International Journal of Cancer Medicine

Clinical Research | Vol 6 Issue S8

Regenerating Family Member 4 Expression was a Potential Marker for Carcinogenesis, Aggressiveness and Prognosis of Gastric Cancer

Cong-yu Zhang[1], Li Zhang[2], Hong-zhi Sun[1], Min-wen Ha[1], Zheng-guo Cui[3] and Hua-chuan Zheng[1*]

[1]*Cancer Center, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China*

[2]*Department of Oncology, The Affiliated Hospital of Chengde Medical University, Chengde, China*

[3]*Department of Environmental Health, University of Fukui School of Medical Sciences, Fukui, Japan*

Correspondence should be addressed to Hua-chuan Zheng, Cancer Center, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou 121001, China

Received: August 14, 2023; Accepted: September 07, 2023; Published: September 14, 2023

ABSTRACT

INTRODUCTION

REG4 might activate EGFR/Akt/AP-1 pathway, and be regarded as a potential marker for neuroendocrine tumors and mucin-producing adenocarcinomas.

METHODS

We combined meta-, bioinformatics and pathological analyses to explore the clinicopathological significances of REG4 expression in gastric cancer (GC). The effects of REG4 on the aggressive phenotypes and relevant molecular mechanisms were also investigated in GC cells.

RESULTS

Compared with normal mucosa, up-regulated REG4 expression was found in GC at either mRNA or protein level ($p < 0.05$), and negatively associated with the histological grading of GCs ($p < 0.05$). The REG4-related signal pathways included neuroactive ligand-receptor interaction, digestion and absorption, endoplasmic reticulum lumen, hormone, glycan processing and binding, glycosylation, extracellular matrix, bindings of carbohydrate and cAMP response element, Golgi apparatus, transmembrane transporter activity, cytidine metabolism and so on ($p < 0.05$). REG4 expression was positively related to depth of invasion, lymph node metastasis, TNM staging and dedifferentiation of GCs ($p < 0.05$). Recombinant REG4 exposure or Full-length REG4 overexpression promoted proliferation, anti-apoptosis, migration, and invasion of GC cells in an autocrine or paracrine manner by activating EGFR-PI3K-Akt-NF-κB pathway, while non-signal-peptide REG4 didn't have these biological effects and anti-REG4 antibody blocked the effects of REG4 overexpression. REG4 was involved in chemoresistance of GC cells not through de novo lipogenesis, but through lipid droplet assembly. REG4 induced proteasomal degradation of ACC1 or ACLY in GC cells.

Citation: Cong-yu Zhang, Regenerating Family Member 4 Expression was a Potential Marker for Carcinogenesis, Aggressiveness and Prognosis of Gastric Cancer. Int J Can Med 6(S8): 30-48.

CONCLUSION

REG4 may be involved in tumorigenesis and aggressiveness of GC by EGFR-PI3K-Akt-NF-κB pathway, and chemoresistance through lipid droplet assembly. REG4 attenuates the expression of de novo lipid synthesis key enzymes by promoting the ubiquitination- mediated proteasomal degradation.

KEYWORDS

Gastric cancer; REG4; Bioinformatics analysis; Meta-analysis; Aggressiveness; Chemoresistance; Lipid droplet formation

INTRODUCTION

The regenerating (Reg) gene family produces the calcium-depending lectins, which function as acute phase reactants, anti-apoptotic factors, and growth agents [1]. REG4 activates EGFR/Akt/AP-1 pathway and is considered as a potential marker for neuroendocrine tumors and mucin-producing adenocarcinoma [2-4]. SP1, CDX2 and GATA6 are proven to be transcription factors responsible for the gene expression of REG4, which is required for proliferation and carcinogenesis of gastrointestinal cancers [5-7]. In lung cancer, K-ras-induced REG4 promoted the properties of cancer stem cells by activating WNT/β-catenin signal pathway [8]. In colorectal cancer (CRC), Bishnupuri et al. [9] reported that REG4-mediated mitogenesis was initiated by Akt-GSK-3β-β-catenin-TCF-4 pathway. REG4 was also involved in the invasion of pancreatic cancer cells by up-regulating the expression of both MMP-7 and MMP-9 proteins [10]. In GC, REG4 activated MAPK/ERK/BIM signal pathway to inhibit cell apoptosis and enhance the chemoresistance to 5-FU [11].

Reportedly, REG4 phosphorylated the EGFR and inhibited 5-FU-induced apoptosis in GC cells [12]. Bishnupuri et al. [4,13] demonstrated that REG4 might protect colon cancer and normal intestinal crypt cells from apoptotic induction by up-regulating the expression of survivin, Bcl-2 and Bcl-xl proteins. Chen et al. [14] showed that REG4 overexpression and recombinant REG4 protein strengthened proliferation, anti-apoptosis, G2/S progression and invasion with Bax under-expression and Wnt5a, survivin, p70s6k and VEGF overexpression.

Overexpression of REG4 triggered a positive feedback loop made up of REG4, SP1, EGFR, TGF-α, ADAM17 and GPR37, which could promote peritoneal metastasis of GC [5]. Moreover, REG4 deteriorated migration and invasion of CRC cells in either autocrine or paracrine manner, while anti-REG4 antibody blocked the biological event [15]. In pancreatic cancer cells, Takehara et al. [16] found that REG4 silencing weakened cell viability, and rhREG4 promoted proliferation at a dose-dependent manner. In prostate cancer cells, REG4 hypoexpression was found to up-regulate p21 expression and down-regulate Cyclin D1 expression, finally to block the G1/S transition and proliferation [17]. REG4 knockdown in lung cancer might induce cell cycle arrested at G2/M to inhibit cell proliferation [18].

REG4 mRNA was strongly observed in inflammatory and dysplastic epithelium, and malignant lesions of ulcerative colitis, and closely linked to the mRNA expression of HGF and bFGF that in turn enhanced REG4 expression in CRC cells [19,20]. REG4 mRNA overexpression was detectable in pancreatic, hepatocellular, colorectal and prostate cancers as well [21-25]. In our previous study, REG4 protein might be regarded as a potential biomarker for gastric intestinal metaplasia and was especially related to the histogenesis of gastric signet ring cell carcinoma [26]. Here, we carried out meta, bioinformatics, and pathological analyses to clarify the clinicopathological significances of REG4 expression in GC. The effects of REG4 on the phenotypes of GC cells were also studied as well as the detailed mechanisms explored.

METHODS

Cell Culture and Transfection

GC cell lines were kindly presented by Prof. Takano Yasuo from The School of Medicine, University of Toyama. They were maintained in RPMI 1640 (MKN45, MKN28 and KATOIII), HAM F-12 (AGS), MEM (HCG-27) and DMEM (GT-3TB) medium supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA), 100 units/ml penicillin, and 100 units/ml streptomycin (Beyotime, China), in a humidified atmosphere of 5% CO_2 at 37°C. All the cell lines used in the experiment have been verified by STR analysis. The cells were transfected at 70% confluence 20 hours to 24 hours after seeding on dishes according to the manufacturer's instructions. The cells were transfected with pcDNA3.1-REG4 (full-length, FL-REG4), pcDNA3.1-REG4 (non-signal-peptide, NSP-REG4) using Lipofectamine 3000 (Thermo Fisher Scientific, USA). The cells were treated with high glucose (HG, 4.5 mg/L), recombinant human REG4 protein (rhREG4, R&D system) or anti-REG4 antibody (R&D system). The cells were also exposed to 5-Fluorouracil (5-FU, MCE, USA), cisplatin (DDP, MCE, USA), cycloheximide (CHX, a selective inhibitor of protein synthesis), MG132 (proteasome inhibitor), ACC1 (acetyl-CoA carboxylase 1) or ACLY (ATP-citrate lyase) inhibitor (Abmol, USA), Cetuximab (Anti-EGFR antibody, MCE, USA), Erlotinib (tyrosine kinase inhibitor, MCE, USA), PI3K inhibitor LY294002 (Beyotime, China), and Akt inhibitor AZD536 (Beyotime, China). These drugs were used to treat cells and observe the alteration of cellular phenotypes and their relevant protein expression. Centrifuged cells were washed with PBS, and total protein or RNA extraction was conducted. Some cells were subjected to proteasome extract using minute nuclear or cytosolic proteasome enrichment kit (Invent Biotechnologies, USA).

Assessment of Cell Proliferation by CCK-8 Assay

Cell Counting Kit-8 (CCK-8) was used to determine cellular viability. Briefly, we seed 2.5×10^3 cells/well into 96-well plate and allowed them to adhere. At different time points, 10 μL of CCK-8 reagents was dispensed into each well. After incubation for 3 hours in the incubator at 37°C, we measured optical density on a microplate reader at a wavelength of 490 nm.

Apoptosis Assay by Flow Cytometry

We detected phosphatidylserine externalization using fluorescein isothiocyanate (FITC)-labeled Annexin V (Beyotime, China) and propidium iodide (PI) to measure the proportion of early apoptosis. After treatment, any remaining intact cells were collected, washed with cooled PBS at 4°C and centrifuged at 700 × g for 3 minutes. The 490 μL cell suspension was gently mixed with 5 μL FITC-labeled Annexin-V and 5 μL PI. After incubation at 4°C for 10 minutes, the cell suspension was detected by flow cytometry [23].

Wound Healing Assay

Cells (5.0×10^5 per well) were plated into 6-well culture plates. When the cells reached 75%-80% confluence, they were scraped with a tip, washed four times in PBS to reduce the number of broken cells, and cultured in FBS-free HAMs F-12 medium. We measured the area of the scratch immediately and took photos at 0 hour, 24 hours and 48 hours using Image J.

