Antitumor effect of memantine is related to the formation of the splicing isoform of GLG1, a decoy FGF-binding protein

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Abstract. Drug repositioning is a strategy for repurposing the approved or investigational drugs that are outside the scope of the original medical indication. Memantine is used as a non-competitive *N*-methyl-D-aspartate receptor antagonist to prevent glutamate-mediated excitotoxicity in Alzheimer's disease, and is one of the promising agents which is utilized for the purpose of cancer therapy. However, the association between memantine and Golgi glycoprotein 1 (GLG1), an intracellular fibroblast growth factor receptor, in cancers has not yet been clarified. The present study analyzed the expression and location of GLG1 in tumor cells treated with memantine. Memantine was found to suppress the growth of malignant glioma and breast cancer cells in a concentration-dependent manner. The mRNA expression of GLG1 was upregulated in a concentration-dependent manner, and the splicing variant profiles were altered in all cell lines examined. The results of western blot analysis revealed an increase in the full-length and truncated forms of GLG1. Moreover, GLG1 spread in the cytosol of memantine-treated cells, whereas it localized in the Golgi apparatus in control cells. Since GLG1 functions as a decoy FGF receptor, the modulation of GLG1 may prove to be one of the mechanisms underlying the cancer-suppressive effects of memantine.

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Abbreviations: GLG1, Golgi glycoprotein 1; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HSPG, heparan sulfate proteoglycan; NMDA, N-methyl-D-aspartate; CFR, chicken cysteine-rich fibroblast growth factor receptor; ESL-1, E-selectin-ligand 1

Key words: memantine, Golgi glycoprotein 1, glioma, breast cancer, fibroblast growth factor

Introduction

Drug repositioning is a strategy for repurposing approved or investigational drugs outside the scope of their original medical indication. Memantine is a promising agent for cancer therapy (1-3). Memantine, an antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, exerts beneficial effects and is widely used in the treatment of Alzheimer's disease. Memantine acts on the glutamatergic system by blocking NMDA receptors and inhibiting glutamate overstimulation (4). Of note, NMDA receptors have been found in several types of cancer, such as glioma (5), medulloblastoma, neuroblastoma (6), oral squamous cell carcinoma (7), laryngeal cancer (8), gastric cancer (9,10) and prostate cancer (11). There is increasing evidence to suggest that memantine regulates tumor growth, invasion and metastasis in a number of types of cancer, such as high-grade glioma (5), neuroblastoma (12), lung cancer (13), breast cancer (11), prostate cancer (2,11), colon cancer (11), skin cancer (14), and leukemia (15). One of its tumor-suppressive effects is considered to be the blockade of the NMDA receptor followed by glutamine depletion in cancer cells (16).

Fibroblast growth factors (FGFs) are potent regulators of cell proliferation and differentiation (17,18). Accordingly, the FGF receptor (FGFR) pathway plays a major role in several biological processes during oncogenesis (19,20). Several aberrations, including gene amplifications, point mutations and chromosomal translocations, have been reported in various types of cancer (21,22). Moreover, the upregulation of FGFR signaling is a common event in a number of tumor types. Thus, the FGFR pathway is a promising target for cancer treatment (23). Several FGFR inhibitors are currently used in the clinical setting (20,24).

In gliomas, the expression levels of FGFs and their receptors (FGFRs) are elevated, serving as autocrine or paracrine growth accelerators (25-27). In addition, the upregulation of FGFs and FGFRs in breast cancer has been reported to result in brain metastasis and treatment-resistant cancer (28,29). Of note, FGFs bind to three distinct types of molecules: i) FGF receptor tyrosine kinases (FGFR1-4); ii) heparan sulfate proteoglycans (HSPGs); and iii) Golgi glycoprotein 1 [GLG1; also known as MG-160, cysteine-rich FGF receptor and E-selectin-ligand 1 (ESL-1)] (30,31).

In particular, GLG1 is a 160-kDa membrane sialoglycoprotein, originally isolated from the Golgi apparatus of rat neurons (32,33). Two homologs, the chicken cysteine-rich fibroblast growth factor receptor (CFR) (34) and ESL-1 (35-37), were identified in embryonic chick and murine myeloid cells, respectively. This FGF-binding protein has 16 cysteine-rich repeats in the extracellular luminal amino-terminal region, 21 amino acids in the transmembrane domain, and 13 amino acids in the intracellular cytoplasmic carboxy-terminal domain (Fig. 1A) (38). Three major Glg1 splice variants are known. Variant 1 is a full-length form. Compared with variant 1, GLG1 variant 2 lacks an in-frame coding exon, resulting in lack of an internal segment. Compared with variant 1, GLG1 variant 3 has an additional segment in the 3' region, resulting in a shorter C-terminus region. GLG1 (Fig. 1B). GLG1 is a conserved membrane sialoglycoprotein of the Golgi apparatus in the majority of cells, displaying >90% amino acid sequence identity with CFR (30) and ESL-1 (30,31,33-35,39). The *GLG1* gene was assigned to human chromosome 16q22-23 (40).

FGF1, 2, 3, 4 and 18 are known to bind to this protein (30,39). Although GLG1 does not have a tyrosine kinase domain, which plays a main role in a variety of cellular processes, including growth, motility, differentiation and metabolism, it has been reported to participate in the intracellular trafficking of FGF that is integrated into the cell following ligand-receptor conjugation (40-43). According to a previous study, the overexpression of GLG1 induces cell death (42). However, the pattern of expression of GLG1 and its function in tumors remain unexplored.

In the present study, in order to gain insight into the possible involvement of GLG1 in the treatment of neoplasia with memantine, the changes in its expression were analyzed in several types of memantine-treated human glioma and breast cancer cells known to frequently metastasize to the brain. All memantine-treated tumor cells exhibited an upregulated expression of GLG1. The induction of the differential expression of GLG1 variants and changes in its intracellular distribution in memantine-treated cells were also identified. The results presented herein suggest the possibility that memantine exerts a suppressive effect on cell proliferation partly through the modulation of the expression of GLG1, which has an FGF traffic control function. The aim of the present study was to elucidate the intracellular behavior of tumor growth-related factors under treatment with memantine.

