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

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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.

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 $48 \pm 3\%$ (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′-CCT-GC 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′-CAC-GG 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′-TT-GAC 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	Oligodendroglioma 1pLOH (+)	Oligodendroglioma 1pLOH (-)	Astrocytoma	GBM	Normal whole brain	Locus
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.
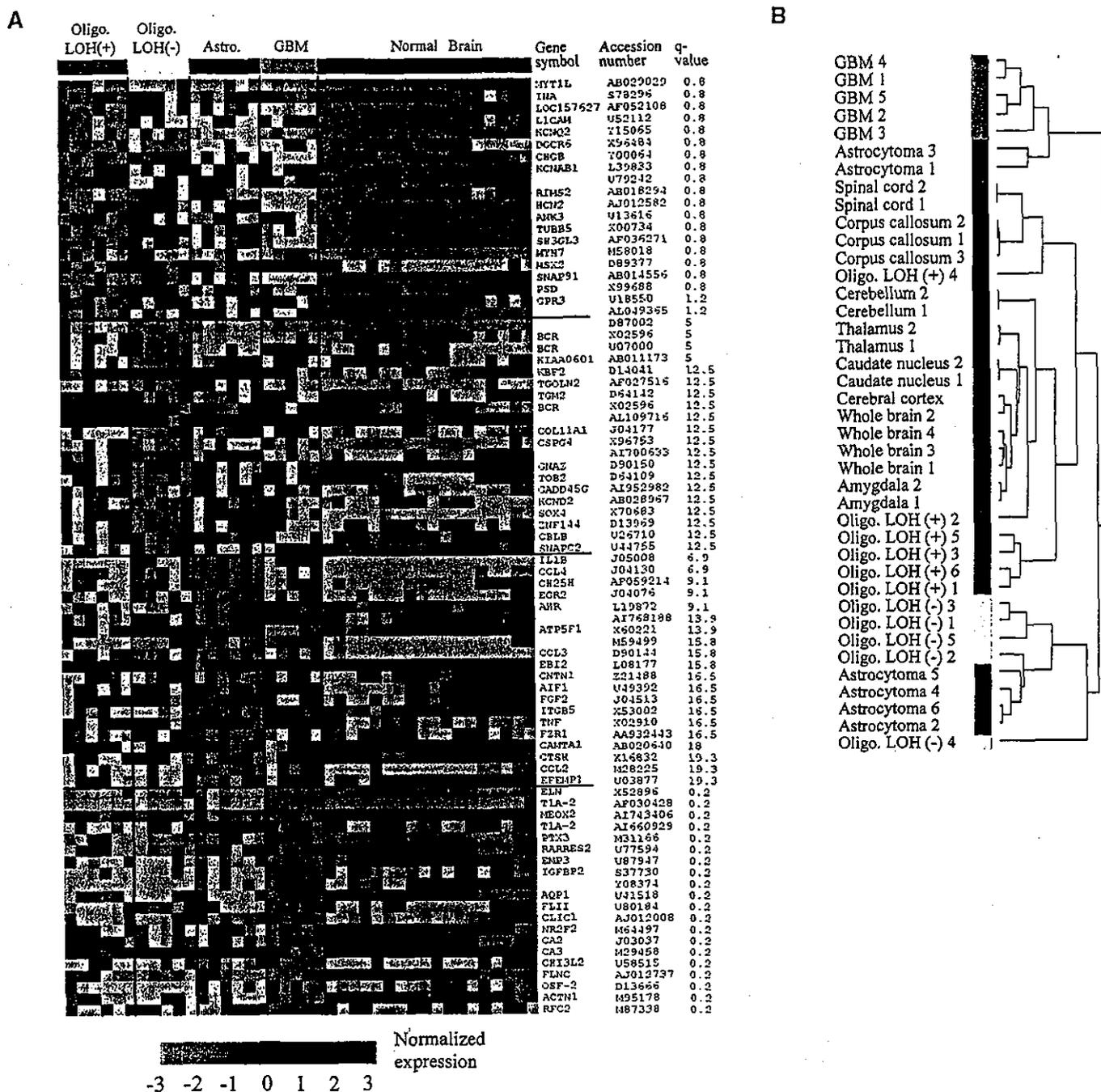* The genes examined by qPCR are indicated by *.

