Prognostic significance of S-phase fractions in peritumoral invading zone analyzed by laser scanning cytometry in patients with high-grade glioma: A preliminary study

SYOICHI NAKAJIMA[1,2], KEN MORII[1], HITOSHI TAKAHASHI[3], YUKIHIKO FUJII[1] and RYUYA YAMANAKA[4]

[1]Department of Neurosurgery, Brain Research Institute, Niigata University, Niigata 951-8585; [2]Department of Neurosurgery, Niigata Neurosurgical Hospital, Yamada, Niigata 950-1101; [3]Department of Pathology, Brain Research Institute, Niigata University, Niigata 951-8585; [4]Laboratory of Molecular Target Therapy for Cancer, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

Abstract. The predominant characteristic of malignant glioma is the presence of invading tumor cells in the peritumoral zone. Distinguishing between tumor cells and normal cells in a peritumoral lesion is challenging. Therefore, the aim of the present study was to investigate the cell-cycle phase measurements of fixed paraffin-embedded specimens from the peritumoral invading zone of high-grade gliomas using laser scanning cytometry. A total of 12 high-grade gliomas (2 anaplastic astrocytomas and 10 glioblastomas) were studied. The tumor core and peritumoral invading zone of each tumor specimen were investigated. Tissue sections (50 μm) from the paraffin blocks were deparaffinized, rehydrated and enzymatically disintegrated, and the cells in suspension were stained with propidium iodide and placed on microscope slides. A slight trend for an increased S-phase fraction in the peritumoral invading zone compared with the tumor core was observed (P=0.24). Additionally, there was a trend for a decrease in the overall survival time of patients with increasing peritumoral invading zone S-phase fraction (P=0.12). These data suggest that laser scanning cytometry is a powerful and clinically relevant tool for the objective analysis of the cell cycle in malignant gliomas.

Introduction

High-grade gliomas are the most prevalent type of primary tumor of the central nervous system in adults (1,2). Although progress has been made in brain tumor therapies, the prognosis for patients with malignant glioma remains extremely poor (1). The standard treatment for patients with recently diagnosed glioblastoma, which comprises temozolomide and radiotherapy, has increased the median overall survival (OS) time by 15-20 months (1); however, tumor recurrence remains inevitable. Salvage treatments for tumor recurrence are palliative at best, and rarely provide the patient with any notable survival benefit (1). The poor prognosis of the disease may be attributed to the difficulty of early detection and to the high rate of recurrence following initial treatment. Thus, it is essential to further elucidate the biological features of malignant gliomas in order to improve diagnosis and treatment of the disease.

There are a number of histological grading schemes for glioblastoma, of which the World Health Organization (WHO) system (2) is the most commonly used at present. A high WHO grade is associated with clinical progression and a reduced survival rate (2); however, outcomes may vary between individuals within diagnostic categories, even those with grade IV glioma (1-3). This indicates the requirement for additional markers of prognosis. The inadequacy of histopathological grading is partially demonstrated by its inability to prospectively recognize patients (3).

The S-phase index has been used as a prognostic indicator for several types of tumor, and the S-phase fraction estimated from tumor tissues has been previously demonstrated to be the most important prognostic indicator (4-9). Numerous studies that have utilized flow cytometric analysis of brain tumors have also indicated that the DNA ploidy index or S-phase fraction is associated with the grade of malignancy (10-15). These previous studies focused on investigating the core of the tumor; however, the predominant characteristic of malignant glioma is the presence of invading tumor cells in the peritumoral zone. In addition, distinguishing tumor cells from normal cells in the peritumoral lesion is challenging. Therefore, the aim of the present study was to investigate the cell-cycle phase measurements of fixed paraffin-embedded specimens from the peritumoral invading zone of high-grade gliomas using laser scanning cytometry (LSC).

Correspondence to: Dr Ryuya Yamanaka, Laboratory of Molecular Target Therapy for Cancer, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kyoto 602-8566, Japan
E-mail: ryaman@koto.kpu-m.ac.jp

Table I. Patient characteristics.

