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GNAO1 as a novel biomarker in colorectal cancer

조선대학교 대학원

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이 논문을 의학박사학위신청 논문으로 제출함

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목차

도목차

초 록

직결장암에서 GNA01의 종양억제유전자로서의 역할과 임상적 의의

GNA01 as a novel biomarker in colorectal cancer

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서론

직결장암은 우리나라에서 높은 발생률를 보이며, 사망률도 폐암, 위암 다음으로 높게 보고된다. 기존의 직결장암에 대한 항암치료와 더불어 새로운 바이오마커에 대한 표적치료를 시행하여 치료효과를 상승시켰으나 4기 직결장의 기대여명은 3년에 불과하다. 많은 연구자들이 암의 발생, 증식, 침윤, 전이 및 예후에 관여하는 새로운 바이오마커를 연구하고 이의 임상적 의의를 찾으려고 하고 있다. 이에 본 저자는 GNA01이 직결장암에서 종양억제유전자로서 역할을 검증하고 임상적의의를 파악하고자 하였다.

방법

GNA01 역할을 확인하기 위하여 RNA sequencing, Proliferation assay, Anchorage independent growth assay, Migration assay, Xenograft animal tumor growth assay, Proteome Array, GNA01 patients tissues assay를 진행하였다. 그리고 human phospho-kinase array assays 를 통하여 GNA01의 분자유전학적 기전을 확인하였다.

결과

RNA-seqencing analysis를 통해 GNA01 gene이 직결장암에서 발현이 억제된다는 것을 확인하였다($p<0.0001$). GNA01의 과발현이 human colon HCT116 과 DLD-1 세포주에서 암세포의 증식, 세포의 이동을 억제시킨다는 것을 확인하였다. 또한 종양의 발생과 GNA01 발현과의 관계를 보기 위한 동물실험에서도 GNA01이 주입된 그룹에서 종양의 생성이 억제되는 것을 확인할 수 있었다. 또한, 암의 진행병기에 따른 GNA01발현의 정도는 병기가 높아질수록 발현이 억제됨을 확인할 수 있었다. GNA01 gene의 종양억제 역할에 p70-S6 kinsae pathway가 관여한다는 것을 확인할 수 있었다.

결론

저자는 GNA01의 발현과 직결장암의 발생과의 연관성에 대해서 연구를 진행하였고 GNA01이 직결장암에서 종양억제유전자로서의 역할을 확인하였다. 또한 직결장암의 병기와 GNA01 발현정도의 상관관계를 증명하여 추후 예후를 예측하는 새로운 바이오마커로서의 가능성도 확인할 수 있었다. 마지막으로 직결장암의 발병기전으로 알려진 mTOR-S6K1 pathway에 GNA01이 종양억제 유전자로서의 역할을 한다는 것을 일부 증명하여 직결장암의 치료 표적으로써 임상적 의의를 확인할 수 있었다.

Key word: 직결장암, 종양억제유전자, GNA01, mTOR-S6K1 pathway

I. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide. Korea has one of the highest CRC incidences compared to many countries worldwide. In 2018, the CRC incidence was 44.5/100,000, the second highest after Hungary [1]. In Korea, the CRC incidence has been decreasing since 2012 due to fecal occult blood tests and colonoscopy; however, it is still the third most common cancer in both men and women, along with lung and liver cancer, and is one of the main causes of cancer death [2]. Surgical resection is the only curative method in the early stage; however, chemotherapy combined with targeted therapy is performed as a standard method in inoperable advance stage patients with cancer metastasis or recurrence[3,4]. The mechanism of traditional chemotherapy is to kill cancer cells by attacking the cells that rapidly divide and multiply with 'cytotoxic anticancer drugs'. These mechanisms of chemotherapy cause many side effects, including damage to normal cells in the body. Methods to attack cancer cells selectively have always been considered to eliminate damage to normal tissues. The molecular biological properties of cancer have been studied. Specific proteins or genes are more expressed in cancer cells than in normal cells. Therefore, we refer to these biomarkers. Molecular targeted therapy attacks these biomarkers to kill cancer cells by blocking signals involved in the development and growth of cancer [4-6]. Recently, research on tumoral genetic abnormalities and carcinogenetic mechanisms in colorectal cancer has been rapidly progressing, and various target agents have been developed. Additionally, various clinical studies have been conducted to target the treatment of targeted tumor markers or tumor suppressor genes [7].

Therefore, the combination of cytotoxic chemotherapy with molecular target agents for epidermal growth factor receptor (EGFR) and vascular

endothelial growth factor receptor (VEGFR) has become the standard chemotherapy for advanced, metastatic, and recurrent colorectal cancer. The standard combination chemotherapy increased the response rate and dramatically improved the survival rate. However, despite many recent studies, molecular targets other than EGFR and VEGFR have not been identified yet, and the number of studies have slightly decreased [8].

Recently, large-scale gene projects such as The Cancer Genome Atlas(TCGA) have rejuvenated research on molecular targets as data on various genes that are uniquely mutated in tumors have begun to be analyzed. The discovery of tumor-specific mutant genes may lead to the development of not only therapeutic markers, but also biomarkers that can change diagnosis, prognosis, and treatment methods. Therefore, many researchers have analyzed new biomarkers involved in the development, proliferation, invasion, metastasis, and prognosis of cancer and determined their clinical significance [7,9].

