Original article

Diversity of glial cell components in pilocytic astrocytoma.

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To characterize the cellular density and proliferative activity of glial fibrillary acidic protein (GFAP)-negative cells in pilocytic astrocytoma (PA), surgically excised tissues of PAs (n=37) and diffuse astrocytomas (DAs) (n=11) were examined morphologically and immunohistochemically using antibodies against GFAP, Olig2, Iba1 and Ki-67 (MIB-1). In PA, Olig2 immunoreactivity was significantly expressed in protoplasmic astrocytes in microcystic, loose areas and cells in oligodendroglioma-like areas. Iba1-positive, activated microglia/macrophages were also commonly observed in microcystic areas. In compact areas, a prominent reaction for GFAP was observed, but for Olig2 and Iba1 to a lesser degree. On semiquantitative analysis, the number of Olig2-positive cells was significantly higher in PAs (mean labeling index (LI) ± standard deviation (SD): 46.8 ± 15.4%) than in DAs (13.3 ± 7.8%) (P<0.001). Many Iba1-positive, microglia/macrophages were observed in PAs (19.9 ± 6.5%), similarly to DAs (20.9 ± 9.9%). Re-immunostaining of PA demonstrated that most Ki-67-positive, proliferating cells expressed Olig2, whereas GFAP or Iba1 expression in Ki-67-positive cells was less frequent (14.7 ± 13.7%, and 8.8 ± 13.6%) in a double immunostaining study. Conversely, the percentage of Olig2-positive, proliferating cells in total Olig2-positive cells (7.2 ± 3.9%) was higher than that of Iba1-positive, proliferating cells in total Iba1-positive cells (0.9 ± 0.6%). In conclusion, the present study found that PA consisted of numerous GFAP-negative cells, including Olig2-positive cells with high proliferation. Semiquantitative analysis of Olig2 immunohistochemistry in microcystic areas might therefore be useful for the differential diagnosis of PA and DA.

Key words: pilocytic astrocytoma, diffuse astrocytoma, Olig2, Iba1, immunohistochemistry

INTRODUCTION

Pilocytic astrocytoma (PA) is a histologically well-characterized glial tumor. In addition to characteristic biphasic pattern, such as compact and microcystic areas, there are sometimes oligodendroglioma-like areas that closely resemble oligodendrogliomas (ODs). Several previous studies elucidated the immunohistochemical characterization of PA and there is little doubt that many Olig2-positive cells are distributed in PA.[1,2] It is also well known that a high number of microglia/macrophages are consistently seen in gliomas.[3-5] Sasaki *et al*.[4] found that PA often contains high numbers of glucose transporter 5 (GLUT5)-positive microglia/macrophages when compared to other astrocytic and oligodendroglial tumors. In addition, Klein *et al*.[6] reported the highest levels of proliferating microglia in PA among astrocytic tumors. However, despite the pronounced existence of Olig2-positive cells and microglia/macrophages in PA, there have been no detailed semiquantitative analysis of their proportion and proliferative activity.

Development of novel immunohistochemical markers such as the oligodendroglial marker Olig2 and microglial marker Iba1 has been pivotal in the diagnosis and research of brain tumors. Olig2 is a transcription factor that regulates the phenotype specification of cells of oligodendroglial lineage.[7,8] Antibodies to Olig2 have demonstrated utility in paraffin sections for recognizing normal oligodendrocytes, oligodendrocytic tumors, and astrocytic tumors to some extent.[1,9,10] Ionized calcium binding adaptor molecule 1, Iba1 mediates calcium signals in the monocytic lineage,[11] and its antibody detects monocytic cells and macrophages including microglia. Iba1 is expressed in both ramified and activated microglia.[12] Its antigenicity is preserved in paraffin-embedded tissue sections after considerably long fixation.[12] Previous studies have selected other microglial markers such as GLUT5,[4] CD68 (Ki-M1p),[13] major histocompatibility complex (MHC) class II[14]; Iba1 has only been employed by a few studies.[15]

PA shows intense glial fibrillary acidic protein (GFAP) positivity in compact areas, but less so in microcystic areas. Furthermore, cells in oligodendroglioma-like area are not stained with GFAP antibody at all. To solve the underlying mechanism of this heterogeneity, we elucidated the characteristics and distribution of GFAP- negative cells in PA by immunohistochemistry using Olig2 and Iba1 antibodies, and the mean labeling index (LI) of PAs was compared with that of diffuse astrocytomas (DAs), for which

differential diagnosis is sometimes needed. In addition, we examined the proliferative activity of GFAP-, Iba1-, or Olig2-positive cells in PA by double or re-immunoperoxidase staining. In a case with numerous Olig2-positive cells, fluorescence *in situ* hybridization (FISH) analysis was performed to exclude genetic changes frequently seen in OD. Finally, we addressed tumor cell origin and growth mechanism in PA.