Materials and methods

Cancer cell lines. The U87MG cell line was established from glioblastoma of unknown origin. T98G is a glioblastoma cell line. The MDA-MB-231 cell line was established from triple-negative breast adenocarcinoma. These cell lines were purchased from the American Type Culture Collection (ATCC). The catalog numbers for these cell lines are HTB-14 for U87MG, CRL-1690 for T98G, and CRM-HTB-26 for MDA-MB-231.

SNB19 is a glioblastoma cell line. The characterization of this cell line has been precisely studied by Welch *et al* (44). The SNB19 cell line used in the present study was a gift from Professor Richard S. Morrison, Department of Neurological Surgery, University of Washington, Seattle, WA, USA, who is one of the authors of the aforementioned study. In their laboratory, the authors of the present study used the early passage of dispensed frozen cells. The authenticity of the SNB-19 cell line was confirmed by STR analysis (45).

All cell lines were cultured in Dulbecco's modified Eagle's medium (cat. no. 11885084, DMEM, Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS; Biosera) in a humidified atmosphere of 5% CO_2 at 37˚C and harvested when a confluency of ~70-80% was achieved.

Memantine. Memantine hydrochloride (1-amino-3,5-dimethyladamantane hydrochloride) was purchased from FUJIFILM Wako Pure Chemical Corporation. A 10-mM stock solution was prepared in distilled H_2O, filtered through a 0.22-μm PES syringe filter (PES013022; Membrane Solutions LLC), and stored at -80˚C until use.

Memantine treatment. The cells ($4x10^5$) grown in a 30-mm culture dish were exposed to memantine at final concentrations of 0-1,000 μM for 3 days.

Cell viability assay. A cell viability assay was performed by a trypan blue exclusion assay using 0.4%-Trypan blue solution (15250-061; Gibco; Thermo Fisher Scientific, Inc.). The cells were suspended in 0.2% trypan blue and counted using a Bio-Rad cell counter TC20 (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol.

Microscopic observation. Morphological changes in treated tumor cells were observed under a microscope (TS100; Nikon Corporation) and recorded using a Nikon Digital Sight 1000 (Nikon Corporation).

mRNA extraction, cDNA preparation and reverse transcription. Total RNA was extracted from the tumor cells using a RNeasy Mini kit according to the manufacturer's instructions (cat. no. 74134; Qiagen GmbH). Each RNA sample was quantified using a NanoDrop spectrophotometer (serial no. G188; Thermo Fisher Scientific, Inc.). cDNA synthesis was performed with 1 μg total RNA using a PrimeScript RT reagent kit (cat. no. RR037A; Perfect Real Time, Takara Bio Inc.), in which oligo dT primer and random 6mers were used according to the manufacturer's instructions, followed by reverse transcription for 15 min at 37˚C and enzyme inactivation for 5 sec at 85˚C.

Quantitative PCR (qPCR). qPCR was performed using a StepOnePlus™ real-time PCR system (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed using GLG1 primers (Fig. 2 and Table I). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an mRNA loading and integrity control (Table I). SYBR-Green-based qPCR was performed using THUNDERBIRD® SYBR® qPCR Mix (QSP-201; Toyobo Co., Ltd.), following the manufacturer's instructions. Each qPCR assay was performed in a 20-μl reaction volume containing 10 μl 2X one-step SYBR enzyme mix, 0.4 μl ROX reference dye, 0.4 μl (10 μM) of each primer, 7.8 μl RNase-free water and 1.0 μl template. Thermal cycling was performed under the following conditions: 40 cycles at 95˚C for 1 sec and 60˚C for

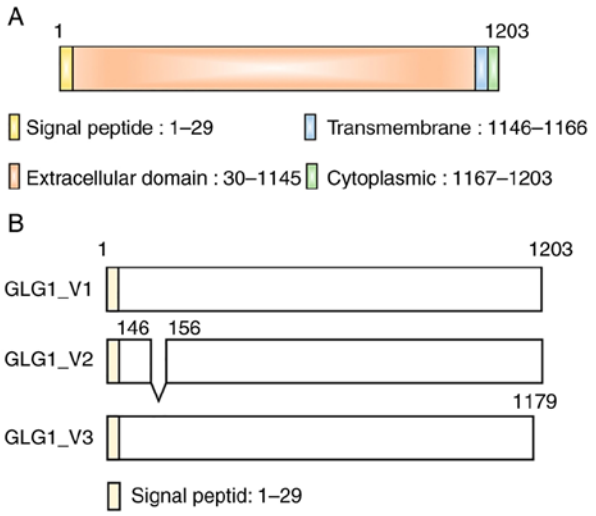

Figure 1. Structure of GLG1. (A) GLG1 has 16 cysteine-rich repeats in the extracellular luminal amino-terminal region, 21 amino acids in the transmembrane domain and 13 amino acids in the intracellular cytoplasmic carboxy-terminal domain. (B) Compared with variant 1, GLG1 variant 2 lacks an in-frame coding exon, resulting in lack of an internal segment. Compared with variant 1, GLG1 variant 3 has an additional segment in the 3' region, resulting in a shorter C-terminus region. GLG1, Golgi glycoprotein 1.

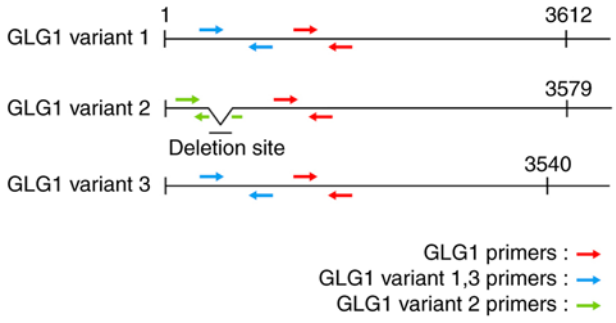

Figure 2. RT-qPCR primer sites. RT-qPCR was performed using GLG1 primers for each splice variant. The amplicon size of GLG1, GLG1 variant 1, 3 and variant 2 is 103, 193 and 71 bp, respectively. RT-qPCR, reverse transcription-quantitative PCR; GLG1, Golgi glycoprotein 1.

20 sec. Each experiment was performed in triplicate. Relative gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (46). The levels of expression of target genes were normalized against those of GAPDH using StepOne™ software (v2.3; Thermo Fisher Scientific, Inc.). Relative quantity (RQ) values were standardized by setting the same threshold values.

