Artesunate attenuates glioma proliferation, migration and invasion by affecting cellular mechanical properties

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Abstract. Glioma is one of the most common malignant brain tumors. Current chemotherapy is far from providing satisfactory clinical outcomes for patients with glioma. More efficient drugs are urgently needed. Artesunate (ART) is clinically used as an anti-malarial agent and exhibits potent antiproliferative activity as a traditional Chinese medicine. In addition, ART has been shown to exert a profound cytotoxic effect on various tumor cell lines, presenting a novel candidate for cancer chemotherapy. However, its anticancer effect on glioma by altering cell biomechanical properties remains unclear. The present study aimed to identify the anticancer effects of ART on human glioma SHG44 cells by assessing cell proliferation, migration/invasion, the expression of claudin-1 and the biomechanical properties of ART-treated SHG44 cells. The proliferation of the SHG44 cells was assessed by MTT assay. The cell apoptosis was detected by flow cytometry. For cell migration and invasion assays, the Transwell was used. The expression of the gene claudin-1 was detected by polymerase chain reaction. The cell membrane and biomechanical properties, as targets of ART action, were investigated by atomic force microscopy (AFM). ART significantly inhibited the proliferation of SHG44 cells in a dose- and time-dependent manner. After treatment with 30 mg/l ART, the level of cell apoptosis was significantly increased (from 6.88±0.062 to 23.7±4.16%). Furthermore, the cell migration and invasion abilities of the SHG44 cells were markedly inhibited after treatment with 30 mg/l ART. Compared with the control group (0 mg/l ART), the SHG44 cells treated with 30 mg/l ART exhibited upregulated expression of claudin-1, increased adhesive force (from 2,400±300 to 3,600±500 pN), increased high connection among SHG44 cells, increased cytomembrane roughness (from 0.118±0.011 to 0.269±0.015 μm) and reduced elasticity (from 23±8 to 3.5±1.1 MPa). The present study demonstrated that ART could alter the biomechanical properties of the glioma cells to inhibit cell proliferation, migration and invasion.

Introduction

Glioma is the most common malignant tumor of the neural epithelium (1). Due to the progress of comprehensive treatments, the survival rate of glioma has recently increased. However, the relapse rate is still almost 100% within 2 years and the prognosis of this malignant disease remains poor (2). Current cancer treatments are based on chemotherapy, radiation therapy and surgery. However, the severe side-effects of chemotherapy and drug resistance are unresolved problems which still exist in clinical oncology and decrease successful therapeutic outcomes of chemotherapy (3,4). Therefore, comprehensive studies on the mechanisms of this type of cancer and the identification of improved novel anticancer compounds are urgently needed.

Artesunate (ART) is an effective and safe anti-malarial drug which is extracted from the Chinese herb *Artemisia annua* (5). Previous studies have reported that ART is active against

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different types of cancer cells, and proliferation analysis indicated that >70 cell lines from different tumors were inhibited by ART (6-13). A previously published study indicated that esophageal squamous cell carcinoma could be resisted by ART by changing the cytoskeleton (14).

However, the effects of ART on glioma cell biomechanical properties which could influence the growth, apoptosis, migration and invasion have not yet been reported.

Atomic force microscopy (AFM) has become a powerful tool in the biological field for decades, and is a tool to obtain high-resolution ultrastructural data from the cellular membrane (15), and explore the shape and biomechanical properties of a single cell or cell cluster (16,17). AFM has been frequently used to detect tumor cell membranes and biomechanical properties in the context of anticancer drugs (18-20).

The effects of ART on glioma cell behavior and the change in mechanical properties require investigation.

In the present study, the effects of ART on the change in glioma cell behaviors in regards to biomechanical properties were evaluated by AFM. In addition, the abnormal expression of the gene claudin-1, responsible for the tight junction disruption in cancer and associated with cell migration and invasion (21), was detected after treatment with ART in glioma cells.

Materials and methods

Cell culture conditions. Human glioma cell line SHG44, purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China), was grown in Dulbecco's modified Eagle's medium:nutrient mixture F-12, (DMEM/F12) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and incubated in a humidified atmosphere at 37˚C with 5% CO_2. The cells were subcultured every 2 days.