MATERIALS AND METHODS

Tissue specimens

Tissue specimens were obtained from 37 cases of PAs (0-66 years old, mean age 24.2 ±21.2 years, 15 males and 22 females, 9 supratentorial and 28 infratentorial) between 1980 and 2005 and 11 cases of DAs (27-79 years old, mean age 43.5 ±14.5 years, 6 males and 5 females) between 1995 and 2004. Informed consent was obtained from all patients whose tumor materials were used, and consent was also obtained from the local ethics committee. Patients underwent craniotomy and were pathologically diagnosed, having been identified from the pathology files of Gunma University Hospital and an affiliated hospital. All histologic materials were reviewed according to the World Health Organization (WHO) classification,[16,17] and selected when there were available paraffin blocks containing sufficient tissue for analysis.

Immunohistochemistry

Single immunoperoxidase staining

Routine immunohistochemical examination using the biotin-streptavidin immunoperoxidase method (Histofine kit, Nichirei, Tokyo, Japan) was performed in formalin-fixed, paraffin embedded tissue sections. Anti-GFAP (polyclonal, our own, 1:5000)[18], anti-Olig2-C (Olig2, polyclonal, our own, 1:5000)[9], anti-Iba1 (polyclonal, Wako pure chemical industries, Japan, 1:250), and anti-Ki-67 (MIB-1, monoclonal, Dako, Glostrup, Denmark, 1:50) were used. For antigen retrieval before staining against Olig2, Iba1, and Ki-67, sections were autoclaved (121ºC, 5 min, in phosphate buffered saline pH7.4). After visualization with diaminobenzidine (DAB), tissue sections were briefly counterstained with hematoxylin.

For semiquantitative analysis, LI of Olig2, Iba1, or Ki-67 was calculated as the percentage of positive cells in the total number of tumor cells in microscopic fields using the optical grid of a 40x objective lens. We used sections where at least total 3000 cells positive for Olig2 or Iba1 and 2000 cells for Ki-67 could be counted, and LI was calculated in the area of maximal labeling.

Double immunoperoxidase staining and re-immunoperoxidase staining

First, 2 arbitrarily selected PA cases were analyzed by double immunostaining to examine the intracellular localization and the possibility of colocalization of these antigens. Double immunostaining was performed according to the method reported previously [19] using DAB (brown) for the first antigen and 4-chloro-1-naphthol (dark purple) for the second antigen. The combinations of antibodies were GFAP with Olig2, Olig2 with Iba1, and Iba1 with GFAP.

Second, double immunostaining for Ki-67 with GFAP, Ki-67 with Iba1, and re-immunostaining for Ki-67 with Olig2 were performed in all PA cases to examine cellular proliferation. The double immunostaining method was the same as mentioned above: Ki-67 for the first antigen and GFAP or Iba1 for the second antigen, respectively. Briefly, for the re-immunostaining method, immediately after the first step using Ki-67 with the 4- chloro- 1-naphthol reaction, digital pictures were taken and saved on computer. Thereafter, sections were treated with 0.1 M glycine-HCl buffer following a peroxidase reaction with DAB for the second antigen Olig2. Hematoxylin counterstaining was performed on the re-stained sections, and they were compared with the digital pictures of the same microscopic fields taken beforehand.

For semiquantitative analysis, the first approach was applied to GFAP and Iba1 antibodies. In sections where at least 100 Ki-67-positive nuclei could be counted, LI was calculated in a way that the number of double-positive, proliferating cells was divided by the estimated number of total Ki-67-positive nuclei, and multiplied by 100. The second approach was applied to Iba1 and Olig2 antibodies. In sections where least 1000 Iba1- or Olig2-positive cells could be counted, LI was calculated in a way that the number of double- or re-positive, proliferating cells were divided by the estimated number of total Iba1- or Olig2-positive cells, and multiplied by 100.

