Prognostic implications of the subcellular localization of survivin in glioblastomas treated with radiotherapy plus concomitant and adjuvant temozolomide

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OBJECTIVE Currently, the standard treatment protocol for patients with newly diagnosed glioblastoma (GBM) includes surgery, radiotherapy, and concomitant and adjuvant temozolomide (TMZ). Various prognostic biomarkers for GBM have been described, including survivin expression. The aim of this study was to determine whether the subcellular localization of survivin correlates with GBM prognosis in patients who received the standard treatment protocol.

METHODS The authors retrospectively examined the subcellular localization of survivin (nuclear, cytoplasmic, or both) using immunohistochemistry in 50 patients with GBM who had received the standard treatment. The relationship between survivin localization and overall survival (OS) was assessed with uni- and multivariate analyses including other clinicopathological factors (age, sex, Karnofsky Performance Scale [KPS] score, extent of resection, the use of second-line bevacizumab, O6-methylguanine-DNA methyltransferase [MGMT] status, and MIB-1 labeling index).

RESULTS Log-rank tests revealed that patient age, KPS score, extent of resection, MGMT status, and survivin localization (p < 0.0001) significantly correlated with OS. Multivariate analysis indicated that patient age, MGMT status, and survivin localization significantly correlated with OS. Patients with nuclear localization of survivin had a significantly shorter OS than those in whom survivin expression was exclusively cytoplasmic (median OS 19.5 vs 31.7 months, respectively, HR 5.690, 95% CI 2.068–17.612, p = 0.0006). There was no significant difference in OS between patients whose survivin expression was exclusively nuclear or nuclear/cytoplasmic.

CONCLUSIONS Nuclear expression of survivin is a factor for a poor prognosis in GBM patients. Subcellular localization of survivin can help to predict OS in GBM patients treated with the standard protocol.

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KEY WORDS survivin; glioblastoma; subcellular localization; temozolomide; prognosis; oncology

Glioblastoma (GBM), the most common type of malignant brain tumor in adults, is highly resistant to chemo- and radiotherapy. Currently, the standard treatment for patients with newly diagnosed GBM involves resection, a 6-week cycle of external beam radiation therapy, and concomitant oral temozolomide (TMZ), followed by adjuvant TMZ.30 While TMZ is currently the most important chemotherapy agent for controlling GBM progression, the prognosis of GBM patients remains poor, with a median survival of only 12–15 months.14,30 At present, patients treated with radiation plus TMZ live 2.5 months longer than those treated with radiation alone.30 Moreover, O6-methylguanine-DNA methyltransferase (MGMT) status is considered a powerful predictor of the response to TMZ in patients with GBM.30 Various other prognostic biomarkers for GBM have been described, including survivin expression,2,27,29 which has been linked to a poor prognosis in patients with GBM who did not receive TMZ.2,27,29 However, the role of survivin expression in GBMs treated with the standard protocol of surgery and...
Survivin is a member of the inhibitor of apoptosis protein (IAP) family and is involved in the regulation of cell division and apoptosis. Immunohistochemical studies have suggested that subcellular localization of survivin correlates with GBM prognosis. While Shirai et al. reported that nuclear survivin expression predicts a poorer prognosis in GBM, we found that the simultaneous expression of survivin in the nucleus and cytoplasm correlates with a poor prognosis in high-grade astrocytoma, including GBM. In these studies, all patients underwent surgery and radiotherapy, and some patients received chemotherapy, but TMZ was not administered. Prior in vitro data suggested that nuclear accumulation of survivin enhances radiation-induced DNA double-strand break repair capability in GBM cell lines. This result indicated that nuclear survivin is a radiation resistance factor in GBM. Additionally, survivin expression has been linked to the chemoresistance of glioma cells. However, the association between the subcellular localization of survivin and TMZ chemoresistance in GBM has not been elucidated.

The prediction of sensitivity to the standard treatment of surgery, radiotherapy, and TMZ is essential for improving the clinical management of GBM. The aim of the present study was to determine whether the subcellular localization of survivin correlates with GBM prognosis in patients who received the standard treatment protocol.

Methods

Patient Selection and Treatment

We retrospectively studied the records of 50 patients with newly diagnosed supratentorial GBM who had undergone resection and the standard radiotherapy and TMZ treatment protocol at Hiroshima University Hospital in the period from October 2005 to July 2015. One of the authors (V.J.A.), a neuropathologist, had diagnosed the tumors according to the 2007 WHO criteria. The study population comprised 26 males and 24 females, who ranged in age from 31 to 79 years (mean age 60 years). Fifty-three patients had been diagnosed with supratentorial GBM and had undergone resection during this period. We excluded the 3 patients who did not receive the standard treatment protocol because of a low performance status (Karnofsky Performance Scale [KPS] score ≤ 40). This retrospective study protocol was approved by the institutional review board of Hiroshima University. Because the study was retrospective, the institutional review board waived the requirement for informed consent. To protect patient privacy, we removed all identifiers from our records upon completion of our analyses.

