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Clinicopathological features and global genomic copy number alterations of pilomyxoid astrocytoma in the hypothalamus/optic pathway: comparative analysis with pilocytic astrocytoma using array-based comparative genomic hybridization

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Pilomyxoid astrocytoma is a recently identified variant of pilocytic astrocytoma. We studied 11 circumscribed astrocytomas with focal ($n=5$) or diffuse ($n=6$) pilomyxoid features and compared them with 17 pilocytic astrocytomas from the hypothalamic/chiasmatic region in children. In one patient, a tumor that recurred after initial surgery had changed from pure-form pilomyxoid astrocytoma to the mixed form. The presence of a pilomyxoid area was associated with shorter survival. Next, we compared the comprehensive genome copy number changes in the pilomyxoid astrocytoma ($n=4$) with those in pilocytic astrocytoma ($n=6$) cases by array-based comparative genomic hybridization. The number of lost clones was larger in pilomyxoid astrocytoma than in pilocytic astrocytoma. Clones located in chromosome 8q24.3 were frequently gained in pilocytic astrocytoma (four of six) and in pilomyxoid astrocytoma (one of four). Clones located in 9p24.3 and 15q26.3 were lost in all of the pilomyxoid astrocytomas and in five of the pilocytic astrocytomas. Those in 8p23.3 showed a copy number loss in three of the pilomyxoid astrocytomas and four of the pilocytic astrocytomas. The frequency of copy number changes was significantly different between pilomyxoid astrocytoma and pilocytic astrocytoma in 47 (3.6%) clones, 20 of them having been located in 2p, 10 in 2q, and 11 in 3q. An unsupervised hierarchical clustering analysis classified the cases into three clusters: one pilomyxoid astrocytoma patient into one cluster, two pilomyxoid astrocytoma patients into another cluster, and six pilocytic astrocytoma patients and one pilomyxoid astrocytoma patient into the third cluster. In conclusion, the presence of mixed-form pilomyxoid astrocytoma, the acquisition of pilocytic astrocytoma features in a recurrent tumor in pure-form pilomyxoid astrocytoma, and the above results of the genome-wide gene copy number analysis suggest that pilomyxoid astrocytoma might be a pathologically and genetically related, aggressive variant of pilocytic astrocytoma with partially different genetic alterations.
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Keywords: pilomyxoid astrocytoma; pilocytic astrocytoma; array CGH

Pilomyxoid astrocytoma is a recently identified variant of pilocytic astrocytoma, which is generally encountered in the hypothalamic and optic chiasmatic area and occurs in younger patients than does pilocytic astrocytoma. Pilomyxoid astrocytoma has unique histological features, including a monophasic/monomorphic pattern of elongated pilocytic cells in a prominent myxoid background, as well as frequent angiocentricity of tumor cells. Unlike typical pilocytic astrocytoma, pilomyxoid astrocytoma lacks a biphasic pattern, protoplasmic cells, an oligodendroglioma-like pattern, Rosenthal fibers, and eosinophilic granular bodies. Pilocytic astrocytoma in

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the hypothalamic/chiasmatic region was found to exhibit more aggressive behavior than pilocytic astrocytoma in other regions. However, in studies that matched patients by age and location, pilomyxoid astrocytoma has been reported to show more aggressive behavior, with more frequent cerebrospinal fluid (CSF) dissemination than pilocytic astrocytoma.[1–6] In recent years, pilomyxoid astrocytomas primarily involving extra-hypothalamic/chiasmatic regions such as the spinal cord and cerebellum, and cases occurring in adults, have also been described.[1,7–10] Pilomyxoid astrocytoma and pilocytic astrocytoma have even been encountered in patients with neurofibromatosis type I.[3,11] In terms of the nature of pilomyxoid astrocytoma neoplastic cells, it has been postulated that pilomyxoid astrocytoma might be derived from astrocytic cells or radial glia existing in the embryonic optic chiasm.[5] However, some researchers have found ependymal differentiation using electron microscopy and have suggested an ependymal derivation of the tumor.[12] The several shared morphological features of pilomyxoid astrocytoma and pilocytic astrocytoma, as well as reports that recurrent pilomyxoid astrocytoma after several years achieved typical histopathological features of pilocytic astrocytoma, or 'maturated' or 'differentiated' to pilocytic astrocytoma, support the postulation that pilomyxoid astrocytoma might be closely related to pilocytic astrocytoma.[1,3,5,10] Therefore, some authors have suggested that pilomyxoid astrocytoma is an infantile or juvenile form of pilocytic astrocytoma, whereas others have suggested that pilomyxoid astrocytoma is an extreme morphological subtype within the pilocytic astrocytoma continuum with different clinical behaviors.[1,5,10] However, the pathogenesis of pilomyxoid astrocytoma and the relationship with pilocytic astrocytoma remains to be elucidated. In addition, there have been few comparative cytogenetic or molecular studies on pilomyxoid astrocytoma and pilocytic astrocytoma.

Array-based comparative genomic hybridization (array CGH) has been widely used to measure the specific changes in the genomic copy number of whole chromosomes with a high resolution.[13,14] Array CGH has also been applied to the neoplasm of the nervous system to find common genetic tumor aberrations to determine novel tumor suppressor genes that had been deleted and oncogenes that had been amplified, or to identify the tumor-specific and clinically relevant molecular genetic signatures.[15–19]

In this study, we investigated the clinicopathological features of 11 circumscribed astrocytomas with focal ($n=5$) or diffuse ($n=6$) pilomyxoid features and compared them with 17 pilocytic astrocytomas from the hypothalamic/chiasmatic region in young children. In addition, to further delineate the genetic characteristics of pilomyxoid astrocytoma and the possible association with pilocytic astrocytoma, we compared the comprehensive genome copy number changes in pilomyxoid astrocytoma with those in pilocytic astrocytoma by high-resolution array CGH.

