Signaling of ghrelin and its functional receptor, the growth hormone secretagogue receptor, promote tumor growth in glioblastomas

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Running title: Ghrelin/GHS-R signaling in glioblastomas

ABSTRACT

Ghrelin is a 28-amino-acid peptide that is the endogenous ligand for the pituitary growth hormone secretagogue receptor (GHS-R). Ghrelin is mainly produced from the stomach, but it is also expressed by various other tissues including the central nervous system under normal conditions. Physiologically, ghrelin regulates appetite, gut motility, and GH release from the anterior pituitary, as well as cardiovascular and immune systems. Recent studies also indicate that ghrelin and the GHS-R may play an important autocrine/paracrine role in neoplastic conditions. In order to clarify the role of ghrelin/GHS-R in gliomas, the present study assessed the expression of ghrelin and its functional receptor, GHS-R1a, in 39 glioblastomas (GBs), 13 anaplastic astrocytomas (AAs), and 11 diffuse astrocytomas (DAs) using immunohistochemical analyses. Immunohistochemical staining was evaluated as follows: no staining; 1+, 0-10% positive cells; 2+, 10-50% positive cells; 3+, >50% positive cells. Ghrelin expression was detected in 52 of 63 cases of which 38, 13, and 1 were scored as 3+, 2+, and 1+, respectively. GHS-R1a expression was detected in 45 of 63 cases of which 29, 15, and 1 were scored as 3+, 2+, and 1+, respectively. Ghrelin immunoreactivity was observed in 38 of 39 GBs, 12 of 13 AAs, and 2 of 11 DAs. GHS-R1a immunoreactivity was observed in 39 of 39 GBs, 5 of 13 AAs, and 1 of 11 DAs. AAs and GBs showed moderate or strong immunostaining of ghrelin/GHS-R1a in the tumor cells and in proliferating microvessels.

Patients were classified into lower to moderate-score, and high-score ghrelin/GHS-R categories according to the principal component and cluster analyses. Multivariate analysis of overall survival indicated that there was a significant difference (p=0.0001) in the survival rate between these two groups. The combined results indicated that expression of the ghrelin/GHS-R1a axis increases the growth of AAs and GBs through an autocrine/paracrine mechanism.

Key words: autocrine/paracrine, ghrelin, glioblastoma, growth hormone secretagogue receptor, prognosis

INTRODUCTION

Kojima et al. identified ghrelin, a 28-amino acid peptide secreted by the stomach, as the endogenous ligand for the pituitary growth hormone secretagogue receptor (GHS-R).[1,2] The organ expressing the highest amount of ghrelin is the stomach, where it is synthesized by and secreted from an endocrine cell in the submucosal layer of the stomach.[1-3] However, ghrelin expression is not limited to the gastrointestinal tract, and ghrelin is distributed throughout the entire body, including the central nervous system. [1-4] GHS-R is a transmembrane G protein-coupled receptor for ghrelin and is also expressed in various organs, including in brain regions. [1,2] In addition to the regulation of GH secretion, the ghrelin/ GHS-R axis has many other functions, including the regulation of appetite and gut motility and of growth hormone release from the anterior pituitary and also plays roles in the cardiovascular and immune systems. [1-3] The discovery of ghrelin is a typical instance of reverse pharmacology because GHS-R was identified before its ligand, ghrelin. [5,6] Different isoforms of GHS-Rs have been cloned that are named GHS-R1a and GHS-R1b. GHS-R 1a encodes a full-length biologically active receptor; however, the GHS-R1b encodes a truncated receptor that is widely expressed throughout the entire body. [5-7]

Recent studies have indicated that ghrelin is highly expressed in various tumor tissues in which GHS-R is expressed along with ghrelin. [5-14] Accordingly, the possibility of an autocrine/paracrine role of ghrelin/GHS-R signaling in the regulation of cancer cell proliferation has aroused much interest. Regarding astrocytic tumors, Dixit et al. reported that ghrelin, acting via the functional

GHS-R 1a, increased intracellular calcium mobilization and led to membrane ruffling, which resulted in high motility and invasion of astrocytoma cells. [13] In addition, in an in vitro study, Chen et al. reported that ghrelin induced cell migration through a GHS-R, CaMKII, AMPK, and NF-κB signaling pathway in glioma cells. [14] To the best of our knowledge, however, immunohistochemical analysis of the specific localization of ghrelin/GHS-R1a expression in astrocytic tumors, particularly in glioblastomas (GBs), has not yet been studied. In the present study, therefore, we addressed the expression pattern of ghrelin/GHS-R1a in specimens of GBs and other astrocytic tumors and the correlation between ghrelin/GHS-R axis expression and patient prognosis, in order to clarify the role of ghrelin/GHS-R in the tumorigenesis of gliomas, particularly of GBs.

MATERIALS and METHODS

Cases

Surgical tissue samples (n=63) from 63 patients with astrocytic tumors were analyzed in the present study. These astrocytic tumors consisted of 39 GBs, 13 anaplastic astrocytomas (AAs), and 11 diffuse astrocytomas (DAs). All tumor specimens were retrieved from the archives of Kurume University and its affiliated hospitals between 2000 and 2012. Clinical information for the 63 patients was also retrieved from these archives. This study was carried out in accordance with the principles of the Helsinki Declaration and was approved by the ethics committee of our institution. Tissue samples were fixed in 10% buffered formalin, embedded in paraffin, and processed using conventional histological and immunohistochemical methods. Sections (5 μm) were stained with hematoxylin and eosin (HE) for histological evaluation. The remaining serial unstained sections were used for immunohistochemistry. All specimens were histologically diagnosed according to the World Health Organization criteria for tumours of the

central nervous system. [15] Immunohistochemical studies were performed on paraffin sections following heat-induced antigen retrieval, staining with the appropriate antibodies and signal detection with immunoperoxidase methods (ChemMate ENVISION kit/HRP(DAB), DakoCytomation) using an autostainer (Dako autostainer universal staining system). Primary antibodies were directed toward Ghrelin (dilution 1:200; AbD serotec, Oxford, UK), GHS-R1a (dilution 1:400; Santa Cruz Biotechnology Inc., Santa Cruz, USA), endoglin (CD105, dilution 1:50; Novocastra, Newcastle, UK), and Ki-67 (MIB-1, dilution 1:100;immunotech, Marseille, France). The MIB-1 labeling index was the percentage of nuclear area stained in areas of maximum labeling. The immunohistochemical studies were evaluated as follows; no staining; 1+, 0-10% positive cells ; 2+, 10-50% positive cells; 3+, >50% positive cells. The immunohistochemical evaluation was performed by two observers (Y.O and Y.S.) in independent readings. Cases that varied significantly between readers were re-evaluated in order to arrive at a consensus.

