

Antitumor effect of lenalidomide in malignant glioma cell lines

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Abstract. Glioblastoma is a malignant brain tumor exhibiting highly aggressive proliferation and invasion capacities. Despite treatment by aggressive surgical resection and adjuvant therapy including temozolomide and radiation therapy, patient prognosis remains poor. Lenalidomide, a derivative of thalidomide, is known to be an immunomodulatory agent that has been used to treat hematopoietic malignancies. There are numerous studies revealing an antitumor effect of lenalidomide in hematopoietic cells, but not in glioma cells. The present study aimed to demonstrate the antitumor effect of lenalidomide on malignant glioma cell lines. The growth inhibition of malignant glioma cells (A-172, AM-38, T98G, U-138MG, U-251MG, and YH-13) by lenalidomide was assessed using a Coulter counter. The mechanism of the antitumor effect of lenalidomide was examined employing a fluorescence-activated cell sorter, western blot analysis, and quantitative real-time reverse transcriptional polymerase chain reaction (RT-qPCR) in malignant glioma cell lines (A-172, AM-38). The results revealed that the number of malignant glioma cells was decreased in a concentration-dependent manner by lenalidomide. DNA flow cytometric analysis demonstrated an increase in the ratio of cells at the G₀/G₁ phase following lenalidomide treatment. Western blot analysis and RT-qPCR revealed that p53 activation and the expression of p21 were increased in glioma cells treated with lenalidomide. Western blot analysis revealed that cleavage of PARP did not occur; however, increased expression of Bax protein, cleavage of caspase-9 and cleavage of caspase-3 were confirmed. Analysis

by FACS also supported the conclusion that little apoptosis induction occurred following lenalidomide treatment of malignant glioma cell lines. In conclusion, lenalidomide exerts an antitumor effect on glioma cells due to alterations in cell cycle distribution.

Introduction

Glioblastoma is the most common primary tumor of the central nervous system in adults. Despite treatment including maximal surgical resection, radiotherapy and temozolomide chemotherapy, overall survival (OS) of patients remains at 14.6 months (1,2). Although several clinical trials have emerged as potential novel treatments, there have been few advances in treatment options during the last 3 decades.

Recently, drug repurposing has been successful in various different therapeutic fields, including those involving aspirin, sildenafil, erythromycin, minoxidil and thalidomide. Thalidomide was originally used for the treatment of morning sickness in the 1950s. However, in 1961, it was banned because of potential teratogenicity such as phocomelia. In 1999, thalidomide was found to exert an antitumor effect on multiple myeloma (3). Subsequently, it was again used and the side effects are now considered acceptable if the thalidomide is administered to patients with a life-threatening condition, such as multiple myeloma (4-7).

On the other hand, lenalidomide is a thalidomide derivative and was developed in order to reduce the side effects and enhance the antitumor effect of thalidomide. Clinically, the antitumor effect of lenalidomide was shown to be more effective than that of thalidomide in hematopoietic malignancies (8-12). Recently, lenalidomide has been widely used clinically as an antitumor drug for hematopoietic malignancies including multiple myeloma and myelodysplastic syndrome (13-15). Lenalidomide has been reported to display a multiple antitumor effect including immunological activity, an inhibitory effect on angiogenesis, a growth inhibitory effect and induction of apoptosis (16). The main antitumor mechanism of lenalidomide has been proposed to involve induction of growth arrest due to an increased expression of

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p21 (16). It was reported recently that cell cycle arrest induced by p21 is related to cereblon protein as a primary target of lenalidomide (17), and cereblon expression is required for the antitumor activity of lenalidomide (18).

Thalidomide has been previously reported to exert an antitumor effect on glioma cells. Several clinical trials since then have revealed an effect of thalidomide on malignant glioma patients (19-21). However, these trials failed to demonstrate an extended median survival time. An antitumor effect of lenalidomide in glioblastoma can also be expected, as lenalidomide is superior to thalidomide in cases of hematopoietic malignancies clinically. Furthermore, lenalidomide has a low molecular weight of 259.26 g/mol and can therefore pass through the blood-brain barrier. Lenalidomide may display an antitumor effect by prolonging progression-free survival and overall survival of patients with glioblastoma, and Phase I trials using lenalidomide for central nervous system tumors were reported in 2007 and 2011 (22,23). In these studies, lenalidomide was well tolerated in patients including children with recurrent central nervous system tumors, although the toxicity due to lenalidomide mainly involved myelosuppression and thromboembolism. Preliminary data indicated that the effect of lenalidomide was favorable. However, its effect on malignant glioma cells has remained controversial. In the present study, we aimed to elucidate the antitumor effect of lenalidomide on malignant glioblastoma cells.

Materials and methods

Materials. Lenalidomide (Celgene Corp.) was employed for the experiments. Lenalidomide was dissolved in DMSO, which was used as a control at corresponding concentrations.

Cell lines and culture. Human malignant glioma cell lines A-172 (cell no. JCRB0228; lot no. 021999), AM-38 (cell no. IFO05492; lot no. 12082003), T98G (cell no. IFO50303; lot no. 1007), U-251MG (cell no. IFO 50288; lot no. 12132002), and YH-13 (cell no. IFO50493; lot no. 1164) (purchased from Health Science Research Resources Bank), and U-138MG (cat. no. HTB-16; lot no. 1104428; purchased from American Type Culture Collection) were employed in the present study. These cells were regularly cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical) supplemented with 5% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc.) using plastic flasks (Corning) in a humidified incubator at 37°C with an atmosphere containing 5% CO₂, as described previously (24).

Coulter counter assays. The growth inhibition of malignant glioma cells by lenalidomide was evaluated by counting the numbers of cells using a Z1 Coulter Counter® (Beckman Coulter). Each well was seeded at 1x10⁴ cells in 24 plates and cultured for 24 h. The cells were incubated in the medium with 0.1, 1, 10 and 100 μM of lenalidomide, respectively. After 72 h of exposure to the various concentrations of lenalidomide, the cells were trypsinized and counted with the cell Coulter counter. The experiments were repeated 4 times at each concentration.

