Abstract. Glioma is the most common primary brain tumor and represents one of the most aggressive and lethal types of human cancer. Recent advances have implicated long noncoding RNAs (lncRNAs) as crucial mediators of cancer development and progression. The present study aimed to investigate the role of a newly-discovered lncRNA, termed eosinophil granule ontogeny transcript (EGOT), in the aggressive abilities of cells in human glioma. It was initially found that the relative transcription level of EGOT in glioma cancerous tissues was significantly lower than that in adjacent non-cancerous tissues. EGOT was differentially expressed in a series of glioma cell lines, with its lowest level in high aggressive U251 and U87 cells. When EGOT was overexpressed by an expression plasmid, cell viability was significantly inhibited in U251 and U87 cells. Furthermore, with EGOT overexpression, the cell cycle was arrested at G0/G1 phase and consequently, cell apoptosis was significantly promoted along with the activities of caspase-3 and caspase-9. The migration abilities of EGOT-overexpressed cells were inhibited by 71.4% in U251 cells and by 69.5% in U87 cells. These data suggest that overexpression of EGOT inhibits cell proliferation and migration, and promotes cell apoptosis in glioma. Therefore, EGOT has potent anticancer activity and may function as a tumor suppressor in human glioma.

Introduction

Glioma is the most common brain tumor, accounting for >50% of all brain tumors (1). According to a population-based investigation, the overall median survival duration for patients with malignant glioma is ~15 months (2). Despite significant advances in the therapeutic strategies of glioma, including neurosurgical approaches, chemotherapy and radiotherapy, the prognosis of patients with malignant glioma remains poor, primarily due to the fact that malignant glioma cells are highly aggressive and capable of infiltrating into adjacent normal brain tissue, leading to the eventual failure of complete surgical dissection of the tumor (3,4). Recurrence and resistance to chemotherapy are to be claimed two influential factors for prognosis (5,6). Therefore, developing novel strategies and elucidating innovative therapeutic agents for patients suffering from glioma are imperative and urgent tasks.

Long noncoding RNAs (lncRNAs) are a type of RNAs that widely exist in the nuclei and cytoplasm of eukaryotic cells. These RNAs are non-protein coding and >200 nucleotides in length (7,8). With continuous advances in research approaches, research focused on lncRNAs has recently undergone a rapid expansion. LncRNAs are closely associated with tumor development (9,10) and particularly, have been implicated in glioma progression (11). For example, the lncRNA, MALAT1, has been demonstrated to have crucial roles in the cell proliferation, metastasis and apoptosis processes in glioma (12-14). MALAT1 was proposed as a tumor suppressor in glioma (14). Knockdown of MALAT1 increases the blood-tumor barrier permeability (15), and affects proliferation and the expression of stemness markers in glioma (12). However, only a small proportion of lncRNAs have been functionally characterized in glioma.

LncRNA eosinophil granule ontogeny transcript (EGOT) contains two exons and is nested within an intron of the inositol triphosphate receptor type 1 gene in 3p26.1. EGOT is conserved at the nucleotide level and was initially reported to be involved in eosinophil development and expressed in mature eosinophils (16). Furthermore, EGOT is highly expressed in bone marrow, indicating its role in the development from bone marrow hematopoietic stem cells (17). Recently, an association
between aberrant expression of EGOT with malignant status and poor prognosis has been detected in breast cancer (17). EGOT was downregulated in breast cancer and its low levels were significantly associated with larger tumor size, lymph node metastasis and worse prognosis. This pioneering study implied that EGOT may exert potential anticancer activity in human tumorigenesis. However, the functional role of EGOT in other types of cancer remains largely unknown.

Preliminary microarray analysis demonstrated that EGOT was differentially expressed in glioma and non-glioma tissues (data not shown). Based on this finding, the present study aimed to investigate the role of EGOT in cell proliferation and metastasis in glioma. The transcription level of EGOT was initially examined in clinical glioma tissues, followed by a systematic assessment of the biological activities of EGOT in glioma in vitro. The present report represents the first experimental study to elucidate the functional role of EGOT in human cancer.