GNAO1 (guanine nucleotide-binding protein, α-activating activity polypeptide O; G-α-o), a G protein, is a molecular switch that internally transmits chemical signals outside the cell [10]. In 2013, epileptic encephalopathy in four patients was reported as a disease caused by a GNAO1 mutation in Japan. Oncogenesis of mutant GNAO1 has also been studied [11]. The role of GNAO1 as a tumor suppressor gene in prostate cancer, breast cancer, hepatocellular carcinoma, and gastric cancer has been reported [12,13]. However, the expression and role of GNAO1 in rectal cancer have not yet been established. This study aims to demonstrate the following hypotheses: to investigate the role of GNAO1 as a tumor suppressor in colorectal cancer, determine the value of biomarkers, predict prognosis, and reveal the possibility of targeted therapy by demonstrating the mechanism of the GNAO1 pathway.

II. Materials and Methods

RNA-seq and alignments

To evaluate transcriptome features in colorectal cancer at the nucleotide-wise resolution, we performed RNA-seq analysis of poly(A+) RNAs isolated from normal colon cells and colon cancer cells. A total of 67,219,518 paired-end reads for normal colon cells and 71,840,146 paired-end reads for colon cancer cells were produced from the Hi-Seq pipeline with a length of 50 bp at each end. The short reads were aligned to the mm10 reference genome using TopHat, with up to two mismatches admitted. The unmapped reads were trimmed to eliminate poly-A/T tails (repeats of [A/N] s or [T/N] s) from read ends/starts and then aligned to the reference genome. It is worth noting that we only retained reads with at least 30 bp at both ends after trimming. In this study, 88.2% of short reads from normal colon cells and 88.7% of sequence reads from colon cancer cells were mapped to the reference genome using TopHat for APA analysis.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined using the 3-(4, 5-Dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to a standard protocol. Following the treatment, the cells were incubated with 10 μL MTT (1 mg/mL) in PBS in 96-well plates for 4 h at 37 ° C. Thereafter, the medium containing MTT was eliminated, and 100 μL dimethyl sulfoxide (DMSO) was added. The cells were incubated for an additional 10 min at 37 ° C with gentle shaking. The absorbance was read on an ELISA plate reader (Tecan, Trading AG, Switzerland) with

a 570 nm filter. The degree of cell viability was measured based on the relative color intensities of the treated and untreated samples.

In Vitro Migration assay using transwell

In vitro migration experiments were performed using Transwell (Costar), consisting of 24-well companion plate with cell culture inserts containing 8 mm pore size filters. Briefly, transfected HCT116-control cells and HCT116-GNA01 cells (5×10^4) in serum-free medium were added to each insert (upper chamber), and the chemoattractant (10% FCS) was placed in each well of the 24-well companion plate (lower chamber). After 24 h incubation at 37 ° C in a 5% CO_2 incubator, the upper surface of the filter was wiped with a cotton-tipped applicator to remove cells not migrated. Cells migrated through the filter pores and attached to the undersurface of the filter. These cells were fixed and stained. The membranes were mounted on glass slides, and cells from 10 random microscopic fields were calculated. All experiments were performed in triplicates.

Soft agar colony formation assay

Soft agar assays were performed on 6-well plates. The base layer of each well consisted of 2 ml with final concentrations of 1× medium and 0.6% low-melting-point agarose (Duchefa, Haarlem, The Netherlands). Plates were chilled at 4 ° C until the contents solidified. Thereafter, a 1 ml growth agar layer was poured, consisting of 5×10^4 cells suspended in 1× medium and 0.3% low-melting-point agarose. Plates were again chilled at 4 ° C until the growth layer congealed. Furthermore, 1 ml of 1× medium without agarose was inserted on top of the growth

layer. Cell cultures were maintained at 37 ° C for 14 d by exchanging the media every 3 d. Total colonies were stained with 0.005% crystal violet (Sigma, St Louis, MO, USA) and calculated in five random fields. Images were analyzed using Image-Pro Plus 4.5 software (Media Cybernetics).

Tumor formation in nude mice

In this study, six weeks old male BALB/c nude mice (Orient Bio Inc. Seongnam, Korea) were used and housed in our pathogen-free facility. The mice were handled in accordance with standard protocols and animal welfare regulations. Control-HCT116 cells (1×10^6 cells) and GNA01-HCT116 cells (1×10^6 cells) were resuspended in serum-free Iscove's modified Dulbecco's medium and injected subcutaneously into the left flank of BALB/c nude mice. Tumor volume was measured using digital calipers every alternate day and calculated according to the following formula: [length × (width) 2]/2. After three weeks of therapy, all mice were euthanized with disproof according to the institutional guidelines, and local tumors were resected and analyzed.

Immunoblotting

Cells were washed with 1× PBS and lysed in lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM Na_3VO_4, and 5 mM NaF). Concentration of protein was determined using a dye-binding microassay (Bio-Rad, Hercules, CA, USA). Equal concentrations of tissue or cells extracts were resolved using 8-12% sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer of the protein bands onto a polyvinylidene difluoride membrane (PALL Life Sciences, NY, USA). The membranes were blocked for 1 h with Tris-buffered saline-Tween (TBS-T; 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat milk and incubated with specific primary antibodies overnight at 4 ° C. The blots were washed with TBS-T four times for 15 min per wash and incubated for 1 h with the corresponding peroxidase-conjugated secondary antibodies (1:4000 dilution; Jackson ImmunoResearch Inc., West Grove, PA, USA). The blots were washed four times with TBS-T and developed using enhanced chemiluminescence detection system (iNtRON Biotechnology, Gyeonggi, Korea). The antibodies used for western blotting were as follows: rabbit anti-GNAO1 (ab154001 Abcam Cambridge, UK), p70 S6 kinase Substrate antibody sampler kit (#2903 Cell Signaling Technologies, USA), and mTOR Pathway Antibody Sampler Kit (#9964 Cell Signaling Technologies, USA).

