Identification of lncRNA EGOT as a tumor suppressor in renal cell carcinoma

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Abstract. Renal cell carcinoma (RCC) is the most common type of kidney cancer and the prognosis of metastatic RCC remains poor, with a high rate of recurrence and mortality. Long non-coding RNA (lncRNA) is a class of RNA which serves important roles in multiple cellular processes and tumorigenesis. In the present study, the expression and function of lncRNA eosinophil granule ontogeny transcript (EGOT) were examined in RCC. In 24 paired tissues (RCC tissues and adjacent normal tissues) the results of reverse transcription-quantitative polymerase chain reaction analysis revealed that EGOT was downregulated in 22 RCC tissues compared with paired tissues. Upregulation of lncRNA EGOT by transfection of 786-O and ACHN RCC cells with pcDNA3.1-EGOT suppressed cell proliferation, migration and invasion, and induced RCC cell apoptosis. The results demonstrated that EGOT may serve as a tumor suppressor in RCC and may be a potential prognostic biomarker of RCC.

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer and accounts for ~2-3% of all cases of cancer in adults (1). RCC may be resistant to chemotherapy and conventional radiation therapy, although surgical resection is an effective treatment (2-4). However, 30% of patients with RCC who undergo surgical resection suffer from recurrence (3,5). The prognosis of patients with RCC remains unfavorable, particularly for those with advanced tumors (6). It is essential to diagnose RCC as early as possible and to identify novel biomarkers for RCC.

Long non-coding RNAs (lncRNAs) are a group of non-protein coding transcripts that have a length of >200 nucleotides (7,8). lncRNAs were regarded as 'transcriptional noise' when identified initially. However, accumulating evidence has demonstrated that lncRNAs may influence gene expression via transcriptional, post-transcriptional or epigenetic regulation (9). With the development of whole-genome sequencing technologies, it is now clear that non-coding RNAs account for ≥90% of the human genome and serve important roles in different biological processes (10,11), including genome rearrangement (12), chromatin modifications (13), gene imprinting (14), cell cycle control (15) and X-chromosome inactivation (16). The lncRNA eosinophil granule ontogeny transcript (EGOT) is located at 3p26.1. A previous study revealed that lncRNA EGOT was downregulated in breast cancer, and its expression was associated with malignant status and poor prognosis (17). In addition, next-generation deep sequencing revealed that lncRNA EGOT may be functional in clear cell carcinoma (18); however, there appears to be no associated studies about lncRNA EGOT in RCC. In the present study, the expression and function of lncRNA EGOT in RCC was examined.

Materials and methods

Sample collection. A total of 24 paired RCC tissues (RCC and adjacent normal tissues) were collected from Peking University Shenzhen Hospital (Shenzhen, China) from December 2012 to December 2014. The adjacent normal tissues were 2 cm away from visible RCC lesions and negative for tumor tissues, as determined by microscopy. Tissues were immersed in RNAlater (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for ≥30 min while being dissected, and subsequently stored at -80˚C until further use. Written informed consent was obtained from all patients. Collection and usage of the samples were reviewed and approved by the Ethics Committees of Peking University Shenzhen Hospital (Shenzhen, China). The

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tissues collected were reviewed and classified by hematoxylin and eosin staining. The clinical and pathological characteristics of the patients are presented in Table I.

Cell culture. The 2 RCC cell lines, 786-O and ACHN, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in a humidified incubator containing 5% CO_2 at a temperature of 37˚C in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% glutamine (Gibco; Thermo Fisher Scientific, Inc.), 1 μl/ml penicillin and 1 μg/ml streptomycin sulfates.

Cell transfection. For overexpression of EGOT, the polymerase chain reaction (PCR)-amplified EGOT digested with *Bam*HI and *Eco*RI restriction enzymes was subcloned into the pcDNA3.1 mammalian expression vector (Genepharm, Inc., Sunnyvale, CA, USA). The EGOT expression vector, pcDNA3.1-EGOT, was verified by sequencing. Synthesized pcDNA3.1-EGOT or mock (pcDNA3.1) was transfected (0.2 μg/well in 96-well plates; 4.0 μg/well in 6-well plates) into 80% confluent RCC cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), which was mixed with Opti-MEM® I Reduced Serum medium (Gibco; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The transfection assay was performed at 37˚C for 24 h. Reverse transcription-quantitative PCR (RT-qPCR) analysis was performed to detect the expression of EGOT in cells following transfection.