Materials and methods

Human tissues. A total of 50 glioma tissues and their adjacent non-cancerous tissues were collected from patients (age, 50±5, 27 male:23 female) who underwent neurosurgical dissection at the Department of Oncology, The Second Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China) between May 2015 and May 2016. Infiltrating cases, in which clinicians could not differentiate between cancerous and adjacent non-cancerous tissues, were excluded from this study. These patients had not received chemotherapy nor radiotherapy prior to surgical resection. All cases were diagnosed with glioma by two independent pathologists, without any controversy and the Glioma Diagnosis and Treatment Guidelines of Central Nervous System in China were followed. Written consent was obtained from each patient. Protocols for using these samples for research purposes were approved by the Institutional Review Board at The Second Affiliated Hospital of Zhejiang Chinese Medical University.

Cell lines, cell culture and cell transfection. Normal glial HEB cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Four glioma cell lines, including A172, U251, U87 and SHG44, were obtained from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; ATCC) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Briefly, U251 and U87 cells (1x10^4 cells) were plated into 6-well plates (Corning, Inc., NY, USA) and transfected with vector or EGOT expression plasmid for 48 h, as described. Following this, 1x10^4 cells were collected and fixed with cold ethanol (70%; 10 min on ice) prior to washing and re-suspension in cold PBS and incubation at 37°C for 30 min with 10 mg/ml RNase and 1 mg/ml propidium iodide (PI; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). DNA content was analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). The percentage of cells in each phase of the cell cycle was determined using Cell Quest acquisition software (version 2.9; BD Biosciences).

Flow cytometric analysis of cell apoptosis. Annexin V/PI assay was performed according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, U251 and U87 cells (1x10^4 cells) were plated into 6-well plates (Corning, Inc., NY, USA) and transfected with vector or EGOT expression plasmid, as described. Cells were subsequently washed with cold PBS, centrifuged (1,000 x g for 5 min at 4°C), and re-suspended in 100 μl binding buffer containing 2.5 μl fluorescein isothiocyanate-conjugated Annexin V and 1 μl PI (100 μg/ml). Cells were incubated at room temperature for 15 min in the dark. At least 10,000 events were collected and analyzed by flow cytometry (BD Biosciences) and Cell Quest acquisition software (version 2.9; BD Biosciences) was used.

Cell viability determination. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega Corp., Pittsburgh, PA, USA) was used to measure cell growth abilities according to the manufacturer's protocol. Briefly,
U251 and U87 cells were seeded in 96-well plates at an initial density of $5 \times 10^4$/well in DMEM supplemented with 10% FBS in a humidified incubator containing 5% CO$_2$. Each experimental group of cells was spread into six wells and the cultural medium was replaced every other day. Cell viability was assessed for five consecutive days. At each evaluation point (1, 2, 3, 4 and 5 days post-transfection), cell viability was detected using a microplate reader (Tecan Group, Männedorf, Switzerland) at an absorbance of 490 nm. Absorbance of control cells at day 1 was set as 1. Other absorbance values were normalized to the control cells at day 1.

Transwell migration assay. U251 and U87 cells that were transfected with vector or EGOT expression plasmid, respectively, were harvested with serum-free DMEM and 150 µl of cell suspension ($6 \times 10^4$ cells) was seeded into the upper chamber (Corning, Inc., Corning, NY, USA), whereas the lower chamber was filled with 600 µl cultural medium supplied with 10% FBS. Following incubation for 12 h at 37°C, cells in each group were fixed with ice-cold methanol for 10 min and stained with crystal violet for 5 min at room temperature. Images were captured under a microscope (magnification, x200; Nikon Corp., Tokyo, Japan).