Fluorescence *in situ* hybridization (FISH)

In a case of PA with numerous Olig2-positive cells, dual-probe hybridization was performed on paraffin-embedded 3-μm-thick tissue sections using a previously published FISH protocol.[20] We used the 1p probe (RP11-260I13, 1p32) labeled with spectrum green and the 1q probe(RP11-184E11, 1q42) labeled with spectrum orange. Signals of one hundred nonoverlapping nuclei were counted, and a ratio of green signals to orange signals of <0.8 was considered to constitute an allelic loss of 1p, as described previously.[21]

Statistical analysis

Mean LIs of PAs and DAs for Olig2-positive cells or Iba1-positive cells were calculated, and the Mann-Whitney U-test using JMP-IN 5.1 software (SAS Institute Inc) was used to determine statistical significance.

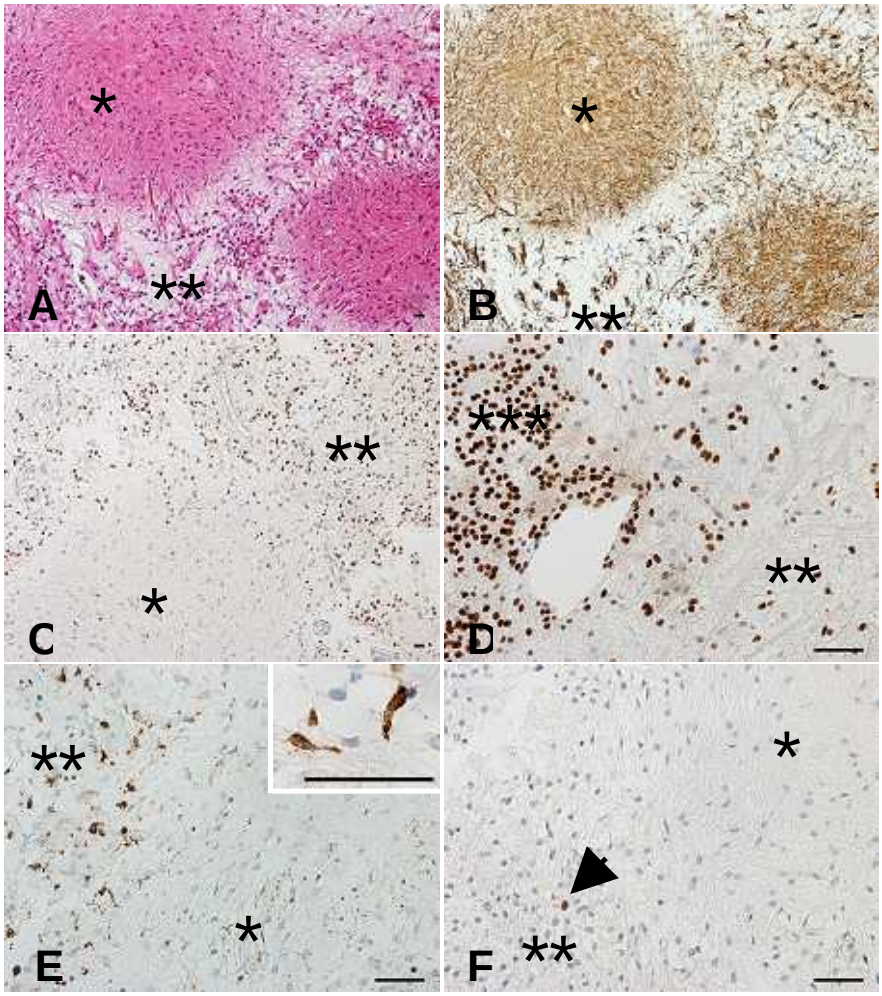

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Fig. 1 Histological and immunohistochemical findings in PA. Compact areas and microcystic areas are marked with a single asterisk (*) and a double asterisk (**), respectively. (A) Characteristic biphasic pattern seen on HE staining. (B) Long, bipolar tumor cells in the compact area show strong immunoreactivity for GFAP. (C) Tumor cells with ambiguous processes in the microcystic area show strong reactivity for Olig2. (D) Nuclei of honeycomb cells in oligodendroglioma-like area (***) show strong immunoreactivity for Olig2. (E) Iba1-positive microglia/macrophages are commonly distributed in microcystic area. The inset shows a higher magnification of Iba1-positive, activated microglia. (F) A small number of Ki-67-positive proliferating cells (arrow) was mainly distributed in microcystic area. Bars=50μm.

