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ORIGINAL PAPER

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Systematic immunohistochemical profiling of 378 brain tumors with 37 antibodies using tissue microarray technology

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Abstract We performed a systematic immunohistochemical study on 378 brain tumors using 37 antibodies and tissue microarray (TMA) technology. The aim of this study was to find new diagnostic biomarkers using antibodies established in our laboratory. Our TMA consisted of a grid of 1.5-mm cores that were extracted from individual donor blocks. Staining for each antibody was scored using a three-point system. We used hierarchical clustering analysis to interpret these data, which resulted in separation of all the brain tumors into seven groups. Although there were some exceptions, cases with the same histological diagnosis were generally grouped together. We then carried out statistical analyses to find the most useful antibodies for grouping of brain tumors. Ten antibodies [glial fibrillary acidic protein (GFAP), Olig2, vimentin, epithelial membrane antigen (EMA), cytokeratin (AE1/AE3), alpha-internexin, nestin, pinealocytes PP5, aquaporin-4 (AQP4) M13d and AQP4M13e] discriminated between astrocytomas and oligodendroglial tumors. Six antibodies [EMA, AE1/AE3, TUJ1, nestin, neurofilament protein-MH (NF-MH) and perivascular cells GP-1] showed significant differences between high-grade and low-grade gliomas. Our data have revealed new antibodies with potential diagnostic utility (Olig2, PP5, GP-1) and demonstrate that TMA technology is highly useful for evaluating newly established antibodies in brain-tumor research.

Keywords Brain tumors · Tissue microarray · Immunohistochemistry

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Introduction

Tissue microarrays (TMAs) are powerful, large-scale tools for tumor analysis [12], which have markedly increased the number of available specimens for tumor research. Since staining conditions for all the tissue cores on each TMA slide are identical, technical variation among cases is avoided. Furthermore, the use of reagents is minimized, which leads to cost-effectiveness. TMA technology is still being developed and improved. While at present TMAs are mainly used for immunohistochemistry, they have also been utilized for DNA fluorescence in situ hybridization on paraffin blocks (TMA-FISH) [2, 5, 10], for frozen tissue [4, 14], and cell lines [7].

Brain tumors include many histological subtypes. In the current World Health Organization (WHO) classification, brain tumors are classified into as many as 126 types [11]. Since therapeutic strategies and prognosis are highly dependent on the histological type of tumors, accurate pathological diagnosis is indispensable. Therefore, pathologists need to have adequate knowledge of the morphological and immunohistochemical features of brain tumors, but considerable pathological variety and rareness (eight to ten cases occur per 100,000 Japanese per year) of brain tumors seem to have prevented large-scale immunohistochemical analyses. Some publications applied TMAs to brain-tumor profiling [5, 16, 20, 22, 23]; however, there is still a need for analysis of larger numbers of cases and antibodies. For research, development and application of new diagnostic biomarkers are necessary. Our laboratory has established many polyclonal and monoclonal antibodies against nervous-system antigens [1, 17–19, 26–31]. We are already using some of these antibodies for routine pathological diagnosis. However, the expression patterns of our biomarkers among many types of brain tumors remain unknown.

In this study, we applied TMA technology for exhaustive immunoprofiling of brain tumors in an attempt to find new biomarkers with diagnostic utility.

Materials and methods

Case materials

Paraffin blocks of primary brain tumors were acquired from the pathology archives of Gunma University Hospital and Kantoh Neurosurgical Hospital. The cases dated from 1991 to 2005. Tumors with marked heat degeneration at surgery or marked calcification were not used. The total of 378 cases listed in Table 1 were selected to build a TMA.

TMA construction

Tissue microarray production started with a pathological review of all selected cases. A representative area of each tumor was marked both on a hematoxylin–eosin (H&E) section and an original donor block. One tissue core of 1.5-mm diameter was extracted from the marked area of each donor block using an arraying machine (MTA-1, Beecher Instruments, Sun Prairie, WI, USA). The core was fit into a vertical hole that was bored in a recipient paraffin block in advance. This sampling procedure for each case was repeated many times to produce TMA blocks. Finally, recipient blocks were incubated at 60°C for 5 min, pressed on a hot plate for 3 min, and cooled in ice water. This procedure made the paraffin melt and congeal, which enabled tissue cores to integrate into the recipient block and thereby prevented tissue core loss at sectioning. Sections of 3-μm thickness were cut from each array block. Unused sections were stored at minus 80°C with paraffin coating.