A

Oligo. LOH(+) | Oligo. LOH(-) | Astro. | GBM | Normal Brain

Gene symbol	Accession number	q-value
MYT1L	AB029029	0.8
INA	S78296	0.8
LOC157627	AF052106	0.8
L1CAM	U52112	0.8
KCNQ2	Y15065	0.8
DGCR5	X96484	0.8
CHGB	Y00064	0.8
KCNAB1	L39833	0.8
	U79242	0.8
RIMS2	AB018294	0.8
HCN2	AJ012582	0.8
NRK3	U13616	0.8
TUBB5	X00734	0.8
SH3GL3	AF036271	0.8
MTN7	M58018	0.8
NSK2	D89377	0.8
SNAP91	AB014556	0.8
PSD	X99688	0.8
GPR3	U18550	1.2
	AL049355	1.2
	D87002	5
BCR	X02596	5
BCR	U07000	5
KIAA0601	AB011173	5
KBF2	D14041	12.5
TGOLN2	AF027516	12.5
TGM2	D64142	12.5
BCR	X02596	12.5
	AL109716	12.5
COL11A1	J04177	12.5
CSPG4	X96753	12.5
	AI700633	12.5
GNAZ	D90150	12.5
TOB2	D64109	12.5
GADD45G	AI952982	12.5
KCND2	AB028967	12.5
SOX4	X70683	12.5
ZNF144	D13969	12.5
CBLB	U26710	12.5
SNAPC2	U44755	12.5
IL1B	J05008	6.9
CCL4	J04130	6.9
CH25H	AF059214	9.1
EGR2	J04076	9.1
AHR	L19872	9.1
	AI768188	13.9
ATP5F1	X60221	13.9
	M59499	15.8
CCL3	D90144	15.8
EBI2	L08177	15.8
CNTN1	Z21488	16.5
AIF1	U49392	16.5
FGF2	J04513	16.5
ITGB5	X53002	16.5
TNF	X02910	16.5
F2R1	AA932443	16.5
CAMTA1	AB020640	18
CTSK	X16832	19.3
CCL2	M28225	19.3
EFEMP1	U03877	19.3
ELN	X52896	0.2
T1A-2	AF030428	0.2
MEOX2	AI743406	0.2
T1A-2	AI660929	0.2
PTX3	M31166	0.2
RARRES2	U77594	0.2
EMP3	U87947	0.2
IGFBP2	S37730	0.2
	Y08374	0.2
AQP1	U41518	0.2
FLII	U80184	0.2
CLIC1	AJ012008	0.2
NR2F2	M64497	0.2
CA2	J03037	0.2
CA3	M29458	0.2
CHI3L2	U58515	0.2
FLNC	AJ012737	0.2
OSF-2	D13666	0.2
ACTN1	M95178	0.2
RFC2	M87338	0.2

Normalized expression

-3 -2 -1 0 1 2 3

B

GBM 4
GBM 1
GBM 5
GBM 2
GBM 3
Astrocytoma 3
Astrocytoma 1
Spinal cord 2
Spinal cord 1
Corpus callosum 2
Corpus callosum 1
Corpus callosum 3
Oligo. LOH (+) 4
Cerebellum 2
Cerebellum 1
Thalamus 2
Thalamus 1
Caudate nucleus 2
Caudate nucleus 1
Cerebral cortex
Whole brain 2
Whole brain 4
Whole brain 3
Whole brain 1
Amygdala 2
Amygdala 1
Oligo. LOH (+) 2
Oligo. LOH (+) 5
Oligo. LOH (+) 3
Oligo. LOH (+) 6
Oligo. LOH (+) 1
Oligo. LOH (-) 3
Oligo. LOH (-) 1
Oligo. LOH (-) 5
Oligo. LOH (-) 2
Astrocytoma 5
Astrocytoma 4
Astrocytoma 6
Astrocytoma 2
Oligo. LOH (-) 4