RESULTS

Pilocytic astrocytoma

Immunoreactivity for GFAP, Olig2, and Iba1 was detected in all 37 cases of PA. A biphasic pattern was seen in 34 cases (92.0%) (Fig. 1A). A prominent reaction for GFAP was observed in compact, fiber-rich areas and only a weak reaction was observed in microcystic, loose areas (Fig. 1B). Immunostaining for Olig2 was confined to nuclei, and was consistently present in cells with small nuclei and few flaccid processes in microcystic areas, but not significant in compact areas (Fig. 1C). A large number of Olig2-positive cells was observed in tumor areas with honeycomb cells resembling OD (Fig. 1D). Immunostaining for Iba1 was predominantly cytoplasmic and weakly nuclear. Iba1-positive cells were commonly observed in microcystic areas, whereas only a minority of them was distributed in compact areas (Fig. 1E). Iba1-positive cells were morphologically compatible with activated microglia and macrophages (microglia/macrophages), including unipolar cells. Ramified, resting microglia were scarcely seen in the tumor tissues. Ki-67-positive cells were few and predominantly distributed in microcystic areas (Fig. 1F). There was no correlation between the number and intensity of immunoreactivity and clinicopathological findings (patient age, sex and tumor location, data not shown).

Double immunoperoxidase staining using combinations of two antibodies between GFAP, Iba1, and Olig2 in a randomly selected two cases of PA demonstrated that there was no double labeling and that these antibodies were mutually exclusive (Fig. 2A-C). Double immunostaining showed brown nuclear staining for Ki-67 with DAB and dark purple cytoplasmic staining for GFAP and Iba1 with 4-chloro-1 naphtol (Fig. 2D, E). In the re-immunoperoxidase staining method, almost all Ki-67-positive nuclei were also stained for Olig2 (Fig. 2F, G). Double-positive proliferating cells were predominantly distributed in microcystic and oligodendroglioma -like areas, and to a lesser degree in compact areas.

Semiquantitative analyses of Olig2- and Iba1- positive cells were performed in 24 cases. Thirteen cases that were small specimen without microcystic or oligodendroglioma-like areas were excluded. The LI of GFAP-positive cells was not counted because of the complex arrangement of counterstained nuclei and GFAP-positive processes. As a result, the mean LI for Olig2 was 46.8 ±15.4% (SD) and the mean LI for Iba1 was 19.9 ± 6.9% (Fig.

3A).

Semiquantitative analyses of double immunostaining for GFAP with Ki-67 or Iba1 with Ki-67 was performed in 8 cases. The mean Ki-67 LI was 3.6 ± 2.2% (Fig. 3B). The mean LI of double-positive, proliferating cells in total Ki-67-positive, proliferating cells was 8.8± 13.6% for GFAP, and 14.7 ± 13.7% for Iba1, respectively (Fig. 3B). The number of Ki-67-positive cells in serial sections of double immunostaining with GFAP or Iba1 was almost equal in each section. However, the number of Ki-67-positive cells was fewer in re-immunostaining with Ki-67 and Olig2 than in double immunostaining for GFAP or Iba1, probably due to weak staining in the former. Thus, we could not perform comparative analysis of Olig2-positive cells in total proliferating cells.

Conversely, the mean LI of Iba1-positive, proliferating cells in total Iba1-positive cells was 0.9 ± 0.6% and that of Olig2-positive, proliferating cells in total Olig2-positive cells was 7.2 ± 4.0% (Fig. 3C). In each PA case, the individual LI of Olig2-positive, proliferating cells in total Olig2-positive cells tended to be higher than that of Iba1-positive, proliferating cells in total Iba1-positive cells.

Diffuse astrocytoma

Immunoreactivity for GFAP, Olig2, and Iba1 was detected in all 11 DA cases. Corresponding to the many processes of neoplastic astrocytes (Fig. 4A) the GFAP staining showed a diffuse, strong reaction (Fig. 4B), whereas Olig2- and Iba1- positive cells were also diffusely distributed but few in number (Fig. 4C, D). Iba1-positive cells were morphologically compatible with microglia/macrophages and almost all of them showed ramified, resting form.

All 11 DA cases had satisfactory tissue for semiquantitative analysis. The mean LI of Olig2 was 13.8 ± 7.8% and that of Iba1 was 20.9 ± 9.9% (Fig. 3A).

Statistical analysis

For Olig2-positive cells, the mean LI in PA was significantly higher than that in DA ($P<0.001$). In contrast, there was no significant difference in the mean LI of Iba1-positive cells between PA and DA (Fig. 3A).

FISH

In a case of PA with a high Olig2 LI (76.4%), 100 nuclei were counted and almost all nuclei had both 2 green and 2 orange signals. The ratio of green/orange signals was >0.8, and the case was judged to show no evidence of allelic loss of 1p (Fig. 5).