Table 1 Brain tumors in the tissue microarray block

Tumor type	Grade I	Grade II	Grade III	Grade IV
Astrocytic tumors	7	13	7	23
Oligodendroglial tumors	0	20	31	0
Ependymal tumors	0	5	0	0
Choroid plexus papilloma	1	0	0	0
Central neurocytomas	0	4	0	0
Pineal parenchymal tumors of intermediate differentiation[a]	0	0	4	0
Medulloblastomas	0	0	0	7
Schwannomas	39	0	0	0
Meningiomas	100	25	2	0
Hemangioblastomas	6	0	0	0
Hemangiopericytomas	0	0	7	0
Malignant lymphomas	0	0	0	13
Germinomas	0	0	9	0
Craniopharyngiomas	8	0	0	0
Pituitary adenomas	47	0	0	0

[a]We clinicopathologically interpreted pineal parenchymal tumors of intermediate differentiation as grade III tumors

Immunohistochemistry

Immunohistochemical studies were carried out by a streptavidin–biotin–immunoperoxidase technique (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan). The 37 antibodies listed in Table 2 were used in this study. Various antibodies we had established in our laboratory were chosen, including antibodies against pinealocytes (PP1–PP7), pineal interstitial cells (PI1, PI2, and PX1), perivascular cells of the central nervous system (GP-1, GP-2), Schwann cells (Schwann/2E), protoplasmic astrocytes (PRAS-1, PRAS-4), oligodendrocytes (Olig2), and neurofilament protein (NF1D).

Sections of 3-μm thickness were deparaffinized, rehydrated, treated with 0.3% H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity, and rinsed in phosphate buffered saline (PBS). For antigen retrieval before staining with some antibodies, pretreatment was performed as follows: (1) autoclaving in PBS or 0.1 mol/l citrate buffer pH 6.0 for 10 min at 121°C; (2) pronase treatment (0.05% protease, type XXVII, Sigma, St. Louis, MO, USA) for 5 min at room temperature. Sections were then covered with 10% normal goat or rabbit serum for 30 min. Sections were overlaid with optimally diluted antibodies and incubated overnight in a moist chamber at 4°C. Sections were washed three times with PBS and incubated with biotinylated anti-mouse or anti-rabbit IgG for 30 min. After three washes in PBS, sections were incubated with peroxidase-conjugated streptavidin for 30 min. Finally, peroxidase activity was visualized by incubation with 0.02% 3,3′-diaminobenzidine-4HCl (Wako Pure Chemicals, Osaka, Japan) in 0.05 mol/l Tris–HCl buffer (pH 7.6) containing 0.005% H_2O_2 for 5 min. Sections were washed, counterstained lightly with hematoxylin, dehydrated, and mounted. Incubation, except that with primary antibodies, was carried out at room temperature.

Scoring of immunostaining data

Each tumor core was considered to be suitable for evaluation if the tumor occupied more than 10% of the core area. The scoring system was as follows: score 0 (less than 5% of tumor cells stained), score 1 (5–25% of tumor cells stained), and score 2 (more than 25% of tumor cells stained).

Data analysis and statistics

Staining data were recorded directly into Microsoft Excel worksheets and reformatted into a suitable form for hierarchical clustering analysis. This reformat procedure was performed using the TMA-Deconvoluter program, freely available at the Stanford TMA software Web site (http://www.genome-www.stanford.edu/TMA/) [15]. The hierarchical clustering analysis was performed with the Cluster program, and the results

