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Abbreviations used in this paper: FRT = fractionated radiotherapy; GBM = glioblastoma multiforme; GRZB = Granzyme B; IDH1 = isocitrate dehydrogenase-1; IHC = immunohistochemistry; MGMT = O6-methylguanine-DNA methyltransferase; MHC = major histocompatibility complex; PD-1 = Programmed cell death 1; PD-L1 = PD-1 ligand; AFTV = autologous formalin-fixed tumor vaccine; OS = overall survival; PFS = progression-free survival; TIL = tumor-infiltrating lymphocytes; TMZ = temozolomide.

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Abstract

Background

Glioblastoma (GBM) is the most common type of malignant brain tumor and has a very poor prognosis. Most patients relapse within 12 months despite aggressive treatment and patient outcome after recurrent is extremely worse. This study was designed to clarify the change of the molecular expression, including programmed cell death 1 (PD-1) and PD-ligand 1 (PD-L1), on the initial and secondary resected tumor specimens and to address the influence of these expressions for patient outcome after second surgery of glioblastoma.

Methods

We investigated 16 patients, ranging in age from 14 to 65 years, with histologically verified WHO grade IV GBM, whose original tumor was resected between 2008 and 2014, and treated with fractionated radiotherapy (FRT) and temozolomide (TMZ). Four patients who were treated with immunotherapy using autologous formalin-fixed tumor vaccine were enrolled. All of the patients underwent secondary resection after tumor recurrence within 24 months. We carried out an immunohistochemical examination of the initial and secondary resected tumors from patients using a panel of immune system molecular markers, and assessed whether marker expression correlated with clinical outcomes.

Results

CD3, CD8 and PD-1 on tumor-infiltrating lymphocytes (TILs) was significantly increased in secondary resected specimens compared with initially resected specimens ($p \leq 0.05$). All patients expressed PD-L1 on tumor cells in initial and secondary resection specimens. Patients were divided into high or low expression group by median IHC score of PD-1 on initial or secondary resected specimens. No significant differences in patient outcomes were observed between high and low PD-1 or PD-L1 groups of initially resected specimens. In high expression group of secondary resected specimens, most patients score had increased

which compared with initial resected tumor specimens. The PD-1 high expression score group of secondary resected specimens was associated with long progression-free survival and short survival after recurrence.

Conclusion

PD-L1 expression was detected in almost all initial and secondary specimens. Patients with high PD-1 expression of secondary specimen had bad prognosis after secondary resection. PD-1/PD-L1 pathway may be associated with patient outcome after second surgery of glioblastoma.

Introduction

Glioblastoma (GBM) is the most common type of malignant brain tumor [1-3]. Despite aggressive treatment, including tumor resection, fractionated radiotherapy (FRT) and temozolomide (TMZ), the overall survival (OS) of patients with GBM is only 14.6 months [4]. Furthermore, patient outcomes after recurrence are generally dismal, with a median OS of less than 10.8 months [5-8]. Various studies have shown that anti-angiogenic antibodies or intraoperative chemotherapeutic implantation do not dramatically improve patient outcomes [9, 10]. Although *isocitrate dehydrogenase-1* (*IDH-1*) mutation, O^6-methylguanine-methyltransferase (MGMT) methylation status and Ki-67 index for initially diagnosed GBM are predictive biomarkers associated with suitability for additional treatment or prognosis, there are few biomarkers that are useful after recurrence [8, 11]. Previous studies showed that expression of TP53, MDM2, MSH2 and EGFRvIII was down-regulated in recurrent GBM [12, 13]. In other types of recurrent malignant tumors, including breast cancer and colorectal cancer, patient outcomes were influenced by the tumor immune microenvironment [14, 15].

We have performed immunotherapy using autologous formalin-fixed tumor vaccine (AFTV) for initially diagnosed GBM [16-19]. In these studies, we found that a part of patients treated with AFTV had very good prognosis, while some patients had unfavorable outcomes. However, any molecular marker relating to patient prognosis was not determined.

More recently, immune checkpoint inhibitors are attracting attention as a new treatment strategy that helps immune cells recognize and attack cancer cells [20, 21]. In patients with malignant melanoma and non-small cell lung cancer, which carry a relatively high number of somatic mutations, treatment with an anti- PD-1 antibody induced a significant and durable reduction in tumor volume [20, 21]. A high number of somatic mutations is thought to be a predictive marker of effectiveness for checkpoint inhibitor immunotherapy, because the degree of mutation represents the height of antigenicity and to be considered induction of cytotoxic T cell lymphocyte (CTL) repertoire [22]. Although there have been a few studies addressing the associations between PD-1/PD-1 ligand (PD-L1) expression in GBM and patient outcomes [23-25], the value of PD-1/PD-L1 expression as a prognostic marker remains controversial. We studied the immune system molecular markers by immunohistochemical examination of initially and secondary resected tissues from patients with GBM treated with FRT and TMZ at our institution, and assessed whether marker expression correlated with clinical outcomes after second surgery.

Materials and methods

Patients and GBM specimens

We retrospectively investigated 16 patients with histologically verified WHO grade IV GBM, ranging in age from 14 to 65 years, who received the first surgical tumor removal between 2008 and 2014, and treated with FRT and TMZ at our institute (Department of Neurosurgery, Faculty of Medicine, University of Tsukuba). All patients, including four patients who were

treated with AFTV, and other four patients who were treated with proton therapy, underwent secondary resection after tumor recurrence within 24 months. The study included three patients registered in the University Hospital Medical Information Network (UMIN) clinical trials number 000001426, the study design and treatment protocol of which were approved by the ethics committees of our institute [17], and a patient treated with AFTV who was reported in a previous paper [19]. The AFTV was prepared from autologous formalin-fixed tumor tissue and administered with adjuvant microparticles as described previously [16, 18].