RNA extraction, cDNA synthesis and the RT-qPCR. RNAiso Plus reagent (Takara Bio, Inc., Otsu, Japan) was used to extract total RNA from tissues and cells, according to the manufacturer's protocol. The concentration of RNA was measured using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, 1 μg RNA was used to generate a cDNA library by performing RT with the PrimeScript™ RT reagent kit (Takara Bio, Inc.). The expression of EGOT was detected by qPCR with Premix Ex Taq™ II (Takara Bio, Inc.) on a Roche light cycler 480 Real-Time PCR system (Roche Applied Science, Penzberg, Germany). The qPCR thermocycling conditions were as follows: 95˚C for 1 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec, 70˚C for 30 sec. GAPDH was used as an internal control and the sequences of the primers are listed in Table II. The expression level of EGOT in tissues and cells was analyzed via the $2^{-\Delta\Delta Cq}$ method (19). RT-qPCR analysis was performed in triplicate.

Cell proliferation assay. The MTT and Cell Counting Kit-8 (CCK-8) assays were performed to assess the proliferative ability of the cells. After ~12 h seeding of ~3,000 cells/well in a 96-well plate, cells were transfected with pcDNA3.1-EGOT or mock. For the CCK-8 assay, following transfection, 10 μl CCK-8 (Beyotime Institute of Biotechnology, Haimen, China) was added into the wells for 1 h, prior to measuring the optical density (OD) value of each well using an ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 490 nm. For the MTT assay, 20 μl MTT solution (5 mg/ml; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to the wells for 4 h. The solution was subsequently replaced with 150 μl dimethyl sulfoxide (Sigma-Aldrich, Merck KGaA), and the OD value was measured at a wavelength of 490 nm following agitation of the wells for 15 min at room temperature. The experiments were performed in replicates of six and repeated ≥3 times.

Table I. Clinicopathological features of renal cell carcinoma patients.

Clinical characteristic	No. patients
Age (years)	
Mean (range)	51 (25-70)
Sex	
Male	15
Female	9
Histological type	
Clear cell	21
Papillary	3
TNM stage	
T1	15
T2	7
T3 + 4	2
AJCC clinical stages	
I	14
II	7
III + IV	3

AJCC, American Joint Committee on Cancer; TNM, tumor node metastasis.

Table II. Sequences of primers.

Primer name	Sequence
lncRNA EGOT	F: 5'-CACTGCACAGGGAAACACAAA-3'
	R: 5'-ACCCTGTTCATAAGCCCTGATG-3'
GAPDH	F: 5'-GGTCTCCTCTGACTTCAACA-3'
	R: 5'-GTGAGGGTCTCTCTCTTCCT-3'

EGOT, eosinophil granule ontogeny transcript; lncRNA, long coding RNA; F, forward; R, reverse.

Cell mobility assay. Cell scratch assay was performed to assess the cell mobility of 786-O and ACHN. Cells (~3x10^5 cells/well) were seeded into a 6-well plate and 24 h later were transfected with pcDNA3.1-EGOT or mock as aforementioned. The cell monolayer was scratched 24 h later with a sterile 200 μl pipette tip to generate a wound. Cells were then rinsed with phosphate-buffered saline to remove the floating cells, and then cells were cultured in DMEM supplemented with 5% FBS. Scratches were imaged at 0 h and after 24 h with a digital camera system. The experiments were performed in triplicate and repeated 3 times.

Transwell invasive and migratory assays were performed to assess the cell migratory and invasive ability. Transwell

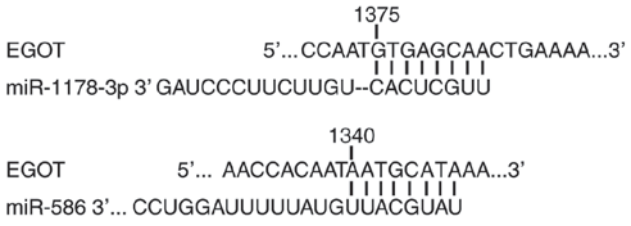

Figure 1. Prediction of EGOT-associated miRs. miR-1178-3p and miR-586 were predicted to regulate EGOT expression, as demonstrated by the complementary binding sites. EGOT, eosinophil granule ontogeny transcript; miR, microRNA.