Immunohistochemical Staining

Histopathological assessment was performed according to the WHO criteria. All tumor specimens had been obtained at the initial surgery and fixed in 10% formalin before paraffin processing. The expression of survivin, Ki-67, mutant IDH1, and MGMT was assayed in the formalin-fixed, paraffin-embedded tumor samples. The primary antibodies used were a polyclonal survivin antibody (1:200 dilution, Santa Cruz Biotech), a monoclonal Ki-67 antibody (clone MIB-1, 1:100 dilution, DAKO), an IDH1 R132H antibody (1:100 dilution, Dianova), and a monoclonal MGMT antibody (1:100 dilution, Millipore, Thermo Fisher, UK). Bound antibodies were detected using avidin-biotin immunoperoxidase or Simple Stain MAX-peroxidase (Nichirei). In accordance with a previous report, we determined the percentage of MGMT-positive tumor cells in individual cases by continuously counting over 400 nuclei in high magnification views. When the proportion of labeled nuclei/all tumor nuclei was 30% or more, the case was regarded as “positive”; the case was considered “negative” when the proportion was less than 30%. The MIB-1 labeling index (LI) was determined by counting 1000 tumor cell nuclei. We selected the mean value of the MIB-1 LI as the cutoff point to classify the patients into two groups.

Evaluating the Localization of Survivin Expression

The specimens were independently scored for nuclear and cytoplasmic staining by two of the authors (T.S. and K.S.) who were blinded to patient outcome. At least 1000 tumor cells per sample from randomly selected fields were counted at magnification ×400. Samples in which ≥ 10% of the tumor cells stained positive for survivin (in the nucleus and/or cytoplasm) were recorded as positive, and samples in which < 10% of the tumor cells stained positive were scored as negative, as previously reported. The positive specimens were categorized into 3 groups according to the localization of survivin expression: nuclear-positive group (N group, only nucleus positive), cytoplasmic-positive group (C group, only cytoplasm positive), and nuclear/cytoplasmic-positive group (B group, both nucleus and cytoplasm positive).

Analyzing Extent of Resection

Tumor volume and extent of resection were assessed with MRI performed within 72 hours of surgery. We used 3D Slicer 4.0, which is freely downloadable from the website http://www.slicer.org, for semi-automatic volumetry to determine the extent of resection. We measured the tumor volume and extent of resection using the contrast-enhanced area on the contrast-enhanced T1-weighted images.

Statistical Analysis

Data were analyzed using the SPSS (version 16.0) software package (SPSS Inc.). Associations between the localization of survivin expression and clinicopathological characteristics (age, sex, KPS score, extent of resection, use of second-line bevacizumab, MGMT status, and MIB-1 LI) were calculated using the chi-square test. Survival was estimated using the Kaplan-Meier method. Correlations between overall survival (OS) and age (< 60 vs ≥ 60 years), KPS (< 80 vs ≥ 80), extent of resection (total vs partial and biopsy), use of second-line bevacizumab, MGMT status, MIB-1 LI (mean 41.6%; < 41.6% vs ≥ 41.6%), and survivin localization (N vs C group, B vs C group, and B vs N group) were estimated using the log-rank test (univariate analysis). Patients who were alive on July 31, 2016, were censored for OS analysis. Multivariate survival anal-
ysis was performed using the Cox proportional-hazards regression model, which included the following factors: age, sex, KPS score, extent of resection, MGMT status, and survivin localization. A p value < 0.05 indicated a statistically significant difference in all analyses.

Results

Among the 50 patients, only one showed positive for mutant IDH1 immunostaining.

Localization of Survivin Expression and Clinicopathological Characteristics of Patients

All 50 GBM specimens were positive for survivin (N group 20 cases, C group 19 cases, B group 11 cases). A representative specimen for each group is shown in Fig. 1. Table 1 shows the distribution of survivin localization and clinicopathological characteristics for the 50 patients. The localization of survivin significantly correlated with patient sex and KPS score, but not with age, extent of resection, use of second-line bevacizumab, MGMT status, and MIB-1 LI.