Pathological examination

Histopathological diagnosis was confirmed according to the 2007 WHO criteria [26] by pathologists at Tsukuba University Hospital or the Japan Brain Tumor Reference Center at Gunma University (Maebashi, Japan) using paraffin-embedded tissue sections stained with hematoxylin and eosin (H&E). Formalin-fixed paraffin embedded (FFPE) sections (2 μm) were deparaffinized in xylene and rehydrated through graded alcohols (99.5%–70%). Antigen retrieval was carried out using target retrieval solution (pH 6.0; Dako) for PD-L1 or citric acid buffer (pH 6.0) for other molecules for 10 minutes in a microwave (specimens were put in a microwavable pressure cooker when using microwave). Endogenous peroxidase activity was quenched by immersion in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature and the sections were then incubated with primary antibodies at 4°C overnight. The next day, the slides were incubated with a secondary biotinylated antibody (LSAB2 Kit; Dako) at room temperature for 10 minutes. After another 10 minutes incubation with streptavidin-horse radish peroxidase (LSAB2 kit; Dako), reactions were developed using a Liquid DAB Substrate Chromogen system (Dako). The slides were counterstained with 50% Mayer's hematoxylin and dehydrated through graded alcohols (80%–99.5%) and xylene, then cover slipped with EUKITT (mounting reagent).

Monoclonal antibodies used for immunohistochemistry (IHC) included anti-Ki-67 (MIB-1, Dako), anti-TP53 (DO-7, Dako), anti-MHC class I (W6/32, Santa Cruz Biotechnology), anti-MHC class II (LN3, Novus), anti-IDH-1R132H (D299-3, MBL), anti-CD3 (SP7, Novus), anti-CD8 (SP16, Gene Tex), anti-CD20 (BV11, Novus), anti-CD45RO (UCH-L1, Santa Cruz Biotechnology) and anti-PD-L1 (28-8, Abcam). Polyclonal antibodies included anti-Granzyme B (GRZB) (E2582, Spring Bioscience), anti-PD-1 (E18662, Spring Bioscience) and anti-ATRX (HPA001906, Sigma Aldrich).

Staining indices were calculated as the average number of positive cells in randomly chosen pairs of vascular rich and relatively hypo-vascular areas with appropriate staining condition from the entire section, with a total number of cells not less than 1000. The Ki-67 and TP53 indices were expressed as percentages. For category analysis, cases with 10% or more positive cells were deemed positive, and cases with fewer than 10% positive cells were deemed negative for TP53 [27]. For IDH1-R132H and ATRX, cases with 50% or more positive cells were rated as positive, and cases with fewer than 50% positive cells were rated as negative. Expression of CD3, CD8, CD20, CD45RO, GRZB and PD-1 in infiltrating immune cells was scored as 1 point (0 to 4 cells per high power field, x400), 2 points (5 to 8 cells), 3 points (9 to 12 cells) and 4 points (13 cells or more). Staining scores, which were the sum (2 to 8 points) of two median values of each of three points in the vascular-rich areas (1 to 4 points) and the relatively hypo-vascular areas (1 to 4 points) were used for analysis, modifying a method described previously [15]. Expression of MHC class I and PD-L1 in tumor cells was graded as '-' (absence of staining), '+' (up to 25% of cells stained), '++' (25–50% of cells stained) or '+++' (more than 50% of cells stained) [27, 28]. The IHC evaluation was performed by one of the authors (T. M.) with no reference to other clinical data.

DNA extraction and bisulfite treatment

Genomic DNA was extracted from freshly frozen tissue using a DNeasy® Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. Genomic DNA (1 µg) was bisulfite modified by the MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures), following the manufacturer's protocol. Modified DNA was purified and used immediately. Bisulfite modified genomic DNAs from Hela and U87 cell lines were used as positive controls for unmethylated and methylated DNA, respectively. Distilled water was used as negative control.

Methylation Specific PCR (MSPs)

MSPs were carried out with 50 ng of bisulfite modified DNA in a total volume of 10 µl, which contained 5 µl of 2x AmpliTaq Gold™ Fast PCR Master Mix (Applied Biosystems), in a Veriti thermocycler (Applied Biosystems). Oligonucleotide primers for methylated or unmethylated MGMT were described previously [(29)]. PCR reactions were 95°C for 10 min, followed by 40 cycles of 30 seconds denaturation at 96°C, 30 seconds annealing at 62°C, 30 seconds extension at 68°C and a final extension step at 72°C for 30 seconds. PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide at a final concentration of 0.1 µg/ml.

Statistical analysis

All results are presented as absolute number (%) or median (range). Statistical analyses were performed using standard statistical software (IBM SPSS Statistics version 24.0 for Windows; SPSS, Chicago, Illinois, USA). For comparison of IHC score and grade, Wilcoxon tests between initial and secondary specimens and Mann-Whitney U tests between initial specimens of vaccine group and control group were used, respectively. OS from diagnosis to death or last follow-up was estimated using the Kaplan-Meier limit method. The log-rank

test was used to assess group differences. P values of less than 0.05 were considered statistically significant.

Results

The characteristics, IHC pathological status at the first surgery, and treatment histories after surgery of the 16 patients are presented in Table 1. Two patients had IDH-1 mutation and diagnosed with secondary GBM.

Fig. 1 shows micrographs of CD8 and PD-1 membranous expression on tumor-infiltrating lymphocytes (TILs) in initially resected and secondary resected specimens from a representative recurrent case after AFTV treatment. Although double-staining could not be done due to the host type of antibody used in this study, most (approximately 74 %) of CD8+ T cells co-expressed PD-1 in evaluable TILs using consecutive tissue sections. Remaining CD8+ T cells were PD-1 negative cells. High and low PD-L1 membranous expression on tumor cells, and PD-1 expression on TILs of representative cases are shown in Fig. 2. Table 2 shows IHC indices, staining scores and grades (*see Material and Methods)* of TILs and tumor cells for specimens obtained from first and second surgeries. Ki-67, P53, ATRX, IDH1R132H, HLA-ABC, HLA-DR and PD-L1 staining scores or indices were not significantly different. CD3, CD8 and PD-1 staining scores were significantly increased in secondary resected specimens compared with initially resected specimens ($p \leq 0.05$), and CD20, CD45RO and GRZB staining scores were unaltered.

