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Prognostic Significance of Expression Patterns of EGFR Family, p21 and p27 in High-grade Astrocytoma

Shinya NABIKA[1,*], Katsuzo KIYA[1], Hideki SATOH[1], Tatsuya MIZOUE[1], Hiroshi KONDO[1], Masaya KATAGIRI[1], Takashi NISHISAKA[2], Kazuhiko SUGIYAMA[3] and Kaoru KURISU[3]

1) Department of Neurosurgery, Hiroshima Prefectural Hospital, Hiroshima 734-8530, Japan
2) Department of Pathology, Hiroshima Prefectural Hospital, Hiroshima 734-8530, Japan
3) Department of Neurosurgery, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8551, Japan

ABSTRACT

The goal of this study was to investigate the relationship among immunohistochemical expression of epithelial growth factor receptor (EGFR) family proteins, p21, p27 and prognosis in patients with high-grade astrocytoma. Expression of EGFR family proteins (c-erbB-1, c-erbB-2, c-erbB-3, c-erbB-4), p21 and p27 and Ki-67 labeling index (LI) were studied in 59 samples of high-grade astrocytoma. Expression of protein levels was analyzed by immunohistochemical staining of formalin-fixed and paraffin-embedded sections. Results were analyzed in relation to age, gender and survival. Overexpression of c-erbB-1, c-erbB-2, c-erbB-3 and c-erbB-4 was found in 40 (67.8%), 17 (28.8%), 3 (5.1%) and 42 (75.0%) samples, respectively. Similarly, low expression of p21 and p27 was observed in 50 (84.8%) and 27 (45.8%) samples. Mean Ki-67 LI was 17.3 ± 1.1. Cox multiple regression analysis showed that c-erbB-1 (Hazard rate(HR) 1.57, 95% Confidence interval (CI) 1.08-2.36; p=0.017), c-erbB-4 (HR 1.79, 95%CI 1.20-2.74; p=0.004) and p27 (HR 0.50, 95%CI 0.30-0.82; p=0.006) were significantly associated with survival. High expression of c-erbB-1 and c-erbB-4 and low expression of p27 were associated with poor prognosis in these patients.

Key words: *EGFR, p27, p21, High-grade astrocytoma*

The epidermal growth factor receptor (EGFR) family comprises 4 closely related receptor proteins, c-erbB-1 (c-erbB1/HER or EGFR), c-erbB-2 (HER2/neu), c-erbB-3 (HER3) and c-erbB-4 (HER4). When these 4 receptor proteins are activated by epidermal growth factor and other ligands, 10 possible homo- and hetero-dimerizations can be formed and then subsequently phosphorylated. A variety of intracellular signaling pathways can then be triggered, such as via PI3K/Akt and Ras/MAPK. These processes subsequently regulate cell-cycle progression, accelerating progression of G1 into S phase[23]. This process requires phosphorylation and activation of cyclin-dependent kinase (CDKs) by cyclins. As a counterbalance to this, CDK inhibitors such as p21 and p27 can interact with CDK-cyclin complexes and inhibit their activities. The role of c-erbB-1 expression in the malignant progression of astrocytoma and its effect on progression-free survival and overall survival have been extensively debated[3,5,20,21]. However, few studies have examined c-erbB-2-4 and the clinical significance of simultaneous expression of all 4 members in glioma has not yet been well investigated[1,10,17]. The objective of this study was to establish the prognostic significance of the expression of EGFR family members in patients with high-grade astrocytoma. In addition, correlations between the expression of EGFR family members and CDK inhibitors (p21, p27) were also investigated, despite the fact that these markers alone may offer better prognostic indicators in patients with high-grade astrocytoma[8].