Wound-healing assay. Wound-healing assays were performed by creating identical wound areas for anchorage-dependent cells U251 and U87 using 10-µl sterile pipette tips. Briefly, cells were seeded into 6-well plates and co-incubated with the same quantity of plasmids in the presence or absence of EGOT overexpression for 24 h at 37°C. Afterwards, cells were washed with PBS and a cross was scraped in the center of each well, washed with PBS again and immediately replaced with fresh serum-free medium. Images of the cells were captured once the scratch was made. After 24 h growth, cells were observed and images were captured under a Nikon microscope at a magnification of x200 for each group.

Determination of caspase activities. Activities of caspase-3, caspase-8 and caspase-9 were determined using caspase activity kits according to the instructions provided by Beyotime Institute of Biotechnology (Haimen, China). Briefly, cell lysates were collected from each group of cells. Equal amounts of protein (10 µl) from cell lysates were added into 96-well plates and mixed with an aliquot of 80 µl reaction buffer supplemented with caspase substrate (2 mM). Following incubation for 4 h at 37°C, caspase activities were determined via a TECAN reader at an absorbance of 450 nm.

Western blot analysis. U251 and U87 cells (1x10$^4$ cells) were treated with EGOT expressing plasmid and cultured for additional 48 h. Following this, total proteins were extracted with NP40 lysis buffer (Beyotime Institute of Biotechnology) and quantified via the standard BCA method (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts of protein from each sample (50 µg) were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose (NC) membranes. Following blocking with TBST supplied with 5% milk, the membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies against caspase-9 (sc-8297), caspase-3 (sc-271028), cytochrome c (cyt c), GAPDH and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA) and was used in a dilution of 1:1,000.

Statistical analysis. All data were expressed as the mean ± standard deviation. Each experiment was repeated at least three times in triplicate. Student's t-test was used to compare the difference between groups under normal distribution. Wilcoxon's test was used for the clinical paired samples and Mann-Whitney test was used to compare values on the cell line experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

EGOT is downregulated in glioma and differentially expressed in glioma cell lines. Initially, the transcription level of EGOT was examined in 50 cases of clinical glioma tissues as well as their matched adjacent non-cancerous tissues were evaluated via reverse transcription-quantitative polymerase chain reaction analysis. (B) Transcription levels of EGOT were analyzed in five cell lines, including the normal glial HEB cells and four glioma cell lines: A172, U251, U87 and SHG44. Glioma cell lines exhibited significantly lower transcription levels of EGOT than the normal HEB cells and among the four glioma cell lines, highly aggressive U251 and U87 cells exhibited the lowest levels of EGOT. All data are expressed as the mean ± standard deviation. *P<0.05 vs. HEB; **P<0.01. EGOT, eosinophil granule ontogeny transcript.
glioma cell lines consistently exhibited lower transcription levels of EGOT (Fig. 1B). Among the four glioma cell lines, U251 and U87 cells, which are highly aggressive, exhibited the lowest levels of EGOT (Fig. 1B), indicating the negative role of EGOT expression in the aggressiveness of glioma cells. These data suggest that EGOT is downregulated in glioma.

Overexpression of EGOT inhibits the proliferation of U251 and U87 cells. Effects of EGOT overexpression on cell biological activities were assessed in U251 cells and U87 cells. Following transfection of the EGOT expression plasmid, relative EGOT transcription levels significantly increased in U251 cells (4-fold) and U87 cells (3.5-fold; Fig. 2A), as compared with the controls, indicating the successful upregulation of EGOT in glioma cells. Subsequently, cell viability was determined. In U251 cells, while all groups of cells remained constantly proliferative, EGOT-overexpressed cells exhibited a significantly slower proliferative rate, as compared with the controls, from day 4 (Fig. 2B). In U87 cells, it was similarly observed that cell proliferative rates were not significantly different among the three groups of cells in the first 3 days. However, the cell proliferative rate in EGOT-overexpressed cells was significantly inhibited by up to 42% on day 5, as compared with the control cells (Fig. 2C). These data suggest that overexpression of EGOT inhibits cell viability in glioma cells.