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
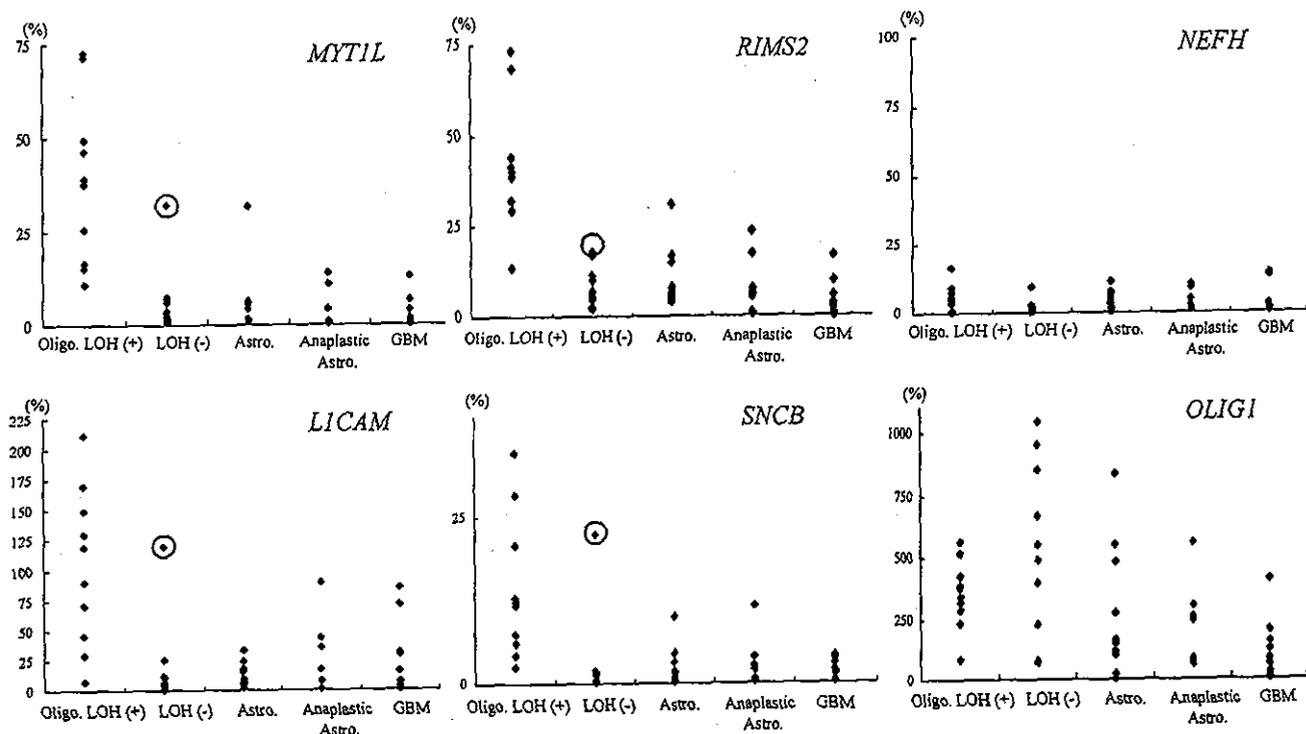
Figure 2. Quantitative real-time PCR analysis on oligodendrogliomas with 1pLOH (n=10), without 1pLOH (n=10), low-grade diffuse astrocytomas (n=9), anaplastic astrocytomas (n=7), glioblastomas (n=9) and normal brains (n=2). Note that Y-axis represents the relative gene expression level to the average 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
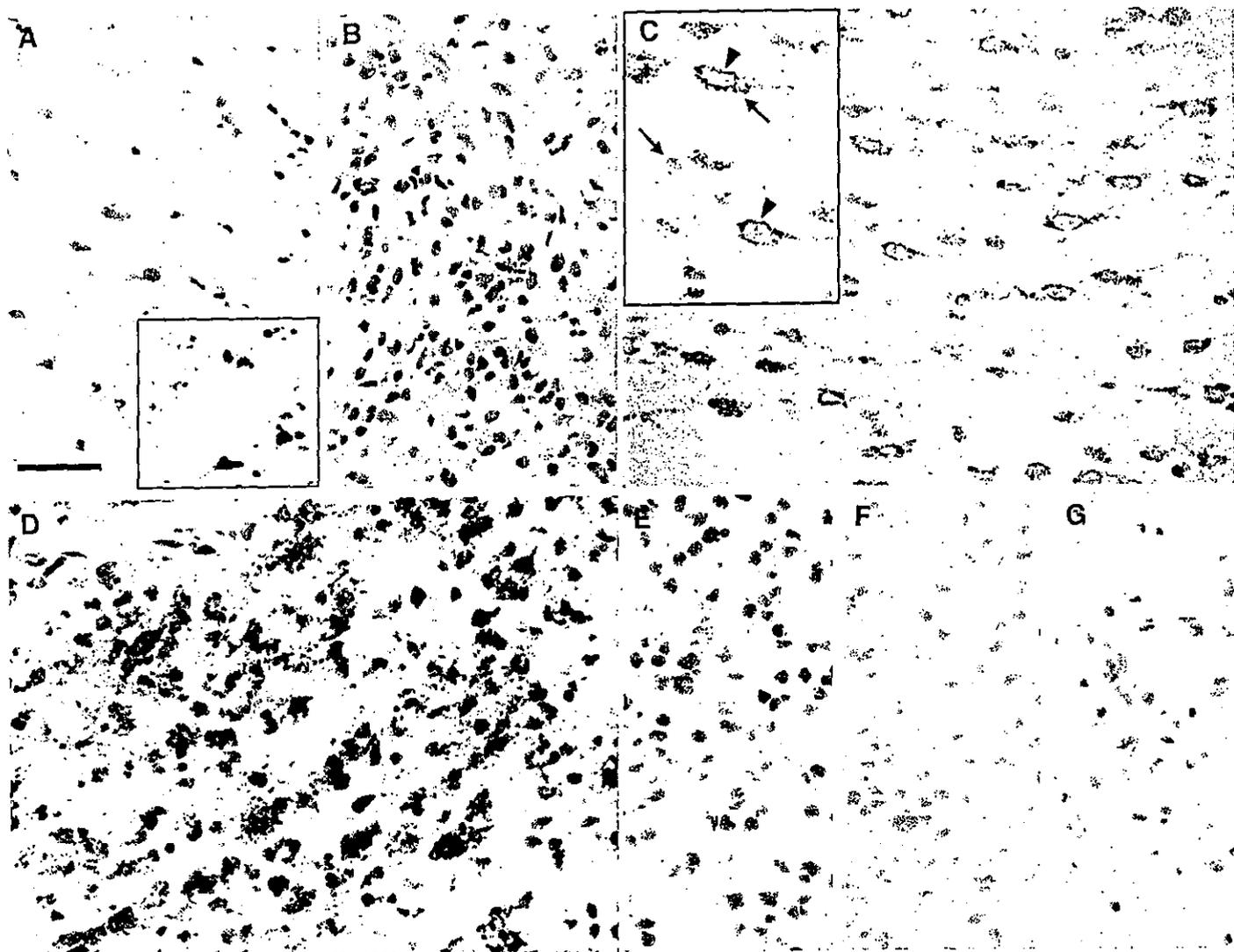
Figure 3. In situ hybridization for *MYT1L* transcripts, with corresponding H&E staining of normal brain (**A**) and oligodendroglioma with 1pLOH (**B**) on frozen section. *MYT1L* expressions were strongly observed in neurons (arrowheads), which contain large nuclei, comparing with the surrounding cells 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 oligodendro-gliomas 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