MATERIALS AND METHODS

Tumor samples

Data from 59 patients with high-grade astrocytoma treated at our hospital between 1995 and 2005 were obtained from clinical records. Patients

*Address all correspondence to: Shinya Nabika,
Department of Neurosurgery, Matsue Red Cross Hospital, 200 Horomachi, Matsue, Shimane 690-8506, JAPAN
Tel: +81-852-24-2111 Fax: +81-852-31-9783 E-mail: snabika@go4.enjoy.ne.jp

were followed for at least 2 years after resection or until death. All tumors were situated in the supratentorial cavity and tissue specimens were obtained from initial surgery and classified according to WHO criteria by one of the authors (T.N.). Tumors originating in the brainstem or thalamus were excluded from this study. Patients who had undergone biopsy were excluded. Twenty-four tumors were classified as anaplastic astrocytomas (Grade 3), and 35 tumors were graded as glioblastoma (Grade 4). All patients underwent postoperative radio- and/or chemotherapy. Patient information was handled in accordance with Institutional Review Board regulations.

Immunohistochemistry

Specimens were fixed routinely in 4% phosphate-buffered formaldehyde and embedded in paraffin. The most representative blocks were selected and sectioned at 4-μm thickness on positively charged slides. Tumor samples were used to determine the expression of EGFR family members, p21, p27 and Ki-67. Mouse monoclonal antibody was employed as a primary antibody at 1:1000 dilution for p27 (Dakocytomation Denmark A/S, Glostrup, Denmark), 1:10 dilution for c-erbB-1 (Novocastra Laboratories, Newcastle, UK), 1:40 dilution for c-erbB2 (Novocastra Laboratories), 1:30 dilution for c-erbB3 (Novocastra Laboratories), and 1:100 dilution for Ki-67 (Dakocytomation Denmark A/S). Similarly, mouse polyclonal antibody was employed as a primary antibody at 1:50 dilution for c-erbB4 (LAB Vision, Fremont, CA, USA) and 1:150 dilution for p21 (Santa Cruz Biotechnology, Heidelberg, Germany). Pathological specimens (4 m thick) were deparaffinized by treatment with xylene for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min. Each specimen was washed 3 times in phosphate-buffered saline (PBS) and incubated overnight with primary antibodies at 4°C. After washing 3 times in PBS, secondary antibody was applied according to the instructions of the manufacturer. Detection of secondary antibody was achieved using a solution of diaminobenzidine in 0.05 M Tris buffer (pH 7.6) containing 0.003% hydrogen peroxide for 5 min. To facilitate cytoplasmic visualization of immunostained product, slides were counterstained with Mayer hematoxylin. Samples of breast tumor tissue were used as positive controls. Negative controls were obtained by omitting the primary antibody. All slides stained for EGFR family, p21, p27 and Ki-67 were reviewed and scored by the same author, who was unaware of pathological diagnosis and other clinical and radiological data. Labeling index (LI) for all proteins was determined by counting the number of positive cells per 1000 tumor cells.

Statistical Analysis

Correlations were determined between the expression pattern of each protein and survival of the 59 high-grade astrocytoma patients. Statistical analyses were performed using SAS software ver.8.2 (SAS Institute, Cary, NC, USA). Values of $p<0.05$ were considered statistically significant. Survival was estimated in days beginning from the date of the diagnostic surgical procedure to death or the end of follow-up. EGFR family members were categorized immunohistochemically as negative (LI 30%) or positive (LI >30%). Immunopositivity for p21 and p27 was categorized into 3 groups: LI <30% (low expression), LI=30-50%, and LI >50%. Comparison of relative risk factors was evaluated by multiple regression analysis using Cox proportional hazards modeling. For univariate survival analysis, survival curves were computed using the Kaplan-Meier (KM) product-limit method and compared using the log-rank test.