MTT assay. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, purchased from Sigma Corporation (St. Louis, MO, USA) was used to assess SHG44 cell proliferation after treatment with different concentrations of ART (Guilin Pharmaceutical Co., Ltd., Guangxi, China). In brief, SHG44 cells were seeded into a 96-well plate (Corning, Inc., New York, NY, USA) at a density of $5x10^3$ cells/well. Then, different concentrations of ART (0, 10, 30 and 50 mg/l) were added to the SHG44 cells for a final volume of 200 μl/well. SHG44 cell proliferation was measured at 3 time points, 24, 36 and 48 h. After incubation, 20 μl of MTT (5 mg/ml) was added into each well, and the cells were incubated at 37˚C for 4 h. Then, the medium was removed from each well mentioned above and 200 μl dimethyl sulfoxide (DMSO; Sigma Corporation) was added into each well. The optical density (OD) was determined at 490 nm with a microplate reader (Bio-Rad 550; 94547; Bio-Rad, Hercules, CA, USA). Each assay was run in triplicate.

Cell apoptosis assay by flow cytometry. Compared with the control group (0 mg/l ART), the optimal concentration of ART (30 mg/l, based on the results of MTT assay) was used, and the apoptosis rate of the SHG44 cells was assessed. Briefly, after being cultured with ART for 48 h, SHG44 cells were collected at a density of $1x10^6$/ml, and washed 2 times with phosphate-buffered saline (PBS). For the apoptosis assay, the cells was suspended in 500 μl of binding buffer. Then, 5 μl of Annexin V and 5 μl of propidium iodide (PI) (both from Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) were added to each sample for 15 min in the dark. Finally, the cells were analyzed using BD FACSCalibur flow cytometry with CellQuest software (version 5.1; BD Biosciences, Franklin Lakes, NJ, USA). Each sample was repeated 3 times.

Table I. RT-PCR primer sequences used for the amplification of specific genes.

Genes		Primer sequence (5'-3')	Product size (bp)	Annealing temp. (˚C)
GAPDH (22)	F	TTCGTACCTGGCATTGACTGG	225	57
	R	GAAGGTGAAGGTCGGAGT		
Claudin-1 (22)	F	GATGAGGTGCAGAAGATGAGG	200	63
	R	AGAAGGCAGAGAGAAGCAGC		

F, forward; R, reverse; temp, temperature.

Cell migration and invasion assays. SHG44 cells were grown to 85% confluency in 25 cm^2 culture flasks (Corning, Inc.) and treated with different concentrations of ART (0, 30 mg/l) for 48 h. For the migration assays, $1x10^5$ live cells in serum-free media were seeded onto an inserted chamber (8 μm; Corning, Inc.) not coated with Matrigel. The lower chamber contained culture media with 10% FBS. Twenty-four hours later, invasive cells were stained by crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and counted under a microscope (IX71; Olympus, Tokyo, Japan). Experiments were repeated 3 times. For the invasion assays, experiments were carried out with Matrigel basement membrane matrix (BD Biosciences)-coated Transwell migration chambers; the other steps were the same as the migration assays.

Atomic force microscopy (AFM) analysis. The 3D morphology and biomechanical properties of the SHG44 cells treated with ART (0, 30 mg/l) were detected by AFM. AFM was performed using protocols as previously described (14).

Analysis of claudin-1 expression. Transcriptase-polymerase chain reaction (PCR) and western blotting were performed to detect claudin-1 expression with specific primers and antibodies. We started reverse transcriptase-polymerase chain reaction (RT-PCR) with cell RNA. Then, we performed PCR with cDNA from cell RNA. Each PCR product (10 μl) was electrophoresed on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide. The primer sequences used for the amplification of specific genes are provided in Table I. Western blotting was used to detect claudin-1 expression at the protein level. The protein extracted from the control and 30 mg/l ART groups was run on and extracted from an SDS-PAGE gel. The corresponding claudin-1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated in 10%

