Expression of the Ghrelin/GHS-R axis and its functional role in promoting tumor growth in primary CNS lymphomas

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Running title: Ghrelin/GHS-R expression in PCNSLs

Abstract

Ghrelin and its receptor, growth hormone secretagogue receptor (GHS-R), have been found in a variety of malignant tumor tissues, suggesting a biological function of the ghrelin/GHS-R axis in tumor growth and progression. Amongst CNS tumors, primary central nervous system lymphomas (PCNSLs) are relatively rare and characterized by a rapid progression and poor prognosis. In order to clarify ghrelin expression and its functional role in promoting tumor growth and progression in PCNSLs, we undertook an immunohistochemical investigation for ghrelin and GHS-R expression in 43 patients and tested the effect of ghrelin inhibition on lymphoma cells. Furthermore, we investigated the expression of CD105, a marker for tumor angiogenesis, to explore its association with the ghrelin/GHS-R axis. The Kaplan-Meier method and Cox's proportional hazards regression model were used to determine the association of ghrelin/GHS-R expression with overall survival rate. The immunohistochemical study showed moderate/strong immunostaining of cells for ghrelin and GHS-R in 40 patients (93.0%) and 39 patients (90.7%), respectively. A ghrelin inhibitor did not affect tumor cell proliferation *in vitro*. Expression levels of ghrelin and GHS-R were divided into high and low groups by the rate of moderate-strong staining cell to tumor cell. The survival rate was significantly lower in patients with high GHS-R expression (p=0.0368,

log-rank test and p=0.0219, Wilcoxon test). In addition, multivariate analysis of overall survival using Cox's proportional hazards regression model indicated that GHS-R was a significant independent prognostic factor (p=0.0426). CD105 expression on tumor vessels was positive in 33 patients (33/37, 89.2%). There was a positive correlation between the moderate-strong staining rate of ghrelin and CD105-positive vessel count. These results indicated that the ghrelin/GHS-R axis plays a potential role in promoting tumor growth and progression through neoangiogenesis, rather than the proliferation of tumor cells.

Key words: ghrelin, growth hormone secretagogue receptor, PCNSLs, prognosis, tumor growth

Introduction

Ghrelin is a peptide hormone that was identified by Kojima et al. in 1998.[1] It is a ligand of growth hormone secretagogue receptor (GHS-R).[1] Ghrelin is mainly produced in the endocrine cells of the stomach, but it is also expressed in a variety of tissues, and in both physiological and malignant conditions. Ghrelin is highly expressed in various tumor tissues, along with GHS-R.[2-5] Thus, the functional role of the ghrelin/GHS-R axis in promoting tumor growth and progression has been explored previously.[4-6]

The possibility of an autocrine/paracrine role of ghrelin/GHS-R signaling in the regulation of cancer cell proliferation has been indicated in hormone-dependent cancers such as prostate and breast cancers.[5] Dixit et al. determined that high levels of GHS-R and ghrelin expression were more common in high, as opposed to low, grade CNS tumors using a microarray of astrocytoma tissue. Thus, the ghrelin axis constitutes a novel autocrine pathway in astrocytomas.[4] Chen et al. also reported that ghrelin increased GHS-R up-regulation and enhanced ghrelin-induced glioma cell motility, which was markedly inhibited by a GHS-R antagonist.[6] Recently, Okada et al. reported that ghrelin/GHS-R axis expression level was associated with prognosis, suggesting that the expression of the ghrelin/GHS-R axis increases the growth of gliomas through an

autocrine/paracrine mechanism.[7] In their study, the proportionate rate of ghrelin expression was correlated with histological grading of diffuse gliomas.