Furthermore, to evaluate the underlying mechanisms of the antitumor effect of lenalidomide, we undertook further experiments employing A-172 and AM-38 human glioma cell lines. The cells were incubated for 24 h to achieve attachment and exposed to lenalidomide at a concentration of 10 μM in the new medium.

Cell cycle distribution analysis. A-172 and AM-38 cells were harvested using 0.25% trypsin-EDTA solution (Invitrogen; Thermo Fisher Scientific, Inc.) after 0, 8, 24, 48 and 72 h of lenalidomide treatment, fixed in ice-cold 70% of ethanol, and treated with 0.5% of RNase A (Roche Diagnostics). The treated cells were stained with 1 μg/ml propidium iodide (PI) (Miltenyi Biotec) for 30 min. The fluorescence was measured with a FACS-Calibur flow cytometer (BD Biosciences) at a wavelength of 610 nm (FL3). The DNA histograms were analyzed employing FlowJo software (v.9.9.6; BioLegend, Inc.).

Determination of apoptotic cells. The apoptosis induced by lenalidomide was evaluated by flow cytometry. The cells were incubated in the medium with 0.1, 1, 10 and 100 μM of lenalidomide, respectively. Following lenalidomide treatment, the cells were harvested using 0.25% trypsin-EDTA solution. After rinsing twice with phosphate-buffered saline (PBS) (-) (Nissui Pharmaceutical), the fixed cells were treated with RNase A for 1 h, and propidium iodide (PI) and Annexin V Alexa Flour 488 conjugate (Thermo Fisher Scientific, Inc.) were added. Flow cytometry was performed with a FACS-Calibur flow cytometer (BD Biosciences). The fluorescence was detected with FL1 (510 nm) and FL3 (610 nm). The apoptosis was analyzed employing FlowJo software (BioLegend, Inc.).

Western blot analysis. Soluble protein lysates of sub-confluent glioma cells were obtained using radioimmunoprecipitation assay (RIPA) buffer (Wako Pure Chemical) containing protease inhibitors (Compleat Mini, EDTA-free, Roche Diagnostics) for 20 min on ice. After centrifugation at 20,400 x g for 1 h at 4°C, the protein content of the separated supernatants was determined by BCA assay employing a Pierce BCA protein assay kit (Thermo Scientific, Inc.). The protein (100 μg proteins) was loaded and separated by 12% polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories) for 1 h at 15 V with a Bio-Rad transblot (Bio-Rad Laboratories). The membrane was treated with blocking buffer (PBS/0.05% containing 1% skimmed milk) for 1 h at room temperature, and subsequently treated with primary antibodies (Table I) in new blocking buffer for 24 h at 4°C. After washing the membrane with washing buffer (PBS/0.05% Tween 20), treatment with the secondary antibody (Table I) conjugated with horseradish peroxidase was undertaken for 1 h in room air. The transferred membrane was washed 3 times with washing buffer and visualized using LAS-4000 (GE Healthcare) after treatment with ECL Prime Western Blotting detection reagent (dilution 1:1,000) (GE Healthcare). The proteins (Bax and cleaved PARP in A-172 cells, caspase-9 and caspase-8 in A-172 cells, and cleaved PARP and caspase-8 in AM-38 cells) were probed from the same membranes.

Table I. Primary and secondary antibodies for the western blot analysis.

Antibody name	Species	Dilution	Supplier
Primary antibody			
Anti-cereblon antibody	Rabbit polyclonal	1:1,000	Proteintech
Anti-p53 antibody	Mouse monoclonal	1:500	Santa Cruz Biotechnology
Anti-p-p53 antibody	Mouse monoclonal	1:500	Santa Cruz Biotechnology
Anti-p21 antibody	Mouse monoclonal	1:500	Santa Cruz Biotechnology
Anti-Bcl-2-associated x protein (Bax) antibody	Mouse monoclonal	1:500	Santa Cruz Biotechnology
Anti-caspase-9 antibody	Mouse monoclonal	1:1,000	Cell Signaling Technology
Anti-caspase-3 antibody	Mouse monoclonal	1:500	Santa Cruz Biotechnology
Anti-caspase-8 antibody	Mouse monoclonal	1:1,000	Cell Signaling Technology
-β-actin antibody	Mouse monoclonal	1:2,000	Wako Pure Chemical Industries, Ltd.
Anti-cleaved PARP antibody	Rabbit polyclonal	1:500	Cell Signaling Technology
Secondary antibodies			
Conjugated anti-mouse IgG peroxidase			Sigma-Aldrich; Merck KGaA
Conjugated anti-rabbit IgG peroxidase			Cell Signaling Technology

Quantification of mRNA by real-time quantitative reverse transcriptional-PCR (RT-qPCR). Cells were cultured in 25 cm² collagen-coated flasks. After treatment with 10 μM lenalidomide, the total RNA was extracted from the cells by employing a RNeasy Mini kit (Qiagen, Inc.). RT-qPCR was performed on a Step-One Real-time PCR system (Applied Biosystems) with SYBR-Green PCR Mix (Toyobo). The PCR conditions were as follows: 1st stage, 90°C for 30 sec, 61°C for 20 min, and 95°C for 1 min; 2nd stage, 45 cycles at 95°C for 15 sec, 55°C for 15 sec and 74°C for 45 sec; and 3rd stage, 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Glyceraldehyde-3-phosphatase dehydrogenase (*GADPH*) mRNA expression levels were employed as the quantitative internal control. The expression levels were calculated employing the following equations by comparing the threshold cycle (Cq): ΔCq=Cq of p21 or p53-Cq of GAPDH, ΔΔCq (target cell line)-ΔCq (reference cell line), and ratio=2^{-ΔΔCq} (25). Fold changes of the related mRNA expression were calculated by using the comparative 2^{-ΔΔCq} method to normalize the mRNA level. The sequences of primers that were utilized for the PCR are shown in Table II.

