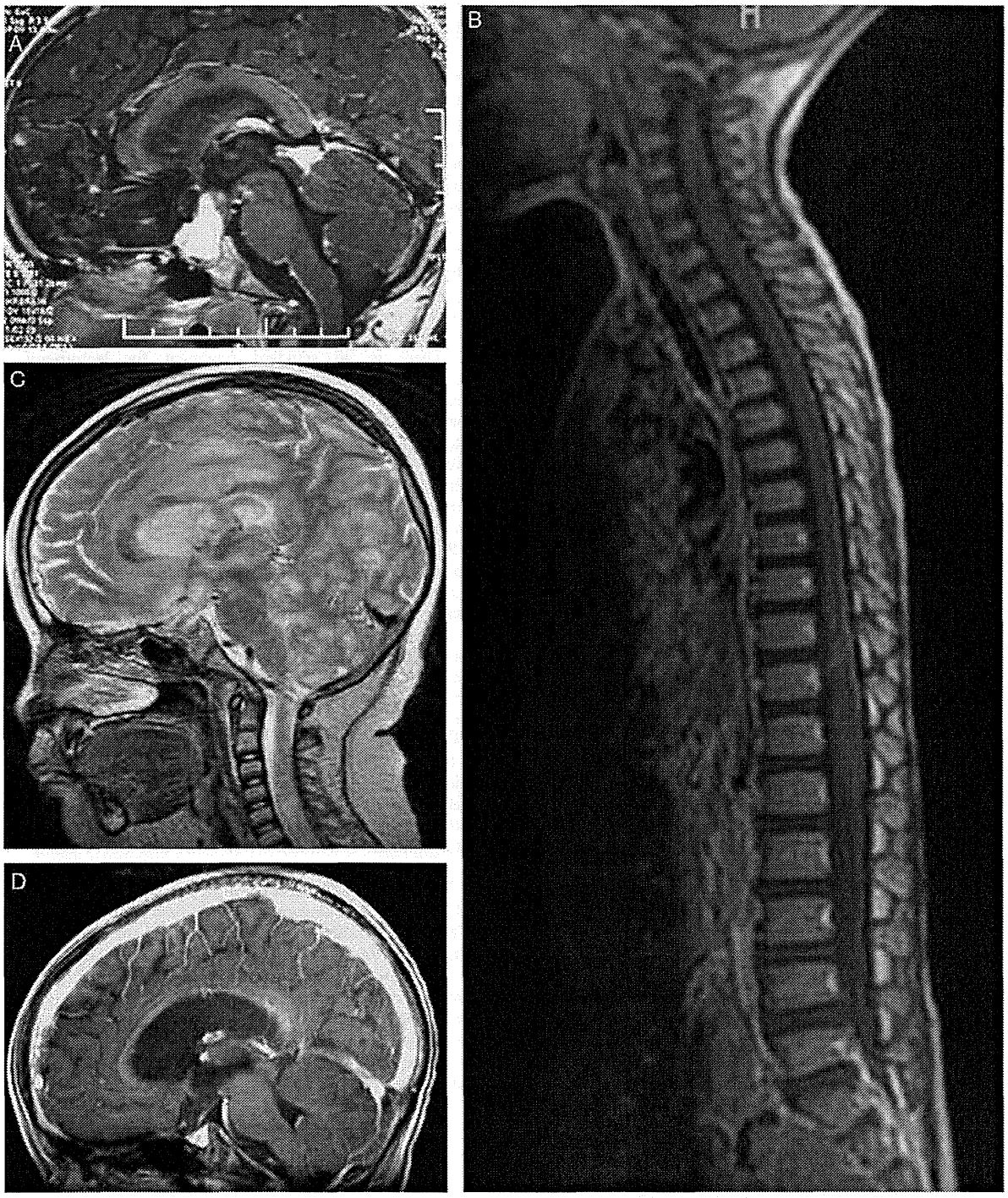

FIGURE 1. Radiographic findings: (A) Sagittal, T1-weighted, contrast-enhanced magnetic resonance imaging (MRI) taken at initial presentation of the 5-year-old patient. The optic nerve is seen to enter the enhanced portion of the tumor, and patchy, linear enhancement of the leptomeninges is visible. B, Sagittal, T1-weighted, contrast-enhanced MRI of the spine showing diffuse extramedullary gadolinium enhancement. C, Sagittal T2-weighted image after 5 cycles of maintenance chemotherapy. No enhanced lesion of the optic nerve is visible, but global growth of the T2-prolonged lesions is evident. D, Sagittal, T1-weighted, contrast-enhanced image of the patient at 7 years of age, after 3 months of maintenance temozolomide treatment. The T1-weighted contrast-enhanced lesion and T2-prolonged lesions show a complete response.

expansive growth of the gliomatosis lesions (Fig. 1C). Because his neurological status worsened with decreasing speech production, loss of vision, and gait ataxia, the patient was then treated with craniospinal radiation at a dose of 24 Gy (1.8 Gy/fraction, 5 fractions/wk) with concomitant TMZ at a dose of 75 mg/m^2/d, given orally 7 days per week from the first day of radiotherapy until the last day of radiotherapy. MR scans at 3 weeks after completion of radiotherapy showed disappearance of the leptomeningeal gliomatosis lesions. After a 4-week break, we initiated adjuvant therapy with TMZ at a dose of 150 to 200 mg/m^2 orally on days 1 to 5, every 28 days. After the third course of adjuvant TMZ, we saw no evidence of disease on MR images or CSF samples, in which protein level had normalized (16 mg/dL) (Fig. 1D). TMZ treatment was discontinued after 4 courses. The patient is blind but alive with no evidence of disease 2 years after the start of treatment. Neuropsychological testing revealed a verbal intelligence quotient of 53, hence the patient is taught in a special education setting.

The patient's parents gave informed consent before all treatments and testing. All the protocols we used were approved by our hospital's institutional review board.

DISCUSSION

We describe a child with optic nerve and leptomeningeal dissemination of PMA presenting as leptomeningeal gliomatosis. PMA was originally described by Jänisch et al[3] in 1985 as "diencephalic pilocytic astrocytoma with clinical onset in infancy," with the term "pilomyxoid astrocytoma" first introduced in 1999 by Tihan et al.[1] PMA is a newly defined distinct glioma listed as grade II in the 2007 World Health Organization classification.[7] PMA is found typically in the hypothalamic/chiasmatic regions, sites that are also affected by classical PA. Predominantly affecting infants and children (median age, 10 mo), PMA seems to have a less favorable prognosis than PA. Local recurrences and cerebrospinal spread are more likely to occur in PMA than in PA, despite multimodal chemotherapy. Although distinguishable on clinical and histologic grounds, a closer relationship between the 2 diseases may exist based on the cellular origins of PMA and PA.[4] The present case highlights the propensity of this tumor to disseminate through the CSF and neuraxis.

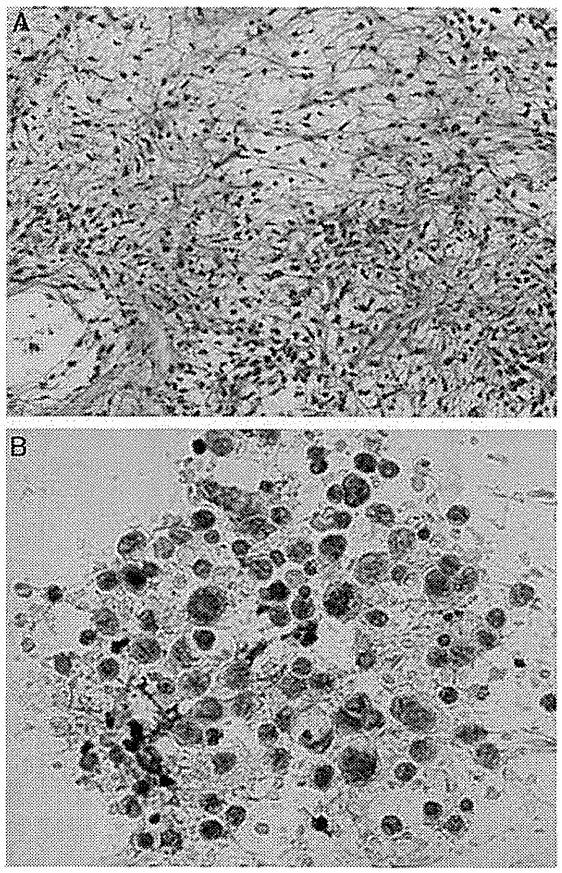

FIGURE 2. A, Biopsy sample stained with hematoxylin and eosin showing monomorphous piloid astrocytes embedded in a predominantly myxoid background (original magnification × 100). B, The cells in the cerebrospinal fluid displayed high-grade features, including round-to-oval hyperchromatic, pleomorphic nuclei. Variable amounts of cytoplasm staining with glial fibrillary acidic protein were observed (original magnification × 400).

There is no standard treatment for leptomeningeal gliomatosis; indeed, treatment of this condition has largely been unsuccessful in previous studies.[8] The most frequent approach is to use CSI as the main treatment with adjuvant systemic and/or intrathecal chemotherapy. In a series of 18 patients with leptomeningeal gliomatosis who underwent multimodal treatment, the median survival from the initiation of treatment was only 3.5 months.[9] For the present patient, we initially used frontline chemotherapy consisting of CBDCA and VCR, a regimen described by Packer et al.[6] They reported that 2 of 3 cases with diffuse leptomeningeal lesions responded to this regimen.[6] The primary rationale for using frontline chemotherapy for children with progressive or unresectable gliomas has been to delay the need for radiotherapy. None of the patients described by Packer and colleagues, however, had leptomeningeal gliomatosis arising from PMA. Because our initial frontline regimen failed to stop the growth of leptomeningeal lesions (8 mo after the initiation of treatment), we decided to switch to radiotherapy, given as CSI, combined with TMZ according to the Stupp regimen for glioblastoma multiforme.[10] Clinical and radiographic improvements were considerable, and the first cerebral MR imaging showed a complete response. Our patient represents the first report of a child with optic nerve and leptomeningeal dissemination of PMA and CSF seeding who was treated with low-dose CSI and concurrent TMZ.

We successfully treated the present patient with low-dose CSI (24 Gy) and concurrent TMZ. To date, no consensus exists on the recommended dose of CSI for the treatment of PMA with leptomeningeal gliomatosis. Moreover, although 35 Gy is most commonly used when CSI can be delivered alone, no studies have reported the impact of treatment with CSI and concurrent TMZ for this condition. Enting et al[5] reported on a patient with leptomeningeal PMA who was treated with a combination of whole-brain radiotherapy and TMZ. Distant spinal recurrence was noted after whole-brain radiotherapy. Even in leptomeningeal gliomatosis arising from PA, both the activity of chemotherapy and prognosis are highly variable.[11,12] Given that TMZ may produce the biological effect of radiosensitization, has shown some activity with only mild toxicity in this disease, and is easy to administer,[13,14] we opted for systemic treatment with TMZ combined with low-dose CSI in the present patient. This low dose of CSI combined with chemotherapy was well tolerated in other central nervous system malignancies,[15,16] and dose reduction of CSI seems to significantly reduce the severity of cognitive loss, particularly in younger patients who receive CSI to eradicate disease involving the leptomeninges.[17]

The present report contributes to our understanding of the diagnostic profile of PMA and describes the combination of

CSI and TMZ as an alternative for leptomeningeal dissemination of PMA. Leptomeningeal dissemination is a globally aggressive neoplasm with different management requirements from intraparenchymal glioma that develops from PA or PMA. Although leptomeningeal gliomatosis arising from PMA is a rare phenomenon, its histopathologic and clinical characteristics can be distinguished, and we suggest aggressive treatment with low-dose CSI as an effective option. More work is needed to address the issue of optimal therapy and the relative value of more intensive chemotherapy, as compared with CSI or radiotherapy, for this enigmatic pediatric neoplasm. This report is the first to show that leptomeningeal gliomatosis arising from PMA can be successfully treated with a combination of CSI and TMZ after failure of frontline chemotherapy.

