Acta Pharmaceutica Sinica B 2024;14(2):698–711

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com

ORIGINAL ARTICLE

GLUL stabilizes N-Cadherin by antagonizing β-Catenin to inhibit the progresses of gastric cancer

Qiwei Jiang[a,†], Yong Li[b,†], Songwang Cai[c,†], Xingyuan Shi[d,†], Yang Yang[a], Zihao Xing[a], Zhenjie He[a], Shengte Wang[a], Yubin Su[a], Meiwan Chen[e], Zhesheng Chen[f], Zhi Shi[a,*]

[a]*Department of Cell Biology & Institute of Biomedicine, National Engineering Research Center of Genetic Medicine, State Key Laboratory of Bioactive Molecules and Druggability Assessment, MOE Key Laboratory of Tumor Molecular Biology, Guangdong Provincial Key Laboratory of Bioengineering Medicine, College of Life Science and Technology, Jinan University, Guangzhou 510632, China*
[b]*Department of Gastrointestinal Surgery & General Surgery, the Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou 510080, China*
[c]*Department of Thoracic Surgery, The First Affiliated Hospital of Jinan University, Guangzhou 510632, China*
[d]*Department of Radiation Oncology, The Fifth Hospital of Guangzhou Medical University, Guangzhou 510150, China*
[e]*State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau 519000, China*
[f]*Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, St. John's University, Queens, NY 11439, USA*

Received 3 June 2023; received in revised form 25 September 2023; accepted 6 November 2023

KEY WORDS

Gastric cancer;
GLUL;
N-Cadherin;
β-Catenin;
Enzyme;

Abstract Glutamate-ammonia ligase (GLUL, also known as glutamine synthetase) is a crucial enzyme that catalyzes ammonium and glutamate into glutamine in the ATP-dependent condensation. Although GLUL plays a critical role in multiple cancers, the expression and function of GLUL in gastric cancer remain unclear. In the present study, we have found that the expression level of GLUL was significantly lower in gastric cancer tissues compared with adjacent normal tissues, and correlated with N stage and TNM stage, and low GLUL expression predicted poor survival for gastric cancer patients. Knockdown

*Corresponding author.
E-mail address: tshizhi@jnu.edu.cn (Zhi Shi).
[†]These authors made equal contributions to this work.
Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2023.11.008

Protein-protein interaction;
Protein stability;
Ubiquitination

of GLUL promoted the growth, migration, invasion and metastasis of gastric cancer cells *in vitro* and *in vivo*, and *vice versa*, which was independent of its enzyme activity. Mechanistically, GLUL competed with β-Catenin to bind to N-Cadherin, increased the stability of N-Cadherin and decreased the stability of β-Catenin by alerting their ubiquitination. Furthermore, there were lower N-Cadherin and higher β-Catenin expression levels in gastric cancer tissues compared with adjacent normal tissues. GLUL protein expression was correlated with that of N-Cadherin, and could be the independent prognostic factor in gastric cancer. Our findings reveal that GLUL stabilizes N-Cadherin by antagonizing β-Catenin to inhibit the progress of gastric cancer.

1. Introduction

Gastric cancer is not only the most common cancer in the digestive system, but also one of the highest morbidity and mortality in the world, which displays poor prognosis due to lack of early detection and effective therapy[1,2]. Extensive invasion and metastasis are the major causes of treatment failure and death in advanced gastric cancer patients[3,4]. Although lots of important factors participated in the regulation of cancer proliferation, invasion and metastasis through multiple signaling pathways[5,6], the main molecular mechanism remains unclear. Therefore, a better elucidation of the molecular mechanism contributing to gastric cancer invasion and metastasis is warranted with the hope of improving early diagnosis and therapy efficacy.

Glutamine is a remarkable amino acid in the human body, it plays a predominant role in nitrogen metabolism for many cell systems, especially for glutamine-addicted cancer cells[7]. Glutamate-ammonia ligase (GLUL, also known as glutamine synthetase) is a crucial enzyme involved in the process of nitrogen metabolism[8]. It catalyzes the synthesis of glutamine from glutamate and ammonia in an ATP-dependent reaction[9]. Mutation or deregulation of GLUL has been linked to several human diseases, including congenital glutamine deficiency[10], neurological disorders[11], as well as cancers[12]. GLUL is overexpressed and promotes the progress in various cancers, including liver cancer[13,14], lung cancer[15,16], breast cancer[17,18], pancreas cancer[19,20], ovarian cancer[21] and colon cancer[22]. Thus, high expression of GLUL contributes to the progress of these cancers, which is partially due to the reasons that these types of cancers belong to glutamine-dependent. However, it has been reported that GLUL expression is downregulated in the gastric cancer tissues, possibly because gastric cancer is so aggressive that it takes an amount of glutamine from the external environment, resulting in there is no need for self-synthesis of glutamine[7]. Additionally, in astrocytes, down-regulation of GLUL expression significantly increases the migratory and invasive capacity[23]. Furthermore, GLUL over-expression in C6 glioma cells results in growth suppression, motility spreading and attachment inhibition[24].