Survivin Localization and OS of GBM Patients Who Received the Standard Treatment

Of the 50 GBM patients who received the standard treatment protocol, 20 underwent bevacizumab treatment for lesion recurrence or progression. Log-rank tests revealed that patient age, KPS score, extent of resection, MGMT status, and survivin localization significantly correlated with OS (p < 0.0001), whereas sex, use of second-line bevacizumab, and MIB-1 LI did not (Table 2). Median OS in the C group was 31.7 months, significantly longer than survival in the N (p = 0.0011) and B (p < 0.0001) groups (Fig. 2 left). Additionally, the B group had significantly shorter OS than the N group (p = 0.0157; Fig. 2 right).

Multivariate analysis indicated that patient age, MGMT status, and survivin localization significantly correlated with OS (Table 3). The N group had significantly shorter OS than the C group (HR 5.690, 95% CI 2.068–17.612, p = 0.0006). Similarly, the B group had significantly shorter OS than the C group (HR 11.368, 95% CI 3.624–39.587, p < 0.0001). There was no significant difference in OS between the B and N groups (HR 1.998, 95% CI 0.782–5.030, p = 0.1454).

Discussion

In this study, we first investigated the association between survivin localization and sensitivity to the standard treatment in GBM patients. Prior studies have indicated that functions of survivin and its effects on oncogenesis vary depending on its subcellular localization. For example, survivin’s roles in mitosis regulation and apoptosis inhibition are linked to its nuclear and cytoplasmic localization, respectively. Survivin has 3 splice variants, including survivin-ΔEx3, expressed in the nucleus, and wild-type survivin and survivin-2B, which occur predominantly in the cytoplasm. Prior reports have indicated that nuclear or simultaneous nuclear and cytoplasmic survivin expression predicts a poorer prognosis in GBM. However, in these earlier studies, the chemotherapy protocols were not standardized and TMZ was not adminis-

![FIG. 1. Immunohistochemical staining for survivin expression in GBM. A: Expression only in the cytoplasm (cytoplasmic-positive group). B: Expression only in the nucleus (nuclear-positive group). C: Expression in both nucleus and cytoplasm (nuclear/cytoplasmic-positive group). Original magnification ×400. Figure is available in color online only.](image-url)
tered. Therefore, the correlation between survivin localization and sensitivity to the standard treatment (radiotherapy and TMZ after surgery) could not be evaluated. In the current study, we assessed only GBM patients who had been treated with the standard protocol of surgery and radiotherapy plus concomitant and adjuvant TMZ and found that survivin localization significantly correlated with OS in these patients. Our findings suggest that the nuclear localization of survivin is an important marker of a poor prognosis in patients with GBM who receive the standard treatment protocol.

Previous studies have shown that survivin enhances radioresistance in GBM cells. Ex vivo assays indicated that radiotherapy induces the formation of double-strand DNA breaks, resulting in the activation of repair and response machinery. Chakravarti et al. first reported that survivin suppresses apoptotic cell death in a caspase-independent manner after radiation exposure in GBM cells. They suggested that survivin may enhance tumor cell survival after radiation exposure by the activation of double-strand DNA break repair and the upregulation of tumor cell metabolism, which were most evident in the radiation-resistant GBM cell lines. Additionally, they demonstrated that radiation appears to cause a shift in survivin cellular localization from the cytoplasm to the nucleus, particularly in the radioresistant GBM cells. This appeared to be correlated with enhanced double-strand DNA repair abilities compared with more radiosensitive cells. Consistent with these data, Reichert et al. reported that nuclear accumulation of survivin and its interaction with components of the DNA double-strand break repair machinery indicate that survivin is involved in the upregulation of DNA double-strand break damage repair that leads to a significant improvement in GBM cell survival. These results suggest that nuclear expression of survivin correlates with radioresistance in patients with GBM who received the standard treatment protocol.

It has also been reported that survivin expression confers TMZ chemoresistance to GBM cells. Temozolomide is an alkylating agent that inhibits cell proliferation and induces G2/M arrest and apoptosis in GBM cell lines. It produces cell cycle arrest at G2/M through the activation of ATM/ATR-Chk1/2, which are G2/M checkpoint kinases. Chk1/2 can activate Wee1, the kinase that phosphorylates Cdk1, and can inhibit Cdc25, thus leading to cell cycle arrest before mitosis. Xie et al. found that the majority of GBM cells were aneuploid and noted a close association