Fluorescence immunohistochemical staining

In two cases of glioblastoma, fluorescent double immunostaining of ghrelin and GHS-R1a expression, of endoglin (CD105) and GHS-R1a expression, and of Ki-67 and GHS-R1a expression in GB tissues was performed. Co-staining was performed by staining GHS-R1a with rabbit anti-GHS-R1a antibodies, and by co-staining for ghrelin, endoglin (CD105) or Ki-67 with with mouse anti-ghrelin, anti-endoglin (CD105) or anti-Ki-67 (MIB-1) antibodies, respectively, followed by their respective staining by addition of anti-rabbit IgG-Texas Red (TR) (sc-2780, Santa Cruz Biotechnology) and anti-mouse IgG-Alexa488 (Life Technologies, CA, USA) as appropriate. Fluorescence was analyzed using a

fluorescent microscope (BX51FL, Olympus, Tokyo, Japan) and a CCD camera (DP71, Olympus, Tokyo, Japan).

Statistical analysis

To gain a better understanding of the correlation between expression of ghrelin/GHS-R in GBs and patient prognosis, statistical analyses among the relevant groups were performed using SAS 9.4 (SAS Institute, Cary, NC, USA). Survival rates were computed using the Kaplan-Meier method. Patients were censored on loss-to-follow-up at the time of analysis. When multivariate analysis of overall survival was studied, principal component analysis was conducted to assess patterns in ghrelin, GHS-R1a expression, and patient age. [16] In addition, cluster analysis was done for new indices. [17] A log-rank test and a Cox proportional hazards regression model were used for univariate and multivariate analyses of overall survival, respectively. In addition, to determine the relationship between the levels of expression of Ghrelin/ GHSR-1a and the MIB-1 labelling index, we performed a Kendall tau rank correlation test between Ghrelin and the MIB-1 labelling index in gliomas, or between GHSR-1a and the MIB-1 labelling index in gliomas using SAS 9.4 (SAS Institute, Cary, NC, USA). Statistical significance was set at the level of $P<0.01$.

RESULTS

The patient data and immunohistochemical findings of the 63 cases studied are summarized in Table 1 and Table 2. All patients with GBs and AAs had received radiation therapy and chemotherapy. All patients with ASs had received radiation therapy. The staining intensity of tumor cells was correlated with the percentage of tumor cells immunostained for both ghrelin and GHS-R1a expression. Ghrelin expression was detected in 52 of 63 cases of which 38, 13, and 1 cases were scored as a staining intensity of 3+, 2+, and 1+, respectively. GHS-R1a expression was

detected in 45 of 63 cases of which 29, 15, and 1 cases were scored as a staining intensity of 3+, 2+, and 1+, respectively. Ghrelin immunoreactivity was observed in 38 of 39 GBs, 12 of 13 AAs, and 2 of 11 DAs. GHS-R1a immunoreactivity was observed in 39 of 39 GBs, 5 of 13 AAs, and 1 of 11 DAs.

In GBs, ghrelin expression was detected in 38 of 39 cases of which 29 and 9 cases were scored as a staining intensity of 3+ and 2+, respectively. Cases that were scored as 3+ and 2+ showed strong immunostaining either of the pseudopalisading cells or of proliferating multilayered microvessels (Fig. 1a, 1b). GHS-R1a expression was detected in 39 of 39 cases of which 26 and 13 cases were scored as a staining intensity of 3+ and 2+, respectively. Cases that were scored as 3+ and 2+ also showed strong immunostaining either of the pseudopalisading cells or of proliferating multilayered microvessels (Fig. 1c,1d).

In AAs, ghrelin expression was detected in 12 of 13 cases of which 9 and 3 cases were scored as a staining intensity of 3+ and 2+, respectively. Cases that were scored as 3+ and 2+ showed strong immunostaining in proliferating microvessels and their surrounding tumor cells (Fig. 2a). GHS-R1a expression in AAs was detected in 5 of 13 cases of which 3 and 2 cases were scored as a staining intensity of 3+ and 2+, respectively. Cases that were scored as 3+ and 2+ also showed strong immunostaining of proliferating microvessels and their surrounding tumor cells (Fig. 2b).

In DAs, ghrelin expression was detected in 2 of 11 cases, and these 2 cases were each scored as a staining intensity of 1+ and 2+, respectively. The cases that were scored as 1+ showed weak immunostaining in tumor cells and negative immunostaining in proliferating microvessels (Fig. 2c). GHS-R1a expression in DAs was detected in 1 of 11 cases and this case was scored as a staining intensity of 1+. Representative immunohistochemical staining of GHS-R1a in DA tissue showed no GHS-R1a immunostaining of tumor cells or of endothelial cells of

proliferating microvessels (Fig. 2d). In summary, strong expression of ghrelin/GHS-R1a was frequently detected in tumor cells and in proliferating microvessels of GBs and AAs, but little expression of ghrelin/GHS-R1a was detected in DAs. The frequency of ghrelin expression was almost the same in GBs and AAs (38/39, 97% and 12/13, 92%, respectively), but that of GHS-R1a expression was clearly different (39/39, 100% and 5/13, 38%, respectively). With regard to proliferative activity, GBs, AAs, and DAs showed MIB-1 labeling indices ranging from 4 to 56 (mean: 22.3$\pm$12.9), 5 to 40 (mean: 15.9$\pm$14.0), and 1 to 7 (mean: 2.2$\pm$1.8), respectively (Table 1). Fluorescent double immunostaining of ghrelin and the GHS-R1a in GB tissue confirmed positive staining of ghrelin and the GHS-R1a in the same tumor cells (Fig. 3a-3d). In addition, fluorescent double immunostaining of endoglin (CD105) and GHS-R1a confirmed positive staining of endoglin (CD105) and GHS-R1a in the same endothelial cells of proliferating microvessels (Fig. 3e-3f). On the other hand, fluorescent double immunostaining of GHS-R1a and Ki-67 in GB tissue did not confirm positive staining of the GHS-R1a and Ki-67 in the same tumor cells (Fig. 3i-3l).