Patients were divided into high (7 or more) or low PD-1 score group (Less than 7) by PD-1 staining scores on secondary specimens. PD-1 (as an activated/exhausted lymphocyte marker) expression scores of the high PD-1 score group was significantly increased in secondary resection specimens compared with initial resection specimens ($p \leq 0.05$) (Fig. 3A). All patients treated with AFTV increased PD-1 score on secondary specimens compared

with initial specimens. CD3 (T cell receptor), CD8 (Cytotoxic lymphocyte marker) and CD45RO (Memory T cell marker) expression scores were also significantly increased in high PD-1 score group (data not shown). There was no significant differences in molecular marker expression on TILs between the initial and secondary specimens of low PD-1 score group. No significant difference was seen between high and low PD-1 score groups on the initial specimens.

To assess the association between marker expression and patient outcomes, univariate analyses of PFS, survival from secondary surgery and OS (from initial surgery) in high- and low-expression groups were performed. Fig. 3B shows the survival curves of the high and low PD-1 score groups on secondary specimens. In log-rank analysis, high PD-1 score after the secondary surgery was a significant factor associated with favorable PFS, and low PD-1 score after the secondary surgery was also significant for improved survival. PD-1 score and other molecular score including Ki67 score and P53 score did not correlate in this study. High CD8 score after the secondary surgery was also significant poor prognostic factor of survival after second resection as high PD-1 score (p=0.005), and high CD3 score was trend of poor prognostic factor of survival after second resection (p=0.065). In tumor markers, high PD-L1 grading also show trend of poor prognostic factor of PFS (p=0.095). Univariate log-rank analysis of other IHC data, including ATRX, IDH-1R132H and others, revealed that positive ATRX staining after the initial surgery was the only significant factor ($p<0.05$) associated with favorable PFS. No significant differences were observed for other factors, including IDH-1R132H staining status, probably due to low patient numbers.

Discussion

The balance of cytotoxic CD8+TILs and negative immune regulators, such as PD-1/PD-L1 expression and regulatory T cells in the tumor microenvironment, are critical determinants of

patient outcome associated with both the intrinsic and immunotherapy mediated immune response [30]. In this study, the IHC expression scores of CD3, CD8 and PD-1 on TILs were significantly increased in the secondary resection specimens compared with the initial resection specimens, although CD20 and GRZB scores were not different. These results indicated that various stimulations including AFTV treatment induce the recruitment of large numbers of T cell type TILs, consisting mostly of CD8+ cytotoxic T cells. However, most of the CD8+ TILs co-expressed PD-1. PD-1 positive TILs including these CD8 T cells were in the exhausted state at the recurrent stage. Earlier research revealed that inflammatory cytokines, such as interferon alfa (IFN α) and interferon gamma (IFN γ) secreted by activated T cells, mediate PD-1 expression on TILs and PD-L1 expression on tumor cells, respectively. This mechanism is considered to be an important factor associated with tumor recurrence [31, 32]. In this study, PD-L1 expression on tumor cells was detected in all specimens, and the secretion of inflammatory cytokines accumulated TILs may be linked to this phenomenon. In addition, GRZB downregulation was observed in PD-1 expressing TILs as shown in Fig. 1. We speculate that activated TILs were attenuated by inhibitory factors, such as accumulating regulatory T cells or myeloid derived suppressor cells, PD-1/PD-L1, inhibitory factors (TGF-beta, IL-10, IDO) or tryptophan catabolites, all of which are found in various tumor microenvironments [33, 34].

The results of our previous Phase IIa clinical study of FRT, TMZ and AFTV immunotherapy for initially diagnosed GBM patients included a 33% two-year progression-free ratio, 22.2 months OS and 38% three-year survival ratio [17]. In other words, a third of patients had a long PFS of over 24 months resulting in an extremely favorable outcome, and half of the rest of the patients had an unfavorable outcome. Thus, it will be very valuable to identify the mechanisms underlying early AFTV treatment failure. In the present study, all of the tumors in the AFTV group recurred within 24 months, and there were

no patients with a PFS over 24 months. In our experience, most patients with a PFS over 24 months do not have a recurrence within the follow-up period of 3.1 years. Instead, typically they may have a small mass in a new location distal from the original lesion that is treatable with stereotactic radiotherapy. For this reason, the present study includes only AFTV failure patients with a relatively short PFS.

Our data showed that cases with increased numbers of PD-1 positive TILs at the secondary surgery tended to have a favorable PFS. We speculate that PFS was extended by spontaneously or AFTV induced PD-1 negative CD8 TILs that attack the remaining tumor cells after the initial surgery and inhibit tumor progression. On the other hand, TILs had a limited capacity to infiltrate tumor tissue and influence tumor growth. However, the PD-1 low group had better survival after secondary surgery compared with the high group. We speculate that expansion of PD-1 negative CD8 T cells probably extend the interval to recurrence. After TILs including CD8 T cells beginning to express PD-1, tumor may recur rapidly. Almost all TILs express PD-1 after secondary surgery in the high groups, and there were minimal numbers of active TILs that were able to influence tumor progression consistent with very poor prognosis. Median survival from secondary surgery for the PD-1 High group and PD-1 Low group was 13.8 months and 18.4 months, respectively.

There are few studies addressing the association between PD-1/PD-L1 expression in GBM and patient outcomes. Nduom and co-authors examined 92 GBMs by IHC and reported that high expression of PD-L1 on GBM cells was a poor prognostic marker [25]. In contrast, Berghoff and co-authors examined 117 initially diagnosed and 18 recurrent GBMs by IHC and did not find a correlation between high PD-L1 expression and prognosis [23]. In regard to other mechanisms that may be related to AFTV treatment failure, we speculate that some hyper-proliferative tumors will recur until acquired immunity is established after AFTV administration.

Our results indicate that anti-PD-1 antibody therapy may effective for preventing tumor recurrence after initial surgery. In a mouse breast cancer model (TUBO cell xenograft), combination therapy with a multi-peptide vaccine and anti-PD-1 antibody prolonged survival of tumor-bearing mice and enhanced TIL antigen reactivity [35]. Further, in colon cancer (CT26 cell xenograft) and ovarian cancer (ID8-VEGF cell xenograft) mouse models, the combination therapy of vaccination or adaptive transfer of TILs and double immune checkpoint blockade (CTLA-4 and PD-1) blocked tumor growth more effectively than monotherapy or combination therapy with single immune checkpoint blockade [36]. Anti-CTLA-4 antibody inhibits the negative regulation by CTLA-4 signaling in the activation phase of the APC-T cell interaction, and enhances anti-tumor effects as a result of T cell activation and proliferation [37]. However, it has been reported that the side effects of double immune checkpoint blockade combination therapy are significant [38]. In our cohort, AFTV induced high numbers of TILs with no or very weak side effects (e.g. redness and/or induration in the administration skin site, and mild fever). In addition, combination therapy with AFTV, pembrolizumab (anti-PD-1 antibody) and radiation was effective for the treatment of chemo-refractory liver-metastasized uterine cervical small cell carcinoma [39]. Thus, we expect that combination therapy with AFTV and anti-PD-1 antibody is a more effective and safer method for cancer immunotherapy.