Cell Migration and Invasion Assays

Assays for migration were carried out by suspending 1.2×10^5 cells per 200 μL in FBS-free HAMs F-12 and seeding the upper chamber of each transwell (Corning, USA). As a chemoattractant, 12% FBS was added to each lower compartment. After incubation for 24 hours, we scrubbed the upper surface of the transwell membrane using cotton swab, washed the transwell chamber with PBS 3 times and

fixed cells in 100% cold methanol. The membrane was stained with crystal violet for several minutes. For the invasive assay, the same process as mentioned above was repeated except that each transwell insert was coated with matrigel (Corning, USA).

Nile Red Staining

GC cells were cultured on coverslips for 12 hours, fixed in 4% neutral paraformaldehyde for 28 minutes and stained with Nile red (Invitrogen, Carlsbad, CA, USA; 1:1000) for 14 minutes. Finally, we stained slides with 4',6-diamidino-2-phenylindole (DAPI) and covered them with SlowFade® Gold anti-fade reagent (Thermo Fisher Scientific, Waltham, MA, USA). The two software programs (Image J and Icy) were used to acquire and analyze pictures.

Name	Dilution	Source	Company	Catalog Number
GAPDH	1:2000 for WB	Mouse	Proteintech, USA	60004-1-Ig
REG4	1:1000 for WB; 1:100 for IHC; 1:50 for IF	Goat	R&D, USA	AF1379
EGFR	1:10000 for WB	Mouse	Proteintech, USA	66455-1-Ig
EGFR Tyr992	1:1000 for WB	Rabbit	CST, USA	2235S
EGFR Tyr1068	1:1000 for WB	Rabbit	CST, USA	2234S
EGFR Tyr1148	1:1000 for WB	Rabbit	CST, USA	4404S
EGFR Tyr1173	1:1000 for WB	Rabbit	CST, USA	4407S
PI3K	1:1000 for WB	Mouse	CST, USA	4249S
P-PI3K	1:1000 for WB	Rabbit	CST, USA	4228S
AKT	1:1000 for WB	Rabbit	Proteintech, USA	10176-2-AP
P-AKT	1:1000 for WB	Rabbit	Proteintech, USA	4058S
NF-KB	1:2000 for WB	Rabbit	Proteintech, USA	10745-1-AP
P-NF-KB	1:1000 for WB	Rabbit	CST, USA	3033S
BCL-2	1:2000 for WB	Rabbit	Proteintech, USA	12789-1-AP
BCL-X/L	1:1000 for WB	Rabbit	Santa Cruz, USA	sc-56021
ACLY	1:2000 for WB; 10 ug per co-IP	Rabbit	Proteintech, USA	15421-1-AP
ACC1	1:2000 for WB; 10 ug per co-IP	Rabbit	Proteintech, USA	21923-1-AP
P-ACC1	1:1000 for WB	Rabbit	CST, USA	11818S
AC-H3	1:2000 for WB	Rabbit	Proteintech, USA	61637
AC-H4	1:2000 for WB	Rabbit	Proteintech, USA	39925
ING5	1:2000 for WB	Rabbit	Proteintech, USA	10665-1-AP
HDAC	1:20000 for WB	Mouse	Proteintech, USA	66085-1-Ig
SREBP-1	1:200 for WB	Mouse	Santa Cruz, USA	66875-1-Ig
ACAT	1:2000 for WB	Rabbit	Proteintech, USA	16215-1-AP
ADRP	1:2000 for WB	Rabbit	Proteintech, USA	15294-1-AP
CIDE A	1:1000 for WB	Rabbit	Proteintech, USA	13170-1-AP
CIDE B	1:2000 for WB	Rabbit	Proteintech, USA	27600-1-AP
CIDE C	1:2000 for WB	Rabbit	Proteintech, USA	12287-1-AP
Perilipin 5	1:2000 for WB	Rabbit	Proteintech, USA	26951-1-AP
TIP47	1:2000 for WB	Rabbit	Proteintech, USA	10694-1-AP
NEDD4	1:200 for WB	Mouse	Santa Cruz, USA	sc-518160
NEDD4L	1:200 for WB	Mouse	Santa Cruz, USA	sc-514954
CBL	1:200 for WB	Mouse	Santa Cruz, USA	sc-1651
SYVN1	1:200 for WB	Mouse	Santa Cruz, USA	sc-293484
COP1	1:200 for WB	Mouse	Santa Cruz, USA	sc-166799
PSMC1	1:500 For WB	Rabbit	Proteintech, USA	11196-1-AP
Ubiquitin	1:1000 for WB; 10 ug per co-IP	Rabbit	Proteintech, USA	10201-2-AP
β-actin	1:1000 for WB	Rabbit	Proteintech, USA	20536-1-AP
Lamin B	1:5000 for WB	Rabbit	Proteintech, USA	12987-1-AP

Note: WB: Western Blot; IHC: Immunohistochemistry; co-IP: co-Immunoprecipitation; IF: Immunofluorescence

Supplementary Table 1: The primary antibodies used in this study.

Immunofluorescence

GC cells were cultured on glass slides, fixed for 10 minutes with 4% neutral formaldehyde, and then permeabilized for 10 minutes with 0.5% Triton X-100. The cells were rinsed with PBS three times and then treated with anti-goat REG4 (R&D Systems, USA; 1:100) antibody in 4°C overnight. After that, the cells were incubated with Alexa Fluor 488 (green) anti-goat IgG (Invitrogen, USA) for 55 minutes.

Slides were then mounted using SlowFade® Gold anti-fade reagent after DAPI was stained.

Co-immunoprecipitation (co-IP)

Eight µg of primary antibody (Supplemenatry Table 1) was added to 1.5 mg of protein and subjected to gentle rotation at 4°C overnight. One hundred µL of agarose A beads (50% v/v) were then added, and the mixture was gently rotated at 4°C overnight. In order to exclude non-specific binding proteins, the beads were centrifuged and washed with 1% NP40 lysis buffer four times. The pellet was mixed using 50 µL of 2 × SDS sample buffer, and heated at 100°C for 10 minutes. The sample supernatant was used for the following western blot.

Subjects

Gastric primary cancers (n = 566), intestinal metaplasia (n = 152), gastritis (n = 132), adenoma (n = 89) and metastatic cancer in lymph node (n = 76) were collected in our hospital between 2013 and 2023. The cancer patients were 398 men and 168 women (27~90 years, mean = 64.3 years). Among them, 279 cases have lymph node metastasis. Thirty-seven cases of GC and matched normal mucosa were obtained from our hospital and frozen at -80°C until protein or RNA extraction by homogenization. Before surgery, none of the patients had undergone radiation or chemotherapy. The ethics committee of our institution authorized the research plan after giving unanimous approval to utilize tumor tissues for clinical research.

Pathology and Tissue Microarray

All tissues of primary and metastatic cancers, precancerous lesion and normal mucosa were preserved in 20% neutral formalin, embedded in paraffin, and sectioned into 4 µm-thick pieces. Hematoxylin-and-eosin (HE) staining was used to validate the histological diagnosis and features of these sections. In HE-stained sections of chosen cases, representative portions of solid tumors were selected under a microscope. Using a tissue microarray, a two-mm tissue core from each donor block was punched out and transferred to a recipient block with a maximum of 48 cores (Azumaya kin-1, Japan). From the recipient block, 4 µm-thick portions were sequentially cut and placed on glass poly-lysine-treated slides.

Western blot

One hundred or eighty microgram samples of denatured protein were separated on SDS-polyacrylamide gels (11 percent acrylamide) and then transferred to a Hybond membrane (Amersham, Chicago, IL, USA). The membrane was then blocked overnight in 4.5% skim milk in Tris Buffered Saline with Tween 20 (TBST). The membrane was treated with primary antibody (Supplementary Table 1) for immunoblotting for 1 hour. Following TBST rinse for three times, it was incubated for 1 hour with anti-goat, anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Dako, Glostrup, Denmark). After wash with TBST for three times, bands were observed using C300 imaging system (AZURE, Peking, CHINA) and ECL-Plus detection reagents (Santa Cruz, Dallas, TX, USA).

RT-PCR

Total RNA was extracted from GC cells and tissues using QIAGEN RNase mini kit (Germany) in accordance with the manufacturer's instructions. AMV transcriptase and a random primer were employed to create cDNA from 2 ug of total RNA (Takara, Kusatsu, Japan). The primers for REG4 were forward: 5’-CCTTTCCACAGTATCCTTCTTCCCT-3’ and reverse: 5’-TATGGCCAAAGACCCAGCTGTT-3’ (104 bp). The primers for GAPDH were forward: 5'-CAATGACCCCTTCATTGACC-3' and reverse: 5'-TGGAAGATGGTGATGGGATT-3' (135 bp) for GAPDH. Quantitative reverse transcription (RT)-PCR amplification was performed using a SYBR Premix Ex Taq™ II kit (Takara).

Immunohistochemistry

After deparaffinization with xylene and dehydration with alcohol, successive slices in a target retrieval solution (TRS; Dako, Denmark) were microwaved for 20 minutes. Hydrogen peroxide at 3% in methanol was used to block endogenous peroxidase activity. To stop non-specific binding, we immersed the slides in 4% bovine serum albumin for 5 minutes. The sections were treated with anti-goat conjugated to horseradish peroxidase (Dako, 1:100) antibodies for 25 minutes after being incubated with goat anti-human REG4 antibody (R&D Systems, 1:50) for 17 minutes. To enable previously reported intermittent irradiation, all incubations were carried out in a microwave oven [14]. REG4 immunoreactivity was localized in the cytoplasm. According to the coloring degree of positive cells, immunohistochemical degree was divided into: Blue, negative; pale yellow, weakly positive; brown, moderately positive; dark brown, strongly positive. From five typical fields in each region, 100 cells were randomly chosen and counted by two independent observers blinded to the samples (Zhang CY and Zheng HC). Semi-quantitative two-tier grading was used to determine the positive proportion of counted cells: positive (+), 6%-100% and negative (-), 0%-5%.