To gain a better understanding of the relationship between the ghrelin/GHS-R1a axis and the prognosis of patients with astrocytic tumors, statistical analyses were performed. Univariate analysis of overall survival indicated that, when ghrelin was used a marker, there was no significant difference in the survival rate between any of the three ghrelin groups (A: no staining and 1+, B: 2+, C: 3+, p=0.2310 for all comparisons) (Fig. 4a). In contrast, when GHS-R1a was used a marker, there was a significant difference in the survival rate between all three ghrelin groups (A: no staining and 1+, B: 2+, C: 3+, p=0.0080 for all comparisons) (Fig. 4b).

Considering age and ghrelin/GHS-R expression, patients were classified into lower-score, moderate, and high-score ghrelin/GHS-R categories or lower-score,

and high-score ghrelin/GHS-R categories according to principal component analysis and cluster analysis. Multivariate analysis of overall survival indicated that there was a significant difference between the lower score groups (B) and the high grade score groups (A) or between the moderate score groups (C) and the high grade score groups (A), for each comparison (p=0.0005) (Fig. 4c). In addition, there was a significant difference in overall survival between the lower to moderate-score group (B) and the high-score group (A) (p=0.0001) (Fig. 4d).

Correlation between Ghrelin/ GHSR-1a expression and the MIB-1 labelling index in gliomas was investigated using the Kendall tau rank correlation test. There was no significant correlation between either Ghrelin or GHSR-1a expression and the MIB-1 labelling index in gliomas (Table 3).

DISCUSSION

Many studies of the ghrelin/GHS-R signaling system have been carried out, primarily in the field of the regulation of appetite and gut motility, growth hormone release from the anterior pituitary, and its roles in the cardiovascular and immune systems. [1-4] In the central nervous system, ghrelin has been found in the hypothalamic arcuate nucleus, an important region for controlling appetite. In addition, the presence of ghrelin has been reported in hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei, where it plays a role in controlling food intake.[1-4] Furthermore, several other functions of ghrelin such as stimulation of neurogenesis[18] and inhibition of oligodendrocytic cell death in the central nervous system have been reported. [19]

Recent investigations have shown that ghrelin and the GHS-R may also play an important autocrine/paracrine role in many of various neoplasms. [5-14] For

example, in the human gastric cell line, Tian et al. have shown that ghrelin signaling promotes expression of the CDK6 oncogene and represses expression of the tumor suppressor gene p53. In addition, they reported that ghrelin activates expression of the metastasis factor MMP2 in gastric cancer cells via a GHS-R/NF-κB signaling pathway and promotes the proliferation of tumor cells. [10] In an immunohistochemical study, Omoto et al. showed that ghrelin expression correlated with tumor depth and tumor differentiation, suggesting an important role for ghrelin in tumor growth in esophageal carcinoma. [11] Interestingly, Lin et al. reported that ghrelin could activate Snail, a transcriptional repressor of E-cadherin via the GHS-R-P13K-Akt axis, which might contribute to renal cell carcinoma metastasis. [12]

On the other hand, some reports have indicated that ghrelin inhibits the proliferation of tumor cells. [20,21] Thus, Rotondo et al. reported that ghrelin immunopositivity did not serve as a biomarker of biologic behavior, prognosis, or therapeutic responsiveness in pituitary adenoma.[20] Xu et al. reported that ghrelin inhibited ovarian epithelial carcinoma *in vitro*.[21] The structure of ghrelin is unique in that specific acyl-modification of its third serine is necessary for ghrelin to bind to GHS-R1a and exert biologic activity. The enzyme responsible for acylation of ghrelin is highly expressed in the stomach, lung, pituitary, adrenal cortex, and spleen, but is very weakly expressed in the ovary. Therefore, regarding the discrepancy in the function of ghrelin/GHS-R1a between other cancers and ovarian cancers, Xu et al. speculated that the effect of active acyl ghrelin on ovarian carcinoma might still occur through an endocrine rather than a paracrine/autocrine pathway.

The role of the ghrelin/GHS-R axis in the development of gliomas has not been investigated in detail. In the present study, we detected ghrelin/GHS-R1a expression by various astrocytic tumor cells using immunohistochemistry.

However, although strong expression of ghrelin/GHS-R1a was frequently detected in tumor cells and proliferating microvessels of GBs and AAs, little expression of ghrelin/GHS-R1a was detected in DAs. The rate of ghrelin expression was almost the same in GBs and AAs (38/39, 97% and 12/13, 92%, respectively), but the rate of GHS-R1a expression was clearly different (39/39, 100% and 5/13, 38%, respectively). These combined results indicated that the ghrelin/GHS-R1a axis plays an important role in the tumorigenesis of high grade gliomas, particularly of GBs. These data also suggest that ghrelin/GHS-R1a signaling is not as effective in AAs compared with GBs. GBs are characterized by pseudopalisade necrosis and increased levels of angiogenesis. These features are pathophysiologically linked and mechanistically instrumental to disease progression. Recent investigations have shown that the initial events of pseudopalisade formation in GBs are as follows.[22,23] First, vascular occlusion related to endothelial apoptosis and intravascular thrombosis leads to ischemia and subsequently to hypoxia in the region around the vessels. Secondly, hypoxic-induced tumor cells begin to move away from the hypoxic zone, creating a peripherally moving wave of cells and forming a pseudopalisade with central necrosis. Third, a strong angiogenic response results in microvascular formation in regions peripheral to the central necrosis. Interestingly, in the present study, the percentage of tumor cells that expressed ghrelin and the GHS-R correlated with the WHO grade of gliomas. In addition, GBs showed moderate to intense immunostaining of both ghrelin and GHS-R1a, with immunostaining being particularly observed in the pseudopalisading cells and in CD-105 labeled proliferating microvessels.