Lin and Hsiao showed high expression of ghrelin in many cancers including large B cell lymphoma, suggesting a pathological role of this gene in cancer.[8] They also showed the relative ghrelin and GHS-R expression in pan-cancer patients from The Cancer Genome Atlas, suggesting the potential pathological role of the axis in cancers. However, to the best of our knowledge, the present study is the first to investigate immunohistochemical expression of ghrelin and GHS-R in lymphoma; and to study the association of the ghrelin/GHS-R axis with prognosis in patients with primary central nervous system lymphomas (PCNSLs). Amongst CNS tumors, PCNSLs are relatively rare and characterized by rapid tumor progression and poor prognosis.

Regarding prognostic factors associated with PCNSLs, CD105 (also known as endoglin), a marker of angiogenesis, is reportedly a reliable prognostic marker.[9] Therefore, in order to clarify the ghrelin/GHS-R axis expression and its functional role in promoting tumor growth in PCNSLs, we investigated the correlation between ghrelin/GHS-R and CD105 in PCNSL tissue, and carried out cell viability assays using PCNSL cell lines treated with a ghrelin inhibitor. Furthermore, we explored whether the levels of expression of ghrelin/GHS-R in tumor tissues influenced the survival of

patients with PCNSLs.

Materials and methods

Cases

Surgical tissue samples (n=43) from 43 patients with PCNSLs, 37 patients with diffuse large B-cell lymphoma (DLBCL) and six patients with marginal zone B-cell lymphoma (MZBCL), were analyzed in the present study. All tumor specimens were retrieved from the archives of Kurume University and its affiliated hospitals, and Niigata University between 2011 and 2017. Clinical information for the 43 patients was also retrieved from the archives of Kurume University and Niigata University. This study was carried out in accordance with the principles of the Helsinki declaration and was approved by the ethics committee at our institutions.

All specimens were histologically diagnosed according to World Health Organization (WHO) criteria for CNS tumors.[10] Although the primary diffuse large B-cell lymphomas are designated as PCNSLs by the WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues,[11] a variety of other B-cell lymphomas have been highlighted in recent years.[12,13] One of these is extranodal MZBCL, which is a subtype of B-cell lymphoma that originates primarily in the marginal zone. We therefore

included MZBCL in this study.

Immunohistochemistry

Tissue samples obtained from patients were fixed in 10% neutral buffered formalin, embedded in paraffin, and then processed using conventional histological and immunohistochemical methods. For histological evaluation, 5 μm sections were stained with hematoxylin and eosin (H & E). All specimens were histologically diagnosed according to the WHO criteria for CNS tumors.

Immunohistochemical studies were performed on paraffin-embedded tissue sections following heat-induced antigen retrieval and stained using specific antibodies and immunoperoxidase kits (ChemMate EnVision kit/HRP [DAB], DakoCytomation Tokyo, Japan). The primary antibodies were raised against ghrelin (dilution 1:400; AbD Serotec, Oxford, UK), GHS-R (dilution, 1:400; prepared in-house) and endoglin (CD105, dilution 1:50; Novocastra, Newcastle, United Kingdom). The manuscript detailing the method for generation of anti-GHS-R antibodies is in preparation; briefly, we immunized MRL/lpr mice with purified GHS-R to obtain anti-GHS-R antibodies; specificity of the generated antibodies was confirmed by enzyme-linked immunosorbent assay.

We evaluated the staining intensity of ghrelin and GHS-R in samples from 43 patients based on a 4-tier grading system: not stained (grade 0), weak (grade 1), moderate (grade 2), and strong (grade 3) (Fig. 1). The expression level of ghrelin/GHS-R was assessed as the proportion of the moderate-strong immunostaining (grade 2-3 staining) cell to total tumor cell from three randomly-selected high-power fields (HPFs). The samples were divided according to high and low expression levels based on the grade 2-3 staining rate (cut off value, 50%).

We evaluated CD105-positive vessels in three HPFs within the tissue sections. The average count of CD105-positive vessels from the three areas was recorded as a marker of tumor angiogenesis. It was possible to evaluate CD105 in 37 cases.

Immunohistochemical evaluations were performed by two independent observers (H.M., Y.S.). The cases where evaluations varied significantly between the readers were re-evaluated until a consensus was reached.

