G protein subunit α q regulates gastric cancer growth via the p53/p21 and MEK/ERK pathways

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Abstract. Genetic alterations in G protein subunit α q (GNAQ) have been reported in numerous types of human cancer. However, the role of GNAQ in human gastric cancer (GC) has not been explored. In the present study, we found that GNAQ was highly expressed in GC patient samples and GNAQ expression was related to patient age, GC differentiation status and adjuvant therapy, as determined by immunohistochemical assay. Lentivirus delivery of short hairpin RNA (shRNA) targeting GNAQ was used to explore the function of GNAQ in GC cells. Silencing of GNAQ markedly suppressed proliferation and colony formation in GC cells, and arrested the cell cycle at the S phase. Mechanistic analysis revealed that knockdown of GNAQ significantly increased the expression of p53 and p21, and decreased cyclin A and p-CDK2 protein expression. Moreover, the phosphorylation of ERK and MEK was also decreased after knockdown of GNAQ as determined by western blotting assay. Overall, our results suggest that GNAQ plays a critical role in regulating GC cell growth and survival via canonical oncogenic signaling pathways including MAPK and p53, and therefore serves as a promising new therapeutic target in GC.

Introduction

Gastric cancer (GC) is the 4th most common cause of cancer-related deaths worldwide. In 2012, there were almost 1,000,000 new cases and over 720,000 deaths (1). Currently, the standard care for GC patients includes surgery, chemotherapy, radiotherapy and targeted therapy. However, even after multimodal therapy most patients still suffer a high rate of disease recurrence, metastasis and progression (2). Insight into the molecular mechanisms of gastric carcinogenesis may offer novel, more effective treatment options. Several studies in GC have investigated inhibition of targets such as HER-2, EGFR, VEGFR, mTOR, C-Met and HGF alone or in combination with chemotherapy (3-7). However, to date, only two studies have shown promising clinical results of molecular-target agents in GC. First, the TOGA study established trastuzumab in combination with chemotherapy as a new standard of care for patients with HER2-neu-positive advanced or metastatic GC (5). Second, the REGARD trial demonstrated a survival benefit for ramucirumab in patients with advanced gastric or esophagogastric junction (EGJ) adenocarcinoma after progression following first-line chemotherapy (3). Overall, however, the population of patients who can benefit from available targeted therapies is very limited. Therefore, identification of novel GC therapeutic targets is essential.

Alterations at chromosomal position 9q21 have been detected in numerous types of human cancer, including breast and lung cancer, melanoma and glioblastoma (8-11). G protein subunit α q (GNAQ) is a protein coding gene and the oncogenic potential of GNAQ was revealed by a systematic analysis of the transforming potential of G proteins and GPCRs (12). Additionally gain of function mutations in GNAQ or GNA11 oncogenes, encoding persistently active GNAQ, have been previously demonstrated to drive uveal melanoma growth (13). However, the precise molecular mechanism of the GNAQ contribution to oncogenesis remains unknown and its potential role in GC has yet to be examined. In the present study, we investigated the relationship between GNAQ overexpression and the clinicopathological features of patients with GC, and also determined the biological functions of GNAQ in GC.

Materials and methods

Patient samples. GC patients treated with curative gastrectomy at the First Hospital of Jilin University (Changchun, China) were enrolled in the present study. Tumor specimens were collected from patients between 2011 and 2013. The World Health Organization Classification of Tumors was used for histological grading. Tumors were staged according to the TNM classification of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC). Survival status was updated in November 2015. Overall survival (OS) was calculated from the date of surgery

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to the date of death or to the date of the most recent follow-up. Disease-free survival (DFS) was calculated from the date of curative surgery to the date of progression, or to the date of the most recent follow-up. The present study, was retrospectively performed and approved by the Institutional Review Board of the First Hospital of Jilin University. All patients provided informed consent for the use of their clinical specimens in the present study.

Tissue sample immunohistochemistry. Tumor specimens containing normal and carcinoma tissues were obtained from the patients after surgery. Histology of the surgical specimens was observed using the streptavidin-peroxidase (SP) assay according to the manufacturer's instructions (Histostain-Bulk-SP kit; Zymed, South San Francisco, CA, USA). Primary anti-GNAQ antibody (diluted 1:50; Proteintech, Chicago, IL, USA) was used for immunohistochemical staining. Specimen staining results were evaluated by two independent pathologists blinded to the clinicopathological data. Scores for each specimen were calculated by multiplying the staining intensity and the distribution area of GNAQ-positively stained cells. The specimen staining intensity was divided into light yellow, yellow, brown yellow and reddish brown which were scored as 0, 1, 2 and 3, respectively. The distribution area of the positively-stained cells (0%; 1-20%; 21-60%; 61-100%) was scored as 0, 1, 2 and 3, respectively. The final total score was determined as negative (scores 0-1) and positive (scores 2-6).

Cell culture. Human GC cell lines MGC80-3, SGC7901, AGS and human embryonic kidney cell line 293T (HEK293T) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MGC80-3 and SGC7901 cells were maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel). AGS cells were cultured in Ham's F12 medium (Gibco-BRL, Grand Island, NY, USA) and 10% FBS. HEK293T cells were grown to confluence in Dulbecco's modified Eagle's medium (HyClone) supplemented with 5% FBS. These cell lines were maintained in a 5% CO_2-humidified atmosphere at 37˚C.