Western blot analysis. Samples were prepared using the same number of treated cells that were homogenized in 100 μl whole-cell lysis buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA and 1% NP40) supplemented with a complete protease inhibitor cocktail (cat. no. 05056489001; Roche Diagnostics), and then lysed on ice. Equal amounts of protein samples (30 μg) measured with the Pierce BCA Protein Assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.) were subjected to 7.5% SDS-PAGE Mini-PROTEAN® TGX™ precast gels (cat. no. 4561024, Bio-Rad Laboratories, Inc.) and transferred to polyvinylidene difluoride membranes (cat. no. 1704156, mini PVDF transfer packs; Bio-Rad Laboratories, Inc.) and blocked using 5% BSA for 1 h at room temperature. The membranes were then incubated with primary antibodies against GLG1 (cat. no. MAB78791, monoclonal mouse IgG1, clone #858238; R&D Systems, Inc.; 1:1,000), GAPDH (cat. no. ab181602; Abcam; 1:1,000) at 4˚C overnight or anti-mouse secondary antibody (cat. no. 330; Medical and Biological Laboratories Co., Ltd.; 1:10,000) for 1 h at room temperature. Signals were visualized using the ECL™ Prime Western Blotting System (cat. no. RPN2232, Cytiva), and the FUSION SL-chemiluminescence imaging system (Vilber-Lourmat, Collégien) was used as the digital image processor. Quantitative analysis of relative protein expression and band size were performed with the software provided with FUSION SL.

Immunofluorescence staining. All cells ($2x10^4$ cells per well) were grown in 4-chamber slides (Watson Co., Ltd.) for 2 days. Following treatment with memantine for 2 days, the cells were fixed with 4% PFA (cat. no. 09154-85; Nacalai Tesque, Inc.), permeabilized with 0.1% Triton X-100 (cat. no. 35501-15; Nacalai Tesque, Inc.), blocked with 2% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, and incubated overnight at 4˚C with the following antibody: anti-GLG1 mouse monoclonal antibody (cat. no. MAB78791; R&D Systems, Inc.; 1:1,000). Subsequently, the slides were incubated with Alexa Fluor 488-labeled goat anti-mouse (cat. no. A-11017; Thermo Fisher Scientific, Inc.; 1:10,000) secondary antibody for 1 h at room temperature. Nuclei were counterstained with DAPI (cat. no. D523, Cellstain® DAPI solution; Dojindo Laboratories, Inc.) for 5 min at room temperature. Samples were analyzed using the BZ-X800 all-in-one fluorescence microscope (Keyence Corporation).

Statistical analysis. Experimental data are presented as the mean ± SEM from three or more independent experiments (unless otherwise indicated). The levels of significance (one-way ANOVA followed by Tukey's HSD test) were calculated in each treatment, and the 50% effective concentration (EC_{50}) value were determined in four parameter logistic models using GraphPad Prism 9 software (GraphPad Software, Inc.).

Results

Suppressive effects of memantine on cell proliferation. To confirm the suppressive effects of memantine on the proliferation of cancer cells, three glioma cell lines and one breast cancer cell line were cultured in the presence of memantine at various concentrations (0-1,000 μM) for 3 days and the number of live cells was counted. It was found that memantine suppressed the growth of malignant glioma and breast cancer cells in a concentration-dependent manner (Fig. 3). As regards the SNB19 glioma cells, it was observed that treatment with 100 μM memantine suppressed cell growth to 53.0%. This phenomenon was more apparent in the MDA-MB-231 cells, which exhibited an 67.1% decrease in proliferation (Fig. 3). It was also noted that the MDA-MB-231 breast cancer cells were more prominently affected by memantine than the glioma cells. Exogenously applied memantine suppressed the growth of all cell lines, exhibiting half maximal effective concentration (EC_{50}) values of 87.54, 131.2, 201.6 and 98.26 μM in the SNB19, U87MG, T98G and MDA-MB-231 cells, respectively (Fig. S1).

Table I. Sequences of primers used for RT-qPCR.

Gene symbol	Forward primer	Reverse primer
GLG1	CCAAGATGACGGCCATCATTT	AGCCGAATACTGCCACATTTC
GLG1 variant 1-3	GTGAGGGAGCCTGAAAATGAA	GGTGATCCACCAAGCAGGAA
GLG1 variant 2	CCTAAGCACACCTGGAGCAA	TTCCACAACAACTCCCTCACA
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

GLG1 primers recognize all GLG1 variants. GLG1 variant 1-3 primers detect GLG1 variant 1 and 3. GLG1 variant 2 primers detect only GLG1 variant 2. GLG1, Golgi glycoprotein 1.