Assessment of RNA expression by in situ hybridization

In three representative cases of DLBCL, ghrelin mRNA *in situ* hybridization (ISH) was performed with formalin-fixed paraffin-embedded tissue using the RNAscope® 2.5 HD Reagent Kit-BROWN (catalog: 322300, Advanced Cell Diagnosis

[ACD]; Hayward, CA, USA) and RNAscope-Target Probe HA-GHRL (catalog:455131, ACD) according to the manufacturer's protocols. Tissue sections that were 5 μm thick were deparaffinized and dehydrated, followed by inhibition of endogenous peroxidase activity. Incubation was performed by boiling the samples in the target retrieval reagent for 10 min, rinsing them in deionized water, dipping them in 99% ethanol, and allowing them to dry. The samples then underwent treatment with a protease reagent at 40 °C for 30 min in a HybEZ hybridization oven (Advanced Cell Diagnostics, Hayward, CA.), [14] hybridization with the target probes, 6-step amplification, visualization with DAB and counterstaining with hematoxylin. Ghrelin mRNA appeared as brown dots in the ISH.

Cell Culture

HKBML cells (RIKEN Cell Bank, Tsukuba Science City, Japan) were used to culture PCNSL cells in DMEM and Ham's F12 media. Complete media was supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Cell proliferation assay

Proliferation of the cell line was evaluated in triplicate using a colorimetric

WST-1 assay (Roche, Mannheim, Germany) according to the manufacturer's protocol. Approximately 1,000 cells of each population were seeded in 96-well plastic plates in 200 μL of culture medium supplemented with 0.1% FBS. The plates were incubated for 4 hours at 37 °C. Twenty μL of WST-1 (10% of total volume) was added to the cells, which were then incubated. The plate was examined using a DS2 plate reader (Dynex Technologies, Chantilly, VA) by measuring the absorbance of the dye at 450 nm, with the reference wavelength set at 600 nm. Average absorbance values were calculated and plotted. The cells were treated with various concentrations of a ghrelin inhibitor (compound 20, a kind gift from Dr. Kojima M, a professor of Kurume University School of Medicine, Japan) which acts as a specific antagonist to GHS-R.[15]

Statistical analysis

Descriptive and statistical analysis was carried out using JMP Pro 13 and SAS 9.4. We calculated the correlation coefficient between the rate of grade 2-3 cell staining of ghrelin/GHS-R and CD105-positive vessel count using the Spearman rank correlation test. The overall survival rate was calculated using the Kaplan-Meier method. Patients were censored on loss-to-follow-up at the time of analysis. A Cox's proportional hazards regression model was used for uni/multivariate analysis of ghrelin,

GHS-R, age, and sex for overall survival. The level for statistical significance was set at $p<0.05$.

Results

Clinical information about the patients and the results of the immunohistochemical staining for ghrelin/GHS-R and CD105 are shown in Table 1. Out of 43 cases, grade 2-3 immunostaining of ghrelin/GHS-R was shown in 40 (93.0%) and 39 (90.7%) specimens, respectively. The number and percentage of samples with high expression levels for ghrelin and GHS-R are shown in Table 1. In DLBCL, the proportions of specimens showing high ghrelin/GHS-R expression were 16.2% and 27.0%, respectively. No specimens showed high expression level for ghrelin/GHS-R in MZBCL. CD105-positive tumor vessels were found in 33 out of 37 (89.2%) specimens. Representative immunohistochemical staining for ghrelin/GHS-R are shown in Fig. 1. Additionally, immunohistochemical staining using commercially available anti-GHS-R antibody (dilution 1:3000; Abcam, Cambridge, United Kingdom) was performed in 30 of the 43 cases to confirm the quality of our in-house generated anti-GHS-R antibody. Immunohistochemical staining for GHS-R showed similar findings with both anti-GHS-R antibodies.