Table 2 List of antibodies used for immunohistochemistry

Antibody	Source	Category	Dilution	Antigen retrieval method
PP1	[19]	Mouse monoclonal	1:10	None
PP2	[19]	Mouse monoclonal	1:50	None
PP3	[19]	Mouse monoclonal	1:300	None
PP4	[19]	Mouse monoclonal	1:50	None
PP5	[19]	Mouse monoclonal	1:800	None
PP6	[19]	Mouse monoclonal	1:200	None
PP7	[19]	Mouse monoclonal	1:100	None
PI1	[19]	Mouse monoclonal	1:100	None
PI2	[19]	Mouse monoclonal	1:100	None
PX1	[19]	Mouse monoclonal	1:10	None
GP-1	[30]	Mouse monoclonal	1:50	None
GP-2	[30]	Mouse monoclonal	1:100	None
Schwann/2E	[1]	Mouse monoclonal	1:50	None
PRAS-1	[28]	Mouse monoclonal	1:50	None
PRAS-4	[29]	Mouse monoclonal	1:50	None
S-100	[26]	Rabbit polyclonal	1:20,000	None
GFAP	[17]	Rabbit polyclonal	1:10,000	None
Olig2	[31]	Rabbit polyclonal	1:10,000	Autoclaving (citrate buffer)
NF1D	[18]	Mouse monoclonal	1:50	None
NF70/200k	SCYTEK, Logan, UT, USA	Mouse monoclonal	1:50	None
NF-MH	Zymed, San Francisco, CA, USA	Mouse monoclonal	1:5	Autoclaving (PBS)
Chromogranin A	DakoCytomation, Glostrup, Denmark	Rabbit polyclonal	1:1,000	None
α-Internexin	NOVOCASTRA, Newcastle upon Tyne, UK	Mouse monoclonal	1:50	Autoclaving (citrate buffer)
TUJ1	Covance Research Products, CA, USA	Mouse monoclonal	1:1,500	None
Nestin	IBL, Gunma, Japan	Rabbit polyclonal	1:50	None
Vimentin	DakoCytomation, Glostrup, Denmark	Mouse monoclonal	1:200	None
c-kit	DakoCytomation Japan, Kyoto, Japan	Rabbit polyclonal	1:50	Autoclaving (citrate buffer)
AE1/AE3	Boehringer Mannheim	Mouse monoclonal	1:500	Pronase
MIC2	DakoCytomation, Carpinteria, CA, USA	Mouse monoclonal	1:50	None
CD68	DakoCytomation, Glostrup, Denmark	Mouse monoclonal	1:50	Pronase
NeuN	Chemicon, Temecula, CA, USA	Mouse monoclonal	1:1,000	Autoclaving (citrate buffer)
PLAP	DakoCytomation, Glostrup, Denmark	Rabbit polyclonal	1:1,000	None
CD34	Nichirei Corporation, Tokyo, Japan	Mouse monoclonal	1:200	None
EMA	DakoCytomation, Glostrup, Denmark	Mouse monoclonal	1:100	None
AQP4M13d	Courtesy of Prof. K. Takata	Rabbit polyclonal	1:4,000	None
AQP4M13e	Courtesy of Prof. K. Takata	Rabbit polyclonal	1:8,000	None
Glut5	IBL, Gunma, Japan	Rabbit polyclonal	1:400	Autoclaving (citrate buffer)

GFAP glial fibrillary acidic protein, *NF* neurofilament, *TUJ1* neuronal class III beta tubulin clone TUJ1, *PLAP* placental alkaline phosphatase, *EMA* epithelial membrane antigen, *AQP* aquaporin, *PBS* phosphate buffered saline

were visualized using the TreeView program. Both programs were freely available at the Eisen Lab Web site (http://www.rana.lbl.gov/EisenSoftware.htm). Clustered data were displayed with antibodies on the horizontal axis and cases on the vertical axis. Cases or antibodies were placed next to each other if they had the most similar expression profiles. Length of branches was inversely proportional to profiling similarity. Cases with 50% or less interpretable scores were excluded from the hierarchical clustering analysis.

Staining results were compared between two tumor types by Fisher's exact test using StatView for Windows version 5.0 software (SAS Institute Inc.). A significant difference was declared if the P value was less than 0.05. If multiple significance tests are conducted in parallel, inflation of the overall type-1 error may occur. Because the significance level is assumed to be 5% in this study, 5% of results are expected to be false positives. Usually, no strict correction of significance levels of the single tests is carried out in statistical analyses of TMA data.

Among all conducted tests, a certain number of false positive results is accepted.