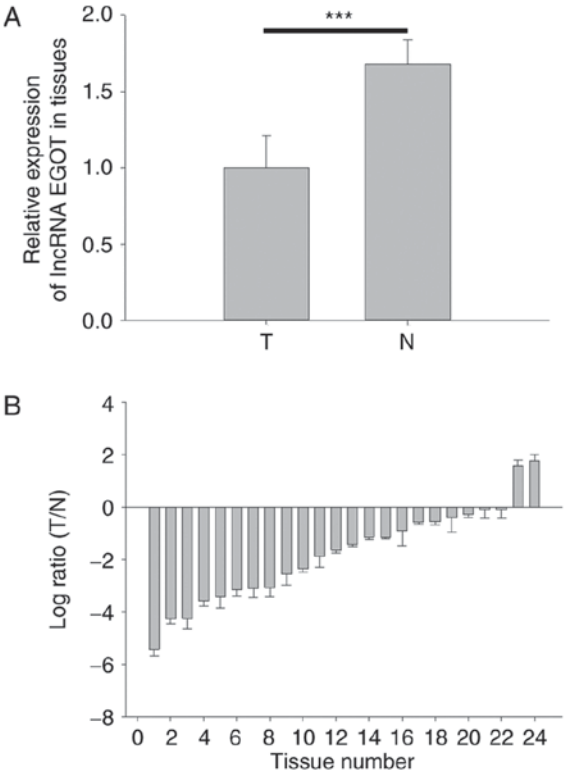

Figure 2. Expression of lncRNA EGOT in RCC or normal adjacent tissues. (A) Expression of EGOT was downregulated in RCC tissues compared with normal adjacent tissues. (B) Log2 ratio (T/N) of EGOT expression in 24 paired tissues. EGOT was downregulated in 22 RCC tissues. ***P<0.001. RCC, renal cell carcinoma; T, renal cell carcinoma tissues; N, normal adjacent tissues; lncRNA, long coding RNA; EGOT, eosinophil granule ontogeny transcript.

chamber inserts with (for migration) or without (for invasion) Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used according to the manufacturer's protocol. Cells were transfected as aforementioned, resuspended in 200 μl DMEM without serum and seeded at a density of $1x10^4$ cells/well into the upper channel of the inserts. The lower chamber contained DMEM supplemented with 10% serum. Cells were allowed to migrate for 36 h and invade for 48 h. The cells that had migrated or invaded to the bottom of the inserts were stained with crystal violet for 15 min at room temperature and counted using an inverted microscope. The experiments were performed in in triplicate and repeated at least 3 times.

Flow cytometric analysis. Flow cytometry was performed to detect the percentage of apoptotic cells following transfection with an Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit

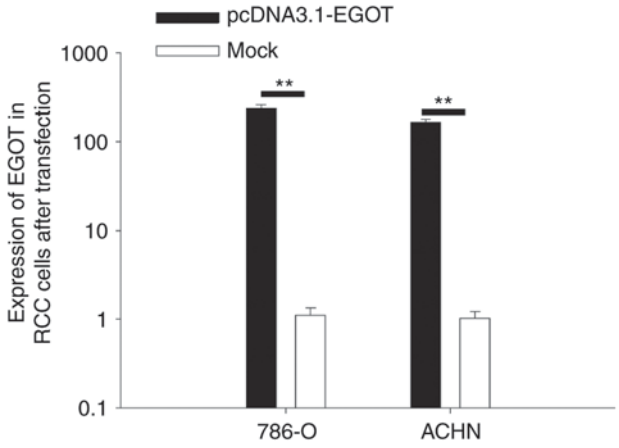

Figure 3. Relative expression levels of EGOT in 786-O and ACHN RCC cells following transfection with either a pcDNA3.1-EGOT plasmid or a mock control plasmid. **P<0.01. RCC, renal cell carcinoma; EGOT, eosinophil granule ontogeny transcript.

(Invitrogen; Thermo Fisher Scientific, Inc.). Following seeding of ~$3x10^5$ cells/well in a six-well plate and incubation for 12 h, the cells were transfected with pcDNA3.1-EGOT or mock. Following 48 h of transfection, all the cells were harvested and stained according to the manufacturer's protocol, and the percentage of apoptotic cells was determined by flow cytometry and analyzed using FlowJo version X (FlowJo LLC, Ashland, OR, USA). The experiments were performed in triplicate and repeated ≥3 times.

Prediction of EGOT-associated microRNA (miRNA/miR). Prediction of associated miRNAs was determined by computational algorithms according to base-pairing rules between miRNA and mRNA target sites, location of binding sequences within the 3'-untranslated region of the target and conservation of target binding sequences. In the present study, miRDB (mirdb.org/miRDB/index.html) and miRWalk (www.umm.uni-heidelberg.de/apps/zmf/mirwalk) were used. Putative miRNAs were miR-1178-3p (previous name miR-1178) and miR-586, and the binding site is displayed in Fig. 1.