The immunohistochemical findings in sequential sections for ghrelin, GHS-R, and CD105 are shown in Fig. 2. Fig. 2A and B showed co-localization of ghrelin and GHS-R in the same tumor cells. Fig. 2C showed representative CD105-positive endothelial cells of tumor vessels, with co-localization of ghrelin and GHS-R.

In situ hybridization mRNA analysis demonstrated that DLBCL cells were positive for ghrelin in all three representative cases (Fig. 3).

The cell proliferation assay of a PCNSL cell line showed no effect from changing the concentration of the ghrelin inhibitor on the measured absorbance (which corresponds with cell proliferation) (Fig. 4).

In order to determine whether there was an association between the levels of expression of ghrelin/GHS-R and survival rate, the patients with PCNSL were divided into two groups depending on expression level. The numbers of specimens with high and low levels of ghrelin expression were 6 and 37, respectively. The numbers of specimens with high and low levels of expression for GHS-R were 10 and 33, respectively. There was no significant difference in survival rate between patients with low and high levels of ghrelin expression. However, the survival rate was significantly lower in patients with high compared to low GHS-R expression ($p=0.0368$ by log-rank test and $p=0.0219$, Wilcoxon test, Fig. 5). Multivariate analysis of ghrelin, GHS-R, age

and sex for overall survival using a Cox's proportional hazards regression model demonstrated that GHS-R was an independent prognostic factor (p=0.0426, Table 2). There was a significant positive correlation between the grade 2-3 staining rate of ghrelin and CD105-positive vessel count (Table 3).

Discussion

Recent studies have shown that ghrelin/GHS-R is highly expressed in various tumor tissues, suggesting the possibility of an autocrine/paracrine role in tumor progression and growth.[2-5] Amongst CNS glial tumors, the proportion of ghrelin/GHS-R-expressing cells varied on a histological grading. The rate of ghrelin expression was almost the same in glioblastomas (GBs) and astrocytomas (AAs), but the expression of GHS-R1a, encoded as a full-length biologically active receptor, was higher in GBs than in AAs.[7] High ghrelin/GHS-R expression was more common in specimens from high-grade astrocytomas compared to that in low-grade astrocytomas.[4] These results suggest that the ghrelin/GHS-R axis plays an important role in the tumorigenesis of high grade gliomas.[7] However, there have been no studies of ghrelin/GHS-R expression in PCNSLs. Interestingly, in the present study, immunohistochemical investigation showed grade 2-3 immunostaining of ghrelin/GHS-R in most PCNSL cells. In addition, DLBCL

and MZBCL samples showed distinct levels of ghrelin/GHS-R expression. High levels of ghrelin and GHS-R expression were not observed in patients with MZBCL. Extranodal MZBCL is a subtype of B-cell lymphoma that originates primarily in the marginal zone.[12, 13] There have been very few case reports on MZBCLs in the CNS.[16, 17] CNS extranodal marginal zone lymphoma is an indolent, low grade, and radiosensitive lymphoma associated with good treatment outcomes and prognosis.[12] In our study, there were no MZBCL specimens with high ghrelin/GHS-R expression. The immunohistochemical results we obtained from PCNSLs are therefore consistent with previous studies of astrocytomas.[4]

We investigated RNA expression in PCNSL cells. RNAscope is a novel RNA *in situ* hybridization method that uses formalin-fixed, paraffin-embedded tissues (FFPE). This new method can detect and visualize a single molecule of mRNA by simultaneous signal amplification and background suppression using a specifically designed probe.[14] There have been several studies into RNA identification in cancer cells using this method. Bishop et al. detected transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma using the RNAscope method.[18] They described the development of RNA *in situ* hybridization probes which are complementary to E6/E7 mRNA and permit the direct visualization of viral transcripts in routinely

processed tissues. Bunker et al. reported the clinical utility for measuring intra-tumoral gene expression of the potential prognostic markers GFT1 and TNFRSF11A in colorectal cancer.[19] They analyzed 112 consecutively collected colorectal cancer tumor samples and found that RNAscope enabled evaluation of mRNA expression at the single cell level. We therefore chose to confirm ghrelin mRNA expression in PCNSL cells using the RNAscope, in conjunction with immunohistochemical staining to assess protein expression.