Lentivirus construction and transfection. Targeting human GNAQ (NM_002072.4) shRNA (S1) sequence (5'-GATCCC TATGATAGACGACGAGAATACTCGAGTATTCTCGTCG TCTATCATAGTTTTTG-3'), shRNA (S2) sequence (5'-GAT CCCTATGATAGACGACGAGAATACTCGAGAGATATTC TCGTCGTCTATCATTTTTTG-3') and the control shRNA sequence (5'-GATCCTTCTCCGAACGTGTCACGTCTCGA GACGACGCACTGGCGGAGAATTTTTG-3') were designed and inserted into the pFH lentivirus vector (Hollybio, Shanghai, China). The recombinant GNAQ silencing and control plasmid were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, the recombinant lentivirus encoding shRNA against GNAQ or the control shRNA were harvested and purified. Short hairpin RNA (shRNA) interference vector pFH-L containing an H1 promoter upstream of the shRNA, lentivirus packaging vector pVSVG-I and pCMV4R8.92 were obtained from Shanghai Hollybio (Shanghai, China), which uses green fluorescent protein (GFP) as an internal control with an independent promoter. For the generation of MGC80-3-shGNAQ (S1), MGC80-3-shGNAQ (S2), MGC80-3-shControl, SGC7901-shGNAQ (S1) and SGC7901-shControl cells, the MGC80-3 and SGC7901 cells were added together with shGNAQ or shCon lentivirus at a multiplicity of infection (MOI) of 40, respectively. A fluorescence microscope was used to verify recombinant lentiviral transduction efficiency. After 120 h of infection, cells were observed and photographed under a fluorescence microscope (BX50; Olympus, Tokyo, Japan) and then harvested to assess GNAQ silencing efficiency using qRT-PCR and western blotting.

Quantitative real-time PCR (qRT-PCR) analysis. Total RNA from the cultured MGC80-3 and SGC7901 cell lines were reversely transcribed into cDNA using oligo(dT) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's protocol. The mixture of 10 μl 2X SYBR Premix Ex Taq, 0.8 μl forward and reverse primers (2.5 μM), 5 μl cDNA and 4.2 μl ddH_2O was added to qRT-PCR reactions using the ABI 7300 cycler (Applied Biosystems, Foster City, CA, USA). The β-actin gene was used to normalize expression levels in subsequent quantitative analyses. To amplify the target genes, the following primers were used: GNAQ forward, 5'-GACACCATCCTCCAGTTG AACC-3' and reverse, 5'-ACACGCTCACACAGAGTCCAG-3'; β-actin forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAACGCAACTA-3'.

Western blotting. Cell samples were harvested in protein lysis buffer 2X SDS lysis buffer containing 100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS and 10% glycine. Protein supernatants were obtained by centrifugation at 12,000 rpm for 10 min at 4˚C. Protein quantification was carried out using a BCA protein assay kit (PF205629; Thermo Fisher Scientific, Waltham, MA USA). Protein was separated using SDS-PAGE and was blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk in a Tris-buffered saline (TBS) buffer, the membrane was probed with a primary antibody for GNAQ (#13927-1-AP), p53 (#10442-1-AP; both from Proteintech), p21 (#2947; Cell Signaling Technology, Inc., Danvers, MA, USA), cyclin A (#18202-1-AP; Proteintech), p-CDK2 (#2561), CDK2 (#2546), p-ERK (#4370), ERK (#Sc-154) (all from Cell Signaling Technology, Inc.,), p-MEK (#11205) and MEK (#21428) [both from Signalway Antibody (SAB), College Park, MD, USA] with a dilution of 1:1,000, respectively, and GAPDH (#10494-1-AP; Proteintech) as a control with a dilution of 1:100,000 at 4˚C overnight. After rinsing three times with TBS solution buffer, the membrane was incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG antibody (#Sc-2054; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and then washed, followed by visualization using a chemiluminescence analysis system (Tanon-4200; Tianneng, Shanghai, China).

Cell growth assay. Cellular growth of the MGC80-3 control (Con group), MGC80-3-transfected (shCon and shGNAQ groups), SGC7901 control (Con group) and SGC7901

transfected cells (shCon and shGNAQ groups), were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 72 h of incubation, the cells were trypsinized, resuspended, counted and plated in a 96-well plate at a density of $2x10^3$ cells/well for five time-points (day 1, 2, 3, 4 and 5). At each time point, MTT solution was added for 4 h followed by acidic isopropanol overnight. The absorbance at 595 nm was assessed to evaluate cell growth. The experiment was performed in triplicate for each data point.

Cell colony formation assay. A total of 500 cells for each group including the MGC80-3 control cells (Con group), MGC80-3 transfected cells (shCon and shGNAQ groups), SGC7901 control cells (Con group) and SGC7901-transfected cells (shCon and shGNAQ groups), were seeded into each well of a 6-well plate. The culture medium was changed every three days, and cells were cultured for 9 days at 37˚C. Subsequently, the cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 min, and then stained with 0.1% crystal violet for 20 min. The cell colonies were then counted under a light microscope (CH-2; Olympus) and photographed under a digital camera (Nikon, Tokyo, Japan). The experiments were performed in triplicate.

Cell cycle analysis. The MGC80-3 control cells (Con group), MGC80-3-transfected cells (shCon and shGNAQ groups), SGC7901 control cells (Con group) and SGC7901-transfected cells (shCon and shGNAQ groups) were trypsinized, washed twice in cold PBS with 0.1% bovine serum albumin, and fixed with 75% ethanol overnight. Before being run on the flow cytometer, the cells were washed twice again as aforementioned and incubated with 5 μl RNase (200 U/ml, DNase-free) for 15 min. The cells were then stained with 10 μg/ml propidium iodide for at least 1 h in the dark. The cell cycle distribution was analyzed by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Each experiment was performed in triplicate.

Statistical analysis. Results were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± SD. Statistical significance was determined by Student's t-test. The characteristics of two GC patient groups were compared using the χ^2 test. DFS and OS curves were estimated using the Kaplan-Meier method. A p-value <0.05 was considered statistically significant.

Results

GNAQ expression in GC patient samples is significantly higher in the regions with carcinoma vs. normal tissues. To examine GNAQ expression in the surgical tissue of GC patients, we performed immunohistochemistry (IHC) on surgical specimens from 280 GC patients with a history of gastrectomy. Higher expression of the GNAQ protein was observed in the regions with carcinoma as compared to normal adjacent tissues >5 cm distance from the tumor tissue (76.4 vs. 50.7%, p<0.001; Table I). GNAQ overexpression was observed in all stages (I-IV) of GC and was not significantly different between the stages (Fig. 1). These data suggest that GNAQ expression is dysregulated in GC patient samples.