Statistical analysis. All studies were carried out more than 3 times. The resultant data are expressed as the means ± SE (standard error) and were considered significant at P<0.05 or P<0.01. The statistical significance was evaluated by the Student's t-test and one-way analysis of variance (ANOVA) with Tukey's post hoc test. Data analyses were performed employing the statistical software IBM SPSS statistics v.21.0 (International Business Machines Corporation).

Results

Coulter counter assay of glioma cells exposed to lenalidomide. To evaluate the antitumor effect of lenalidomide, the six malignant glioma cell lines (A-172, AM-38, T98 G, U-138MG, U-251MG, and YH-13) were incubated in medium

with 0.1, 1.0, 10.0 and 100.0 μM of lenalidomide, respectively. Lenalidomide inhibited the cell counts of all malignant glioma cells in a concentration-dependent manner (Fig. 1). The cell growth inhibition effect tended to be confirmed at 10 and 100 μM exposure to lenalidomide.

Effects of lenalidomide on the proliferation of A-172 and AM-38 cells. Hallmarks of cell cycle arrest including p53, p-p53 and p21 were determined in the glioma cells treated with lenalidomide using western blot analysis (Fig. 2A). The expression levels of p-p53 and p21 protein were increased at 4 h of treatment, while the expression of p53 protein was unchanged in the A-172 cells. In AM-38 cells, the expression of p-p53 protein was increased at 8 h of treatment, while the expression of p53 protein was decreased at 8 h of treatment and increased at 24 h of treatment. The expression of p21 protein was increased at 24 h after lenalidomide treatment (Fig. 2A). The RT-qPCR data showed that the *p53* and *p21* mRNA expression levels were significantly increased in the A-172 and AM-38 cells receiving 3 h of lenalidomide treatment (Fig. 2B). DNA flow cytometric analysis demonstrated a significantly increased population of cells in the G₀/G₁ phase at 24 h after treatment with lenalidomide. These findings indicated that alterations in the cell cycle distribution had occurred in the A-172 and AM-38 cells (Fig. 3A and B).

Expression of cereblon. Furthermore, to evaluate the mechanism underlying the cell cycle arrest induced by lenalidomide, we examined the expression of cereblon protein which is considered the primary target of lenalidomide. Cereblon protein was expressed in all cell lines, but the degree of expression was varied (Fig. 4). There was no obvious relationship between the expression level of cereblon and inhibition of malignant glioma due to lenalidomide.

Effects of lenalidomide on the apoptosis of the A-172 and AM-38 cells. To assess the apoptosis induced by lenalidomide,

Table II. Primer sets.

Gene	Primer
<i>GAPDH</i>	Forward; 5'-CAGAACATCATCCCTGCCTCT-3' Reverse; 5'-GCTTGACAAAGTGGTCGTTGAG-3'
<i>p53</i>	Forward; 5'-GGCCCACTTCACCGTACTAA-3' Reverse; 5'-GTGGTTTCAAGGCCAGATGT-3'
<i>p21</i>	Forward; 5'-TGGAGACTCTCAGGGTCGAAA-3' Reverse; 5'-GGCGTTTGGAGTGGTAGAAATC-3'
<i>Bax</i>	Forward; 5'-TTTGCTTCAGGGTTTCATCC-3' Reverse; 5'-CAGTTGAAGTTGCCGTCAGA-3'
<i>Caspase-9</i>	Forward; 5'-TTCCAGGTTTTGTTTCCTG-3' Reverse; 5'-CCTTTCACCGAAACAGCATT-3'
<i>Caspase-3</i>	Forward; 5'-AGAAGTGGACTGTGGCATTGAG-3' Reverse; 5'-GCTTGTCCGCATACTGTTTCAG-3'

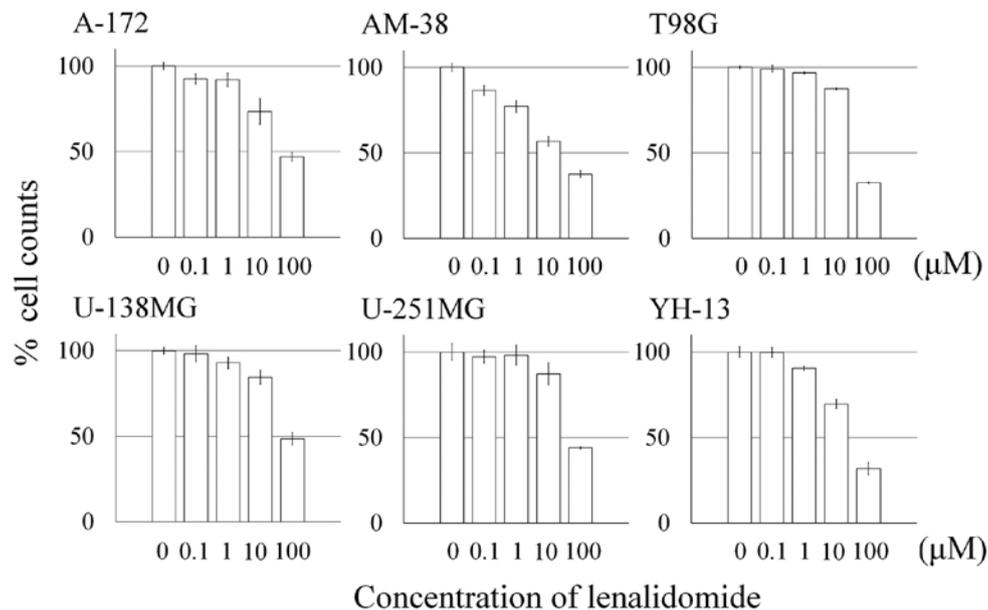


Figure 1. Percentage of the cell counts of glioma cells following treatment with lenalidomide. Six malignant glioma cell lines (A-172, AM-38, T98G, U138MG, U-251MG and YH-13) were incubated in medium with 0.1, 1.0, 10.0 and 100.0 μM of lenalidomide, respectively. After 72 h of exposure to the various concentrations of lenalidomide, the cells were trypsinized and counted using a Z1 Coulter Counter. The number of all malignant glioma cells was inhibited in a concentration-dependent manner. Lenalidomide exerted an antitumor effect in the malignant glioma cell lines, and in most cell lines, this antitumor effect was confirmed at a concentration of $>10 \mu\text{M}$. The results are expressed as the means \pm SE.