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REFERENCES

1. Tihan T, Fisher PG, Kepner JL, et al. Pediatric astrocytomas with monomorphous pilomyxoid features and a less favorable outcome. *J Neuropathol Exp Neurol.* 1999;58:1061–1068.
2. Komotar RJ, Mocco J, Jones JE, et al. Pilomyxoid astrocytoma: diagnosis, prognosis, and management. *Neurosurg Focus.* 2005; 18:1–4.
3. Jänisch W, Schreiber D, Martin H, et al. Diencephalic pilocytic astrocytoma with clinical onset in infancy. Biological behavior and pathomorphological findings in 11 children. *Zentralbl Allg Pathol.* 1985;130:31–43.
4. Ceppa EP, Bouffet E, Griebel R, et al. The pilomyxoid astrocytoma and its relationship to pilocytic astrocytoma: report of a case and a critical review of the entity. *J Neurooncol.* 2007; 81:191–196.
5. Enting RH, van der Graaf WT, Kros JM, et al. Radiotherapy plus concomitant and adjuvant temozolomide for leptomeningeal pilomyxoid astrocytoma: a case study. *J Neurooncol.* 2006;80: 107–108.
6. Packer RJ, Ater J, Allen J, et al. Carboplatin and vincristine chemotherapy for children with newly diagnosed progressive low-grade gliomas. *J Neurosurg.* 1997;86:747–754.
7. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 2007;114:97–109.
8. Awad I, Bay JW, Rogers L. Leptomeningeal metastasis from supratentorial malignant gliomas. *Neurosurgery.* 1986;19: 247–251.
9. Chamberlain MC. Combined-modality treatment of leptomeningeal gliomatosis. *Neurosurgery.* 2003;52:324–329; discussion 330.
10. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med.* 2005;352:987–996.
11. Perilongo G, Garre ML, Giangaspero F. Low-grade gliomas and leptomeningeal dissemination: a poorly understood phenomenon. *Childs Nerv Syst.* 2003;19:197–203.
12. Bohner G, Masuhr F, Distl R, et al. Pilocytic astrocytoma presenting as primary diffuse leptomeningeal gliomatosis: report of a unique case and review of the literature. *Acta Neuropathol.* 2005;110:306–311.
13. Franceschi E, Cavallo G, Scopece L, et al. Temozolomide-induced partial response in a patient with primary diffuse leptomeningeal gliomatosis. *J Neurooncol.* 2005;73:261–264.
14. Kil WJ, Cerna D, Burgan WE, et al. In vitro and in vivo radiosensitization induced by the DNA methylating agent temozolomide. *Clin Cancer Res.* 2008;14:931–938.
15. Yasuda K, Taguchi H, Sawamura Y, et al. Low-dose craniospinal irradiation and ifosfamide, cisplatin and etoposide for non-metastatic embryonal tumors in the central nervous system. *Jpn J Clin Oncol.* 2008;38:486–492.
16. Chintagumpala M, Hassall T, Palmer S, et al. A pilot study of risk-adapted radiotherapy and chemotherapy in patients with supratentorial PNET. *Neuro-oncol.* 2009;11:33–40.
17. Packer RJ, Sutton LN, Atkins TE, et al. A prospective study of cognitive function in children receiving whole-brain radiotherapy and chemotherapy: 2-year results. *J Neurosurg.* 1989;70: 707–713.

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Immunological evaluation of personalized peptide vaccination in refractory small cell lung cancer

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Since the prognosis of small cell lung cancer (SCLC) remains poor, development of new therapeutic approaches, including immunotherapies, would be desirable. In the current study, to evaluate immunological responses in refractory SCLC patients, we conducted a small scale phase II clinical trial of personalized peptide vaccination (PPV), in which vaccine antigens are selected based on pre-existing host immunity. Ten refractory SCLC patients, who had failed to respond to chemo- and/or chemoradiotherapies (median number of regimens, 2.5; median duration, 20.5 months), were enrolled. A maximum of four human leukocyte antigen (HLA)-matched peptides showing higher antigen-specific humoral responses were subcutaneously administered (weekly for six consecutive weeks and then bi-weekly thereafter). PPV was terminated before the 3rd administration in four patients because of rapid disease progression, whereas the remaining six patients completed at least one cycle (six times) of vaccinations. Peptide-specific immunological boosting was observed in all of the six patients at the end of the first cycle of vaccinations, with their survival time of 25, 24.5 (alive), 10 (alive), 9.5, 6.5, and 6 months. Number of previous chemotherapy regimens and frequency of $CD3^+CD26^+$ cells in peripheral blood were potentially prognostic in the vaccinated patients (hazard ratio [HR] = 2.540, 95% confidence interval [CI] = 1.188–5.431, P = 0.016; HR = 0.941, 95% CI = 0.878–1.008, P = 0.084; respectively). Based on the feasible immune responses in refractory SCLC patients who received at least one cycle (six times) of vaccinations, PPV could be recommended for a next stage of larger-scale, prospective clinical trials. (*Cancer Sci* 2012; 103: 638–644)

Although recent advances in chemotherapies contributed to improved clinical outcomes in refractory small cell lung cancer (SCLC) patients, their prognosis still remains very poor with a median survival time of 6–10 months.[1–3] Several clinical trials of immunotherapies have been attempted in refractory SCLC patients,[4,5] but none of them demonstrated a meaningful therapeutic benefit to patients. We have developed a novel regime of personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered based on the pre-existing host immunity before vaccination.[6–13] For example, a recently conducted randomized clinical trial in advanced prostate cancer patients showed a promising clinical benefit of PPV.[7] In the current study, to address if refractory SCLC patients have the capability to respond to cancer vaccines, we conducted a small scale phase II study of PPV and evaluated immunological responses in the vaccinated patients.

Materials and Methods

Patients. Patients with histological diagnosis of SCLC were eligible for inclusion in the current study, if they had failed to respond to previous chemotherapies and/or chemoradiotherapies. They also had to possess positive humoral responses to at least two of the 31 different vaccine candidate peptides (Table S1), determined by both human leukocyte antigen (HLA) class I types and the titers of IgG against each peptide. The other inclusion criteria as well as exclusion criteria were not largely different from those of the previously reported clinical studies;[6–9] an age between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for HLA-A2, -A3, -A11, -A24, -A26, -A31, or -A33; life expectancy of at least 12 weeks; adequate hematologic, renal, and hepatic function. Patients with lymphocyte counts of <1000 cells/μL were excluded from the study, since we previously reported that pre-vaccination lymphopenia is an un-favorable factor for overall survival (OS) in cancer patients receiving PPV.[11] Other exclusion criteria included pulmonary, cardiac, or other systemic diseases; an acute infection; a history of severe allergic reactions; pregnancy or nursing; or other inappropriate conditions for enrollment judged by clinicians. The protocol was approved by the Kurume University Ethical Committee and conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). It was registered in the UMIN Clinical Trials Registry (UMIN# 2984). After full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Clinical protocol. This was an open-label phase-II study, in which the primary and secondary endpoints were to identify biomarkers for OS and to evaluate safety in refractory SCLC patients who received PPV, respectively. Thirty-one peptides (PolyPeptide Laboratories, San Diego, CA, USA; American Peptide Company, Vista, CA, USA), whose safety and immunological effects had been confirmed in previously conducted clinical studies,[6–13] were used for vaccination (Table S1). The frequencies of expression of the parent proteins, from which the vaccine peptides were derived, in SCLC tissues were examined by immunohistochemistry (Fig. S1) and shown in Table S1. The right peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, assessed by the titers of IgG specific to each of the 31 different vaccine candidates, as previously described.[14] Although the prostate-related antigens, including prostate-specific antigen (PSA), prostatic acid

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phosphatase (PAP), and prostate-specific membrane antigen (PSMA), have been reported to be expressed not only by prostate cancer but also by other types of cancers,[15–18] the expression frequencies of these molecules in SCLC tissues were low (Table S2). Therefore, the peptides derived from them were selected only when pre-existing IgG responses to other remaining peptides were absent. A maximum of four peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, were subcutaneously administered with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for consecutive 6 weeks. After the first cycle of six vaccinations, up to four antigen peptides, which were re-selected according to the titers of peptide-specific IgG at every cycle of six vaccinations, were administered every 2 weeks up to four cycles (24 vaccinations). Combined chemotherapy and/or radiotherapy were allowed during the vaccination. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC Ver 3.0). The clinical responses were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST 1.1) after the first cycle of vaccinations or at premature termination from the study. Pre-vaccination blood samples (PBMCs and plasma) were available from all of the enrolled patients (n = 10). Post-vaccination blood samples were available from six and four patients, who completed the first and second cycles of vaccinations, respectively.

Measurement of humoral and T cell responses. The humoral responses specific to each of the 31 peptide candidates (Table S1) were determined by peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX, USA), as previously reported.[14] If the titers of peptide-specific IgG to at least one of the vaccine peptides in the post-vaccination plasma were more than twofold higher than those in the pre-vaccination plasma, the changes were considered to be significant.

T cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ ELISPOT assay (MBL, Nagoya, Japan). Briefly, PBMCs (2.5×10^4 cells/well) were incubated in 384-well microculture plates (IWAKI, Tokyo, Japan) with 25 μL of medium (OpTmizer T Cell Expansion SFM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (MP Biologicals, Solon, OH), interleukin (IL)-2 (20 IU/mL; AbD serotec, Kidlington, UK), and each peptide (10 μM). Half of the medium was replaced with new medium containing the corresponding peptide (20 μM) at day 3. After incubation for the following 6 days, the cells were harvested and tested for their ability to produce IFN-γ in response to either the corresponding peptides or negative control peptides from human immunodeficiency virus (HIV). Antigen-specific IFN-γ secretion after 18-h incubation was determined by ELISPOT assay with an ELISPOT reader (ImmunoSpot S5 Versa Analyzer; Cellular Technology Ltd, Shaker Heights, OH, USA). Means of the triplicate samples were used for analyses. Antigen-specific T cell responses were evaluated by the differences between the spot numbers in response to the corresponding peptides and those to the control peptide; differences of at least 10 spot numbers per 10^5 PBMCs were considered as positive. If the spot numbers in response to at least one of the vaccine peptides in the post-vaccination PBMCs were more than twofold higher than those in the pre-vaccination PBMCs, the changes were considered as significant.

Measurement of C-reactive protein, serum amyloid A, and cytokines. C-reactive protein (CRP), serum amyloid A (SAA), and IL-6 in plasma were examined by ELISA using the kits from R&D systems (Minneapolis, MN, USA), Invitrogen, and eBioscience (San Diego, CA, USA), respectively. Multiplexed bead-based Luminex assays were used to measure Th1/Th2 cytokines, including IL-2, IL-4, IL-5, and IFN-γ (Invitrogen). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions. Means of the duplicate samples were used for analyses.

Flow cytometric analysis of immune cell subsets in PBMCs. A suppressive immune cell subset, myeloid-derived suppressor cells (MDSCs), in PBMCs was examined by flow cytometry. For analysis of MDSCs, PBMCs (0.5×10^6) were incubated for 30 min at 4°C with mAbs against lineage markers (CD3, CD14, CD19, CD56), CD33, and HLA-DR. In the cell subset negative for the lineage markers and HLA-DR, MDSCs were identified as positive for CD33. The frequency of MDSCs in the mononuclear cell gate defined by the forward scatter and side scatter was calculated. In addition, the expression of CD26 in PBMCs was analyzed, since the gene expression level of this molecule assessed by DNA microarray analysis was prognostic for OS in the prostate cancer patients receiving PPV (Sasada T , Komatsu N, Itoh K, unpublished observation). PBMCs were stained with anti-CD26 and anti-CD3 mAbs followed by calculation of the frequencies of $CD26^+$ subset in $CD3^+$ cells. The samples were run on a FACSCanto II (BD biosciences, San Diego, CA, USA), and data were analyzed using the Diva software (BD biosciences). All mAbs were purchased from Biolegend (San Diego, CA, USA).

Immunohistochemistry. Anti-tumor immune responses were examined by immunohistochemistry (IHC) in tumor tissues resected from SCLC patients treated with PPV (n = 1, Patient No. 5) or without PPV (n = 3). Paraffin-embedded tissue samples were cut into 4-μm sections, and labeled on the BenchMark XT (Ventata Automated Systems Inc., Tucson, AZ, USA) with anti-CD3 (clone LN10; Novocastra, Newcastle, UK), anti-CD4 (clone 4B12, Novocastra), and anti-CD8 (clone 4B11, Novocastra) mAb. The streptavidin-biotin complex method with 3,3′-diaminobenzidine tetrachloride (DAB) was used as a chromogen (Ventana iVIEW DAB Detection Kit). The expressions of vaccine antigens SART3 and p56lck in the tumor tissue from the patient treated with PPV (Patient No. 5) were also examined by IHC with anti-SART3 (rabbit polyclonal; Abcam, Cambridge, UK) and anti-p56lck (rabbit polyclonal, Abcam) Abs.