There have been both positive and negative reports on associations between ghrelin expression and survival rate. The association is not significant in most cancer studies except in those on kidney cancer, lung cancer, and acute myeloid lymphoma (AML).[8] Okada et al. reported that overall survival rate was not associated with the level of ghrelin expression, but it was significantly associated with the level of GHS-R expression in gliomas.[7] Although overall survival rate of the patients was not associated with ghrelin expression level, patients with high levels of expression of GHS-R in our study had a significantly poorer prognosis. There are two possible speculations for the observed results. First, the exogenous circulating ghrelin may also function as a GHS-R axis component. Second, the effects of GHS-R on the clinical prognosis of the patients with PCNSLs could be due to the functional role of the ghrelin/GHS-R axis in

promoting tumor growth.

It has been reported that, ghrelin promotes prostate cancer cell proliferation[20] and oral tumor cell proliferation through the regulation of glucose metabolism.[21] However, ghrelin *also* inhibits cancer cell proliferation.[22, 23] Lin and Hsiao suggested that these discrepancies might be partially due to the ghrelin concentrations used to treat cancer cell lines.[8] Thus, the correlation between ghrelin expression and cancer cell proliferation remains controversial in cell line studies.[24] We therefore carried out an *in vitro* study to determine if ghrelin promotes the proliferation of PCNSL cells. Our results showed that PCNSL cell proliferation was not inhibited by a ghrelin inhibitor. Thus, we suggest that ghrelin does not directly promote tumor cell proliferation in PCNSLs.

If not for promoting proliferation, what is the involvement of ghrelin in PCNSL cells? Dixit et al. reported that ghrelin increased intracellular calcium mobilization and led to membrane ruffling which resulted in high motility and invasion of astrocytoma cells.[4] In addition, Chen et al. reported that ghrelin induced cell migration through the GHS-R, CaMK, adenosine monophosphate-activated protein kinase and the NF-κB signaling pathway in glioma cells.[6]

In the present study, 33 cases showed positive immunohistochemical staining for

CD105. The immunohistochemical findings in sequential sections for ghrelin and GHS-R suggested the co-localization in tumor cell and CD105-positive endothelial cells. In addition, there was a significant positive correlation between the grade 2-3 staining rate of ghrelin and CD105-positive vessel count, intratumoral micro vessel density. Taken together, these results indicate that ghrelin may promote tumor growth through intratumoral neoangiogenesis in PCNSLs. Regarding the role of ghrelin in angiogenesis, Wang et al. explained that ghrelin activates its receptor on endothelial cells to promote angiogenesis and migration in the human umbilical vein through extracellular regulated protein kinases signaling pathway.[25] Okada et al. detected high expression of ghrelin/GHS-R1a in tumor cells and proliferating microvessels in AAs and GBs.[7] They also found that GHS-R1a co-localized with CD105 in proliferating microvessels, suggesting that ghrelin/GHS-R1a interactions play an important role in growth, invasion, and neoangiogenesis in astrocytomas. Sugita et al. reported that CD105 was expressed in endothelial cells and that intratumoral microvessel density correlated with survival rate in PCNSLs. [9] They also described that the high microvessel density in PCNSLs could play a significant role in the ability of tumors to infiltrate surrounding tissues. Furthermore, considering the results of the present *in vitro* study, ghrelin may promote tumor growth and progression by PCNSL cell invasiveness rather than PCNSL

cell proliferation.

This study was limited by the small study sample. Although PCNSLs are rare, further studies are necessary to clarify the biological effects of the ghrelin axis in tumor growth and progression.

In conclusion, the results of both the *in vitro* study and the clinical prognosis analysis suggested that the ghrelin/GHS-R axis plays a potential role in promoting tumor growth and progression through neoangiogenesis, rather than by proliferation of tumor cells.