Bioinformatics Analysis

We used the xiantao platform (https://www.xiantaozi.com/, keywords: REG4) and The University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) database (http://ualcan.path.uab.edu, keywords: REG4) to analyze the expression, relevant genes, and signaling pathways of the REG4 gene. A Kaplan–Meier plotter (http://kmplot.com/, keywords: 223447_at) was used to evaluate the prognostic value of REG4. Additionally, we discovered the genes that were differentially expressed by Xiantao and subjected these to protein-protein interaction (PPI) network analysis and a search of critical hub genes. Subsequently, Gene Ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) analyses were conducted on these genes.

Statistics Analysis

Student t test was employed to compare the means of different groups and $\chi 2$ test to compare the positive rates. A log-rank statistic was used to compare survival curves and create Kaplan-Meier survival curves. SPSS 10.0 was used to handle all data and statistical significance was defined as two-sided $p < 0.05$.

RESULTS

The Clinicopathological Significances of REG4 Protein Expression in GC

A lower REG4 mRNA expression was found in normal tissues than GC according to real-time RT-PCR (Figure 1A, $p < 0.05$), xiantao (Figure 1B, $p < 0.05$) and UALCAN databases (Figure 1C, $p < 0.05$). According to Western blot, non-neoplastic mucosa showed a higher REG4 expression than GC (Figure 1D, $p < 0.05$). As indicated in Figure 1E, REG4 was strongly expressed in gastric deep gland, intestinal metaplasia (IM) and adenomas, well-, moderately- and poorly-differentiated adenocarcinoma, signet ring cell carcinoma and metastatic cancer in lymph node. Overall, the positive rate of REG4 expression was 34.8% for gastritis, 100.0% for IM, 92.1% for adenoma, 62.7% for primary cancer and 13.2% for metastatic cancer (Table 1). Statistically, REG4 expression was higher in IM than in gastritis, adenoma, primary cancer and metastatic cancer; in adenoma than in gastritis, primary cancer and metastatic cancer; in primary cancer than in gastritis and metastatic cancer (Table 1, $p < 0.05$). As summarized in Table 2, REG4 expression was positively correlated with depth of invasion, venous invasion, lymph node metastasis, and TNM staging ($p < 0.05$), but versa with age. Among histological subtypes of WHO, signet ring cell carcinoma more frequently expressed REG4 than well-, moderately- or poorly differentiated carcinoma ($p < 0.05$). The mucinous or moderately differentiated subtype displayed a

higher REG4 expression than the well- or poorly differentiated carcinoma (Table 3, p <0.05).

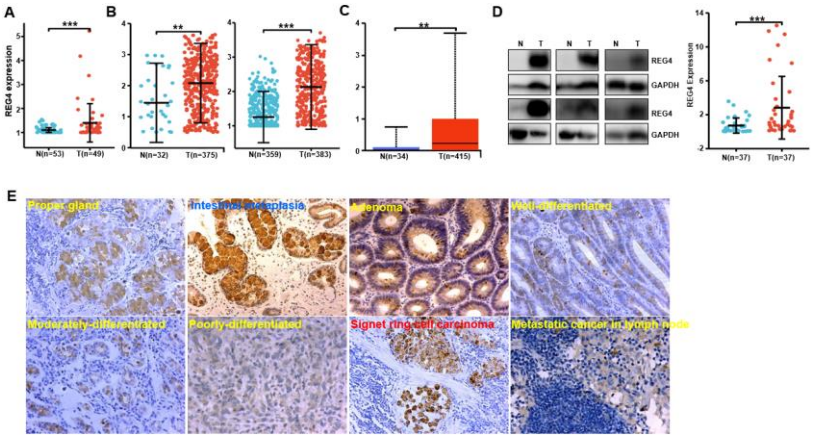


Figure 1: The clinicopathological significance REG4 expression in gastric cancer.
A higher REG4 mRNA expression was detectable in gastric cancer than that in normal gastric mucosa according to the real-time PCR **(A)**, Xiantao **(B)** and UALCAN's database **(C)**. Western blot was used to detect REG4 protein level in gastric cancer. Densimetric analysis showed its higher expression in gastric cancer than normal tissues **(D)** (p <0.05). Immunohistochemically, REG4 protein was positively expressed in the cytoplasm of gastritis, intestinal metaplasia, adenoma, primary and metastatic cancer **(E)**.
Note: N: Normal; T: Tumor; **, p <0.01; ***, p <0.001

Groups	n	REG4 expression		
		-	+	PR（%）
Gastritis	132	86	46	34.8
Intestinal Metaplasia	152	0	152	100.0[a]
Adenoma	89	7	82	92.1[b]
Primary Cancer	566	211	355	62.7[c]
Metastatic Cancer in Lymph Node	76	66	10	13.2

Note: PR: Positive Rate
[a]Compared with gastritis, adenoma, primary cancer and metastatic cancer, P <0.001
[b]Compared with gastritis, primary cancer and metastatic cancer, P <0.001
[c]Compared with gastritis and metastatic cancer, P <0.001
Table 1: REG4 expression during gastric carcinogenesis and subsequent progression.

Clinicopathological Features	n	REG4 Expression			
		-	+	PR（%）	P value
Age (Years)					0.009
<65	279	90	189	67.7	
≥65	287	121	166	57.8	
Sex					0.416
Male	398	150	248	62.3	
Female	168	61	107	63.7	
Tumor Size (cm)					0.417
<4	261	99	162	62.1	
≥4	305	112	193	63.3	
Depth of Invasion					0.001
Tis, T1	197	92	105	53.3	
T2-4	369	119	250	67.8	
Lymphatic Invasion					0.378
-	352	129	223	63.4	
+	214	82	132	61.7	
Venous Invasion					0.022
-	431	171	260	60.3	
+	135	40	95	70.4	
Lymph Node Metastasis					0.001
-	287	126	161	56.1	
+	279	85	194	69.5	
TNM Staging					0.001
0-I	224	101	123	54.9	

II-IV	342	110	232	67.8	
Lauren's Classification					0.168
Intestinal Type	295	116	179	60.7	
Diffuse Type	271	95	176	64.9	

Note: Tis: Carcinoma in Situ; T1: Lamina Propria and Submucosa; T2: Muscularis Propria and Subserosa; T3: Exposure to Serosa; T4: Invasion into Serosa

Table 2: Relationship between REG4 expression and clinicopathological features of gastric cancer.

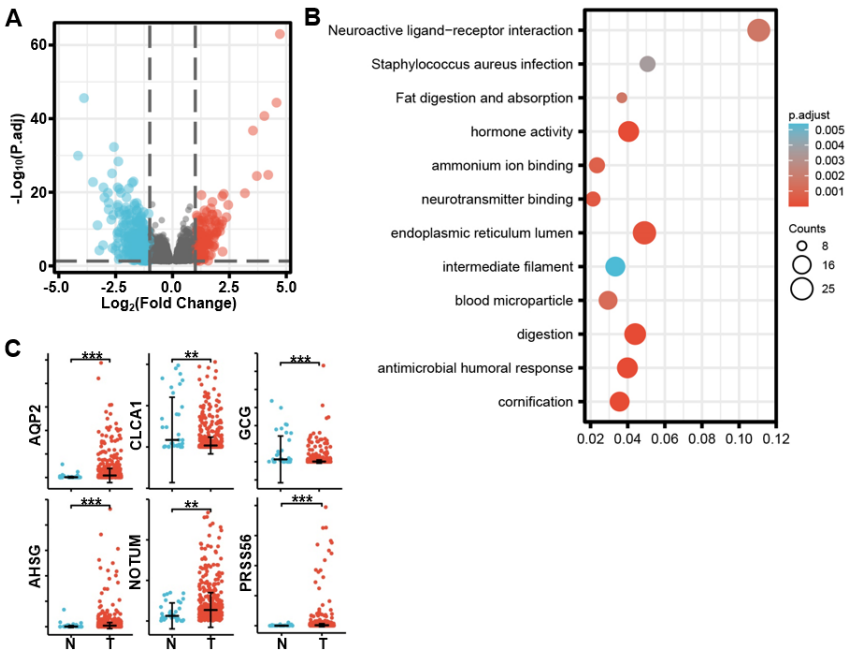
Histologic subtypes of WHO	n	REG4 expression		
		-	+	PR（%）
Papillary	6	1	5	83.3
Well-differentiated	166	87	79	47.6
Moderately differentiated	136	29	107	78.7[a]
Poorly differentiated	191	91	100	52.4
Mucinous	13	0	13	100[b]
Signet Ring Cell	54	3	51	94.4[c]

[a]Compared with well- or poorly differentiated carcinoma, p <0.001
[b]Compared with well- or poorly differentiated carcinoma, p <0.001
[c]Compared with well-, moderately, or poorly differentiated carcinoma, p <0.05

Table 3: REG4 expression in the different subtypes of gastric adenocarcinoma according to WHO classification.