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SPECIAL ARTICLE

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Immunohistochemical analysis of the mutant epidermal growth factor, ΔEGFR, in glioblastoma

Abstract The naturally occurring mutated form of the epidermal growth factor receptor, ΔEGFR (also named EGFRvIII and de2-7EGFR), greatly enhances glioblastoma (GBM) cell growth in vivo through several activities, such as down-regulating p27 and up-regulating BclX(L) while increasing signaling through the RAS-MAPK and PI3-K cascades. More than half of GBMs, especially of the de novo type, overexpress EGFR, and 50%–70% of these express ΔEGFR. However, little is known about the distribution of ΔEGFR-expressing tumor cells within surgical specimens. In order to address this clinically important issue, we performed immunohistochemical analyses of 53 GBMs obtained during surgery using the anti- ΔEGFR monoclonal antibody, DH8.3. We also simultaneously analyzed wild-type EGFR expression in these tissues using the anti-EGFR monoclonal antibody, EGFR.113. ΔEGFR and wild-type EGFR expression were observed in 20/53 (38%) and 29/53 (55%), respectively. Nineteen (95%) of the ΔEGFR-positive tumors also expressed wild-type EGFR; one case was ΔEGFR-positive but wild-type EGFR-negative. In 13/20 (65%) of the ΔEGFR-positive tumors, tumor cells were scattered diffusely within the tumors, 6/20 showed geographical distribution of ΔEGFR-positive tumor cells, and one case showed homogeneous staining. In the wild-type EGFR-positive cases, almost all tumor cells expressed EGFR. The differential distribution of cells expressing the two receptors observed here may suggest either that ΔEGFR arises at a low frequency from wild-type EGFR-expressing cells, perhaps during the process of gene amplification, or that there is a paracrine-type of interaction between them.

Key words Epidermal growth factor receptor · Glioblastoma · Immunohistochemistry

Indroduction

The epidermal growth factor receptor (EGFR) gene is amplified at the DNA level and overexpressed at the level of mRNA or protein expression in tumor tissues of about 40%–50% of human glioblastoma (GBM) cases.[1-3] This EGFR gene amplification is often followed or accompanied by further gene rearrangement. About two-thirds of such rearrangements result in a particular mutant form called-ΔEGFR, de2-7EGFR, or EGFRvIII, an in-frame deletion of exons 2–7 resulting in a deletion of 267 amino acids.[4-6] The resulting mutant protein is ligand independent, constitutively phosphorylated, and localized primarily to the cell surface.[7-9] ΔEGFR promotes the tumorigenesis of GBM cells in vivo by increasing cellular proliferation,[10] decreasing cellular apoptosis,[11] and promoting tumor cell invasion.[12] However, these conclusions have derived from the analysis of the behavior of xenografts that arose after inoculation of cells expressing relatively homogeneous levels of ΔEGFR, and little is known about the distribution of ΔEGFR-expressing tumor cells in surgical specimens. Since this limits understanding of the clinical significance of ΔEGFR, we performed immunohistochemical analysis of ΔEGFR and wild-type EGFR expression in primary GBM tissues. Our results revealed differential distribution of tumor cells expressing these receptors, suggesting potential interactions between them.

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Materials and methods

Brain tumor samples

Fifty-three GBM specimens that were surgically resected between 1989 and 2002 at Saitama Medical School were subjected to investigation. All tumors were diagnosed according to the World Health Organization (WHO) classification of brain tumors.[13] Tissue samples were fixed in 10% formalin and embedded in paraffin for histological as well as immunohistochemical examination. For Western blotting, samples were snap-frozen in liquid nitrogen in the operating rooms and stored at −80°C until use. Twenty samples were available for this series of studies.

Immunohistochemistry

The sections were deparaffinized, rehydrated, and incubated in hydrogen peroxide to block endogenous peroxidase activity, followed by antigen retrieval in a steam cooker and preincubation in normal goat serum. An anti-ΔEGFR monoclonal antibody, DH8.3, raised against a synthetic peptide spanning the unique junctional sequence of the deleted part of ΔEGFR,[14] was diluted 1:50 and applied to the samples for 1 h at room temperature. DH8.3 does not react with wild-type EGFR.[14] A standard ABC method was performed according to the manufacturer's recommendations (Vectastain, Vector Laboratories, Burlingame, CA, USA), and diaminobenzidine tetrahydrochloride was used to visualize the immunoreactivities. The slides were lightly counterstained with hematoxylin. Anti-EGFR monoclonal antibody, EGFR.113, raised against the extracellular domain of the wild-type EGFR was purchased from Novocastra (Newcastle, UK) and used for immunohistochemistry according to the manufacturer's recommendations.