Accumulating data of how GLUL participated in regulating cancer progression are gradually reported. YAP1 can directly bind to the transcriptional start site of the GLUL promoter and elevates GLUL activity and expression. Thus, the knockdown of YAP1 reduces GLUL expression and then inhibits liver cancer cell growth[25]. Oncogenic c-Myc upregulates GLUL activity and expression by directly activating GLUL promoter demethylation[26]. Increased GLUL expression promotes the biosynthesis of ribonucleotides and asparagine, as well as amino acid transport in breast cancer cells. And inhibition of GLUL expression suppresses cell survival, proliferation and oncogenesis in c-Myc-driven cancers[27]. However, the role of GLUL in gastric cancer remains unclear.

In this study, we investigated the expression and function of GLUL in gastric cancer, and demonstrated a novel function of GLUL in gastric cancer.

2. Materials and methods

2.1. Patients and specimens

Specimens including 97 pairs of gastric cancer and matched adjacent normal tissues were obtained from patients who received curative surgery in the Department of Gastrointestinal Surgery & General Surgery, Guangdong General Hospital, Guangdong Academy of Medical Sciences (Guangzhou, China). Signed informed consent was obtained from all patients or their relatives. The study was approved by the ethics committee of Guangdong General Hospital (Guangzhou, China).

2.2. Plasmids and antibodies

The shRNA targeting human GLUL mRNA (5′-GACAATGCCC-GACGTCTAA-3′) was cloned into pLKO.1-GFP lentiviral vector. Full-length of GLUL-WT/or R324C, N-Cadherin and deletion mutants were cloned into pDest27-GST or pCDH-Neo-Venus/Dest vectors as previously described[28]. Anti-GLUL (Cat#D122427) and anti-GFP (Cat#D110008) antibodies were purchased from Sangon Biotech (Shanghai, China). Anti-N-Cadherin (Cat#610920), anti-β-Catenin (Cat#610154), anti-c-Myc (Cat#551102) and anti-Cyclin D1 (Cat#514181) antibodies were purchased from BD Bioscience (NJ, USA). Anti-Vimentin (Cat#5741) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-GST (Cat#SC-459) and anti-HA (Cat#SC-7392) antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-Flag (Cat#F7452) antibody was purchased from Sigma−Aldrich (St. Louis, MO, USA). Anti-GAPDH (Cat#KM9002T), anti-β-actin (Cat#KM9001T) and anti-Tubulin (Cat#KM9007T) antibodies were purchased from Sungene Biotech (Tianjin, China).

2.3. Cell culture and lentivirus infection

Human gastric cancer cell lines (AGS, BGC823, MGC803, MKN45, SGC7901 and KATO III), human gastric epithelium cell

line GES-1 and human normal embryonic kidney cell line HEK293T were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, NY, USA), penicillin (100 U/mL) and streptomycin (100 ng/mL) (Sangon Biotech, Shanghai, China) in a humidified incubator at 37 °C with 5% CO_2. Lentivirus was packaged in HEK293T cells and collected from the medium supernatant. Stable cell lines were established by infecting lentivirus into cells, followed by puromycin or G418 selection (Sigma−Aldrich, St. Louis, MO, USA).

2.4. Cell proliferation assay

Cells were firstly seeded into a 96-well plate at a density of 3000 cells/well, and incubated for different times (0, 12, 24, 48, 72 and 96 h). Then methyl thiazolyl tetrazolium was added to each well at a final concentration of 0.5 mg/mL. After incubation for 4 h, formazan crystals were dissolved in 100 μL of DMSO, and the absorbance at 570 nm was measured by a plate reader (Multiskan Sky, Thermo Scientific, 51119670).

2.5. Soft-agar colony formation assay

Cells with a density of 5×10^3 cells/well were resuspended in DMEM containing 0.3% low-melting agarose and 10% FBS and seeded onto a coating of 0.5% low-melting agarose in DMEM containing 10% FBS into 6-well plate. After cultured for 3 weeks, cell colonies were analyzed microscopically (Olympus, CKX53).

2.6. Wound healing assay

Cells with a density of 5×10^5 cells/well were seeded into a 6-well plate. Till the cells reached 90% confluence, the cell monolayer was wounded using a sterilized 10 μL pipette tip and washed with PBS for two times. Cells were allowed to migrate for 24 and 48 h in serum-free medium, and the wounds were observed and captured. The gap lengths were measured from the photomicrographs.