Statistical analysis. A paired t-test was used to compare the expression levels of EGOT in matched tumor/normal tissues, and in cells following transfection. A Student's t-test was used to characterize the phenotypes of cells. All statistical analyses were performed using the SPSS version 19.0 statistical software package (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA EGOT is downregulated in RCC tissues. To detect the expression level of lncRNA EGOT in RCC tissues and paired normal tissues, RT-qPCR analysis was performed. The expression levels of lncRNA EGOT in RCC tissues (1±0.213) was significantly decreased compared with adjacent normal tissues (1.68±0.16) (Fig. 2A; P<0.001). The relative expression levels of lncRNA EGOT in the 24 tissues demonstrated that lncRNA EGOT was downregulated in

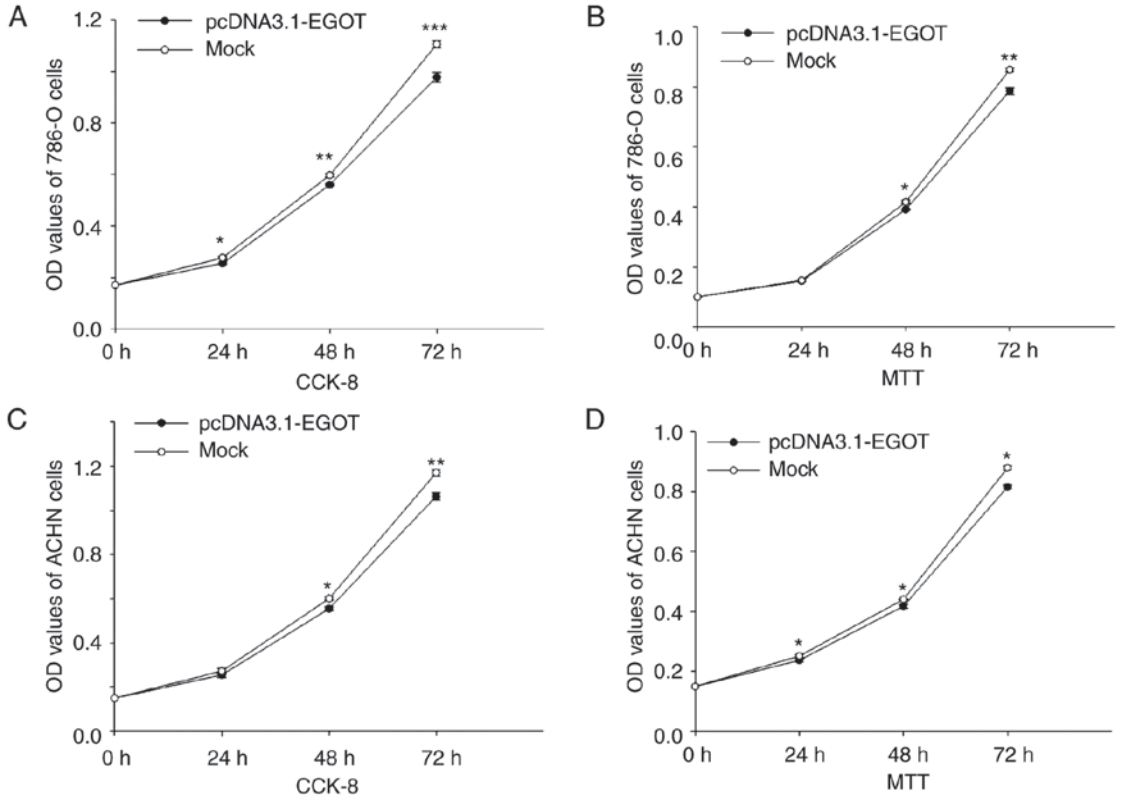

Figure 4. Cell proliferation assay in RCC cells. The proliferation of 786-O and ACHN cells transfected with the pcDNA3.1-EGOT plasmid or the mock control plasmid was measured by MTT and CCK-8 assays. (A) Results of the CCK-8 assay in 786-O cells. (B) Results of the MTT assay in 786-O cells. (C) Results of the CCK-8 assay in ACHN cells. (D) Results of the MTT assay in ACHN cells. *P<0.05, **P<0.01 and ***P<0.001 vs. respective mock group. RCC, renal cell carcinoma; EGOT, eosinophil granule ontogeny transcript; OD, optical density; CCK-8, Cell Counting Kit-8.