Statistical analysis. The Wilcoxon test was used to compare differences between pre- and post-vaccination measurements. All tests were two-sided, and differences at $P < 0.05$ were considered to be statistically significant. OS time was calculated from the first day of peptide vaccination until the date of death or the last date when the patient was known to be alive. Curves for OS were estimated by the Kaplan–Meier method. Potentially prognostic factors were evaluated by the Cox proportional hazards model. A value of $P < 0.1$ was used to identify potentially significant variables. All statistical analyses were conducted using the JMP version 9 or SAS version 9.1 software package (SAS Institute Inc., Cary, NC, USA).

Results

Patients' characteristics. Between March 2009 and October 2010, 10 patients with histology of SCLC were enrolled in this study. Table 1 shows the clinicopathological characteristics of the enrolled patients. All patients were male subjects with a median age of 63.5 years, ranging from 48 to 69. They had advanced stages of cancer (limited-stage disease [LD] at diagnosis, n = 5; extended-stage disease [ED] at diagnosis, n = 5), which had been refractory to previous treatments. Before enrollment, they failed to respond to one (n = 3), two (n = 2), three (n = 2), or more than 4 (n = 3) regimen(s) of chemotherapies and/or chemoradiotherapies. Median duration of these preceding regimens prior to the PPV was 20.5 months, ranging from 1 to 51. Performance status at the time of enrollment was grade 0 (n = 7) or grade 1 (n = 3). The numbers of peptides

Table 1. Characteristics of the enrolled patients with refractory SCLC (n = 10)

Patient No.	HLA Type	Gender	Age	Stage at diagnosis	PS	No. previous regimens	Previous treatment period (months)	Disease location (tumor size) before vaccination	No. vaccinations	Combined therapy	Treatment response†	OS (days)
1	A2/A26	M	58	ED	0	2	32	Mediastinal LN (28 mm), cervical LN‡, brain‡	24	CBDCA, PTX	PD	771
2	A24	M	68	LD	0	3	26	Pleural dissemination‡	2	(–)	PD	17
3	A24	M	62	LD	0	4	19	Cervical LN‡, liver (13 mm)	11	VNR	PD	178
4	A24/A26	M	52	ED	1	6	22	Liver (30 mm), bone (spine)‡, atelectasis‡	2	CBDCA, PTX	PD	16
5	A31/A33	M	67	LD	0	1	51	Lung (36 mm), brain‡	24	CDDP, VP16, WBRT	SD	746§
6	A2/A26	M	51	ED	0	2	5	Mediastinal LN‡, bone (spine)‡	10	AMR	Non-CR/ non-PD	285
7	A26/A31	M	65	LD	0	5	31	Lung (39 mm), adrenal (40 mm, 18 mm), brain (10 mm), mediastinal LN‡	2	CPT11, PTX	PD	33
8	A2/A24	M	69	ED	1	3	10	Pancreas (19 mm), mediastinal LN (15 mm)	14	(–)	PD	195
9	A2/A26	M	69	ED	1	1	3	Lung (50 mm), brain‡	1	(–)	PD	89
10	A2/A24	M	48	LD	0	1	1	Mediastinal LN (16 mm)	21¶	AMR, TPT, SRT	SD	306§

†Evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). ‡Non-measurable lesion. §Patients alive (censored data). ¶Under treatment. AMR, amrubicin; CBDCA, carboplatin; CDDP, cisplatin; CPT11, irinotecan; CR, complete response; ED, extensive-stage disease; LD, limited-stage disease; LN, lymph node; M, male; OS, overall survival; PD, progressive disease; PS, performance status; PTX, paclitaxel; SCLC, small cell lung cancer; SD, stable disease; SRT, stereotactic radiotherapy; TPT, topotecan; VNR, vinorelbine; VP16, etoposide; WBRT, whole brain radiotherapy.

vaccinated to the patients at the first cycle of vaccinations were four peptides in eight patients and two in two patients. Of the 10 patients, six completed the first cycle of six vaccinations, whereas the remaining four patients failed before the 3rd vaccinations due to rapid disease progression. The median number of vaccinations was 10.5 with a range of 1–24. During the PPV, seven patients were treated in combination with chemotherapies and/or radiotherapy, and the remaining three patients did not tolerate them. None had a complete response (CR) or partial response (PR). The best response, seen in two patients, was stable disease (SD), whereas seven patients had progressive disease (PD). A patient without measurable lesions (Patient No. 6) had Non-CR/non-PD.

Toxicities. Toxicities are shown in Table 2. The most frequent adverse events were dermatological reactions at injection sites (n = 7), hematological toxicity (n = 10), and hypoalbuminemia (n = 8). Grade 3 serious adverse events (SAE) were as follows: dyspnea (n = 1), anemia (n = 1), leukocytopenia (n = 1), and lymphopenia (n = 1). The Grade 3 hematological SAE, including anemia, leukocytopenia, and lymphopenia, were transiently observed in the Patient No. 1 during PPV, just after he started receiving a concomitant chemotherapy with carboplatin and paclitaxel. But these SAE disappeared soon after stopping the concomitant chemotherapy, and did not recur even if he restarted the vaccinations after his recovery from the SAE. In addition, he showed no hematological SAE before this episode, while he received no concomitant chemotherapies. Based on these observations, the independent safety evaluation committee for this trial concluded that these SAE might not be directly associated with the vaccinations, but with the concomitant chemotherapy. The Grade 3 dyspnea was observed in Patient No. 2, who rapidly developed pleural effusion due to pleural dissemination and required hospitalization for oxygen supplementation. Since this symptom was highly likely to be caused by the rapidly progressing disease, the independent safety evaluation committee concluded that it might not be directly associated with the vaccinations.

Immune responses to the vaccine peptides. Both IgG and T cell responses specific to the vaccine peptides were analyzed in blood samples before and after vaccinations (Table 3). Plasma samples were obtained from 10, six and four patients before and at the end of the first (six vaccinations) and second (12 vaccinations) cycles of vaccinations, respectively. For monitoring of humoral responses, the titers of peptide-specific IgG reactive to each of 31 different peptides were measured by bead-based multiplex assay. The IgG responses specific to at least one of the vaccine peptides were augmented in five of six patients (83%) and in all of four patients (100%) examined at the end of the first and second cycles of vaccinations, respectively.

T cell responses to the vaccine peptides were also measured by IFN-γ ELISPOT assay (Table 3). PBMCs were available

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Table 2. Toxicities

	Grade 1	Grade 2	Grade 3	Grade 4	Total
Injection site reaction	3	4	0	0	7
Constitutional symptom					
Fever	0	1	0	0	1
Fatigue	2	0	0	0	2
Gastrointestinal					
Anorexia	2	0	0	0	2
Nausea	1	0	0	0	1
Pulmonary/Upper respiratory					
Dyspnea	0	0	1	0	1
Blood/Bone marrow					
Anemia	8	1	1	0	10
Leukocytopenia	3	0	1	0	4
Neutropenia	0	1	0	0	1
Lymphopenia	3	0	1	0	4
Thrombocytopenia	1	0	0	0	1
Laboratory					
AST elevation	0	1	0	0	1
ALT elevation	1	1	0	0	2
γ-GTP elevation	1	0	0	0	1
Creatinine elevation	1	1	0	0	2
Hypoalbuminemia	8	0	0	0	8
Hyperkalemia	1	0	0	0	1
Hyponatremia	1	0	0	0	1
Hyperglycemia	1	0	0	0	1
Hyperuricemia	1	0	0	0	1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase.

Table 3. Immunological responses to the vaccine peptides

Patient No.	Peptide	IgG response†			T cell response‡		
		Before	1st	2nd	Before	1st	2nd
1	Lck-422	185	252	0	0	1000	2050
	HNRPL-140	428	723	1155	0	119	447
	SART3-109	224	657	2028	1309	294	186
	WHSC2-103	554	1332	16987	0	264	543
	MAP-432§	176	290	0	0	53	949
2	SART2-93	6609	NA	NA	0	NA	NA
	PSA-248	8975	NA	NA	0	NA	NA
	SART2-161	7979	NA	NA	0	NA	NA
	PSMA-624	7555	NA	NA	0	NA	NA
3	SART2-93	80	0	NA	146	0	NA
	MRP3-503	410	3040	NA	0	2389	NA
	SART2-161	166	0	NA	125	0	NA
	Lck-486	76	413	NA	0	364	NA
	PAP-213§	0	146	NA	NA	NA	NA
	PSMA-624§	38	42	NA	NA	NA	NA
4	PAP-213	552	NA	NA	0	NA	NA
	PSMA-624	266	NA	NA	333	NA	NA
	MAP-432	200	NA	NA	1333	NA	NA
	WHSC2-103	591	NA	NA	0	NA	NA
5	SART3-734	2142	11371	54795	1833	188	5390
	Lck-449	45	31	21708	600	944	9500
	SART3-109§	0	50	1854	NA	NA	0
	SART3-511§	0	28	1328	NA	NA	107
6	MAP-432	43	0	NA	0	227	NA
	HNRPL-501	104	446	NA	0	444	NA
	UBE2V-43	241	0	NA	157	71	NA
	SART3-109	2075	2621	NA	0	694	NA
7	SART3-109	174	NA	NA	117	NA	NA
	SART3-511	25	NA	NA	42	NA	NA
	Lck-90	85	NA	NA	0	NA	NA
	HNRPL-501	294	NA	NA	41	NA	NA
8	SART2-93	20	22	9222	0	56	NA¶
	PAP-213	208	187	12293	86	0	NA¶
	PSA-248	25	3856	18849	6	33	NA¶
	Lck-486	35	67	17704	15	16	NA¶
9	CypB-129	136	NA	NA	121	NA	NA
	Lck-422	34	NA	NA	13	NA	NA
10	Lck-246	74	63	3725	0	729	515
	WHSC2-141	77	58	455	0	75	0
	PAP-213	25	0	16345	0	89	166
	Lck-486	41	0	1378	0	102	0
	CypB-129§	70	86	81	0	0	19
	HNRPL-140§	43	48	24	0	34	64

†Values indicate the fluorescence intensity unit (FIU) of plasma IgG reactive with the corresponding peptides before and after the 1st and 2nd cycles of vaccinations. The augmented IgG responses are underlined. ‡Values indicate the number of spots per 10^5 peripheral blood mononuclear cells (PBMCs) reactive with the corresponding peptides in IFN-γ ELISPOT assay before and after the 1st and 2nd cycles of vaccinations. When the number of spots was <10 per 10^5 PBMCs, the data are shown as "0". The augmented T cell responses are underlined. §Peptides used for the 2nd cycle of vaccinations. ¶PBMCs unavailable. NA, not assessed.

from 10, six and three patients before and at the end of the first and second cycles of vaccinations, respectively. Antigen-specific T cell responses to at least one of the vaccine peptides were detectable in eight of 10 patients (80%) before vaccination, and augmented in five of six patients (83%) and in all of three patients (100%) tested at the end of the first and second cycles of vaccinations, respectively.

Collectively, at the end of the first cycle of six vaccinations, peptide-specific immunological boosting assessed by IgG and/or T cell responses was observed in all of the six patients who received at least six vaccinations, with their survival time of 25, 24.5 (alive), 10 (alive), 9.5, 6.5, and 6 months.

Cytokines and inflammation markers. We then measured cytokines (IL-2, IL-4, IL-5, IL-6, and IFN-γ) and inflammation markers (CRP and SSA) in the plasma before and at the end of the first cycle of vaccinations (Table 4). IL-6 was detectable in five of 10 patients (50%) before vaccination with median of 0.5 pg/mL, ranging from 0 to 7 pg/mL. IL-6 levels were increased, decreased, or unchanged in 2, 1, or 3 patients tested, respectively. There was no significant difference in the level of IL-6 between before and after vaccinations ($P = 0.500$; Wilcoxon test). Other cytokines, including IL-2, IL-4, IL-5, and IFN-γ, were rarely detectable in either pre- or post-vaccination plasma (data not shown).

An inflammation marker, CRP, was detectable in pre-vaccination plasma from the majority of patients (nine of 10 patients [90%]), with median value of 0.46 mg/dL (ranging from 0 to 1.04 mg/dL). Plasma CRP levels were increased or decreased in four or two patients, respectively. Another inflammation marker, SAA, was also detected in pre-vaccination plasma from all of the patients (100%) with median value of 5.475 mg/dL (ranging from 0.13 to 15.37 mg/dL). Plasma SAA levels were increased or decreased in three or three patients, respectively. There were no significant differences in the levels of CRP as well as SAA between before and after vaccinations ($P = 0.910$ and $P = 0.924$, respectively; Wilcoxon test).