The Related Genes and Signal Pathways of REG4 in GC

In Xiantao platform, we found the differential genes between low and high expression groups of REG4 mRNA in GC and build up the volcano map as Figure 2A. KEGG analysis showed that the top signal pathway included neuroactive ligand-receptor interaction, digestion and absorption, endoplasmic reticulum lumen, hormone and so on (Figure 2B, p <0.05). Among these top differential genes, the expression of AQP2, AHSG, NOTUM, and PRSS56 was higher in GC than normal mucosa, while opposite for CLCA1 and GCG (Figure 2C, p <0.05). In addition, we performed the string and Cytoscape and found the hub genes of REG4 in GC (Figure 3A). The top hub genes were shown in Figure 3B. According to Xiantao database, CPB2, F2, AHSG, APOA2, KNG1, FGB, SERPINA1, and CRP were more expressed in GC than in normal tissues (Figure 3C, p <0.05), while the opposite was seen in APOPA1 and TTR (Figure 3C, p <0.05).



Note: N: Normal; T: Tumor; **, p <0.01; ***, p <0.001

Figure 2: The differential genes and related signal pathways about REG4 expression in colorectal cancer. The volcano map of the differential genes about REG4 was shown in gastric cancer **(A)**. These genes were subjected to the signal pathway analysis using KEGG **(B)**. The differential genes were compared between gastric mucosa and cancer **(C)**.

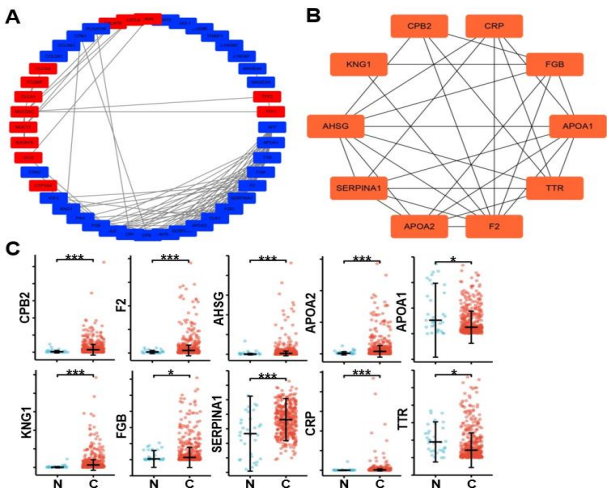


Figure 3: PPI network and module analysis about differential genes of REG4 in gastric cancer.
STRING was used to identify the protein-protein interaction network of differential genes about REG4 in gastric cancer **(A)**. Cytoscape was employed to find out the top 10 hub nodes ranked by degree **(B)**. The hub genes were compared between gastric cancer and normal tissues **(C)**.
Note: N: Normal; T: Tumor; *, p <0.05; **, p <0.01; ***, p <0.001.

According to Xiantao, the positively correlated genes of REG4 in GC were shown in the hop map of Figure 4A (p <0.05), and involved in GC, glycan processing and binding, and glycosylation, extracellular matrix, bindings of carbohydrate and cAMP response element, Golgi apparatus, and so forth (Figure 4B). The negatively-correlated genes of REG4 in GC were shown in the hop map of Figure 4C (p <0.05), and involved in gastric cancer, transmembrane transporter activity, cytidine metabolism and so forth (Figure 4D). The REG4-correlated genes (AGR2, MUC2, PATZ1, PBX2, SMIM31, LGALS4, KCTD17, APOBEC3F, CREB3L1, MUC3A, SLC22A11, SLC35E3) were more expressed in GC than normal tissue (Figure 4E, p <0.05). TCEA2 expression was negatively correlated with overall, disease-specific and progression-free survival of the GC patients (Figure 4F, p <0.05). It was the same between PFS and SLC22A11, and between DSS and PBX2 in the GC patients (Figure 4F, p <0.05).

The Effects of REG4 on the Phenotypes of GC Cells

REG4 protein was positively expressed in MKN45 cells, while its mRNA was strongly expressed in all GC cells by Western blot (Figure 5A) and RT-PCR respectively (Figure 5B). According to Western blot (Figure 5C), immunofluorescence (Figure 5D) and PCR (Figure 5E, p <0.05), AGS cells were successfully transfected with its FL-REG4 plasmid. After the treatment with rhREG4, the cell viability of AGS was increased in a dose-dependent manner (Figure 5F, p <0.05). The exposure to anti-REG4 antibody decreased the cell viability of FL-REG4-overexpressing AGS cells at a dose-dependent manner (Figure 5G, p <0.05). The rhREG4 exposure or FL-REG4 overexpression resulted in a higher viability (Fig. 5H, p<0.05), chemoresistance against DDP and 5-FU (Figure 5I), anti-apoptosis (Figure 5J, p <0.05), migration and invasion (Figure 5K and Figure 5L, p <0.05) and a high lipid droplet formation (Figure 5M, p <0.05) in comparison with AGS. Additionally, FL-REG4 transfection and its rhREG4 exposure enhanced the expression of REG4, EGFR-Tyr992, -Tyr1068, -Tyr1148 and -Tyr1173, p-PI3K, AKT, p-Akt, NF-κB, p-NF-κB, Bcl-2 and Bcl-X/L, compared with AGS cells by Western blot (Figure 5J). However, anti-REG4 antibody blocked the effects of FL-REG4 overexpression (Figure 5H - Figure 5N). According to quantitative RT-PCR (Figure 6A), western blot (Figure 6B) and immunofluorescence (Figure 6C), NSP-REG4 was successfully transfected into AGS cells, evidenced by higher REG4 level in NSP-REG4 transfectant than AGS

cells. There were no effects of NSP-REG4 on the aggressive phenotypes and their related proteins' expression in AGS cells, different from FL-REG4 expression (Figure 6D - Figure 6H).

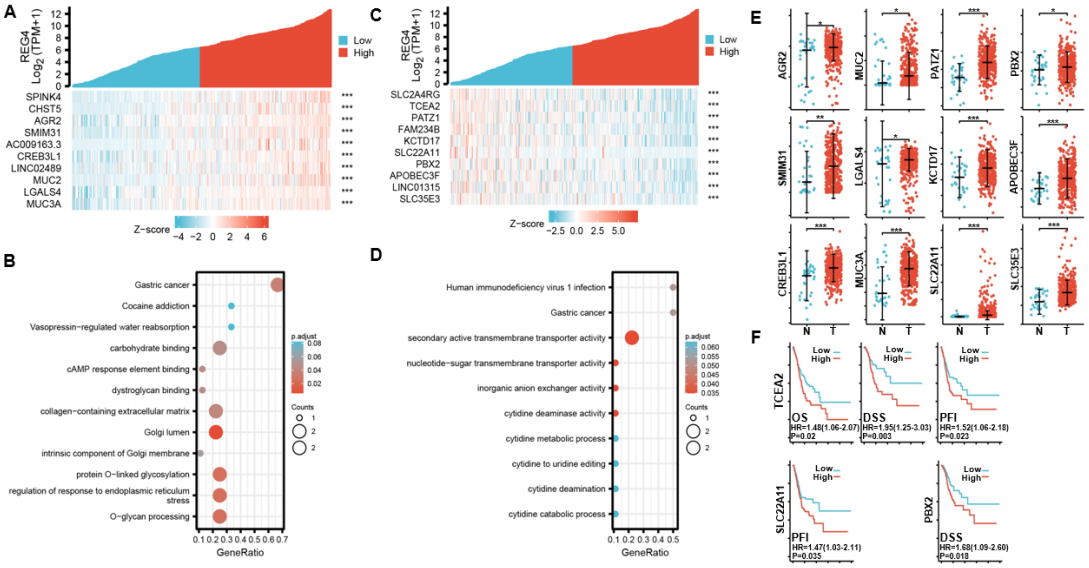


Figure 4: The REG4-related genes and signal pathways in gastric cancer.
The positively related genes of REG4 were screened **(A)** and were classified into the signal pathway using xiantao database **(B)**. The negatively related genes of REG4 were screened **(C)** and were classified into the signal pathway using xiantao database **(D)**. The genes were compared between gastric cancer and normal tissue using Xiantao platform **(E)** and correlated with the overall survival (OS), disease-specific survival (DSS) and progression-free interval (PFI) of the cancer patients **(F)**.
Note: N: Normal; T: Tumor; *, p <0.05; **, p <0.01; ***, p <0.001.