Patient	Age, years	Gender	Histology	Initial/Recurring	Overall survival, days
1	75	Female	Glioblastoma	Initial	193
2	51	Male	Glioblastoma	Initial	530
3	64	Male	Glioblastoma	Initial	85
4	74	Male	Glioblastoma	Initial	509
5	48	Male	Glioblastoma	Recurring	441
6	60	Male	Glioblastoma	Initial	372
7	32	Male	Glioblastoma	Recurring	300
8	50	Female	Anaplastic astrocytoma	Recurring	527
9	56	Male	Anaplastic astrocytoma	Initial	762
10	69	Female	Glioblastoma	Initial	568
11	70	Male	Glioblastoma	Initial	467
12	61	Female	Glioblastoma	Initial	815

Materials and methods

Tumor specimens. Tumor specimens were obtained from patients who underwent neurosurgery for primary brain tumors at the Department of Neurosurgery, Brain Research Institute, Niigata University (Niigata, Japan) between 1997 and 2000. Samples were specifically re-reviewed according to the WHO grading system for tumors of the central nervous system (2). The research protocol was approved by the local ethics committee (Kyoto Prefectural University of Medicine, Kyoto, Japan; approval no. RBMR-G-123-1). Overall survival was measured between the date of diagnosis at surgery, and the date of mortality.

Specimen analysis. A total of 4 specimens from 2 anaplastic astrocytomas and 24 specimens from 10 glioblastomas were analyzed at the tumor core and peritumoral invading zone, respectively. Paraffin-embedded sections (50-μm thick) were deparaffinized in xylene, rehydrated in decreasing ethanol concentrations (99.5, 90, 70 and 50%), and enzymatically disintegrated with trypsin and protease (Sigma-Aldrich Co., LLC, Tokyo, Japan). The single cells were subsequently stained with propidium iodide (Sigma-Aldrich Co., LLC) and placed on microscope slides.

LSC. The slides were scanned using an iCys LSC instrument (CompuCyte, Cambridge, MA, USA) equipped with an Argon (Ar; 488 nm) and a Helium-Neon (HeNe; 633 nm) laser and WinCyte® software (CompuCyte). DNA staining based on propidium iodide served as the trigger/contouring parameter. The following channels and settings were used for data collection: Argon Green [photomultiplier tubes (PMT), 22-32%; offset, 2,030; gain, 255], HeNe LongRed (LR) (PMT, 19-22; offset, 2,000-2,010; gain, 255), threshold on HeNe LR, 2,000-2,700 units; integration contour, +4 pixels; dynamic background with inner/outer contour, 4/6 pixels; and minimum event area, 25 μm^2. Equal areas of 3,500x5,000 μm were scanned in the middle of each well using a x40 objective lens. DNA histograms were exported and analyzed using ModFit LT™ (Verity, Topsham, ME, USA). The histograms are presented with a smoothing standard deviation of 2. The cell-cycle phase fraction was calculated using a using the MultiCycle program (Phoenix Flow Systems Inc, San Diego, CA, USA).

Statistical analysis. Analysis of the differences in cell-cycle phase between the tumor core and the peritumoral invading zone were performed based on three independent experiments, using the paired two-tailed Student's *t*-test (two-tailed distribution or as indicated). All analyses were completed using the JMP statistical software package, version 10 (SAS Institute Japan Ltd. Tokyo, Japan). Statistical tests were two-tailed with type one error of 0.05. Linear correlations between the results were estimated using the product moment correlation coefficient.

Results

Patients. A total of 2 anaplastic astrocytoma (WHO grade III) and 10 glioblastoma (WHO grade IV) specimens were obtained from patients who underwent surgical resections (Table I). The median age of the patients was 61.5 years (range, 32-75 years), and the group comprised 8 male and 4 female patients. Following maximal surgical tumor resections, patients received courses of external beam radiation therapy (standard total dose, 60 Gy; applied to the tumor with a 2 cm margin) and, for patients aged <65 years, first-line chemotherapy (methyl chloroethyl nitroso urea, MCNU, 100 mg/m^2, Vincristine 1.0 mg, intravenously, 3 cycles). The patients were monitored for tumor recurrences during the initial and maintenance therapy using magnetic resonance imaging (MRI) or computed tomography scans. All treatments were performed at the Department of Neurosurgery, Niigata University Hospital (Niigata, Japan).

