Research Article

GNA14 silencing suppresses the proliferation of endometrial carcinoma cells through inducing apoptosis and G₂/M cell cycle arrest

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Endometrial carcinoma is the most common gynecological malignancy. The pathological factors triggering this disease are largely unknown. Although the role of guanine nucleotide-binding protein subunit α (GNA) 11 (GNA11) in melanoma has been described, the involvement of GNA14 in endometrial carcinoma remains to be determined. Here, we found that GNA14 expression was increased in endometrial carcinoma tissues compared with simple hyperplasia tissues. Based on lentivirus-mediated knockdown assay, we showed that GNA14 silencing significantly suppressed the proliferation of both HEC-1-A and Ishikawa cells. The caspase 3/caspase 7 activity and apoptosis were enhanced by GNA14 knockdown. GNA14 depletion led to cell cycle arrest at the G₂/M phase. In addition, Apoptosis Array analysis revealed that caspase-3 and Fas were up-regulated by GNA14 knockdown. Our study suggests that GNA14 silencing blunts endometrial carcinoma cell proliferation. Targetting GNA14 may bring help for the patients of endometrial carcinoma.

Introduction

Endometrial carcinoma is one of the commonest gynecological malignancies, accounting for 20–30% of all the female reproductive cancers [1,2]. Due to its biological and histopathological variables, endometrial carcinoma is classified into two subtypes [3]. Type I endometrial carcinoma, the more frequent one, is usually well-differentiated and pathologically associated with estrogen. By contrast, the less common type II endometrial cancer is estrogen independent and often poorly differentiated and more aggressive [3,4]. Evidences have shown that type I endometrial carcinoma is closely associated with the abnormalities of KRAS, PTEN, and β-catenin [5-8], while type II endometrial cancers are linked to dysregulation of TP53 and ERBB2 [9-11]. These findings are specific but still limited as these genes seem to contribute to the disease progression in condition of estrogen or not. Therefore, widely used biomarkers and common pathological genes are in need to be identified for endometrial cancer. Essential molecular events triggering this deadly disease should be explored.

Guanine nucleotide-binding protein subunit α (GNA) is a member of G-protein. It plays an important role in transducing extracellular signals that activate phospholipase C, finally leading to activation of protein kinase C. Evidences have shown that approximately 80% of primary uveal melanomas exhibit GNAQ or GNA11 mutation that contributes to mitogen activated kinase-like protein (MAPK) pathway activation in this disease [12-14]. MAPK activation in GNAQ mutant melanomas is caused by overexpressed RasGRP3 [15]. The role of GNA’s other family members in tumor development has also been reported. GNA15 is highly expressed in gastroenteropancreatic neuroendocrine neoplasia (GEP-NEN) cells and its depletion causes suppressed proliferation and induction of apoptosis [16]. Mutation of GNA14 is associated with congenital and sporadic vascular tumors and anastomosing hemangiomas [17,18]. These results suggest that GNA14 may play...
an important role in cancer development. However, the function of GNA14 in endometrial carcinoma is unclear.

In the present study, we attempted to investigate the role of GNA14 in endometrial carcinoma. We provided the first evidence of higher GNA14 level in endometrial carcinoma tissues compared with that in simple hyperplasia tissues. Efficient silencing of GNA14 suppressed the proliferation of HEC-1-A and Ishikawa cells. Caspase 3/caspase 7 activity, apoptosis, and G2/M cell cycle arrest were all induced by GNA14 knockdown in both cells. Additionally, up-regulation of Fas and caspase 3 may explain the enhanced apoptosis in GNA14 silencing HEC-1-A and Ishikawa cells.

Materials and methods
Human endometrial carcinoma tissues
Human endometrial carcinoma tissues (88) and simple hyperplasia tissues (41) were collected from The First Hospital of Lanzhou University. Written informed consent was obtained from all the patients and participants. The present study was approved by the Ethics Committee of The First Hospital of Lanzhou University.