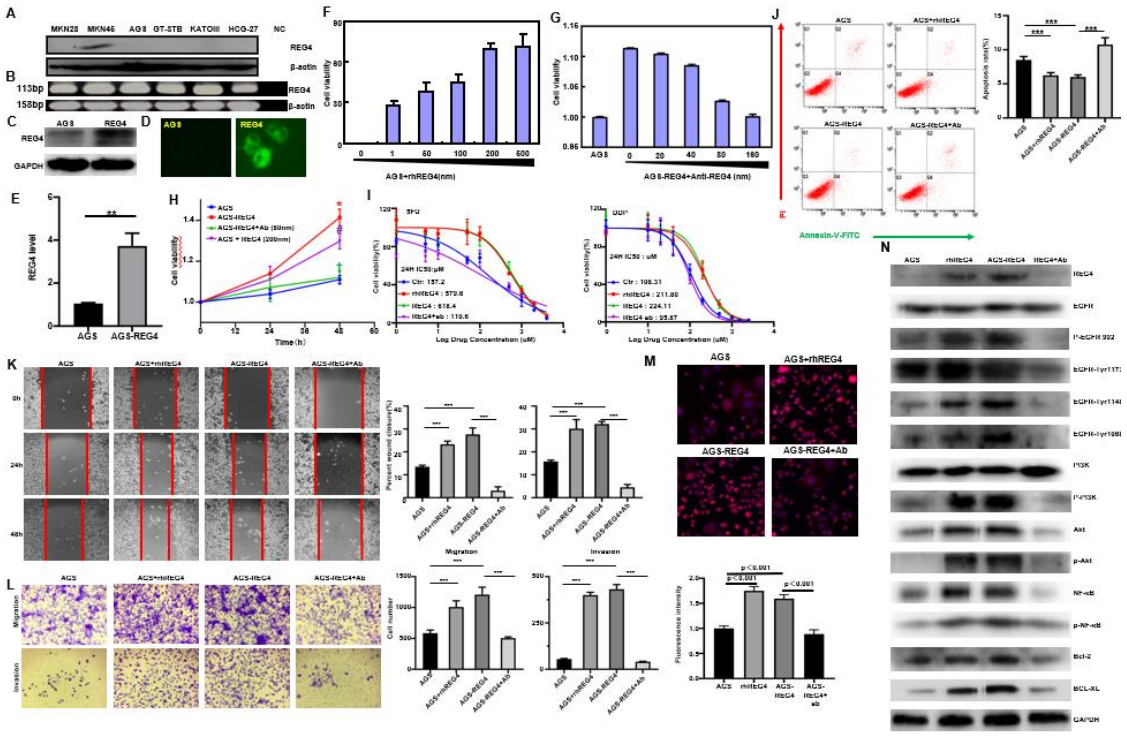
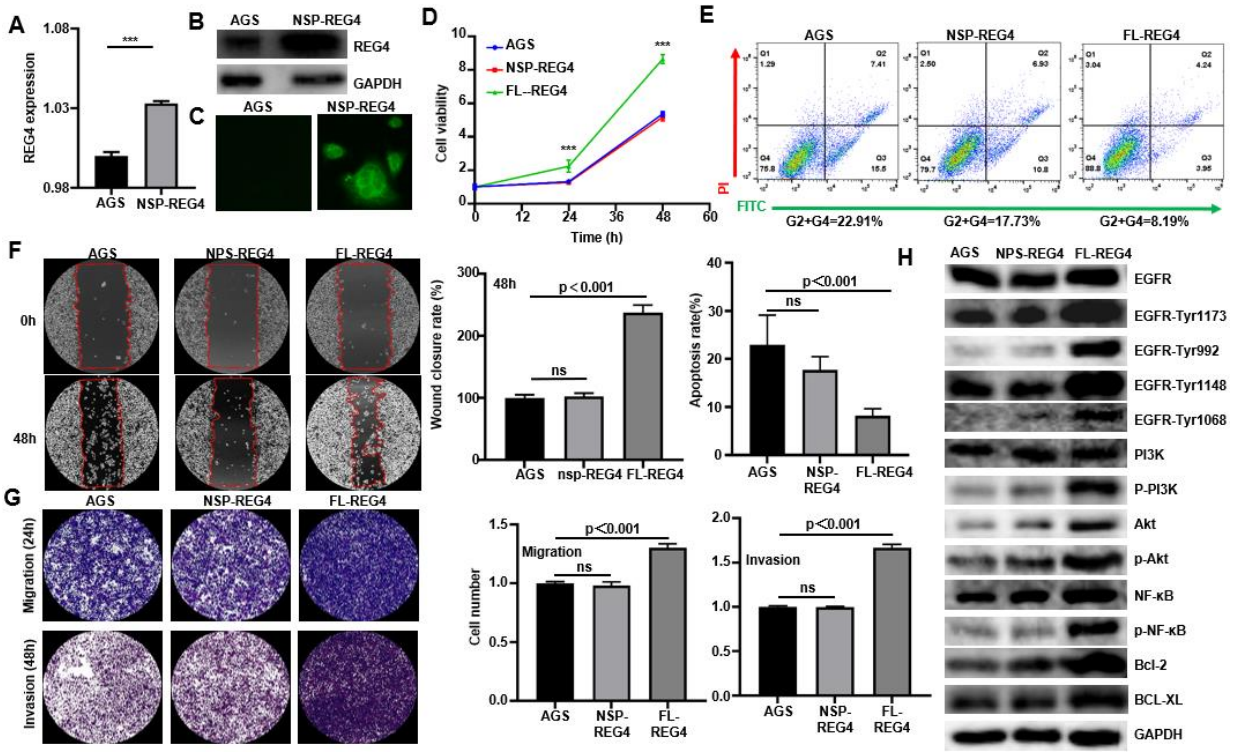


Figure 5: The effects of full-length (FL) REG4 on the phenotypes of AGS cells.
REG4 expression was detected in gastric cancer cells by Western blot **(A)** and PCR **(B)**. AGS cells were successfully transfected with FL-REG4-expressing plasmid, evidenced by Western blot **(C)**, immunofluorescence **(D)** and RT-PCR **(E)**. AGS cells were incubated with increasing doses of rhREG4 (0 nmol/L - 500 nmol/L) for 48 hours and subjected to cell proliferation assay by CCK-8 method **(F)**. Treatment with anti-REG4 antibody produced a dose-dependent decrease in cell number of FL-REG4-overexpressing AGS cells **(G)**. The

FL-REG4 transfectants showed a high proliferation **(H)**, chemoresistance to DDP and 5-FU **(I)**, a low apoptosis **(J)**, a strong migration or invasion **(K,L)**, and a high lipid droplet formation **(M)** in comparison with AGS in line with rhREG4 exposure, which was blocked by anti-REG4 antibody by CCK-8, Annexin-V-FITC/PI staining, wound healing, transwell and Nile red staining. The phenotype-related proteins were screened by Western blot **(N)**.

Note: ab: Anti-REG4 Antibody; rhREG4: Recombinant Human REG4; **, p <0.01; ***, p <0.001.



Note: NSP: Non-Signal-Peptide; ns: Not Significant; ***, p <0.001

Figure 6: The effects of non-signal-peptide (NSP) REG4 on the phenotypes of AGS cells

AGS cells were successfully transfected with NSP-REG4-expressing plasmid, evidenced by quantitative RT-PCR **(A)**, Western blot **(B)** and Immunofluorscence **(C)**. AGS, NSP- and full length (FL) REG4 transfectants were subjected to REG4 secretion, proliferation, apoptosis, migration and invasion by CCK-8 **(D)**, Annexin-V-FITC/PI staining **(E)**, wound healing **(F)**, and transwell **(G)**. The phenotype-related proteins were screened by Western blot **(H)**.

The Effects of REG4 on Chemoresistance and Droplet Formation of GC Cancer Cells

rhREG4 treatment, FL-REG4 overexpression and the exposure of anti-REG4 antibody to REG4 transfectants had no effects on IC50 of ACC1 or ACLY inhibitor in AGS cells (Figure 7A). However, ACC1 or ACLY inhibitor weakened the chemosensitivity to DDP or 5-FU (Figure 7B) and lipid droplet formation (Figure 7C) in AGS and its REG4 transfectant, while HG had the opposite effects (Figure 7B and Figure 7C). Additionally, FL-REG4 overexpression enhanced the expression of ACAT (Acyl coenzyme A-cholesterol acyltransferase), CIDE (cell death-inducing DFF45-like effector) C, SREBP-1 and TIP47 (tail-interacting protein 47), but weakened the expression of ACLY, ACC1, p-ACC1, HDAC, AC-histone 3 (H3), AC-histone 4 (H4), ING5, perilipin 5, ADRP (adipose differentiation-related protein), CIDE A and B in AGS cells (Figure 7D).

REG4 Deteriorated the Cellular Aggressiveness of AGS by EGFR-PI3K-Akt Pathway

The treatment with Cetuximab, Erlotinib, PI3K or Akt inhibitor inhibited the proliferation (Figure 8A, p <0.05), migration (Figure 8B and Figure 8C, p <0.05), invasion (Figure 8C, p<0.05), chemoresistance to DDP or 5-FU (Figure 8D) and lipid droplet formation (Figure 8E, p <0.05) in AGS cells overexpressing REG4 or treated with rhREG4. Cetuximab and Erlotinib decreased the

expression of EGFR-Tyr 992, -Tyr1068, -Tyr1148 and -Tyr1173, p-PI3K, p-Akt, ACAT, CIDEC and TIP47, PI3K inhibitor did the expression of p-PI3K, p-Akt, ACAT, CIDEC and TIP47, while Akt inhibitor did the expression of p-Akt, ACAT, CIDEC and TIP47 (Figure 8F).