Western blotting

The tissue specimens were lysed in extraction buffer (50 mM Tris-HCl [pH 7.6], 50 mM NaCl, 2% NP-40, 0.5% deoxycholic acid, 0.2% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5 mM Na_3VO_4) and sonicated. The lysates were centrifuged at 8000 g for 5 min, and the supernatants were collected. Each protein sample (20 μg) was separated with 7.5% polyacrylamide/SDS gels and electroblotted onto nitrocellulose membranes (ECL membrane, Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking with 5% skim milk in Tris-buffered saline with 0.05% Tween 20, the membranes were incubated with an anti-EGFR monoclonal antibody, C13, which reacts with both wild-type EGFR and ΔEGFR (a kind gift from Dr. Gordon Gill, University of California at San Diego), and then incubated with a horseradish peroxidase-conjugated antimouse secondary antibody (Vector Laboratories), and subjected to chemiluminescence detection (ECL, Amersham Pharmacia Biotech).

Image intensification

The images from immunohistochemistry and Western blotting were captured with a digital camera and processed with Adobe Photoshop 7.0 on an Apple Macintosh computer.

Results

Immunohistochemistry

Positive immunoreactivities for DH 8.3 and EGFR.113 were observed in 20/53 (38%) and 29/53 (55%), respectively. Of those 20 cases positive for DH8.3, 19 were also positive and 1 was negative for EGFR.113. Typical examples of DH8.3-immunoreactivity are shown in Fig. 1A–E. Immunoreactivities for DH8.3 were observed on the cell membrane or in the cytoplasm of tumor cells that were similar to those for EGFR.113 (Fig. 1A and F). Immunoreactive cells were scattered diffusely in the 13 of 20 (65%) specimens positive for DH8.3 (Fig. 1A and B). In six specimens (30%), cells immunoreactive for DH8.3 were geographically distributed (Fig. 1C). The remaining specimen showed a homogeneous distribution of immunoreactive cells. In one specimen, DH8.3-immunoreactive cells were observed adhering to tumor vessels (Fig. 1D and E). On the other hand, in the tumors immunoreactive for EGFR.113, almost all tumor cells had homogeneous immunoreactivities, as shown in Fig. 1F, except for one case that showed geographical distribution of EGFR-expressing cells. Figure 1F is from the same area of the same specimen shown in Fig. 1B.

Western blotting

Significant amounts of ΔEGFR and full-length EGFR were detected in 9 of 20 (45%; lanes 5, 6, 8, 9, 10, 11, 13, 17, and 20 in Fig. 2) and 12 of 20 (60%; lanes 2, 5, 6, 7, 8, 9, 10, 11, 13, 15, 17, and 20 in Fig. 2) samples by Western blotting, respectively. Three samples (lanes 2, 11, and 15 in Fig. 2) were negative for EGFR.113 by immunohistochemistry but positive for full-length EGFR by Western blotting. None of the samples negative for full-length EGFR by Western blotting was positive for EGFR.113 by immunohistochemistry, and both results were concordant in 17/20 (85%) of the samples. Four samples (lanes 5, 9, 11, and 17 in Fig. 2) were negative for DH8.3 by immunohistochemistry and positive for ΔEGFR by Western blotting, and both results were concordant in 16/20 (80%).