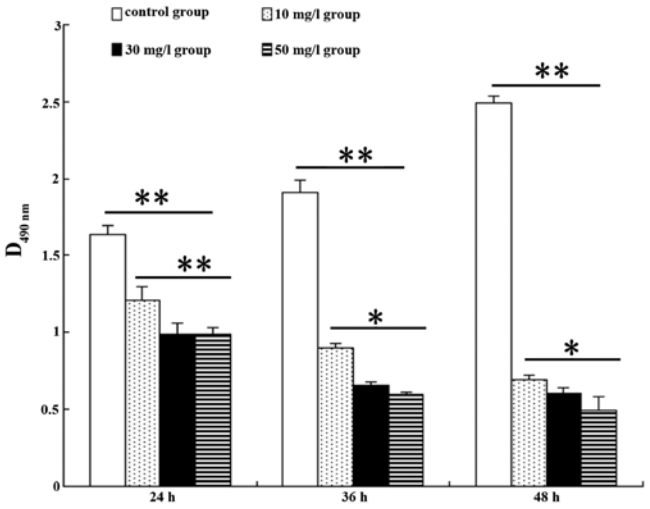

Figure 1. Proliferation of the SHG44 cells treated with different concentrations of ART. SHG44 cells were treated with ART for 24, 36 and 48 h. The proliferation level of the control group was significantly higher than that of the ART-treated groups; and that of the 10 mg/l ART group was significantly higher than that of the 30 and 50 mg/l ART groups. All data are presented as the mean ± standard deviation; *P<0.05, **P<0.01. ART, artesunate.

BSA/PBST. Western blotting was performed using standard protocols as previously described (22). The results of agarose gel electrophores were detected by gray scale analysis with software ImageJ [National Institutes of Health (NHI)].

Statistical analysis. The mechanical properties of the SHG44 cells were subjected to an arcsine transformation. The transformed data were then analyzed by analysis of variance (ANOVA; General Linear Model; SPSS 11.0) (SPSS, Inc., Chicago, IL, USA) (23).

For the analysis of other data, the data were also subjected to arcsine transformation and analyzed by either the Student's t-test (the cell apoptosis rate and the results of cell migration/invasion) or ANOVA (MTT analysis). P<0.05 was considered to indicate a statistically significant result. All data are expressed as mean ± standard deviation (SD).

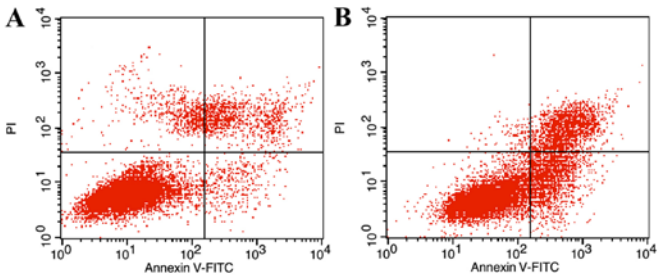

Figure 2. Cell apoptosis analysis. Cell apoptosis analysis of the (A) control and (B) 30 mg/l ART-treated SHG44 cell group. ART, artesunate.

Results

Effects of ART on cell growth and apoptosis in glioma cells. The proliferation of the SHG44 cells incubated with different concentrations of ART (10, 30 and 50 mg/l) was significantly reduced than that noted in the control SHG44 cells (0 mg/l ART-treated) at 3 time points (Fig. 1). In addition, the proliferation levels of the 30 and 50 mg/l ART-treated groups were significantly lower than that of the 10 mg/l ART-treated group at all time points. However, there was no significant difference between the 30 and 50 mg/l ART-treated groups in regards to cell proliferation at all time points (Fig. 1). The growth of the SHG44 cells was markedly inhibited by ART in a time- and dose-dependent manner.

Based on the results of the cell proliferation assay, the optimal concentration of ART (30 mg/l) was used to treat the SHG44 cells for 48 h and the cell apoptosis rates were assessed. The results showed that the apoptosis rate of the 30 mg/l ART-treated group was significantly