Table I. GNAQ expression in surgical specimens of the gastric cancer patients (n=280).

	GNAQ in normal tissues		
Group	(-)	(+)	P-values
GNAQ in carcinoma tissues			
(-)	27	39	**<0.001**[a]
(+)	111	103	
Total	138	142	

[a]P<0.001. GNAQ, G protein subunit α q.

Table II. Association between patient characteristics and GNAQ expression in gastric carcinoma tissues (n=280).

	GNAQ expression in carcinoma tissues		
Patient characteristics	(+), n (%)	(-), n (%)	P-values
Gender			0.256
Male	158 (78.2)	44 (21.8)	
Female	56 (71.8)	22 (28.2)	
Age, years			**<0.001**[b]
<60	114 (68.3)	53 (31.7)	
≥60	100 (88.5)	13 (11.5)	
Stage (AJCC/UICC)			0.264
I, II	132 (79.5)	34 (20.5)	
III, IV	82 (71.9)	32 (28)	
Location			0.106
GEJ	37 (86)	6 (14)	
N-GEJ	177 (74.7)	60 (25.3)	
Differentiation			**<0.001**[b]
Intermediate	116 (69)	52 (31)	
Poor	98 (87.5)	14 (12.5)	
Adjuvant therapy			**0.002**[a]
Yes	84 (67.7)	40 (32.3)	
No	126 (83.4)	25 (16.6)	

[a]P<0.01, [b]p<0.001. GNAQ, G protein subunit α q; AJCC, American Joint Committee on Cancer; UICC, Union for International Cancer Control; GEJ, gastroesophageal junction; N-GEJ; non-gastroesophageal junction.

GNAQ overexpression is associated with the age and histological subtype of GC patients. A total of 280 GC patients treated with gastrectomy were enrolled in this retrospective, pilot cohort study. We evaluated the clinicopathological features of GC patients according to the GNAQ expression status in the cancer tissues. No significant difference was found in gender, stage and tumor location between patients according to the GNAQ expression status (p=0.256, 0.264

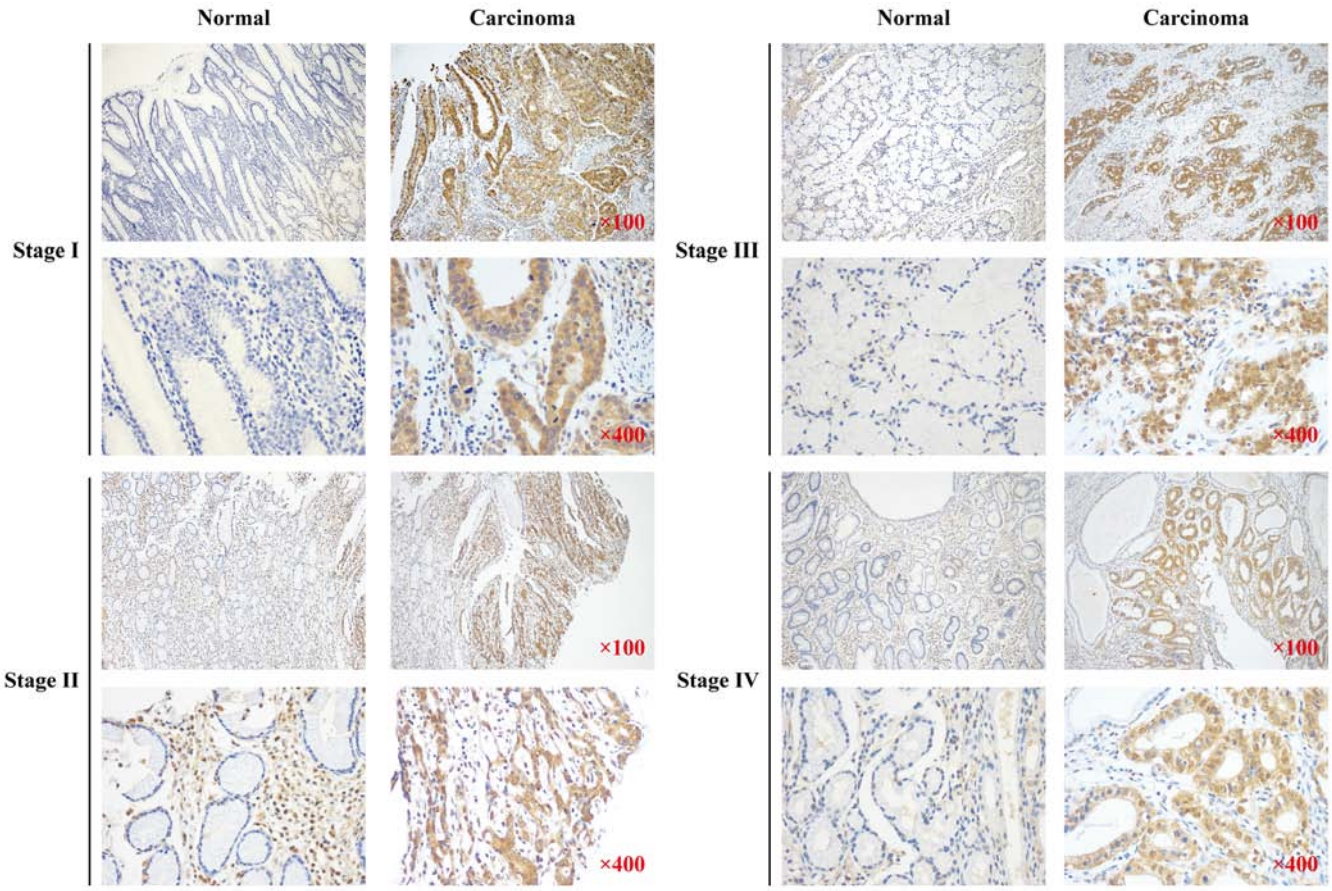

Figure 1. GNAQ is overexpressed in GC surgical specimens of different stages. Histology of GC surgical specimens by SP assays. GNAQ staining shows that there is no significant difference in GNAQ expression in samples from patients with earlier stages (I and II) and later stages (III and IV). GNAQ, G protein subunit α q; GC, gastric cancer; SP, streptavidin-peroxidase.

and 0.106, respectively; Table II), however, the age and histological differentiation were significantly associated with GNAQ expression. Higher expression levels of GNAQ were found in the tumor tissues of older patients (≥60 years) compared with younger patients (<60 years, 88.5 vs. 68.3%, p<0.001; Table II). Furthermore, GNAQ overexpression was observed in patients with worse histological characteristics (87.5 vs. 69%, p<0.001; Table II).