Materials and methods

Patients and Tumor Samples

A total of 28 pediatric cases that had been diagnosed with pilocytic astrocytoma in the hypothalamic area and optic pathway between 1987 and 2005 were retrieved from the brain tumor registry archive at Seoul National University Hospital. Pathological material was obtained from a stereotactic biopsy in two patients, and from surgical resection in the others. All of the H&E slides were reviewed by two pathologists (YKJ and SHP) according to the previous histological description of pilomyxoid astrocytoma.[1] Cases showing a typical monophasic myxoid area with perivascular radiating pilocytic cells in more than one-fourth of the tumor area were reclassified as pilomyxoid astrocytoma. In the diagnosis, 11 (39%) of the 28 cases were deemed to be pilomyxoid astrocytoma; more specifically, 6 pure-form (of entirely pilomyxoid astrocytoma-like characters) and 5 mixed-form (showing both pilomyxoid astrocytoma- and pilocytic astrocytoma-like characters) pilomyxoid astrocytoma. The pilomyxoid astrocytoma patients ($n=11$) were younger than the pilocytic astrocytoma patients ($n=17$). In pilomyxoid astrocytoma patients, the age at diagnosis ranged from 3 to 156 months, with a median of 36 months (mean ± s.d., 53.36 ± 50.61), whereas in pilocytic astrocytoma, the age at diagnosis ranged from 9 to 416 months with a median of 120 months (131.06 ± 97.30) ($P=0.031$). There was a slight female predominance in pilomyxoid astrocytoma, the male/female ratio being 2:9, as compared with 7:10 in pilocytic astrocytoma. A clinician blinded to the pathological results collected clinical data. Formalin-fixed paraffin-embedded tissue blocks adequate for DNA extraction were available in four pilomyxoid astrocytomas (one pure form and three mixed forms) and six pilocytic astrocytomas, which were further subjected to array CGH analysis.

Immunohistochemistry

Immunohistochemical staining for p53 (DAKO, Glostrup, Denmark), GFAP (DAKO), and Ki-67 (DAKO) was performed automatically using the TechMate™ 500 Plus (DAKO) according to the protocol of the manufacturer, on the basis of the conventional streptavidin–biotin–peroxidase method.

Array CGH

Bacterial artificial chromosome (BAC) clones were first bioinformatically selected from Macrogen's proprietary BAC library (http://www.macrogen.com) to give an average genomic coverage of 2 Mb

resolutions.[20] All of the clones were two end-sequenced using Applied Biosystems 3700 sequencers (Foster City, CA, USA) and their sequences were BLAST-searched and mapped according to their positions in the University of California, Santa Cruz (UCSC) human genome database (http://www.genome.ucsc.edu). Confirmation of the locus specificity of the chosen clones was performed by removing multiple loci-binding clones and individually examining them under standard fluorescence *in situ* hybridization (FISH), as described previously.[21] Each of the BAC clones was represented as triplicated spots on an array. Finally, the array used in this study consisted of 1440 human BACs spaced approximately 2.3 Mb across the whole genome (MacArray™ Karyo 1400 from Macrogen Inc., Seoul, Republic of Korea).

DNA was extracted from each of the tumor tissues using the PureGene kit (Gentra Systems Inc., Minneapolis, MN, USA). The labeling and hybridization protocols described by Pinkel *et al*[22] were used, with some modification. In brief, 21 μl of solution containing 500 ng of normal DNA (reference) or test DNA, 20 μl of BioPrime® aCGH Labeling System random primers solution (Invitrogen, Carlsbad, CA, USA), and water were combined and incubated for 5 min at 95 °C and were subsequently cooled on ice. After the addition of 5 μl of 10 × dNTPs labeling mix, 3 μl of 1 mM Cy3-dCTP or Cy5-dCTP (PerkinElmer, Foster City, CA, USA) and 40 U of BioPrime aCGH Labeling System Klenow fragment (Invitrogen), the mixture was incubated overnight at 37 °C. After labeling, unincorporated fluorescent nucleotides were removed using the BioPrime aCGH Purification Module (Invitrogen). In one tube, the Cy3-labeled sample and Cy5-labeled reference DNAs were mixed together, and 70 μg of human Cot I DNA (Invitrogen), 20 μl of 3 M sodium acetate, and 600 μl of cold 100% ethanol were precipitated.

The pellet was resuspended in 40 μl of hybridization solution containing 50% formamide, 10% dextran sulfate, 2 × SSC, 4% SDS, and 200 μg yeast tRNA. The hybridization solution was denatured and subsequently incubated for 1 h at 37 °C to block repetitive sequences. Hybridization was performed over 48 h at 37 °C. After post-hybridization washing and drying, the arrays were scanned using a GenePix4200A two-color fluorescent scanner (Axon Instruments) and were then quantitated using GenePix software (Axon Instruments). The median ratio of three replicate spots for each clone was calculated. Clones with >70% missing or poor values were excluded, and a total of 1294 different BAC clones were used in the final analysis. The $\log_2$-transformed fluorescent ratios were calculated using the background-subtracted median intensity values. These ratios were used to perform LOWESS normalization before copy number calculations.[23] Macrogen's MAC viewer, array CGH analysis software, MS Excel VBA, and avadis3.3 Prophetic were used for graphical illustration and image analysis of the array-CGH data.

Statistical Analysis

To detect the gain or loss of spots in each group, we performed an analysis of copy errors in the data set.[24] In addition, the differences between pilomyxoid astrocytoma and pilocytic astrocytoma were assessed using R software, according to the array CGH data and a Fisher's exact test adjusted for multiple comparisons with a False Discovery Rate of <0.037. We performed complete linkage hierarchical clustering on the basis of a Euclidian distance measure of all of the samples using the BAC clones. A nonparametric test was performed to analyze the difference in the mean values of the gene copy number changes between pilomyxoid astrocytoma and pilocytic astrocytoma. A χ^2 test was used to compare the frequency of the specific findings between the groups. A survival analysis was performed using the Kaplan–Meier method along with a log-rank test using the SPSS (version 11.5.0) software package (Chicago, IL, USA).