Cell culture
Human endometrial carcinoma cells HEC-1-A and Ishikawa cells were obtained from American Type Culture Collection (ATCC) and were cultured in 1640 medium (Invitrogen, Carlsbad, CA, U.S.A.), supplied with 10% FBS (Gibco, California, U.S.A.) and 1% penicillin/streptomycin (Corning, New York, U.S.A.). All the cell cultures were maintained in 37°C incubators containing 5% CO2.

Immunohistochemistry analysis of GNA14
FFPE sections (4 μm) of endometrial carcinoma tissues and simple hyperplasia tissues were first subjected to deparaffinization in xylene and hydration in graded alcohol. Citrate buffer (pH = 6) and 3% hydrogen peroxide were used for antigen retrieval and endogenous peroxidase blockage, respectively. The slides were then blocked with 10% serum for 60 min, followed by incubation of GNA14 antibody (Abnova, catalog number H00009630-M06, Taiwan) at 4°C overnight. Secondary antibodies (Santa Cruz Biotechnology, CA, U.S.A.) were incubated at room temperature for half an hour. DAB was used as a chromogen. The staining of GNA14 positive signal was determined by Zeiss microscope and ImageJ software. The intensity of GNA14 expression was graded as follows: negative, 0; weak, 1; moderate, 2; and strong, 3. The extent of GNA14 staining was grouped according to the percentage of high-staining cells in the cancer area: negative, 0; 1–25%, 1; 26–50%, 2; 51–75%, 3; and 76–100%, 4. The final quantitation of each staining was obtained by multiplying the two scores. Score ≤6 represents GNA14 low expression (−), and score >6 represents GNA14 high expression (+).

Lentivirus-mediated GNA14 knockdown in HEC-1-A and Ishikawa cells
pGCSIL-GFP lentivirus vector was used to silence GNA14 in HEC-1-A and Ishikawa cells. Briefly, shRNA against GNA14 (5′-TTCTCCGAACGTGTCGCTTGA-3′) or shCtrl (5′-TTCTCCGAACGTGTCGCTTTTA-3′) was synthesized and cloned into pGCSIL-GFP vector. Then, the mixture of pGCSIL-shGNA14-GFP or pGCSIL-shCtrl-GFP and packing vectors pH Helper1.0 and Helper2.0 were co-transfected into 293T cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocols. Forty-eight hours after transfection, lentivirus supernatants were collected and filtered through a 0.45-μm filter and were immediately subjected to infection of HEC-1-A and Ishikawa cells. Quantitative real-time PCR (qRT-PCR) and Western blot assay were used for the evaluation of infection efficiency.

Total mRNA isolation and qRT-PCR
Total RNA was isolated from shGNA14 and shCtrl HEC-1-A or Ishikawa cells using TRIsol reagent (Invitrogen) and RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Total RNA (0.5–1 μg) was subjected to reverse transcription using M-MLV reverse transcriptase (Promega, WI, U.S.A.). qRT-PCR was performed using SYBR master mixture (Takara, Japan) on an IQ-5 machine. The real-time PCR primer sequences were as follows: GNA14 forward: 5′-CACCCTTCCCTGTAATCTGT-3′ and reverse: 5′-CCCTTCCACTCCGTGTCATGT-3′; Fas forward: 5′-CTTGTTTTGTTACGCTTGGTT-3′ and reverse: 5′-CTGTTGCTTCCTGCTGTTT-3′; Caspase-3 forward: 5′-CATGGAAGCGAATATGACTGA-3′ and reverse: 5′-TGTTTCCCGGAGTATGTC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5′-TGACTTCCACACGACACCCA-3′ and reverse: 5′-CACCCTGTTGCTTAGCACA-3′. GAPDH served as the internal control.
Western blot
shGNA14 and shCtrl HEC-1-A or Ishikawa cells were lysed in lysis buffer (Beyotime, Beijing, China). The concentration of total protein was determined by BCA protein assay kit (Beyotime). Sixty micrograms of protein was separated on SDS/PAGE (10 or 12% gel), followed by transferring on to PVDF membrane (Millipore, Germany). The membranes were blocked with 5% skim milk for at least 60 min at room temperature and incubated with primary antibodies at 4°C overnight. After incubating with secondary antibodies at room temperature for 2 h, the immune-activity of indicated proteins was detected with ECL-Plus kit (Amersham Biosciences, General Electric, U.S.A.). Antibody against GNA14 was purchased from Abnova (H00009630-M06). Antibody against caspase-3 and Fas were from Abcam (ab32351 and ab133619, respectively, Cambridge, United Kingdom). Antibody against GAPDH (SC-32233) and all the secondary antibodies were from Santa Cruz Biotechnology.