RESULTS

Clinical data

The 59 patients comprised 35 men and 24 women. Mean age at diagnosis was 61.2 ± 13.3 years (range, 28-94 years). Tumors comprised 24 cases of anaplastic astrocytoma and 35 cases of glioblastoma. Median total dose of postoperative

Table 1. Immunohistochemistry of marker proteins in high-grade astrocytoma

Marker proteins	No. of samples (%) with LI >30%		
c-erbB1	40/59 (67.8)		
c-erbB2	17/59 (28.8)		
c-erbB3	3/59 (5.1)		
c-erbB4	42/59 (75)		
	No. of samples (%)		
	LI <30%	LI=30-50%	LI >50%
p21	50/59 (84.8)	9/59 (15.3)	0/59 (0.0)
p27	27/59 (45.8)	23/59 (39.0)	9/59 (15.3)

Table 2. Cox Regression multivariate analysis for survival in high-grade astrocytomas

	Survival		
Variable	Hazard Ratio	95%CI	p
Age	1.02	0.99-1.05	0.159
Gender	0.82	0.56-1.18	0.285
HER 1	1.57	1.08-2.36	0.017
HER 2	0.71	0.46-1.07	0.100
HER 3	0.81	0.32-1.57	0.570
HER 4	1.79	1.20-2.74	0.004
Ki-67	1.02	0.98-1.04	0.310
p21	1.06	0.55-2.05	0.851
p27	0.50	0.30-0.82	0.006

radiotherapy was 56 Gy, with 2 Gy daily fractions for all patients.

Immunohistochemical analysis

Overexpression of c-erbB-1, c-erbB-2, c-erbB-3 and c-erbB-4 was found in 40 (67.8%), 17 (28.8%), 3 (5.1%), and 42 (75.0%) tumor samples, respectively. The p21 LI was <30% in 50 samples (84.8%), 30-50% in 9 samples (15.3%), and >50% in 0 samples (0%). The p27 LI was <30% in 27 samples, (45.8%), 30-50% in 23 samples (39.0%), and >50% in 9 samples (15.3%). Mean Ki-67 LI was 17.3 1.1 (Table 1). Overexpression of c-erbB-2 was found in 5 anaplastic astrocytomas (20.8%) and 12 blioboastoma (34.3%).

Correlations between markers

Cox multiple regression analysis showed that c-erbB-1 (hazard ratio (HR), 1.57, 95% confidence interval (CI) 1.08-2.36; p=0.017), c-erbB-4 (HR 1.79, 95%CI 1.20-2.74; p=0.004) and p27 (HR 0.50, 95%CI 0.30-0.82; p=0.006) were significantly associated with survival (Table 2). Representative pathological and immunohistochemical findings are shown in Fig. 1a-c. KM survival curves for patients with overexpression of c-erbB-1, c-erbB-4 and p27 are presented in Fig. 2a-c. Survival of patients with c-erbB-1-positive tumors was significantly shorter than that of patients with c-erbB-1-negative tumors (p=0.023). Survival of patients with p27-positive tumors was significantly longer than that of patients with p27-negative tumors (p=0.028). Although no significant correlation existed between survival and c-erbB-4 expression according to the KM survival curve, multivariate analysis with the Cox regression test showed a strong relationship.

DISCUSSION

This is the first study to investigate the relationship between expression patterns of EGFR family members (including cyclin-dependent kinase inhibitors, p21 and p27) and survival in patients with high-grade astrocytoma. The results suggest that high expression of c-erbB-1 and c-erbB-4 and low expression of p27 suggest poor outcomes for patients with high-grade astrocytoma.