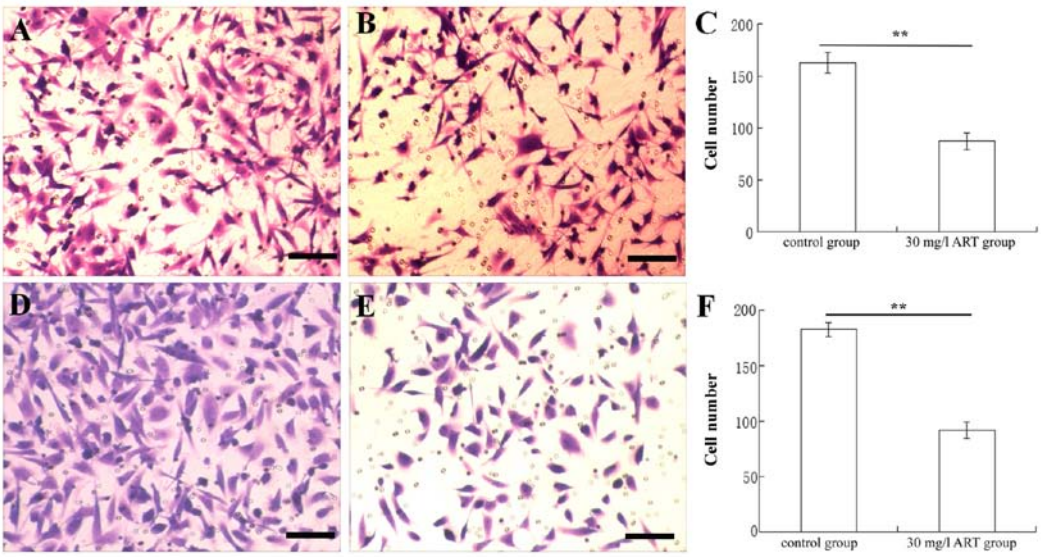

Figure 3. Transwell migration and invasion assays of the SHG44 cells following treatment with ART for 24 h. Migration of the (A) control and (B) 30 mg/l ART-treated groups. (C) Statistical analysis of migration. Invasion of the (D) control and (E) 30 mg/l ART-treated groups. (F) Statistical analysis of invasion. The relative ratio of invasive cells/field is presented. Bar, 100 μm. All data are presented as the mean ± standard deviation; **P<0.01. ART, artesunate.

