Kaori Shiina for histological studies, and Reiko Matsuura, Chizu Kobayashi, Hiroko Meguro, Erio Ashihara, Saori Fukui and Junko Yagi for their technical assistance.

REFERENCES

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

2. Clayton DF, George JM (1998) The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci* 21:249-254.

3. Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95:14863-14868.

4. Fuller GN, Rhee CH, Hess KR, Caskey LS, Wang R, Bruner JM, Yung WK, Zhang W (1999) Reactivation of insulin-like growth factor binding protein 2 expression in glioblastoma multiforme: a revelation by parallel gene expression profiling. *Cancer Res* 59:4228-4232.

5. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 286:531-537.

6. Gutmann DH, Hedrick NM, Li J, Nagarajan R, Perry A, Watson MA (2002) Comparative gene expression profile analysis of neurofibromatosis 1-associated and sporadic pilocytic astrocytomas. *Cancer Res* 62:2085-2091.

7. Huang H, Colella S, Kurrer M, Yonekawa Y, Kleihues P, Ohgaki H (2000) Gene expression profiling of low-grade diffuse astrocytomas by cDNA arrays. *Cancer Res* 60:6868-6874.

8. Ino Y, Betensky RA, Zlatescu MC, Sasaki H, Macdonald DR, Stemmer-Rachamimov AO, Ramsay DA, Cairncross JG, Louis DN (2001) Molecular subtypes of anaplastic oligodendroglioma: implications for patient management at diagnosis. *Clin Cancer Res* 7:839-845.

9. Ishii M, Hashimoto S, Tsutsumi S, Wada Y, Matsushima K, Kodama T, Aburatani H (2000) Direct comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis. *Genomics* 68:136-143.

10. Kim JG, Armstrong RC, v Agoston D, Robinsky A, Wiese C, Nagle J, Hudson LD (1997) Myelin transcription factor 1 (Myt1) of the oligodendrocyte lineage, along with a closely related CCHC zinc finger, is expressed in developing neurons in the mammalian central nervous system. *J Neurosci Res* 50:272-290.

11. Kleihues P, Cavenee WK (eds.) (2000) *Pathology and Genetics of Tumours of the Nervous System*. IARC Press, Lyon.

12. Lu QR, Park JK, Noll E, Chan JA, Alberta J, Yuk D, Alzamora MG, Louis DN, Stiles CD, Rowitch DH, Black PM (2001) Oligodendrocyte lineage genes (OLIG) as molecular markers for human glial brain tumors. *Proc Natl Acad Sci U S A* 98: 10851-10856.

13. Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH (2002) Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* 109: 75-86.

14. Marie Y, Sanson M, Mokhtari K, Leuraud P, Kujas M, Delattre JY, Poirier J, Zalc B, Hoang-Xuan K (2001) OLIG2 as a specific marker of oligodendroglial tumour cells. *Lancet* 358:298-300.

15. McGraw TS, Mickle JP, Shaw G, Streit WJ (2002) Axonally transported peripheral signals regulate alpha-internexin expression in regenerating motoneurons. *J Neurosci* 22:4955-4963.

16. Mukasa A, Ueki K, Matsumoto S, Tsutsumi S, Nishikawa R, Fujimaki T, Asai A, Kirino T, Aburatani H (2002) Distinction in gene expression profiles of oligodendrogliomas with and without allelic loss of 1p. *Oncogene* 21:3961-3968.

17. Nutt CL, Mani DR, Betensky RA, Tamayo P, Cairncross JG, Ladd C, Pohl U, Hartmann C, McLaughlin ME, Batchelor TT, Black PM, von Deimling A, Pomeroy SL, Golub TR, Louis DN (2003) Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. *Cancer Res* 63: 1602-1607.

18. Patt S, Labrakakis C, Bernstein M, Weydt P, Cervos-Navarro J, Nisch G, Kettenmann H (1996) Neuron-like physiological properties of cells from human oligodendroglial tumors. *Neuroscience* 71:601-611.

19. Perry A, Scheithauer BW, Macaulay RJ, Raffel C, Roth KA, Kros JM (2002) Oligodendrogliomas with neurocytic differentiation. A report of 4 cases with diagnostic and histogenetic implications. *J Neuropathol Exp Neurol* 61:947-955.

20. Rickman DS, Bobek MP, Misek DE, Kuick R, Blaivas M, Kurnit DM, Taylor J, Hanash SM (2001) Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis. *Cancer Res* 61:6885-6891.

21. Sallinen SL, Sallinen PK, Haapasalo HK, Helin HJ, Helen PT, Schraml P, Kallioniemi OP, Kononen J (2000) Identification of differentially expressed genes in human gliomas by DNA microarray and tissue chip techniques. *Cancer Res* 60:6617-6622.

22. Su AI, Cooke MP, Ching KA, Hakak Y, Walker JR, Wiltshire T, Orth AP, Vega RG, Sapinoso LM, Moqrich A, Patapoutian A, Hampton GM, Schultz PG, Hogenesch JB (2002) Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci U S A* 99:4465-4470.

23. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 98:5116-5121.

24. Ueki K, Nishikawa R, Nakazato Y, Hirose T, Hirato J, Funada N, Fujimaki T, Hojo S, Kubo O, Ide T, Usui M, Ochiai C, Ito S, Takahashi H, Mukasa A, Asai A, Kirino T (2002) Correlation of histology and molecular genetic analysis of 1p, 19q, 10q, TP53, EGFR, CDK4, and CDKN2A in 91 astrocytic and oligodendroglial tumors. *Clin Cancer Res* 8: 196-201.

25. Watson MA, Perry A, Budhjara V, Hicks C, Shannon WD, Rich KM (2001) Gene expression profiling with oligonucleotide microarrays distinguishes World Health Organization grade of oligodendrogliomas. *Cancer Res* 61:1825-1829.

26. Wharton SB, Chan KK, Hamilton FA, Anderson JR (1998) Expression of neuronal markers in

oligodendrogliomas: an immunohistochemical study. *Neuropathol Appl Neurobiol* 24:302-308.

27. Wolf HK, Buslei R, Blumcke I, Wiestler OD, Pietsch T (1997) Neural antigens in oligodendrogliomas and dysembryoplastic neuroepithelial tumors. *Acta Neuropathol (Berl)* 94:436-443.

28. Zhou Q, Anderson DJ (2002) The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* 109:61-73.