Angiogenesis is the process by which tumors induce a blood supply, and it plays a crucial role in both tumor growth and tumor progression. [24,25] Endoglin (CD105) is predominantly expressed on cellular lineages within the vascular

system. Endoglin (CD105) is overexpressed by proliferating endothelial cells that participate in tumor angiogenesis, whereas it is either weakly or not expressed in the vascular endothelium of normal tissues. Yao et al. have shown that endoglin (CD105) is a more specific and sensitive marker of microvessels in astrocytic tumors than other commonly used pan-endothelial antibodies.[25] Milewski et al. have recently shown that ghrelin/GHS-R1a are expressed both in glandular endometrioid epithelium and in some stromal cells, particularly in some fibroblasts, blood vessels, and infiltrating leukocytes in ovarian endometrioma.[26] Based on their results, they speculated that co-localization of ghrelin/GHS-R1a affects the development and growth of endometriotic lesions and influences local inflammatory and angiogenic responses. In the present study, we also detected high expression of ghrelin/GHS-R1a in tumor cells and proliferating microvessels of AAs and GBs. We also found that GHS-R1a co-localized with endoglin (CD 105) in proliferating microvessels. These data therefore suggest that ghrelin/GHS-R1a interactions play an important role in growth, invasion, and neoangiogenesis in high grade astrocytic tumors through both autocrine and paracrine mechanisms.

Hirano et al. suggested that insulin-like growth factor-1 (IGF-1) signaling occurs early in astroglial tumorigenesis in the setting of cell proliferation. They further suggested that the distinctive correlative patterns of IGF-1 and insulin-like growth factor-1 receptor (IGF-1 R) signaling have an association with the development of malignant phenotypes related to aberrant angiogenesis and invasive tumor interactions with reactive brain.[27] Many tumors are responsive to the mitogenic influence of components of the growth hormone (GH) axis. There is increasing evidence that GH contributes both directly and indirectly (via its tissue biomediator, IGF-1) to neoplastic tissue growth. Ghrelin is known to enhance the GH/IGF axis via activation of the GHS-R, resulting in increased GH and IGF-1

production.[8] These findings therefore suggested that ghrelin plays an important role in growth, invasion, and neoangiogenesis in high grade astrocytic tumors via activation of the GH/IGF axis.

Dixit et al. demonstrated that ligation of the GHS-R on cells of an astrocytoma cell line by ghrelin resulted in an increase in intracellular calcium mobilization, protein kinase C activation, actin polymerization, matrix metalloproteinase-2 activity, and astrocytoma motility. In addition, their analysis of a central nervous system tumor tissue microarray showed that strong GHS-R and ghrelin expression was significantly more common in high grade tumors compared with low grade tumors.[13] Based on these results, they considered that the ghrelin/GHS-R axis plays a role in astrocytoma cell migration and invasiveness. Chen et al. have shown that ghrelin increases GHS-R up-regulation, and that the enhancement of ghrelin-induced glioma cell motility is markedly inhibited by a GHS-R antagonist in vitro.[14] They therefore suggested that ghrelin-enhanced migration of glioma cells is mainly regulated by a GHS-R, CaMKII, AMPK, and NF-κB pathway.

In the present study, there was no significant correlation between either ghrelin or GHSR-1a expression and the MIB-1 labelling index in gliomas. In addition, fluorescent double immunostaining of GHS-R1a and Ki-67 in GB tissue did not confirm positive staining of the GHS-R1a and Ki-67 in the same tumor cells. Ki-67 is an antigen that corresponds to a nuclear nonhistone protein expressed by cells in the cell cycle proliferative phases of G1, G2, M, and S.[28] In addition, a monoclonal antibody (MIB-1) recognizes a formalin-resistant epitope of Ki-67.[28] Therefore, these results indicated two possibilities regarding the role of Ghrelin/ GHS-R in the tumorigenesis of gliomas, in particular in GBs. First, upon stimulation of Ghrelin/ GHS-R signaling from quiescent tumor cells themselves, the quiescent tumor cells (in G0) might enter G1, after they progress to cell

division. Second, Ghrelin/ GHS-R might play an important role in the migration and invasiveness of tumor cells rather than in the proliferation of tumor cells.

We also showed that the survival rate of patients with tumors that showed lower-expression of ghrelin/GHS-R was significantly higher than that for patients with tumors that showed higher-expression of ghrelin/GHS-R. In addition, in multivariate analysis, the ghrelin/GHS-R1a axis emerged as an independent prognostic factor in glioma patients. We thus considered that interactions of ghrelin/GHS-R are necessary for the growth of high-grade astrocytic tumors, particularly of GBs.

In conclusion, the high expression of the ghrelin/GHS-R axis by GB cells and endothelial cells of proliferating microvessels that was determined in the present study may indicate an interaction between tumor cells and the tumor microenvironment, which could explain the pathological processes of GBs. In addition, the ghrelin/GHS-R pathway may be regarded as a potential therapeutic target for various cancers. The present study could thus eventually lead to therapeutic trials of treatments for patients with GBs in the future.

Disclosure and acknowledgment

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Figure Legends

Figure 1

Representative immunohistochemical staining of ghrelin and GHS-R1a in glioblastoma.

a. Strong immunostaining of ghrelin in the pseudopalisading cells is shown (asterisk, necrosis; bar =20 μm).
b. Strong immunostaining of ghrelin in glioblastoma cells and in endothelial cells of proliferating microvessels (arrows) is shown. (bar =20 μm).
c. Strong immunostaining of GHS-R1a in the pseudopalisading cells is shown (asterisk, necrosis; bar =20 μm).
d. Strong immunostaining of GHS-R1a in glioblastoma cells and in endothelial cells of proliferating microvessels (arrows) is shown. bar =20 μm).

Figure 2

Representative immunohistochemical staining of ghrelin and GHS-R1a in anaplastic astrocytomas and in diffuse astrocytoma tissues.

a. Strong immunostaining of ghrelin in anaplastic astrocytoma cells and endothelial cells of proliferating microvessels (arrow) is shown. (bar =20 μm).
b. Strong immunostaining of GHS-R1a in anaplastic astrocytoma cells and in endothelial cells of proliferating microvessels (arrow) is shown. (bar =20 μm).
c. Weak immunostaining of ghrelin in diffuse astrocytoma cells is shown. No staining is seen in endothelial cells of proliferating microvessels (arrow;_bar =20 μm).
d. No immunostaining of GHS-R1a of diffuse astrocytoma cells and endothelial cells of proliferating microvessels (arrow) was observed (bar =20 μm).