2.7. Transwell assay

The assay was performed in an invasion chamber consisting of a 24-well tissue culture plate with 12 cell culture inserts (Corning). Cells with a density of 2×10^4 cells/well in serum-free culture medium were added to the inserts, and each insert was placed in the lower chamber containing 10% FBS culture medium. After being cultured for 24 h, cells on the upper surface of the membrane were wiped off, and cells that invaded the bottom surface were photographed and counted.

2.8. Phalloidin staining assay

Cells were seeded on glass coverslips for 24 h and then fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. The coverslips were incubated in the dark with 100 nmol/L rhodamine-phalloidin at room temperature for 30 min. Nuclei were stained with 10 μg/mL DAPI. The coverslips were rinsed in PBS and inverted on a drop of anti-fade mounting medium on a glass slide. Then, these slides were sealed with neutral balsam and viewed under the confocal microscope (ZEISS LSM880, ZENblue).

2.9. Western blot assay

Proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% BSA and incubated with the indicated primary antibodies. Corresponding horseradish peroxidase-conjugated secondary antibodies were used against each primary antibody. Signals were detected with the ChemiDoc XRS chemiluminescent gel imaging system (Analytik Jena).

2.10. GST pull-down and co-immunoprecipitation assay

Cells were lysed in lysis buffer (1% Nonidet P-40, 150 mmol/L NaCl, 100 mmol/L HEPES, 5 mmol/L $Na_4P_2O_7$, 5 mmol/L NaF, 2 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonylfluoride, 10 mg/L aprotinin, 10 mg/L leupeptin). Cleared cell lysates were incubated with glutathione or Protein G conjugated sepharose (GE Healthcare) and the appropriate antibodies for 2 h at 4 °C. Then, the sepharose was washed three times with lysis buffer and boiled in 6 × SDS sample buffer for Western blot analysis.

2.11. Immunohistochemistry assay

Tissues were paraffin-embedded, sectioned and stained with antibodies using a microwave-enhanced avidin-biotin staining method as previously described[29]. For quantitation of the protein expression, the following formula was used: Immunohistochemistry score = % positive cells × intensity score. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, intense. An immunohistochemistry score of 100 or greater was considered as positive.

2.12. GLUL activity assay

GLUL activity was measured using Micro Glutamine Synthetase Assay Kit (Solarbio, Beijing, China). Briefly, cells were harvested and washed with PBS for two times, and then extracted with the extraction buffer. The mixture was sonicated for 20 s and centrifuged at 8000 × *g* at 4 °C for 10 min. The supernatant was collected, and the absorbance at 520 nm was read for the calculation of GLUL activity (Multiskan Sky, Thermo Scientific, 51119670).

2.13. Measurements of intracellular glutamine levels

Measurements of intracellular glutamine levels were performed with Glutamine measurement kit (COIBO BIO, Shanghai, China). Briefly, cells were harvested and washed with PBS for two times, repeated freeze−thaw cycles. The cells were damaged and intracellular components were released. After centrifugation for 20 min at the speed of 2000−3000 rpm (5420, Eppendorf, Oldenburg, Germany), the supernatant were collected. Glutamine levels were then measured by reading ELISA plate at OD450 nm (Multiskan Sky, Thermo Scientific, 51119670).

2.14. Dual luciferase reporter assay

The TOPflash and FOPflash plasmids were transiently transfected using Lipofectamine into cells along with *Renilla*-TK luciferase vector (pRL-TKRenilla, Promega) as the internal control. Meanwhile, Flag-β-Catenin or GST-GLUL were co-transfected into cells. Cells were harvested after 48 h, and the luciferase activity

was determined by the dual-luciferase assay system (Cat. E1910, Promega) according to the manufacturer's protocol. The luciferase activity was normalized to the *Renilla* luciferase activity, and the β-Catenin transcription activity was measured as *TOP*/*FOP* ratio (SkanIt Software 6.1, Thermo Scientific). All experiments were performed three times.

2.15. Nude mice tumorigenesis and lung metastasis assay

The female BALB/c nude mice (5 weeks old, 18.0−20.0 g, purchased from The Shanghai SLAC Laboratory Animal Co.) were randomly divided into indicated groups. The mice were subcutaneously injected with the indicated cells (5×10^6 cells in a volume of 100 μL DMEM, $n = 7$ mice), or injected through lateral tail vein (2×10^6 cells in a volume of 100 μL PBS, $n = 5$ mice). Tumors and lungs were weighed after mice were anesthetized. Hematoxylin and eosin staining were used to identify cancer tissue and normal tissue. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Jinan University.