Flow cytometric analysis of immune subsets in PBMCs. Immune cell subsets in both pre-vaccination and post-vaccination PBMCs were examined by flow cytometry (Table 4). The median frequency of MDSCs in pre- and post-vaccination PBMCs was 0.2% (range from 0 to 0.8%, $n = 10$) and 0.3% (range from 0 to 0.9%, $n = 6$), respectively. The median

Table 4. Laboratory data before and after vaccination†

Patient No.	IL-6 (pg/mL)		CRP (mg/dL)		SAA (mg/dL)		MDSCs (%)		$CD3^+CD26^+$ (%)	
	Before	After	Before	After	Before	After	Before	After	Before	After
1	0	0	0.39	0.56	8.58	7.78	0.3	0.6	48.2	58.4
2	7	NA	0.92	NA	12.65	NA	0.1	NA	29.8	NA
3	3	1	0.54	0.52	3.10	0.00	0.0	0.0	15.3	24.6
4	0	NA	0.47	NA	1.17	NA	0.1	NA	21.0	NA
5	1	2	0	0.56	0.28	3.99	0.2	0.1	32.9	34.8
6	3	9	0.39	0.61	5.47	11.95	0.2	0.5	49.7	57.3
7	1	NA	0.40	NA	5.48	NA	0.8	NA	19.0	NA
8	0	0	1.04	0.17	12.36	6.73	0.6	0.9	51.1	39.0
9	0	NA	0.94	NA	15.37	NA	0.4	NA	15.6	NA
10	0	0	0.45	0.53	0.13	0.55	0.1	0.1	39.4	28.3

†Values before and after the 1st cycle of vaccinations are shown. CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells; NA, not assessed; SAA, serum amyloid A.

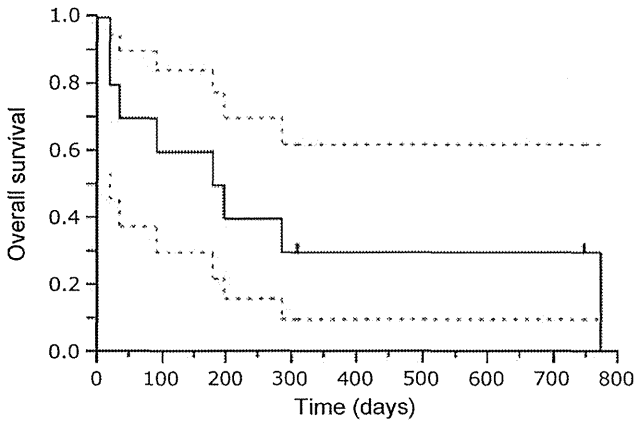

Fig. 1. Kaplan–Meier survival analysis in the enrolled patients. The median overall survival of patients who received personalized peptide vaccination (PPV) (n = 10; solid line) was 186.5 days and the 1 year survival rate was 30%. Dotted lines show 95% confidence intervals.

Table 5. Statistical analysis with clinical findings and laboratory data

Factor	Hazard ratio (95% CI)†	P-value†
Age	1.047 (0.943–1.163)	0.393
Limited-stage disease at diagnosis	1.250 (0.278–5.625)	0.771
Performance status (PS)	3.270 (0.651–16.427)	0.150
Number of previous treatment regimens	2.540 (1.188–5.431)	0.016
Previous treatment period (months)	0.989 (0.945–1.035)	0.637
Combined treatment (+)	0.336 (0.066–1.698)	0.187
IL-6 (pg/mL)	1.299 (0.900-1.877)	0.163
CRP (mg/dL)	7.459 (0.608–91.517)	0.116
SAA (mg/dL)	1.095 (0.940–1.275)	0.246
MDSCs (%)	2.872 (0.094–87.379)	0.545
$CD3^+CD26^+$ (%)	0.941 (0.878–1.008)	0.084

†Evaluated by the Cox proportional hazards model. CI, confidence interval; CRP, C-reactive protein; IL, interleukin; MDSCs, myeloid-derived suppressor cells; SAA, serum amyloid A.

frequency of $CD3^+CD26^+$ cells in pre- and post-vaccination PBMCs was 31.35% (range from 15.3 to 51.1%) and 36.9% (range from 24.6 to 58.4%), respectively. No significant differences were found in the frequencies of MDSCs and $CD3^+CD26^+$ between before and after the vaccinations (P = 0.140 and P = 0.825, respectively; Wilcoxon test).

Potentially prognostic factors in SCLC patients undergoing PPV. Median OS of the 10 patients was 186.5 days, with 1 year survival rate of 30% (Fig. 1). To identify potentially prognostic factors in refractory SCLC patients undergoing PPV, statistical analyses were carried out by the Cox proportional hazards model with clinical findings or laboratory data. As shown in Table 5, the number of previous chemotherapy regimens and frequency of $CD3^+CD26^+$ cells in PBMCs before vaccination were potentially prognostic in the patients receiving PPV (hazard ratio [HR] = 2.540, 95% confidence interval [CI] = 1.188–5.431, P = 0.016; HR = 0.941, 95% CI = 0.878–1.008, P = 0.084; respectively).

Accumulation of tumor-infiltrating lymphocytes in a patient undergoing tumor resection after PPV. A patient (Patient No. 5), who had good immune responses to vaccine antigens and showed stable disease (24.5 months alive), underwent resection of the primary tumor after 24 vaccinations. The parent proteins for the used peptides, SART3 and p56lck, were expressed in the tumor tissue resected after the vaccinations (Fig. 2). To know the immune responses to the tumor following the vaccinations, tumor-infiltrating lymphocytes were assessed by IHC using antibodies specific to immunological markers, including CD3, CD4, and CD8. In the tumor from this patient treated with PPV, $CD3^+$ cells infiltrated densely not only within the cancer stroma but also within the cancer cell nest (Fig. 3a). These tumor-infiltrating lymphocytes consisted of both $CD4^+$ and $CD8^+$ cells (Fig. 3b,c). In contrast, when the tumors from SCLC patients without PPV treatment (n = 3) were examined by IHC as a control, only a few cells positive for CD3, CD4, or CD8 accumulated within the tumors from all patients examined (representative data were shown in Fig. 3d–f). These results suggest the possibility that PPV induced anti-tumor immunity mediated by $CD4^+$ and $CD8^+$ T cells, leading to better clinical outcomes.

Discussion

Despite recent advances in chemotherapies for refractory SCLC patients, novel treatment modalities, including immunotherapies, still remain to be developed.[1–3] However, there have been a few reports available regarding immunotherapies against SCLC.[4,5] For example, a DC-based vaccine targeting p53 was reported to show a feasible result in a subset of SCLC patients, who had positive immune responses against p53. However, the induction rate of anti-p53 immunity was relatively low.[19,20] Vaccinations with cell surface glycolipid antigens to induce antigen-specific Ab responses were also attempted in several clinical studies.[21,22] However, only a

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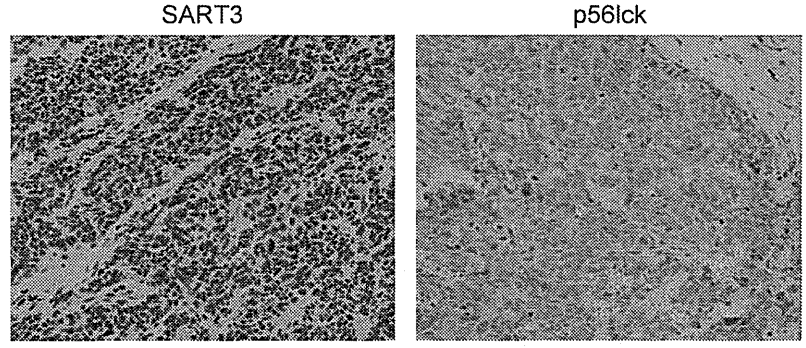

Fig. 2. Expression of the vaccine antigens in the tumor from a small cell lung cancer (SCLC) patient undergoing surgery after personalized peptide vaccination (PPV) treatment. The vaccine antigens SART3 and p56lck were detected by immunohistochemistry (IHC) with the antibodies specific to these molecules in the tumor tissue from a patient undergoing surgery after PPV treatment (Patient No. 5). Both sections, ×200.

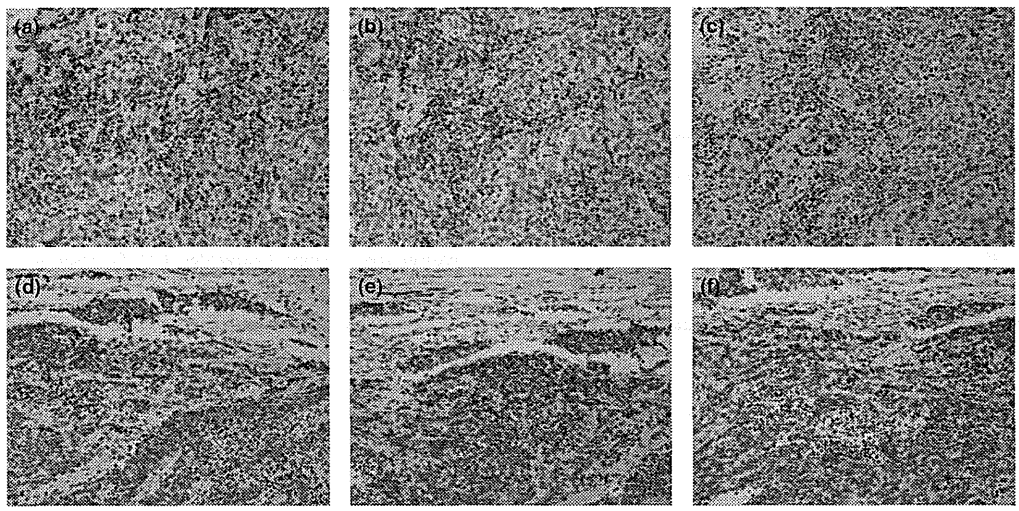

Fig. 3. Detection of tumor-infiltrating lymphocytes in tumors from small cell lung cancer (SCLC) patients treated with or without personalized peptide vaccination (PPV). Immune cells infiltrating within tumors were detected by immunohistochemistry (IHC) with the antibodies against CD3 (a and d), CD4 (b and e), and CD8 (c and f). All sections, ×100. (a–c) Tumor from a SCLC patient after PPV treatment (Patient No. 5). (d–f) Tumor from a SCLC patient without PPV treatment. Since the tumors from three SCLC patients without PPV treatment showed similar findings, representative data are shown.

limited number of patients developed a detectable Ab response, and there was no impact on clinical outcomes. In the current study, we addressed if refractory SCLC patients could have pre-existing IgG responses to 31 different vaccine candidates and well respond to these peptide vaccines. Notably, our results demonstrated that pre-vaccination plasma from all of the refractory SCLC patients had detectable levels of IgG specific to the cancer vaccine candidates, suggesting that they had the capability to show secondary immune responses to vaccine antigens. Furthermore, immunological boosting of T cell or IgG responses was observed in all of the patients, who completed at least one cycle of six vaccinations. Toxicity of PPV was mainly skin reactions at injection sites, and no SAE directly associated with the vaccinations were observed. These findings suggest the feasibility of PPV for refractory SCLC.

Interestingly, in a patient undergoing tumor resection after PPV, both $CD4^+$ and $CD8^+$ T cells infiltrated densely not only within the cancer stroma but also within the cancer cell nest. Since the vaccine antigens SART3 and p56lck were expressed in the tumor cells, it may be possible that T cells specific to these molecules infiltrated and accumulated within tumors. SART3 was strongly and homogeneously expressed in the tumor cells, whereas expression of p56lck was weak and heterogeneous. This heterogeneous expression of p56lck may be attributed to the immune escape mechanism of tumor cells following PPV, although the pre-vaccination tumor tissue of this patient was unavailable to demonstrate this possibility.