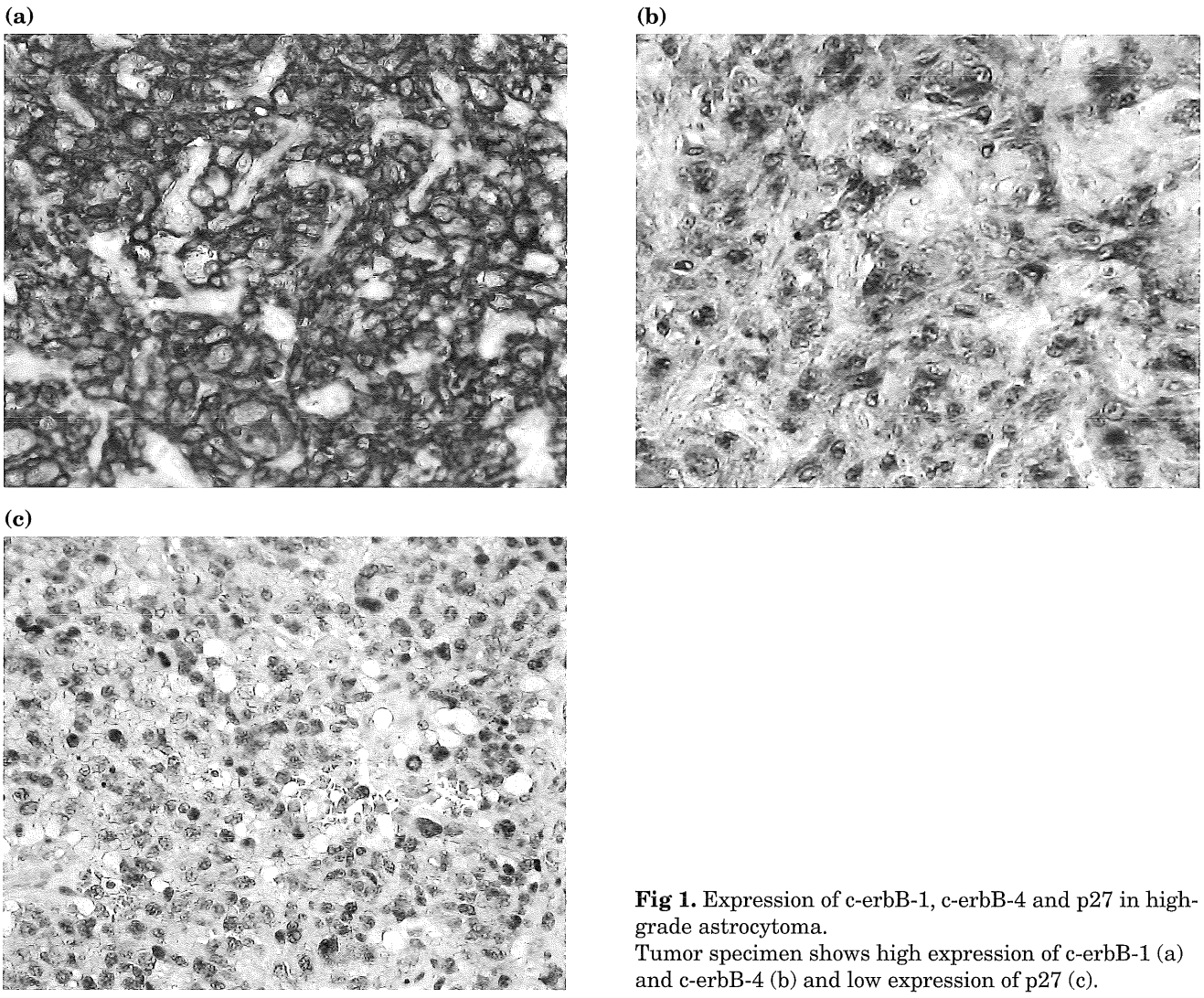

Fig 1. Expression of c-erbB-1, c-erbB-4 and p27 in high-grade astrocytoma.
Tumor specimen shows high expression of c-erbB-1 (a) and c-erbB-4 (b) and low expression of p27 (c).

Although the present and other previous studies have reported that c-erbB-1 overexpression is associated with worse prognosis in patients with astrocytic neoplasms of multiple grades[3,11,12,19], other studies have reported that c-erbB-1 overexpression or amplification does not correlate with prognosis in this population[5,18]. Differences in age distributions of patients may explain the lack of consistent findings among different studies. Heimberger et al showed that overexpressed c-erbB-1 was indicative of poor prognosis in younger patients[5]. On the other hand, Simmons et al reported that c-erbB-1 overexpression tended to be associated with decreased survival in younger patients, but increased survival in older patients. These data suggested that the effect of c-erbB-1 might differ depending on age group. This shows that c-erbB-1 has significant effects on survival whatever the consequence may be[20]. In the present study, c-erbB-1 overexpression indicated worse prognosis irrespective of patient age.