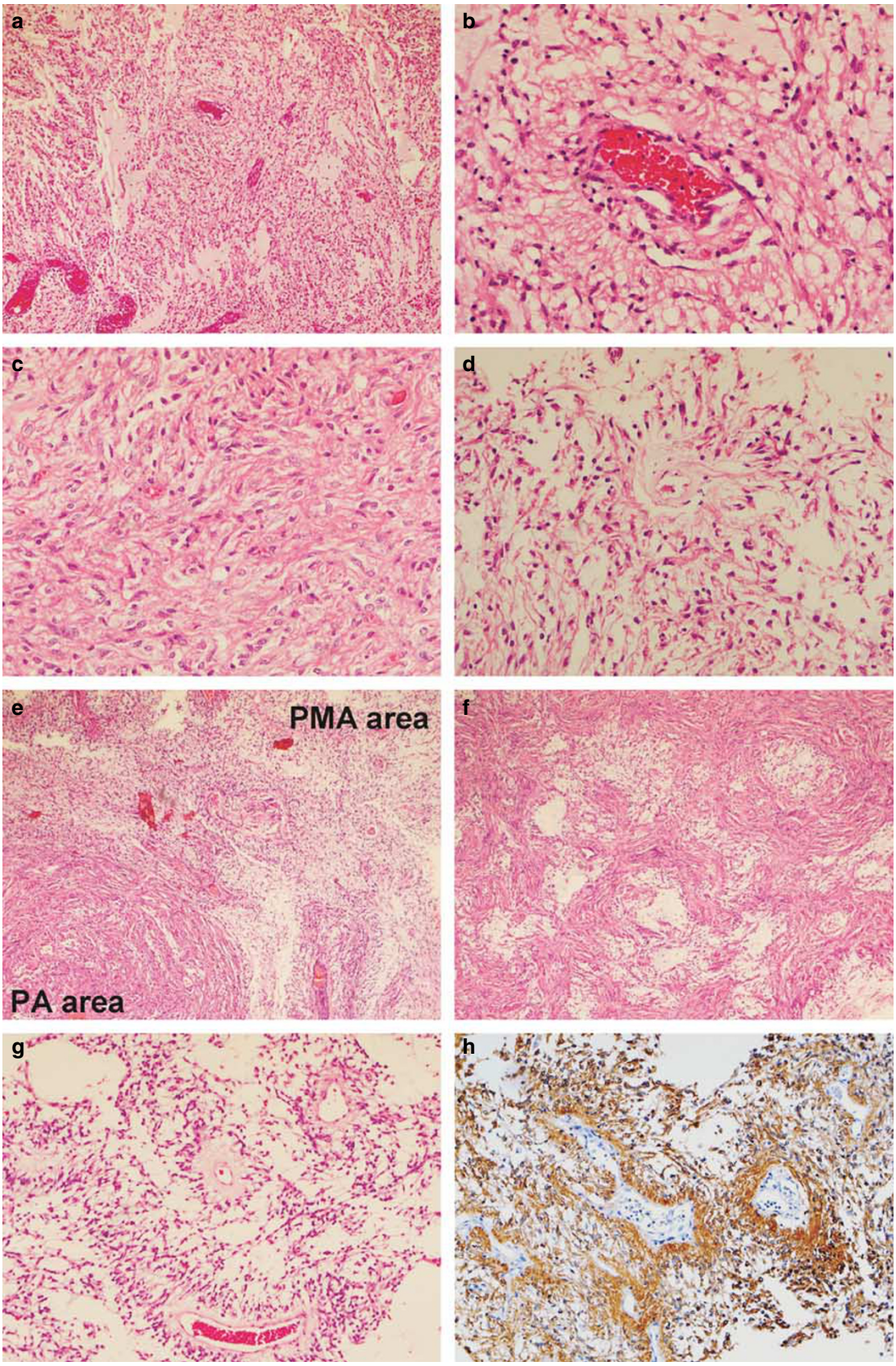
Results

Clinical Features of Pilomyxoid Astrocytoma and Pilocytic Astrocytoma Patients

The demographic features of pilomyxoid astrocytoma and pilocytic astrocytoma patients are summarized in Materials and methods. Interestingly, one patient (case no. P2) had been diagnosed with pure-form pilomyxoid astrocytoma at the time of initial surgery; however, a recurrent mass that was found 3 years later exhibited the histology of mixed-form pilomyxoid astrocytoma (Figure 1a–d). Except for two patients (one pilomyxoid astrocytoma and one pilocytic astrocytoma patient) from whom only biopsy material was obtained, all of the patients were initially treated by surgical resection of the tumor. Of the 17 pilocytic astrocytoma patients, a near-total resection was performed in 6 cases (35%) and a subtotal resection in 10 (59%). However, near-total resection could not be achieved in any of the cases of pilomyxoid astrocytoma ($n=11$). Instead, a subtotal resection was carried out in 8 (73%) cases, and only a partial resection was possible for 2 (18%) of the patients.

Pathological Features of Pilomyxoid Astrocytoma

In the case of mixed-form pilomyxoid astrocytoma ($n=5$), the proportion of the monophasic myxoid pilomyxoid astrocytoma area varied from one-fourths to three-fourths (25–75%) of the tumor area (Figure 1e–g). The most characteristic and consistent histological features of pilomyxoid astrocytoma were the presence of a monophasic prominent


a
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h

myxoid area and the angiocentric radiating arrangement of piloid cells. As shown in Table 1, glial islet, Rosenthal fibers, eosinophilic granular bodies, hemorrhage, necrosis, lymphocytic infiltration, vascular endothelial hyperplasia, mitosis, and nuclear atypism were infrequently observed. Immunohistochemically, the tumor cells were positive for GFAP in all of the pilomyxoid astrocytoma and pilocytic astrocytoma cases. Nuclear accumulation of p53 protein was not observed in any of the cases of pilomyxoid astrocytoma or pilocytic astrocytoma. There was no difference in the Ki-67 labeling index between pilomyxoid astrocytoma (mean ± s.d., 0.43 ± 0.65 (range 0–2)) ($n = 10$) and pilocytic astrocytoma (0.39 ± 0.55 (0–1.8)) ($n = 15$).

Radiological Features of Pilomyxoid Astrocytoma

We retrospectively reviewed the preoperative magnetic resonance (MR) ($n = 5$) and computed tomography (CT) ($n = 6$) findings in the six children with pilomyxoid astrocytoma. The MR imaging showed a well-defined, lobulating-contoured mass in the suprasellar area, with subfrontal ($n = 2$) and prepontine ($n = 2$) extension. These tumors showed high signal intensity on T2WI and mixed low signal intensity on T1WI (Figure 2). On contrast-enhanced T1WI, a frond-like peripheral enhancement and a central non-enhancing portion suggesting myxoid degeneration were observed in three patients (Figure 2f). Obvious cystic degeneration was evident in two patients, and these patients showed a dense enhancement in the solid portion, along with a bubbly microcystic component (Figure 2h). CSF seeding of the tumor was noted in one patient (Figure 2f). Extension to the bilateral optic tract was present in one other patient. On precontrast CT, the tumors of all patients showed slightly low attenuation compared with the brain parenchyma. Curvilinear calcification was noted in one patient, and focal high attenuation suggestive of hemorrhage was observed in one other patient (Figure 2c).