22 RCC tissues compared with normal adjacent tissues (Fig. 2B).

Validation of the expression of lncRNA EGOT following transfection. RT-qPCR analysis was performed to detect the expression of lncRNA EGOT in RCC cells following transfection. The results demonstrated that the expression of lncRNA EGOT was increased 236.39-fold (786-O cells) and 167.15-fold (ACHN cells) in cells transfected with pcDNA3.1-EGOT compared with cells transfected with mock (empty pcDNA3.1) (P<0.01; Fig. 3).

lncRNA EGOT suppresses cell proliferation. MTT and CCK-8 assays were performed to assess cell proliferation. In 786-O cells, compared with the mock group, proliferation was reduced by 7.75% (24 h; P<0.05), 6.27% (48 h; P<0.01) and 11.67% (72 h; P<0.001) when measured by the CCK-8 assay (Fig. 4A), and by 1.43% (24 h), 5.98% (48 h; P<0.05) and 8.24% (72 h; P<0.01) when measured by the MTT assay (Fig. 4B), following upregulation of lncRNA EGOT using the pcDNA3.1-EGOT plasmid. In addition, upregulation of lncRNA EGOT using the pcDNA3.1-EGOT plasmid suppressed ACHN cell proliferation by 6.26% (24 h), 7.60% (48 h; P<0.05) and 9.11% (72 h; P<0.01) when measured by the CCK-8 assay (Fig. 4C), and by 5.58% (24 h; P<0.05), 5.14% (48 h; P<0.05) and 7.21% (72 h; P<0.05) when measured by the MTT assay (Fig. 4D). These results suggested that lncRNA EGOT may suppress RCC cell proliferation.

lncRNA EGOT suppresses RCC cell mobility. Wound-healing, Transwell migratory and Transwell invasive assays were performed in the present study. The results of the wound-healing assay demonstrated that at 24 h, the migratory distance of cells transfected with pcDNA3.1-EGOT was reduced by 54.23% compared with the mock group in 786-O cells (P<0.01), and by 26.60% in ACHN cells (P<0.01) (Fig. 5).

The results of the Transwell assay revealed that in 786-O cells, upregulation of lncRNA EGOT reduced invasive ability by 25.45% (P<0.05) and migratory ability by 22.42% (P<0.01). For ACHN cells, upregulation of lncRNA EGOT reduced invasive the number of cells by 32.80% (P<0.05) and migratory ability by 34.84% (P<0.05) (Fig. 6). The results suggested that lncRNA EGOT suppressed RCC cell mobility.

lncRNA EGOT induces cell apoptosis. The percentage of apoptotic cells was quantified by flow cytometry (Fig. 7). The results demonstrated that the apoptotic rate in 786-O cells transfected with pcDNA3.1-EGOT was 34.50%, compared with an apoptotic rate of 10.93% in mock cells (P<0.01). In ACHN cells, the apoptosis rate of cells transfected with pcDNA3.1-EGOT was 16.96% compared with an apoptotic rate of 8.89% in mock cells (P<0.01). These results suggested that EGOT may induce cellular apoptosis in RCC cell lines.

Discussion

lncRNAs have been implicated in multiple molecular mechanisms, including transcriptional regulation, post-transcriptional regulation and processing of other short lncRNAs (20). Accumulating evidence has demonstrated that lncRNA serves