Our study has some limitations. The characteristics of our patient group were not typical for standard GBM patients because only recurrent cases treated with repeated surgery were included. The past study did not show a correlation between PD-L1 expression and MGMT methylation status in 117 newly diagnosed GBMs [23]. Another previous analysis of 222 GBMs also found no relationship between MGMT methylation status, TP53 expression, or enhancement of PD-1 positive TILs or PD-L1 expression on tumor cells at the tumor site [40]. So we speculate that bias based on MGMT methylation status and TP53 expression might

not be related to the presence of PD-1-positive TILs**.** We could not prove that PD-1 positive TILs were reduced in long PFS (over 24 months) patients treated with AFTV, or patients with no tumor recurrence who had a co-incidental second operation, because it is very difficult to obtain TILs from these patients. We speculate that recurrence is not observed in long PFS patients because tumor cells have already been eliminated even if some PD-1 positive TILs are induced. Moreover, GBM heterogeneity might make it difficult to evaluate with precision whether high PD-1/PD-L1 expression after secondary surgery shortens OS, and the small number of patients is also a limitation of this study, and further analysis using a larger number of patients is necessary.

Conclusion

PD-L1 expression was detected in almost all initial and secondary specimens. In the vaccinated group, the expression of PD-1 on TILs was significantly upregulated in secondary resected specimens compared with the initially resected tumors ($p \leq 0.05$). The patients with high PD-1 expression in the secondary specimens had long PFS, and short survival after recurrence. PD-1/PD-L1 pathway may be associated with patient outcome after second surgery of glioblastoma.

References

1. Committee of Brain Tumor Registry of Japan (2009) Report of brain tumor registry of Japan (1984–2000). Neurol Med Chir (tokyo) 49:PS1–96
2. Crocetti E, Trama A, Stiller C, Caldarella A, Soffietti R, Jaal J, Weber DC, Ricardi U, Slowinski J, Brandes A; RARECARE working group (2012) Epidemiology of glial and non-glial brain tumours in Europe. Eur J Cancer 48(10):1532-1542
3. Ostrom QT, Gittleman H, Fulop J, Liu M, Blanda R, Kromer C, Wolinsky Y, Kruchko C, Barnholtz-Sloan JS (2015) CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2008–2012. Neuro Oncol 17(Suppl 4):iv1–iv62
4. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO, European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups, National Cancer Institute of Canada Clinical Trials Group (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987–996
5. Gorlia T, Stupp R, Brandes AA, Rampling RR, Fumoleau P, Dittrich C, Campone MM, Twelves CC, Raymond E, Hegi ME, Lacombe D, van den Bent MJ (2012) New prognostic factors and calculators for outcome prediction in patients with recurrent glioblastoma: a pooled analysis of EORTC Brain Tumour Group phase I and II clinical trials. Eur J Cancer 48(8):1176–1184
6. Park JK, Hodges T, Arko L, Shen M, Dello Iacono D, McNabb A, Olsen Bailey N, Kreisl TN, Iwamoto FM, Sul J, Auh S, Park GE, Fine HA, Black PM (2010) Scale to predict

survival after surgery for recurrent glioblastoma multiforme. J Clin Oncol 28(24):3838-3843

7. Vredenburgh JJ, Desjardins A, Herndon JE, Marcello J, Reardon DA, Quinn JA, Rich JN, Sathornsumetee S, Gururangan S, Sampson J, Wagner M, Bailey L, Bigner DD, Friedman AH, Friedman HS (2007) Bevacizumab plus irinotecan in recurrent glioblastoma multiforme. J Clin Oncol 25(30):4722-4729
8. Weller M, Tabatabai G, Kästner B, Felsberg J, Steinbach JP, Wick A, Schnell O, Hau P, Herrlinger U, Sabel MC, Wirsching HG, Ketter R, Bähr O, Platten M, Tonn JC, Schlegel U, Marosi C, Goldbrunner R, Stupp R, Homicsko K, Pichler J, Nikkhah G, Meixensberger J, Vajkoczy P, Kollias S, Hüsing J, Reifenberger G, Wick W; DIRECTOR Study Group (2015) MGMT Promoter Methylation Is a Strong Prognostic Biomarker for Benefit from Dose-Intensified Temozolomide Rechallenge in Progressive Glioblastoma: The DIRECTOR Trial. Clin Cancer Res 21(9):2057-2064
9. Sandmann T, Bourgon R, Garcia J, Li C, Cloughesy T, Chinot OL, Wick W, Nishikawa R, Mason W, Henriksson R, Saran F, Lai A, Moore N, Kharbanda S, Peale F, Hegde P, Abrey LE, Phillips HS, Bais C (2015) Patients With Proneural Glioblastoma May Derive Overall Survival Benefit From the Addition of Bevacizumab to First-Line Radiotherapy and Temozolomide: Retrospective Analysis of the AVAglio Trial. J Clin Oncol 33(25):2735-2744
10. Westphal M, Hilt DC, Bortey E, Delavault P, Olivares R, Warnke PC, Whittle IR, Jääskeläinen J, Ram Z (2003) A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. Neuro Oncol 5(2):79-88
11. Karsy M, Neil JA, Guan J, Mahan MA, Colman H, Jensen RL (2015) A practical review of prognostic correlations of molecular biomarkers in glioblastoma. Neurosurg Focus