Survival Analysis

During the follow-up period (1–264 months), 7 (64%) pilomyxoid astrocytoma and 4 (24%) pilocytic astrocytoma patients suffered from local tumor recurrences, and 3 (27%) pilomyxoid astrocytoma and 1 (6%) pilocytic astrocytoma patients died of their conditions. The median disease-free survival time was 11 ± 2 months for pilomyxoid astrocytoma, whereas the median disease-free survival was not achieved for pilocytic astrocytoma during the follow-up period. In the Kaplan–Meier analysis, pilomyxoid astrocytoma patients showed a tendency toward shorter failure (recurrence or death)-free survival and overall survival than pilocytic astrocytoma patients ($P = 0.058$ and $P = 0.089$, respectively; Figure 3a and b). After separately analyzing the mixed-form and pure-form pilomyxoid astrocytoma, the mixed-form pilomyxoid astrocytoma, as compared with pilocytic astrocytoma, also exhibited poor failure-

Table 1 Histological features of pilomyxoid astrocytoma

No.	*Sex*	*Age (mo)*	*Type*	*Monophasic myxoid*	*Angiocentric pattern*	*Glial islet*	*RF/EGB*	*H/N/C/L*	*VEH/atypia*	*Mitosis/Ki-67 LI*
P1	F	3	Pure	+	+	−	−/−	−/−/−/−	−/−	+ (1)[a]/0.1
P2	F	20	Pure	+	+	−	−/−	+/+/−/+	+/−	−/0.1
P3	M	82	Pure	+	+	−	−/−	+/+/−/−	−/+	−/0.1
P4	F	132	Pure	+	++	+	+/−	−/−/−/−	−/−	−/2
P5	F	156	Pure	+	++	−	+/+	−/−/−/−	−/−	−/0.1
P6	F	36	Pure	+	++	−	−/−	−/−/−/−	−/−	−/0.4
M1	F	62	Mixed	+ (50%)	+	+	+/−	−/−/−/−	−/−	−/NA
M2	F	34	Mixed	+ (50%)	+	−	+/−	−/−/−/−	−/−	−/0
M3	F	9	Mixed	+ (25%)	+	−	−/−	−/−/−/−	−/−	−/0.1
M4	M	20	Mixed	+ (75%)	++	−	−/+	+/+/−/−	−/−	−/1.2
M5	F	86	Mixed	+ (25%)	+	+	−/−	−/−/−/−	−/−	−/0.2

Atypia, nuclear atypia; H/N/C/L, hemorrhage/necrosis/calcification/lymphocytic infiltration; Ki-67 LI, Ki-67 labeling index (%); mo, months; NA, not available; RF/EGB, Rosenthal fiber/eosinophilic granular body; VEH, vascular endothelial hyperplasia.
[a]Mitotic count: 1/10 HPF.

Figure 1 Pathological features of pilomyxoid astrocytoma (pilomyxoid astrocytoma). In one patient (case no. P2), the initial mass exhibited monophasic piloid cell proliferation in a myxoid background (**a**) with frequent angiocentricity (**b**). However, a recurrent tumor that was found three years later revealed both a pilocytic astrocytoma (pilocytic astrocytoma) area with Rosenthal fibers in a glial background (**c**) and a pilomyxoid astrocytoma area (**d**). The tumor of mixed-form pilomyxoid astrocytoma (case no. M4) was composed of both pilocytic astrocytoma (pilocytic astrocytoma)- and pilomyxoid astrocytoma-like areas (**e**). The pilocytic astrocytoma area showed a characteristic biphasic pattern (**f**). In contrast, in the pilomyxoid astrocytoma area, piloid cells were arranged with a perivascular-radiating pattern in a prominent myxoid background (**g**). The piloid cells were positive for GFAP (**h**).

free survival or overall survival ($P=0.045$ and $P=0.072$, respectively). Next, to exclude the confounding impact on the patient outcome in terms of the extent of surgical resection, we performed a survival analysis on pilomyxoid astrocytoma and pilocytic astrocytoma patients undergoing subtotal resection. In the result, failure-free survival and overall survival were shorter in pilomyxoid astrocytoma than in pilocytic astrocytoma ($P=0.085$ and $P=0.015$, respectively, data not shown).