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Disclosure

Authors declare no conflict of interests related to this article.

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Figure legends

Fig. 1. Representative immunohistochemical staining of ghrelin and GHS-R in PCNSLs. (A) Weak immunostaining of ghrelin in PCNSLs (grade 1). (B) Moderate immunostaining of ghrelin in PCNSLs (grade 2). (C) Strong immunostaining of ghrelin in PCNSLs (grade 3). (D) Weak immunostaining of GHS-R in PCNSLs (grade 1). (E) Moderate immunostaining of GHS-R in PCNSLs (grade 2). (F) Strong immunostaining of GHS-R in PCNSLs (grade 3). In each panel, the scale bar represents 20 μm.

Fig. 2. Immunohistochemical findings in sequential sections for ghrelin, GHS-R, and CD105 in PCNSLs. (A) The immunostaining of ghrelin in tumor cells and endothelial cells of microvessels. (B) The immunostaining of GHS-R in tumor cells and endothelial cells of microvessels. (C) The immunostaining of CD105 in the endothelial cells of microvessels. In each panel, arrows show endothelial cells and the scale bar represents 20 μm.

Fig. 3. Lymphoma cells showing positive signals for ghrelin mRNA by using RNA scope. The scale bar represents 20 μm.

Fig. 4. Effect of treatment with the ghrelin inhibitor Compound 20 on PCNSL cell viability. The concentrations investigated did not significantly alter the viability of PCNSL cells.

Fig. 5. Univariate analysis of overall survival curves (Kaplan-Meier). (A, B) Univariate analysis of overall survival curves according to ghrelin and growth hormone secretagogue receptor (GHS-R) expression. The patients are divided into either high or low expression levels depending on their individual grade 2-3 staining rate. Six patients showed high expression levels, and 37 showed low expression levels of ghrelin. Ten patients showed high GHS-R expression and 33 showed low GHS-R levels. There was no significant difference in survival rate between patients with low and high levels of ghrelin expression. However, the survival rate was significantly lower in the high GHS-R expression group, compared to the low expression level group ($p=0.0368$ by log-rank test and $p=0.0219$, Wilcoxon test).

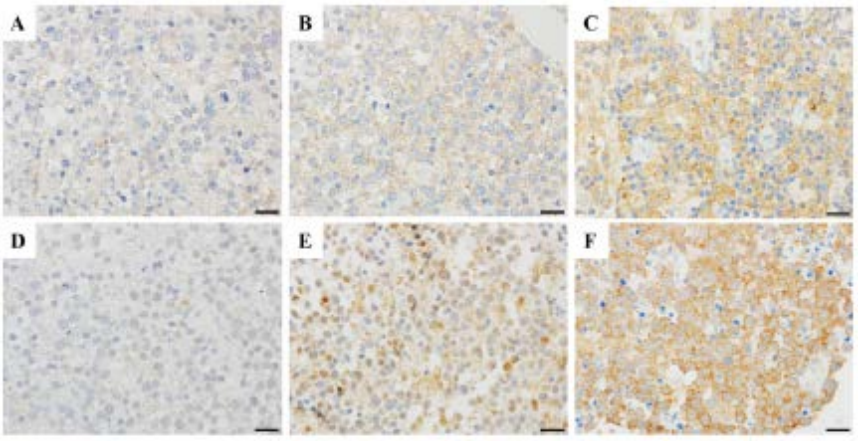

Fig. 1. Representative immunohistochemical staining of ghrelin and GHS-R in PCNSLs.