Overexpression of EGOT arrests cell cycle at G0/G1 phase. Cell cycle analysis demonstrated that, following EGOT plasmid transfection, U251 cells were disturbed in each cell cycle phase. The cell percentage in the G0/G1 phase was significantly increased by ~20% in the G0/G1 phase and decreased correspondingly in the S and G2/M phases. (B) U87 cells were also subjected to cell cycle analysis with or without EGOT overexpression. Cell percentages in each phase were indicated. All data are expressed as the mean ± standard deviation. *P<0.05 vs. matched control U251 cells; †P<0.05 vs. matched control U87 cells. EGOT, eosinophil granule ontogeny transcript.
S phase or G2/M phase (Fig. 3B). These observations suggest that overexpression of EGOT promotes cell cycle arrest at G0/G1 phase.

Overexpression of EGOT inhibits cell migration in glioma. In our preliminary tests, it was found that cell apoptosis was insignificant within 24 h among distinct groups (data not shown). Therefore, to exclude the possible effect of cell apoptosis, cell migration was determined within 24 h in the present study. Cell migration capacity was then assessed using Transwell migration assays. Following transfection with the EGOT plasmid, it was visually observed that migrated cells were markedly less stained in U251 and U87 cells (Fig. 4A). Counting the migrated cells further demonstrated that a mean of ~96 cells had migrated to the lower surface after EGOT overexpression in U251 cells, which was significantly fewer than the ~350 control U251 cells that had successfully migrated (Fig. 4B). In U87 cells, while a mean of 326 control cells migrated, only 84 EGOT-overexpressing cells migrated to the lower surface, demonstrating a significant inhibition of 69.5% in U87 cells (Fig. 4B).

A wound-healing assay was subsequently performed to further explore the role of EGOT in human glioma. As shown in Fig. 4C and D, overexpression of EGOT in U251 cells retarded cell migration by ~50% compared to the control cells, whereas the migration potential of U87 cells was also inhibited by ~50% when cells were transfected with EGOT-expressing plasmids. These data suggest that overexpression of EGOT inhibits cell migration in glioma.

Overexpression of EGOT induces cell apoptosis and increases the activities of caspase-3 and caspase-9. In consideration of the cell cycle arrest detected at G0/G1 phase, cell apoptosis was detected in both cell lines. As expected, it was observed that the EGOT plasmid significantly increased the apoptotic rates in both cell lines (Fig. 5A). Furthermore, with the upregulation of EGOT, the activity of caspase-3 was significantly increased (2-fold) in U251 cells and U87 cells (1.8-fold; Fig. 5B). Caspase-8 activity remained consistent among the different groups (Fig. 5C); whereas the activity of caspase-9 significantly increased following EGOT transfection in both cell lines (Fig. 5D). Subsequent western blot analysis was used to detect the protein expression levels of caspase-3, caspase-9 and cyt c. As shown in Fig. 6, in U251 and U87 cells, transfection of EGOT plasmid increased the protein expression levels of caspase-3, caspase-9 and cyt c. Consistent with the increased activities of caspase-3 and caspase-9, these findings support the hypothesis that EGOT induces cell apoptosis in glioma cells.

Discussion

Malignant brain tumors are one of the most lethal types of cancer in adults. As the most common brain tumor, glioma is
Survival of patients with malignant glioma after surgery and radiotherapy is <1 year (5). Temozolomide chemotherapy may prolong survival; however, research has demonstrated that the majority of patients succumb to their symptoms within two years (6). Therefore, there is an urgent need for novel strategies for the treatment of glioma.