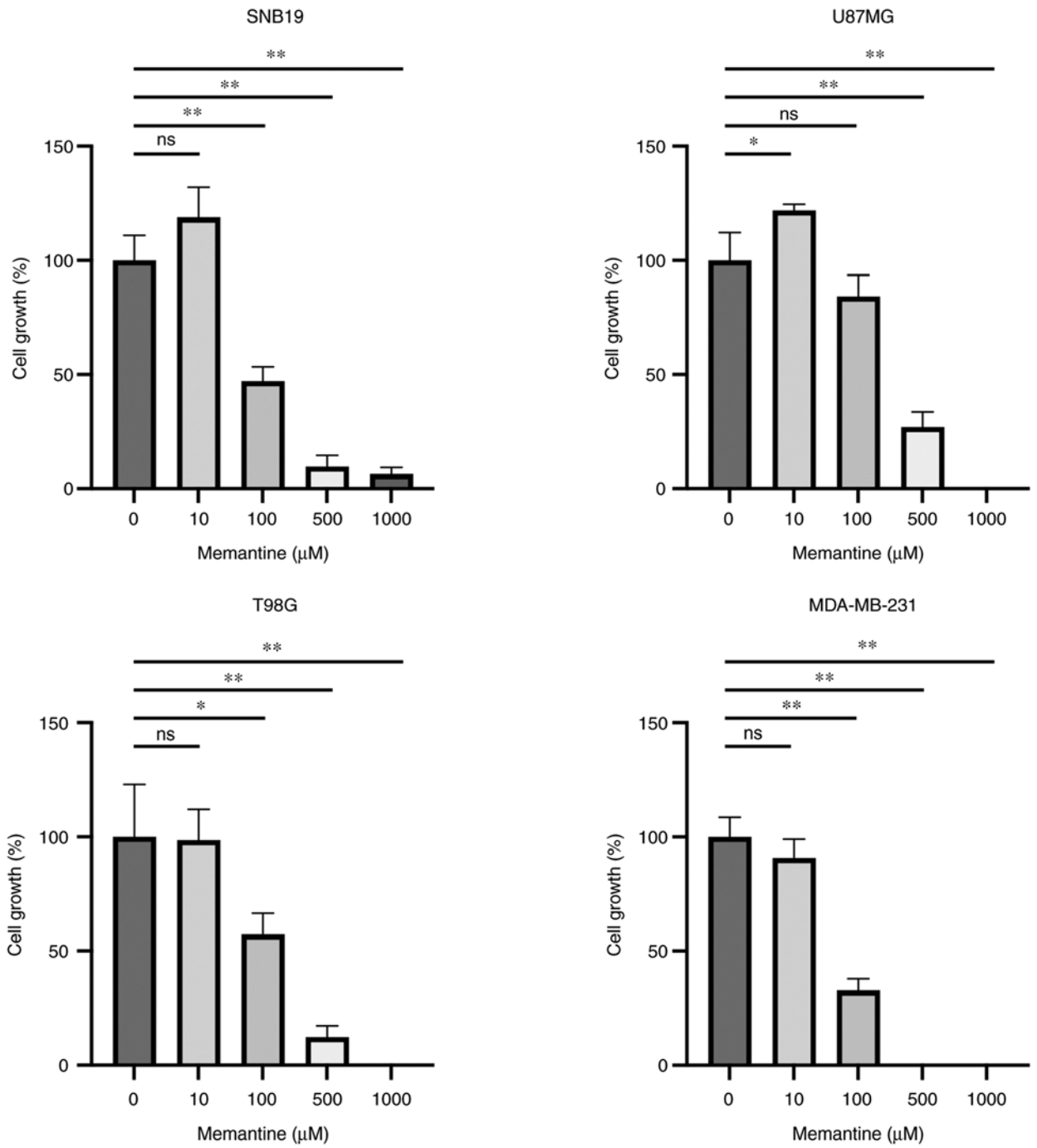

Figure 3. Growth suppressive effects of memantine on tumor cells. Memantine suppressed the growth of malignant glioma cells and breast cancer cells in a concentration-dependent manner. Bars represent the mean ± SEM. *P<0.05 and **P<0.01, significant differences between treated and untreated cells, as determined using ANOVA followed by Tukey's test. ns, not significant.

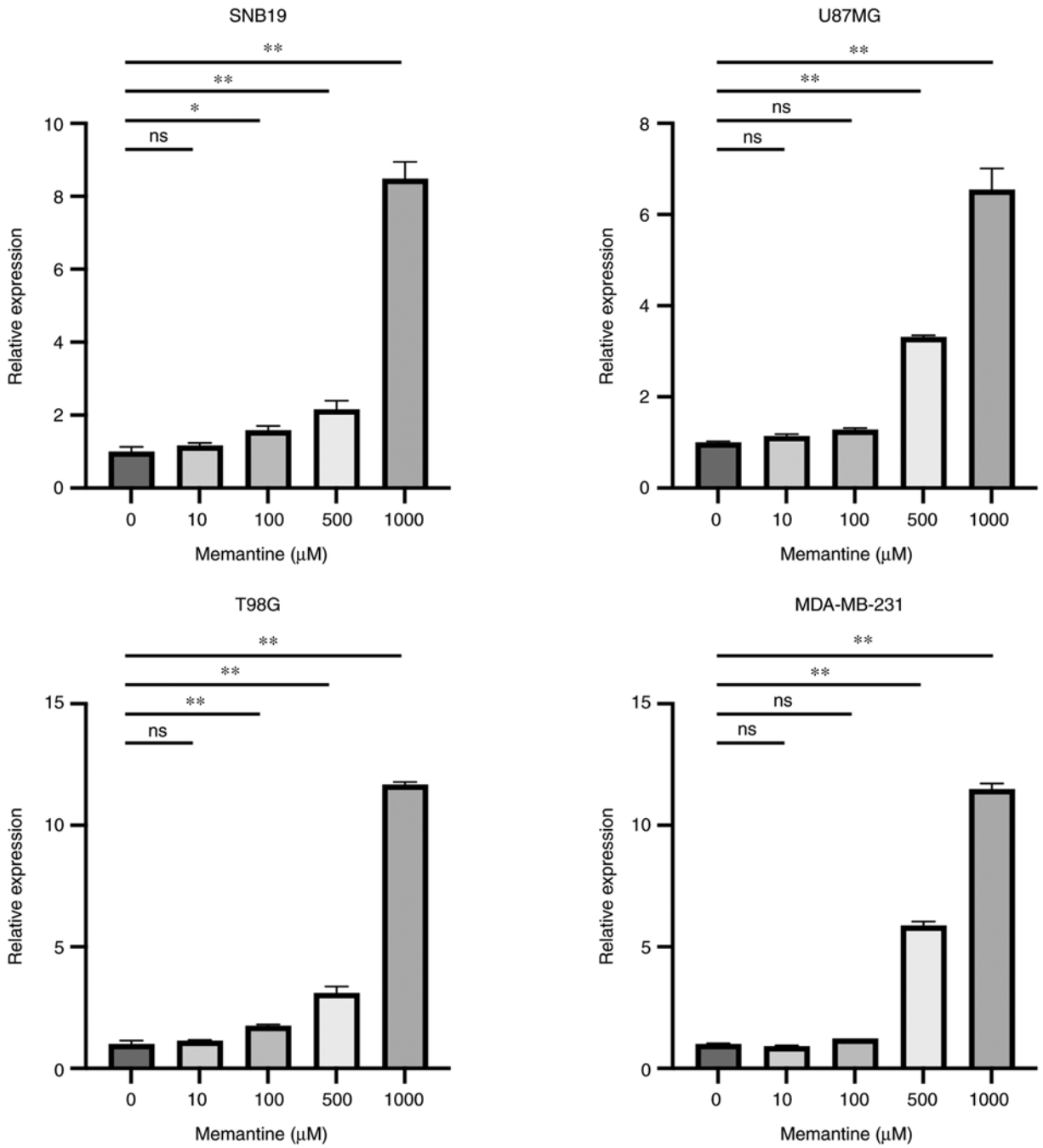

Figure 4. Changes in GLG1 expression following treatment with memantine. The mRNA expression of GLG1 increased following treatment with memantine in a concentration-dependent manner, both in malignant glioma and breast cancer cells. Bars represent the mean ± SEM. *P<0.05 and **P<0.01, significant differences between treated and untreated cells, as determined using ANOVA followed by Tukey's test. ns, not significant; GLG1, Golgi glycoprotein 1.