2.16. Mass spectrometry analyses

For mass spectrometry analysis, anti-Flag immunoprecipitations were performed with the WCL derived from three 10 cm dishes of HEK293T cells transfected with Flag-GLUL. The proteins were resolved by SDS-PAGE, and identified by Coomassie staining. The band containing GLUL was reduced with 10 mmol/L DTT for 30 min, alkylated with 55 mmol/L iodoacetamide for 45 min, and in-gel-digested with trypsin enzymes. The tryptically digested peptides were desalted with monospin C18 column (SHIMADZU-GL), and then analyzed on an Easy-nLC1200 system equipped with a homemade reverse phase C18 column (75 μm × 300 mm, 1.9 μm). The peptides were separated with a 120 min gradient from 5% to 100% of buffer B (buffer A: 0.1% formic acid in water; buffer B: 0.1% formic acid in 80% acetonitrile) at 300 nL/min. The eluted peptides were ionized and directly introduced into a Q-Exactive mass spectrometer (ThermoScientific, San Jose, CA, USA) using a nano-spray source with the application of a distal −2.5 kV spray voltage. A cycle of one full-scan MS spectrum (m/z 300−1500) was acquired followed by top 20 MS/MS events, sequentially generated on the first to the twentieth most intense ions selected from the full MS spectrum at a 30% normalized collision energy.

The acquired MS/MS data were analyzed against a homemade database (including all target proteins) using PEAKS Studio 8.5. Cysteine alkylation by iodoacetamide was specified as fixed modification with mass shift 57.02146 and methionine oxidation, protein N-terminal acetylation as variable. Additionally, phosphorylation was set as dynamic modification with mass shift 79.9663. In order to accurately estimate peptide probabilities and false discovery rates, we used a decoy database containing the reversed sequences of all the proteins appended to the target database.

2.17. Statistical analysis

Comparisons between the two groups were performed using Student's t-test. Overall survival and disease-free survival curves were obtained by the Kaplan−Meier method, and differences were compared by the log-rank test. The Chi-Square test was calculated to show the correlation. P-value of less than 0.05 was considered statistically significance.

3. Results

3.1. GLUL is downregulated and correlated with poor prognosis in gastric cancer

To investigate the protein expressions of GLUL in gastric cancer, we first detected the protein expression of GLUL in human gastric cancer cell lines and tissues by Western blot. As shown in Fig. 1A, the protein expressions of GLUL in all human gastric cancer cells (AGS, BGC823, MGC803, MKN45, SGC7901 and KATOIII) were lower than that of normal gastric epithelium cells (GES-1). Moreover, the protein expressions of GLUL in most human gastric tumor tissues (T) were lower than those of paired adjacent normal tissues (N) (Fig. 1B and C). Furthermore, the immunohistochemistry results of tissue arrays showed that the protein expressions of GLUL in human gastric tumor tissues (T) were also lower than those of paired adjacent normal tissues (N), and negative GLUL expression was present in 65 (67.01%) out of 97 tumor tissues (Fig. 1D). In addition, GLUL expression was associated with N stage and TNM stage but not with age, gender, Lauren type, T stage, lymph node metastasis, differentiation, tumor diameter longitudinal and vessel carcinoma embolus (Supporting Information Table S1). Kaplan−Meier analysis revealed that patients with low GLUL expression showed poor overall survival and disease-free survival (Fig. 1E and F). These results suggest that GLUL is downregulated in gastric cancer and correlated with poor prognosis.

3.2. GLUL inhibits the proliferation, migration, invasion and metastasis of gastric cancer

To explore the effect of GLUL on gastric cancer, we first selected two cell lines (AGS and MKN45) with relatively high GLUL expression to generate stable knockdown of endogenous GLUL, and then these cells were re-expressed exogenous GLUL (Fig. 2A and C). The MTT and soft-agar colony formation results showed that GLUL knockdown enhanced cell proliferation, cell sphere numbers and sizes, and GLUL re-expression weakened cell proliferation, cell sphere numbers and sizes (Fig. 2B and D−F). Moreover, the subcutaneous tumor models in nude mice revealed that GLUL knockdown promoted tumor growth, and GLUL re-expression inhibited tumor growth (Fig. 2G and H). The further wound healing and trans-well results presented that GLUL knockdown accelerated cell migration and invasion, and GLUL re-expression suppressed cell migration and invasion (Fig. 3A−E). The phalloidin staining results exhibited that GLUL knockdown promoted the formation of pseudopodia-like membrane protrusions which indicates an epithelial-mesenchymal transition (EMT) phenotype, and GLUL re-expression blocked the formation of pseudopodia-like membrane protrusions (Supporting information Fig. S1A). The results of Western blot displayed that GLUL knockdown upregulated the expression of EMT biomarkers such as β-Catenin, as well as the downstream genes cyclin D1 and c-Myc, and *vice versa* (Fig. S1B). In addition, GLUL decreased the β-Catenin transcription activity (Fig. S1C), which means GLUL may partially participate in Wnt signal transduction. Furthermore, the model of lung metastasis through tail vein injection in nude mice demonstrated that GLUL knockdown increased lung weights and the numbers of lung metastatic tumors, and GLUL re-expression decreased lung weights and the numbers of lung metastatic tumors (Fig. 3F−I). Hence, these