the expression levels of apoptotic hallmark proteins including Bax, caspase-9, caspase-3, cleaved poly-ADP-ribose polymerases (PARP) and caspase-8 were evaluated by western blot analysis. The data showed that there was little induction of apoptosis in the A-172 and AM-38 cells (Fig. 5). No band of cleaved PARP was detected, although the apoptotic hallmarks of the intrinsic pathway such as Bax and caspase-9 were accumulated over time following treatment with 10 μM lenalidomide. Cleaved caspase-9 and cleaved caspase-3 were accumulated during the same time course. The apoptotic hallmarks of the extrinsic pathway such as caspase-8 and cleaved caspase-8 remained unaffected (Fig. 6). The induction of apoptosis by lenalidomide in A-172 and AM-38 cells was evaluated by flow cytometry using Annexin V/PI double staining.

This analysis revealed no increased population of early-stage apoptosis or late-phase apoptosis. After 24, 48, and 72 h of treatment with 0.1, 1.0, 10.0, and 100.0 μM of lenalidomide in the medium, the FACS data indicated that little apoptosis had occurred (Fig. 7).

Discussion

The antitumor activity of lenalidomide has been previously reported in hematopoietic cells. Kuramitsu *et al* demonstrated an effect of lenalidomide on the immune system *in vitro* and *in vivo* (26). Lenalidomide exerts an antitumor effect due to enhancement of the cytotoxic effect in T cell interferon- γ secretion. Kuhnol *et al* reported that the immune reaction