38(3):E4

12. Martin J, van den Bent, Ya Gao, Melissa Kerkhof, Johan M. Kros, Thierry Gorlia, Kitty van Zwieten, Jory Prince, Sjoerd van Duinen, Peter A. Sillevis Smitt, Martin Taphoorn, and Pim J. French (2015) Changes in the EGFR amplification and EGFRvIII expression between paired primary and recurrent glioblastomas. Neuro-Oncology 17(7):935– 941
13. Stark AM, Witzel P, Strege RJ, Hugo H-H, Mehdorn HM (2003) p53, mdm2, EGFR, and msh2 expression in paired initial and recurrent glioblastoma multiforme. J Neurol Neurosurg Psychiatry 74:779–783
14. Brown JR, Wimberly H, Lannin DR, Nixon C, Rimm DL, Bossuyt V (2014) Multiplexed quantitative analysis of CD3, CD8, and CD20 predicts response to neoadjuvant chemotherapy in breast cancer. Clin Cancer Res 20(23):5995-6005
15. Dahlin AM, Henriksson ML, Van Guelpen B, Stenling R, Oberg A, Rutegård J, Palmqvist R (2011) Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. Mod Pathol 24(5):671-682
16. Ishikawa E, Muragaki Y, Yamamoto T, Maruyama T, Tsuboi K, Ikuta S, Hashimoto K, Uemae Y, Ishihara T, Matsuda M, Matsutani M, Karasawa K, Nakazato Y, Abe T, Ohno T, Matsumura A (2014) Phase I/IIa trial of fractionated radiotherapy, temozolomide, and autologous formalin-fixed tumor vaccine for newly diagnosed glioblastoma. J Neurosurg 121(3):543-53
17. Ishikawa E, Tsuboi K, Yamamoto T, Muroi A, Takano S, Enomoto T, Matsumura A, Ohno T (2007) Clinical trial of autologous formalin-fixed tumor vaccine for glioblastoma multiforme patients. Cancer Sci 98(8):1226-1233
18. Muragaki Y, Maruyama T, Iseki H, Tanaka M, Shinohara C, Takakura K, Tsuboi K, Yamamoto T, Matsumura A, Matsutani M, Karasawa K, Shimada K, Yamaguchi N, Nakazato Y, Sato K, Uemae Y, Ohno T, Okada Y, Hori T (2011) Phase I/IIa trial of

autologous formalin-fixed tumor vaccine concomitant with fractionated radiotherapy for newly diagnosed glioblastoma. Clinical article. J Neurosurg 115(2):248-255

19. Sakamoto N, Ishikawa E, Yamamoto T, Satomi K, Nakai K, Sato M, Enomoto T, Morishita Y, Takano S, Ohno T, Tsuboi K, Matsumura A (2011) Pathological changes after autologous formalin-fixed tumor vaccine therapy combined with temozolomide for glioblastoma - three case reports - . Neurol Med Chir (Tokyo) 51(4):319-325
20. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, Barlesi F, Kohlhäufl M, Arrieta O, Burgio MA, Fayette J, Lena H, Poddubskaya E, Gerber DE, Gettinger SN, Rudin CM, Rizvi N, Crinò L, Blumenschein GR Jr, Antonia SJ, Dorange C, Harbison CT, Graf Finckenstein F, Brahmer JR (2015) Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. N Engl J Med 373(17):1627-1639
21. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, Hassel JC, Rutkowski P, McNeil C, Kalinka-Warzocha E, Savage KJ, Hernberg MM, Lebbé C, Charles J, Mihalcioiu C, Chiarion-Sileni V, Mauch C, Cognetti F, Arance A, Schmidt H, Schadendorf D, Gogas H, Lundgren-Eriksson L, Horak C, Sharkey B, Waxman IM, Atkinson V, Ascierto PA (2015) Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med 372(4):320-330
22. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjörd JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Ilicic T, Imbeaud S, Imielinski M, Jäger N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, López-Otín C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN, Span PN,

Teague JW, Totoki Y, Tutt AN, Valdés-Mas R, van Buuren MM, van 't Veer L, Vincent-Salomon A, Waddell N, Yates LR; Australian Pancreatic Cancer Genome Initiative; ICGC Breast Cancer Consortium; ICGC MMML-Seq Consortium; ICGC PedBrain, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR (2013) Signatures of mutational processes in human cancer. Nature 500(7463):415-421

23. Berghoff AS, Kiesel B, Widhalm G, Rajky O, Ricken G, Wöhrer A, Dieckmann K, Filipits M, Brandstetter A, Weller M, Kurscheid S, Hegi ME, Zielinski CC, Marosi C, Hainfellner JA, Preusser M, Wick W (2015) Programmed death ligand 1 expression and tumor-infiltrating lymphocytes in glioblastoma. Neuro Oncol 17(8):1064-1075
24. Liu Y, Carlsson R, Ambjørn M, Hasan M, Badn W, Darabi A, Siesjö P, Issazadeh-Navikas S (2013) PD-L1 expression by neurons nearby tumors indicates better prognosis in glioblastoma patients. J Neurosci 33(35):14231-14245
25. Nduom EK, Wei J, Yaghi NK, Huang N, Kong LY, Gabrusiewicz K, Ling X, Zhou S, Ivan C, Chen JQ, Burks JK, Fuller GN, Calin GA, Conrad CA, Creasy C, Ritthipichai K, Radvanyi L, Heimberger AB (2016) PD-L1 expression and prognostic impact in glioblastoma. Neuro Oncol 18(2):195-205
26. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P (2007) The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 114(2):97-109
27. Lotfi M, Afsharnezhad S, Raziee HR, Ghaffarzadegan K, Sharif S, Shamsara J, Lary S, Behravan J (2011) Immunohistochemical assessment of MGMT expression and p53 mutation in glioblastoma multiforme. Tumori 97(1):104-108
28. Yeung JT, Hamilton RL, Ohnishi K, Ikeura M, Potter DM, Nikiforova MN, Ferrone S, Jakacki RI, Pollack IF, Okada H (2013) LOH in the HLA Class I region at 6p21 is

Associated with Shorter Survival in Newly Diagnosed Adult Glioblastoma. Clin Cancer Res 19(7):1816-1826