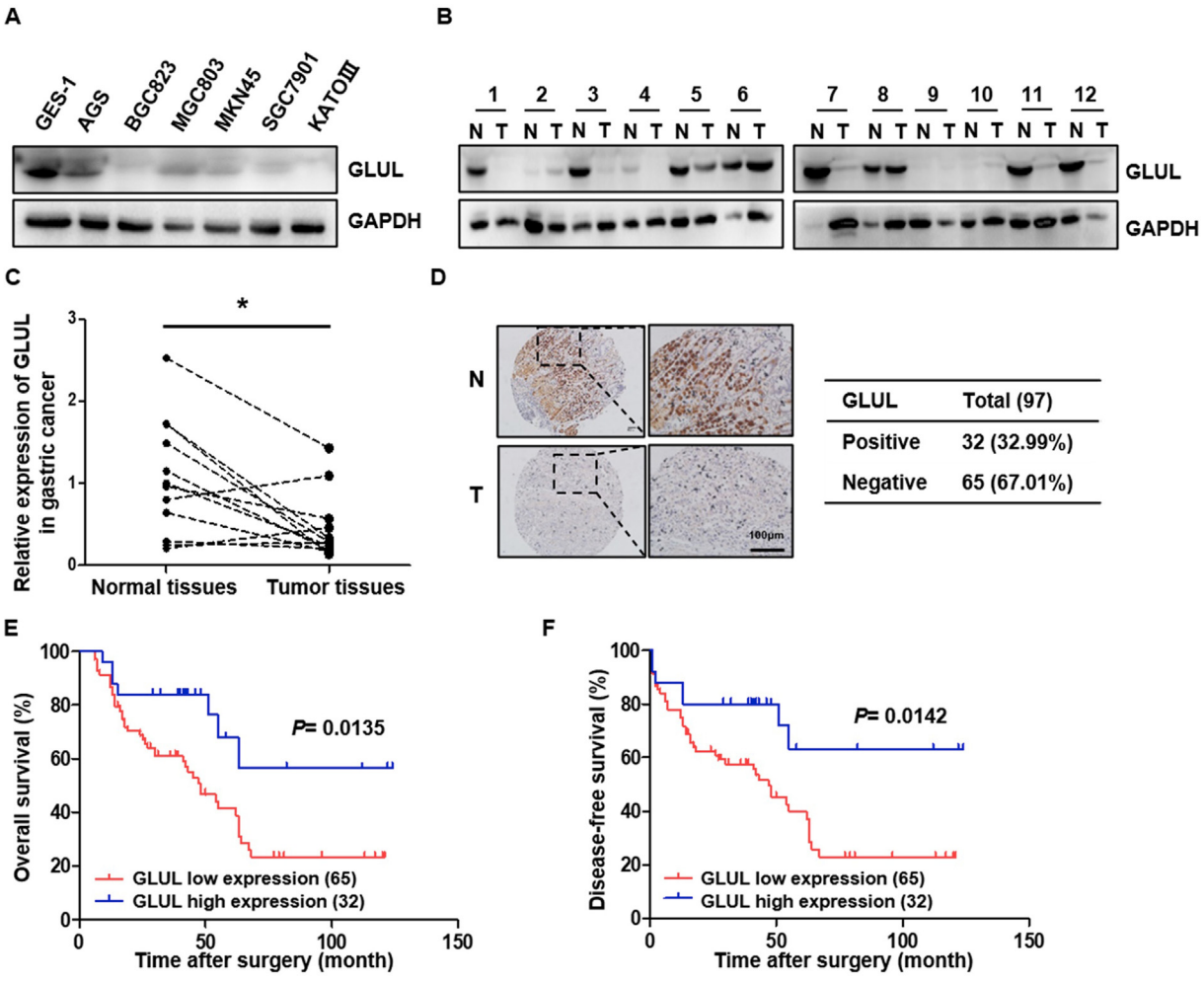

Figure 1 GLUL is downregulated and correlated with poor prognosis in gastric cancer. (A) Western blot analysis of GLUL expression in the indicated cells. (B) Western blot analysis of GLUL expression in 12 pairs of human adjacent normal tissues (N) and gastric cancer (T) tissues. (C) The quantification of the protein expressions of GLUL relative to GAPDH (B) is shown. (D) GLUL expression in 97 pairs of human adjacent normal tissues (N) and gastric cancer (T) tissues were examined with immunohistochemistry assay. (E)−(F) Kaplan−Meier analysis of overall and disease-free survival rates of 97 gastric cancer patients based on their expression of GLUL. *$P < 0.05$.

findings together suggest that GLUL inhibits the growth and metastasis of gastric cancer *in vitro* and *in vivo*.