Results

Hierarchical clustering analysis

Nineteen cases were excluded from the hierarchical clustering analysis because their data were insufficient (i.e., interpretable scores were 50% or less). The reasons for insufficient data were mainly inadequate tumor volume or tissue core loss at sectioning. The clustering of the remaining 359 cases produced seven groups, group A to group G (Fig. 1a). All the groups included more than one histological type. The number of cases and the major histological type in each group were, respectively, as follows: group A (eight cases, germinomas); group B (95 cases, gliomas); group C (19 cases, malignant lymphomas and hemangioblastomas); group D (131 cases, meningiomas); group E (37 cases, schwannomas); group F (50 cases, pituitary adenomas); group G (19 cases, medulloblastomas, neuronal tumors and pineal parenchymal tumors). Occasionally, cases with the same histological diagnosis were placed into different groups. For example, two of 126 meningiomas were placed into group C or E, not into group D. The other results of clustering were as follows: ependymomas were placed into group B; hemangiopericytomas were placed into group F; craniopharyngiomas were placed into group F.

In the same way, clustering analysis of the 37 antibodies used for immunohistochemistry was carried out (Fig. 1b). The length of branches above antibody names in the figure is inversely proportional to the profiling similarity. Two biomarkers with a close relationship were placed next to each other [for example, neurofilament (NF) 1D and NF70/200k, antibodies against neurofilament protein]. However, two adjacent antibodies did not always have a close relationship (for example, Glut5 and vimentin). A close-up view of the clustering analysis results for a part of group B is shown in Fig. 1c. Gliomas of different histological types are placed therein. However, gliomas were not completely divided in accord with their cell of origin or grade. To find biomarkers that further differentiate gliomas, we adopted another method: statistical analysis.

Statistical analysis

All gliomas were examined in terms of cell of origin and grade. Ten antibodies [glial fibrillary acidic protein (GFAP), Olig2, vimentin, epithelial membrane antigen (EMA), cytokeratin (AE1/AE3), alpha-internexin, nestin, PP5, aquaporin-4 (AQP4) M13d and AQP4M13e] significantly distinguished between astrocytomas and oligodendrogliomas. The expression of these biomarkers in astrocytic tumors and oligodendroglial tumors is shown in Table 3a, Fig. 2a, and Table 3b, Fig. 2b, respectively (p values are indicated in Table 4).

Six antibodies [EMA, AE1/AE3, neuronal class III beta tubulin clone TUJ1, nestin, neurofilament protein-MH (NF-MH) and GP-1] differentiated between high-grade (WHO grade III or IV) and low-grade (WHO grade I or II) gliomas. The expression of these biomarkers in high- and low-grade gliomas is shown in Table 5a, Fig. 3a and Table 5b, Fig. 3b, respectively (P values are indicated in Table 6).

Discussion

We applied TMA for exhaustive profiling of a wide variety of brain tumors. As a result, we observed distinct immunostaining features with several antibodies. Specifically, some antibodies obtained previously in our laboratory were found to be useful for subtyping or grading of gliomas, including Olig2, PP5, and GP-1. In addition, immunostaining results we obtained using commonly used antibodies were in accord with previous findings (for example, schwannomas were positive for S-100). We also found that some antibodies yielded unexpected clues with potential diagnostic usefulness. Additional experiments will be needed to draw definitive conclusions regarding these issues.

For discrimination between astrocytic and oligodendroglial tumors, ten biomarkers were useful (Tables 3, 4; Fig. 2). The distinction between these two types of tumors is important because oligodendroglial tumors often show chemosensitivity to procarbazine, lomustine (CCNU), and vincristine (PCV) therapy and a more favorable clinical behavior. A previous study showed that the expression of several oligodendroglial lineage genes was frequently increased in gliomas; nevertheless, no significant expression difference between astrocytic tumors and oligodendroglial tumors was detected [21]. In our study, we found Olig2 to be useful for distinguishing between these tumors. Olig2 recognizes a transcription factor that regulates oligodendroglial development. Olig2 labels the nuclei of oligodendrocytes and oligodendroglial tumors, and besides, astrocytomas are also positive for Olig2 to a lesser extent [31]. Our data are in accord with that finding. Another useful antibody we had obtained was PP5, an antibody against human pineal antigens [19, 27].