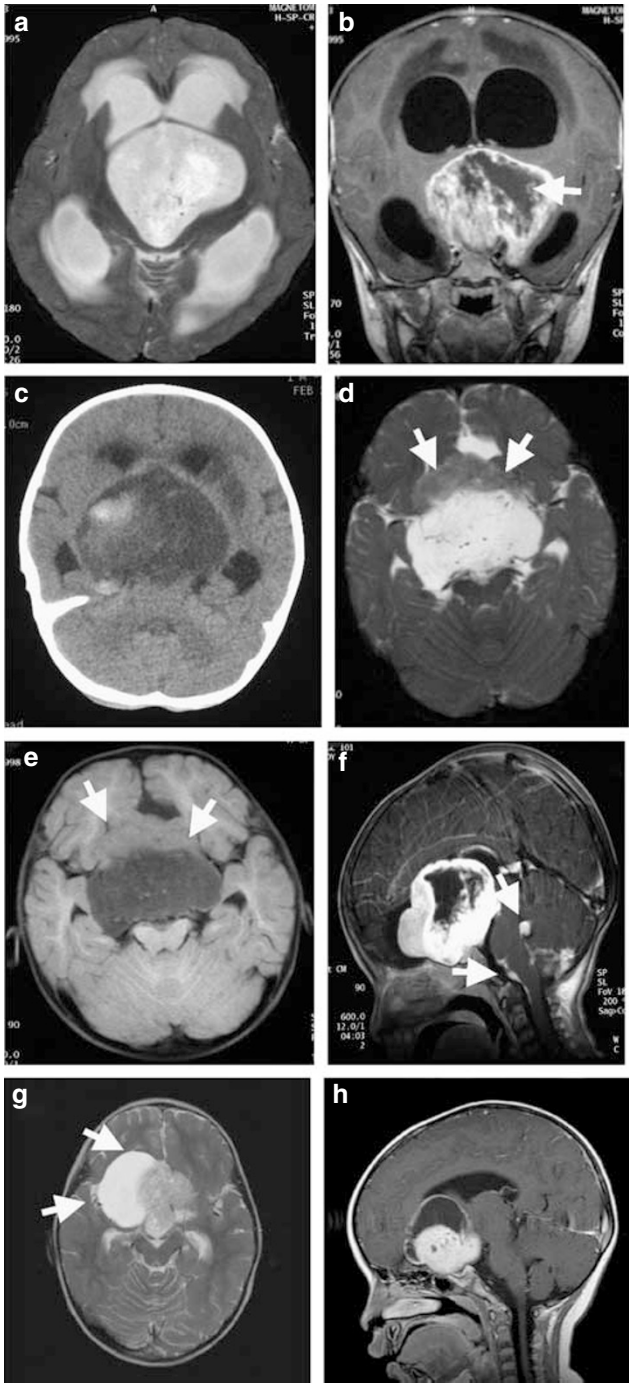

Figure 2 Representative radiological features of pilomyxoid astrocytoma. Patient no. P2 (**a**, **b**), Patient no. M4 (**c**–**f**), and Patient no. P6 (**g**, **h**). (**a**) The T2-weighted axial image shows a well-defined, heterogeneously high signal intensity mass in the suprasellar area with the hydrocephalus. (**b**) On the contrast-enhanced coronal T1WI, the tumor shows a heterogeneous peripheral enhancement with the central non-enhancing portion (arrow). (**c**) The precontrast CT scan shows a well-defined low-attenuated mass in the suprasellar area with a focal high attenuating portion suggesting hemorrhage. (**d**) The T2-weighted axial image shows a heterogeneous high signal intensity mass with a focal iso-signal intensity portion (arrow) in the suprasellar area. (**e**) On the T1WI axial image, the tumor shows a heterogeneous low signal intensity with a focal iso-signal intensity portion (arrow). (**f**) After contrast enhancement, the mass shows a frond-like peripheral enhancement and non-enhancing central portion. Note the nodular or linear enhancement along the 4th ventricle and cisternal space, suggesting CSF tumor seeding (arrows). (**g**, **h**) An obvious unilocular cystic portion (arrow) and bubbly microcystic lesions are visible within the solid portion.

Change in the Gene Copy Number of Pilocytic Astrocytoma and Pilomyxoid Astrocytoma Detected by Array CGH

The array CGH analysis results for six pilocytic astrocytoma and four pilomyxoid astrocytoma (one pure form and three mixed forms) cases are summarized in Figure 4 and Table 2. Each tumor showed variable gene copy number alterations, and the mean frequency of clones with copy number gains and losses was 12.9 ± 23.8 ($1\pm1.8\%$) (range: 0–79) and 160.2 ± 219.1 ($12.4\pm16.9\%$) (range: 4–636). We then compared the gene copy number profiles between pilocytic astrocytoma and pilomyxoid astrocytoma using the Mann–Whitney *U*-test and found no significant differences in the number of gained clones, with 6.8 ± 6.8 ($0.5\pm0.5\%$) in pilocytic astrocytoma and 22 ± 38 ($1.7\pm2.9\%$) in pilomyxoid astrocytoma. A larger number of lost clones was observed in pilomyxoid astrocytoma, with 333.8 ± 263.5 ($25\pm20.4\%$), than in pilocytic astrocytoma, with 44.5 ± 68 ($3.4\pm5.3\%$), but the difference was not statistically significant ($P=0.08$). Although most of the clones with gene copy number changes were randomly observed in individual cases, seven clones showed frequent gains or losses in more than half of the cases of pilocytic astrocytoma, as summarized in Table 3. In the case of pilomyxoid astrocytoma, 115 clones were lost in 75% of cases (three of the four pilomyxoid astrocytoma patients), and those clones were located in chromosomes 2p, 2q, 3p, 3q, 6q, 7p, 7q, 8p, 9p, 9q, 15q, 20q, and 22q. We found only 47 (3.6%) clones showing a significantly different frequency of copy number alterations between the pilomyxoid astrocytoma and pilocytic astrocytoma group, with a *P*-value of less than 0.05 in a χ^2 test. Specifically, all of the clones were frequently lost in pilomyxoid astrocytoma cases; however, there were no copy number changes of those clones in any of the pilocytic astrocytoma cases. Furthermore, 20 of the

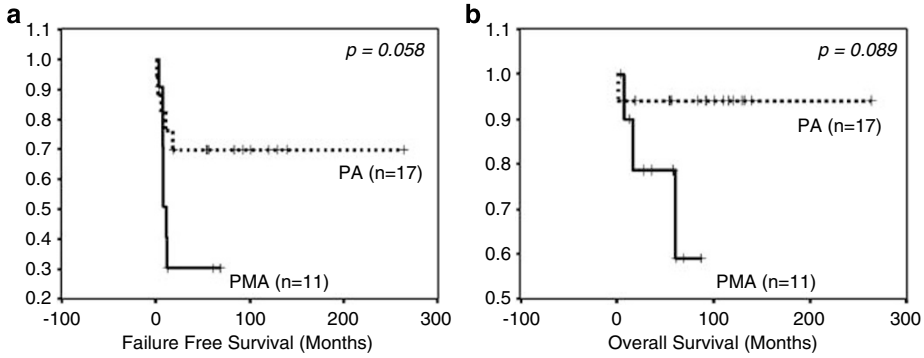

Figure 3 Failure-free and overall survival curves for patients according to histology.