Discussion

Expression of ΔEGFR was observed in 20/53 (38%) of GBM samples by immunohistochemistry and 9/20 (45%) by Western blotting. Those frequencies were similar to those
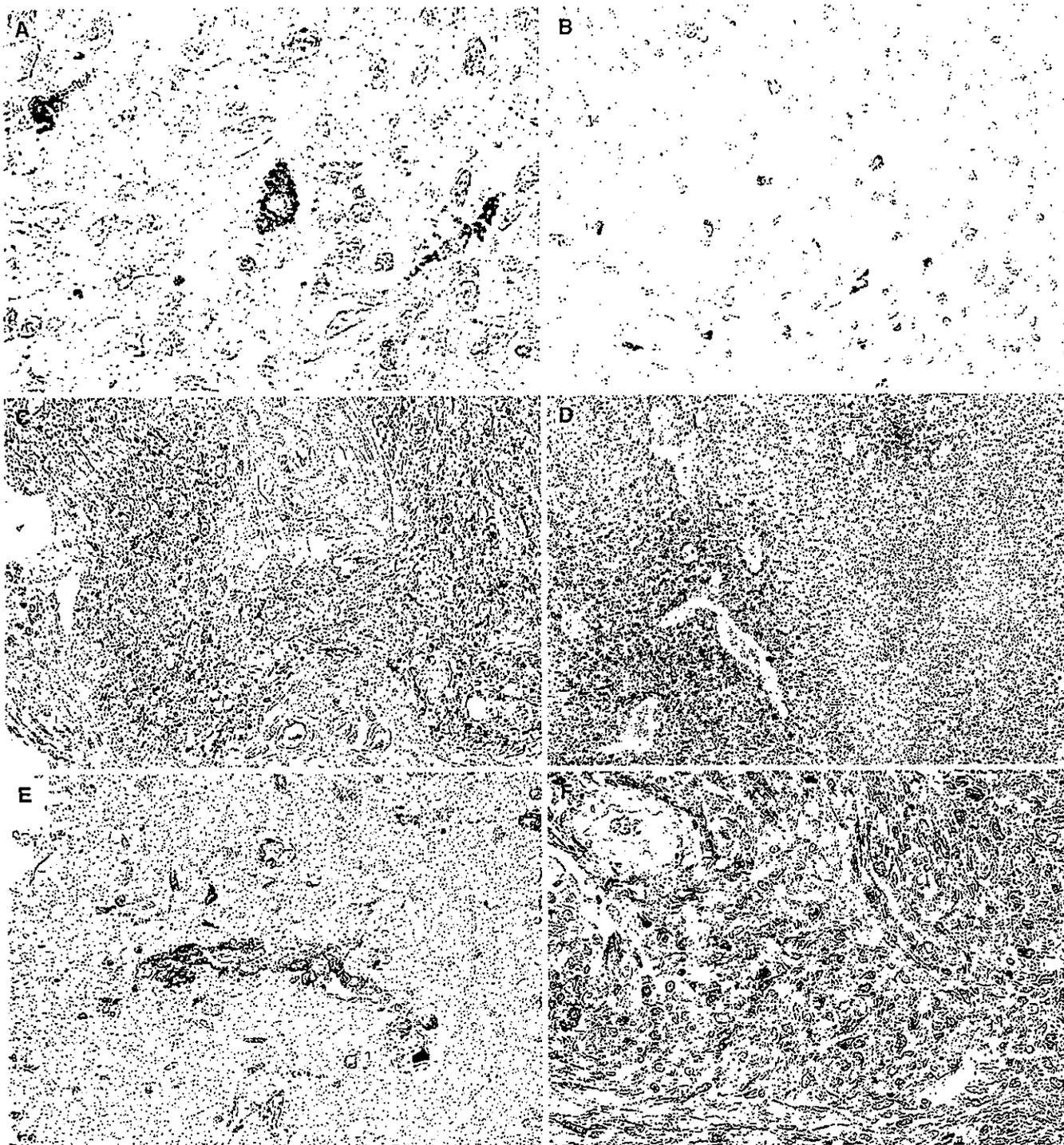
Fig. 1. Immunohistochemical detection of mutated epidermal growth factor receptor (ΔEGFR) (A–E) and wild-type EGFR (F) expression in glioblastoma specimens. Original magnifications are (A) ×1000, (B, C, E, F) ×400, and (D) ×100
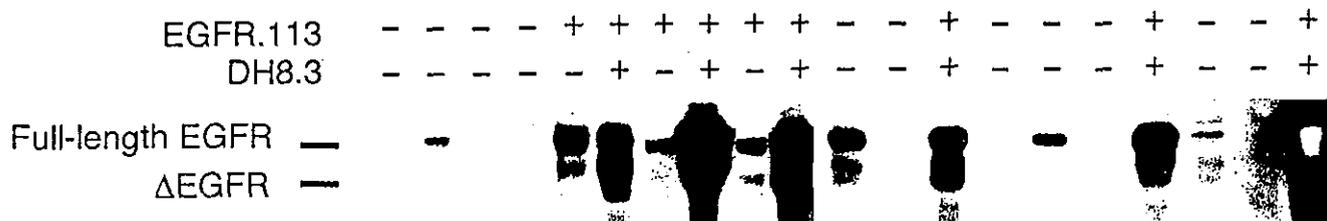

Fig. 2. Western blotting analysis of ΔEGFR and wild-type EGFR expression in glioblastoma specimens. + or − indicates immunoreactivities for DH8.3 and EGFR.113 by immunohistochemistry

in reported series: 7/12 (58%) by Western blotting,[5] and 8/12 (67%)[5] or 12/21 (62%)[3] by immunohistochemistry. Among the 20 cases in which frozen samples were available for Western blotting, the results of Western blotting and immunohistochemistry were concordant in 16 cases (80%). Expression of ΔEGFR in the remaining four cases was positive by Western blotting but negative by immunohistochemistry, which would indicate either that Western blotting was more sensitive for the detection of low levels of ΔEGFR expression, or that there were sampling errors due to the heterogeneous location of positive cells.

Each of the 20 cases that were immunoreactive for DH8.3 by immunohistochemistry was also immunoreactive for EGFR.113, with one exception. Among the 29 cases positive for EGFR.113, 19 (66%) were positive for DH8.3. It has been reported that GBMs with ΔEGFR mutation always harbor increased *EGFR* gene dosage, and frequency of ΔEGFR found in GBMs with *EGFR* amplification was 67%.[6] These data suggest that amplification of the *EGFR* gene precedes *EGFR* mutation.