High-content screening assay
shGNA14 and shCtrl HEC-1-A cells were seeded in 96-well plates and cultured for 5 days. The images of cultured cells were scanned every day by fluorescence-imaging microscope under 20× objective filters. The stained cells were analyzed by the ArrayScan™ HCS software (Cellomics Inc, Thermo Fisher, PA, U.S.A.). The cell viability was described as cell count and fold change of the cell number.

MTT assay
shGNA14 and shCtrl HEC-1-A or Ishikawa cells at a density of 3000 cells/well were seeded into 96-well plates containing 200-μl culture medium. At 1, 2, 3, 4, and 5 days after seeding, 20 μl of MTT solution (5 mg/ml) was added into each well and incubated at 37°C for 2–4 h. After removing the supernatants from each well, 100 μl DMSO was added. The optical density (OD) was measured at 490 nm by a microplate reader. Cell viability was determined by OD_{490} value of indicated time/OD_{490} of day 1.

Apoptosis analysis
Cell apoptosis was detected using Annexin V-APC apoptosis detection kit (Ebioscience, Thermo Fisher). Seventy-two hours after seeding, shGNA14 and shCtrl HEC-1-A or Ishikawa cells were harvested, washed with PBS, and resuspended in staining buffer at a density of 1 × 10^6 cells/ml. Five microliters of Annexin V-APC was added into 100 μl of the cell suspension. The mixture was incubated at room temperature for 15 min, and then subjected to flow cytometry analysis (FACSCalibur, Becton Dickinson, U.S.A.).

Caspase 3/7 activity measurement
Caspase-Glo reagent (Promega) was prepared following the manufacturer's instructions. shGNA14 and shCtrl HEC-1-A or Ishikawa cells at a density of 10000 cells/well were seeded into 96-well plates. Seventy-two hours later, 100 μl of Caspase-Glo reagent was added into each well in the plates. After rotating at 300–500 rpm for 30 min, the plates were maintained at room temperature for 90 min. Then, the activity of caspase 3/caspase 7 was detected by a microplate reader.

Cell cycle assay
The cell cycle distribution of shGNA14 and shCtrl HEC-1-A or Ishikawa cells was checked using Propidium Iodide (PI, Sigma–Aldrich, MO, U.S.A.) staining on a flow cytometer. The cells were seeded in six-well plates and cultured for 72 h. When cell density reached 80%, nuclei of the cells were analyzed by PI staining. Then PI absorbance was determined by FACS on a flow cytometer (FACSCalibur, Becton Dickinson, U.S.A.).

Statistical analysis
Statistical analysis was performed using GraphPad Prism 6.0 software. The data were presented as mean ± S.E.M. of at least three independent repeats. Student's t test was used to analyze the difference between two groups. One-way ANOVA was used when there were more than two groups. The difference was defined as statistically significant when P<0.05.

Results
GNA14 expression is increased in endometrial carcinoma tissues
To investigate the clinical relevance of GNA14 in endometrial carcinoma, we collected 88 endometrial carcinoma tissues and 41 simple hyperplasia tissues. Using immunohistochemistry assay, we found that endometrial carcinoma
Figure 1. GNA14 is up-regulated in endometrial carcinoma tissues
Immunohistochemistry analysis of GNA14 in endometrial carcinoma tissues (n=88) and weakly hyperplasia tissues (n=41).