29. Lorente A, Mueller W, Urdangarín E, Lázcoz P, von Deimling A, Castresana JS (2008) Detection of methylation in promoter sequences by melting curve analysis-based semiquantitative real time PCR. BMC Cancer 8:61
30. Hodi FS, Dranoff G (2010) The biologic importance of tumor-infiltrating lymphocytes. J Cutan Pathol 37:48-53
31. Mandai M, Hamanishi J, Abiko K, Matsumura N, Baba T, Konishi I (2016) Dual Faces of IFNγ in Cancer Progression: A Role of PD-L1 Induction in the Determination of Pro- and Antitumor Immunity. Clin Cancer Res 22(10):2329-2334
32. Terawaki S, Chikuma S, Shibayama S, Hayashi T, Yoshida T, Okazaki T, Honjo T (2011) IFN-α directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. J Immunol 186(5):2772-2779
33. Duechler M, Peczek L, Zuk K, Zalesna I, Jeziorski A, Czyz M (2014) The heterogeneous immune microenvironment in breast cancer is affected by hypoxia-related genes. Immunobiology 219(2):158-165
34. Platten M, Ochs K, Lemke D, Opitz C, Wick W (2014) Microenvironmental clues for glioma immunotherapy. Curr Neurol Neurosci Rep 14(4):440
35. Karyampudi L, Lamichhane P, Scheid AD, Kalli KR, Shreeder B, Krempski JW, Behrens MD, Knutson KL (2014) Accumulation of memory precursor CD8 T cells in regressing tumors following combination therapy with vaccine and anti-PD-1 antibody. Cancer Res 74(11):2974-2985
36. Duraiswamy J, Freeman GJ, Coukos G (2014) Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors--response. Cancer Res 74(2):633-634

37. Chen L (2004) Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 4(5):336-347.
38. Carlino MS, Long GV (2016) Ipilimumab Combined with Nivolumab: A Standard of Care for the Treatment of Advanced Melanoma? Clin Cancer Res 22(16):3992-3998
39. Miyoshi T, Kataoka T, Asahi A, Maruyama T, Okada R, Uemae Y, Ohno T (2016) A transient increase and subsequent sharp decrease of chemo-refractory liver-metastasized uterine cervical small cell carcinoma to autologous formalin-fixed tumor vaccine plus anti-PD-1 antibody. Clin Case Rep 4(7):687-691
40. Garber ST, Hashimoto Y, Weathers SP, Xiu J, Gatalica Z, Verhaak RG, Zhou S, Fuller GN, Khasraw M, de Groot J, Reddy SK, Spetzler D, Heimberger AB (2016) Immune checkpoint blockade as a potential therapeutic target: surveying CNS malignancies. Neuro Oncol 18(10):1357–1366.

Figure Legends

Fig. 1 Micrographs showing membranous expression of CD8 (A and C) and PD-1 (B and D) on tumor-infiltrating lymphocytes (TILs) in initially resected specimens (A and B) and secondary resected specimens (C and D) in serial sections of a representative case of recurrent glioblastoma (Pt. #4) treated with autologous formalin-fixed tumor vaccine (AFTV) (Magnification x200). Scale bar = 100 μm. White arrows indicate stained cells (A and B).

Fig. 2 Micrograph showing membranous expression of PD-L1 on tumor cells (A and B) and PD-1 on TILs (C and D) (Magnification x400).

A: Low PD-L1 expression area corresponding to grade '+' (up to 25% of cells stained) in the

tumor area. B: High PD-L1 expression area corresponding to grade '+++' (more than 50% of cells stained) in the tumor area, with no PD-L1 expression on blood vessels. C: Low count of TILs with PD-1 expression in the tumor area corresponding to score '1 point' (0-4 cells per high power field). D: High count of TILs expressing PD-1 corresponding to score '4 points' (13 cells or more per high power field). Scale bar = 50 µm.

Fig. 3A Scatter plot depicting the expression scores of PD-1 on TILs in initial and secondary resected specimens. High and low PD-1 expression on secondary resection specimens grouped as higher or lower than median IHC score (Low PD-1 score group (Less than 7), n=7; High PD-1 score group (7 or more), n=9). White circles show 4 patients treated with AFTV. For comparisons of IHC score, the Wilcoxon test between initial and secondary specimens and Mann-Whitney U test between initial specimens of the low group versus high group were used, respectively. $P < 0.05$ were considered statistically significant.

3B Kaplan-Meier curves showing PFS, survival after secondary resection and OS compared with high and low PD-1 expression on secondary resection specimens grouped as higher or lower than median IHC score (Blue line : Low PD-1 score group (Less than 7), n=7; Green line : High PD-1 score group (7 or more), n=9). These curves compared with high and low CD8 and PD-L1 expression on secondary resection specimens are also shown.

Table 1. GBM patient characteristics, treatment histories and pathological status determined by immunohistochemistry (IHC).

	Factors	Values
Patient background	Case number	16
	Age (median (range))	50 (14-65)
	Sex (M:F)	10 : 6
IHC score	MIB-1 index (positive cell %, median (range))	24.1 (5.3-45.7)
	MGMT Methylation specific PCR (Methylated / Unmethylated)	3 / 11**
	IDH-1 R132H (positive/negative)	2 / 14
	TP53 (positive cell %, median (range))	4.4 (0.0-60.4)
GBM type	Primary GBM / Secondary GBM*	14 / 2
Treatments after 1st operation	Conventional FRT/ Proton	12 / 4
	TMZ with RT / others	16 / 0
	AFTV treatment (yes / no)	4 / 12

*IDH-1 mutant or clinically diagnosed secondary GBM.

**2 patients were unavailable.

Table 2. Alteration of indices, staining scores and grades based on IHC staining of TILs and tumor cells obtained from initial and secondary resected specimens.

	1st removal	2nd removal	*p* value**
Case number	16	16	
Ki-67 (%, median)	24.1	15.2	0.178
TP53 (%, median)	4.4	5.7	>0.2
MGMT-Methylation specific PCR (Unmethylated / Methylated)*	11 / 3	10 / 4	>0.2
ATRX (negative / positive)	3 / 13	4 / 12	>0.2
IDH1R123H (negative / positive)	14 / 2	14 / 2	>0.2
PD-L1 (-, +, ++, +++, median)	++	+++	0.187
HLA-ABC (-, +, ++, +++, median)	+++	+++	>0.2
HLA-DR (-, +, ++, +++, median)	++	++	>0.2
CD3 (score, median)	5.5	7.0	0.033
CD8 (score, median)	5.0	6.0	0.009
CD20 (score, median)	4.5	4.0	>0.2
CD45RO (score, median)	6.0	7.0	0.148
GRZB (score, median)	5.0	5.0	>0.2
PD-1 (score, median)	5.0	7.0	0.036

*2 patients were unavailable.