Previous studies investigating the prognostic value of c-erbB-2 expression in malignant gliomas have produced mixed findings[1,13,17]. Some reports have indicated that c-erbB-2 expression might be a poor prognostic marker[10,17], whereas most reports including the present one have established no prognostic value of c-erbB-2 expression, although c-erbB-2 expression correlates well with increasing tumor grade[1,13]. Further studies are needed to delineate relationships between tumor grade and prognosis.

In the present study, c-erbB-4 expression was also an independent indicator of poor survival. Expression of c-erbB-4 varies in different types of cancer. For example, c-erbB-4 is strongly down-regulated in renal, prostate and pancreatic cancers[4,15,22], and up-regulated in medulloblastomas and gastric, breast, and colon cancers[2,6,7,14]. In a study by Andersson et al, higher expression of c-erbB-4 in low-grade compared to high-grade glioma might suggest that c-erbB-4 acts as a suppressor for malignant transformation of glioma[1]. Possible explanations for these contradictory observations could be that co-expression of other EGFR family members influences the function of c-erbB-4, or that c-erbB-4 is expressed as a differentiated phenotype in different types of cancers. In the present study, based on the results of multivariate analysis, c-erbB-4 can be presumed to be implicated

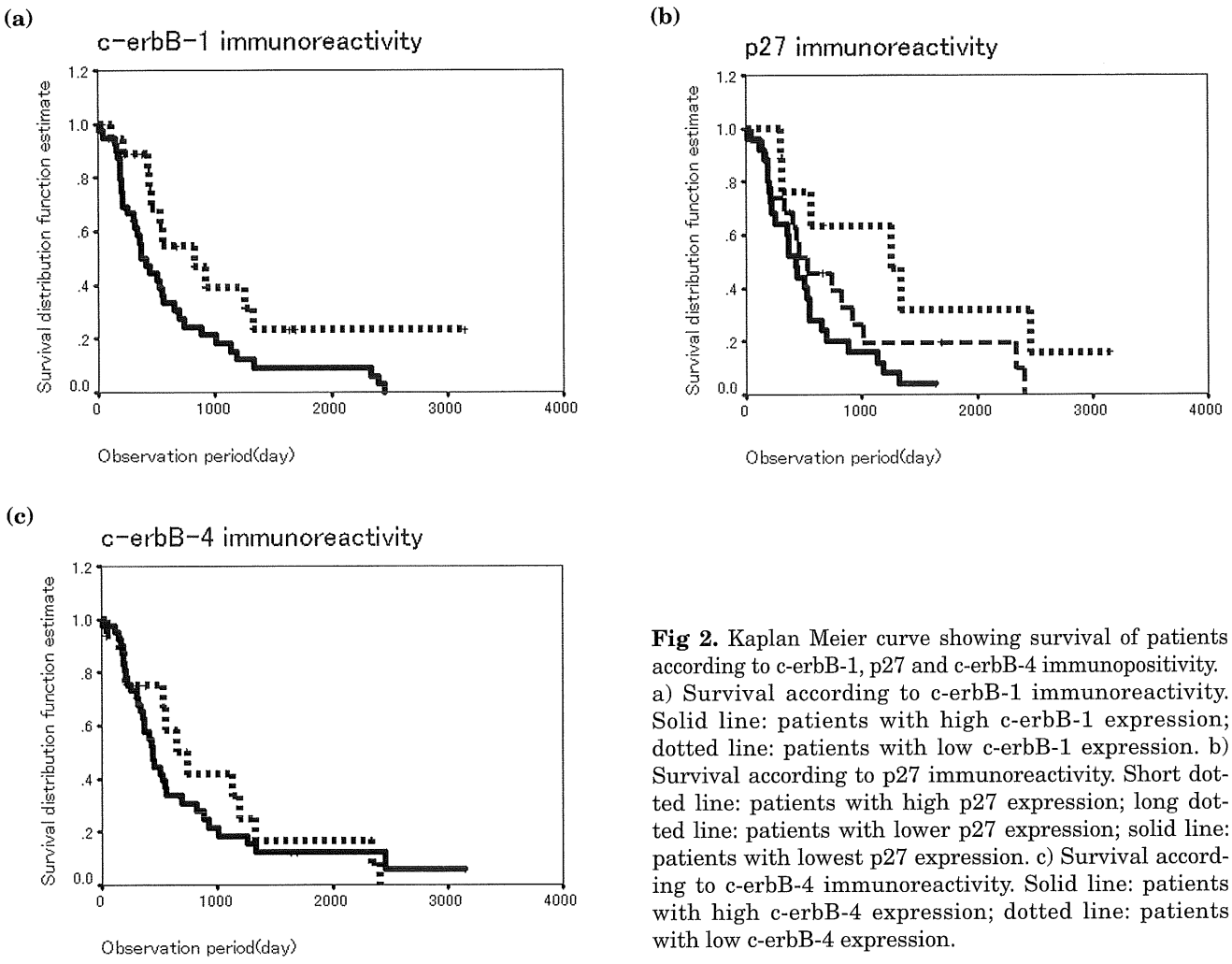

Fig 2. Kaplan Meier curve showing survival of patients according to c-erbB-1, p27 and c-erbB-4 immunopositivity. a) Survival according to c-erbB-1 immunoreactivity. Solid line: patients with high c-erbB-1 expression; dotted line: patients with low c-erbB-1 expression. b) Survival according to p27 immunoreactivity. Short dotted line: patients with high p27 expression; long dotted line: patients with lower p27 expression; solid line: patients with lowest p27 expression. c) Survival according to c-erbB-4 immunoreactivity. Solid line: patients with high c-erbB-4 expression; dotted line: patients with low c-erbB-4 expression.

in the pathogenesis of high-grade astrocytoma. The clinical significance of c-erbB-4 expression remains unknown and needs to be elucidated.