It has previously been demonstrated that ~80% of transcription across the human genome is associated with lncRNAs (8). However, only a small proportion of lncRNAs have been demonstrated to be biologically functional (20). Among the functional lncRNAs, the majority have been implicated in malignant tumors (10,21). The lncRNA, EGOT, was initially thought to be involved in eosinophil development and expressed in mature eosinophils (16); it was subsequently demonstrated that EGOT locus is highly structured, containing several evolutionary conserved regions, and thermodynamic stable secondary structures (22). Notably, the functional roles of EGOT have recently been implicated in ischemic heart diseases (23). Furthermore, another study has found that lncRNA EGOT negatively affects the antiviral response and favors hepatic virus C (HCV) replication (24). These pioneering studies have suggested that EGOT may exert active functional roles in human diseases, including human cancer. For example, the involvement of EGOT in breast cancer (17). It was found that low levels of EGOT were significantly correlated with larger tumor size, lymph node metastasis and a worse prognosis in breast cancer patients.

The present study investigated the role of EGOT in glioma cell activities. EGOT was demonstrated to be downregulated in clinical glioma tissues and, compared with normal glial cells, glioma cells exhibited significantly lower transcription levels of EGOT. These findings were consistent with previous findings, which also revealed the downregulation of EGOT in breast cancer (17). It was found that low levels of EGOT were significantly correlated with larger tumor size, lymph node metastasis and a worse prognosis in breast cancer patients.

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Figure 5. Overexpression of EGOT induces cell apoptosis and increases the activities of caspase-3 and caspase-9. (A) Cell apoptosis was assayed in U251 and U87 cells with or without EGOT overexpression. Activities of (B) caspase-3, (C) caspase-8 and (D) caspase-9 were determined in U251 and U87 cells, respectively. All data are expressed as the mean ± standard deviation. *P<0.05 vs. control U251 cells; #P<0.05 vs. control U87 cells. EGOT, eosinophil granule ontogeny transcript.

Figure 6. Overexpression of EGOT in glioma cells increased the protein levels of caspase-3, caspase-9 and cyt c. Western blot analysis revealed that when U251 and U87 cells were transfected with EGOT plasmid, the expression of caspase-3, caspase-9 and cyt c was markedly increased. All data are expressed as the mean ± standard deviation. EGOT, eosinophil granule ontogeny transcript; cyt c, cytochrome c.
of EGO7 significantly inhibits cell migration in U251 and U87 cells. These findings strongly suggest that EGO7 may inhibit the aggressive activities of glioma cells and function as a tumor suppressor in glioma. However, further investigations are required. In future studies, we will focus on the effects of the depletion of EGO7 on glioma. Specific small interfering RNAs or short hairpin RNAs (lentivirus and adenovirus) will be designed and transfected into the cells prior to injection into mice. Furthermore, our group will to collect clinical data in order to elucidate the association between clinicopathological characteristics and the expression of EGO7 in human glioma.

It remains unclear how EGO7 regulates cell apoptosis in glioma. Cell apoptosis is usually induced by two distinct signal pathways, intrinsic and extrinsic (26). The intrinsic pathway is always initiated by pro-apoptotic factors (B-cell lymphoma-2-associated X protein and Bcl-2-associated death promoter), which leads to increased permeability of the mitochondria membrane, loss of membrane potential and cyt c release into the cytosol. Caspase-3 was thereafter cleaved and activated, followed by caspase-9. By contrast, the extrinsic pathway is associated with the activation of caspase-8. The results of the present study demonstrated that the activity of caspase-8 remained unchanged and the activities of caspase-3 and caspase-9 were significantly increased upon EGO7 overexpression. These analyses suggest that the extrinsic pathway may not be critically involved. Instead, the intrinsic pathway may be involved in the apoptosis induction processes induced by EGO7 overexpression. However, as a recent report indicated that IncRNA EGO7 prefers nuclear localization in HCV-infected cells (24), the subcellular localization of EGO7 in human tumor cells remains to be elucidated and merits further investigation into whether the subcellular location is associated with its regulation of cell apoptosis.

In conclusion, the present study is the first to demonstrate the functional role of IncRNA EGO7 in human tumorigenesis. Overexpression of EGO7 significantly inhibited cell proliferation and migration and induced cell apoptosis in glioma cells. These conclusive findings suggest that EGO7 has potent anticancer activity and may function as a tumor suppressor in human glioma.

References