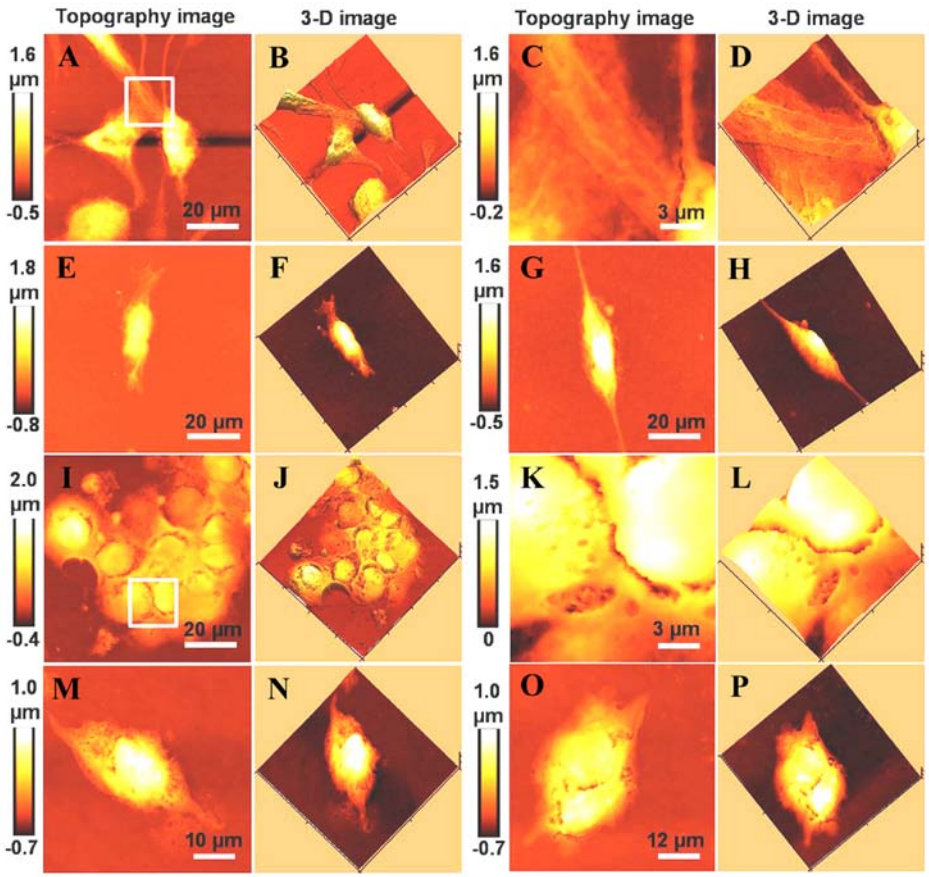

Figure 4. AFM images of SHG44 cell connections. (A) 2D and (B) 3D AFM images of SHG44 cell clusters cultured *in vitro* for 48 h without ART treatment. (C) 2D and (D) 3D ultra-structure AFM images of cell connection in A. (E and G) 2D and (F and H) 3D AFM images of a single SHG44 cell. (I) 2D and (J) 3D AFM images of SHG44 cell cluster cultured *in vitro* for 48 h following treatment with 30 mg/l ART. (K) 2D and (L) 3D ultra-structure AFM images of cell connection in I. (M and O) 2D and (N and P) 3D AFM image of a single SHG44 cell treated with 30 mg/l ART. The connections among SHG44 cell clusters without ART treatment were loose (A-D). The morphology of SHG44 indicated they were slender spindle or polygon in nature (E-H). However, the connection among SHG44 cell clusters treated with 30 mg/l ART cultured *in vitro* for 48 h was very tight (I-L). The morphology of the SHG44 cells treated with 30 mg/l ART changed from a slender spindle/polygon to a wide oval shape (M-P).

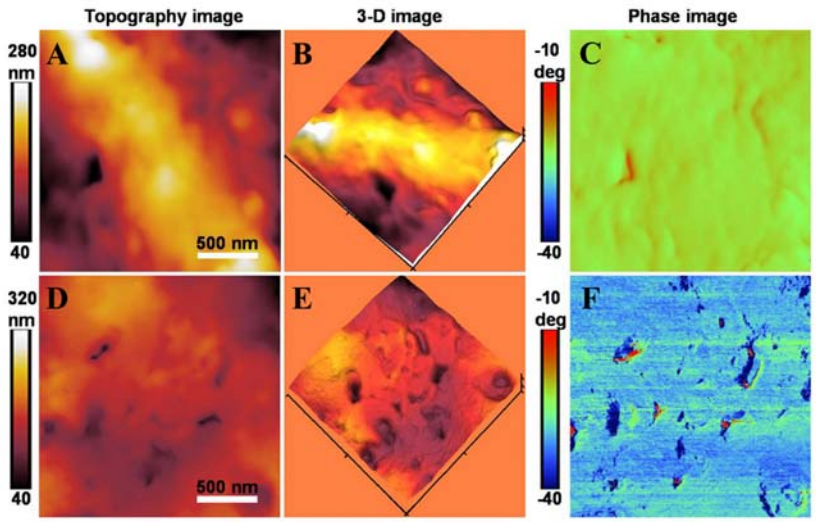

Figure 5. Ultra-structure of SHG44 cells. (A) Topography and (B) 3D images of an untreated SHG44 cell membrane nano-structure which exhibits a smooth membrane (C). (D) Topography and (E) 3D images of an SHG44 cell membrane nano-structure treated with 30 mg/l ART which exhibits a more uneven membrane compared with C (F).

increased (23.7±4.16%) compared with the control group (6.88±0.062%) (Fig. 2A and B).

ART inhibits the migration and invasion of glioma cells. Transwell assay was used to analyze the migration and inva-

Table II. Biomechanical properties of the SHG44 cells after treatment with ART.

	Ra of cyto-membrane (μm)	Adhesive force (pN)	Elasticity (MPa)
Control group (0 mg/l ART)	0.118±0.011[b]	2,400±300[b]	23±8[a]
30 mg/l group	0.269±0.015[a]	3,600±500[a]	3.5±1.1[b]

[a]P<0.05 vs. the control group. pN=10^{-12} Newtons; MPa=10^{6} Pascals. ART, artesunate; Ra, average roughness.

sion of the SHG44 cells following treatment with ART. After incubation with 30 mg/l ART, the migration and invasion of the SHG44 cells were obviously inhibited (Fig. 3). The migration and invasion abilities of the control group were significantly enhanced when compared with these abilities in the 30 mg/l ART-treated group. These results indicate that ART reduced the cell migration and invasion of glioma cells *in vitro*.

Biomechanical properties of SHG44 cells treated with ART are significantly altered as detected by AFM. Different cell types or cells in different physiological conditions possess a unique structure with specific mechanical properties. In the present study, AFM was used to detect changes in the morphology and biomechanical properties of SHG44 cells incubated with ART. Fig. 4 demonstrates without ART treatment, the connection among the SHG44 cell clusters was loose (Fig. 4A-D); but the connection among the SHG44 cell cluster following treatment with 30 mg/l ART cultured *in vitro* for 48 h was extremely tight (Fig. 4I-L); and the SHG44 cells exhibited a slender spindle or polygon morphology (Fig. 4E-H); the SHG44 cells treated with 30 mg/l ART were wide oval in shape (Fig. 4M-P).