Immunohistochemistry

Colorectal cancer tissue samples were purchased from SuperBioChips (Seoul, South Korea). The tissue sections were stained with rabbit anti-GNAO1 antibody (ab154001; Abcam, Cambridge, UK). For IHC, a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) followed by horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA). Following immunolabeling, the specimens were counterstained with hematoxylin. Immunolabeled images were captured using a C-4040Z digital camera and a BX-50 microscope (Olympus Corp, Lake Success, NY, USA). The immunoreactivity of GNAO1 was decided by scoring for staining intensity

(0, none; 1, weak; 2; moderate; and 3, strong) and the percentage of positive cells (0, <5%; 1, 6-25%; 2, 26-50%; 3, 50-75%; and 4, >76%), with the product of the two scores being the final value.

Human colon cancer tissue array staining

A human colon cancer tissue array slide (CDA3) was purchased from SuperBioChips (Seoul, South Korea). The tissue array slide contained 59 samples of colon cancer and normal tissue specimens. In addition, 10 samples were matched to the primary and metastatic tissues from different organs. Immunohistochemistry of the tissue array slides was performed as previously described. The staining results of the tissue array were graded empirically by a pathologist.

Statistical analysis

The results are described as mean ± SD unless otherwise noted. Student's t-test or analysis of variance was used to compare tumor volumes between the two groups. The difference in survival rates between the experimental groups was tested using Kaplan-Meier analysis. *Statistical significance was set at* $p < 0.05$. All experiments were run more than three times repeatedly, and the results from the representative experiments are shown.

III. Results

The down-regulation of GNA01 in human colon cancer

We basically studied and compared the degree of expression of the GNA01 gene in RNA-seq analysis of poly(A+) RNA isolated from normal colon cells and colon cancer cells. A statistically significant decrease in the GNA01 gene expression was observed in colon cancer cells. ($P < 0.0001$; Fig. 1). We also studied the expression levels of GNA01 in eight colon cancer cell lines (HCT-15, SW620, HCT116, DLD-1, SW480, LoVo, CaCo2, and HT29) and normal colon cells (CCD-18co). GNA01 protein levels were measured by western blot analysis and quantified using ImageJ software. The expression of GNA01 was lower in eight colon cancer cell lines than in normal colon cells (Fig. 2).

The effect of GNA01 in cancer cell viability and proliferation

We designed and performed an MTT assay to observe the effect of GNA01 on cancer cell proliferation and viability. Overexpression of GNA01 suppressed the proliferation of HCT116 and DLD-1 human colon cells (Fig. 3).

In Vitro Migration assay using transwell

Control vector and GNA01 overexpressing HCT116 and DLD-1 cells were assessed with cell migration assays performed using a transwell system. We confirmed that overexpression of GNA01 suppresses the migration of human colon HCT116 and DLD-1 cells compared to the control ($p<0.001$; Fig. 4).

Soft agar colony formation assay

Control vector and GNA01 overexpressing HCT116 and DLD-1 cells were

assessed for colony formation in soft agar for 14 days. Overexpression of GNA01 suppresses colony formation of human colon HCT116 and DLD-1 cells compared to the control. ($p<0.001$), respectively (Fig. 5).

GNA01 overexpression on tumor formation in a nude mice HCT116 xenograft model

To observe the effects of GNA01 and tumor formation, we injected HCT116 cells (control) and HCT116 cells mixed GNA01 subcutaneously into the left flank of nude mice. In two groups, tumor volume was measured using digital calipers every 4 day for 40 days. In the control group, an average of 800 mm^2 tumors occurred on day 40. This result proves that tumor formation was inhibited in the GNA01 HCT116 cells mixed GNA01 group (Fig. 6).

Mechanism of GNA01

Overexpression of GNA01 inhibits HCT116 colon cancer cells by phosphorylating p-p70 S6 kinase. To explore the molecular mechanisms of GNA01 in the inhibition of colon cancer, human phospho-kinase array assays (Proteome Profiler; R&D Systems, Minneapolis, MN, USA) were performed in HCT116 cells stably transfected with GNA01 or control. The results of the phospho-kinase array suggested that the activities of p-p70 S6 kinase were inhibited in HCT116-GNA01 cells. The effects of GNA01 overexpression on the activity of p-p70 S6 kinase and Raptor were detected by western blotting (Fig. 7).

Suppression of GNA01 expression according to progression of cancer

Based on the results, it has been shown that the expression of GNA01 inhibits the formation and development of cancer. In addition, we examined the extent of GNA01 expression according to the progression

and severity of cancer. Staining intensities of anti-GNAO1 antibodies in colorectal cancer tissues were stratified into four categories (negative, weak, moderate, and strong). Comparative analysis of matched tissue arrays was conducted by immunohistochemistry with anti-GNAO1 antibody in normal colon (n = 9) and colon cancer tissues, including stage II (n = 11), stage III (n = 15), and stage IV (n = 11). In this study, we confirmed that the expression of GNAO1 was inhibited as colon cancer progressed (Fig. 8).