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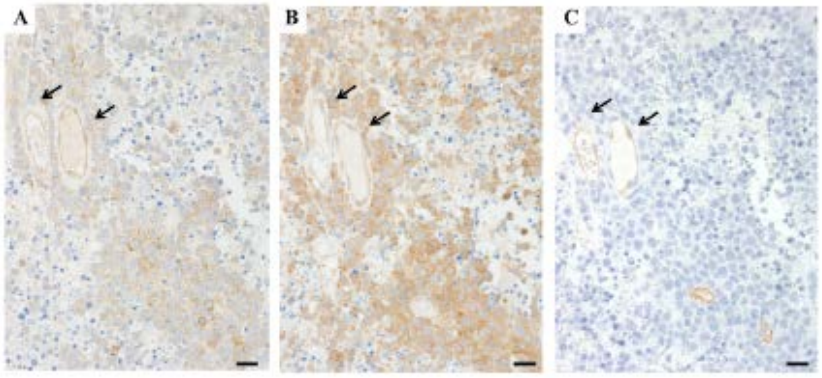

Fig. 2. Immunohistochemical findings in sequential sections for ghrelin, GHS-R, and CD105 in PCNSLs.

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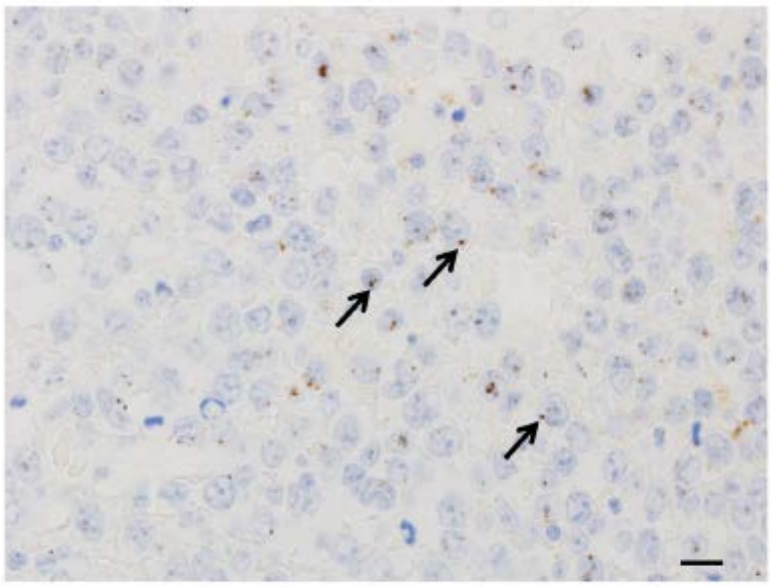
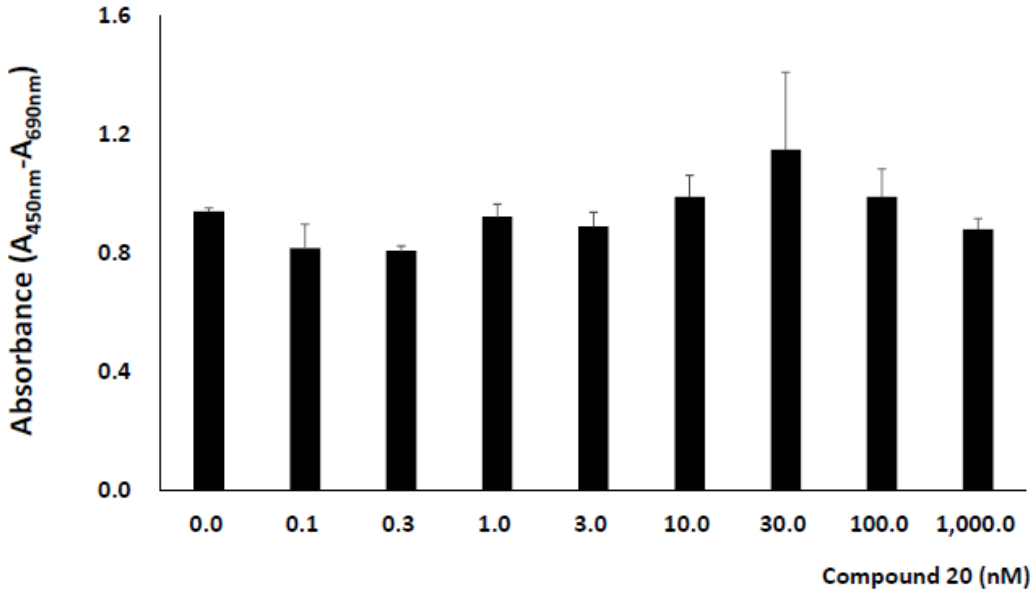


Fig. 3. Lymphoma cells showing positive signals for ghrelin mRNA by using RNA scope.