The roughness of the membrane ultra-structures of the control group cells (Fig. 5A-C) was smoother than that of the 30 mg/l ART-treated group (Fig. 5D-F; Table II). The cell membrane of the 30 mg/l ART-treated group was more uneven and irregular.

In the SHG44 control group (without ART treatment), the adhesive force was 2,400±300 pN (Fig. 6A and B); the elasticity force was 23±8 MPa (Fig. 6C and D); and the average roughness (Ra) was 0.118±0.011 μm. Following treatment with ART at 30 mg/l in the SHG44 cells, the adhesive force was 3,600±500 pN (Fig. 6E and F); the elasticity force was 3.5±1.1 MPa (Fig. 6G and H); and the Ra was 0.269±0.015 μm. Compared with the SHG44 control group, the adhesive force and Ra of the 30 mg/l ART-treated group were significantly higher, whereas the elasticity force of the 30 mg/l ART-treated group was significantly lower than that of the control SHG44 cells (Table II).

ART promotes expression of claudin-1 in glioma. The expression of claudin-1 was significantly higher than that of the control group after treatment with 30 mg/l ART, at the molecular and protein levels (Fig. 7).

Discussion

The incidence and mortality of human glioma are high worldwide, and the treatment outcome is poor. ART is widely used as an effective drug to treat malaria (3-6). Previously, studies have reported that ART decreases tumorigenesis and has low toxicity, and can also pass through the blood-brain barrier. This indicates that ART could be a new potential anti-glioma drug (10-13). In the present study, we detected the effects of the anti-malarial agent ART on SHG44 cells. After being treated with ART, the Ra of the cell membrane, apoptosis rate and the expression of claudin-1 in addition to the adhesive force were significantly increased. Whereas the elasticity of SHG44 cells was significantly decreased after incubation with ART.

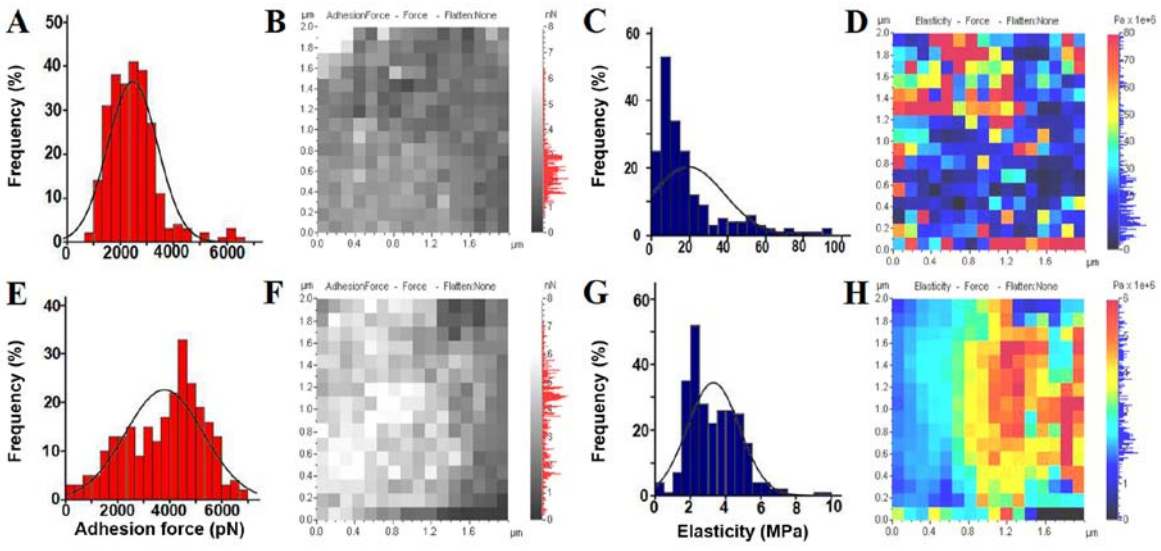

Figure 6. AFM force-distance curve analyses detect adhesive force and elasticity of the SHG44 cells. (A-D) Control group, SHG44 cells without ART treatment: (A) adhesion force histogram (n=256); (B) adhesion force map of the same cell surface area; (C) elasticity histogram (n=256); and (D) elasticity map of the same cell surface area. (E-H) SHG44 cells with 30 mg/l ART-treatment for 48 h: (E) adhesion force histogram (n=256); (F) adhesion force map of the same cell surface area; (G) elasticity histogram (n=256); and (H) elasticity map of the same cell surface area.