IV. Discussion

Epidemiology of Colorectoal Cancer

Worldwide, colorectal cancer(CRC) has the highest cancer incidence followed by lung and breast cancers [14]. In Korea, CRC incidence progressively increased since 1999; however, it has declined since 2011 in men and 2012 in women due to the active implementation of preventive tests such as fecal occult blood test and colonoscopy [15]. However, with regards to domestic incidence and mortality rates, it is still the third most common cancer in both men and women and is one of the main causes of cancer death [14,15]. Similar to other cancers, the survival rate of CRC is gradually improving. According to a recent Korean report, the five-year survival rate of CRC patients diagnosed between 2012 and 2016 was 75.9%, which is better than that of the United States and Japan [15]. This can be attributed to a combination of active preventive check-ups, early diagnosis, and the development of various treatments.

Risk factors of Colorectal Cancer

The risk factors of CRC can be attributed to changes in lifestyle and diet that have occurred for decades. According to data released by the International Agency for Research on Cancer (IARC), drinking, smoking, and processed meat intake were commonly reported as factors related to CRC [16]. Due to the relationship between cancer incidence and drinking, many existing epidemiological investigations and studies have been conducted. Most of them report a positive correlation between the two; a higher intake results in a higher risk [17]. Similar studies conducted on CRC yielded the same results. A meta-analysis of the correlation with smoking showed that smokers have a higher risk of CRC

than that of non-smokers [18]. Previously, the risk of cancer due to red meat intake was as attributed to the relatively high-fat content in red meat. However, recent studies attribute the risk to heterocytic amines produced during cooking at high temperatures, which are converted into mutagenic metabolites in the body, leading to cell damage [17,19]. Additionally, many studies have reported obesity and height as factors that increase the risk of CRC, and many physical activities have been reported to lower the risk of CRC [15]. The risk of CRC associated with obesity was significantly increased in men with a body mass index(BMI) of 25 kg/m^2 or higher. The risk increased by approximately 6% as BMI increased by 5 kg/m^2 [16].

Molecular pathology of colorectal cancer

Colorectal cancer is known to develop from a progenitor lesion, such as adenoma, and is converted into adenocarcinoma. The three most representative mechanisms are also well known and are as follows: Chromosomal instability, microsatellite instability (MSI), and aberrant DNA methylation genetic alteration, and each account for CpG island methylator phenotype up to 85%, around 15%, and 17%, respectively. This abnormality results in cancer development and progression [9,20]. It has also been associated with the interaction of various genetic factors in the development of CRC, and several oncogenes and tumor suppressor genes have been studied. Oncogenes produce peptide growth factors, growth factor receptors, signal transduction factors, tyrosine kinases, and transcription regulatory proteins, which promote cell proliferation. Tumor suppressor genes produce nuclear regulatory proteins and control cell growth. When various risk factors damage these two types of genes, causing mutations and inactivation, cancer can occur [11]. Typical oncogenes include Ras, EGFR (Erb-B1), Erb-B2,

TGF-alpha, and tumor suppressor genes include APC, p53, p27, MSI, LOH 18q, and deletion 5 q allele DNA hypermethylation [9,21]. Ras is the most representative oncogene in CRC, with genetic variation observed in approximately 40%-50% of patients with colorectal cancer [22]. Ras oncogenes produce signals that affect cell proliferation and cell cycle and are believed to be early in the development of colorectal cancer [23]. The EGFR is a soluble protein and a tyrosine kinase that affects intestinal cell torpidity [9]. Genetic mutations in EGFR can lead to EGFR overexpression, which is reported to be associated mainly with lung and colorectal cancers [23]. The APC gene, a representative tumor suppressor gene, is one of the most commonly affected genes in CRC. If APC function is lost due to gene mutation, familial adenomatous polyposis is caused by the Wnt gene, which poses a risk of colorectal cancer in all patients. Known as the "guardian of the genome," P53 located on chromosome 17 and is present in 50% of sporadic CRCs and facilitates carcinogenesis [24,25]. In addition to the two kinds of genes mentioned above, recent studies have reported DNA mismatch repair as genes involved in cancer incidence [26,27]. Recovering the wrong combination, insertion of the bases that occur in DNA replication and recombination fails to restore normal recovery due to errors. These error-prone areas are mutated and accumulate in repeated arrays called microsatellites [28]. It has been reported that more than 80% of hereditary non-polyposis CRCs develop microsatellite instability [28,29].

Chemotherapy development in Colorectal cancer

Since 5-fluorouracil (5-FU) was first used in the 1960s until the early 2000s, cytotoxic chemotherapy with 5-FU and leucovorin folic acid (LV) improved the overall survival rate (OS) in metastatic CRC by more

than 12 months [30-32]. Due to the development of new cytotoxic chemotherapeutic agents (oxaliplatin and irinotecan) since the mid-2000s, clinical studies of new anticancer combinations in metastatic CRC have been conducted. According to the results of two major clinical studies, the combination chemotherapy FOLFOX (Infusional 5-FU and LV with oxaliplatin) and FOLFIRI (Infusional 5-FU and LV with irinotecan) for metastatic CRC was recognized as standard chemotherapy, and the effect was improved with a survival period of more than 20 months [33-35]. Moreover, after it was discovered that chronic myeloid leukemia was caused by BCR-ABL fusion protein in the early 2000s, the results of the dramatic effect of BCR-ABL tyrosine kinase inhibitor name imatinib mesilate) were reported. As a result, studies on the genetic profile and pathogenesis of specific cancers have been conducted even in solid cancers, and various clinical studies have been conducted on therapies targeting molecular or signal transduction steps involved in tumor growth or suppression [36].