Other biomarkers that discriminated between astrocytoma and oligodendroglial tumors included vimentin and AQP4. Antivimentin immunoreactivity has been shown in gliomas and ependymomas but not in neuronal tumors [13]. Our data obtained in the current study were in accord with those findings. Additionally, vimentin was expressed more frequently in astrocytic tumors than in oligodendroglial tumors. AQP4 is a water channel protein that is highly expressed in astrocytes. AQP4

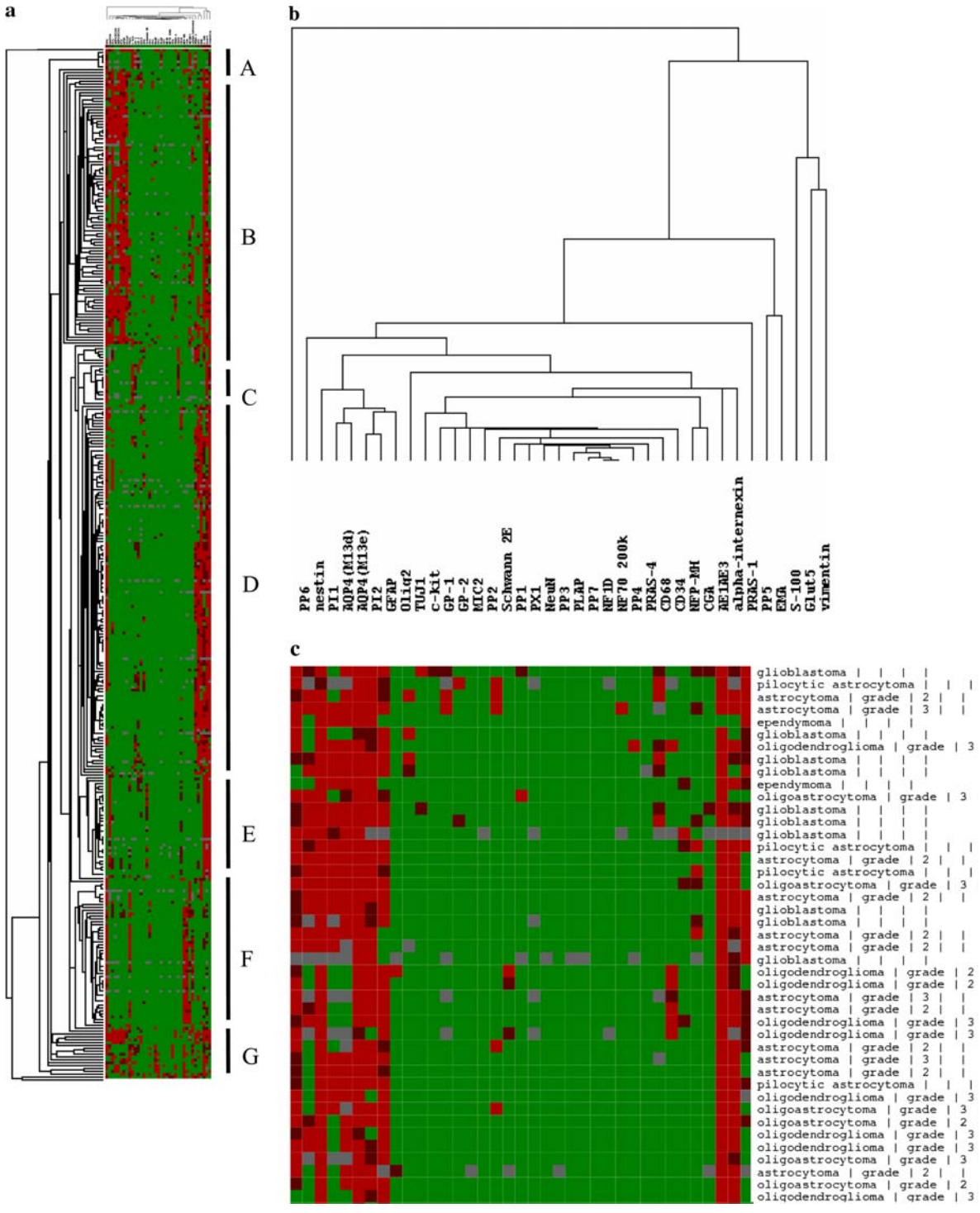

Fig. 1 Hierarchical clustering analysis of brain tumors. **a** A full-length view of the diagram with cases on the *vertical axis* and antibodies on the *horizontal axis*. **b** A close-up view of the diagram of antibody clustering. Two biomarkers with a close relationship are located next to each other. **c** A close-up view of the diagram of clustering analysis of a part of group B. *Red, brown, and green cubes* indicate score 2, score 1, and score 0, respectively. *Grey cubes* indicate the absence of staining data. Gliomas of different cell of origin or grade are placed together

is considered to be involved in regulating water flux between vascular and glial compartments and responsible for development of vasogenic edema in the brain. It has been shown that AQP4 and potassium channel protein Kir4.1 are redistributed in glioblastomas [24], and low- and high-grade gliomas [25], respectively. However, our