Memantine leads to an increased mRNA expression of GLG1. To determine the effects of memantine on the expression of GLG1 in cancer cells, RT-qPCR was performed using primers that detect all three GLG1 mRNA variants. The results revealed that the mRNA expression of GLG1 was increased in a concentration-dependent manner in all cell lines (Fig. 4). Of note, all these changes were apparent in treatments using ≥100 μM memantine. Of note, the highest increase in the mRNA level GLG1 was observed in the T98G glioma cells treated with 1,000 μM memantine, whereas the MDA-MB-231 breast cancer cells exhibited a significant increase in GLG1 expression when treated with ≥500 μM memantine.

Differential mRNA expression of GLG1. The present study also examined the changes in the expression of GLG1 variants following treatment with memantine. It was detected that the expression level of variants 1 and 3 was increased to a greater extent than that of variant 2 in the SNB19 and T98G cells, whereas the expression level of variant 2 was higher than that of variants 1 or 3 in the U87MG and MDA-MB-231 cells (Fig. 5).

GLG1 protein expression. Western blot analysis was then performed to determine whether the protein expression of GLG1 in cancer cells was altered following treatment with

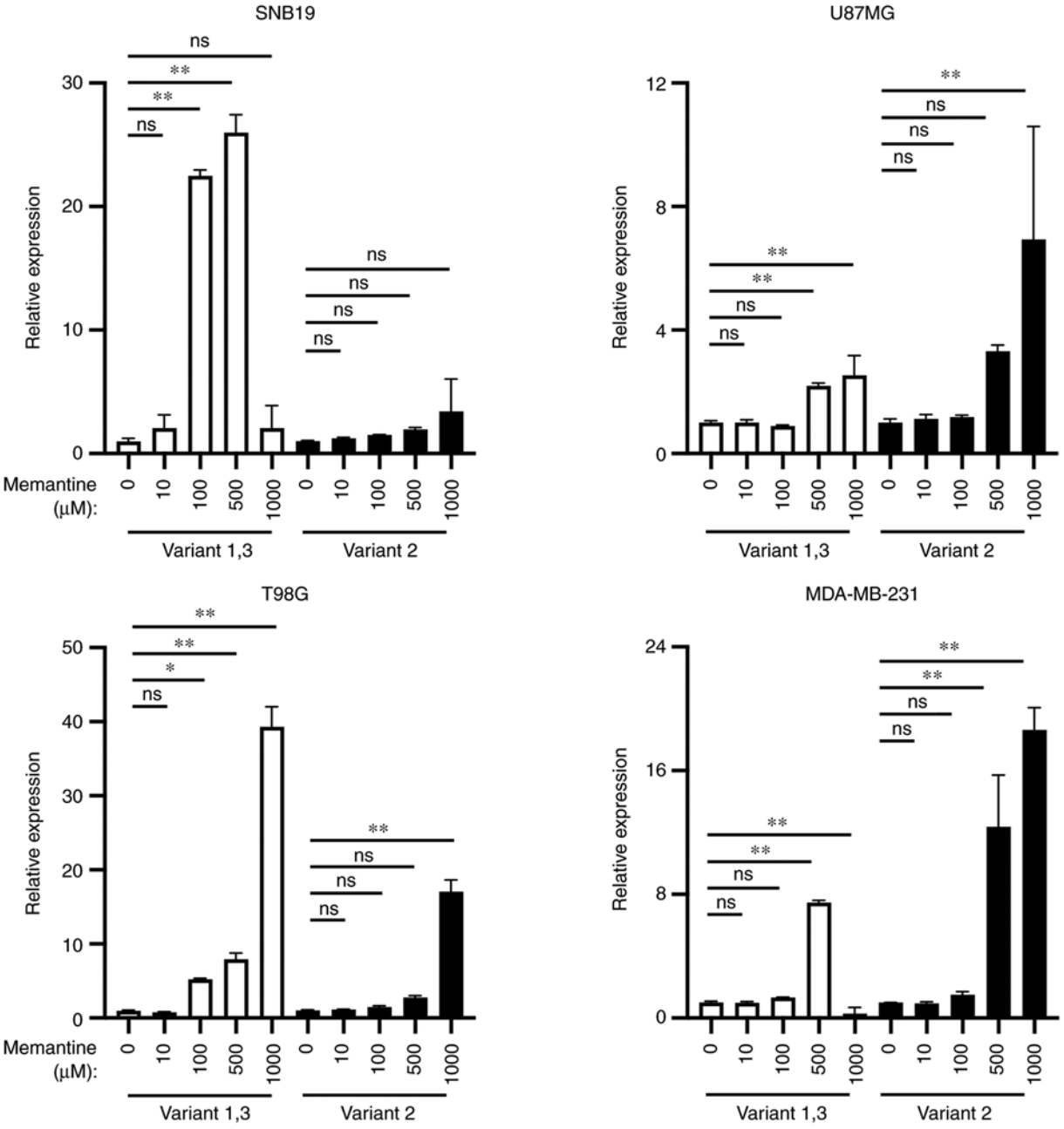

Figure 5. Changes in GLG1 splice variants following treatment with memantine. Reverse transcription-quantitative PCR analysis of the mRNA levels of GLG1 variants 1, 3, or 2 in each cell line treated with memantine for 3 days. Results are presented relative to the expression of GLG1 in the untreated control. Bars represent the mean ± SEM. *P<0.05 and **P<0.01, significant differences between treated and untreated cells, as determined using ANOVA followed by Tukey's test. ns, not significant; GLG1, Golgi glycoprotein 1.