In colorectal cancer, EGFR and VEGF have been classified and studied as the main therapeutic targets. Bevacizumab, a humanized monoclonal antibody of VEGFA, is the main representative drug that targets VEGF. Several clinical studies have reported a significant increase in OS when these agents are added to standard combination cytotoxic doublet chemotherapy (FOLFOX and FOLFIRI)[3,4,5,37,38]. For RAS and BRAF wild-type CRC, the combination of cetuximab (a biological EGFR resident) with cytotoxic doublet chemotherapy also improved the OS and response rate [39]. The current standard chemotherapy of metastatic CRC is combination cytotoxic agents (5-FU/LV, capecitabine, irinotecan, and oxaliplatin), with several target signaling magent (bevacizumab, cetuximab, panitumumab, ziv-aflibercept, and regorafenib), the efficacy of these combination has improved over 30 months [3-5].

Definition and Role of GNAO1 gene

G proteins are molecular switches that internally transmit chemical signals from the outside of the cell [25]. Each subunit of the G protein is encoded by a member of one of three corresponding gene families: 16 alpha (α) subunit family, 5 beta (β) subunit family, and 11 gamma (γ) subunit family [10]. GNAO1 is a subunit of the Gα protein [40-42]. The disorder related to GNAO1 was initially reported as early infantile epileptic encephalopathy in Japan [43]. According to a study reported by Nakamura in 2013, heterozygous mutations in GNAO1, which encodes a Gαo subunit of heterotrimeric G proteins in four epileptic encephalopathy patients through whole-exome sequencing and subsequent mutation screening. Epileptic encephalopathy is a progressive cognitive and intellectual disability that includes motor development delay and impairment behavior. Ohtahara syndrome is the most severe type of epileptic encephalopathy and three out of four patients are diagnosed with it. Nakamura explained that Gαo encoded by GNAO1 is expressed in the cerebrum in abundance and the Gαo-mediated signaling pathway induced by norepinephrine. They presented a calcium-channel modulator as a new drug for treatment [11].

Research has also progressed since the 1990s with an interest in oncogenic mutations associated with various G protein subtypes. Landis reported mutations in codons 201 and 227 of GNAS (Gαs) in 25% of pituitary adenomas [44]. Forbes reported that mutation of the small G-protein Ras was 20% of all tumors in the same study in 2008[45]. Mutations in the Gα subunits of heterotrimeric G proteins, such as mutations in GNAI2 (Gai2) and GNAQ (Gaq), have also been reported in human tumors [24]. Landis explained the molecular mechanism of deactivation of the G protein by damaging its intrinsic ability to hydrolyze GTP [44].

The first somatic mutation of GNAO1 was described in breast cancer by Kan et al., and the molecular basis for its mechanism was studied by Garcia-Marcos [24,43]. Kan analyzed 2,576 somatic mutations across ~1,800 Mb of DNA, representing 1,507 coding genes from 441 tumors

comprising breast, lung, ovarian, and prostate cancer. They revealed that GNAS was amplified in 12% (6 of 49) of ovarian cancers, 20% (10 of 50) HER2 positive breast cancer, and 13% (7 of 53) of HR-positive breast cancers [43]. Garcia-Marcos reported that the novel R234H mutation conveys oncogenic properties to Gao by promoting nucleotide exchange and making it constitutively active, thereby reinforcing signaling pathways responsible for neoplastic transformation [24]. It has also been shown to promote oncogenic transformation, possibly through signal transducer and activator of transcription 3 (STAT3) signaling in vitro [42]. Furthermore, recent studies on the correlation between GNAO1 and cancer development and proliferation have been reported in hepatocellular carcinoma and gastric cancer [46]. Compared to the cancers mentioned above, the molecular mechanisms involved in the pathogenesis of colorectal cancer associated with GNAO1 are poorly understood.

This study presented several results on the potential of GNAO1 as a tumor suppressor gene in colorectal cancer. First, the downregulation of the mRNA level of GNAO1 was demonstrated in tissues from patients with CRC. We also found that the overexpression of GNAO1 suppresses cancer cell development, proliferation, and migration in vitro. We also found that GNAO1 inhibits tumor production and proliferation in vivo, which has sufficiently demonstrated its role as a tumor suppressor gene in colorectal cancer. We confirmed that the expression of GNAO1 and the degree of cancer exacerbation are related. As cancer deteriorated, the expression of GNAO1 decreased. This result can pave the way for a new biomarker to predict prognosis. Therefore, GNAO1 can be an important indication for determining the prognosis of patients with reduced expression and overexpression.