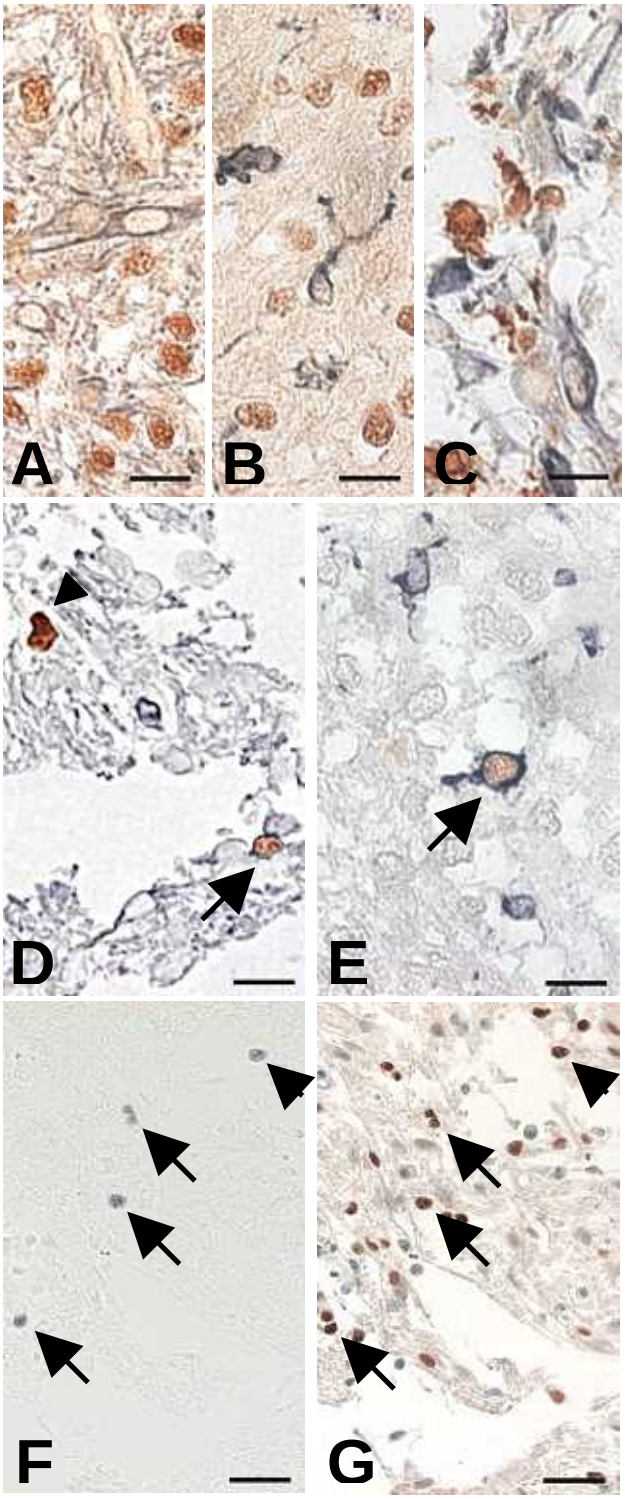

Fig. 2 Double- or re-immunostaining in PA. (A-C) Comparative double immunoperoxidase staining using GFAP (dark purple) with Olig2 (brown) (A), Olig2 (brown) with Iba1 (dark purple) (B), and Iba1 (brown) with GFAP (dark purple) (C) showed no double labeling. (D,E) Double-positive, proliferating cells have brown nuclei for Ki-67 and dark purple cytoplasm for GFAP (D) or Iba1 (E) (arrows). GFAP- negative, proliferating cells had only Ki-67-positive nuclei (arrowhead). (F,G) Re-immunostaining in the same section using 4-chloro-1 naphtol for Ki-67(F) and DAB for Olig2 (G). Almost all Ki-67-positive, dark purple nuclei were re-stained for Olig2 (arrows). In re-stained section, Olig2-negative nuclei were counterstained with hematoxylin. Bars=20μm.