Table 3 Biomarker expression in astrocytic tumors and oligodendroglial tumors

Antibody	Score 0	Score 1	Score 2
(a) Astrocytic tumors			
GFAP	0 (0%)	3 (6.4%)	44 (93.6%)
Olig2	8 (17.4%)	13 (28.3%)	25 (54.3%)
Vimentin	5 (10.9%)	10 (21.7%)	31 (67.4%)
EMA	39 (84.8%)	5 (10.9%)	2 (4.3%)
AE1/AE3	24 (55.8%)	7 (16.3%)	12 (27.9%)
α-Internexin	42 (93.4%)	1 (2.2%)	2 (4.4%)
Nestin	9 (20.4%)	5 (12.3%)	30 (68.3%)
PP5	28 (58.3%)	6 (12.5%)	14 (29.2%)
AQP4M13d	14 (32.5%)	3 (7.0%)	26 (60.5%)
AQP4M13e	3 (7.1%)	4 (9.5%)	35 (83.4%)
(b) Oligodendroglial tumors			
GFAP	10 (20.8%)	12 (25.0%)	26 (54.2%)
Olig2	1 (2.0%)	2 (4.0%)	47 (94.0%)
Vimentin	31 (64.5%)	13 (27.0%)	4 (8.5%)
EMA	49 (100%)	0 (0%)	0 (0%)
AE1/AE3	40 (80.0%)	6 (12.0%)	4 (8.0%)
α-Internexin	22 (45.8%)	1 (2.1%)	25 (52.1%)
Nestin	21 (45.6%)	7 (15.2%)	18 (39.2%)
PP5	44 (88.0%)	2 (4.0%)	4 (8.0%)
AQP4M13d	32 (71.1%)	0 (0%)	13 (28.9%)
AQP4M13e	17 (38.6%)	2 (4.5%)	25 (56.9%)

For antibody definitions, see Table 2

study showed significant differences of the expression profiles of AQP4 between astrocytic tumors and oligodendroglial tumors rather than between high- and low-grade gliomas.

Glioma grade markedly affects patients' prognosis. This work showed that six biomarkers were significantly able to discriminate between high- and low-grade gliomas (Tables 5, 6; Fig. 3). Interestingly, most of these biomarkers were epithelial markers (EMA, AE1/AE3) or neuronal markers (TUJ1, nestin and NFP-MH). Gliomas tend to have EMA immunoreactivity more frequently as their grade becomes higher [6]. In addition, according to a previous study, TUJ1 immunoreactivity is significantly stronger in high-grade astrocytomas than in low-grade astrocytomas [9]. Our data were in accord with those findings. The antigens reacting with GP-1 were expressed more frequently in high-grade gliomas than in low-grade gliomas. GP-1 is an antibody that recognizes lysosomal proteins in the perivascular cells of the central nervous system [30].

In this study, we addressed two technical issues related to TMA technology, namely, TMA construction and data analysis. One limitation of TMAs is that a given tissue core is not always representative of the whole tumor. To overcome this problem, in most recent studies, multiple 0.6-mm cores were prepared at tissue extraction. We attached importance to consistent, reproducible histological observation rather than to examining a number of cores mounted on one recipient block. To ensure the adequate representation of diverse brain tumor tissues, we extracted one 1.5-mm core instead of two or three 0.6-mm cores. Although the tissue of a 1.5-mm core is 6.25 times as large as that of a 0.6-mm core [$(1.5/0.6)^2 = 6.25$], the tissue loss in each donor block was trivial. We paid careful attention to the conditions for storage of unused TMA sections. A previous study demonstrated that each core on a section lost its antigenicity within 1 month if a section was stored unwrapped in room air [3]. That study indicated that frozen storage of slides with paraffin coating held antigenicity loss to a minimum. Using this method, we obtained satisfactory immunostaining results even 10 months after sectioning.