The prognosis of refractory SCLC patients remains very poor with a median survival time of around 6–10 months.[1–3] Therefore, it could be worthwhile to discuss the clinical efficacy of PPV, although it was not the main objective of this study. In 10 refractory SCLC patients receiving PPV, the median OS was 186.5 days, with 1 year survival rate of 30%. In particular, six patients who received at least one cycle of six vaccinations survived for 25, 24.5 (alive), 10 (alive), 9.5, 6.5, and 6 months (median OS, 528 days), although survival time of the remaining four patients without completing six vaccinations was only 0.5, 0.5, 1, and 3 months (median OS, 25 days). Statistically analyses with clinical findings and laboratory data were performed to identify potentially prognostic factors, although the result was preliminary due to the small number of patients and its clinical utility needs to be confirmed in future studies. In the analysis of clinical findings, greater numbers of previous chemotherapy regimens might be associated with worse prognosis, suggesting that PPV should be considered before repeated failures of multiple chemotherapeutic regimens. Similar to our finding, the ability to mount an immune response to therapeutic vaccines was reported to be directly correlated with fewer prior chemotherapy regimens.[23] In addition, the statistical analysis with pre-vaccination laboratory data demonstrated that the frequency of $CD3^+CD26^+$

cells in PBMCs was potentially prognostic in patients receiving PPV. The frequency of $CD3^+CD26^+$ cells has not been previously reported as a biomarker in SCLC patients. CD26 is a cell surface glycoprotein that functions as a proteolytic enzyme, dipeptidyl peptidase IV (DPP IV), and has been reported to play a critical role in signal transduction.[24] Since this molecule is highly expressed on activated T cells,[24] the increased frequency of $CD3^+CD26^+$ might contribute to better immune responses against the vaccine antigens. The role of $CD26^+$ activated T cells in cancer vaccines remains to be determined.

In summary, the current study demonstrated that immune responses to the vaccine antigens were substantially induced without SAE in refractory SCLC patients who received at least one cycle (six times) of vaccinations. Nevertheless, due to the small number of patients and the short term of observation in this early phase trial, clinical efficacy of PPV for refractory SCLC remains to be confirmed in a next step of larger-scale, prospective trials.

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Disclosure Statement

The authors have no conflict of interest.

References

1 William WN, Glisson BS. Novel strategies for the treatment of small-cell lung carcinoma. *Nat Rev Clin Oncol* 2011; **8**: 611–9.
2 Kim YH, Mishima M. Second-line chemotherapy for small-cell lung cancer (SCLC). *Cancer Treat Rev* 2011; **37**: 143–50.
3 Puglisi M, Dolly S, Faria A, Myerson JS, Popat S, O'Brien ME. Treatment options for small cell lung cancer – do we have more choice? *Br J Cancer* 2010; **102**: 629–38.
4 Bridle BW. Neuroendocrine cancer vaccines in clinical trials. *Expert Rev Vaccines* 2011; **10**: 811–23.
5 Simmons O, Magee M, Nemunaitis J. Current vaccine updates for lung cancer. *Expert Rev Vaccines* 2010; **9**: 323–35.
6 Terasaki M, Shibui S, Narita Y *et al.* Phase I trial of a personalized peptide vaccine for patients positive for human leukocyte antigen-A24 with recurrent or progressive glioblastoma multiforme. *J Clin Oncol* 2011; **29**: 337–44.
7 Noguchi M, Kakuma T, Uemura H *et al.* A randomized phase II trial of personalized peptide vaccine plus low dose estramustine phosphate (EMP) versus standard dose EMP in patients with castration resistant prostate cancer. *Cancer Immunol Immunother* 2010; **59**: 1001–9.
8 Hattori T, Mine T, Komatsu N *et al.* Immunological evaluation of personalized peptide vaccination in combination with UFT and UZEL for metastatic colorectal carcinoma patients. *Cancer Immunol Immunother* 2009; **58**: 1843–52.
9 Yanagimoto H, Shiomi H, Satoi S *et al.* A phase II study of personalized peptide vaccination combined with gemcitabine for non-resectable pancreatic cancer patients. *Oncol Rep* 2010; **24**: 795–801.
10 Mine T, Sato Y, Noguchi M *et al.* Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses. *Clin Cancer Res* 2004; **10**: 929–37.
11 Noguchi M, Mine T, Komatsu N *et al.* Assessment of immunological biomarkers in patients with advanced cancer treated by personalized peptide vaccination. *Cancer Biol Ther* 2011; **10**: 1266–79.
12 Yoshida K, Noguchi M, Mine T *et al.* Characteristics of severe adverse events after peptide vaccination for advanced cancer patients: analysis of 500 cases. *Oncol Rep* 2011; **25**: 57–62.
13 Itoh K, Yamada A. Personalized peptide vaccines: a new therapeutic modality for cancer. *Cancer Sci* 2006; **97**: 970–6.
14 Komatsu N, Shichijo S, Nakagawa M, Itoh K. New multiplexed flow cytometric assay to measure anti-peptide antibody: a novel tool for monitoring immune responses to peptides used for immunization. *Scand J Clin Lab Invest* 2004; **64**: 535–45.
15 Kinoshita Y, Kuratsukuri K, Landas S *et al.* Expression of prostate-specific membrane antigen in normal and malignant human tissues. *World J Surg* 2006; **30**: 628–36.
16 Quintero IB, Araujo CL, Pulkka AE *et al.* Prostatic acid phosphatase is not a prostate specific target. *Cancer Res* 2007; **67**: 6549–54.
17 Wang Y, Harada M, Yano H *et al.* Prostatic acid phosphatase as a target molecule in specific immunotherapy for patients with nonprostate adenocarcinoma. *J Immunother* 2005; **28**: 535–41.
18 Wang Y, Harada M, Yano H *et al.* Prostate-specific antigen-reactive cytotoxic T lymphocyte precursors in colon cancer patients. *Oncol Rep* 2006; **15**: 317–21.
19 Antonia SJ, Mirza N, Fricke I *et al.* Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. *Clin Cancer Res* 2006; **12**: 878–87.
20 Chiappori AA, Soliman H, Janssen WE, Antonia SJ, Gabrilovich DI. INGN-225: a dendritic cell-based p53 vaccine (Ad.p53-DC) in small cell lung cancer: observed association between immune response and enhanced chemotherapy effect. *Expert Opin Biol Ther* 2010; **10**: 983–91.
21 Neninger E, Díaz RM, de la Torre A *et al.* Active immunotherapy with 1E10 anti-idiotype vaccine in patients with small cell lung cancer: report of a phase I trial. *Cancer Biol Ther* 2007; **6**: 145–50.
22 Giaccone G, Debruyne C, Felip E *et al.* Phase III study of adjuvant vaccination with Bec2/bacille Calmette-Guerin in responding patients with limited-disease small-cell lung cancer (European Organisation for Research and Treatment of Cancer 08971-08971B; Silva Study). *J Clin Oncol* 2005; **23**: 6854–64.
23 von Mehren M, Arlen P, Gulley J *et al.* The influence of granulocyte macrophage colony-stimulating factor and prior chemotherapy on the immunological response to a vaccine (ALVAC-CEA B7.1) in patients with metastatic carcinoma. *Clin Cancer Res* 2001; **7**: 1181–91.
24 Ohnuma K, Dang NH, Morimoto C. Revisiting an old acquaintance: CD26 and its molecular mechanisms in T cell function. *Trends Immunol* 2008; **29**: 295–301.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Immunohistochemical analysis of vaccine antigens in small cell lung cancer (SCLC) tissues.

Table S1. Peptide candidates for cancer vaccination.

Table S2. Frequency of expression of vaccine antigens in small cell lung cancer (SCLC) tissues.

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ORIGINAL ARTICLE

IDH1/2 gene status defines the prognosis and molecular profiles in patients with grade III gliomas

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Abstract
Background The discovery of isocitrate dehydrogenase 1 and 2 gene (*IDH1/2*) mutations has enabled grade III glioma to be divided into mutated and wild-type *IDH1/2* groups, which are known to carry different prognosis and molecular features. However, detailed subgroup analysis of grade III glioma is limited. To address this, we investigated molecular and prognostic features of grade III glioma with and without *IDH1/2* mutation.
Methods We retrospectively analyzed 115 grade III glioma patients. Clinical parameters were obtained from medical records. The mutation of *IDH1/2* and *TP53* was analyzed by direct sequencing. O^6-methylguanine methyltransferase gene (*MGMT*) gene promoter methylation status was determined by methylation-specific polymerase chain reaction. Detection of chromosome copy number changes of 1p, 7p (*EGFR*), 9p (*CDKN2A*), 10q (*PTEN*), and 19q was carried out by multiple ligation-dependent probe amplification. Patients were divided into two groups, mutated *IDH1/2* and wild-type *IDH1/2*, for correlation with the factors analyzed.
Results In our series, as previously reported, *IDH1/2* mutation was an independent prognostic marker for improved progression-free and overall survival (OS) ($P < 0.0001$ and $P < 0.0001$, respectively) in patients with grade III gliomas. Subgroup analysis found that incomplete resection, 7p gain, and *TP53* mutation were independent prognostic factors of poor outcome in grade III glioma patients with mutated *IDH1/2* ($P = 0.0092$, $P = 0.015$ and $P = 0.026$, respectively), while there were none in patients with wild-type *IDH1/2*.
Conclusions *IDH1/2* gene status was significantly associated with prognosis in grade III gliomas. Subgroup analysis found that poor prognostic factors existed even in patients with *IDH1/2* mutation.

Keywords Grade III gliomas · *IDH1/2* · 1p/19q · *TP53* · 7p gain

Electronic supplementary material The online version of this article (doi:10.1007/s10147-011-0323-2) contains supplementary material, which is available to authorized users.

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Introduction

Recently, total genome analysis of glioblastoma (grade IV) identified somatic mutations at codon 132 of the isocitrate dehydrogenase 1 gene (*IDH1*) and codon 172 of the isocitrate dehydrogenase 2 gene (*IDH2*) [1–3]. These gene mutations occur at a very early stage of gliomagenesis [1, 4], and therefore the status of the *IDH1/2* gene can be used to classify glioma into two distinct types, mutated *IDH1/2* and wild-type *IDH1/2*, which have implications for

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the scheme of the glioma development [5, 6]. Currently, these two distinct types carry significantly different molecular characteristics, as Yan et al. [6] described, so that they may be different subtypes of disease. In grade III gliomas, it has been already shown that patients with mutated *IDH1/2* carry 1p/19q codeletion, *TP53* gene mutation, and O^6-methylguanine methyltransferase gene (*MGMT*) promoter methylation, while those with wild-type *IDH1/2* carry gain of 7p (*EGFR*), loss of 10q (*PTEN*), and homozygous deletion of 9p (*CDKN2A*) [3, 7, 8].

Patients with grade III gliomas present relatively poor overall survival (OS), ranging from 30 to 70 months [9, 10], and *IDH1/2* mutations were frequently found, ranging from 62 to 78% [1]. Therefore, patients in this grade obtain the most benefit from detailed analysis of *IDH1/2* gene status compared to patients with grade II glioma, who carry 71–78% of *IDH1/2* mutations [1] but present favorable prognosis (OS: over 100 months) [11, 12], and patients with primary glioblastoma, who carry only 7% of *IDH1/2* mutations [1]. Here, we conducted a retrospective study targeting detailed analysis of patients with grade III glioma. They were divided into two groups, mutated and wild-type *IDH1/2*, and each subgroup was investigated regarding associated prognostic factors. The factors analyzed were age, sex, preoperative Karnofsky performance status (KPS), extent of resection, oligodendroglial component, *MGMT* promoter methylation, Ki-67 labeling index, *TP53*, 1p/19q codeletion, and alteration of 7p (*EGFR*), 9p (*CDKN2A*), and 10q (*PTEN*), all of which are known prognostic factors of high grade gliomas. This study contains the largest number of analyzed factors, and we hypothesized that this subgroup analysis would enable us to clarify prognostic markers in either mutated or wild-type *IDH1/2* groups of grade III gliomas.

Patients and methods

Patients and samples

All patients underwent radical surgery and postsurgical treatment at Tohoku University Hospital. After the second review by a neuropathologist (H.S.), 8 patients were excluded and in total 115 grade III glioma patients [anaplastic astrocytoma (AA) 56, anaplastic oligoastrocytoma (AOA) 28, anaplastic oligodendroglioma (AO) 31] were analyzed for this study. Clinical profiles of each patient were obtained from the medical records. The resection rate was evaluated by gadolinium-enhanced magnetic resonance imaging (MRI) within 3 days after operation. The postsurgical treatment was chemotherapy [nimustine hydrochloride (ACNU) or temozolomide (TMZ)] and radiation therapy. After the initial treatment, patient observation was performed by enhanced MRI every 2 months for the first 2 years, and every 3–6 months thereafter. Recurrence was judged by progression or new enhanced lesion on MRI. At recurrence, salvage surgery, second line chemotherapy or radiation therapy was performed. Tumor specimens were immediately frozen in liquid nitrogen and stored at −80°C until the extraction of DNA. This retrospective study was conducted with the approval of the ethics committee of Tohoku University School of Medicine, and written informed consent was obtained from all patients.