memantine. Of note, despite differences in its expression levels among cell lines, GLG1 was expressed in all control cell lines. It was found that the U87MG glioma cells exhibited a relatively high expression under normal (untreated) conditions. Following treatment with memantine, the expression of GLG1 was induced in all cell lines (Fig. 6A). Notably, it was found that the protein expression level of GLG1 increased in a concentration-dependent manner in the T98G glioma cells (Fig. 6B). It was also observed that the size of the GLG1 protein detected by the GLG1 antibody in the untreated tumor cells was ~145 kDa. However, it was found that the memantine-treated cells expressed two smaller-size GLG1 proteins. More specifically, it was detected that the molecular weight of each band was ~8 and 15 kDa smaller than that of the full-length GLG1 protein (Fig. 6A). It was thus hypothesized that treatment with memantine induced the expression of truncated proteins, such as GLG1 variants 2 and 3 (Fig. 1B).

Subcellular localization of GLG1 protein. Both GLG1 variants 2 and 3 are widely expressed in cells (41). Therefore, it was hypothesized that treatment with memantine may result in alterations in the cellular localization of GLG1, which is typically the Golgi apparatus. The immunohistochemical staining of GLG1 demonstrated that GLG1 was localized in the Golgi apparatus in untreated cells; however, following treatment with memantine, the localization of GLG1 was

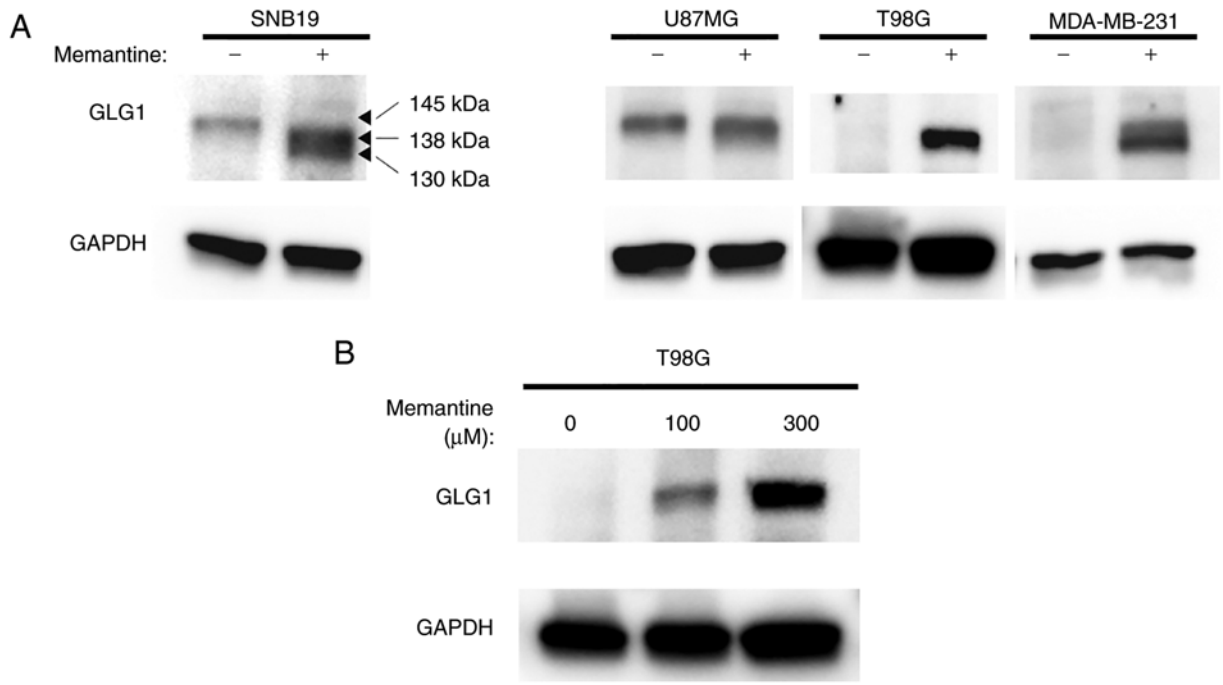

Figure 6. GLG1 protein expression following treatment with memantine. (A) Memantine induced the expression of GLG1 in all cell lines. Memantine-induced GLG1 proteins were smaller than those in untreated cells. Putative variants 2 and 3 were 8 and 15 kDa smaller than the full-length GLG1, respectively. (B) The expression of GLG1 was increased by memantine in a concentration-dependent manner (T98G cells were as a representative cell line). GLG1, Golgi glycoprotein 1.

altered in all cell lines. In particular, it was found that its expression was spread into the cytosol in memantine-treated cells (Fig. 7A). It was further noted that the fluorescence intensity of GLG1 increased following treatment with memantine in a concentration-dependent manner, suggesting an increase in its expression at the protein level (Fig. 7B). In addition, immunofluorescence staining demonstrated that GLG1 was present throughout the cell in memantine-treated SNB19 cells, whereas it was localized near the nucleus in the control cells. GLG1 was observed on the cell surface (Fig. 7C, yellow arrowheads). GLG1 protein detected on the cell surface was considered to be a putative truncated form, consistent with the results of western blot analysis. These results suggest that the memantine-induced increase in the expression of GLG1 and variations in the intracellular distribution of GLG1 play a crucial role in the suppression of tumor growth (Fig. 8).

Discussion

The accumulation of several genetic alterations, such as the loss of tumor suppressor functions and the induction of oncogene functions, results in the transformation of normal cells into cells with highly malignant features (47,48). The deregulation of FGF signaling through the genetic modification or overexpression of FGFs and FGFRs has been observed in numerous tumors, with FGFs playing a key role in tumorigenesis and angiogenesis during tumor growth (28,49,50). There is evidence to indicate that inhibition of FGFR signaling results in anti-proliferative or pro-apoptotic effects (51); thus, an increasing number of drugs against FGF pathways are currently in clinical use (18,23,28).

The high-affinity cell surface FGF receptors belong to a family of receptor tyrosine kinases. Their intracellular tyrosine kinase domain is activated upon ligand binding and induces various intracellular downstream signaling pathways, leading to the positive regulation of cell proliferation. In addition to FGFRs, HSPGs serve as low-affinity receptors for FGFs. It has been suggested that the low-affinity HSPG receptor provides easier access of FGFs to FGFRs, inducing the dimerization of FGFR and the activation of tyrosine kinase inhibitors (52).