Figure 3

Representative double immunofluorescent staining of GHS-R1a and ghrelin or endoglin (CD105) in glioblastoma (a-d). Ghrelin (green, Alexa488) and GHS-R1a (red, Texas Red) were co-stained in glioblastoma tissue and detected by immunofluorescence. Tumor cells showed co-staining of GHS-R1a and ghrelin. a. ghrelin, b. GHS-R1a, c. DAPI, d. Merged (bar=10 μm).

e-h. Representative double immunofluorescent staining of endoglin (CD105; green, Alexa488) and GHS-R1a (red, Texas Red) in glioblastoma tissue. Vascular endothelial cells were co-stained for CD105 and GHS-R1a. e. endoglin (CD105), f. GHS-R1a, g. DAPI, h. Merged (bar=10 μm).

i-l. Representative double immunofluorescent staining of Ki-67 (MIB-1; green, Alexa488) and GHS-R1a (red, Texas Red) in glioblastoma tissue did not confirm positive staining of Ki-67 and the GHS-R1a in the same tumor cells. i. Ki-67, j. GHS-R1a, k. DAPI, l. Merged (bar=10 μm).

Figure 4

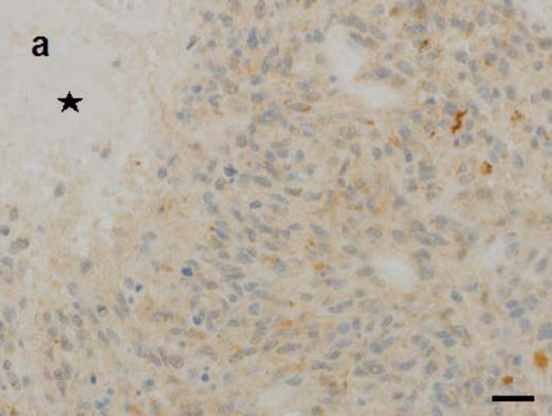
Univariate and multivariate analysis of overall survival according to ghrelin and GHS-R1a expression.

(a, b) Univariate analysis of overall survival curves (Kaplan-Meier).

a. When ghrelin was used a marker, there was no significant difference in the survival rate between any of the three ghrelin groups (A: no staining and 1+, B: 2+, C: 3+, p=0.2310 for all comparisons)
b. When GHS-R1a was used a marker, there was significant difference in the survival rate between any of the three ghrelin groups (A: no staining and 1+, B: 2+, C: 3+, p=0.0080 for all comparisons)

(c, d) Multivariate analysis of overall survival of overall survival curves (Kaplan-Meier).