GNAQ expression is not correlated with clinical outcomes. To estimate the prognostic value of GNAQ overexpression in cancer tissues, we used Kaplan-Meier survival analysis to compare clinical outcomes between GNAQ-positive and -negative patients. All 280 eligible patients were analyzed for DFS and OS. Median follow-up was 40.2 months (from 3.8 to 59.6 months). However, no difference was observed in the DFS and OS of GC patients after gastrectomy between GC patients with GNAQ-positive and -negative tumors (median DFS, 42.288 vs. 40.308 months, p=0.496, and median OS, 45.675 vs. 44.076 months, p=0.430, respectively) (Fig. 2A and B). Subgroup analysis of the survival time according to age, histological differentiation and with the application of adjuvant treatment or without were analyzed in the positive GNAQ expression group and negative GNAQ expression group (Fig. 2C-N). However, no significant difference was observed in the survival time between GNAQ-positive and negative GC patients.

Expression of GNAQ in GC cells. To analyze the expression of GNAQ in different GC cells, we performed western blotting assay to estimate the expression in MGC80-3, SGC7901 and AGS cell lines. As shown in Fig. 3A, The GNAQ expression levels ranked as SGC7901 > MGC80-3 > AGS.

GNAQ knockdown inhibits cellular growth and colony formation. To investigate the function of the GNAQ gene in GC, the lentivirus targeting the GNAQ gene [shGNAQ (S1) group and shGNAQ (S2) group] and negative control (shCon group) was infected into MGC80-3 cells. We performed qRT-PCR and western blotting assay to estimate the GNAQ knockdown efficiency in MGC80-3 cells and found that both the mRNA and protein levels of GNAQ were significantly decreased in the shGNAQ group as compared to the blank control (Con group) and shCon group (p<0.001; Fig. 3A and B). Moreover, the GNAQ protein levels in the SGC7901 cells were significantly decreased in the shGNAQ group compared to the blank control (Con group) and shCon group (Fig. 4A). Subsequently, MTT and colony formation assays were further performed to examine the influence of GNAQ on GC cell growth. As shown in Figs. 3C and 4B, the cellular proliferation in the shGNAQ group was markedly lower compared with the control groups (Con and shCon groups) *in vitro*. Similarly, GNAQ silencing obviously decreased the colony formation ability of the MGC80-3 and SGC7901 cells in size and number when compared with the Con and shCon groups (p<0.001;