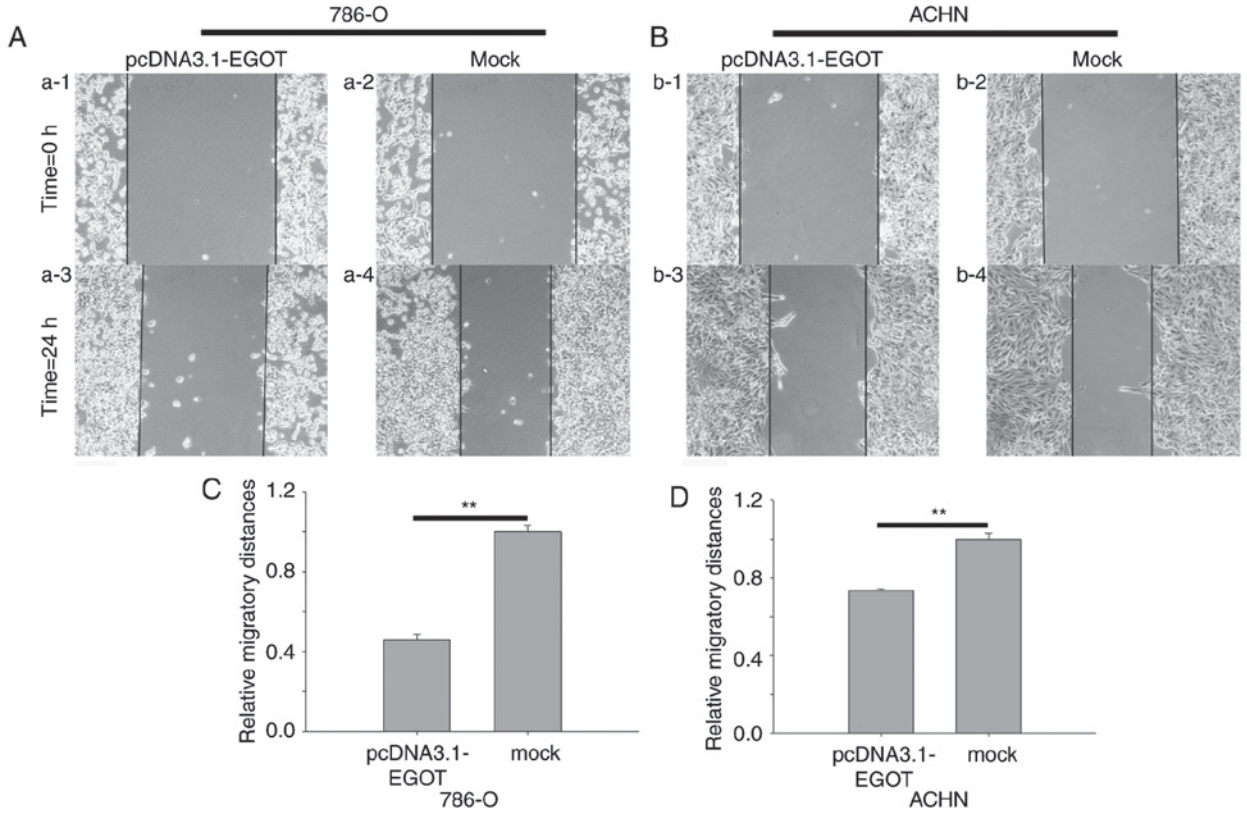

Figure 5. Wound-healing assay of 786-O and ACHN RCC cells. Migration of (A) 786-O cells or (B) ACHN cells following transfection with the pcDNA3.1-EGOT plasmid or the mock control plasmid, at 0 and 24 h. Relative migratory distances of (C) ACHN cells or (D) 786-O cells following transfection. **P<0.01. RCC, renal cell carcinoma; EGOT, eosinophil granule ontogeny transcript.