DISCUSSION

This study showed that Olig2-positive cells were the major cellular component of PA and mainly distributed in two areas: microcystic, textured loose areas and oligodendroglioma-like areas. Previous reports[1,2] have shown Olig2-positive cells in PA, however, this is the first semiquantitative analysis of Olig2-positive cells. Our results indicate that the morphology and immunophenotype of Olig2-positive cells in oligodendroglioma-like areas were similar to those of oligodendroglioma cells, and that Olig2-positive cells in microcystic areas seemed to be compatible with so-called protoplasmic astrocytes with small nuclei and few flaccid processes. The mutual exclusivity of Olig2 and GFAP expression[9] supports our finding that protoplasmic astrocytes with poor glial fibrils expressed Olig2 but not GFAP. Furthermore, the following might explain Olig2 expression in protoplasmic astrocytes: (1) the transcriptional factor Olig2 appears in the oligodendrocyte lineage from oligodendrocyte precursors to adulthood, whereas it was reported that astrocytic lineage cells enigmatically express Olig2 in tumor environments,[1,9,10] (2) Ohnishi *et al.*[1] recently demonstrated that the proportion of Olig2-positive cells was high in protoplasmic astrocytomas.

Our semiquantitative analysis of Olig2-positive cells demonstrated a significant difference between PA and DA. Cytogenetical assessment such as allelic loss at 1p and 19q in OD is sometimes available for diagnosis of some gliomas. However, PA is a clinicopathologically defined tumor, and cytogenetical assessment is not so useful for diagnosis. For example, on analysis of genetic alteration, 64% of PA showed a normal karyotype, despite a tendency toward chromosomal gains, such as chromosomes 5 and 7.[22] In addition, PA lacks definite molecular alterations[23] such as the mutation or deletion of the TP53 gene and abnormal expression of EGFR observed in high grade glioma.[24] PA sometimes closely resembles DA and differential diagnosis is difficult. Immunohistochemistry with glial markers such as GFAP is not so useful in this situation. This study found high Olig2 expression in microcystic and oligodendroglioma-like areas in PA and emphasizes the importance of semiquantitative analysis of Olig2 immunostaining in differential diagnosis of low grade astrocytomas.

We demonstrated that Olig2-positive cells constituted the main proliferating population in PA and that Olig2-positive cells had high proliferative potential. The proportion of Olig2-positive, proliferating cells in total proliferating cells was estimated to be 75% when LIs of GFAP- and Iba1-positive, proliferating cells were considered together. However exact comparison between GFAP-, Iba1-, and Olig2-positive, proliferating cells in PA was impossible due to the different methods of immunohistochemistry. Recently, progenitor cells have been generally believed to be closely