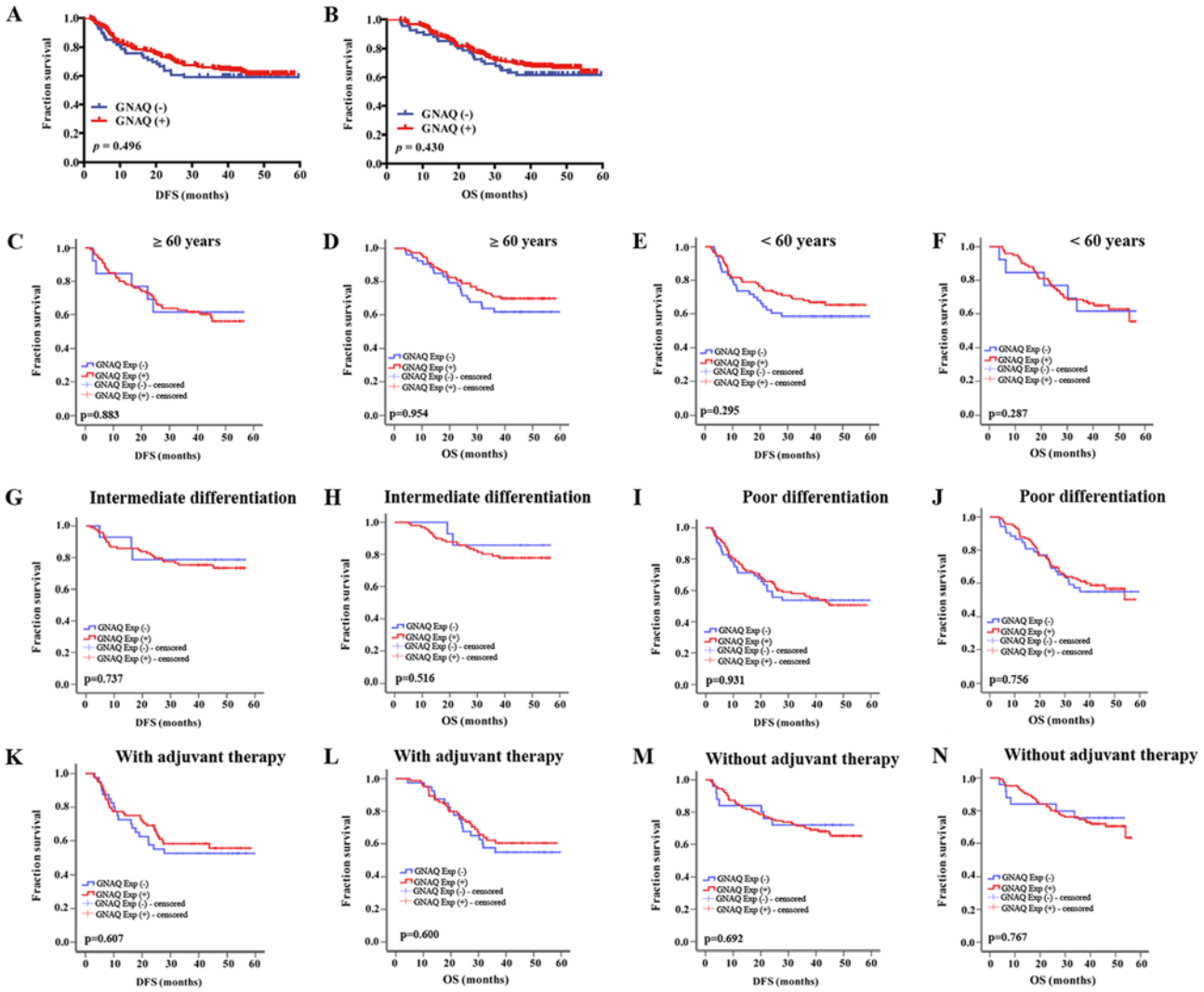

Figure 2. GNAQ expression is not correlated with DFS or OS in GC patients. (A) Patients with GNAQ overexpression (red) in GC surgical specimens exhibited no difference in DFS compared with patients without GNAQ expression (blue) (median DFS, 42.288 vs. 40.308 months; p=0.496). (B) Patients with GNAQ overexpression (red) in GC surgical specimens exhibited no difference in OS compared with patients without overexpression (blue) (median OS, 45.675 vs. 44.076 months; p=0.430). (C) No significant difference was observed in the DFS of elder (≥60 years) GC patients with GNAQ-positive and -negative tumors (p=0.883). (D) No significant difference was observed in the OS of elder (≥60 years) GC patients with GNAQ-positive and -negative tumors (p=0.954). (E) No significant difference was observed in the DFS of younger (<60 years) GC patients with GNAQ-positive and -negative tumors (p=0.295). (F) No significant difference was observed in the OS of younger (<60 years) GC patients with GNAQ-positive and -negative tumors (p=0.287). (G) No significant difference was observed in the DFS of GC patients with intermediate histological differentiation in the positive GNAQ expression and negative groups (p=0.737). (H) No significant difference was observed in the OS of GC patients with intermediate histological differentiation in the positive GNAQ expression and negative groups (p=0.516). (I) No marked difference was displayed in the DFS of GC patients with poor histological differentiation in the GNAQ-positive and -negative group (p=0.931). (J) No marked difference was displayed in the OS of GC patients with poor histological differentiation in the GNAQ-positive and -negative group (p=0.756). (K) No significant difference was observed in the DFS of GC patients who received adjuvant therapy in the GNAQ-positive and -negative group (p=0.607). (L) No significant difference was observed in the OS of GC patients who received adjuvant therapy in the GNAQ-positive and-negative group (p=0.600). (M) No significant difference was observed in the DFS of GC patients who did not receive adjuvant therapy in GNAQ-positive and -negative group (p=0.692). (N) No significant difference was observed in the OS of GC patients who did not receive adjuvant therapy in GNAQ-positive and -negative group (p=0.767). GNAQ, G protein subunit α q; GC, gastric cancer; DFS, disease-free survival; OS, overall survival.

Fig. 3D and E) (p<0.001; Fig. 4C and D). Although, the cellular proliferation as determined by MTT assay in the shGNAQ (S1) group of the SGC7901 cells was lower compared with the control groups (Con and shCon groups) *in vitro* (p<0.001) it was not as decreased as in the shGNAQ (S1) group of the MGC80-3 cells. The aforementioned findings indicate that the GNAQ gene may be involved in the cellular growth of GC.

GNAQ knockdown induces GC cell cycle arrest and promotes apoptosis. To examine the role of GNAQ in GC cell cycle kinetics, we performed flow cytometry. We found that a significantly greater percentage of GNAQ-silenced MGC80-3 cells was observed in the S phase, and cells in the G0/G1 phase presented a decreased population compared with the control groups (Con and shCon groups) (p<0.001; Fig. 5A and B). Moreover, we observed a similar result in the GNAQ-silenced SGC7901 cells. Cells in the S phase were significantly increased whereas cells in the G0/G1 phase presented a decreased population compared with the control groups (Con and shCon groups) (p<0.001; Fig. 4E). The cell cycle assay analysis also revealed that knockdown of GNAQ increased the percentage of sub-G1 cells, indicative of an increase in