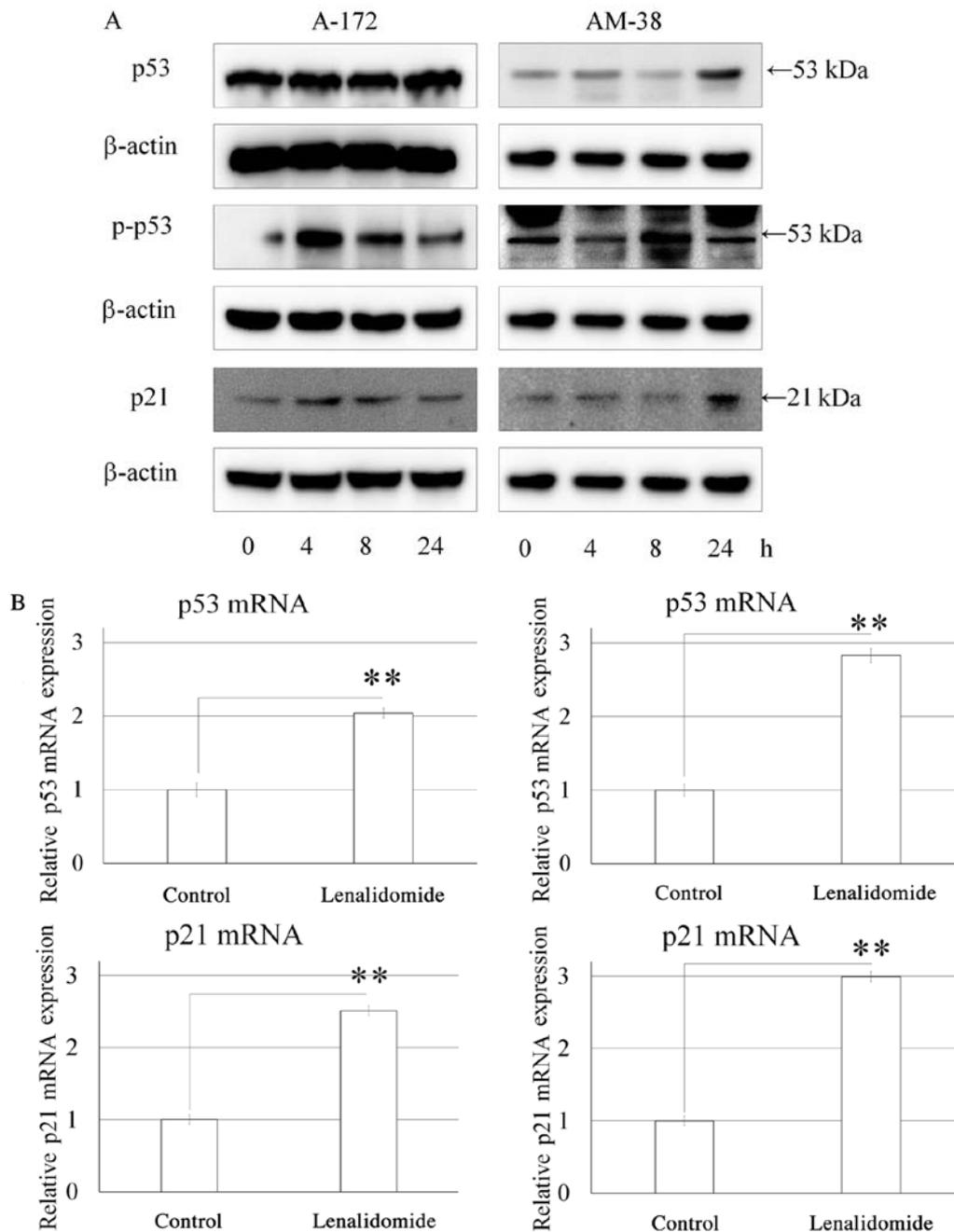


Figure 2. Effects of lenalidomide on the proliferation of A-172 and AM-38 glioma cell lines. (A) Proteins related to cell cycle arrest hallmarks including p53, phosphorylated (p)-p53 and p21 were investigated by western blot analysis at 0, 4, 8 and 24 h after treatment with lenalidomide, respectively. The expression levels of p-p53 and p21 proteins were increased at 4 or 8 h of treatment, while the expression of p53 protein remained unchanged in A-172 cells. The expression of p53 protein was decreased, and the expression of p-p53 protein was increased at 8 h of treatment in AM-38 cells, while the expression of p21 protein was increased at 24 h after lenalidomide treatment in the AM-38 cells. (B) The mRNA expression of *p53* and *p21*, related with cell cycle arrest, was investigated by RT-qPCR. The data obtained showed that *p53* and *p21* mRNA was overexpressed at 3 h after lenalidomide treatment. *GAPDH* was employed as the internal control. The relative expression level of the genes was calculated using $2^{-\Delta\Delta Cq}$ method. The results are expressed as the means \pm SE. ** $P < 0.01$ (Student's t-test).

was enhanced by lenalidomide in *in vitro* models (27). These studies evaluated the antitumor effect of immune cells induced by lenalidomide. However, no effect of lenalidomide on malignant glioma cells has yet been confirmed. We focused on the direct antitumor effects of lenalidomide, such as its influence on the cell cycle and induction of apoptosis in glioma cells. Much of the mechanisms underlying the antitumor effect in glioma cells remains unknown. It was newly discovered that lenalidomide showed a direct antitumor effect of cell cycle arrest in malignant glioma cell lines.

In the Coulter counter assays, the number of malignant glioma cells was decreased in a concentration-dependent manner. In most cell lines, an antitumor effect of lenalidomide was confirmed at a concentration of 10 μ M.

We examined the mechanisms underlying the antitumor effect of lenalidomide using A-172 and AM-38 glioma cell lines. The glioma cells were exposed to lenalidomide, and we evaluated the expression levels of various proteins employing western blot analysis, flow cytometric analysis and mRNA expression analysis using the RT-qPCR to

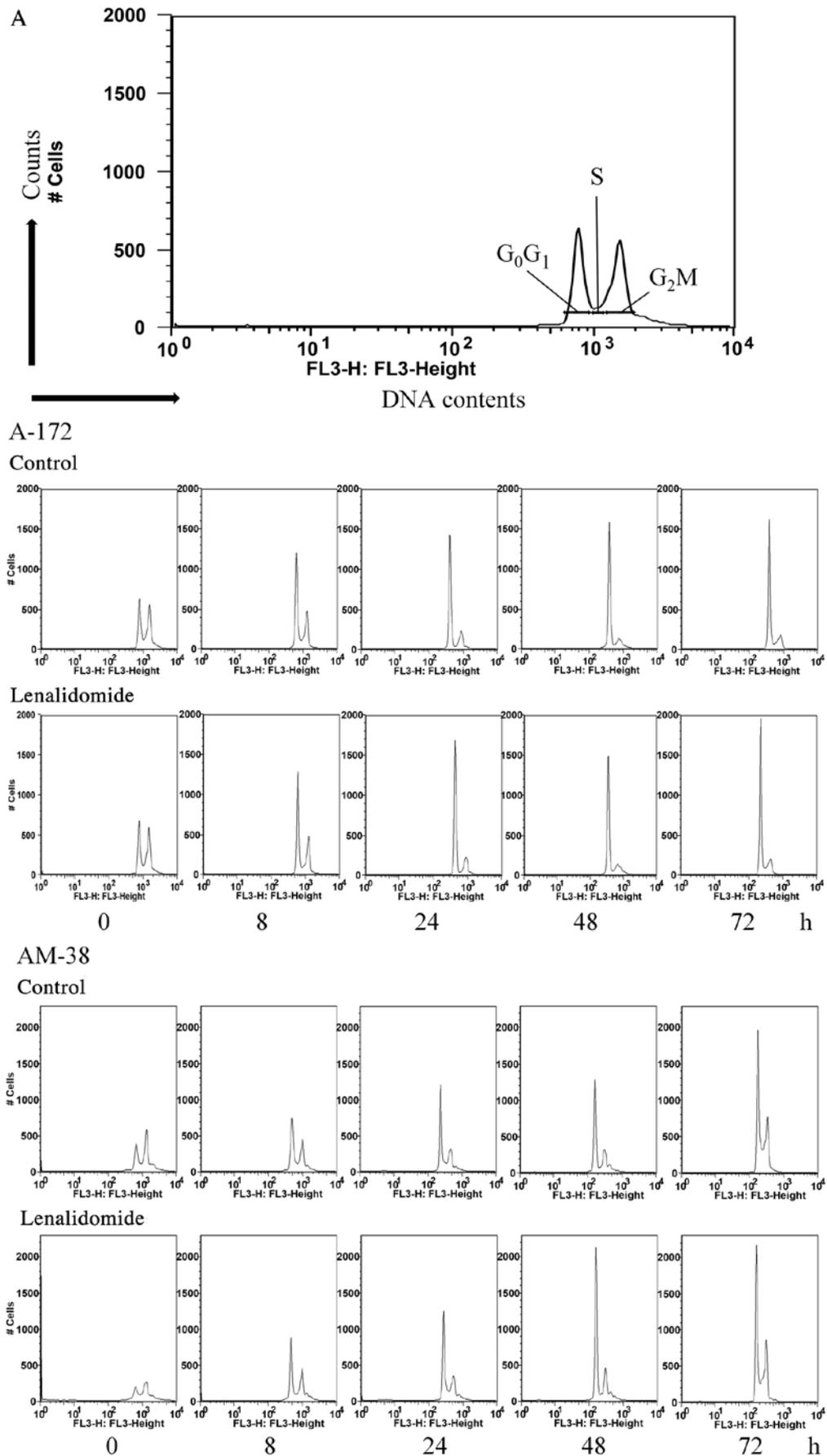


Figure 3. (A and B) Cell cycle distribution analysis of A-172 and AM-38 cells treated with 10 μ M of lenalidomide at 0, 8, 24, 48, and 72 h. A-172 and AM-38 were used for this experiment. These cells were treated with 10 μ M of lenalidomide for 0, 8, 24, 48, and 72 h. A group treated with the same amount of DMSO as that in which the lenalidomide was dissolved was used as the control. The histogram data revealed an increase in population of cells in the G₀/G₁ phase following lenalidomide treatment (* P <0.05, ** P <0.01).