3.3. *Inhibition of the proliferation and migration of gastric cancer is independent of GLUL enzyme activity*

To examine whether the above effects of GLUL on gastric cancer are dependent on its enzyme activity, we first detected the cell proliferation level under the conditions with or without glutamine, and found that the AGS and MKN45 cells could still grow but just slow down in the absence of glutamate (Supporting information Fig. S2A and S2B). Then we treated AGS and MKN45 cells with l-methioninesulfoximine (MSO), a specificity enzyme inhibitor of GLUL. Results showed that MSO indeed decreased the GLUL activity and intracellular glutamine levels (Figs. S2C and S2E), but did not affect the proliferation and migration of AGS and MKN45 cells (Fig. 4A and C). Furthermore, MSO also did not cause changes in the β-Catenin and vimentin expression (Fig. 4B). In addition, GLUL inactive mutant R324C re-expression inhibited the proliferation and migration of AGS/shGLUL and MKN45/shGLUL cells as GLUL-WT re-expression did (Figs. 4D−G, S2D and S2F). These results suggest that inhibition of the proliferation and migration of gastric cancer is independent of GLUL enzyme activity.

3.4. *GLUL competes with* β*-Catenin to bind to N-Cadherin*

To investigate the mechanism of GLUL inhibiting the proliferation, migration, invasion and metastasis of gastric cancer, we performed mass spectrometry analysis of GLUL immunoprecipitation complex and found that N-Cadherin was a potential binding protein of GLUL (Supporting information Fig. S3A). To validate the binding of GLUL and N-Cadherin, we co-transfected GST or GST-GLUL and Venus-N-Cadherin into HEK293T cells and performed GST pull-down assay. As shown in Fig. 5A, Venus-N-Cadherin was only detectable in the GST-GLUL complex but not in the GST complex. We also reversed the fusions and co-transfected GST or GST-N-Cadherin and Venus-GLUL into HEK293T cells. Venus-GLUL was only detectable in the GST-N-Cadherin complex, but not in the GST complex (Fig. 5B). Moreover, endogenous GLUL and N-Cadherin were separately immunoprecipitated from AGS cells, and reciprocal protein