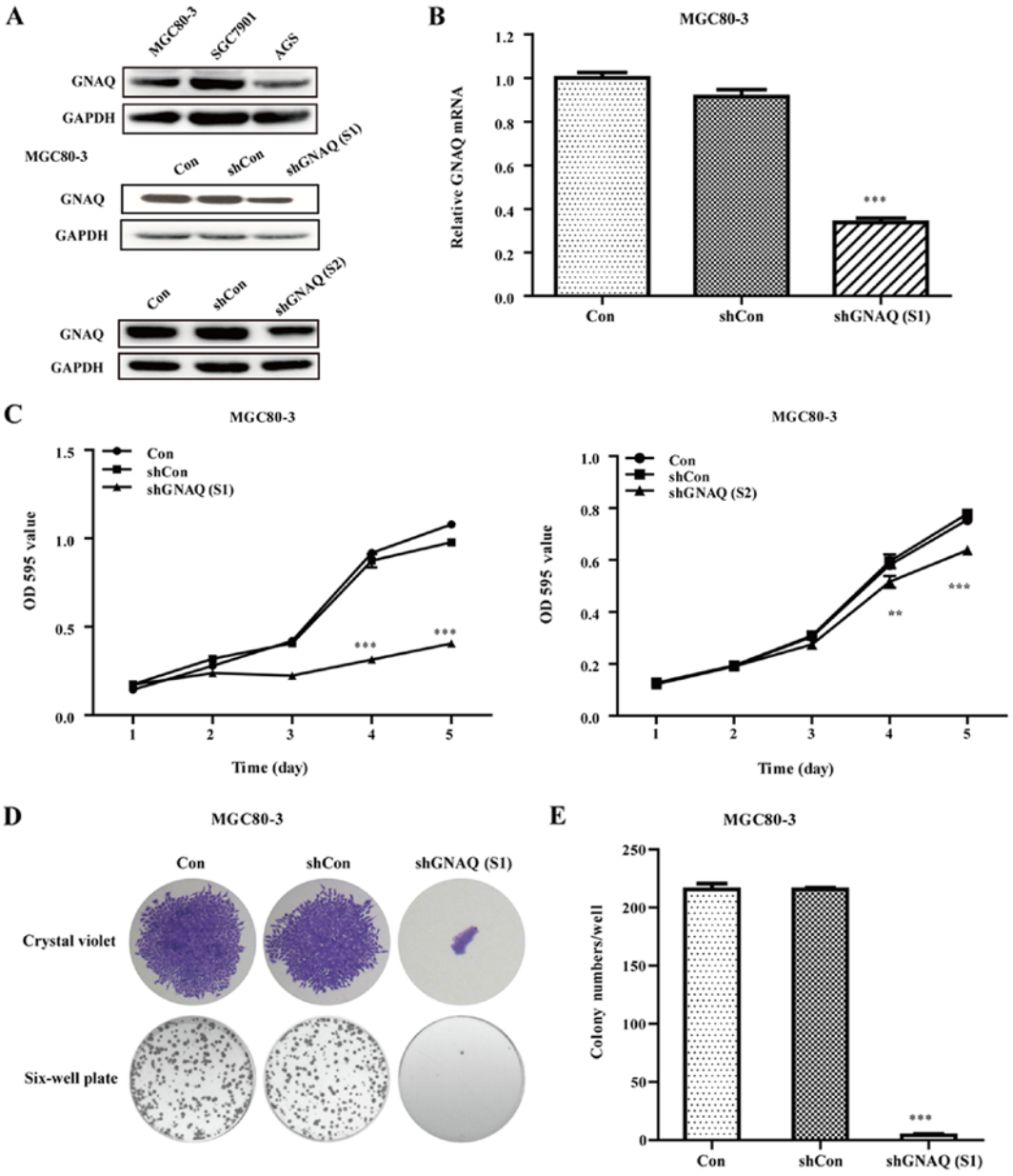

Figure 3. GNAQ silencing inhibits MGC80-3 cell growth and colony formation. (A) The expression of GNAQ in MGC80-3, SGC7901 and AGS cell lines and GNAQ potein expression in the shGNAQ groups was confirmed by western blot assay. GAPDH served as a loading control. (B) GNAQ mRNA expression in the shGNAQ group was examined by qRT-PCR analysis. (C) The rate of cellular growth in the MGC80-3 control cells (Con group) and in the MGC80-3 transfected cells (shCon and shGNAQ groups) was determined by MTT assay. (D) Colony formation assay was performed to confirm the cell colony formation ability in the MGC80-3 cells infected with shGNAQ. (E) Quantification of colony formation in the three different groups (Con, shCon and shGNAQ groups); $^{**}p<0.01$, $^{***}p<0.001$. GNAQ, G protein subunit α q; GC, gastric cancer.

apoptosis (Figs. 5C and 4F). Therefore, GNAQ gene silencing in GC cells led to an S phase arrest and promoted apoptosis.

GNAQ knockdown significantly increases p53 expression and inhibits cell cycle-related proteins. To determine the mechanism by which GNAQ alters the cell cycle and apoptosis, MGC80-3 cell lysates from different groups (Con, shCon or shGNAQ group) were used for the immunoblot analysis of p53, p21, phospho-CDK2 and other cyclin family members. As shown in Fig. 6A, the expression of p53 was significantly increased in the shGNAQ group compared with the Con and shCon groups. Similarly, higher p21 expression was observed in the knockdown GNAQ cells compared with the controls (Con and shCon groups). Furthermore, cyclin A and phospho-CDK2 were decreased in cells infected with shGNAQ compared to the controls (Fig. 6A). Collectively, these data suggested that GNAQ directly or indirectly inhibits p53 and several key regulators of cell cycle signaling.

GNAQ knockdown inhibits MAPK pathway activity. Mitogen-activated protein kinase (MAPK) signaling regulates cell proliferation in numerous tumor and normal cells. We therefore examined the key components of the MAPK pathway by western blotting assay, and found that knockdown of GNAQ decreased the phosphorylation of two key signaling proteins along the MAPK pathway: ERK and MEK (Fig. 6B). These data indicated that GNAQ expression was required for MEK/ERK phosphorylation in MGC80-3 cells.