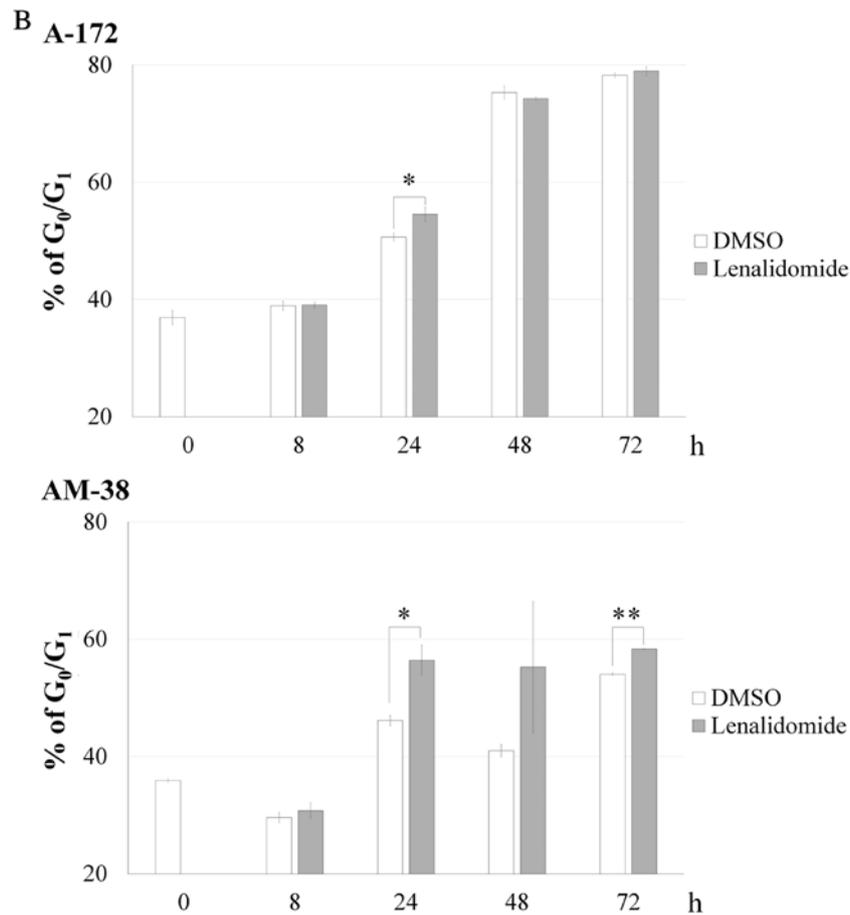


Figure 3. Continued. (A and B) Cell cycle distribution analysis of A-172 and AM-38 cells treated with 10 μ M of lenalidomide at 0, 8, 24, 48, and 72 h. A-172 and AM-38 were used for this experiment. These cells were treated with 10 μ M of lenalidomide for 0, 8, 24, 48, and 72 h. A group treated with the same amount of DMSO as that in which the lenalidomide was dissolved was used as the control. The histogram data revealed an increase in population of cells in the G₀/G₁ phase following lenalidomide treatment (*P<0.05, **P<0.01).

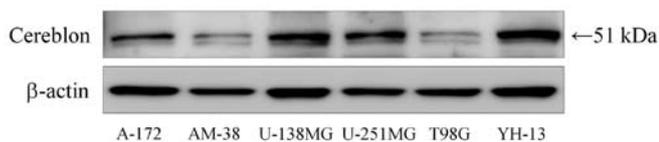


Figure 4. Expression of cereblon. The expression of cereblon in six malignant glioma cells (A-172, AM-38, T98G, U138MG, U-251MG and YH-13) was evaluated by western blot analysis. β -actin was employed as the internal control. Cereblon proteins were expressed in all the glioma cells.

observe alterations occurring in cell cycle distribution and apoptosis.

The concentration utilized for our experiments was 10 μ M, since our results indicated that the antitumor effect of lenalidomide was observed at a concentration less than 10 μ M. In addition, Warren's phase I trial demonstrated that the safety dose of lenalidomide was in the range of 2.5 to 116 mg/m² (23). Following administration of 20 mg/m² of lenalidomide, the blood concentration in the body rises to approximately 2.2 μ M with a peak at 1 h (28). We inferred that 10 μ M of lenalidomide was reasonable for clinical use without harmful side effects.

In previous research concerning lenalidomide employing hematopoietic cancer cells, various antitumor effects including a growth inhibition effect, induction of apoptosis,