GBM cells expressing ΔEGFR have a remarkable in vivo growth advantage.[7] One consequence of this could be a clonal expansion of the cell population. Consonant with this notion are reported experiments showing that injection of a mixture of U87MG.ΔEGFR (a GBM cell line expressing ΔEGFR) and parental U87MG at a 1:50 000 ratio into nude mouse brains showed an outgrowth of U87MG.ΔEGFR cells, with the proportion reaching 83% in the tumors that developed.[11] The geographical distribution of ΔEGFR-expressing tumor cells observed in surgical specimens has been believed to be the result of an analogous clonal expansion. However, our results here show that GBM tumor cells expressing ΔEGFR prefer to distribute diffusely in a scattering pattern in the majority of cases. In contrast, tumor cells expressing wild-type EGFR appeared to distribute in a more homogeneous fashion (Fig. 1F). The differential distribution of ΔEGFR- and wild-type EGFR-expressing tumor cells, together with the fact that almost all tumors expressing ΔEGFR expressed wild-type EGFR, appears to support the hypothesis that ΔEGFR arises at a low frequency from wild-type EGFR-expressing cells. The significantly enhanced biological aggressiveness of the tumor cells expressing ΔEGFR, together with their often diffuse occurrence among other tumor cells, also raises the possibility that they might provide a positive field effect on surrounding tumor cells that overexpress wild-type EGFR. The nature of such interactions requires further study.

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Cell Biology of Brain Tumor :
With Special Emphasis on Tumor Cell Invasion

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Introduction

Treatment results of malignant tumors of various organs have been progressing. The 5-year survival rate as well as cure rate have been improved. The treatment results of malignant brain tumors, however, have not improved dramatically with some exceptions such as medulloblastoma and germ cell tumors. The results of gliomas, especially glioblastoma, are extremely poor. In this report, the research history of malignant brain tumors is reviewed stressing importance of cell biological standpoint.

To establish glioma, there are many steps such as initiation of tumor with genetic changes, tumor establishment escaping from immune system, tumor progression with proliferation of cells and angiogenesis, finally invasion to surrounding tissue. All of these steps are the target for malignant brain tumor research and therapy.

Recent advancement of molecular biology and genetic study of glial tumors are remarkable. However these advancements do not directly mean the improvement of treatment, but also does not have good correlation with clinical situations. The most important point which we should focus on is that the tumor cells exist in the host (patient) in the context of host-tumor interaction. Thus even in the era of major advancement in molecular biology, pathology as well as cell biology are important target to study in the neuro-oncology research. In this brief review, cell biological aspects of malignant glioma will be discussed.

Historical Aspect of Research of Proliferative Potential of Malignant Brain Tumor

Biology of glioma has been studied from the standpoint of growth potential. Hoshino and his colleagues opened and developed this field.[17,18] Based on their and other investigators, it is known that in glioma tissue, proliferating tumor cells are recruited from G0 (resting) cells. Irrespective of grade of the tumor, the cell cycle time of glioma is in a narrow range. Thus the ratio of proliferating cells represents the potential of tumor proliferation itself.[23,29] Historically this study of proliferation was initiated with autoradiography using ^{3}H-thymidine.[17] Then bromodeoxyuridine (BrdU, BudR) as thymidine analogue was used widely. BrdU was given to the patients before excision of the tumor or the tumor tissue was incubated in a medium containing BrdU immediately after tumor excision. Cells which are synthesizing DNA (in S phase) incorporate BrdU and incorporated BrdU was stained using monoclonal antibody. Thus cells in S phase can be differentiated from other cells. The S phase labeling index (LI) of each tumor was counted and their proliferative potential was studied.[23] Later this method was replaced by Ki67 staining or MIB-1 staining which stains all the cells in cell cycle.[11] With these approaches, biological behavior of gliomas were gradually disclosed, i.e,

S phase labeling index and its correlation to prognosis of patients.[16,12] Sensitivity to radiation therapy was also assessed by looking at the viable cells after radiation therapy. These methodology are called cell kinetics.

Resistance to Radiation Therapy

Gliomas are radioresistant. Irrespective of intensive radiation therapy, glioblastoma cannot be controlled for longer period. Cell kinetics study of glioma tissue after irradiation showed that proliferative potential of glioblastoma did not significantly change even after 60 Gy of irradiation. After conformal irradiation therapy up to 90 Gy,[25] the glioblasotma still retain proliferative potential in the area received 70 or 80 Gy of irradiation. Furthermore it was observed that damage to the glioblastoma is rather via vascular damage than direct effect to the tumor cells to show that glioma cells are really radioresistant.[13] Thus it should be noted that radiation therapy to glioma produced still unsatisfactory results and major advancement should be done to concur these barrier.