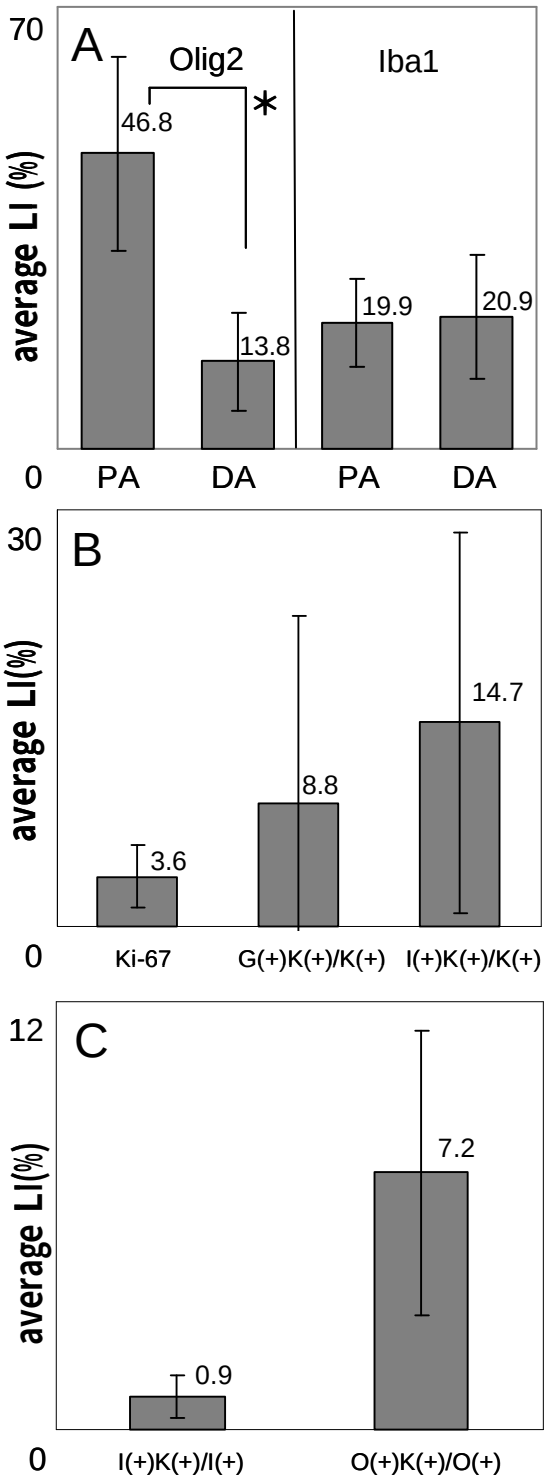

Fig. 3 Semiquantitative analysis of LIs using immunohistochemical markers. (A) LIs of Olig2 and Iba1 between PA and DA. The asterisk (*) indicates a significant difference of $p<0.001$ for PA versus DA in the immunostaining of Olig2. There was no significant difference for Iba1. (B, C) G, K, I, and O represent GFAP, Ki-67, Iba-1, and Olig2, respectively. (B) Mean LI of Ki-67 alone (left), and mean LI of GFAP- (center), Iba1- (right), positive, proliferating cells in total Ki-67-positive, proliferating cells. (C) Mean LI of Iba1-positive, proliferating cells in total Iba1-positive cells (left), and Olig2-positive, proliferating cells in total Olig2-positive cells (right). Values are expressed as mean ± SD (%).

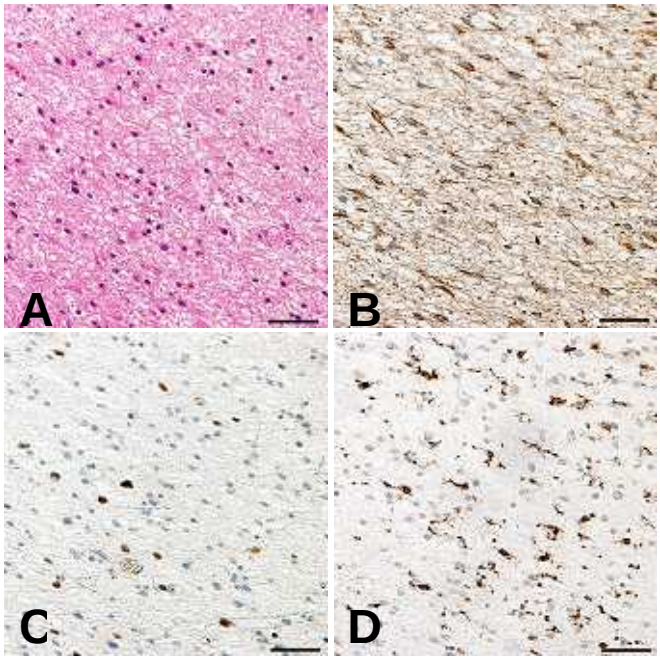

Fig. 4 Histological and immunohistochemical findings in DA. (A) Typical area of fibrillary astrocytoma. (B) A large number of processes of the tumor cells are diffusely and strongly stained with GFAP. (C, D) Olig2- (C) or Iba1-(D) positive cells are also diffusely distributed but the number of positive cells is small. Bars=50μm.

associated with tumorigenesis and proliferation of brain tumors.[25] As reported previously, the high expression of PEN5, a sulfated polylactosamine carbohydrate epitope,[26] and NG2 /PDGFαR[27] in PA and OD indicate the possibility that both tumors are derived from common progenitor cells, oligodendrocyte progenitor cells (OPCs). In addition, the strong expression of SOX10, an oligodendroglial specific transcriptional factor[28], and the expression pattern of proteolipid protein or peripheral myelin protein(PMP)-22[29] show some similarity between PA and oligodendroglial lineages. Therefore, the Olig2-positive, proliferating cells observed in this study may derive from OPCs. In the present study, FISH analysis showed no LOH of 1p, suggesting that PA is not cytogenetically identical to OD. As Shoshan *et al*.[27] pointed out, both PA and OD can be neoplasms generated from OPCs, and they can give rise to neoplasms of clinically and pathologically distinctive phenotypes, probably as a result of the following: different environmental influences within the brain parenchyma, different acquired genetic alterations such as 1p and 19q, and differential factors such as tumor location and host age.

Iba1-positive microglia/macrophages were a cellular component of PA, and they showed characteristic distribution and proliferative potential. They represented approximately 20% of cells in PA, although the number of Iba1-positive cells was not significantly higher in PA than in DA. Previous studies have shown that microglia/macrophages are highly abundant in gliomas,[3,4] and Roggendorf *et al*.[3] suggested a direct correlation between the grade of gliomas and the level of resident tumor microglia/macrophages. Microglia/macrophages in gliomas are thought to be derived from various cells such as resident microglia, perivascular macrophages, and blood monocytes[30] that can be stimulated by chemokines such as monocyte chemoattractant protein-1.[31] In this study, Iba1-positive cells showed various morphological shapes, including monocyte-like cells with round cytoplasm and unipolar processes. Due to the breakdown of the blood-brain barrier in gliomas, blood monocytes might be the most potent candidate for the origin of activated microglia/macrophages.