S6Ks in mTOR/S6K signaling pathway

mTOR (mammalian target of rapamycin) is a protein kinase that controls cell growth by receiving signals from nutrients (amino acids), growth factors (insulin and IGF1), cellular energy (ATP) mTOR exists in

two forms within cells: mTORC1 and mTORC2. When mTORC1 is activated, it activates 4E-BPs (eukaryotic translation initiation factor 4E-binding protein), and S6Ks (ribosomal protein S6 kinase) sequentially, and S6Ks act as effectors of the mTOR pathway, regulating cell growth, survival, and metabolism [47-49]. These mechanisms have shown that the mTOR/S6K signaling pathway is involved in developing diseases such as cancer, diabetes, and obesity [47]. The pathway is involved in the early steps of adipogenesis and glutamine metabolism, which upregulates obesity [47]. S6K is also known to be involved in aging. It is also involved in preventing blood vessel aging by reducing nitric oxide (NO), which causes the aging of endothelial cells [47]. S6K has been reported to improve the activity of cancer cells and increase anticancer drug resistance. If the functional loss of S6K occurs, it is related to cancer. Short isoforms produced by S6K1 alternative splicing have been identified in breast, prostate, and lung cancers. S6K1 alternative splicing results from the oncogenic protein SRSF1 splicing factor [50]. According to Michael Torbenson in 2004, mTOR expression was increased in approximately 5% of hepatocellular carcinoma, whereas activated mTOR or phospho-mTOR, was overexpressed in approximately 15%, and S6K with activated mTOR was also identified at approximately 45% [51]. Shiratori confirmed that the Akt-mTOR pathway was activated in eight human colorectal cancer cell lines (LoVo, SW480, CaCO2, CaRI, COLO205, DLD-1, WiDr, and HT-29) and rapamycin downregulates phosphorylated p70 S6K [52]. It has been reported that S6K inhibition plays an important role in preventing metastatic breast cancer by preventing the migration of cancer cells [45]. Because the S6Ks in the mTOR/S6K signaling pathway plays an important role in developing diseases such as cancer, obesity, and diabetes, they are indicated as specific therapeutic targets and have been studied widely. For example, a specific S6K inhibitor, PF-4708671, which was recently reported, is a potential cell-permeable S6K1 inhibitor with a Ki of 20 nM and an IC50 of 160 nM, which blocks S6K1 selectively and does not block other AGC kinases [47,53]. In this study, using human phospho-kinase array assays, we compared antibodies

involved in various signals in control cells and cells overexpressing GNA01. We observed a decrease in p70 S6 kinase T389 expression in cells overexpressing GNA01. We tested antibodies related mTOR/S6, we found that p-p70 S6 kinase T389 expression decreased in GNA01. As a result, GNA01 can suppress cancer cells by inhibiting p-p70 S6 kinase T389 expression in the mTOR/S6K signaling pathway. GNA01 has been associated with the p70 S6 kinase T389 in the mTOR/S6K signaling pathway. These results may facilitate an understanding of the molecular basis of CRC. It can be concluded that GNA01 has a clinical value as a new therapeutic target in colorectal cancer.

V. Conclusion

In conclusion, we analyzed the role of GNAO1 in CRC and confirmed that GNAO1 was downregulated in cancer cells. GNAO1 inhibited cancer cell formation and proliferation. Based on these results, we have demonstrated that GNAO1 plays an important role in the pathogenesis of CRC and has a potential anticancer effect on CRC. We also provided data to suggest a possible mechanism. Therefore, it is applicable as a new candidate biomarker and a potential therapeutic target for CRC. Further studies are required to investigate its role in the cell signaling pathway for the clinical use of GNAO1; however, this study should serve as a starting point.

Figure 1. Downregulation of GNAO1 in human colon cancer.
Relative expression of GNAO1 gene in colon normal cell and the colon cancer cell is shown in Panel A. '*' stands for *P* value less than 0.0001

Colon Ca	Gene Name	Transcript Name	Transcript Type	Avg. expression in Normal	Avg. expression in Tumor	Tumor/Normal	Log2
	GNAO1	NM_138736	Long Transcript	905.7312729	445.7548056	0.492149072	-1.02283272

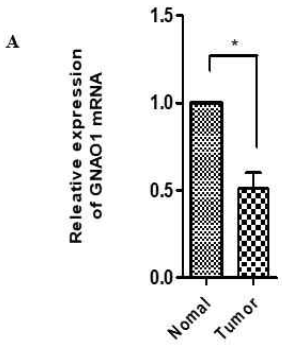


Figure 2. The expression levels of GNAO1 in 8 colon cancer cell lines andnormal colon cell.
GNAO1 protein level was measured by Western bot analysis and quantified using Image J software. Data represent the mean±S.D. from triplicate experiments.

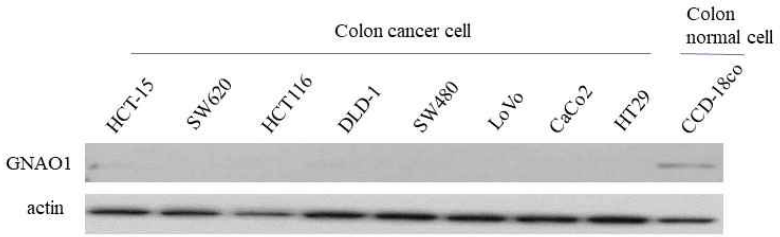


Figure 3. Overexpression of GNAO1 suppresses cell proliferation of human colon HCT116 and DLD-1 cells.
Cell proliferation of HCT116 and DLD-1 cells with GNAO1 overexpression and control was observed by MTT assay.