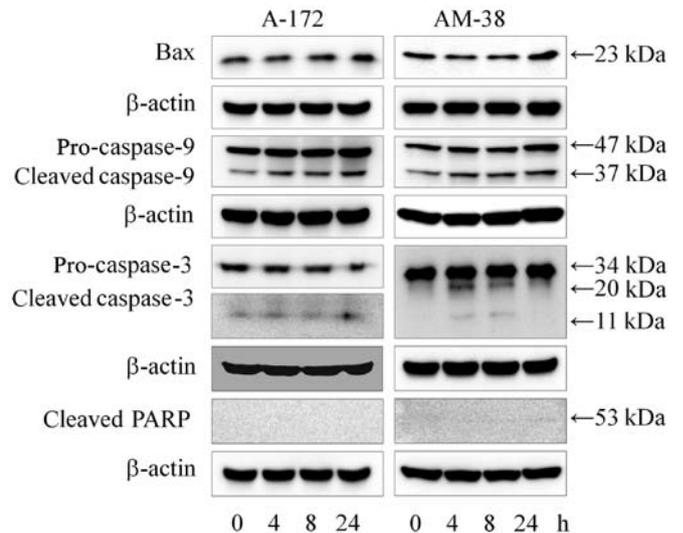


Figure 5. Effects of lenalidomide on the intrinsic pathway of apoptosis. Proteins related with the intrinsic apoptotic pathway hallmarks including Bax, caspase-9 and caspase-3 were investigated by western blot analysis at 0, 4, 8, and 24 h after lenalidomide treatment, respectively. The data obtained revealed that the expression of Bax protein was increased at 24 h in the A-172 and AM-38 cells. Cleavage of caspase-9 protein was increasingly expressed in the time series following 4, 8, and 24 h of treatment. The expression of caspase-3 protein cleavage was increased at 24 h of treatment in the A-172 cells, and increased at 4 and 8 h of treatment in the AM-38 cells. However, cleavage of PARP was not confirmed. PARP, poly(ADP-ribose) polymerase.

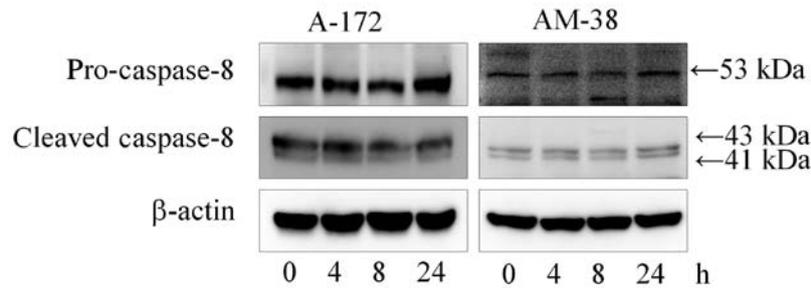


Figure 6. Effects of lenalidomide on the extrinsic pathway of apoptosis. Proteins related with the extrinsic apoptotic pathway hallmarks including caspase-8 were investigated by western blot analysis at 0, 4, 8, and 24 h after lenalidomide treatment, respectively. The data obtained revealed that the expression of caspase-8 protein and degree of cleavage remained unchanged until 24 h after lenalidomide treatment.

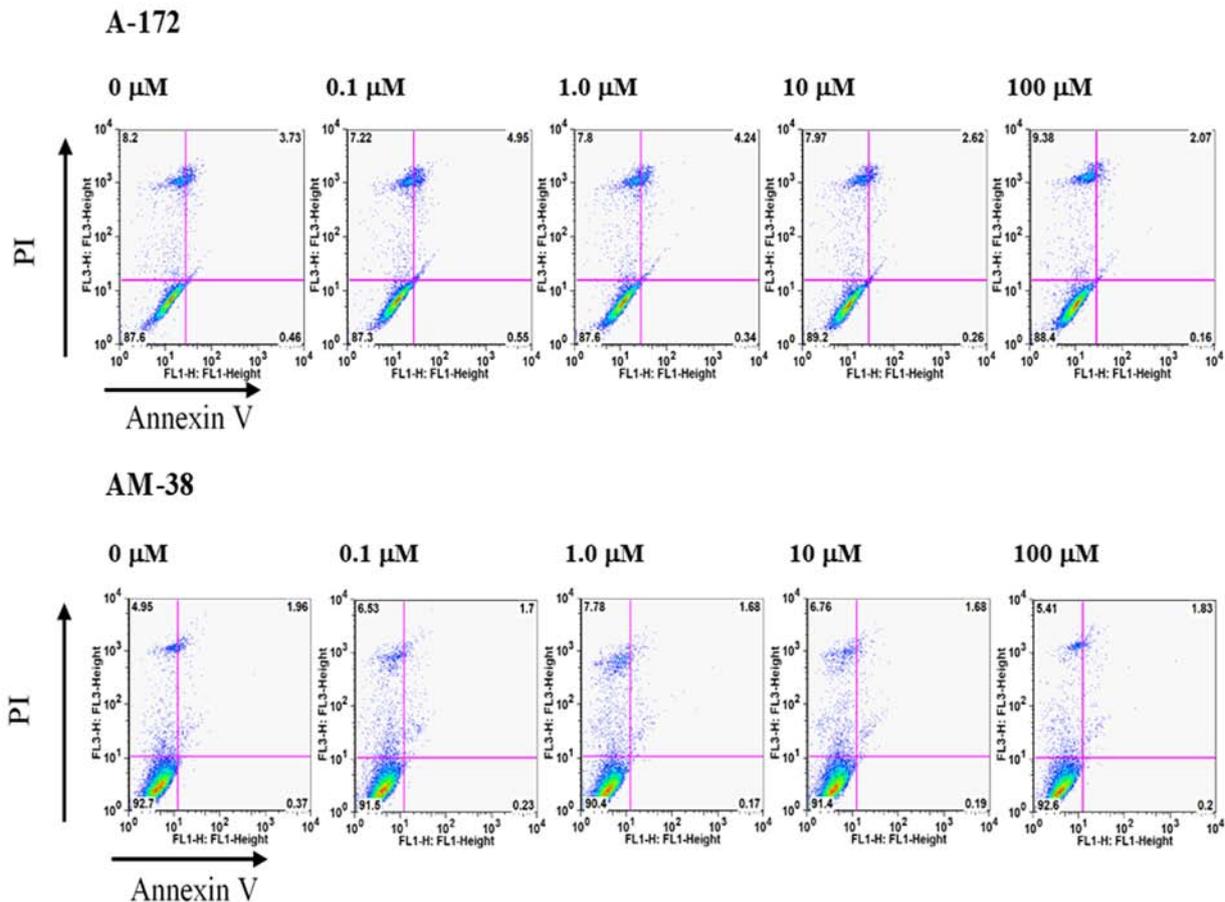


Figure 7. Assessment of apoptotic cells. The induction of apoptosis by lenalidomide in A-172 and AM-38 cells was evaluated by flow cytometry using Annexin V/PI double staining. The data obtained revealed that there were no marked changes after 72 h of lenalidomide treatment, in the medium without lenalidomide and with 0.1, 1.0, 10.0 and 100.0 μ M of lenalidomide, respectively. These findings indicated that little apoptosis had occurred.

anti-angiogenic activity and immunological activity were observed (16,29). In addition, induction of apoptosis was found to be related to caspase-3 activation. In addition to these direct antitumor effects, anti-angiogenesis mediated by suppression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) was described (29,30).