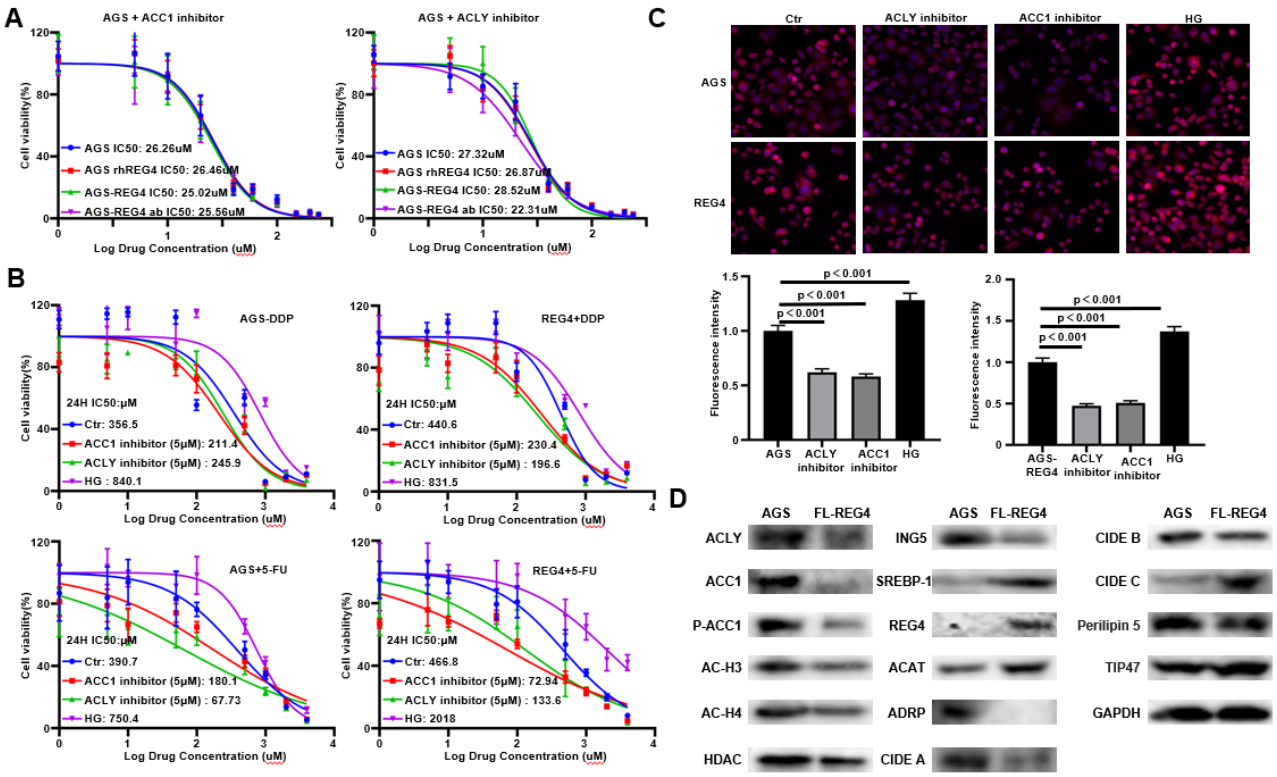


Figure 7: The effects of REG4 on chemoresistance and droplet formation of AGS cells.
After the treatment of ACC1 or ACLY inhibitor, or high glucose (HG), the viability **(A)**, chemosenstivity against DDP and 5-FU **(B)**, and lipid droplet formation **(C)** were measured in AGS, AGS treated with rhREG4 or transfected with full-length (FL)-REG4 plasmid, and FL-REG4- overexpressing AGS treated with anti-REG4 antibody. The expression of lipid-droplet-related proteins were screened by Western blot **(D)**.
Note: **, p <0.01; ***, p <0.001; ab: Anti-REG4 Antibody; REG4: FL-REG4.

REG4 Destabilized ACLY and ACC1 Proteins via Proteasomal Degradation

After treatment with cycloheximide, ACC1 and ACLY expression was lower in FL-REG4 transfectants than in AGS cells (Figure 9A, p <0.05). MG132 also increased the protein expression of ACC1 and ACLY in AGS cells and FL-REG4 transfectants although their expressions were higher in AGS cells than in FL-REG4 transfectants (Figure 9B, p <0.05). There appeared higher ACC1 and ACLY protein in nuclear proteasome of FL-REG4 transfectants than AGS cells, which was strengthened by MG132 treatment (Figure 9C, p <0.05), while the similar levels of ACC1 and ACLY expression in cytosolic proteasome of transfectants and parental cells, even treated with MG132 (Figure 9C, p >0.05). Regarding ubiquitin ligases, we found the expression levels of CBL and NEDD4L were higher in FL-REG4 transfectant than AGS cells, but the opposite for SYVN1 and NEDD4 (Figure 9D). Co-IP showed that ubiquitylated ACC1 and ACLY were higher in FL-REG4 transfectants than AGS cells, which were weakened by MG132 (Figure 9E). ACLY more bound to SYVN1, NEDD4 and NEDD4L in FL-REG4 transfectants than AGS cells, but versa for CBL. ACC1 more interacted with COP1 in REG4 transfectants than AGS cells, but versa for CBL and SYVN1 (Figure 9E).

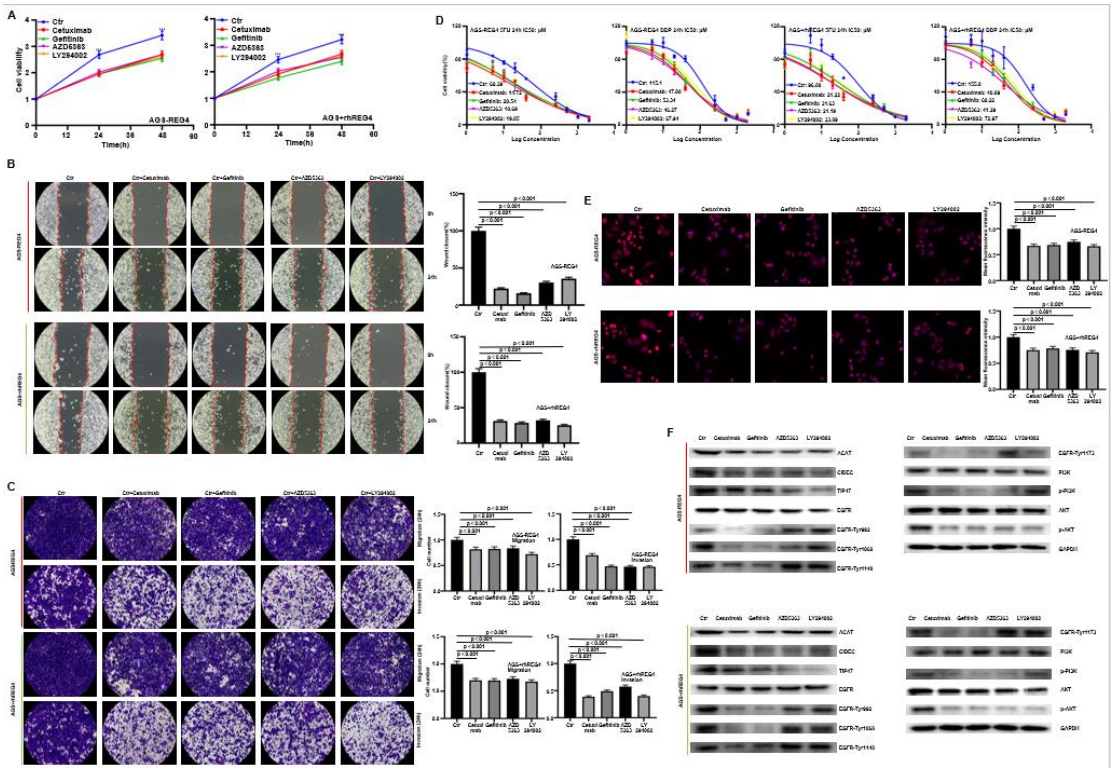


Figure 8: REG4 aggravated the cellular phenotypes by EGFR-PI3K-Akt signal pathways.
We detected the viability **(A)**, migration **(B and C)**, invasion **(C)**, chemosensitivity against cisplatin (DDP) and 5-FU **(D)** and lipid droplet formation **(E)** of FL-REG-overexpressing or rhREG4-exposed AGS cells treated with Cetuximab (10μM), Erlotinib (10μM), PI3K inhibitor LY294002 (10μM) and the Akt inhibitor AZD5363 (3μM) by CCK-8, transwell assay, and Nile red staining respectively. The expression levels of EGFR, p-EGFR, PI3K, p-PI3K, Akt, p-Akt, ACAT, CIDE C and TIP47 were detected by western blotting **(F)**.
Note: FL: Full-Length; *, p <0.05; **, p <0.01; ***, p <0.001.

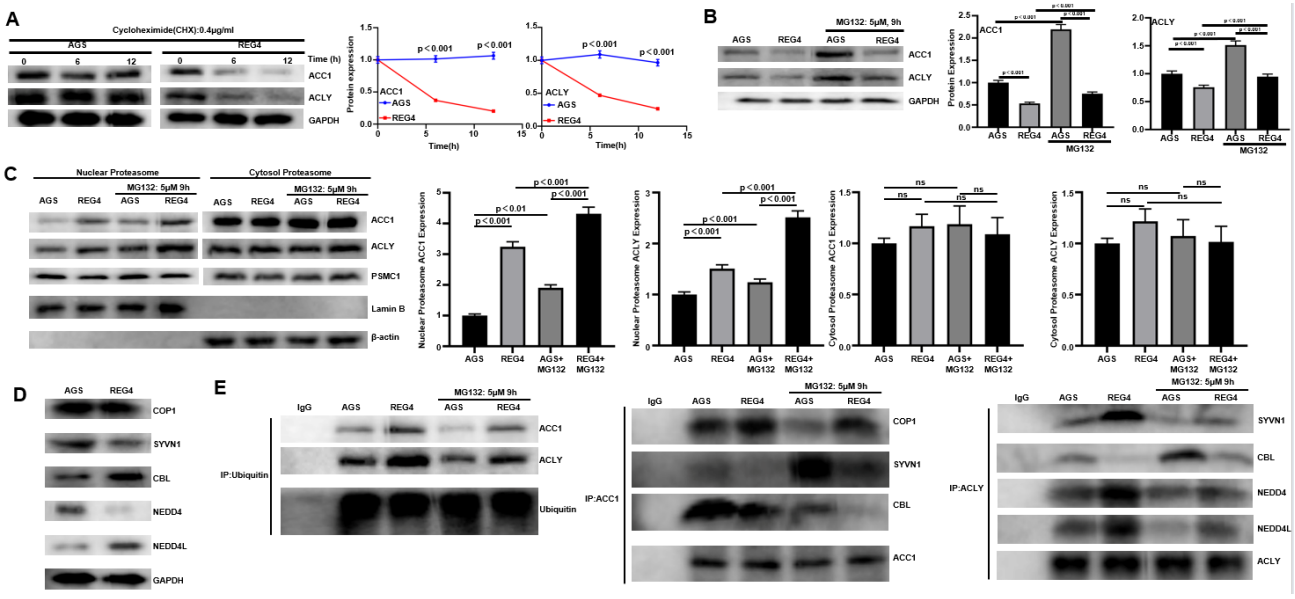


Figure 9: Full-length (FL) REG4 destabilized ACLY and ACC1 proteins via proteasomal degradation.
AGS cells and FL-REG4 transfectants were treated with cycloheximide (CHX, 0.4μg/ml) **(A)** or MG132 (5μM, 9h) **(B)**, and followed by Western blot. AGS cells and FL-REG4 transfectants were subjected to proteasomal extract and subsequent Western blot, even treated with MG132 **(C)**. Ubiquitin transferases (COP1, SYVN1, CBL, NEDD4 and NEDD4L) were detected in AGS and its FL-REG4 transfectants by

western blot **(D)**. After co- immunoprecipitation (Co-IP), Western blot was performed in AGS cells and FL-REG4 transfectants, even treated with MG132 **(E)**.
Note: PSMC1: A Marker for Proteasome; Lamin B: A Marker for Nuclear Fraction; GAPDH and β-actin: A Maker for Cytosolic Fraction; ns: Not Significant; *, p <0.001.