**For comparison of IHC score and grade between 1st versus 2nd specimens, Wilcoxon test was used. Values of $P < 0.05$ were considered statistically significant.

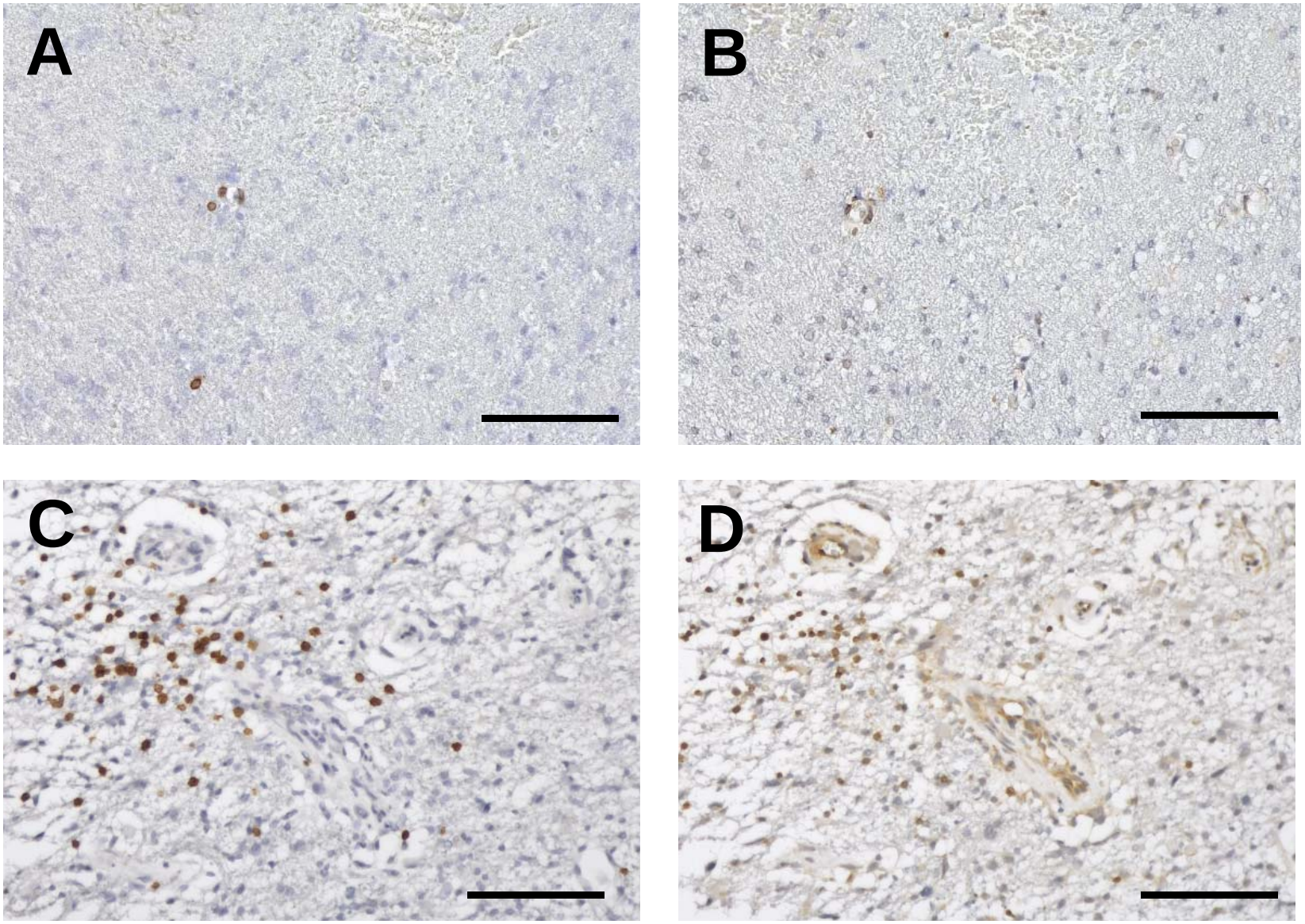

Fig.1

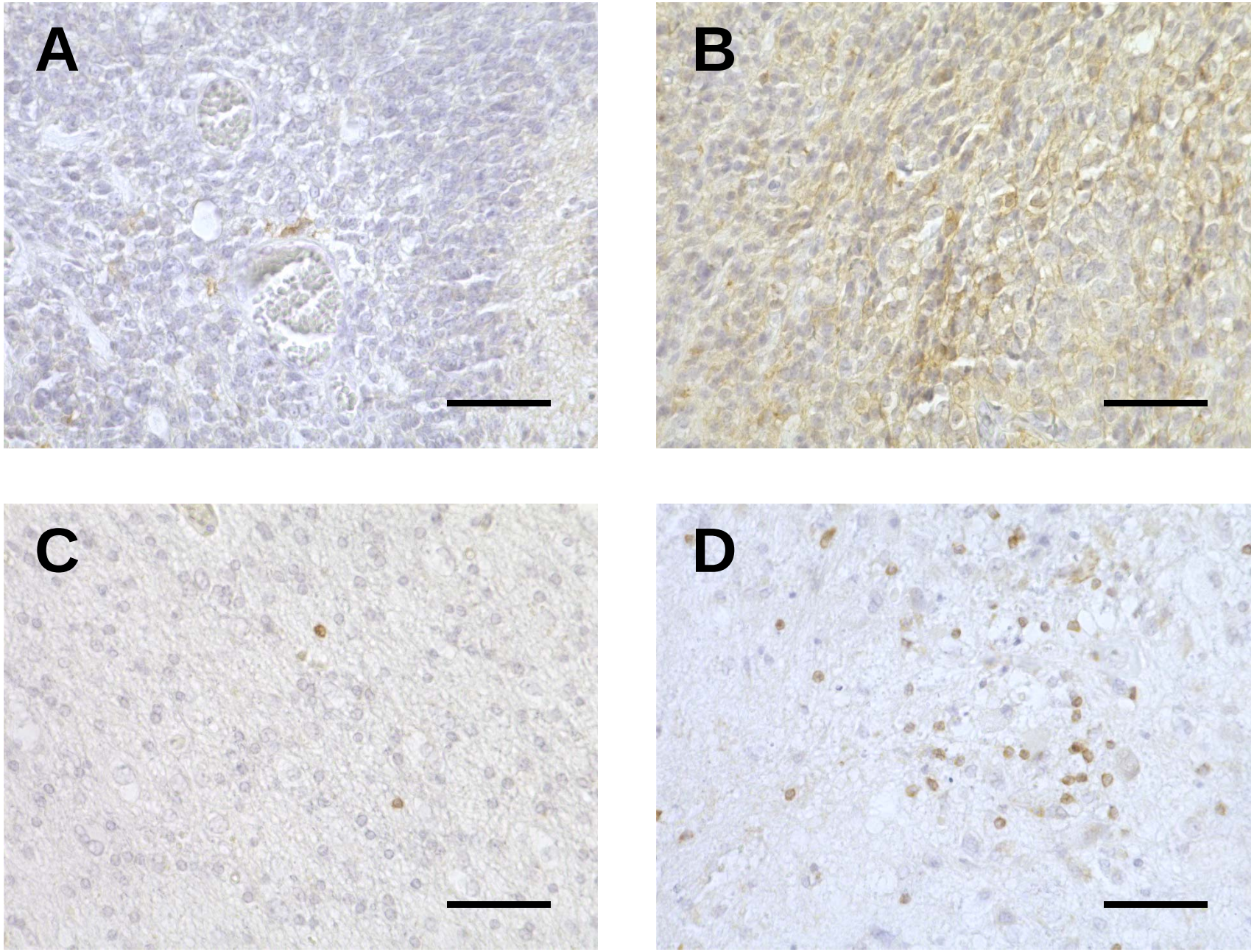

Fig.2