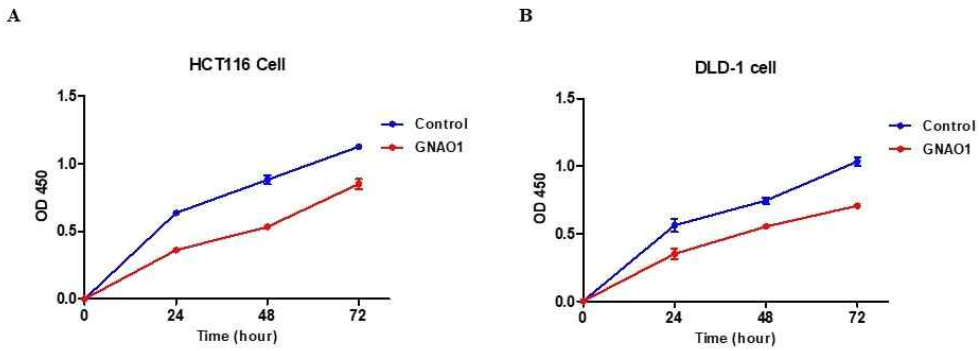


Figure 4. Overexpression of GNAO1 suppresses migration of human colon HCT116 and DLD-1 cells.
(A) Control vector and GNAO1 overexpressing HCT116 cells were assessed for cell Migration assay. Cell migration assays were performed using the Transwellsystem. (B) Control vector and GNAO1 overexpressing DLD-1 cells were assessed for cell Migration assay. Cell migration assays were performed using the Transwellsystem. Data represent the mean cell numbers from 5 fields, $p<0.001$, compared to control.

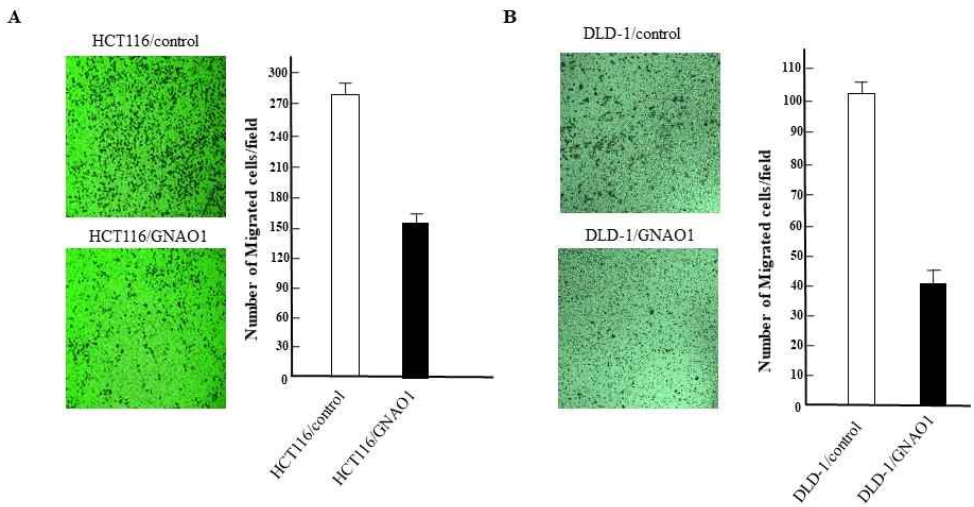


Figure 5. Overexpression of GNAO1 suppresses soft agar of human colon HCT116 and DLD-1 cells.
(A) Control vector and GNAO1 overexpressing HCT116 cells were assessed for colony formation in soft agar for 14 days. (B) Control vector and GNAO1 overexpressing DLD-1 cells were assessed for colony formation in soft agar for 14 days. Data represent the mean cell numbers from 5 fields, $p<0.001$, compared to control.

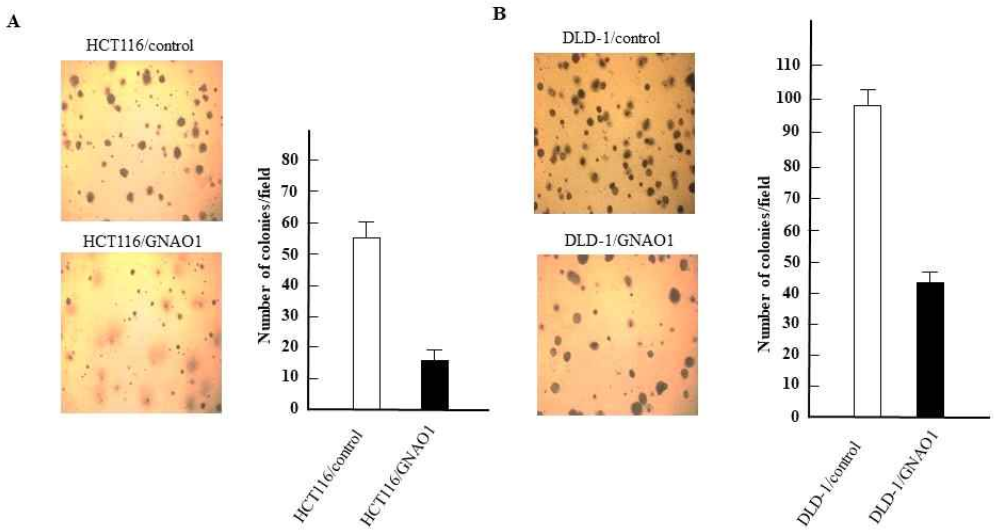


Figure 6. Effects of GNAO1 overexpression on tumor growth cells in a nude mice HCT116 xenograft model.
(A) Control and GNAO1 HCT116 cells were injected subcutaneously into the left flank of nude mice, respectively (n= 3). (B) Tumor volumes were measured every 4 day over a period of 40 days.