Table 1 The expression of GNA14 in human endometrial carcinoma tissues and simple hyperplasia tissues

|                      | Number | GNA14 expression | $\chi^2$ | $P$  |
|----------------------|--------|------------------|----------|------|
| Endometrial carcinoma| 88     |                 |          |      |
| Simple hyperplasia   | 41     | +                | 79.162   | <0.001 |
|                      |        | +                | 78 (88.6)|      |
|                      |        | -                | 10 (11.4)|      |
|                      |        | +                | 3 (7.3)  |      |
|                      |        | -                | 38 (92.7)|      |

Knockdown of GNA14 suppresses the proliferation of HEC-1-A and Ishikawa cells
Since GNA14 was highly expressed in endometrial carcinoma tissues, we next explored the role of GNA14 by knocking down GNA14 in these cells. qRT-PCR and Western blot results showed that GNA14 was efficiently silenced in both HEC-1-A and Ishikawa cells (Figure 2). Then, we subjected these cells to cell proliferation analysis. High-content screening (HCS) results showed that GNA14 silencing significantly reduced the cell viability of HEC-1-A cells (Figure 3A–C). In addition, MTT assay was used to confirm our results and we observed that the cell proliferation rate was largely reduced in shGNA14 HEC-1-A and Ishikawa cells as compared with shCtrl cells (Figure 3D,E). Collectively, GNA14 depletion inhibits the proliferation of both HEC-1-A and Ishikawa cells.
GNA14 knockdown induces apoptosis and G2/M cell cycle arrest of endometrial carcinoma cells

Apoptosis is an important cell death type that negatively correlated with the tumor cell proliferation. We thus determined whether apoptosis participates in GNA14-regulated proliferation of endometrial carcinoma cells using annexin V-APC staining. We found that apoptosis was increased in shGNA14 HEC-1-A and Ishikawa cells as compared with shCtrl cells (Figure 4A–D). We also measured the caspase 3/caspase 7 activity and found that the activity is enhanced by GNA14 silencing in these cells (Figure 4E,F).

Since cell cycle progression is also an important regulator of cell proliferation, we next explore whether GNA14 regulated cell cycle. To address this question, we subjected shCtrl or shGNA14 HEC-1-A and Ishikawa cells to PI staining on a flow cytometer. The results suggested that shGNA14 HEC-1-A cells exhibited higher distribution of G2/M phase and reduced G1- and S-phases of cell cycle (Figure 5A,B). Although no difference was observed in the G1 phase in shCtrl and shGNA14 Ishikawa cells, GNA14 knockdown still led to increased G2/M phase and reduced S phase distribution (Figure 5C,D). Taken together, GNA14 silencing enhances the caspase 3/caspase 7 activity and induces the apoptosis and G2/M cell cycle arrest, which may contribute to the suppressed proliferation of GNA14 knockdown endometrial carcinoma cells.

GNA14 silencing stimulates Fas and caspase 3 expression

To further understand the molecular changes involved in GNA14-mediated apoptosis, shCtrl or shGNA14 HEC-1-A cells were subjected to RayBio C-Series Human Apoptosis Antibody Array C1 analysis. As shown in Figure 6A, this array could determine various factors that were associated with apoptosis. Based on this assay, we found that caspase 3 and Fas were regulated by GNA14 (Figure 6B). The mRNA expression of caspase 3 and Fas were increased in shGNA14 HEC-1-A cells as compared with shCtrl cells (Figure 6C). At the protein level, both Fas and caspase 3 were up-regulated by GNA14 knockdown. Therefore, GNA14 silencing stimulation of Fas and caspase 3 may contribute to its enhancement of apoptosis.
Figure 3. GNA14 knockdown inhibits the proliferation of HEC-1-A and Ishikawa cells