The other issue was the strategy for data analysis. Because the amount of data generated by TMAs is huge,

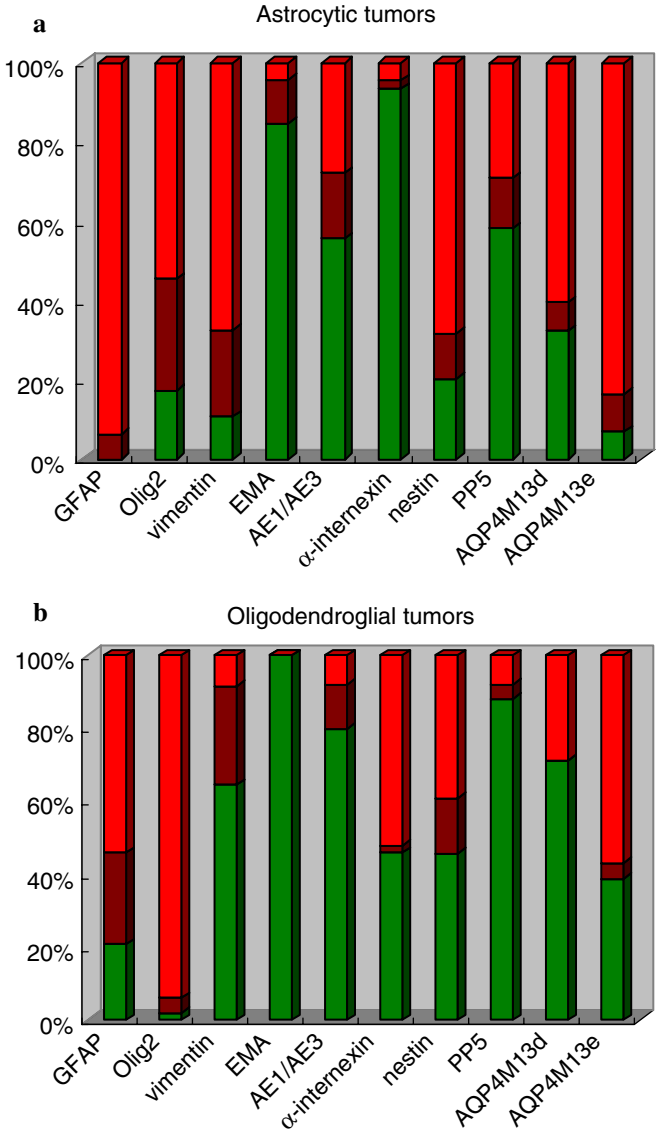

Fig. 2 Comparison of marker expression patterns between astrocytic tumors (**a**) and oligodendroglial tumors (**b**). *Three-step bars* show percentages of tumors positive for each antibody. *Red, brown, and green bars* indicate score 2, 1, and 0, respectively. All the antibodies here showed significant immunostaining ($P < 0.05$)