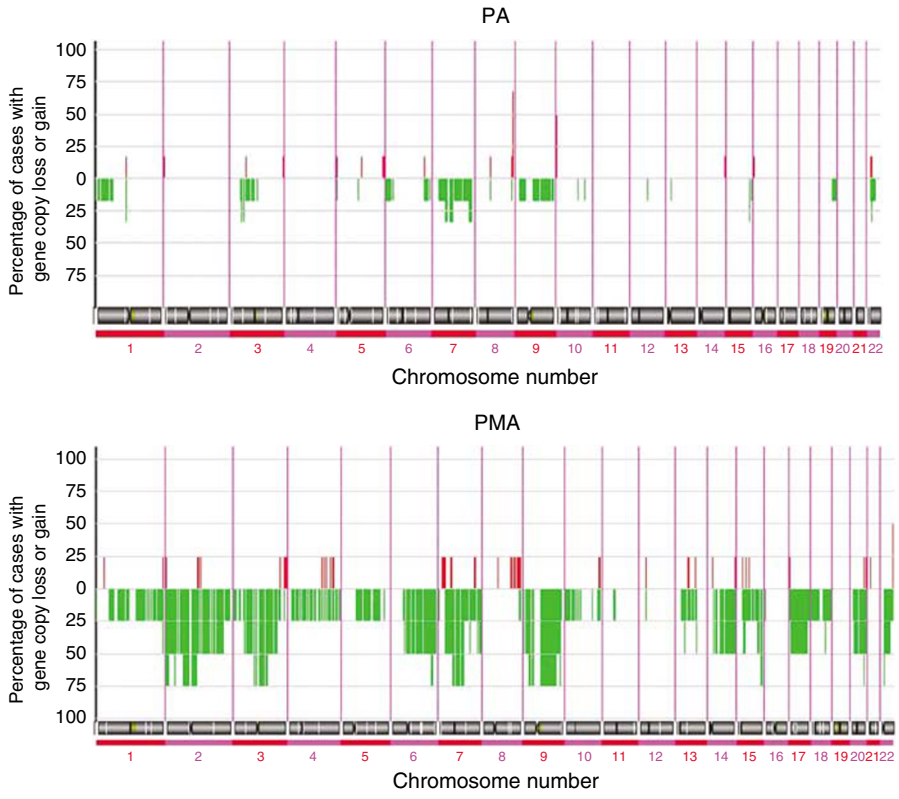

Figure 4 Frequency plots of gains and losses of each chromosomal region in pilocytic astrocytoma (top) and pilomyxoid astrocytoma (bottom) patients. The red- and green-colored bars indicate the percent of patients with a loss or gain in the given chromosomal region.

47 clones were located on chromosome 2p, 10 clones were located on chromosome 2q, and 11 clones were located on chromosome 3q. The others were mapped on 20q (three clones), 7p (one clone), 9q (one clone) and 15q (one clone). The precise chromosomal locations and the representative genes are summarized in Table 4. Otherwise, 42% (20 of 48) of the clones located in chromosome 2p and 18% (10 of 55) of the clones in chromosome 2q exhibited frequent loss of gene copy number in more than half of the cases of pilomyxoid astrocytoma, but none of the pilocytic astrocytoma cases.

Hierarchical Clustering Analysis

Unsupervised hierarchical clustering analysis according to gene copy number profiles revealed a dendrogram composed of three distinct clusters of tumors (Figure 5a). One pilomyxoid astrocytoma (M4) belonged to one cluster, and two pilomyxoid astrocytoma (P3, M3) cases were classified to another cluster. Six pilocytic astrocytomas and one pilomyxoid astrocytoma (M2) belonged to the third cluster, which was further divided into two subclusters, one composed of three pilocytic astrocytomas and the other including three pilocytic

Table 2 Frequency of gene copy number alterations in pilocytic astrocytoma and pilomyxoid astrocytoma in array CGH

Case no.	*Histology*	*Clones with gain*	*Clones with loss*
PA2	Pilocytic astrocytoma	19 (1.5%)	175 (13.5%)
PA3	Pilocytic astrocytoma	10 (0.8%)	5 (0.4%)
PA5	Pilocytic astrocytoma	4 (0.3%)	64 (5%)
PA7	Pilocytic astrocytoma	0	4 (0.3%)
PA12	Pilocytic astrocytoma	2 (0.2%)	5 (0.4%)
PA17	Pilocytic astrocytoma	6 (0.5%)	14 (1.1%)
P3	Pilomyxoid astrocytoma, pure form	2 (0.2%)	636 (49.2%)
M2	Pilomyxoid astrocytoma, mixed form	2 (0.2%)	5 (0.4%)
M3	Pilomyxoid astrocytoma, mixed form	5 (0.4%)	412 (31.8%)
M4	Pilomyxoid astrocytoma, mixed form	79 (6.1%)	282 (21.8%)

Table 3 Chromosomal location and representative genes of BAC clones gained or lost in more than half the cases of pilocytic astrocytoma

Chromosome location	*Representative genes*	*Incidence of GCN changes*			
		Pilocytic astrocytoma (n = *6*)		*Pilomyxoid astrocytoma* (n = *4*)	
		Gain	*Loss*	*Gain*	*Loss*
8q24.3	CYHR1, KIFC2, FOXH1, PPP1R16A, GPT, MFSD3, RECQL4, LRRC14, LRRC24, MGC70857, KIAA1688	4 (67%)	0	1 (25%)	0
8q24.3	ZC3H3, LOC727872, GSDMDC1, C8orf73, NAPRT1, EEF1D	4 (67%)	0	1 (25%)	0
8q24.23-8q24.3	COL22A1	4 (67%)	0	1 (25%)	0
10p15.3	DIP2C	3 (50%)	0	0	0
8p23.3	FBXO25	0	4 (67%)	0	3 (75%)
9p24.3	ANKRD15, LOC642350	0	5 (83%)	0	4 (100%)
15q26.3	LASS3	0	5 (83%)	0	4 (100%)

GCN, gene copy number.

astrocytomas and one pilomyxoid astrocytoma. Hierarchical cluster analysis was performed using 47 clones exhibiting a significantly different frequency of copy number changes between pilomyxoid astrocytoma and pilocytic astrocytoma, and the cases were classified into three clusters composed of one pilomyxoid astrocytoma (M4), two pilomyxoid astrocytomas (P3, M3), and one pilomyxoid astrocytoma (M2) and six pilocytic astrocytoma patients, respectively (Figure 5b).

Discussion

Pilomyxoid astrocytoma, predominantly encountered in the hypothalamic and optic chiasmatic area, has been considered to be an aggressive variant or juvenile form of pilocytic astrocytoma. Ensuing reports suggested a possible close relationship between pilomyxoid astrocytoma and pilocytic astrocytoma on the basis of shared histological features and the observation of the transformation of pilomyxoid astrocytoma to pilocytic astrocytoma with or without chemotherapy.[1–6,10] In this study, 5 of 11 cases of pilomyxoid astrocytoma exhibited both pilomyxoid astrocytoma and pilocytic astrocytoma regions within one tumor at the time of initial surgery. The presence of mixed-form pilomyxoid astrocytoma has already been described by Gottfried *et al*.[25] Consistent with previous reports, pilomyxoid astrocytoma patients showed shorter failure (recurrence or death)-free and overall survival than pilocytic astrocytoma patients in our series, although the difference was not statistically significant. Furthermore, our study demonstrated that mixed-form pilomyxoid astrocytoma and pure-form pilocytic astrocytoma patients also had worse disease-free and overall survival rates than pilocytic astrocytoma patients. These results emphasize the importance of the recognition of the 'pilomyxoid area' to determine the appropriate care of patients, in cases of suspected pilocytic astrocytoma (even at the initial diagnosis) and, in particular, in those occurring in the hypothalamic/chiasmatic area. As reported previously,[3] pilomyxoid astrocytoma was less amenable to complete surgical resection in our series, and this might be the cause of the poor prognosis for pilomyxoid astrocytoma patients. However, the overall survival of pilomyxoid astrocytoma patients was significantly shorter than that