Drug Resistance

Chemotherapy of glioma has been known to have many problems also. However recent advancements highlighted chemotherapy of glioma again. Many studies since Cairncross et al,[7] showed that oligodendroglial tumors are chemosensitive to nitrosourea-vincristine-procarbazine combination chemotherapy.[20] Furthermore, this chemosensitivity has been reported to be related to some genetic changes.[8]

Temozolomide is an another recent topic in the chemotherapy of glioma. Many studies showes that this novel drug has activity against gliomas as well as metastatic brain tumor.[1] For recurrent anaplastic astrocytoma and anaplastic oligoastrocytoma, 45% recurrence-free survival for 6 months have been reported. Radiological study showed 35% response (CR and PR) rate and 26% stable disease.[30] Even in recurrent glioblastoma, 18% of the patients showed 6-months recurrence free period.[3]

Irrespective of these advances in glioma chemotherapy, brain tumors are still difficult target to treat with chemotherapy. The presence of of blood brain barrier (BBB) in the normal tissue is one of the reasons for the difficulty. The center of brain tumors usually lacks BBB, however area of tumor infiltration seems to have intact BBB. Brain tumor model in animals showed that in small tumor of less than 2 mm in diameter[28] or 0.2 mm^2 in area,[31] the BBB is preserved. These data suggest that BBB is intact in small tumors or area of infiltration thus chemotherapeutic agents would not be delivered to tumor cells in these lesions. Success of Temozolomide seems to be, at least partly, due to permeability of this drug through BBB.

Invasion

Invasiveness or infiltration of glioma is one of the major reasons of difficulty in the treatment of glioma. If gliomas were not invasive, the tumors can be cured, of course, by surgery alone, which is not the case.

In malignant glioma, especially in glioblastoma, tumor cells infiltrate far beyond the main tumor mass. The infiltrative tumor cells can be found as far as 3 cm away from the border of the neuroradiological image.[5,6] Before reviewing this invasiveness, general aspects of cancer cell invasion will be discussed.

Invasion of cancer cell is one of the most significant features of malignant tumor together with capability of distant metastasis. In fact, invasion and metastasis are so to say "two sides of the same coin". To establish metastatic foci

cancer cells must proceed sequential multiple steps and at each step invasion mechanisms or part of them work. Initially to create primary tumor mass, tumor cells needs blood supply. Angiogenic process takes place at this point. To leave the primary tumor, the tumor cells must get into the blood or lymphatic stream. To get into the blood or lymphatic vessel, the tumor cells have to degradate basement membrane of vessels, move (motility) and intravasate into vessels. Once get into the blood or lymphatic vessels, the tumor cells should escape from immune system to reach the target organ. At the target organ, the tumor cells have to attach to the endothelium, migrate between the endothelial cells, degradate the basement membrane again and lyse the local extracellar matrix. The tumor cells, then, have to proliferate in the target organ adjusting themselves to the local microenvironment. Interestingly, these are not random processes but certain tumor cells have a specific affinity to certain target organ ("seed and soil" phenomenon).[27] To complete metastatic process, the tumor cells have to have many features, i.e. high motility, high expression of degradating enzymes, high affinity to endothelial cells, higher expression of growth factor receptor which is suitable for the target organ and so on. That means the tumor cells which finally established metastasis were cells with multiple aptitude. Fidler, who is the authority of metastasis research mentioned that "Metastatic tumor cells are champion of decathlon". The word is an apt remark of multiple potential of metastatic cells.

As described above, invasion is one of the most remarkable features of malignant tumor cells. Many techniques *in vitro* and *in vivo* are employed to investigate metastatic phenotype of malignant tumor cells. *In vitro* research includes assay for protein, enzyme expression, and mRNA. *In vitro* experiments using cells to assess their invasiveness of many substrates including proteins consisting basement membrane or intracellular molecules.[2] Motility assay are also employed. *In vitro* experiments to see the interaction between the tumor cells and tissue or *in vivo* experiments to assess tumor host interaction are also important.

These methods were applied in the malignant brain tumor research also and with these many molecules have been reported to be involved in the pathophysiology of invasion of brain cancer. As for extracellular matrix component of brain, where the tumor cells to invade, laminin, collagen IV, tenascin, hyaluronic acid and brain enriched hyaluronan binding (BEHAB)/brevican have been in the list.[19,26,14] Heparan sulfate proteoglycan, collagen I, fibronectin, vitronectin and entactin are found in relation to basement membrane of blood vessels.[14,19,9] Another molecules, which mainly produced by tumor cells and related to tumor cell-matrix adhesion are integrins and hyaluronate receptor CD44.[4] Cadherins, selectins, neural cell adhesion molecule (NCAM), intercellular cell adhision molecule (ICAM) and vascular cell adhesion molecule (VCAM) have also been reported to be related to brain tumor invasion.[14,15] Another type of molecules which play major roles in degradating surrounding substrates are matrix metalloproteases (MMP) including MMP2 and MMP9.[24,21] However role of these MMPs are still controversial in some reports.

Balance between MMP and tissue inhibitor of metalloproteinase (TIMP) was also mentioned to be involved in tumor invasion.[10] Other degradating enzyme, urokinase-type plasminogen activator (uPA) and its receprot (uPAR) might be important.[22]

We have been investigating the role of lysophospholipase D (Lyso PLD)/autotaxin in glioma invasion. This molecule also seems to play some role in glioma invasiveness.

Glioma invasion is a complicated process but without overcoming this, cure of glioma would not be achieved.

Although many factors seem to be related to glioma invasion, further investigation is needed to integrate these findings.

In conclusion, many biological factors are related to malignant behavior of glioma as described here, including tumor cell genetics, proliferation, radiation- and chemosensitivity and invasive nature. Understanding of these factors will lead to better treatment and cure of this devastating disease.

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