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* Statistically significant difference.
between nuclear survivin positivity and tumor aneuploidy.\textsuperscript{22} These findings suggest that nuclear-localized survivin in GBM influences mitotic events and subsequently promotes chromosomal instability by abrogating G2/M checkpoint function. Conversely, Li et al. demonstrated that the silencing of survivin using short interfering RNA (siRNA) can arrest the cell cycle at the G2/M checkpoint and induce cellular apoptosis.\textsuperscript{16} Overall, these data suggest that nuclear survivin opposes the action of G2/M arrest induced by TMZ, thus leading to chemoresistance to TMZ. However, Guzman et al. found that wild-type survivin that is preferentially localized in the cytoplasm protects Wee1 from degradation by blocking caspase-3 activation.\textsuperscript{8} This finding suggests that the cytoplasmic expression of survivin supports G2/M arrest induced by TMZ by promoting Wee1 activation and improves the chemosensitivity to TMZ in patients with GBM. Indeed, in the present study, the median OS of the C group was 31.7 months, significantly longer than that in the other two groups (Fig. 2 and Table 2).

Our results suggest that nuclear survivin expression is associated with chemo- and radioresistance to the standard treatment in GBM. Survivin is highly expressed in most tumor types, including GBM, but is generally absent in terminally differentiated cells.\textsuperscript{26} Therefore, survivin is an attractive target for GBM treatment. Indeed, Kim et al. reported that combination treatment of murine gliomas with low-dose TMZ followed by vaccination with TAT-survivin-pulsed dendritic cells (DCs) enhanced T-cell responses specific for survivin and improved the survival rate, as compared with DC alone or TMZ alone.\textsuperscript{11} Most recently, Cruz et al. demonstrated that survivin downregulation by siRNA delivered using cationic Gemini surfactants, combined with TMZ or etoposide, resulted in a synergistic cytotoxic effect in the U87 human GBM cell line.\textsuperscript{3} The small-molecule 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1H-naphtho(2,3-d)imidazolium bromide (YM155 monobromide) is an inhibitor of the activity of the survivin gene promoter.\textsuperscript{20} The antitumor effects of YM155 were investigated in a wide variety of human malignant tumors, such as lung cancer, prostate cancer, breast cancer, and melanoma.\textsuperscript{4,12,10,20} Survivin inhibition by YM155 elicited cytotoxicity in human GBM cell lines via DNA-protein kinase-independent mechanisms.\textsuperscript{13} and treating U87 GBM cells with YM155 significantly increased radiosensitivity.\textsuperscript{15} Most recently, Guo et al. reported that the inhibition of survivin by YM155 decreased invasion and metastasis, suppressed proliferation, and decelerated the growth rate of U87 GBM cells.\textsuperscript{7} These results suggest that a combination of YM155 and the standard protocol of radiotherapy plus TMZ may be an effective new treatment protocol for GBMs, including those that are chemo- and radioresistant.

The present study has some limitations. First, we could not identify the precise mechanism by which nuclear survivin expression mediated the sensitivity to the standard treatment protocol. Secondly, the study was retrospective with a relatively small sample size; thus, additional larger prospective studies are needed. Thirdly, multivariate survival analysis using the Cox proportional-hazards regression model can reduce bias in causal estimates due to observed differences among 3 groups categorized according to the subcellular localization of survivin but are still subject to biases from other unobserved biomarkers. Fourthly, the study includes only one patient positive for mutant IDH1 immunostaining. Thus, in the future, we should adopt other methods of detecting IDH1 mutation, such as direct DNA sequencing.

### Conclusions

We demonstrated that the subcellular localization of survivin significantly correlates with OS in patients with GBM treated with the standard protocol of surgery, radiotherapy, and concomitant and adjuvant TMZ. Our results suggest that nuclear survivin localization is a factor for a poor prognosis, whereas cytoplasmic localization is associated with a more favorable prognosis. Our data provide added impetus for detailed studies addressing the molecular mechanisms underlying survivin’s prosurvival function in GBM pathogenesis and highlight the potential of targeted survivin therapies for GBM.

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**Disclosures**

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

**Author Contributions**

Conception and design: Saito, Sugiyama, Kawamata. Acquisition of data: Saito, Takeshima, Amatay, Yamasaki, Takayasu, Nosaka. Analysis and interpretation of data: Saito, Sugiyama, Takeshima, Amatay, Yamasaki, Takayasu, Nosaka, Muragaki. Drafting the article: Saito. Critically revising the article: Sugiyama, Muragaki, Kawamata, Kurisu. Reviewed submitted version of manuscript: Takeshima, Amatay, Yamasaki. Approved the final version of the manuscript on behalf of all authors: Saito. Statistical analysis: Muragaki. Administrative/technical/material support: Takayasu, Nosaka. Study supervision: Kawamata, Kurisu.

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