(A–C) Multiparametric HCS was performed to detect the proliferation of shCtrl or shGNA14 HEC-1-A cells from days 1 to 5 after seeding. (A) Representative images of HCS. (B,C) Quantitation of HCS. *P<0.05, **P<0.01, ***P<0.001. The green fluorescence represented viable cells. Scale bar: 100 μm. (D,E) MTT assay was used to determine the proliferation of shCtrl or shGNA14 HEC-1-A (D) and Ishikawa (E) cells from days 1 to 5 after seeding. *P<0.05, **P<0.01, ***P<0.001.
Figure 4. GNA14 silencing increases caspase 3/caspase 7 activity and induces apoptosis

(A) shCtrl or shGNA14 HEC-1-A cells were cultured for 72 h and the apoptosis was determined by annexin V-APC apoptosis detection kit on a flow cytometry. (A) Representative images of the apoptosis analysis. (B) Quantitation of the apoptosis shown in (A). ***P<0.001. (C, D) shCtrl or shGNA14 Ishikawa cells were cultured for 72 h and the apoptosis was determined by annexin V-APC apoptosis detection kit on a flow cytometry. (C) Representative images of the apoptosis analysis. (D) Quantitation of the apoptosis shown in (C). ***P<0.001. (E, F) shCtrl or shGNA14 HEC-1-A and Ishikawa cells were cultured for 72 h and subjected to caspase 3/caspase 7 activity analysis. *P<0.05.

Discussion

Endometrial cancer is a malignant disease with poor prognosis and limited treatment options. So far, numerous oncogenes or tumor suppressors have been found implicated in the pathological process of this malignancy. However, there are still a large amount of endometrial cancer patients with unknown molecular mechanisms. In the present study, we explored the role of GNA14 in endometrial carcinoma. We found that GNA14 abundance was potentiated in endometrial carcinoma tissues comparing with simple hyperplasia tissues. Knockdown of GNA14 suppressed the proliferation of HEC-1-A and Ishikawa cells. Furthermore, GNA14 silencing led to cell cycle arrest at the G2/M phase, enhanced caspase 3/caspase 7 activity, and apoptosis in both HEC-1-A and Ishikawa cells. We suggest that GNA14 is a promising biomarker and an oncogene in endometrial carcinoma. Targetting GNA14 maybe an effective treatment for this disease.
GNA14 is a member of GNA, which is involved in extracellular signal transduction. This process can activate phospholipase C and protein kinase C. Recurrent mutation of GNAQ or GNA11 has been found in a large percentage of primary uveal melanomas and activates MAPK [12-14,19]. Further studies have demonstrated that PKC ε and ε are required for the activation of MAPK depending on RasGRP3-regulated Ras in GNAQ mutant melanomas. Additionally, RasGRP3 is obviously overexpressed in melanomas with GNAQ mutation [15]. Recently, a mouse model with GNA11Q209E mutation was constructed and recapitulated human Gq-associated melanomas [20]. These studies reveal the important role of GNA11 or GNAQ mutation and the potential molecular mechanisms in melanoma development. Similar to GNA11 or GNAQ, mutation of GNA14 is also observed in tumors, including congenital and sporadic vascular tumors and anastomosing hemangiomas [17,18]. However, the expression and potential role of GNA14 in different cancers remain largely unclear. In the present study, we first analyzed the clinical relevance of GNA14 in endometrial carcinoma. The expression of GNA14 was significantly increased in endometrial carcinoma tissue as compared with simple hyperplasia tissues. This suggests that GNA14 may participate in endometrial cancer development. Based on lentivirus-mediated knockdown assay, we illustrated that GNA14 silencing suppressed the proliferation of HEC-1-A and Ishikawa cells, which are commonly used in endometrial cancer studies. We proposed that ectopic expression of GNA14 may promote the progression of endometrial cancer.