DISCUSSION

Reportedly, serum REG4 level was reported to be remarkably higher in esophageal, gastric and pancreatic cancer patients than that in healthy volunteers [27-31]. In line with the findings in ovarian, colon, pancreatic, gallbladder, and prostate cancers and glioma, [14, 29-34] REG4 expression was higher in GC than normal mucosa, and positively associated with depth of invasion, lymph node metastasis, clinicopathological staging and differentiation of GC at either mRNA or protein level, suggesting that REG4 overexpression promoted gastric carcinogenesis and subsequent progression.

Previously, we found that REG4 expression was significantly correlated with the MUC-2 and MUC-5AC expression, and higher in mucin-producing intestinal metaplasia, mucinous carcinoma and signet ring cell carcinoma [20]. REG4 was identified as a potential biomarker of mucinous ovarian cancer at both mRNA and protein levels [14,19,28,35]. Here, we also found both mucinous adenocarcinoma and signet ring cell carcinoma had a higher REG4 protein expression than other histological subtypes, and intestinal metaplasia showed a higher REG4 expression than gastritis, adenoma and GC. Intestinal metaplasia is a precancerous lesion because it can progress into globoid dysplasia, which is called in situ signet ring cell carcinoma. Therefore, REG4 might underlie the molecular mechanism of gastric epithelium-intestinal metaplasia-globoid dysplasia-signet ring cell carcinoma.

Bishnupuri et al. [4] found that the transcriptional activator of D-type cyclins, CD44 intracytoplasmic domain (CD44ICD), was released after REG4 connected with transmembrane CD44 and activated γ-secretase, finally to promote proliferation and stemness of colorectal and pancreatic cancer cells. In this study, we also found that REG4 overexpression and rhREG4 treatment promoted proliferation, anti-apoptosis, migration and invasion of GC cells. Exposure to anti-REG4 antibody inhibited the effects of REG4 overexpression on the phenotypes of GC cells, which is consistent with our earlier result about ovarian cancer cells [32]. Additionally, we also found that REG4 overexpression resulted in the chemoresistance of ovarian cancer cells to cisplatin or taxol by activating PI3K/Akt/mTOR signal pathway [33]. A mutant KRAS-induced factor REG4 increased cancer stemness via Wnt/β-catenin signal [36]. Jin et al. [11] showed that REG4 increased the resistance of GC cells to fluorouracil by stimulating the MAPK/Erk/Bim signal pathway. Here, we found that the expression of REG4, EGFR-Tyr992,-Tyr1068, - Tyr1148 and - Tyr1173, p-PI3K, AKT, p-Akt, NF-κB, p- NF-κB, Bcl-2 and Bcl-x/L was higher in AGS treated rhREG4 or transfected with REG4-overexpressing plasmid than the parental cells, and anti-REG4 antibody, EGRF antibody, TKI, PI3K and Akt inhibitor blocked the effects of REG4 overexpression, indicating that REG4 promoted the aggressive phenotypes by EGFR-PI3K-Akt signal pathway, in line with other reports [7,8,35]. However, no effects of NSP-REG4 on aggressive phenotypes and related signal proteins suggested that REG4 only functioned in GC cells in either autocrine or paracrine manner.

AGS cells developed chemoresistance to 5-FU and DDP as a result of REG4 overexpression, suggesting the role of REG4 in chemoresistance, in line with another two reports [33,37,38]. Reportedly, chemoresistance of colorectal cancer cells was produced by LPCAT2-mediated lipid droplet formation [37] which was also aided by prothymosin α and metastasis-associated in colon cancer, [39] through SREBP-1- and FASN- mediated and

lipogenesis respectively. A crucial enzyme for de novo fatty acid synthesis is either ACC1 or ACLY, which is closely linked to chemoresistance [40]. In the liver and peritoneal tissues, lipid droplet assembly is mediated by ADRP, CIDE, ACAT1, perilipin 5, and TIP47 [41-51]. REG4-mediated lipid droplet formation might be closely linked to the up-regulated expression of ACAT1, CIDC, and TIP47 in REG4 transfectants, but not with de novo lipogenesis, which is evidenced by down-regulated expression ACC1 and ACLY and no effects of REG4 on the chemosensitivity to ACC1 or ACLY inhibitor, which enhanced the sensitivity of AGS to DDP and 5-FU. Moreover, REG4-mediated lipid droplet formation might account for the REG4-induced chemoresistance against 5-FU and DDP, but deteriorated by HG exposure. In combination with these discoveries, we hypothesized that REG4 may have a role in chemoresistance not through de novo lipogenesis, but lipid droplet assembly, which was strengthened by the pretreatment of high-dose glucose.

In the present study, we found that forced REG4 overexpression decreased the expression of AC-H3, AC-H4, ING5, and HDAC in GC cells, while ING5 is involved in the formation of histone acetyltransferase (HAT), suggesting that the regulatory effects of ING5 or HADAC on REG4 expression might be of transcriptional level. Therefore, we also analyzed the modulatory effects of REG4 on the protein stability of ACC1 and ACLY. Firstly, we treated AGS cells and REG4 transfectants with cycloheximide and found that REG4 destabilized ACC1 and ACLY. REG4 could promote the recruitment of ACC1 and ACLY to nuclear proteasome for ubiquitylation-mediated degradation, during which ACLY bound to SYVN1, NEDD4 and NEDD4L, and ACC1 did COP1. REG4 overexpression was found to mediate lipid droplet formation and chemoresistance in AGS cells. These data suggested that REG4 might facilitate the proteasomal degradation of ACC1 and ACLY to suppress the de novo lipogenesis. As a result, REG4 stimulated the lipid droplet formation and subsequently chemoresistance via lipid droplet assembly, but not lipogenesis.

CONCLUSION

In summary, REG4 protein expression was up regulated in GC and positively linked with the degree of invasion and TNM stage, and mucinous differentiation of GC. REG4 aggravated the aggressive phenotypes at autocrine or paracrine manner by EGFR-PI3K-Akt-NF-κB signal pathway. REG4 may be involved in chemoresistance not through de novo lipogenesis, but lipid droplet assembly. REG4 induced proteasomal degradation of ACC1 and ACLY proteins. REG4 may be used as a reliable diagnostic biomarker of aggressiveness, carcinogenesis, and a potential molecule for target therapy in GC.

AUTHOR CONTRIBUTIONS

Cong-yu Zhang: Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing - review and editing (equal). **Li Zhang:** Resources (equal); writing - review and editing (equal). **Hong-zhi Sun:** Resources (equal)writing - review and editing (equal). **Min-wen Ha:** Resources (equal); writing - review and editing (equal). **Zheng-guo Cui:** Resources (equal); writing - review and editing (equal). **Hua-chuan Zheng:** Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); visualization (equal); writing - review and editing (equal).

ACKNOWLEDGMENTS

The present study was supported by Award for Liaoning Distinguished Professor, Natural Science Foundation of Hebei Province (21377772D; H2022406034) and National Natural Scientific Foundation of China (81672700).

CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The trial protocol was approved by an independent ethics committee, all patients provided written informed consent prior to initiation of any study treatment, and the study was conducted in accordance with recognized international scientific and ethical standards, including but not limited to the Declaration of Helsinki.

REFERENCES

1. Hu Y, Pan C, Hu J, et al. (2015) The role of Reg IV in colorectal cancer, as a potential therapeutic target. Contemporary Oncology/Współczesna Onkologia 19(4): 261-264.
2. Oue N, Mitani Y, Aung PP, et al. (2005) Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland 207(2): 185-198.
3. Heiskala K, Andersson LC (2013) Reg IV is differently expressed in enteroendocrine cells of human small intestine and colon. Regulatory Peptides 183: 27-34.
4. Bishnupuri KS, Luo Q, Murmu N, et al. (2006) Reg IV activates the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon adenocarcinomas. Gastroenterology 130(1): 137-149.
5. Wang H, Hu L, Zang M, et al. (2016) REG4 promotes peritoneal metastasis of gastric cancer through GPR37. Oncotarget 7(19): 27874-27888.
6. Kawasaki Y, Matsumura K, Miyamoto M, et al. (2015) REG4 is a transcriptional target of GATA6 and is essential for colorectal tumorigenesis. Scientific Reports 5(1): 14291.
7. Naito Y, Oue N, Hinoi T, et al. (2012) Reg IV is a direct target of intestinal transcriptional factor CDX2 in gastric cancer. PLoS One 7(11): e47545.
8. Hwang JH, Yoon J, Cho YH, et al. (2020) A mutant KRAS-induced factor REG4 promotes cancer stem cell properties via Wnt/β-catenin signaling. International Journal of Cancer 146(10): 2877-2890.
9. Bishnupuri KS, Sainathan SK, Bishnupuri K, et al. (2014) Reg4-induced mitogenesis involves Akt-GSK3β-β-Catenin-TCF-4 signaling in human colorectal cancer. Molecular carcinogenesis 53(S1): E169-E180.
10. He XJ, Jiang XT, Ma YY, et al. (2012) REG4 contributes to the invasiveness of pancreatic cancer by upregulating MMP-7 and MMP-9. Cancer Science 103(12): 2082-2091.
11. Jin J, Lv H, Wu J, et al. (2017) Regenerating family member 4 (Reg4) enhances 5-fluorouracil resistance of gastric cancer through activating MAPK/Erk/Bim signaling pathway. Medical Science Monitor: International Medical Journal of Experimental and Clinical Research 23: 3715-3721.
12. Mitani Y, Oue N, Matsumura S, et al. (2007) Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy. Oncogene 26(30): 4383-4393.
13. Bishnupuri KS, Luo Q, Sainathan SK, et al. (2010) Reg IV regulates normal intestinal and colorectal cancer cell susceptibility to radiation-induced apoptosis. Gastroenterology 138(2): 616-626.