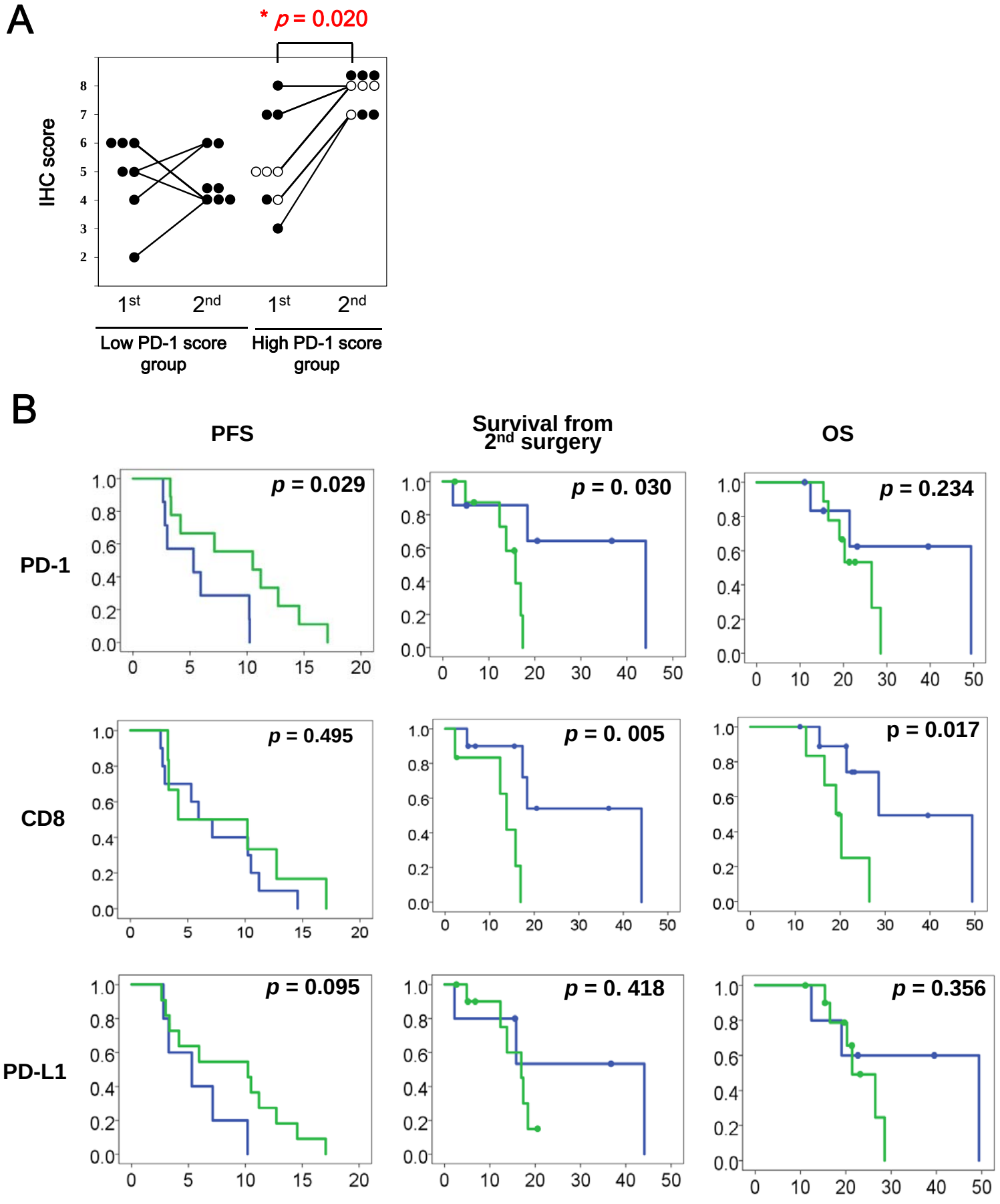
A
* p = 0.020
IHC score
8
7
6
5
4
3
2
1st
2nd
1st
2nd
Low PD-1 score group
High PD-1 score group
B
PFS
Survival from 2nd surgery
OS
PD-1
CD8
PD-L1
p = 0.029
p = 0. 030
p = 0.234
p = 0.495
p = 0. 005
p = 0.017
p = 0.095
p = 0. 418
p = 0.356

Fig.3