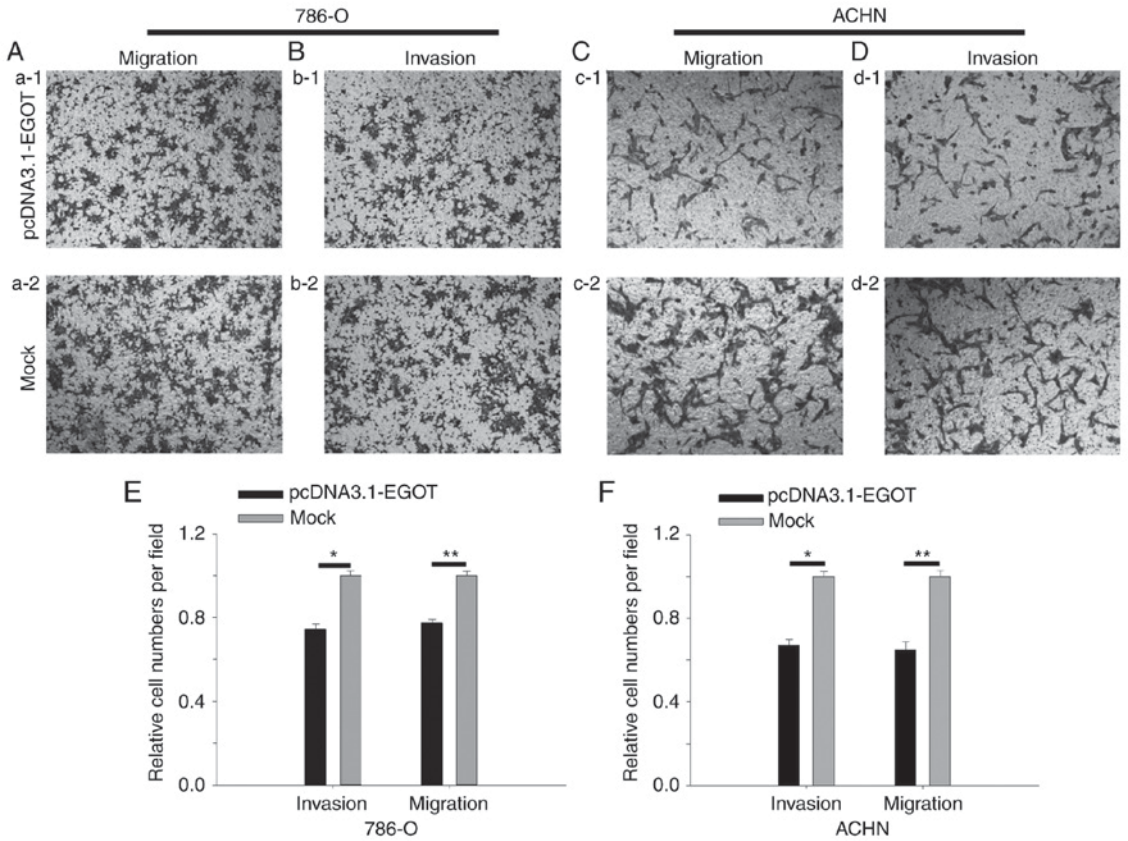

Figure 6. Overexpression of EGOT inhibits RCC cell migration and invasion. Representative images are presented of (A) the migration of 786-O cells, (B) the invasion of 786-O cells, (C) the migration of 786-O cells and (D) the invasion of ACHN cells. Quantification of the invasion and migration of (E) 786-O cells and (F) ACHN cells is displayed. *P<0.05 and **P<0.01. EGOT, eosinophil granule ontogeny transcript; RCC, renal cell carcinoma.