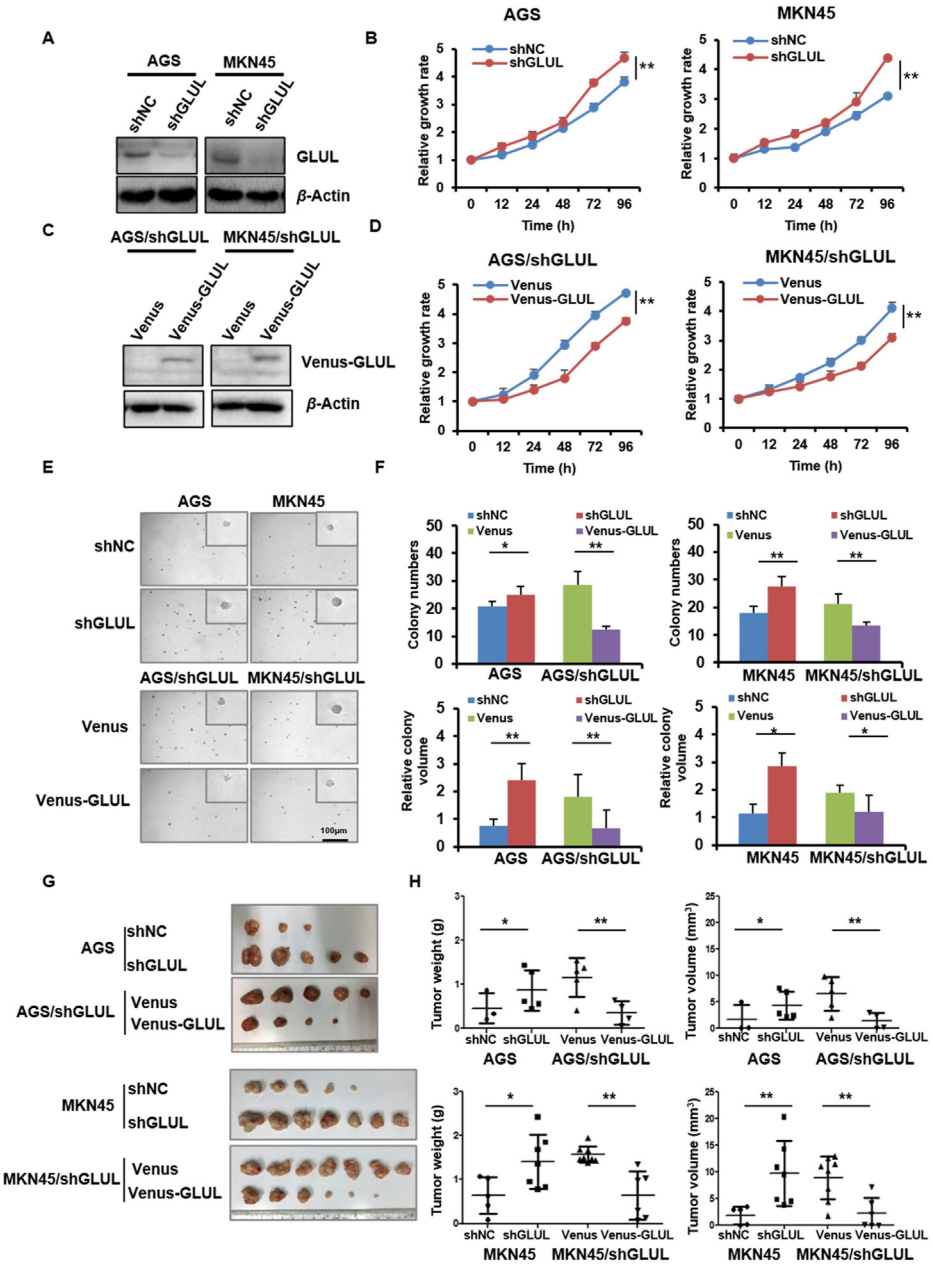

Figure 2 GLUL inhibits gastric cancer cell proliferation *in vitro* and *in vivo*. (A, C) Western blot analysis of GLUL expression in the indicated cells. (B, D) Cell proliferation of indicated cells were determined with MTT assay. Data are presented as mean ± SD. (E) The representative images and (F) quantification of the indicated cells colony numbers and colony volume were determined with soft-agar formation assay. (G) The indicated subcutaneous tumors and (H) quantification of tumor weight and volume are shown. $n = 7$ mice. $^{*}P < 0.05$ and $^{**}P < 0.01$.

detection was performed. Both GLUL and N-Cadherin were detectable in their immunoprecipitated complexes, but not in the control immunoglobulin G complexes (Fig. 5C and D).

To determine the binding specificity and explore which regions in N-Cadherin mediate the binding of GLUL and N-Cadherin, we overexpressed GST, GST-N-Cadherin truncations 1−123, 124−710, 711−906, full-length and Venus-GLUL in HEK293T cells and performed GST pull-down assay. Venus-GLUL was detectable in the complexes for GST-N-Cadherin truncations 124−710, 711−906 and full-length but not in the GST-N-Cadherin truncation 1−123 and GST complexes (Fig. 5E and G). This result indicates that the regions of N-Cadherin responsible for its binding with GLUL are located in both N-Cadherin truncations 124−710 and 711−906. To map the regions of GLUL that bind with N-Cadherin, we generated GLUL truncations 1−109, 110−373 and examined their interaction with N-Cadherin by detecting Venus-N-Cadherin levels in the GST-GLUL truncation complexes from overexpressing lysates. Venus-N-Cadherin was detectable only in the complexes for GST-GLUL full-length but not in the truncations 1−109, 110−373 and GST complexes (Fig. 5F and H). This data suggests that the regions of GLUL responsible for its interaction with N-Cadherin are located in both GLUL truncations 1−109 and 110−373.

Due to the degradation of N-Cadherin is sensitive to extracellular calcium[30], whether the interaction with GLUL renders