Table 4 Chromosomal location and representative genes of BAC clones showing significantly different frequencies of gene copy number losses or gains between pilocytic astrocytoma and pilomyxoid astrocytoma in array CGH[a]

Chromosome location	*Representative genes*	*Incidence of GCN changes*				P-*value*
		Pilocytic astrocytoma (n = *6*)		*Pilomyxoid astrocytoma* (n = *4*)		
		Loss	*Gain*	*Loss*	*Gain*	
2p11.2	DUXAP1	0	0	3	0	0.04
2p12	CTNNA2	0	0	3	0	0.04
2p13.2	NOTO, SMYD5, C2orf7, CCT7, EXOC6B	0	0	3	0	0.04
2p14–2p13.3	TGFA	0	0	3	0	0.04
2p14	CEP68, RAB1A, LOC729317, LOC730198, MEIS1, FLJ16124	0	0	3	0	0.04
2p22.3	MRPL50P1	0	0	3	0	0.04
2p25.1	HPCAL1, ODC1, C2orf48	0	0	3	0	0.04
2q11.2	AFF3, FLJ10081, FER1L5, MAP4K4	0	0	3	0	0.04
2q12.1	SLC9A2, MFSD9	0	0	3	0	0.04
2q12.3	ST6GAL2, LOC729087	0	0	3	0	0.04
2q13	IL1RN, PSD4, pilocytic astrocytomaX8	0	0	3	0	0.04
2q14.1	DPP10	0	0	2	1	0.04
2q14.3	HS6ST1	0	0	2	1	0.04
3q12.2	GPR128, TFG, ABI3BP	0	0	3	0	0.04
3q12.3	SENP7, LOC643412	0	0	3	0	0.04
3q13.11	CBLB	0	0	3	0	0.04
3q13.12	CD47	0	0	3	0	0.04
3q13.2	KIAA2018, NAT13, BOC, WDR52, CCDC52	0	0	3	0	0.04
3q13.33	STXBP5L	0	0	3	0	0.04
3q21.2	SLC12A8	0	0	3	0	0.04
3q21.1–3q21.2	KALRN	0	0	3	0	0.04
7p21.3	FLJ20323	0	0	2	1	0.04
9q34.2	DBH, LOC138948, SARDH, VAV2	0	0	3	0	0.04
15q25.3	NTRK3	0	0	3	0	0.04
20q13.2	DOK5	0	0	3	0	0.04

GCN, gene copy number.
[a]Among the 47 BAC clones with significantly different GCN alterations, representative clones with known genes in the chromosome loci are summarized.

of pilocytic astrocytoma patients when analysis was performed only among the patients undergoing subtotal resection. These results suggest that pilomyxoid astrocytoma histology might act as an independent prognostic factor in hypothalamic/optic chiasmatic pilomyxoid astrocytoma or pilocytic astrocytoma.

We also observed one pilomyxoid astrocytoma patient who manifested a pilocytic astrocytoma histology in a recurrent tumor, as has been reported previously.[1,3,5,10] These results support the hypothesis that pilomyxoid astrocytoma and pilocytic astrocytoma might be biologically related entities rather than distinct diseases. Therefore, to further delineate the biological relevance of pilomyxoid astrocytoma and pilocytic astrocytoma, we comparatively investigated the whole genomic copy number changes using array CGH.

Pilocytic astrocytoma is the most common brain tumor in children. It is a WHO (World Health Organization) grade 1 tumor that does not progress to a higher grade in most cases, and for which the 10-year survival rate among patients is 96%.[26] However, neither the pathogenesis nor the molecular genetic alterations involved in pilocytic astrocytoma have been completely elucidated, and previous reports on the conventional cytogenetics of pilocytic astrocytoma have found only infrequent and inconsistent chromosomal abnormalities, which included gains of chromosomes 5, 6, 7, 12, 17, 19, and 22.[27–36] A previous study analyzing pilocytic astrocytoma by conventional CGH showed a gain of single chromosomes, including 5, 6, 7, and 9, in only 5 (12%) of 41 pediatric pilocytic astrocytomas, as well as multiple complex chromosomal abnormalities, including novel gains of chromosomes 1p and 2p, in 2 (29%) of 7 adult cases.[37] A recent report on genomic alterations of pilocytic astrocytoma using high-resolution array CGH revealed nonrandom multiple whole chromosomal gains in 14 (32%) of