Outcome

The starting point of progression-free survival (PFS) and overall survival (OS) was the day of surgery. Tumor progression at last follow-up examination or death, and the last follow-up examination or death, were the end points for PFS and OS, respectively.

Ki-67 labeling index

The resected specimens were examined by immunohistochemical staining for Ki-67 antigen (Ki-67 antibody; DAKO, Tokyo, Japan). Each slide was individually reviewed and scored by one neuropathologist (M.W.). The Ki-67 labeling index was established by determining the percentage of positive nuclei among 1000 tumor cells. If the tumor had several areas of staining with different percentages, the highest percentage was applied for the analysis.

PCR amplification and direct sequencing

Genomic DNA extraction was carried out using the QIAamp DNA Mini Kit (Qiagen Science, Germantown, Maryland, USA) following the manufacturer's protocol. Exon 4 of the *IDH1* and *IDH2* genes, and exons 4–9 of the *TP53* gene were amplified by PCR as previously described [13, 14]. The primer set of *IDH1/2* was as follows: *IDH1* sense 5′-CGG TCT TCA GAG AAG CCA TT-3′ and antisense 5′-GCA AAA TCA CAT TAT TGC CAA C-3′; *IDH2* sense 5′-CAA GCT GAA GAA GAT GTG GAA-3′ and antisense 5′-CAG AGA CAA GAG GAT GGC TA-3′. All PCR products were purified using a high pure PCR product purification kit (Roche, Basel, Switzerland) before the sequencing reaction. Sequencing analysis was as previously reported [13].

MGMT promoter methylation analysis

Methylation-specific polymerase chain reaction (MSP) was carried out for the *MGMT* gene promoter methylation analysis as previously described [15, 16]. The primer

sequences for methylation were 5′-TTT CGA CGT TCG TAG GTT TTC GC-3′ (sense) and 5′-GCA CTC TTC CGA AAA CGA AAC G-3′ (antisense), and for unmethylation were 5′-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3′ (sense) and 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′ (antisense). MSP products were separated on 4% agarose gel containing ethidium bromide and the bands were visualized with ultraviolet light. Each sample was examined three times.

Detection of DNA copy number changes

Detection of chromosome copy number changes of 1p, 7p (*EGFR*), 9p (*CDKN2A*), 10q (*PTEN*), and 19q was carried out by multiple ligation-dependent probe amplification (MLPA) using a SALSA kit: P088 and kit: P105 probe mix (MRC Holland, Amsterdam, the Netherlands) as previously reported [17–19]. The former probe kit contains 15 1p probes, 8 19q probes, and 15 reference probes, and the latter kit contains 11 *PTEN* probes, 5 *CDKN2A* probes, 8 *TP53* probes, 2 *ERBB2* probes, 11 *EGFR* probes, and 8 reference probes. This technique consists of denaturing, hybridization (over 16 h at 60°C), ligation, amplification, and fragment analysis. Fragment analysis with the CEQ 2000 XL DNA analysis system (Beckman Coulter, Inc., Brea, CA, USA) provides subjective and objective data. Three control samples from healthy humans were kindly provided by FALCO Biosystems (Kyoto, Japan).

For data analysis, each probe amplification product is divided by the average of reference probes to compensate the difference in PCR efficiency, using Microsoft Excel. The data obtained are divided by the corresponding average probe fraction of the normal sample. Above 1.2 or below 0.8 is considered as gain or loss, respectively. In particular, below 0.4 is considered as homozygous deletion [17–20]. A truncated variant of the *EGFR* gene was analyzed as previously reported [18].

Statistical analysis

The relationship between *IDH1/2* gene status and other factors was evaluated by the Mann–Whitney and Fisher's exact tests. Probabilities of PFS and OS were calculated according to the Kaplan–Meier method and compared with the log-rank test. Genetic alterations, together with histology (histological types and Ki-67 labeling index), demographic (age and sex), clinical (preoperative KPS and surgery), and therapeutic variables achieving $P < 0.10$ in univariate analysis, were subsequently introduced in a backward stepwise proportional hazard analysis (Cox model) as independent predictors of survival. Variables which did not achieve $P < 0.10$ were listed as "not applicable (N/A)" (Tables 3, 5). All statistical methods adopted a significance level of $P < 0.050$, using a statistical software package (SPSS, Inc., Chicago, IL, USA).

Results

Patients

Gross total resection was achieved in 48 patients, subtotal in 50 patients, partial in 17 patients and no biopsy. The postsurgical treatment was chemotherapy and radiotherapy in 95 patients (ACNU in 85, TMZ in 10), radiotherapy only in 12, and ACNU chemotherapy only in 5. The other three patients were treated with surgical resection only. There was no significant survival difference in patients treated with ANCU and TMZ (data not shown).

IDH1/2 gene mutation and other prognostic factors

The *IDH1/2* gene mutation was detected in 76 (66%) (70 *IDH1* and 6 *IDH2* gene mutations) of the 115 grade III glioma patients. Frequencies of mutations were 63% (AA), 75% (AOA), and 65% (AO). Among these, 39 patients with *IDH1/2* gene mutation were previously reported [13]. Age, sex, and resection rate were not correlated with *IDH1/2* status (Table 1). The Ki-67 labeling index was analyzed in 87 of the 115 patients. This proliferation index was lower in tumors with mutated *IDH1/2* than in those with wild-type *IDH1/2*, but without significant difference (17.0% and 23.7%, respectively, $P = 0.16$, Table 1).

The *TP53* gene mutation was detected in 51 (44%) of the 115 patients; 36/56 (64%) in AA, 10/28 (36%) in AOA, and 5/31 (16%) in AO. The frequency was 47% in patients with mutated *IDH1/2* and 38% in patients with wild-type *IDH1/2* ($P = 0.43$, Table 1). The *TP53* gene statuses of 22 cases were previously reported [21]. The 1p/19q codeletion was detected in 34 (30%) of 115 patients; 1/56 (1.8%) in AA, 14/28 (50%) in AOA, and 19/31 (61%) in AO. The frequency was 39% in patients with mutated *IDH1/2* and 10% in those with wild-type *IDH1/2* ($P = 0.0011$, Table 1). The 1p/19q statuses of 31 cases by fluorescent in-situ hybridization analysis were previously reported [22] and the same results were obtained by MLPA. One AO patient with *IDH1/2* gene mutation had both *TP53* gene mutation and 1p/19q codeletion, whereas 11 patients with *IDH1/2* gene mutation had neither *TP53* gene mutation nor 1p/19q codeletion (data not shown). *MGMT* promoter methylation was found in 87 (76%) of the 115 patients; 41/56 (73%) in AA, 22/28 (79%) in AOA, and 24/31 (77%) in AO. *MGMT* promoter methylation was significantly associated with *IDH1/2* gene mutation ($P < 0.0001$, Table 1). 7p gain (*EGFR* amplification) and 9p homozygous deletion were detected in 21 (18%) and 9 (7.8%) of

Table 1 Correlation between clinical/genetic factors and *IDH1/2* mutation

Clinical/genetic factor	Total $n = 115$	Mutated *IDH1/2* $n = 76$ (66%)	Wild-type *IDH1/2* $n = 39$ (34%)	*P*
Median age at diagnosis, year (range)	46 (10–77)	41 (10–70)	52 (14–77)	0.14[a]
Sex, female, *n* (%)	45 (39)	34 (45)	11 (28)	0.11[b]
Preoperative KPS $\geq$ 80%, *n* (%)	91 (79)	62 (82)	29 (74)	0.47[b]
Gross total resection, *n* (%)	48 (42)	30 (39)	18 (46)	0.55[b]
Ki-67 labeling index, %	19.4	17.0	23.7	0.16[a]
TP53 mutation, *n* (%)	51 (44)	36 (47)	15 (38)	0.43[b]
1p/19q codeletion, *n* (%)	34 (30)	30 (39)	4 (10)	**0.0011**[b]
7p (*EGFR*) amplification, *n* (%)	21 (18)	8 (11)	13 (33)	**0.0045**[b]
9p (*CDKN2A*) homozygous deletion, *n* (%)	9 (7.8)	3 (3.9)	6 (15)	0.060[b]
10q (*PTEN*) loss, *n* (%)	10 (8.7)	6 (7.9)	4 (10)	0.73[b]
MGMT gene promoter methylation, *n* (%)	87 (76)	69 (91)	18 (46)	**<0.0001**[b]

KPS Karnofsky performance status

Bold indicates statistical significance at $P < 0.050$

[a] Mann–Whitney test

[b] Fisher's exact test

115 cases, respectively. The frequencies of 7p gain were 15/56 (27%) in AA, 6/28 (21%) in AOA, and none in AO, and of 9p homozygous deletion were 6/56 (11%) in AA, none in AOA, and 3/31 (9.7%) in AO. One AOA patient with *IDH1/2* gene mutation harbored a truncated variant of the *EGFR* gene. 7p gain (*EGFR* amplification) was significantly correlated with wild-type *IDH1/2* ($P = 0.0045$, Table 1). 9p homozygous deletion tended to be associated with wild-type *IDH1/2,* but did not reach statistical significance ($P = 0.060$, Table 1). 10q loss was found in 10 (8.7%) of the 115 patients; 7/56 (13%) in AA, 3/28 (11%) in AOA, and none in AO, without correlation with *IDH1/2* gene mutation ($P = 0.73$, Table 1).

Clinical/genetic prognostic factors were also analyzed by MGMT promoter methylation status and chromosome 1p19q status (see Electronic Supplementary Material Tables 1 and 2). *MGMT* promoter methylation was significantly associated with 1p19q codeletion ($P = 0.0003$, Supplementary Tables 1 and 2).

Progression-free survival and overall survival

The median follow-up was 43 months, ranging from 1 to 245 months. 62 patients (54%) remained free from progression. Median PFS was 62 months and 5-year PFS rate was 50.1%. Univariate analysis showed that the factors associated with longer PFS were young age (<50 years) ($P = 0.019$), low Ki-67 labeling index (<15%) ($P = 0.014$), *IDH1/2* gene mutation ($P < 0.0001$), 1p/19q codeletion ($P = 0.0076$), no gain of 7p ($P = 0.0006$), and *MGMT* gene promoter methylation ($P < 0.0001$) (Table 2). Multivariate Cox regression analysis revealed that *IDH1/2* mutation [hazard ratio (HR) 0.11, 95% confidence interval (CI) 0.056–0.23, $P < 0.0001$], low Ki-67 labeling index (<15%) (HR 0.29, 95% CI 0.14–0.57, $P = 0.0004$), and no gain of 7p (HR 0.31, 95% CI 0.14–0.68, $P = 00034$) were independent favorable prognostic factors for PFS (Table 3), while 1p/19q codeletion and *MGMT* gene promoter methylation were not independent prognostic factors for PFS ($P = 0.59$ and 0.21, respectively, data not shown). 77 patients (67%) remained alive. Median OS was not reached and 5-year OS rate was 65.0%. Univariate analysis revealed that female sex ($P = 0.026$), gross total resection ($P = 0.049$), *IDH1/2* gene mutation ($P < 0.0001$) (Fig. 1), 1p/19q codeletion ($P = 0.0092$), no gain of 7p ($P < 0.0001$), and *MGMT* gene promoter methylation ($P = 0.033$) were correlated with longer OS (Table 2). Multivariate Cox regression analysis showed *IDH1/2* mutation (HR 0.16, 95% CI 0.073–0.37, $P < 0.0001$), low Ki-67 labeling index (<15%) (HR 0.24, 95% CI 0.10–0.58, $P = 0.0014$), and no gain of 7p (HR 0.26, 95% CI 0.11–0.60, $P = 0.0018$) were independent favorable prognostic factors for OS (Table 3), but that 1p/19q codeletion and *MGMT* gene promoter methylation were not ($P = 0.63$ and 0.35, respectively, data not shown).

We observed relatively longer PFS and OS (Table 2) compared to previous reports [17]. This is largely due to the high resection rate of our series. In grade III gliomas, it is reported that radical resection is an independent better prognostic factor [17]. We excluded patients who underwent biopsy, and the extent of resection more than subtotal reached about 85%.