Measurement of cell cycle and survival. The tumor core is defined as a densely cellular lesion of neoplastic cells without normal brain tissue. By contrast, the peritumoral invading zone is defined as a low-cellular density lesion of neoplastic cells combined with normal brain tissue close to the tumor core (Fig. 1). The S-phase fraction of the peritumoral invading lesion was increased compared with the tumor core in 9 of the patients. There was a slight trend for an increased S-phase fraction of the peritumoral invading lesion compared with the

Table II. Cell-cycle phase fraction of the tumor core and peritumoral invading zone.

Tumor region	G0/G1	S	G2	M
Tumor core	0.73±0.11	0.12±0.07	0.08±0.04	0.02±0.01
Peritumoral invading zone	0.70±0.10	0.16±0.07	0.08±0.05	0.02±0.01

Data are expressed as the mean (ratio) ± standard deviation.
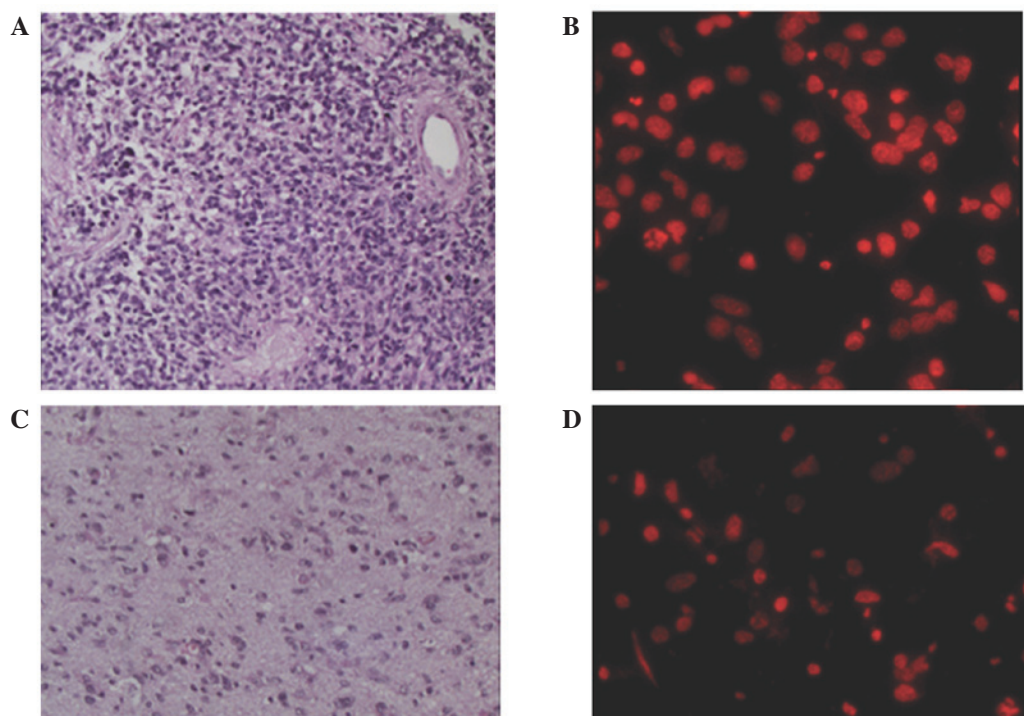