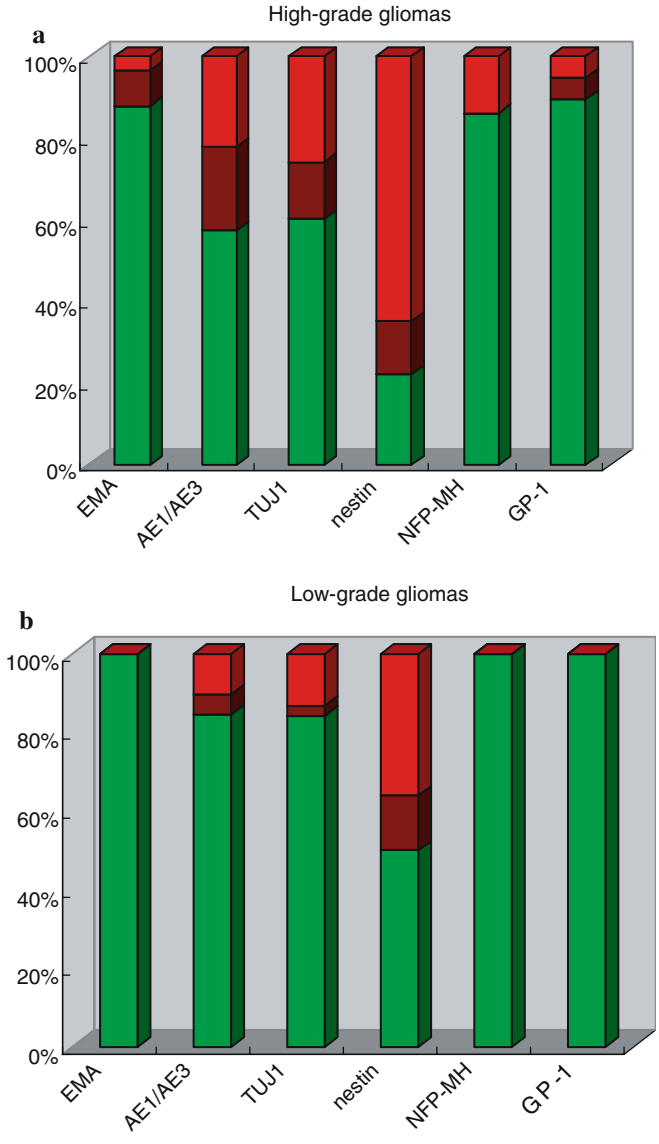

Fig. 3 Comparison of marker expression patterns between high- (**a**) and low-grade gliomas (**b**). All antibodies here showed significant immunostaining ($P < 0.05$)

Table 4 Useful antibodies for discrimination between astrocytic tumors and oligodendroglial tumors

Antibody	*P* value
GFAP	0.0012
Olig2	0.016
Vimentin	< 0.0001
EMA	0.0374
AE1/AE3	0.0037
α-Internexin	< 0.0001
Nestin	0.0143
PP5	0.016
AQP4M13d	0.0006
AQP4M13e	0.0007

For antibody definitions, see Table 2

Table 5 Biomarker expression in high- and low-grade gliomas

Antibody	Score 0	Score 1	Score 2
(a) High-grade gliomas			
EMA	51 (87.9%)	5 (8.6%)	2 (3.5%)
AE1/AE3	31 (57.4%)	11 (20.4%)	12 (22.2%)
TUJ1	35 (60.3%)	8 (13.8%)	15 (25.9%)
Nestin	12 (22.2%)	7 (13.0%)	35 (64.8%)
NFP-MH	50 (86.2%)	0 (0%)	8 (13.8%)
GP-1	51 (89.4%)	3 (5.3%)	3 (5.3%)
(b) Low-grade gliomas			
EMA	37 (100%)	0 (0%)	0 (0%)
AE1/AE3	33 (84.7%)	2 (5.1%)	4 (10.2%)
TUJ1	32 (84.4%)	1 (2.6%)	5 (13.0%)
Nestin	18 (50.0%)	5 (13.9%)	13 (36.1%)
NFP-MH	38 (100%)	0 (0%)	0 (0%)
GP-1	40 (100%)	0 (0%)	0 (0%)

For antibody definitions, see Table 2

Table 6 Useful antibodies for discrimination between high- and low-grade gliomas

Antibody	*P* value
EMA	0.0403
AE1/AE3	0.0064
TUJ1	0.0137
Nestin	0.0113
NFP-MH	0.0203
GP-1	0.0406

For antibody definitions, see Table 2

novel analytic methods are required. In fact, the number of our data points was 13,986 (378 cases with 37 antibodies). To analyze this enormous amount of data, we performed two-step analysis. First, we qualitatively classified all cases with a hierarchical clustering analysis. Then, we quantitatively and statistically evaluated tumor marker expression. This procedure allowed both a simple overview and detailed investigation of all the cases.

Finally, we demonstrated that TMA technology was a powerful method for brain-tumor profiling. On the other hand, TMAs generate such a large amount of data that it is necessary to verify and analyze the data carefully. TMAs can be used for identifying prognostic parameters. For example, loss of heterozygosity on chromosome arms 1p and 19q is predictive of chemosensitivity and longer survival in oligodendroglial tumors [8]. This genetic alteration can be found by TMA with FISH. Our findings demonstrated that TMA technology is highly useful for assessing the usefulness of newly established antibodies for brain-tumor research.

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