Apart from signal-transducing FGFRs, GLG1 is known to bind FGFs. GLG1 was originally identified as an FGF2 receptor, found in the Golgi complex (31). GLG1 binds FGF1, 2, 3, 4 and 18 (30,40); however, it does not have a tyrosine kinase domain, which plays a crucial role in a variety of cellular processes, including growth, motility, differentiation and metabolism.

Therefore, it functions as a so-called 'decoy receptor' that is able to recognize and bind FGFs efficiently; however, it is not structurally able to signal or activate the intended receptor complex. This mechanism is known to regulate the intracellular levels of FGFs (42).

In the present study, it was demonstrated that GLG1 expression was upregulated in a concentration-dependent manner in memantine-treated glioma and breast cancer cells. The behavior of each cell line differed, and this was presumably due to the different reactions in other pathways, such as NMDA-receptor blocking. This phenomenon was observed in three malignant glioma cell lines and one breast cancer cell line. However, other breast cancer cell lines need to be analyzed in the future in order to confirm whether a change in GLG1 expression can be universally detected.

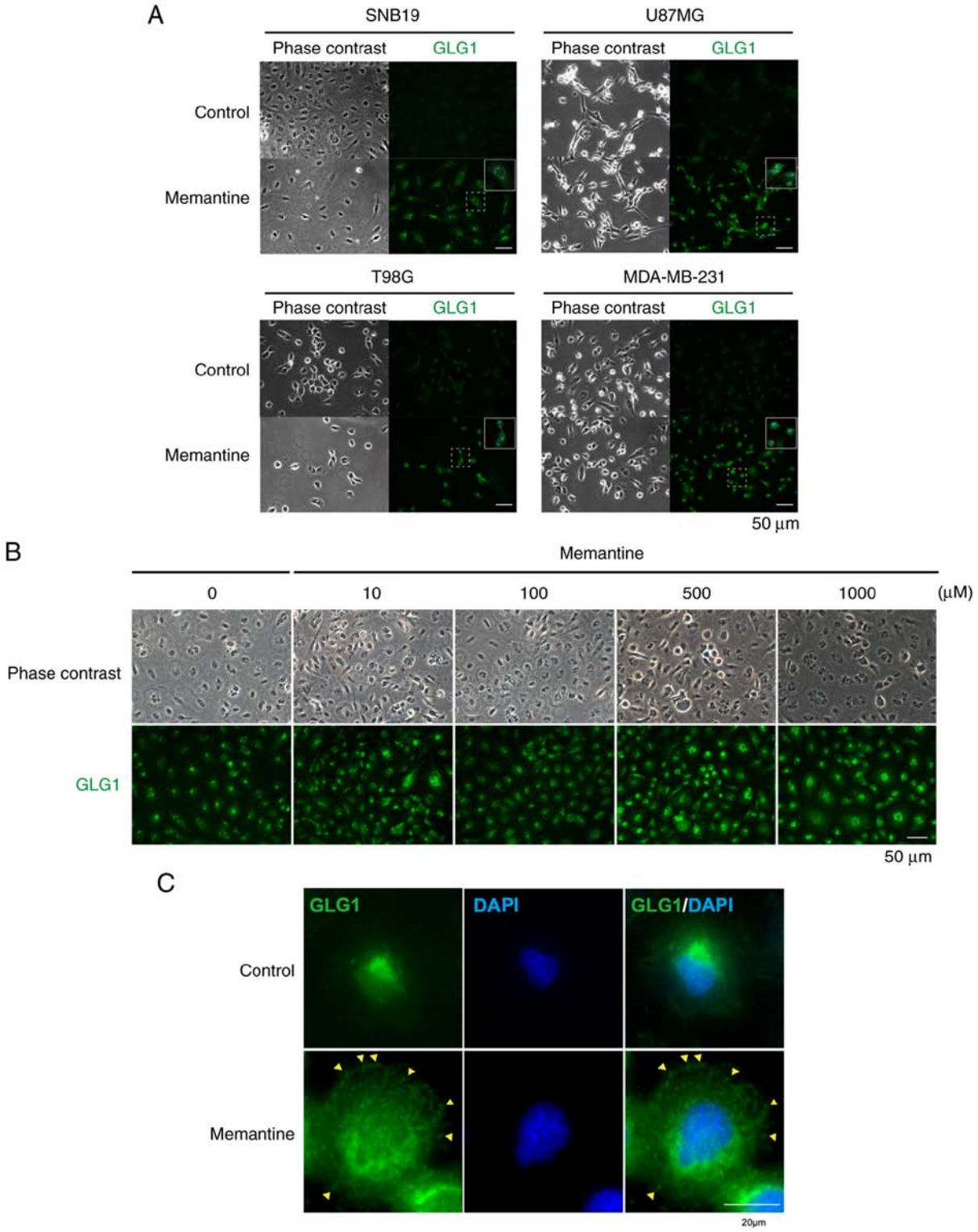

Figure 7. Subcellular localization of GLG1. (A) GLG1 was localized in the Golgi apparatus in untreated cells; however, the localization of GLG1 changed in all cell lines following treatment with memantine. The expression of GLG1 in memantine-treated cells spread into the cytosol. (B) Fluorescence intensity indicating the increase in the level of GLG1 in a memantine concentration-dependent manner, suggesting the increase of its expression at the protein level. (C) Immunohistochemical staining demonstrated that GLG1 was present throughout the cell in memantine-treated SNB19 cells, whereas GLG1 was localized near the nucleus in control cells. GLG1 is displayed on the cell surface (yellow arrowheads). GLG1, Golgi glycoprotein 1.

As GLG1 functions as a decoy to interfere with the tyrosine kinase FGF receptor, this may downregulate the intracellular levels of FGFs, resulting in the loss of proliferative effects, in accordance with the findings of a previous study by the authors reporting that high-grade glioma expressed lower levels of GLG1, whereas low-grade glioma higher levels of GLG1 (53).