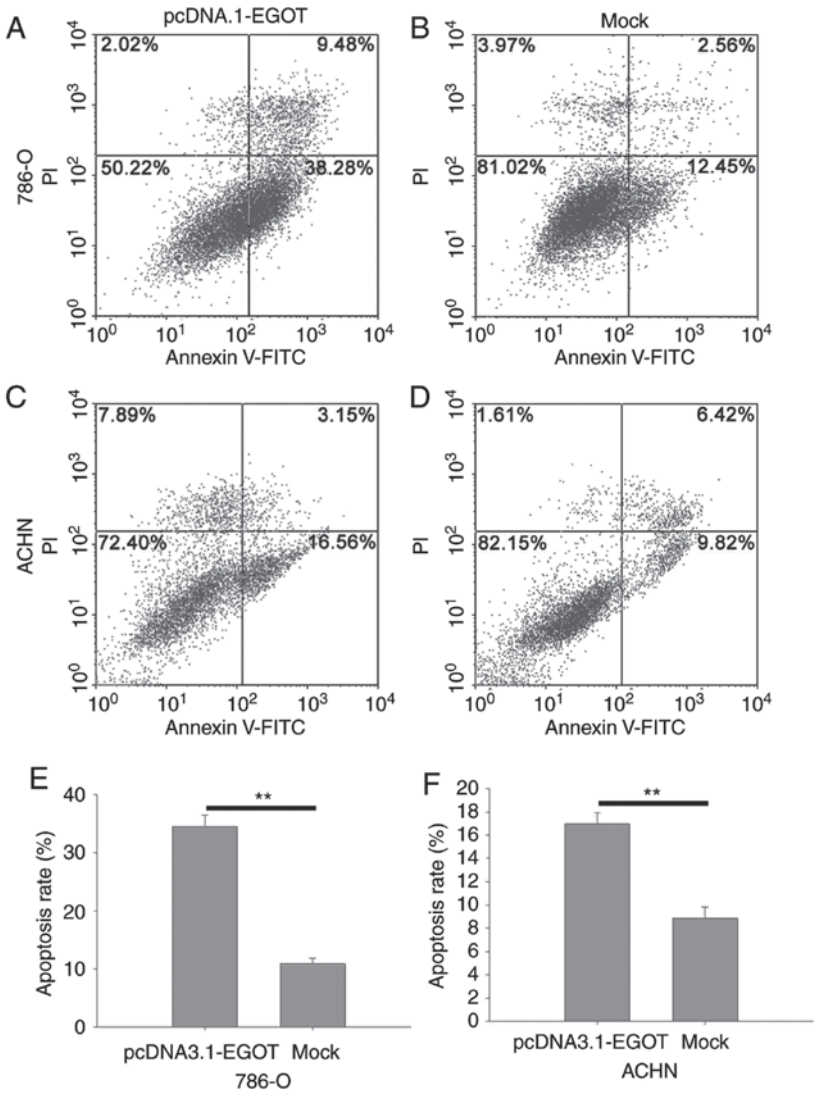

Figure 7. Overexpression of EGOT induced cellular apoptosis in RCC cells. Apoptotic 786-O cells transfected with (A) the pcDNA3.1-EGOT plasmid and (B) mock plasmid. Apoptotic ACHN cells transfected with (C) the pcDNA3.1-EGOT plasmid and (D) mock plasmid. Quantification of apoptotic (E) 786-O cells and (F) ACHN cells. **P<0.01. EGOT, eosinophil granule ontogeny transcript; RCC, renal cell carcinoma; FITC, fluorescein isothiocyanate; PI, propidium iodide.

an important role in tumorigenesis and the development of RCC. Malouf *et al* (18) identified 1,934 expressed lncRNAs by next-generation deep sequencing in 475 cases of primary clear cell (cc)RCC, and the expression of 1,070 lncRNAs demonstrated a strong association with the mRNA expression of their neighboring genes. In another study, Blondeau *et al* (21) reported 1,308 dysregulated lncRNAs in ccRCC. Another microarray assay revealed 27,279 lncRNAs in RCC samples, of which 897 were markedly dysregulated (22). To date, it appears that only a small number of studies have investigated the function or underlying mechanism of lncRNAs in RCC (23-25), and the way in which lncRNAs regulate RCC cell behavior remains to be identified.

lncRNA EGOT, located at 3p26.1, is nested within an intron of the gene encoding inositol triphosphate receptor type 1, and may be involved in eosinophil development and in the maturation of eosinophils, in addition to regulating protein and eosinophil-derived neurotoxin expression (26). A subsequent study (17) revealed that lncRNA EGOT was downregulated in breast cancer, and that decreased expression levels of EGOT were significantly associated with larger tumor size, lymph node metastasis and poor prognosis. However, it appears that no studies have investigated the role of EGOT in RCC. Based on previously published studies (17,18,26), the expression and function of EGOT was examined in RCC in the present study.

In the present study, the expression levels of EGOT in RCC and adjacent normal tissues was detected by RT-qPCR analysis, and the results revealed that EGOT was downregulated in RCC tissues compared with adjacent normal tissues. The function of EGOT in RCC cells (786-O and ACHN) was additionally examined. The results revealed that EGOT upregulation partially suppressed RCC cell proliferation, migration and invasion, and induced RCC cell apoptosis. EGOT upregulation resulted in slight alterations in cell proliferation, although this was not statistically significant, which suggested that EGOT primarily affects RCC cell migration, invasion and apoptosis. Therefore, EGOT may serve as a tumor suppressor in RCC.

There is only a small number of studies that have investigated the expression or function of lncRNAs in RCC. lncRNA urothelial carcinoma-associated 1 (UCA1), metastasis-associated lung

adenocarcinoma transcript-1 (MALAT1), homeobox transcript antisense RNA (HOTAIR) and renal cell carcinoma related transcript-1 (RCCRT1) have been demonstrated to be upregulated in RCC (27-31). In particular, the expression levels of RCCRT1 and MALAT1 were associated with poor prognosis, and UCA1, HOTAIR and RCCRT1 were associated with RCC cell migration, invasion or proliferation (27-31). lncRNA growth arrest-specific transcript 5, maternally expressed 3, cancer susceptibility candidate 2 and TRIM52 antisense RNA 1 were downregulated in RCC, affecting cell function (23,25,32,33). Another study revealed that lncRNA-suppressing androgen receptor in renal cell carcinoma may suppress hypoxic cell cycle progression in von Hippel-Lindau (VHL)-mutant RCC cells; however, SARCC may induce hypoxic cell cycle progression in VHL-restored RCC cells (34), which suggested that lncRNA SARCC may be a potential novel therapeutic biomarker. Therefore, the roles of lncRNAs in RCC remain to be identified and a greater number of studies that focus on this are required.

In conclusion, lncRNA EGOT was downregulated in RCC tissues compared with normal tissues. EGOT may affect RCC cell proliferation, migration, invasion and apoptosis, which indicated that EGOT may serve an important role in the tumorigenesis of RCC. In a future study, a dual-luciferase assay will be performed to explore the association between EGOT and miR-1178-3p or miR-586, which were identified in the present study to be putative regulators of EGOT. The possible downstream effector would be screened by microarray analysis and western blotting.

Acknowledgements

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