Figure 1. Staining of tumor core and peritumoral invading zone samples. The tumor core is defined as a highly cellular lesion of neoplastic cells without normal brain tissue: (A) H&E staining; (B) PI staining. The peritumoral invading zone is defined as a less densely cellular lesion of neoplastic cells admixed with normal brain tissue close to the tumor core: (C) H&E staining; (D) PI staining. (A-D) Original magnification, x400. H&E, hematoxylin and eosin; PI, propidium iodide.
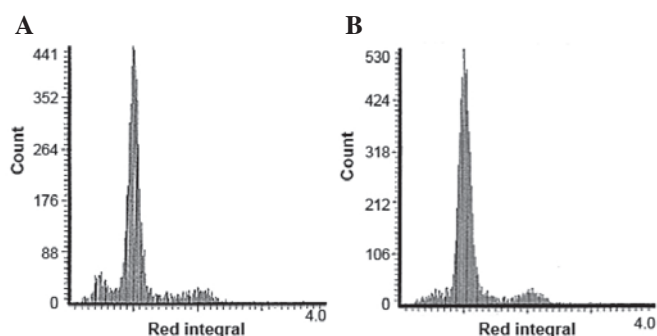

Figure 2. Cell-cycle analysis by laser scanning cytometry. (A) Cell-cycle analysis of the tumor core. A ploidy histogram shows G0/G1 phase = 78.6%, S phase = 11.9%, G2 phase = 6.7%, and M phase = 2.1%. (B) Cell-cycle analysis from the peritumoral invading zone. Ploidy histogram from the patient shows G0/G1 phase = 73.6%, S phase = 11.7%, G2 phase = 9.3%, and M phase = 3.3%.

tumor core (P=0.24) (Table II). The regression coefficient of the peritumoral invading zone S-phase fraction and the OS time was -1,158.2 (P=0.12), indicating a correlation between the peritumoral invading zone S-phase fraction and the OS time.

Illustrative case. A 60-year-old man presented with dizziness and right hemicrania. An intra-axial contrast-enhanced mass was identified in the right temporal lobe on MRI. A subtotal resection of the mass was performed, and a diagnosis of glioblastoma was determined. A total of 60-Gy of local radiotherapy and chemotherapy were administered postoperatively. A cell-cycle analysis based on LSC is shown in Fig. 2.

Discussion

LSC was developed to utilize the sensitivity of fluorescence-based assays and the specificity of on-slide measurements (16-18). LSC involves a microscope-based cytofluorometer that is able to assess flow and image cytometry. The laser-excited fluorescence emitted from individual fluorochromed cells on a microscope slide is rapidly measured at multiple wavelengths with high sensitivity and accuracy. LSC has a number of applications, including the measurement of circulating tumor cells, DNA damage, DNA ploidy and cell-cycle position (19-27). The cells for the study are selected by the operator in a manner that is similar to that for an image analysis system. Alternatively, the entire cell

population on the slide may be measured automatically using small samples, including biopsy specimens. Although flow cytometric analysis requires a large number of cells, LSC may be performed with a relatively small number (17-28).

Retrospective studies of stored paraffin-embedded brain tumor samples may be conducted to investigate the interaction between DNA parameters and patient prognosis. Intraoperative flow cytometric analyses of glioma tissues, in which the differences between the tumor margins and normal tissues were detected using cell-cycle analysis, have been previously reported (29,30). The malignancy index, defined as the ratio of the number of cells with greater than normal DNA content to the total number of cells, was observed to differ between neoplastic and perilesional tissue. An optimal cut-off value of 6.8% was thus used to identify tumors in the specimen (29).

Discriminating between tumor cells in the peritumoral invading zone is challenging; therefore, the quantitative assessment of tumor cells in glioma lesions is important for predicting future recurrence. In addition, the prognostic significance of changes in the malignancy index remains to be determined in follow-up studies involving a greater number of cases and more clinical data. In the present study, although the results were above the usual threshold for statistical significance, LSC was demonstrated to have potential clinical value for the objective analysis of the cell cycle in gliomas, which may provide important information for the characterization of tumor margins. The profiling results of the present study may be useful for the construction of a novel and improved classification scheme for the assessment of clinical malignancies compared with the conventional histological classification system. However, the present study had several limitations. LSC analysis cannot discriminate between glioma cells and normal cells. Therefore, future studies which combine alternative modalities to complement the investigation of the peritumoral invading zone are required. Furthermore, although the assay used was simple, it did not provide sufficient information regarding the molecular characterization of gliomas. In future studies the assay may be expanded to provide more information regarding glioma biology via the addition of immunocytochemical markers, such as cyclins, inhibitors of cyclin-dependent kinases, the tumor suppressor p53 and the Bcl-2 and Bax family of proteins, and by utilizing the full capabilities of fluorescence excitation and emission measurements of LSC. In addition, the results of the present study are based on a cohort of 12 patients and should therefore be considered as preliminary. Thus, additional studies are required that assess a larger cohort of patients in order to further evaluate the role of peritumoral invading zone cell-cycle analysis for gliomas.