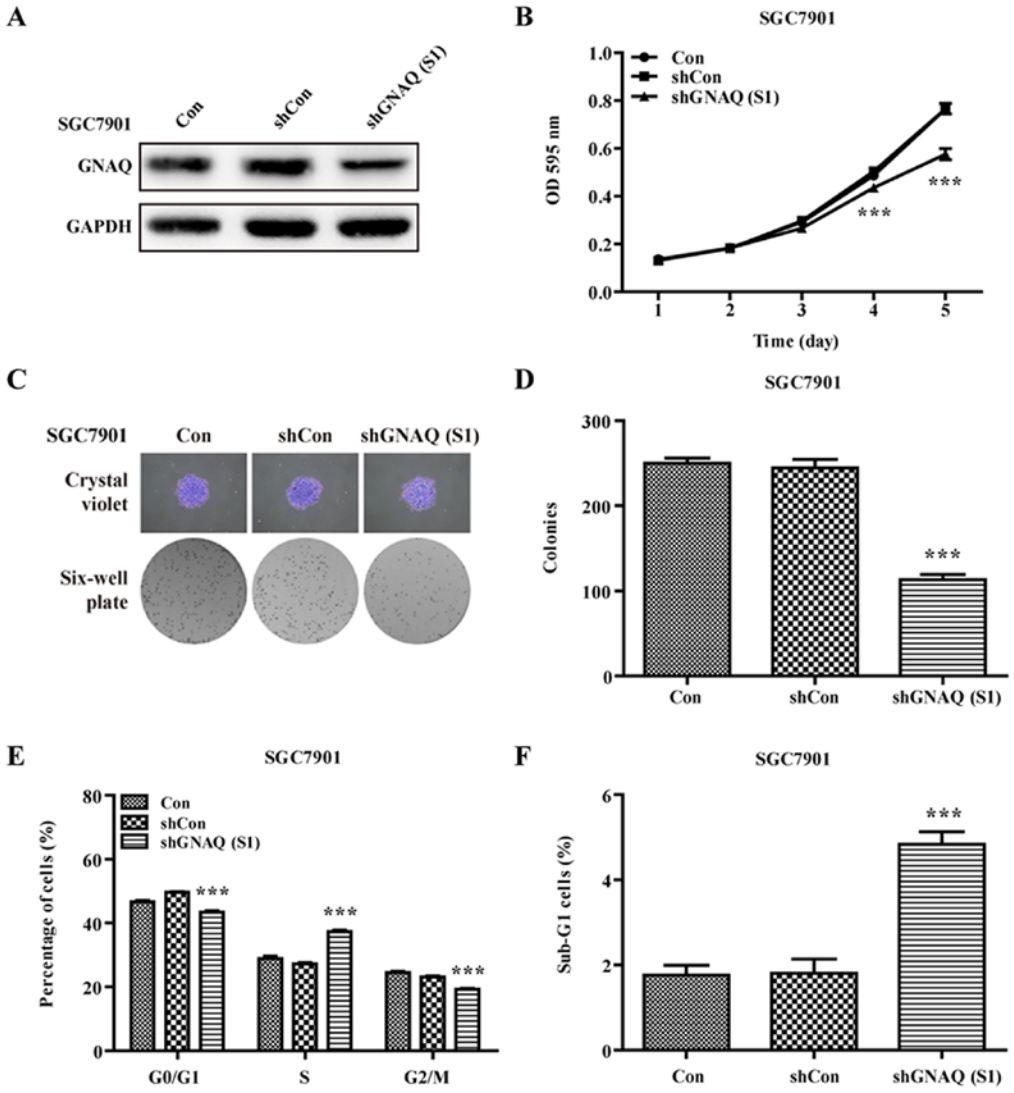

Figure 4. Knockdown of GNAQ inhibits cell proliferation, arrests the cell cycle at the S phase and promotes apoptosis in SGC7901 cells. (A) Knockdown efficiency of GNAQ in SGC7901 cells. (B) The rate of cellular growth in the SGC7901 control cells (Con group) and in the SGC7901 transfected cells (shCon and shGNAQ groups) was determined by MTT assay. (C and D) Colony formation assay was performed to confirm the cell colony formation ability in SGC7901 cells infected with shGNAQ. (E) Knockdown of GNAQ arrests the SGC7901 cell cycle at the S phase. (F) Knockdown of GNAQ promotes apoptosis in SGC7901 cells; $^{***}p<0.001$. GNAQ, G protein subunit α q.

Discussion

G protein-coupled receptor (GPCR) proteins are a large, diverse family of heterotrimeric transmembrane proteins that function as signal transducers from the extracellular environment to the cellular interior. On account of their critical importance and the variety of GPCR signaling to normal homeostatic function, it is perhaps not surprising that alterations in the activity of GPCRs and their downstream effectors are frequently implicated in the pathogenesis of cancer. Nearly 20% of human tumors are associated with GPCR aberrancies (14). The GNAQ gene encodes a G protein α subunit of GPCR and has been identified within chromosomal band 9q21, a region that has been associated with many types of human cancer. However, its explicit function in human cancer particularly in gastric cancer (GC) remains largely unclear.

Our findings revealed that GNAQ was overexpressed in GC patient samples as compared to normal matched tissue. Furthermore, GNAQ expression was found to be associated with poorly differentiated GC, suggesting that GNAQ may be a useful biomarker. We investigated the prognostic value of GNAQ overexpression and determined that it had no predictive value in patient outcome for the population we sampled. To conclusively determine the prognostic value of GNAQ, a larger patient population may be examined and perhaps additional types of cancer may be investigated in future studies.

The GNAQ gene encodes a G protein α subunit that has long been known to activate downstream signaling molecule phospholipase C (PLC), which cleaves phosphotidylinositol diphosphate into inositol triphosphate and diacylglycerol (DAG) (15). Inositol triphosphate and DAG then signal calcium and protein kinase C (PKC) via phosphorylation (16). Phosphorylation of PKC can turn on the mitogen-activated protein kinase (MAPK) pathway, which consists of a cascade of kinases including the GTPase Ras, followed by BRAF, MEK and ERK, leading to activation of various transcription factors involved in normal cell growth and proliferation (17-20). Excessive activation of MAPK signaling is the primary