c. There was a significant difference between the low score groups (B) and high grade score groups (A) and between the moderate score groups (C) and high grade score groups (A) (p=0.0005 for each comparison).
d. There was a significant difference in overall survival between the lower to moderate -score group (B) and the high-score group (A) (p=0.0001)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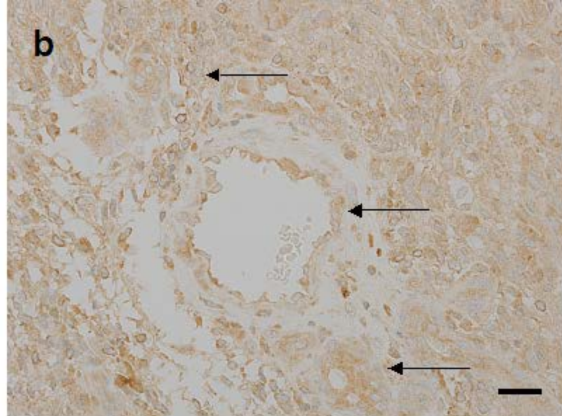
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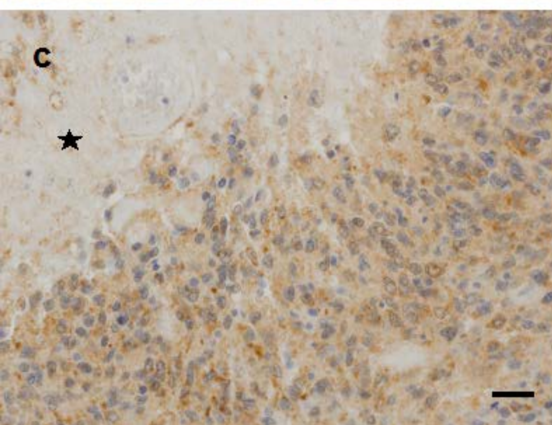
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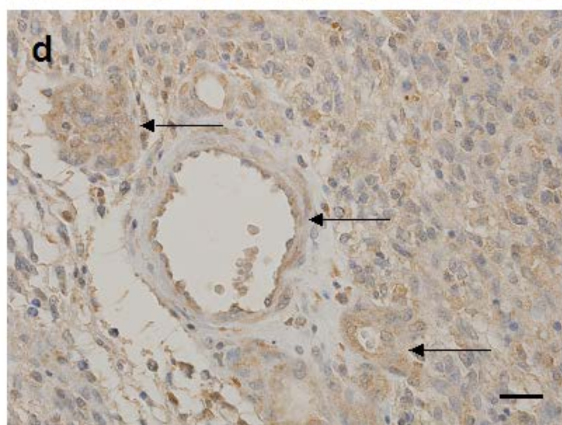
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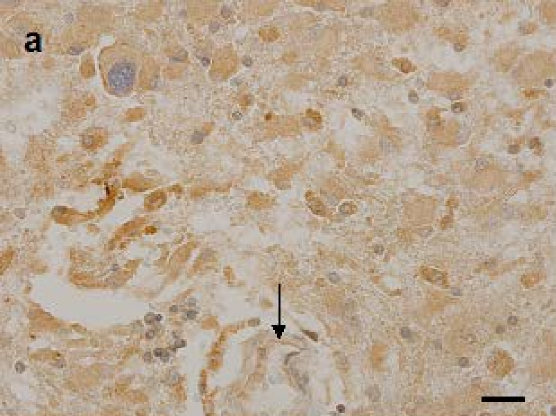
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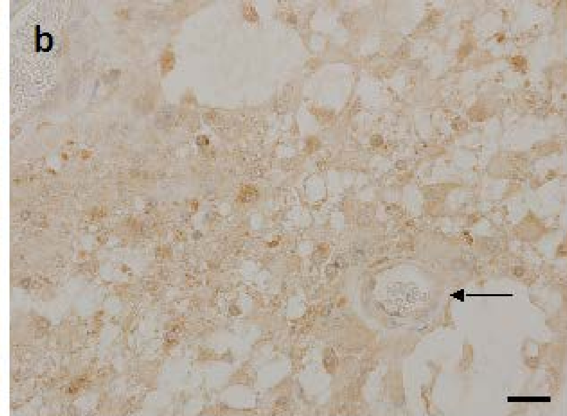
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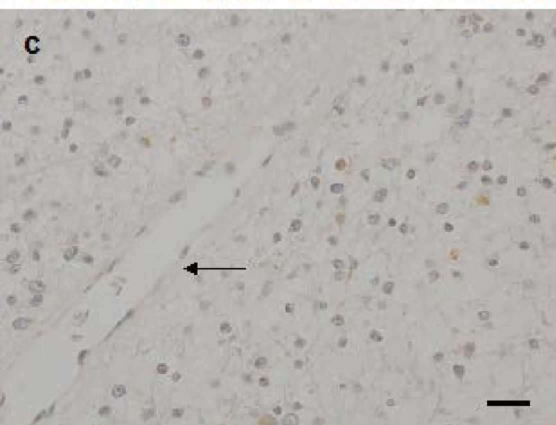
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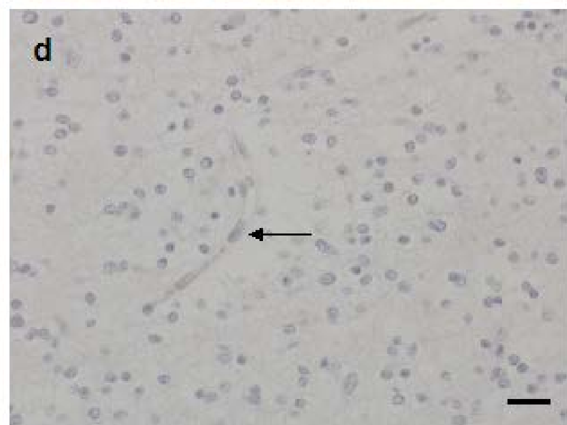
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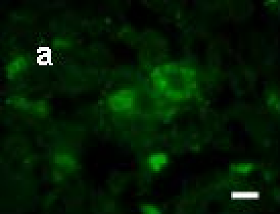