References

1. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, et al; European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group: Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10: 459-466, 2009.

2. Kleihues P, Louis DN, Wiestler OD, Burger PC and Scheithauer BW: WHO grading of tumours of the central nervous system. In: World Health Organization Classification of Tumours of the Nervous System. Louis DN, Ohgaki H, Wiestler OD and Cavenee WK (eds). Vol 1. 4th edition. IARC Press, Lyon, France, pp10-11, 2007.

3. Kawaguchi A, Yajima N, Tsuchiya N, Homma J, Sano M, Natsumeda M, Takahashi H, Fujii Y, Kakuma T and Yamanaka R: Gene expression signature-based prognostic risk score in patients with glioblastoma. Cancer Sci 104: 1205-1210, 2013.

4. Sun XF, Rütten S, Zhang H and Nordenskjöld B: Expression of the deleted in colorectal cancer gene is related to prognosis in DNA diploid and low proliferative colorectal adenocarcinoma. J Clin Oncol 17: 1745-1750, 1999.

5. Kolfschoten GM, Hulscher TM, Pinedo HM and Boven E: Drug resistance features and S-phase fraction as possible determinants for drug response in a panel of human ovarian cancer xenografts. Br J Cancer 83: 921-927, 2000.

6. Russo A, Bazan V, Migliavacca M, Zanna I, Tubiolo C, Tumminello FM, Dardanoni G, Cajozzo M, Bazan P, Modica G, et al: Prognostic significance of DNA ploidy, S-phase fraction, and tissue levels of aspartic, cysteine, and serine proteases in operable gastric carcinoma. Clin Cancer Res 6: 178-184, 2000.

7. Lin JK, Chang SC, Yang SH, Jiang JK, Chen WC and Lin TC: Prognostic value of DNA ploidy patterns of colorectal adenocarcinoma. Hepatogastroenterology 50: 1927-1932, 2003.

8. Ross JS, Linette GP, Stec J, Ross MS, Anwar S and Boguniewicz A: DNA ploidy and cell cycle analysis in breast cancer. Am J Clin Pathol 120 (Suppl): S72-S84, 2003.

9. Kim YT, Zhao M, Kim SH, Lee CS, Kim JH and Kim JW: Prognostic significance of DNA quantification by flow cytometry in ovarian tumors. Int J Gynaecol Obstet 88: 286-291, 2005.

10. Hoshino T, Prados M, Wilson CB, Cho KG, Lee KS and Davis RL: Prognostic implications of the bromodeoxyuridine labeling index of human gliomas. J Neurosurg 71: 335-341, 1989.

11. Vavruch L, Eneström S, Carstensen J, Nordenskjöld B and Wingren S: DNA index and S-phase in primary brain tumors. A comparison between fresh and deparaffinized specimens studied by flow cytometry. J Neurosurg 80: 85-89, 1994.

12. Gilbertson RJ, Jaros E, Perry RH, Kelly PJ, Lunec J and Pearson AD: Mitotic percentage index: A new prognostic factor for childhood medulloblastoma. Eur J Cancer 33: 609-615, 1997.

13. Struikmans H, Rutgers DH, Jansen GH, Tulleken CA, van der Tweel I and Battermann JJ: Prognostic relevance of cell proliferation markers and DNA-ploidy in gliomas. Acta Neurochir (Wien) 140: 140-147, 1998.

14. Weil RJ, Toms SA, Johnson MD and Mealer A: Detection of proliferating S-phase brain tumor cells by in situ DNA replication. J Neurosurg 95: 833-838, 2001.