Although GLG1 is a 150-kDa integral membrane glycoprotein that is primarily located in the cis-medial Golgi complex, a substantial proportion of GLG1 is secreted (40). Structurally,

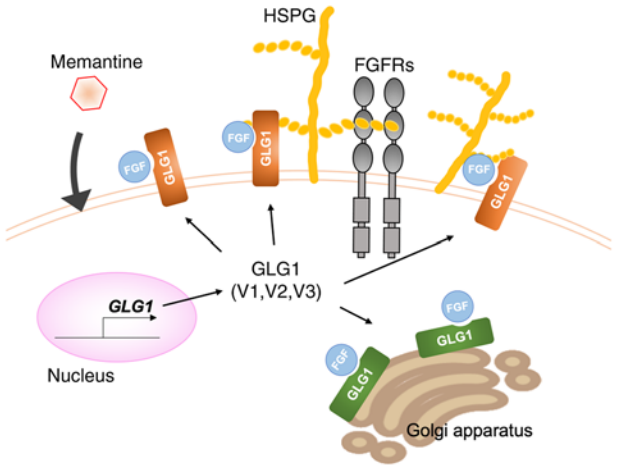

Figure 8. Memantine-induced expression of GLG1 and the putative mechanism of intracellular and extracellular trapping of FGFs. GLG1 expression is upregulated by memantine administration. GLG1 traps FGF in the Golgi apparatus, thus reducing the bioavailability of FGF. Furthermore, GLG1, which is localized on the plasma membrane by memantine treatment and bound to HSPGs, may directly compete with tyrosine kinase receptors (FGFRs) to inhibit FGF binding. GLG1, Golgi glycoprotein 1; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HSPG, heparan sulfate proteoglycan.

compared with variant 1, variant 2 lacks an in-frame coding exon, resulting in the lack of the internal segment of aa 147-157 (Fig. 1B). Conversely, compared with variant 1, variant 3 has an additional segment in the 3' region, resulting in a shorter isoform at the C-terminus, which contains a 14 aa substitution for aa 685-1179 (Fig. 1B). This shorter cytoplasmic segment allows for presentation at the cell membrane, whereas full-length GLG1 is localized in the medial cisternae. These truncated variants are widely distributed in the cell, suggesting that the intraluminal juxtamembrane domain is important for the targeting and retention of GLG1 to the medial Golgis (40).

The present study also found alterations in RNA alternative splicing following the of cancer cells with memantine. RNA splicing is a post-transcriptional process that is estimated to affect the regulation of as many as 60% of all human genes (54). The regulation of alternative splicing is a complex process involving numerous interacting components (55). Of note, alternative splicing has been reported to be associated with principal biological and pathological processes. FGF receptors are subjected to this process and by incorporating different exons into the extracellular binding domain, they are hence affected by both ligand specificity and binding affinity (56-62).

GLG1, a non-tyrosine kinase FGF receptor, is also regulated by RNA splicing (39,41,63). The data of the present study demonstrated that the expression of variants 2 and 3 increased in memantine-treated tumor cells at the mRNA level and putatively at the protein level, in addition to the expression of the GLG1 variant 1. Secreted GLG1 binds to HSPG and traps FGFs, thereby directly competing with tyrosine kinase receptors for FGF binding (40,64). This event inhibits the following dimerization of FGF receptors, thus potentially diminishing the biological availability of FGFs (Fig. 8). The mechanism through which memantine leads to these alterations in alternative splicing remains unknown, and further studies are thus warranted to elucidate this mechanism. To achieve this, factors such as enhancer elements (exonic splicing enhancers and intronic splicing enhancers), activator proteins (SR protein family), silencer elements (exonic splicing silencers and intronic splicing silencers), and repressor proteins (heterogeneous nuclear ribonucleoproteins protein family) need to be analyzed (55).

To date, the mechanism of the tumor growth suppressive effect of memantine has been considered to be attributed to the blockage of the NMDA-receptor activating glutamate. Malignant gliomas and breast cancers are known to exhibit high levels of glutamate, which accelerate cell proliferation following NMDA-receptor stimulation. Therefore, memantine affects tumor cell growth by blocking the NMDA receptor, as reported in a number of studies (2,8,11,13-15,65-67). Albayrak and Demirtas Korkmaz (68) reported that memantine triggered G0/G1 cell cycle arrest; they also examined Caspase-3, Bcl-2 and Bax expression, revealing a change in apoptotic gene expression (69). The present study suggested another mechanism through which the induction of the expression of GLG1 and the generation of additional truncated variants may suppress the FGF and FGFR pathways. The present study revealed the GLG1 expression was altered by memantine. However, the association of this change with cancer proliferation remains unknown.

The safety of memantine has been demonstrated in a randomized, placebo-controlled clinical trial in patients with mild-to-moderate vascular dementia (70). As a result, memantine has been approved for the treatment of moderate-to-severe Alzheimer's disease (71). By contrast, there have been reports on the risk of somnolence, weight gain, confusion, hypertension, nervous system disorders and falling due to the administration of memantine, although it has been shown to be beneficial for patients with Alzheimer's disease as regards the improvement of cognition (72). Therefore, further studies are required to evaluate the clinical use of this medicine for cancer treatment from a safety standpoint. Two clinical trials for glioblastoma are registered in the USA: 'A phase II study of memantine in the treatment of recurrent glioblastoma' (NCT01260467) and 'Temozolomide, memantine hydrochloride, mefloquine, and metformin hydrochloride in treating patients with glioblastoma multiforme after radiation therapy' (NCT01430351). The former trial was terminated, and no adverse effect was reported. The latter phase I trial is currently ongoing, and the results are not available at this moment.

The present study observed a change in the protein and mRNA expression of GLG1. The function of GLG1 and its different variants has been reported in several studies (35,40-42,45,53,63,73). The aim of the present study was to report these phenomena. Determining the extent of the effects of GLG1 on cell proliferation may be the following step in examining its effects under memantine exposure.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FY designed the experiments. FY, SH and SK performed the experiments. TA contributed to sample preparation. FY, SH and YO performed data analyses. SH helped interpret the results and was involved in the preparation of the manuscript. FY wrote the manuscript in consultation with SH and YO. All authors critically revised the manuscript, commented on drafts of the manuscript, and have read and approved the final draft. FY and SH were equal contributors to the study and confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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