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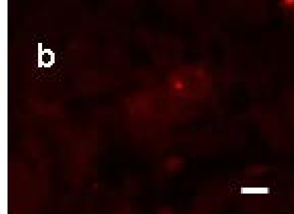
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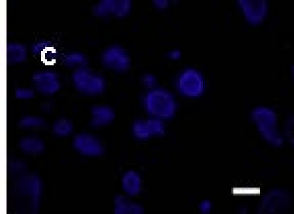
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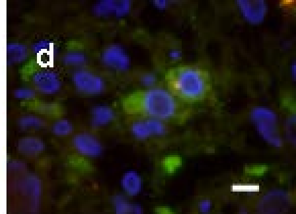
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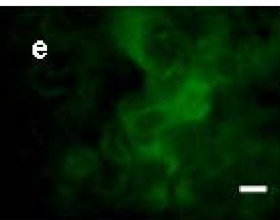
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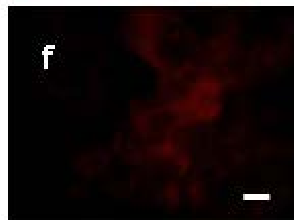
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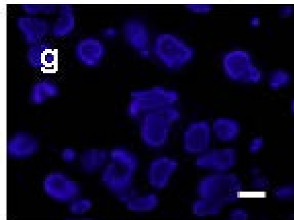
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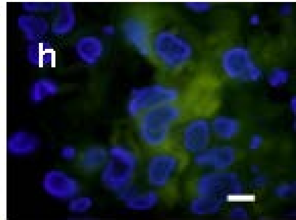
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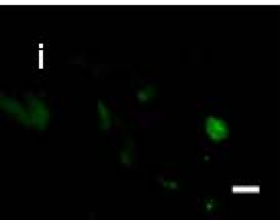
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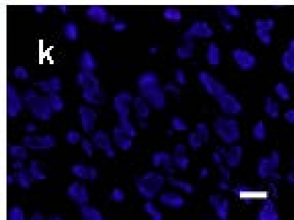
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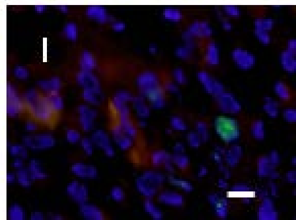
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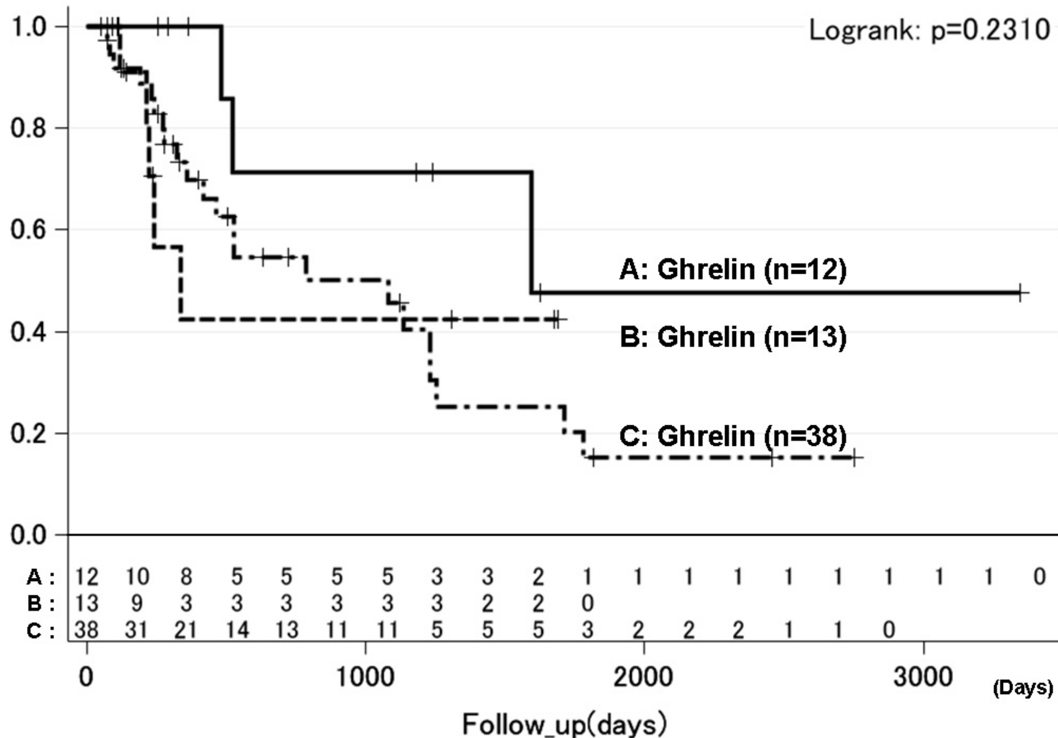
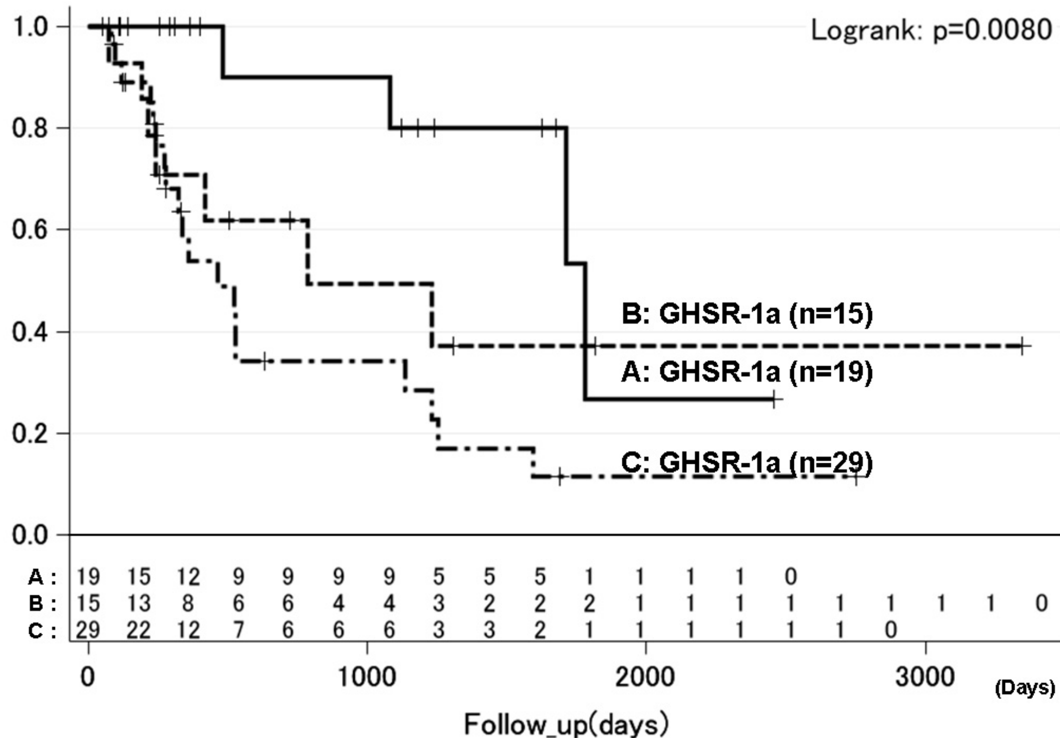
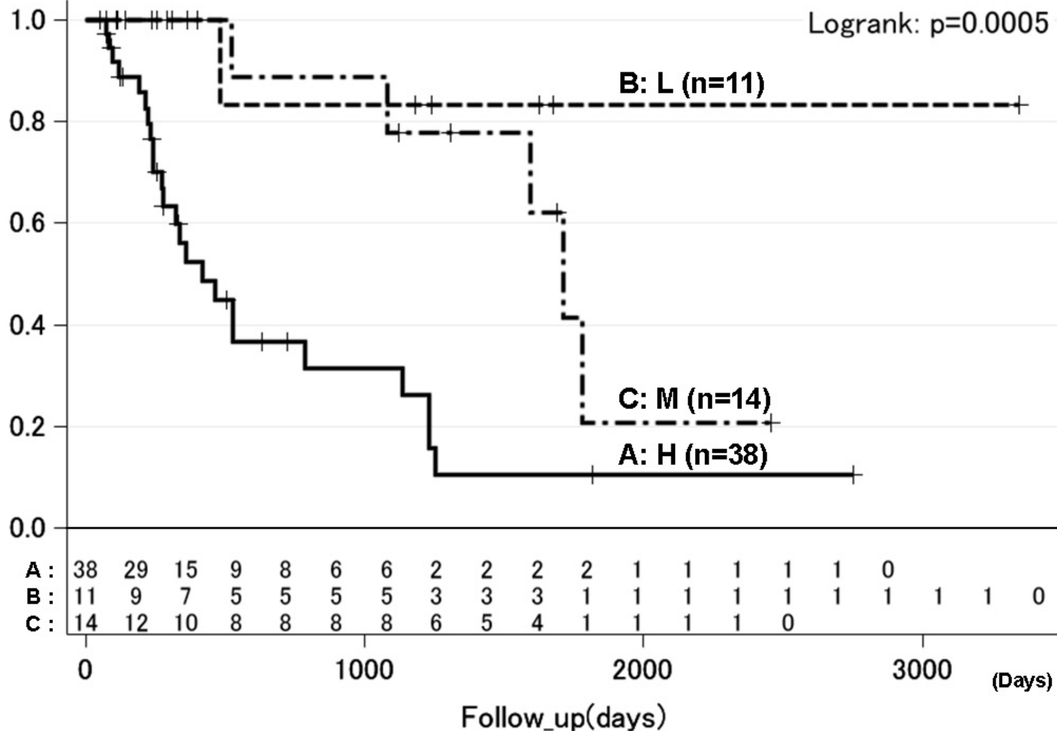
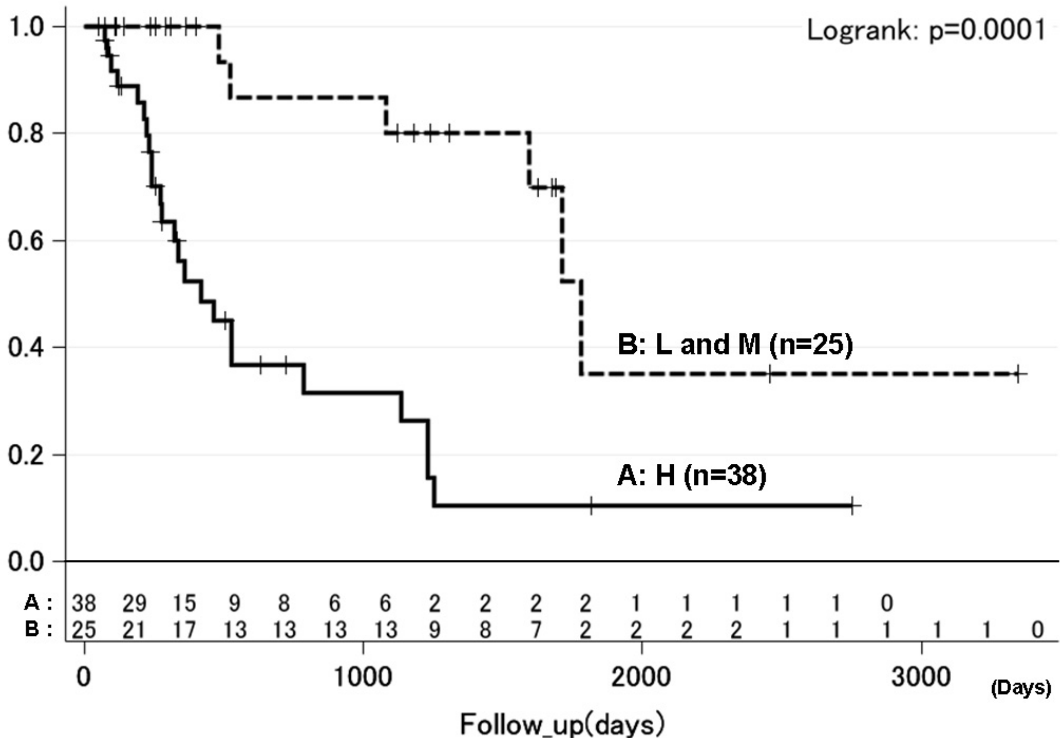