85x63mm (300 x 300 DPI)



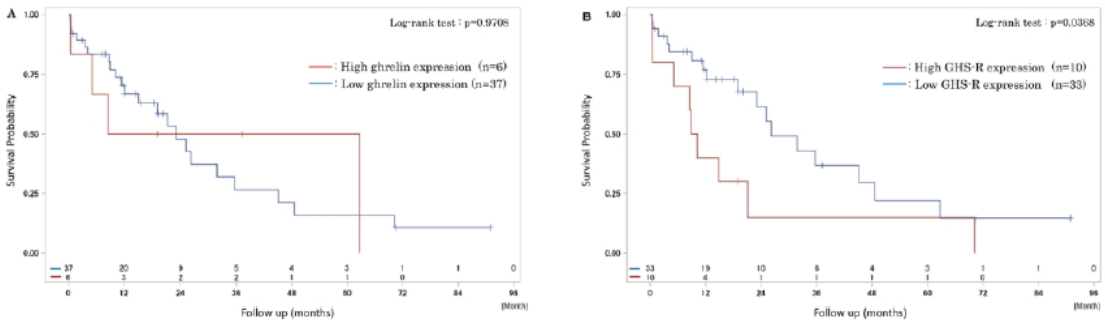


Fig. 5. Univariate analysis of overall survival curves (Kaplan-Meier).

179x54mm (300 x 300 DPI)

Table 1. Characteristics of patients and immunohistochemical expression

Factor		Total	DLBCL	MZBCL
Gender	n of cases	43	37	6
Male		23	18	5
Female		20	19	1
Age	(years)			
Range		35-86	35-85	43-86
Mean ±SD		64.93±13.12	65.68±12.47	60.50±17.33
Moderate-strong immunostaining† n of cases (%)				
Ghrelin		40(93.0)	36(97.3)	4(66.7)
GHS-R		39(90.7)	33(89.2)	6(100)
High expression level‡ n of cases (%)				
Ghrelin		6(14.0)	6(16.2)	0(0.0)
GHS-R		10(23.3)	10(27.0)	0(0.0)
CD105 expression§	n of cases (%)		33(89.2)	

DLBCL, diffuse large B cell lymphoma; MZBCL, marginal zone B cell lymphoma.

† Samples with tumor cells that were stained moderate (grade 2)-strong (grade 3) intensity.

‡ Samples with higher than 50% of grade 2-3 staining-cell rate for total tumor cells calculated on 3 high power fields.

§ Samples with CD105-positive vessels among 37 cases.

Table 2. Result of univariate and multivariate Cox's PH regression (stepwise selection)

	Univariate				Multivariate			
Factor	HR	95%CI		p	HR	95%CI		p
Ghrelin	0.98	0.33	2.87	0.9706				
GHS-R	0.43	0.19	0.97	0.0426	0.43	0.19	0.97	0.0426
Age	0.73	0.31	1.77	0.4904				
Sex	1.01	0.46	2.20	0.9795				

CI, confidence interval; GHS-R, growth hormone secretagogue receptor; HR, hazards ratio.

Table 3. Correlation of expressions between ghrelin/growth hormone secretagogue receptor (GHS-R) and CD105

Variables	Coefficient of correlation	p-value
Ghrelin vs CD105	0.44	0.0033
GHS-R vs CD105	0.20	0.2731

Spearman rank correlation test. PCNSL, primary central nervous system lymphoma.