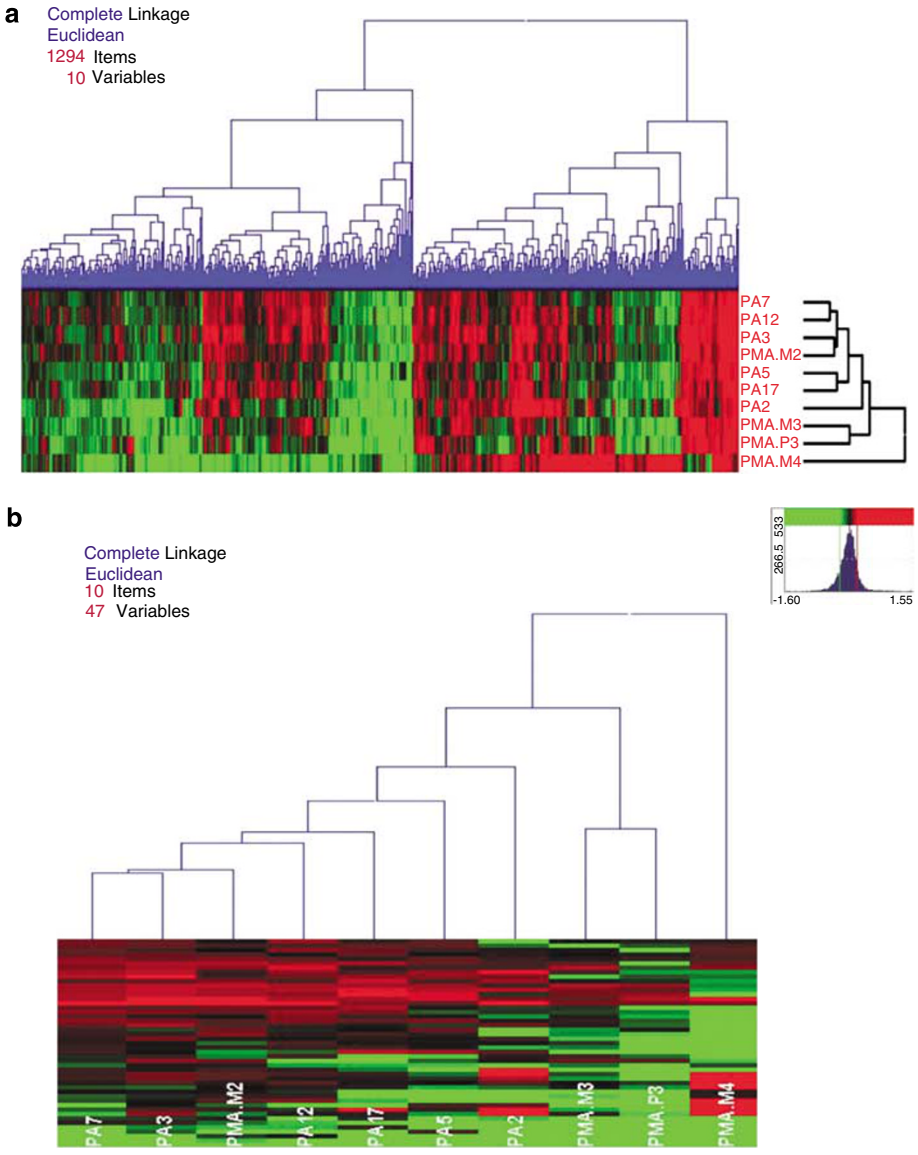

Figure 5 (**a**) Unsupervised hierarchical clustering dendrogram and heat map showing DNA copy number ratios in samples. (**b**) Hierarchical clustering dendrogram and heat map using 47 clones showing a significantly different frequency of copy number gains and losses between pilomyxoid astrocytoma and pilocytic astrocytoma.

44 pilocytic astrocytomas involving chromosomes 5, 7, 6, 11, 15, and 20.[15] Although this study, which employed array-CGH analysis, did not set out to discover the characteristic genetic changes of pilocytic astrocytoma, we compared the previous cytogenetic and molecular genetic results with the results of our study. In the result, we found that chromosome 8q24.3 was gained in the gene copy number in four of six pilocytic astrocytoma patients and one of four pilomyxoid astrocytoma patients. We also found that chromosomes 8p23.3, 9p24.3, and 15q26.3 were commonly lost in 4–5 of the six pilocytic astrocytoma patients. Although it is possible that these genes might be involved in the tumorigenesis of pilocytic astrocytoma, the limited number of patients included in our study makes it difficult to make such a deduction. We could not detect any gains of copy number in chromosome 6. In a previous study on pilocytic astrocytoma using array CGH, a genomic gain or loss of small regions was not a common feature, but whole chromosome gains were observed.[15] However, our study revealed a low frequency of small-region gains or losses as the predominant genomic changes in pilocytic astrocytoma. The difference in the tissue material used, namely fresh frozen tissue in the previous study *vs* formalin-fixed paraffin-embedded tissue in our study, and the application of a different type of array CGH might partly explain the discrepancies.

Few reports are available with respect to the genetic abnormalities of pilomyxoid astrocytoma. Preliminary analysis using CGH, reported by Komotar

et al,[4] showed no abnormalities in pilomyxoid astrocytomas. One pilomyxoid astrocytoma case with *BCR* gene disruption was reported previously.[38] We observed a low frequency of gains or losses of random small regions of variable chromosomes. There were no statistical differences in the frequency of gene copy number alterations between pilomyxoid astrocytoma and pilocytic astrocytoma. Only 47 (3.6%) clones were gained or lost with significantly different frequencies between pilomyxoid astrocytoma and pilocytic astrocytoma. However, although one pilomyxoid astrocytoma case (no. M2) was committed to a subgroup composed of pilocytic astrocytoma patients, unsupervised hierarchical clustering analysis using all 1294 BAC clones was able to classify the pilomyxoid astrocytomas and pilocytic astrocytomas into distinct groups. As three of four pilomyxoid astrocytoma cases subject to array CGH were mixed-form pilomyxoid astrocytomas that also contained a conventional pilocytic astrocytoma region, it might not be possible to clearly identify the genetic aberrations unique to pilomyxoid astrocytoma. This was one of the major weak points of our study, in addition to the small number of patients examined. Whether the 47 clones showing significantly different gene copy numbers between pilomyxoid astrocytoma and pilocytic astrocytoma would provide a specific genetic signature explaining the biological behavior of pilomyxoid astrocytoma should be verified by further study. Notably, chromosomes 15q26.3, 9p24.3, and 8p23.3 were found to have suffered copy number loss in more than 75% of pilocytic astrocytoma and pilomyxoid astrocytoma patients. The representative genes located in these regions and the encoded proteins are as follows: *LASS3* (longevity assurance homolog 3) in 15q26.3; *ANKRD15* (ankyrin repeat domain 15) in 9p24.3; and *FBXO25* (F-box only protein 25) in 8p23.3 (www.genome.ucsc.edu). LASS3 is known as a ceramide synthase that is specifically expressed in testis.[39] FBX25 protein has been strongly expressed in the human brain, and its disruption has been observed in mentally retarded epileptic-seizure patients.[40] Deletion of the *ANKRD15* gene has been reported in a family with parent-of-origin-dependent inheritance of cerebral palsy.[41] Moreover, expression of the *ANKRD15* gene has been found to be suppressed in renal cell carcinoma and, thus, has been suggested as a kind of tumor suppressor gene.[42] This raises the question of the possible role of 9p24.3 (encoding *ANKRD15*) copy number loss in the tumorigenesis of pilocytic astrocytoma and pilomyxoid astrocytoma.

In conclusion, on the basis of the results of clinicopathological examination and global gene copy number analysis, we suggest that pilomyxoid astrocytoma and pilocytic astrocytoma might be pathologically and genetically related entities with partially different genetic alterations manifesting different clinical behaviors rather than distinct diseases.

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Disclosure/conflict of interest

We have no conflicting interests to declare.

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