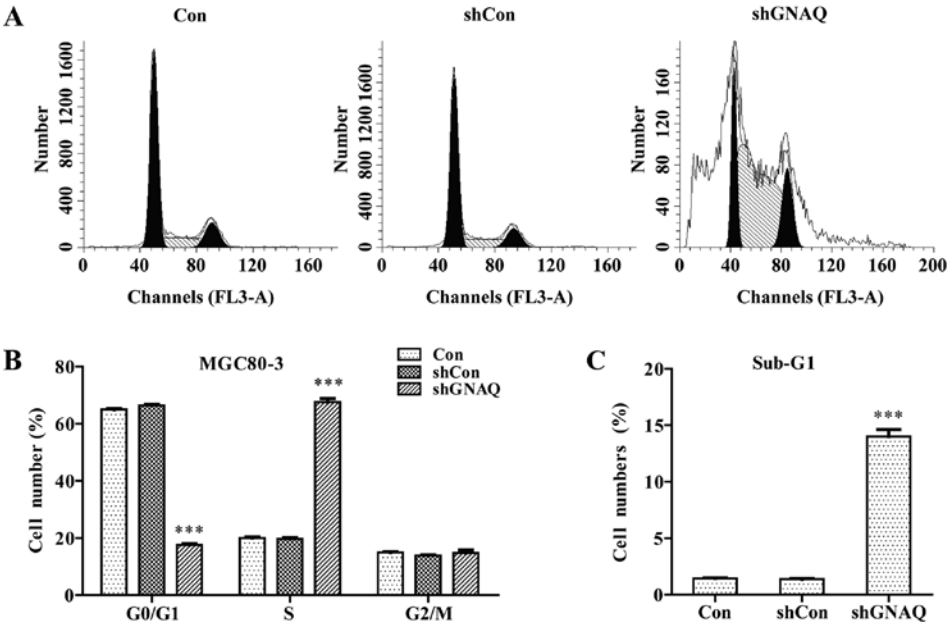

Figure 5. Knockdown of GNAQ arrests MGC80-3 cells at the S phase and promotes apoptosis. (A) Flow cytometric analysis of cell cycle kinetics in the control (Con group) and transfected MGC80-3 cells (shCon and shGNAQ groups). (B) Quantification of different cell cycle phases in the three different groups (Con, shCon and shGNAQ groups). (C) Quantification of the percentage of sub-G1 cells in the shGNAQ group compared with the controls (Con and shCon groups); $^{***}p<0.001$. GNAQ, G protein subunit α q.

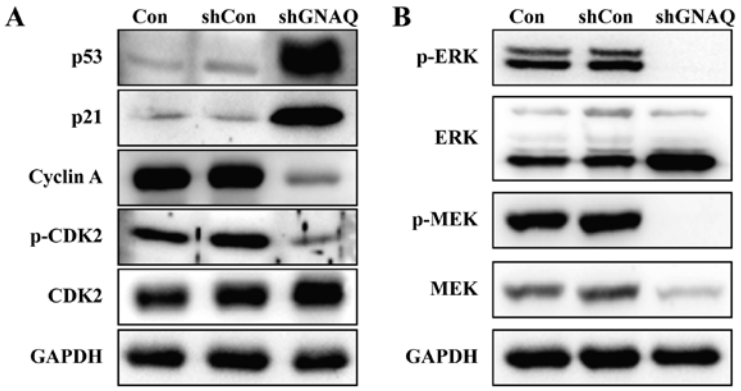

Figure 6. Suppression of GNAQ increases p53 and p21 expression, while inhibiting cell cycle-related proteins, and the MEK/ERK signaling pathway. (A) Immunoblots of the control and transformed cells probing for p53, p21, cyclin A, p-CDK2 and CDK2. (B) Immunoblots of the control and transformed cells probing for p-ERK, ERK, p-MEK and MEK. GAPDH served as a loading control. GNAQ, G protein subunit α q.

driver of cell cycle dysfunction and unregulated proliferation in most cancer cells (20-23). Our data demonstrated that GNAQ regulates GC proliferation, colony formation, the cell cycle, and either directly or indirectly promotes MEK and ERK phosphorylation. Notably, in the Catalogue of Somatic Mutations in Cancer (COSMIC) v69, there is a high prevalence of GNAQ activating mutations in tumor types known to require the MAPK pathway for pathogenesis including uveal melanomas (32%), cutaneous melanomas (1.4%) and colon adenocarcinoma (1.4%). Additionally, a study by Van Raamsdonk *et al* identified activating mutations in GNAQ that could function as oncogenic drivers in models of human uveal melanoma. Furthermore, knockdown of GNAQ in the melanoma cell lines they used, resulted in decreased growth and increased apoptosis, consistent with our findings in GC (24). Ultimately, GNAQ may act as an oncogene in a broad range of human types of cancer by driving MAPK signaling.

p53 is the 'guardian of the genome' and the most commonly mutated tumor suppressor in cancer (25). In normal unstressed cells, p53 is maintained at a low level by its negative regulators, such as MDM2. In response to a wide variety of stress signals, activated p53 transcriptionally regulates the expression of its target genes to modulate various cellular processes, including apoptosis and cell cycle arrest (26). p53 provides a critical barrier to the development of cancer by blocking proliferation or eliminating cancer cells. Over the last two decades, numerous p53-responsive genes have been identified, including the gene encoding cyclin-dependent kinase inhibitor $p21^{Waf1}$ (alternatively $p21^{Cip1}$), which mediates cell cycle arrest at the G1/S and G2/M phase (27,28). CDK2 has high activity during the S phase, which may be due to cyclin A driving the transition from the S phase to mitosis. We observed for the first time that GNAQ is involved in the p53 signaling pathway. Suppression of GNAQ expression in GC cells resulted in

increased p53 and p21 expression. The increased expression of p21 inhibited the activity of the cyclin A/CDK2 complex, slowed down cell proliferation and therefore arrested the cells at the S phase. These data suggest that GNAQ may mediate its effects on the cell cycle and apoptosis through p21 via p53 signaling.

Overall we determined that GNAQ regulates GC proliferation, the cell cycle and apoptosis through a multifactorial mechanism involving downstream pathways including MAPK, p53 and CDKs. In precision medicine, whole genome sequencing data along with related molecular information and individual clinical data, are often employed for selecting appropriate and optimal therapies and for the development of targeted drugs in order to achieve accurate medical care. At present, next generation sequencing (NGS) technology is being applied in some clinical studies. GNAQ, as a target, is detected in the serum of GC patients. The present study indicates that GNAQ is a potential target for the treatment of GC patients. The GNAQ inhibitor is expected to be a novel drug for GC patients with activated gene mutations. These observations provide important insight into the functional significance of GNAQ overexpression in GC and indicate that GNAQ may be a novel target for GC treatment.

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