The function of cell cycle and apoptosis have been widely investigated in cancers. Dysregulated cell cycle progression and apoptosis are correlated with cancer cell proliferation, growth, and tumor development. In endometrial cancer, some oncogenic or suppressive factors, such as PI3K/AKT pathway, CIP2A, RG108, and mir-152 are found to promote Hec-1A and Ishikawa cell proliferation by regulating cell cycle and apoptosis [21-24]. Although GNA14

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**Figure 5. GNA14 knockdown enhances G2/M cell cycle arrest**

(A) shCtrl or shGNA14 HEC-1-A cells were cultured for 72 h and then subjected to flow cytometry analysis of cell cycle using PI staining. (B) Quantitation of the cell cycle of shCtrl or shGNA14 HEC-1-A cells as shown in (A). *P<0.05. (C) shCtrl or shGNA14 Ishikawa cells were cultured for 72 h and then subjected to flow cytometry analysis of cell cycle using PI staining. (D) Quantitation of the cell cycle of shCtrl or shGNA14 Ishikawa cells as shown in (C). *P<0.05.
knockdown suppressed the proliferation of endometrial cancer cells, the function of GNA14 in cell cycle and apoptosis is poorly understood. Here, we found that GNA14 silencing reduced both G1 and S phase in HEC-1-A cells. Even though G1 phase remained unchanged in Ishikawa cells by GNA14 silencing, the S phase decreased. Similarly, G2/M phase was enhanced by GNA14 depletion in both HEC-1-A and Ishikawa cells. We also demonstrated that GNA14 reduction promoted caspase 3/caspase 7 activity and apoptosis in both HEC-1-A and Ishikawa cells. At the molecular level, Fas and caspase-3 were up-regulated after GNA14 knockdown. Fas and caspase-3 are regulated by various factors and their activation contributes to cancer cell apoptosis [25-28]. Therefore, our results indicate that GNA14 silencing suppresses the proliferation of endometrial cancer cells through inducing G2/M cell cycle arrest.
and apoptosis. Enhanced apoptosis maybe correlated with increased expression of Fas and caspase 3. Although we have illustrated the potential mechanisms participating in the GNA14 regulation of endometrial cancer cell proliferation, there are still lack of evidences uncovering how GNA14 knockdown up-regulates Fas and caspase-3 and induces apoptosis and G2/M cell cycle arrest. Therefore, the molecular mechanisms underlying the oncogenic function of GNA14 in endometrial carcinoma need further study.

In summary, we provided for the first time that GNA14 acted as an oncogene for endometrial carcinoma. GNA14 was highly expressed in endometrial carcinoma tissues as compared with the simple hyperplasia tissues. Knockdown of GNA14 enhanced the apoptosis, the activity of caspase 3/caspase 7 and induced the cell cycle arrest at G2/M phase, resulting in reduced cell proliferation in endometrial cancer HEC-1-A and Ishikawa cells. We proposed that GNA14 is a promising diagnostic marker for endometrial carcinoma. Suppression of GNA14 may bring hope for the patients with this lethal disease.

Competing interests
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Author contribution
Y.Y. conceived the study, carried out the experimental design and data interpretation, and prepared and revised the manuscript. J.W. performed most of the experiments. X.L. and F.X. performed the HCS assay. M.W. and C.L. performed the Western blot.

Abbreviations
AKT, AKT serine/threonine kinase; APC, alloglycocyamin; CIP2A, cell proliferation regualtion inhibitor of portein phosphatase 2A; DAB, diaminobenzidine; ERBB2, erb-b2 receptor tyrosine kinase 2; Fas, Fas cell surface death receptor; FFPE, formalin fixed paraffin-embedded; HCS, high-content screening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GNA, guanine nucleotide-binding protein subunit α; KRAS, KRAS proto-oncogene, GTPase; MAPK, mitogen activated kinase-like protein; OD, optical density; PTEN, phosphatase and tensin homolog; PKC, protein kinase C; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta; PI, propidium iodide; RasGRP3, RAS guanyl releasing protein 3; qRT-PCR, quantitative real-time PCR; TP53, tumor protein p53.

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