Abundant microglia/macrophages may play a role in the formation of the characteristic structure of PA. LI of microglia/macrophages were not significantly higher in PA than that in DA, however the state of microglia was different; in PA they were distributed mainly in microcystic areas with a slightly activated morphology, whereas in DA they showed ramified form. In brain immune systems, the role of microglia/macrophages seems to be complex. For example, microglia/macrophages can be neuroprotective/trophic or neurodestructive/toxic in inflammatory, degenerative, vascular or infective situations.[32,33] Microglia/macrophages could be recruited to the glioma to produce a number of cytokines, chemokines, and other mediators like growth factors.[15,30] These mediators might then contribute to successful immune evasion, growth, and invasion of glioma cells. For example, microglia activated by glioblastoma cells produce metalloprotease-2, which supports tumor cell invasion into the matrix.[34] In another study using a glioma tumor model,[35] microglia were a major source of prostaglandin E2 production through the expression of cyclooxygenase-2, and they might closely correlate with peritumoral edema in high grade glioma. Therefore, the unique background structures in microcystic areas (microcystic degeneration, myxoid matrix, edema) and macroscopic cystic formation in PA might be potentially caused by activated microglia/macrophages in addition to increased fluid transport by blood-brain barrier disturbance.[36]

The present study suggests that compact areas are long-standing areas and microcystic areas are developing areas in PA. Compact areas mainly consisted of a population of piloid cells with strong GFAP expression. These cells are a well-known characteristic feature of PA. However, our results showed that this subset is a minor component; the LI of GFAP-positive cells was approximately 35%, as estimated from the LIs of Olig2 and Iba1. In addition, GFAP-positive cells scarcely had any proliferative activity. This finding indicates that compact areas might be neoplastic but formed over a long time course. This is supported by the following evidences[16]: (1) compact areas often include Rosenthal fibers, which are observed in chronic reactive gliosis, (2)

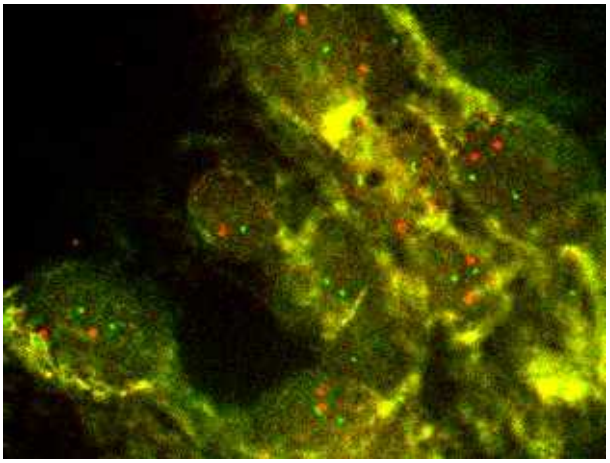

Fig. 5 Fluorescence *in situ* hybridization. In this case, almost all nuclei had 2 red and 2 green signals, demonstrating no allelic loss of 1p.

hair-like piloid cells are similar to reactive astrocytes in chronic pathologic lesions such as hypothalamus, cerebellum, or spinal cord. On the other hand, we demonstrated that microcystic areas contain many proliferating tumor cells and many microglia/macrophages. This result suggests that microcystic areas are responsible for tumor expansion by cellular proliferation, cystic change, and edema. Further studies are required to assess the correlation between mature piloid cells of compact areas and maturing Olig2-positive cells of microcystic areas.

In conclusion, PA is a unique tumor consisting of cells with various glial immunophenotypes. Olig2-positive cells which represent protoplasmic astrocytes, oligodendroglioma-like cells, and to some extent OPCs, are far more prevalent than GFAP-positive, piloid cells. The numerical predominance and high proliferative activity of Olig2-positive cells in microcystic areas suggest their correlation with tumor development. This study also suggests that Iba1-positive microglia/macrophages might be involved in tumor growth and microenvironmental formation. Furthermore, semiquantitative analysis of Olig2 immunohistochemistry in microcystic areas could be helpful for differential diagnosis of PA and DA when the histopathological diagnosis is problematic.

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