14. Chen S, Gou WF, Zhao S, et al. (2015) The role of the REG4 gene and its encoding product in ovarian epithelial carcinoma. BMC Cancer 15(1): 1-12.
15. Rafa L, Dessein AF, Devisme L, et al. (2010) REG4 acts as a mitogenic, motility and pro-invasive factor for colon cancer cells. International Journal of Oncology 36(3): 689-698.
16. Takehara A, Eguchi H, Ohigashi H, et al. (2006) Novel tumor marker REG4 detected in serum of patients with resectable pancreatic cancer and feasibility for antibody therapy targeting REG4. Cancer Science 97(11): 1191-1197.
17. Liu CM, Hsieh CL, He YC, et al. (2013) In vivo targeting of ADAM9 gene expression using lentivirus-delivered shRNA suppresses prostate cancer growth by regulating REG4 dependent cell cycle progression. PloS One 8(1): e53795.
18. Kobayashi Y, Niwa Y, Tajika M, et al. (2010) Serum tumor antigen REG4 as a useful diagnostic biomarker in gastric cancer. Hepato-gastroenterology 57(104): 1631-1634.
19. Nanakin A, Fukui H, Fujii S, et al. (2007) Expression of the REG IV gene in ulcerative colitis. Laboratory Investigation 87(3): 304-314.
20. Oue N, Kuniyasu H, Noguchi T, et al. (2008) Serum concentration of Reg IV in patients with colorectal cancer: Overexpression and high serum levels of Reg IV are associated with liver metastasis. Oncology 72(5-6): 371-380.
21. Zhang Y, Lai M, Lv B, et al. (2003) Overexpression of Reg IV in colorectal adenoma. Cancer Letters 200(1): 69-76.
22. Lasserre C, Colnot C, Bréchot C, et al. (1999) HIP/PAP gene, encoding a C-type lectin overexpressed in primary liver cancer, is expressed in nervous system as well as in intestine and pancreas of the postimplantation mouse embryo. The American Journal of Pathology 154(5): 1601-1610.
23. Lasserre C, Christa L, Simon MT, et al. (1992) A novel gene (HIP) activated in human primary liver cancer. Cancer Research 52(18): 5089-5095.
24. Kimura N, Yonekura H, Okamofo H, et al. (1992) Expression of human regenerating gene mRNA and its product in normal and neoplastic human pancreas. Cancer 70(7): 1857-1863.
25. Ohara S, Oue N, Matsubara A, et al. (2008) Reg IV is an independent prognostic factor for relapse in patients with clinically localized prostate cancer. Cancer Science 99(8): 1570-1577.
26. Zheng HC, Xu XY, Yu M, et al. (2010) The role of Reg IV gene and its encoding product in gastric carcinogenesis. Human Pathology 41(1): 59-69.
27. Sun S, Hu Z, Huang S, et al. (2019) REG4 is an indicator for KRAS mutant lung adenocarcinoma with TTF-1 low expression. Journal of Cancer Research and Clinical Oncology 145: 2273-2283.
28. Oue N, Noguchi T, Anami K, et al. (2011) Serum concentration and expression of Reg IV in patients with esophageal cancer: Age-related elevation of serum Reg IV concentration. Oncology Letters 2(2): 235-239.
29. Takayama R, Nakagawa H, Sawaki A, et al. (2010) Serum tumor antigen REG4 as a diagnostic biomarker in pancreatic ductal adenocarcinoma. Journal of Gastroenterology 45: 52-59.
30. Zhu X, Han Y, Yuan C, et al. (2015) Overexpression of Reg4, alone or combined with MMP-7 overexpression, is predictive of poor prognosis in colorectal cancer. Oncology Reports 33(1): 320-328.
31. Wang Q, Deng J, Yuan J, et al. (2012) Oncogenic reg IV is a novel prognostic marker for glioma patient survival. Diagnostic Pathology 7(1): 1-8.
32. Tamura H, Ohtsuka M, Washiro M, et al. (2009) Reg IV expression and clinicopathologic features of gallbladder carcinoma. Human Pathology 40(12): 1686-1692.

33. Hayashi T, Matsubara A, Ohara S, et al. (2009) Immunohistochemical analysis of Reg IV in urogenital organs: Frequent expression of Reg IV in prostate cancer and potential utility as serum tumor marker. Oncology Reports 21(1): 95-100.
34. Zheng HC, Li J, Zhao S (2018) REG4 expression was a potential marker for carcinogenesis, aggressiveness and prognosis of gastric cancer: A meta and bioinformatics analysis. Oncotarget 5.
35. Eguchi H, Ishikawa O, Ohigashi H, et al. (2009) Serum REG4 level is a predictive biomarker for the response to preoperative chemoradiotherapy in patients with pancreatic cancer. Pancreas 38(7): 791-798.
36. Lehtinen L, Vesterkvist P, Roering P, et al. (2016) REG4 is highly expressed in mucinous ovarian cancer: A potential novel serum biomarker. PLoS One 11(3): e0151590.
37. He HL, Lee YE, Shiue YL, et al. (2014) Overexpression of REG4 confers an independent negative prognosticator in rectal cancers receiving concurrent chemoradiotherapy. Journal of Surgical Oncology 110(8): 1002-1010.
38. Sasahira T, Oue N, Kirita T, et al. (2008) Reg IV expression is associated with cell growth and prognosis of adenoid cystic carcinoma in the salivary gland. Histopathology 53(6): 667-675.
39. Saukkonen K, Hagström J, Mustonen H, et al. (2018) Prognostic and diagnostic value of REG4 serum and tissue expression in pancreatic ductal adenocarcinoma. Tumor Biology 40(3): 1010428318761494.
40. Bishnupuri KS, Sainathan SK, Ciorba MA, et al. (2022) Reg4 interacts with CD44 to regulate proliferation and stemness of colorectal and pancreatic cancer cells. Molecular Cancer Research 20(3): 387-399.
41. Cotte AK, Aires V, Fredon M, et al. (2018) Lysophosphatidylcholine acyltransferase 2-mediated lipid droplet production supports colorectal cancer chemoresistance. Nature Communications 9(1): 322.
42. Jin L, Zhu LY, Pan YL, et al. (2021) Prothymosin α promotes colorectal carcinoma chemoresistance through inducing lipid droplet accumulation. Mitochondrion 59: 123-134.
43. Duan J, Chen L, Zhou M, et al. (2017) MACC1 decreases the chemosensitivity of gastric cancer cells to oxaliplatin by regulating FASN expression. Oncology Reports 37(5): 2583-2592.
44. Shi Y, Fan Y, Hu Y, et al. (2020) α-Mangostin suppresses the de novo lipogenesis and enhances the chemotherapeutic response to gemcitabine in gallbladder carcinoma cells via targeting the AMPK/SREBP1 cascades. Journal of Cellular and Molecular Medicine 24(1): 760-771.
45. Shetty A, Nagesh PK, Setua S, et al. (2020) Novel paclitaxel nanoformulation impairs de novo lipid synthesis in pancreatic cancer cells and enhances gemcitabine efficacy. ACS Omega 5(15): 8982-8991.
46. Sur S, Nakanishi H, Flaveny C, et al. (2019) Inhibition of the key metabolic pathways, glycolysis and lipogenesis, of oral cancer by bitter melon extract. Cell Communication and Signaling 17: 1-13.
47. Fan H, Diao H, Lu Y, et al. (2020) The relation between serum adipose differentiation-related protein and non-alcoholic fatty liver disease in type 2 diabetes mellitus. Therapeutic Advances in Endocrinology and Metabolism 11: 2042018820969025.
48. Kasano-Camones CI, Takizawa M, Iwasaki W, et al. (2020) Synergistic regulation of hepatic Fsp27b expression by HNF4α and CREBH. Biochemical and Biophysical Research Communications 530(2): 432-439.
49. Ayyagari V, Li M, Pasman Z, et al. (2022) Assessment of the diagnostic and prognostic relevance of ACAT1 and CE levels in plasma, peritoneal fluid and tumor tissue of epithelial ovarian cancer patients-a pilot study. BMC Cancer 22(1): 387.

50. Ethem İ and Hacıoğlu C (2022) Effects of perilipin-5 on lipid metabolism and high-sensitivity cardiac troponin I. Revista da Associação Médica Brasileira 68: 1011-1016.
51. Nose F, Yamaguchi T, Kato R, et al. (2013) Crucial role of perilipin-3 (TIP47) in formation of lipid droplets and PGE2 production in HL-60-derived neutrophils. PLoS One 8(8): e71542.