Table 2 Clinical and genetic parameters affecting PFS and OS in grade III gliomas

Characteristic	PFS				OS			
	No. (%)	5-year survival (%)	Median months	*P* (log-rank)	No. (%)	5-year survival (%)	Median months	*P* (log-rank)
	115	50.1	62		115	65.0	NR	
Histology								
AA	56 (49)	44.3	60	0.19 (vs AO, AOA)	56 (49)	58.3	88	0.092 (vs AO, AOA)
AO	31 (27)	54.1	62		31 (27)	79.5	NR	
AOA	28 (24)	57.5	NR		28 (24)	65.5	NR	
Age at diagnosis								
<50 years old	67 (58)	59.0	NR	**0.019**	67 (58)	67.0	NR	0.35
≥50 years old	48 (42)	36.1	47		48 (42)	61.8	81	
Sex								
Female	45 (39)	58.4	NR	0.077	45 (39)	79.3	NR	**0.026**
Male	70 (61)	44.6	47		70 (61)	54.8	88	
Karnofsky performance status								
≥80	91 (79)	51.3	68	0.30	91 (79)	64.4	NR	0.22
<80	24 (21)	46.4	50		24 (21)	65.7	62	
Gross total resection								
Yes	48 (42)	59.3	NR	0.094	48 (42)	71.8	NR	**0.049**
No	67 (58)	42.5	51		67 (58)	60.0	81	
Ki-67 labeling index								
<15%	39 (45)	59.7	95	**0.014**	39 (45)	71.5	NR	0.090
≥15%	48 (55)	32.9	38		48 (55)	56.0	NR	
IDH1/2								
Mutated	76 (66)	70.5	NR	**<0.0001**	76 (66)	82.3	NR	**<0.0001**
Wild-type	39 (34)	10.1	11		39 (34)	26.3	26	
TP53								
Wild-type	64 (56)	53.2	95	0.46	64 (56)	70.0	NR	0.13
Mutated	51 (44)	46.0	54		51 (44)	58.5	88	
1p/19q codeletion								
Yes	34 (30)	69.4	NR	**0.0076**	34 (30)	85.0	NR	**0.0092**
No	81 (70)	42.1	35		81 (70)	56.2	88	
Gain of 7p								
No	94 (82)	57.4	95	**0.0006**	94 (82)	74.9	NR	**<0.0001**
Yes	21 (18)	15.1	18		21 (18)	15.6	27	
Homozygous deletion of 9p								
No	106 (92)	52.3	68	0.17	106 (92)	66.1	NR	0.080
Yes	9 (8)	27.8	22		9 (8)	53.3	64	
Loss of 10q								
No	105 (91)	51.9	68	0.22	105 (91)	66.2	NR	0.31
Yes	10 (9)	32.0	17		10 (9)	56.0	NR	
MGMT								
Methylated	87 (76)	57.2	NR	**<0.0001**	87 (76)	69.6	NR	**0.033**
Unmethylated	28 (24)	24.8	15		28 (24)	44.5	43	

AA anaplastic astrocytoma, *AO* anaplastic oligodendroglioma, *AOA* anaplastic oligoastrocytoma, *NR* not reached, *OS* overall survival, *PFS* progression-free survival

Bold indicates statistical significance at $P < 0.050$

Table 3 Multivariate analysis of independent factors associated with survival in grade III glioma patients

Variable	PFS			OS		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
IDH1/2						
Mutated versus wild-type	0.11	0.056–0.23	**<0.0001**	0.16	0.073–0.37	**<0.0001**
Histological diagnosis						
AO, AOA, vs AA	N/A	N/A	N/A	0.47	0.20–1.12	0.088
Ki-67 labeling index						
<15 vs ≥15%	0.29	0.14–0.57	**0.0004**	0.24	0.10–0.58	**0.0014**
7p (*EGFR*)						
Gained (−) vs (+)	0.31	0.14–0.68	**0.0034**	0.26	0.11–0.60	**0.0018**

N/A not applicable, *OS* overall survival, *PFS* progression-free survival, *CI* confidence interval

Bold indicates statistical significance at $P < 0.050$

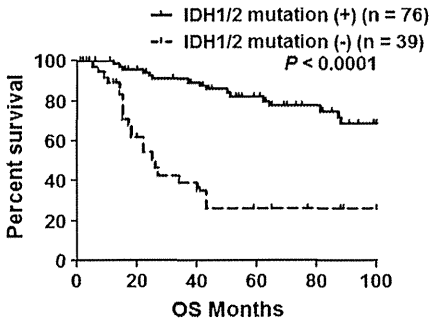

Fig. 1 Overall survival of patients with grade III gliomas by isocitrate dehydrogenase 1 and 2 (*IDH1/2*) gene status. Patients with mutated *IDH1/2* present prolonged survival compared with patients with wild-type *IDH1/2*

Subclassification of Grade III Gliomas Based on *IDH1/2* Status

Patients were divided into two groups by *IDH1/2* gene status. In the mutated *IDH1/2* group, median PFS and OS were not reached, and 5-year survival rate was 70.5 and 82.3%, respectively. Gross total resection was associated with better PFS ($P = 0.040$; Table 4), and gross total resection, wild-type *TP53* gene, and no gain of 7p were associated with longer OS ($P = 0.018$, 0.046, and 0.017, respectively) (Table 4; Fig. 2a–c). Multivariate Cox regression analysis in the mutated *IDH1/2* group revealed that 7p gain (HR 5.3, 95% CI 1.35–20.5, $P = 0.017$) and high Ki-67 labeling index (≥15%) (HR 3.2, 95% CI 1.11–9.05, $P = 0.030$) were independent prognostic factors of poor PFS (Table 5), and incomplete resection (HR 5.7, 95% CI 1.53–20.8, $P = 0.0092$), 7p gain (HR 5.5, 95% CI 1.39–21.5, $P = 0.015$), and *TP53* gene mutation (HR 3.4, 95% CI 1.15–9.84, $P = 0.026$) were independent prognostic factors of poor OS (Table 5).

In the wild-type *IDH1/2* group, median PFS and OS were 13 and 26 months, respectively, and 5-year survival rate was 13.5 and 26.3%, respectively. Low Ki-67 labeling index (<15%) was associated with longer PFS ($P = 0.049$; Table 6) by univariate analysis. Multivariate analysis revealed high Ki-67 labeling index (≥15%) (HR 2.6, 95% CI 1.05–6.16, $P = 0.038$) and 10q loss (HR 3.7, 95% CI 1.01–13.6, $P = 0.047$) were independent prognostic factors of poor PFS (data not shown). For OS in the wild-type *IDH1/2* group, no factor showed statistical significance, but oligodendroglial component and 1p19q codeletion tended to be associated with longer OS by univariate analysis ($P = 0.094$, $P = 0.099$, respectively) (Table 6).

Additionally, grade III patients were divided into two groups by either *MGMT* gene promoter status or chromosome 1p/19q status to see the prognostic effects of *IDH1/2* mutation on each group. Mutated *IDH1/2* subgroups presented better survival in all groups except for 1p19q codeletion & *IDH1/2* wild-type, which did not reach statistical significance due to the small number of patients ($n = 4$) ($P = 0.74$, Supplementary Fig. 1).

Discussion

We mainly focused on the subgroup analysis of grade III glioma based on *IDH1/2* gene status. *TP53* mutation has obtained the status of a hallmark of gliomas [23], but remains a controversial prognostic factor. In one report, *TP53* gene mutation was proposed as a factor in prolonged survival in patients with AA and glioblastoma [24], while in other, *TP53* gene mutation in AA, AOA, and AO was found to indicate shorter survival [25]. Interestingly, we observed that in patients with grade III glioma in the mutated *IDH1/2* group the absence of *TP53* gene mutation was correlated with longer survival (PFS $P = 0.053$, OS $P = 0.046$) (Table 4; Fig. 2b), and was an independent favorable prognostic factor of OS by

Table 4 Clinical and genetic parameters affecting PFS and OS in grade III gliomas with mutated *IDH1/2*

Characteristic	PFS				OS			
	No. (%)	5-year survival (%)	Median months	*P* (log-rank)	No. (%)	5-year survival (%)	Median months	*P* (log-rank)
	76	70.5	NR		76	82.3	NR	
Histology								
AA	35 (46)	71.8	NR	0.86 (vs AOA, AO)	35 (46)	82.6	NR	0.86 (vs AOA, AO)
AO	20 (26)	75.2	NR		20 (26)	89.2	NR	
AOA	21 (28)	64.4	NR		21 (28)	76.0	NR	
Age at diagnosis								
<50 years old	49 (64)	74.0	NR	0.20	49 (64)	81.0	NR	0.39
≥50 years old	27 (36)	61.4	68		27 (36)	85.7	87	
Sex								
Female	34 (45)	72.9	NR	0.29	34 (45)	92.6	NR	0.074
Male	42 (55)	68.7	NR		42 (55)	73.8	NR	
Karnofsky performance status								
≥80	62 (82)	69.2	NR	0.99	62 (82)	80.5	NR	0.45
<80	14 (18)	76.9	62		14 (18)	92.3	87	
Gross total resection								
Yes	30 (39)	80.5	NR	**0.040**	30 (39)	88.4	NR	**0.018**
No	46 (61)	63.0	74		46 (61)	78.5	NR	
Ki-67 labeling index								
<15%	27 (48)	81.7	NR	0.055	27 (48)	90.9	NR	0.15
≥15%	29 (52)	47.1	62		29 (52)	73.9	NR	
TP53								
Wild-type	40 (53)	80.8	NR	0.053	40 (53)	90.2	NR	**0.046**
Mutated	36 (47)	59.3	NR		36 (47)	73.1	NR	
1p/19q codeletion								
Yes	30 (39)	74.3	NR	0.69	30 (39)	87.4	NR	0.48
No	46 (61)	68.1	NR		46 (61)	78.9	NR	
Gain of 7p								
No	68 (89)	73.4	NR	0.089	68 (89)	86.4	NR	**0.017**
Yes	8 (11)	31.3	43		8 (11)	26.7	51	
Homozygous deletion of 9p								
No	73 (96)	71.4	NR	0.93	73 (96)	81.6	NR	0.67
Yes	3 (4)	50.0	77		3 (4)	100	82	
Loss of 10q								
No	70 (92)	71.8	NR	0.70	70 (92)	82.4	NR	0.97
Yes	6 (8)	55.6	NR		6 (8)	83.3	NR	
MGMT								
Methylated	69 (91)	70.2	NR	0.64	69 (91)	81.0	NR	0.82
Unmethylated	7 (9.0)	75.0	74		7 (9.0)	100	88	

AA anaplastic astrocytoma, *AO* anaplastic oligodendroglioma, *AOA* anaplastic oligoastrocytoma, *NR* not reached, *OS* overall survival, *PFS* progression-free survival

Bold indicates statistical significance at $P < 0.050$

multivariate analysis (Table 5). In the wild-type *IDH1/2* group, *TP53* gene status did not affect prognosis (Table 6).