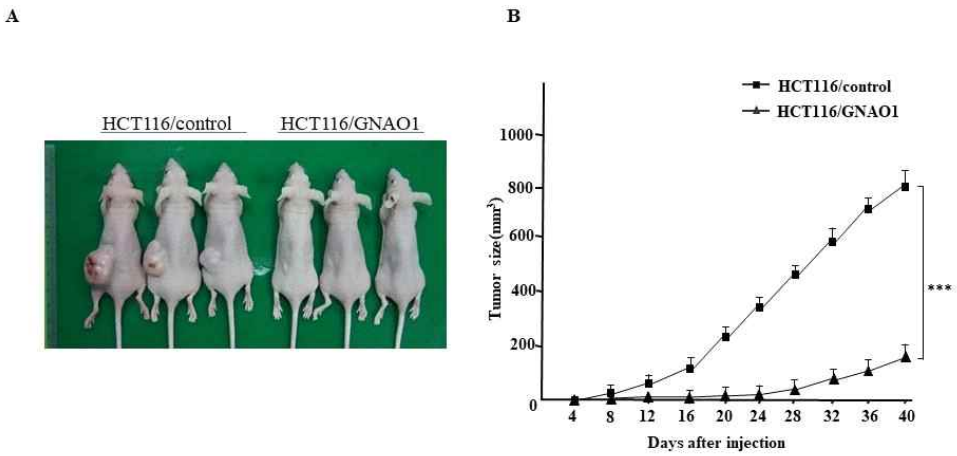


Figure 7 . Overexpression of GNAO1 inhibits HCT116 colon cancer cell the phosphorylation of p-p70 S6 kinase.
In order to explore the molecular mechanisms of GNAO1 in inhibition of colon cancer, human phospho-kinase array assays (Proteome Profiler; R&D Systems, Minneapolis, MN, USA) were performed in HCT116 cells stably transfected with GNAO1 or control. (A) Results of phospho-kinase array suggested that activities of p-p70 S6 kinase were inhibited in HCT116-GNAO1 cells. (B) Impacts of GNAO1 overexpression on the activity of p-p70 S6 kinase and Raptor were detected by western blot assay.

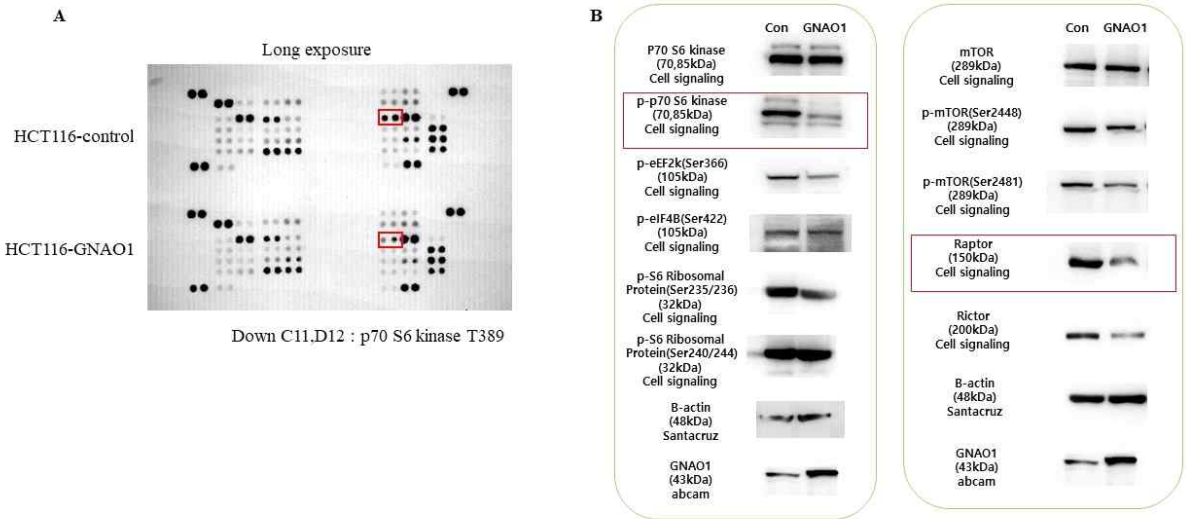
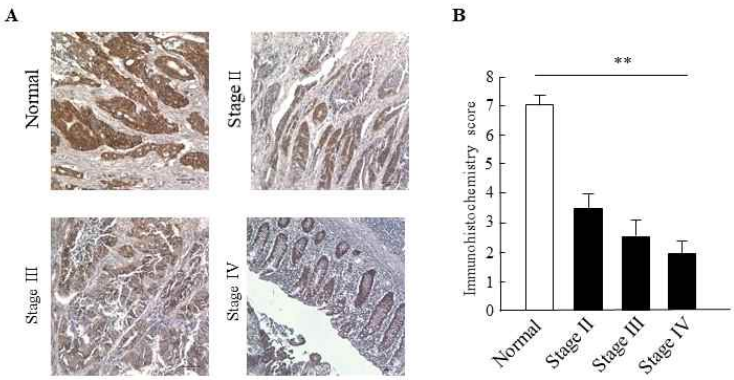


Figure 8. Suppressed GNA01 expression is associated with colorectal cancer tissues.

(A) GNA01 expression was suppressed in colorectal cancer(CRC) tissues compared with normal tissues. Staining intensities of anti-GNA01 antibody in colorectal cancer tissues were stratified into 4 categories(negative, weak, moderate, and strong). Comparative analysis of matched Tissue array was conducted by immunohistochemistry with anti-GNA01 antibody in normal colon (n = 9) and colon cancer tissues including stage II (n = 11), stage III (n = 15) and stage IV (n = 11). Original magnification 40X.



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