Cell cycle progression is regulated by CDKs that interact with cyclins to coordinate the biological sequences resulting in cell division. Cyclin-dependent kinase-inhibitors bind to and inhibit the activity of cyclin/CDK complexes, negatively regulating cell cycle progression. Members of the Cip/Kip family of cyclin-dependent kinase-inhibitors include p21 and p27, and p27 showed independent predictive value for our patients with high-grade astrocytoma. Previous studies of cyclin-dependent kinase-inhibitors have also concluded that low p27 expression correlates with high-grade tumor and is predictive of a poor outcome[8,16]. The suppressive effect of p27 is exerted by the inhibition of pRb phosphorylation, which in turn arrests cells in the G1-phase and prevents entry into the S phase. Furthermore, p27 expression induces apoptosis, implying a second antineoplastic function for this tumor suppressor protein. Interestingly, EGFR positivity was significantly correlated with low p27 protein levels, as previous studies have suggested that EGFR-dependent intracellular signaling pathways promote cell proliferation, inhibition of apoptosis and neo-angiogenesis by downregulating p27 expression. Combined assessment of p27 and the EGFR family, particularly c-erbB-1 and c-erbB-4, may provide more useful prognostic information. Although both p27 and p21 are cell cycle regulators controlling G1-S transition, p21 appears to be less significant in patients with high-grade astrocytoma, as demonstrated in the present and previous studies [8,9]. Kirla et al reported that elevated levels of p21 expression might represent a feedback mechanism for the cell cycle, rather than offering true functional regulation in malignant cell populations[8].

Poor prognosis in high-grade astrocytoma could be attributable to high expression of c-erbB-4, in addition to the well-established fact of high c-erbB-1 expression and low p27 expression. These findings suggest that EGFR-dependent intracellular signaling pathways, mainly c-erbB-1 and c-erbB-4, promote cell proliferation, inhibition of apoptosis and neo-angiogenesis by down-regulating p27 expression. No association was seen between survival and c-erbB-2 and c-erbB-3. Heterogeneity in the expression of different EGFR family members shown by this study may be clinically meaningful, as dimerization between EGFR family is essential for activation. Further research is needed to define the pathogenesis of high-grade astrocytoma, and characterization is then needed to develop further individualized therapeutic strategies for patients.

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