EGFR alterations are frequently observed in grade III and IV gliomas, but the prognostic value is still controversial [23, 25–27]. In our series, 7p gain (*EGFR*

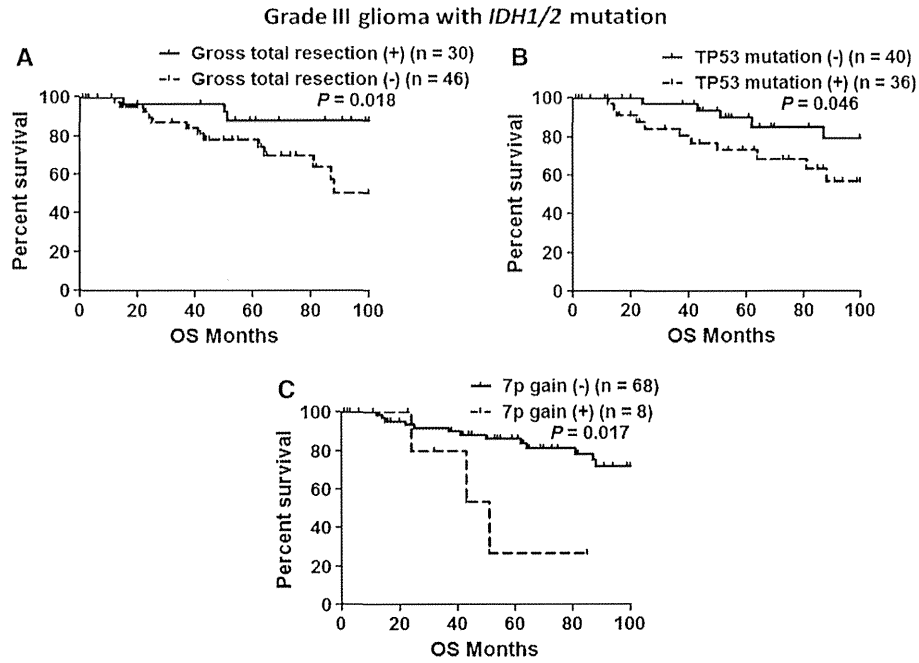

Fig. 2 Overall survival of patients with *IDH1/2*-mutated grade III gliomas by clinical and molecular markers. **a** Patients with gross total resection presented prolonged survival compared with patients with incomplete resection. **b** Patients with *TP53* mutation presented shorter survival compared with patients without *TP53* mutation. **c** Patients with 7p gain presented shorter survival compared with patients without 7p gain

Table 5 Multivariate analysis of independent factors associated with survival in grade III glioma patients with mutated *IDH1/2*

Variable	PFS			OS		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Gross total resection						
Yes vs No			0.27	5.7	1.53–20.8	**0.0092**
7p (*EGFR*)						
Gained vs retained	5.3	1.35–20.5	**0.017**	5.5	1.39–21.5	**0.015**
TP53						
Mutated vs wild-type			0.15	3.4	1.15–9.8	**0.026**
Ki-67 labeling index						
≥15 vs <15%	3.2	1.11–9.05	**0.030**	N/A	N/A	N/A

OS overall survival, *CI* confidence interval, *N/A* not applicable

Bold indicates statistical significance at $P < 0.050$

amplification) was significantly associated with poor survival (Tables 2, 3). Furthermore, from the subgroup analysis, 7p gain was an independent prognostic factor of poor outcome in the mutated *IDH1/2* group (Tables 4, 5), but not in the wild-type *IDH1/2* group (Table 6). The frequency of 7p gain in grade III gliomas in the mutated *IDH1/2* group was small at 11% (Table 4), ranging from 0 to 43% in other reports [1, 3, 7]. Even in the mutated *IDH1/2* group, patients with *EGFR* amplification present poor prognosis, and careful follow-up is needed.

Including our data, 17–41% of grade III glioma patients had wild-type *IDH1/2* [1, 3, 8, 28]. The wild-type *IDH1/2* group consisted of 21 AA, 7 AOA, and 11AO. To exclude the possibility of diagnostic error in the present study, histology was re-reviewed by two neuropathologists. Surprisingly, neither necrosis nor microvascular proliferation was detected in this group, and therefore the wild-type *IDH1/2* group were histologically distinct from glioblastoma. In terms of prognosis, the median OS of patients with grade III gliomas with wild-type *IDH1/2* was 26 months, much shorter than the median OS of patients with grade III glioma with mutated *IDH1/2* (median not reached) (Tables 2, 4, 6), but longer than that of patients with primary glioblastoma (12.5–17 months) [13, 29]. In terms of genetic alterations, grade III gliomas with wild-type *IDH1/2* had only 10% of 10q alteration, 15% of homozygous

Table 6 Clinical and genetic parameters affecting PFS and OS in grade III glioma with wild-type *IDH1/2* group

Characteristic	PFS				OS			
	No. (%)	5-year survival (%)	Median months	*P* (log-rank)	No. (%)	5-year survival (%)	Median months	*P* (log-rank)
	39	13.5	13		39	26.3	26	
Histology								
AA	21 (54)	9.5	11	0.15 (vs AOA, AO)	21 (54)	18.2	18	0.094 (vs AOA, AO)
AO	11 (28)	0	18		11 (28)	46.7	40	
AOA	7 (18)	38.1	25		7 (18)	34.3	43	
Age at diagnosis								
<50 years old	18 (46)	15.7	13	0.71	18 (46)	23.2	18	0.25
≥50 years old	21 (54)	5.9	11		21 (54)	27.5	34	
Sex								
Female	11 (28)	18.2	11	0.60	11 (28)	39.8	43	0.36
Male	28 (72)	5.6	13		28 (72)	18.6	22	
Karnofsky performance status								
≥80	29 (74)	9.9	13	0.48	29 (74)	27.8	25	0.67
<80	10 (26)	10.0	11		10 (26)	29.2	27	
Gross total resection								
Yes	18 (46)	23.8	11	0.32	18 (46)	43.2	26	0.29
No	21 (54)	0	13		21 (54)	9.7	27	
Ki-67 labeling index								
<15%	12 (44)	16.7	21.5	**0.049**	12 (44)	33.3	30.5	0.16
≥15%	15 (56)	0	10		15 (56)	25.6	17	
TP53								
Wild-type	24 (62)	9.9	11	0.98	24 (62)	33.8	34	0.16
Mutated	15 (38)	10.0	17		15 (38)	12.5	25	
1p/19q codeletion								
Yes	4 (10)	33.3	47	0.095	4 (10)	66.7	NR	0.099
No	35 (90)	7.8	11		35 (90)	21.7	22	
Gain of 7p								
No	26 (67)	12.0	11	0.80	26 (67)	38.4	34	0.28
Yes	13 (33)	7.7	13		13 (33)	9.9	22	
Homozygous deletion of 9p								
No	33 (85)	8.5	11	0.93	33 (85)	24.6	26	0.67
Yes	6 (15)	16.7	13		6 (15)	33.3	16	
Loss of 10q								
No	35 (90)	11.5	13	0.086	35 (90)	28.7	26	0.22
Yes	4 (10)	0	8.5		4 (10)	0	20.5	
MGMT								
Methylated	18 (44)	11.8	17	0.48	18 (44)	26.6	25	0.61
Unmethylated	21 (56)	8.4	11		21 (56)	24.0	26	

AA anaplastic astrocytoma, *AO* anaplastic oligodendroglioma, *AOA* anaplastic oligoastrocytoma, *OS* overall survival, *PFS* progression-free survival

Bold indicates statistical significance at $P < 0.050$

deletion of 9p, and 33% of *EGFR* amplification (only one case of truncated variant of *EGFR*) (Tables 2, 6), which were less frequent compared to the majority of glioblastoma carrying 69% of 10q loss [30], 31–57% of homozygous deletion of 9p [30, 31], and 34–39% of *EGFR* amplification (20–50% were truncated variants) [24, 30, 32, 33]. These observations indicate that grade III glioma with wild-type *IDH1/2* is distinct from both grade III

glioma with mutated *IDH1/2* and glioblastoma, and thus cannot be categorized into the current WHO classification. This fact was supported by the evidence that the frequency of *IDH1/2* gene mutation in grade III gliomas (62–69.1%) is lower than that in grade II gliomas (71–82%) [1, 3, 4, 28], indicating that some grade III gliomas may develop through a bypass pathway without *IDH1/2* gene mutation.

In conclusion, subgroup analysis of the clinical and genetic profiles of grade III gliomas by *IDH1/2* gene status helped to understand their concealed background. Grade III glioma patients with *IDH1/2* mutation present favorable prognosis; however, careful follow-up is needed in patients with incomplete resection, 7p gain, and *TP53* gene mutation. Grade III glioma patients with wild-type *IDH1/2* present poor prognosis; however, oligodendroglial component and 1p19q codeletion tend to show better prognosis even in this unfortunate group. These facts may help predict the prognosis of patients with grade III glioma precisely, and be a supportive factor in histological diagnosis.

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Conflict of interest There is no conflict of interest to declare.

References

1. Balss J, Meyer J, Mueller W et al (2008) Analysis of the IDH1 codon 132 mutation in brain tumors. Acta Neuropathol 116(6): 597–602
2. Parsons DW, Jones S, Zhang X et al (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321(5897):1807–1812
3. Yan H, Parsons DW, Jin G et al (2009) IDH1 and IDH2 mutations in gliomas. N Engl J Med 360(8):765–773
4. Watanabe T, Nobusawa S, Kleihues P et al (2009) IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. Am J Pathol 174(4):1149–1153
5. Ohgaki H, Kleihues P (2009) Genetic alterations and signaling pathways in the evolution of gliomas. Cancer Sci 100(12):2235–2241
6. Yan H, Bigner DD, Velculescu V et al (2009) Mutant metabolic enzymes are at the origin of gliomas. Cancer Res 69(24):9157–9159
7. van den Bent MJ, Dubbink HJ, Marie Y et al (2010) IDH1 and IDH2 mutations are prognostic but not predictive for outcome in anaplastic oligodendroglial tumors: a report of the European Organization for Research and Treatment of Cancer Brain Tumor Group. Clin Cancer Res 16(5):1597–1604
8. Ichimura K, Pearson DM, Kocialkowski S et al (2009) IDH1 mutations are present in the majority of common adult gliomas but rare in primary glioblastomas. Neuro Oncol 11(4): 341–347
9. Levin VA, Hess KR, Choucair A et al (2003) Phase III randomized study of postradiotherapy chemotherapy with combination alpha-difluoromethylornithine-PCV versus PCV for anaplastic gliomas. Clin Cancer Res 9(3):981–990
10. van den Bent MJ, Carpentier AF, Brandes AA et al (2006) Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European Organisation for Research and Treatment of Cancer phase III trial. J Clin Oncol 24(18):2715–2722
11. Sanson M, Marie Y, Paris S et al (2009) Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. J Clin Oncol 27(25):4150–4154
12. Houillier C, Mokhtari K, Carpentier C et al (2010) Chromosome 9p and 10q losses predict unfavorable outcome in low-grade gliomas. Neuro Oncol 12(1):2–6
13. Sonoda Y, Kumabe T, Nakamura T et al (2009) Analysis of IDH1 and IDH2 mutations in Japanese glioma patients. Cancer Sci 100(10):1996–1998
14. Mashiyama S, Murakami Y, Yoshimoto T et al (1991) Detection of p53 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. Oncogene 6(8):1313–1318
15. Sonoda Y, Kumabe T, Watanabe M et al (2009) Long-term survivors of glioblastoma: clinical features and molecular analysis. Acta Neurochir (Wien) 151(11):1349–1358
16. Sonoda Y, Yokosawa M, Saito R, et al (2010) O(6)-Methylguanine DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression is correlated with progression-free survival in patients with glioblastoma. Int J Clin Oncol 15:352–358
17. Wick W, Hartmann C, Engel C et al (2009) NOA-04 randomized phase III trial of sequential radiochemotherapy of anaplastic glioma with procarbazine, lomustine, and vincristine or temozolomide. J Clin Oncol 27(35):5874–5880
18. Jeuken J, Sijben A, Alenda C et al (2009) Robust detection of EGFR copy number changes and EGFR variant III: technical aspects and relevance for glioma diagnostics. Brain Pathol 19(4):661–671
19. Jeuken J, Cornelissen S, Boots-Sprenger S et al (2006) Multiplex ligation-dependent probe amplification: a diagnostic tool for simultaneous identification of different genetic markers in glial tumors. J Mol Diagn 8(4):433–443
20. Jeuken JW, Cornelissen SJ, Vriezen M et al (2007) MS-MLPA: an attractive alternative laboratory assay for robust, reliable, and semiquantitative detection of MGMT promoter hypermethylation in gliomas. Lab Invest 87(10):1055–1065
21. Kato H, Kato S, Kumabe T et al (2000) Functional evaluation of p53 and PTEN gene mutations in gliomas. Clin Cancer Res 6(10):3937–3943
22. Kanamori M, Kumabe T, Sonoda Y et al (2009) Predictive factors for overall and progression-free survival, and dissemination in oligodendroglial tumors. J Neurooncol 93(2):219–228
23. Ohgaki H, Kleihues P (2005) Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. J Neuropathol Exp Neurol 64(6): 479–489
24. Smith JS, Tachibana I, Passe SM et al (2001) PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and glioblastoma multiforme. J Natl Cancer Inst 93(16):1246–1256
25. Dehais C, Laigle-Donadey F, Marie Y et al (2006) Prognostic stratification of patients with anaplastic gliomas according to genetic profile. Cancer 107(8):1891–1897
26. Etienne MC, Formento JL, Lebrun-Frenay C et al (1998) Epidermal growth factor receptor and labeling index are independent prognostic factors in glial tumor outcome. Clin Cancer Res 4(10):2383–2390