15. Zamecnik J, Snuderl M, Eckschlager T, Chanova M, Hladikova M, Tichy M and Kodet R: Pediatric intracranial ependymomas: Prognostic relevance of histological, immunohistochemical, and flow cytometric factors. Mod Pathol 16: 980-991, 2003.

16. Darzynkiewicz Z, Halicka HD and Zhao H: Analysis of cellular DNA content by flow and laser scanning cytometry. Adv Exp Med Biol 676: 137-147, 2010.

17. Mach WJ, Thimmesch AR, Orr JA, Slusser JG and Pierce JD: Flow cytometry and laser scanning cytometry, a comparison of techniques. J Clin Monit Comput 24: 251-259, 2010.

18. Pozarowski P, Holden E and Darzynkiewicz Z: Laser scanning cytometry: Principles and applications - an update. Methods Mol Biol 931: 187-212, 2013.

19. Tsukazaki Y, Numa Y, Zhao S and Kawamoto K: Analysis of DNA-ploidy using laser scanning cytometer in brain tumors and its clinical application. Hum Cell 13: 221-228, 2000.

20. Pollice AA, Smith CA, Brown K, Farkas DL, Silverman JF and Shackney SE: Multiparameter analysis of human epithelial tumor cell lines by laser scanning cytometry. Cytometry 42: 347-356, 2000.

21. Amirlak B and Couldwell WT: Apoptosis in glioma cells: Review and analysis of techniques used for study with focus on the laser scanning cytometer. J Neurooncol 63: 129-145, 2003.

22. Zabaglo L, Ormerod MG, Parton M, Ring A, Smith IE and Dowsett M: Cell filtration-laser scanning cytometry for the characterisation of circulating breast cancer cells. Cytometry A 55: 102-108, 2003.

23. Foster SS, Leiman G, Schwarz JE, St John T and Beatty BG: Laser scanning cytometry for the detection of neoplasia in urologic cytology specimens. Cancer 102: 115-123, 2004.

24. Pozarowski P, Huang X, Gong RW, Priebe W and Darzynkiewicz Z: Simple, semiautomatic assay of cytostatic and cytotoxic effects of antitumor drugs by laser scanning cytometry: Effects of the bis-intercalator WP631 on growth and cell cycle of T-24 cells. Cytometry A 57: 113-119, 2004.
25. Liu XP, Sato T, Oga A, Ikemoto K, Kawauchi S, Ikeda E and Sasaki K: Two subtypes of mucinous colorectal carcinoma characterized by laser scanning cytometry and comparative genomic hybridization. Int J Oncol 25: 615-621, 2004.
26. Schwock J, Geddie WR and Hedley DW: Analysis of hypoxia-inducible factor-1alpha accumulation and cell cycle in geldanamycin-treated human cervical carcinoma cells by laser scanning cytometry. Cytometry A 68: 59-70, 2005.
27. Fujiyoshi N, Ushijima K, Kawano K, Fujiyoshi K, Yamaguchi T, Araki Y, Kakuma T, Watanabe S, Kaku T, Nishida T and Kamura T: Radiation effects on DNA content of cervical cancer cells: A rapid evaluation of radiation sensitivity by laser scanning cytometry. Mol Clin Oncol 3: 51-54, 2015.
28. Gerstner AO, Machlitt J, Laffers W, Tárnok A and Bootz F: Analysis of minimal sample volumes from head and neck cancer by laser scanning cytometry. Onkologie 25: 40-46, 2002.
29. Shioyama T, Muragaki Y, Maruyama T, Komori T and Iseki H: Intraoperative flow cytometry analysis of glioma tissue for rapid determination of tumor presence and its histopathological grade: Clinical article. J Neurosurg 118: 1232-1238, 2013.
30. Alexiou GA, Vartholomatos G, Goussia A, Batistatou A, Tsamis K, Voulgaris S and Kyritsis AP: Fast cell cycle analysis for intraoperative characterization of brain tumor margins and malignancy. J Clin Neurosci 22: 129-132, 2015.