In glioma cells, we confirmed that phosphorylated p53 and functional p53 were activated after 4 h of lenalidomide treatment based on western blot analysis. In the present study, we investigated whether the p53/p21 pathway was activated after treatment with lenalidomide, and it was found that increased p53 and p21 protein expression induced cell cycle arrest in

the glioma cells. Fecteau *et al* and Escoubet-Lozach *et al* reported that activation of p21 occurred after treatment with lenalidomide; however, functional p53 was not correlated with p21 activation in multiple myeloma cells and chronic lymphocytic leukemia cells (18). In hematopoietic cancer cells, the expression of p21 protein was increased even when p53 protein was knocked out and loading with lenalidomide treatment was undertaken (31). These observations indicated that the activation of p21 and functional p53 may be independent in hematopoietic cancer cells. In glioma cells, it seemed difficult to conclude that p21 and p53 were dependent; however, lenalidomide did induce an increased expression

of p21, p53, and phosphorylated p53 and cell cycle arrest occurred.

We found that cereblon protein was expressed in all six glioma cell lines. Recently, it was reported that cereblon is a target protein for thalidomide, lenalidomide, and its derivatives (32-34). In hematopoietic malignancies, it was suggested that the expression level of cereblon regulates the antitumor effect via p21 (18). We investigated the expression of cereblon in glioma cells employing western blot analysis. It was difficult to establish the precise relationship between the expression level of cereblon and the antitumor effect. In order to investigate whether cereblon plays a crucial role in the antitumor effect mediated by lenalidomide, research using a glioma cell line with knockdown of cereblon will be carried out.

However, cereblon, a key target protein for lenalidomide, was expressed in the glioma cells. Previous studies have shown that lenalidomide directly binds cereblon and promotes the recruitment of Ikaros (IKZF1) and Aiolos (IKZF3) to the E3 complex containing cereblon. The complex of cereblon with IKZF1 and IKZF4 leads to ubiquitylation and degradation (35-37). In the future, we believe that it will also be necessary to investigate the protein levels of IKZF1 and IKZF3.

We were unable to confirm that lenalidomide exhibited apoptosis-inducing effects in the glioma cells. Previously, it was demonstrated that lenalidomide induced apoptosis in multiple myeloma cells (38). In particular, lenalidomide activated the intrinsic pathway in mantle cell lymphoma cells (39). We hypothesized that lenalidomide may induce apoptosis in glioma cells as well, and investigated the expression changes in apoptosis-related proteins using western blot analysis. However, in the glioma cells, apoptosis was not clearly observed. Western blot analysis showed that apoptotic hallmarks of the intrinsic pathway such as Bax, cleaved-caspase-9, and cleaved-caspase-3 were increased in the A-172 and AM-38 cells. Although the proteins related to intrinsic apoptosis were activated, cleavage of PARP was not accumulated. In addition, there was no change in the expression of the extrinsic pathway such as caspase-8 during the treatment time courses. The FACS data revealed that only a few glioma cells were induced to undergo apoptosis. Lenalidomide is known to be an immunomodulatory agent that has been used to treat hematopoietic malignancies (8-12). It has been frequently reported that lenalidomide exhibits an antitumor effect in hematopoietic cells, but in glioma cells, the situation remains more controversial. The present study was designed to investigate the direct antitumor effect of lenalidomide on malignant glioma cell lines including cell cycle arrest and apoptosis induction. The findings of previous studies indicated that lenalidomide exerted an antitumor effect on gliomas especially enhancement of immune reactions such as interferon secretion from T cells (26,27).

We used a Coulter counter for cell counts. However, the Coulter counter evaluates only the number of cells present, not the viable cells. For proliferation or cell viability assays, the MTT and ATP assays should be employed. We plan to perform further studies using these assays. Our findings were not sufficient to yield a conclusive picture concerning the mechanism of action, and *in vivo* experiments will also need to be undertaken in the future. Additionally, the effective concentration for an antitumor effect remains to be further

evaluated in *in vivo* experiments. In the present study, we examined the antitumor effects of single-use lenalidomide, but not any combination use involving chemotherapy such as with alkylating agents. We need to confirm whether the effect of lenalidomide in combination use may be more effective in the case of glioma cells. Lenalidomide is becoming a key drug in the guidelines for the treatment of hematopoietic cancer including multiple myeloma (15). Several studies have shown that the combination use of lenalidomide with alkylating agents or dexamethasone can be more effective in hematopoietic cancers, and the chemotherapy regimens for hematopoietic cancers usually employ lenalidomide combined with alkylating agents, such as melphalan and cyclophosphamide, and dexamethasone (11,14). The standard protocol of the chemotherapy for glioblastoma also includes temozolomide, which is an alkylating agent. Furthermore, dexamethasone is often used in the postoperative management of glioblastoma (40). We speculate that the combination use of lenalidomide with temozolomide and dexamethasone may have a more potent effect in glioma patients, and this must be further researched.

In conclusion, lenalidomide displays an antitumor effect in malignant glioma cells. It induced increased expression of p21 and p53 and cell cycle arrest did occur, while there was little effect on apoptosis induction in the glioma cells.

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Availability of data and materials

All data and materials during the study are available from the corresponding author on reasonable request.

Authors' contributions

YH and AY designed the study. YH, ES and KS collected the data and drafted the manuscript. YH, ES, YO, CY, JT, SoY and ShY performed the *in vitro* experiments. YH analyzed and interpreted the data and performed the statistical analyses. TN, TU, HH and AY reviewed the manuscript, designed the figures and tables. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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