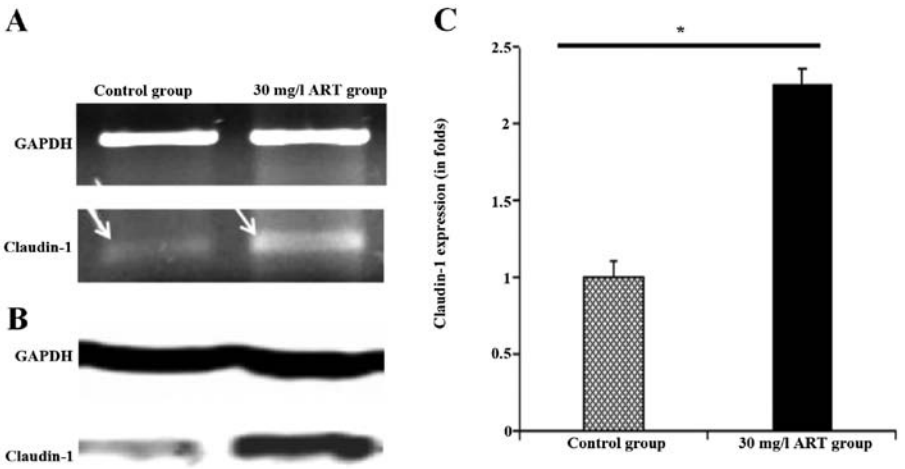

Figure 7. Expression of claudin-1 in human glioma tumor cells treated with different concentrations of ART. After treatment with 30 mg/l ART, the expression of claudin-1 in glioma was significantly higher than that of the control group. (A) Electrophoresed results of claudin-1 expression. (B) Western blot results of claudin-1 expression. (C) Histogram of gray scale analysis of claudin-1 expression levels (based on electrophoresed results). Claudin-1 expression values are represented as mean ± standard deviation, $^{*}P<0.05$. ART, artesunate.

The surface of the cell membrane contains numerous different biological macromolecules, including phospholipids, transmembrane and adsorbed glycoproteins, galactose and glucose as exchange interfaces to transfer signals from outside to inside, which are important to maintain the functionality and integrity of the cell membrane (24,25). Changes in the surface of the cell membrane may affect the integrity of the cell membrane and signal transportation can therefore directly influence cell activities and the cytoskeleton (26,27).

The cytoskeleton could determine the cellular morphology and biomechanical characteristics during different physiological periods (28). A previous study reported that chromosome stability and cell proliferation could be influenced by altered cytoskeleton (29).

Apoptosis is important to maintain normal cell homeostasis, and dysfunction of apoptosis is common in cancer cells (30). The stability of the cytoskeleton is important to maintain cell structural integrity and functionality, which could affect cell apoptosis, and has been studied as a possible target for anticancer therapy; thus, cytoskeleton damage could promote apoptosis (31).

The results of the cell apoptosis analysis and AFM detection indicated that ART may inhibit the proliferation and promote the apoptosis of SHG44 cells by changing the cell membrane-surface integrity, halting signal transfer and affecting the cytoskeleton stability of SHG44 cells.

Tight junctions are necessary for endothelial and epithelial cells, which are formed by the essential protein claudin family (32). Damage of tight junctions could cause increased invasiveness, the loss of cohesion and deletion of differentiation, which promotes tumorigenesis in epithelial cells. The abnormal expression of the claudin family was identified to be responsible for tight junction disruption in cancer (21). Previously published studies indicate that increased claudin-1 expression was found to confer cell death in nasopharyngeal cancers (33), and claudin-1 expression was significantly associated with basal-like breast cancer (34). In glioma, downregulated levels of claudin-1 are associated with progression of the grade of malignancy, and claudin-1 expression is significantly lower in glioma than that in normal cells (22).

Cell elasticity plays a role in tumor cell migration and invasion (35), and may reflect cytoskeleton alterations and may be associated with cell deformation (36).

Claudin-1 gene expression and AFM detection indicated that ART may inhibit the migration and invasion of SHG44 cells by increasing the expression of claudin-1 which may strengthen adhesive force, and cell cluster connections and may suppress cell elasticity.

In the present study, ART was found to have the potential ability to inhibit the proliferation, migration and invasion of glioma cells by affecting the expression of claudin-1 gene, cell morphology and structure. Thus, ART may be a potential new drug for glioma.

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