Logrank: p=0.2310
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
A: Ghrelin (n=12)
B: Ghrelin (n=13)
C: Ghrelin (n=38)
A : 12 10 8 5 5 5 5 3 3 2 1 1 1 1 1 1 1 1 1 0
B : 13 9 3 3 3 3 3 3 2 2 0
C : 38 31 21 14 13 11 11 5 5 5 3 2 2 2 1 1 0
0
1000
2000
3000
(Days)
Follow_up(days)



Logrank: p=0.0080
1.0
0.8
0.6
0.4
0.2
0.0
Survival Probability
B: GHSR-1a (n=15)
A: GHSR-1a (n=19)
C: GHSR-1a (n=29)
A : 19 15 12 9 9 9 9 5 5 5 1 1 1 1 0
B : 15 13 8 6 6 4 4 3 2 2 2 1 1 1 1 1 1 1 1 0
C : 29 22 12 7 6 6 6 3 3 2 1 1 1 1 1 1 0
0
1000
2000
3000
(Days)
Follow_up(days)


Logrank: p=0.0005
B: L (n=11)
C: M (n=14)
A: H (n=38)
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
A : 38 29 15 9 8 6 6 2 2 2 2 1 1 1 1 1 0
B : 11 9 7 5 5 5 5 3 3 3 1 1 1 1 1 1 1 1 1 0
C : 14 12 10 8 8 8 8 6 5 4 1 1 1 1 0
0
1000
2000
3000
(Days)
Follow_up(days)


Logrank: p=0.0001
Survival Probability
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0.8
0.6
0.4
0.2
0.0
B: L and M (n=25)
A: H (n=38)
A : 38 29 15 9 8 6 6 2 2 2 2 1 1 1 1 1 0
B : 25 21 17 13 13 13 13 9 8 7 2 2 2 2 1 1 1 1 1 0
0
1000
2000
3000
(Days)
Follow_up(days)

Table 1. Clinicopathological characteristics of patients

Factor	Number
Gender	
Male	35
Female	28
Histological type	
Glioblastoma	39
Anaplastic astrocytoma	13
Diffuse astrocytoma	11
MIB-1 labelling index	The range (the mean)
Glioblastoma	4 to 56 (22.3±12.9)
Anaplastic astrocytoma	5 to 40 (15.9±14.0)
Diffuse astrocytoma	1 to 7 (2.2±1.8)
Age (yr)	The range (11 to 83)
Glioblastoma	60.8±12.8
Anaplastic astrocytoma	52.7±18.2
Diffuse astrocytoma	34.4±25.2
Tumor location	
Glioblastoma	FR:lt.5,rt.9;TEM:lt.6,rt.8;PA:lt.3,rt.2;OC:lt.1, rt.3;CER:2
Anaplastic astrocytoma	FR:lt.5,rt.3;TEM:rt.1;PA:rt.2; TH: lt.1,rt.1
Diffuse astrocytoma	FR:lt.3,rt.2;TEM:lt.3,rt.2; TH: lt.1

FR, frontal;TEM, temporal;PA, parietal;OC, occipital;TH, thalamus;CER, cerebellum; lt, left;rt, right

Table 2. Proportion of ghrelin/GHSR-1a-expressing cells by histopathological type of gliomas

Factor	Ghrelin				GHSR-1a			
	(-)	1+	2+	3+	(-)	1+	2+	3+
Glioblastoma (n=39)	1	0	9	29	0	0	13	26
Anaplastic astrocytoma (n=13)	1	0	3	9	9	0	2	3
Diffuse astrocytoma (n=11)	9	1	1	0	10	1	0	0

(-), no staining; 1+, 0-10% positive cells; 2+, 10-50% positive cells; 3+, > 50% positive cells

Table 3. Correlation between Ghrelin/GHSR-1a expression and MIB-1 labelling index in gliomas

Variables	Coefficient of correlation	*P* value
Ghrelin vs. MIB-1	-0.2343	0.1139
GHSR-1a vs. MIB-1	-0.1019	0.5013

(Kendall tau rank correlation test)