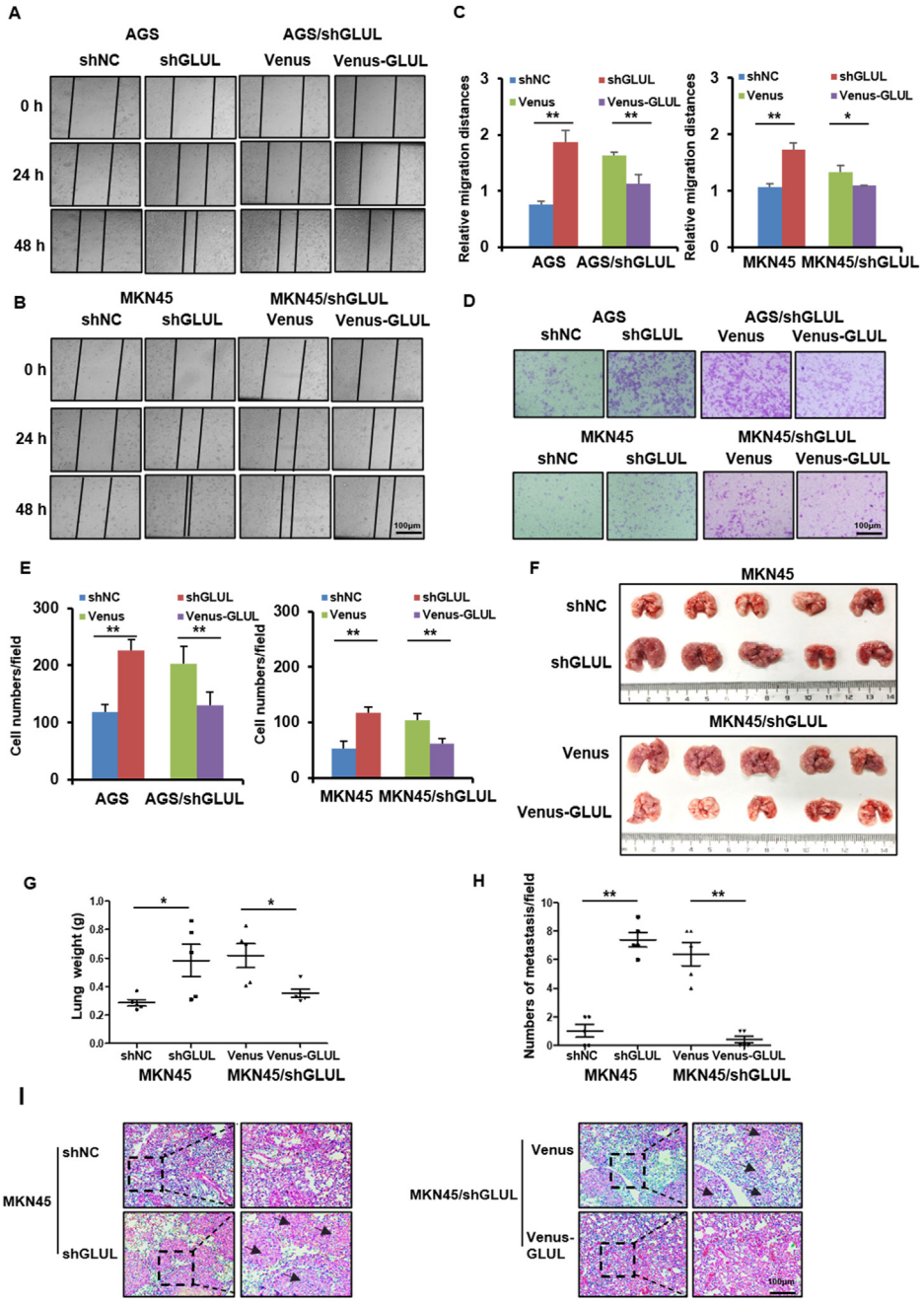

Figure 3 GLUL inhibits gastric cancer cell migration, invasion and metastasis *in vitro* and *in vivo*. (A, B) The representative images and (C) quantification of the indicated cells migration was determined with wound healing assay. (D) The representative images and (E) quantification of the indicated cells invasion were determined with Transwell assay. (F) The indicated images of lung, (G) quantification of lung weight and (H) numbers of lung metastasis are shown. $n = 5$ mice. (I) The representative images of lung metastasis in the indicated lung sections were determined with H&E staining. Data are presented as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$.

N-Cadherin resistant to extracellular calcium. Our results show that calcium reduced N-Cadherin expression, while the down-regulated of N-Cadherin induced by calcium was reversed by adding GLUL (Fig. S3B). In addition, calcium inhibited the interaction between GLUL and N-Cadherin (Fig. 3C). It has demonstrated that the cytoplasmic domain (truncation 711−906) of N-Cadherin mediated its binding with β-Catenin[31]. To explore whether GLUL and β-Catenin competitively bind to N-Cadherin because both can bind with the cytoplasmic domain of N-Cadherin, we co-transfected Flag-β-Catenin, GST-N-Cadherin and Venus-GLUL into HEK293T cells and performed a GST pull-down assay. The level of Venus-GLUL in the GST-N-Cadherin complex gradually decreased when the expression of Flag-β-Catenin was dose-dependently increased, and *vice versa* (Fig. 5I and J). Additionally, knockdown of GLUL increased the interaction between β-Catenin and N-Cadherin (Fig. S3D). These results indicate that GLUL and β-Catenin competitively bind to N-Cadherin.

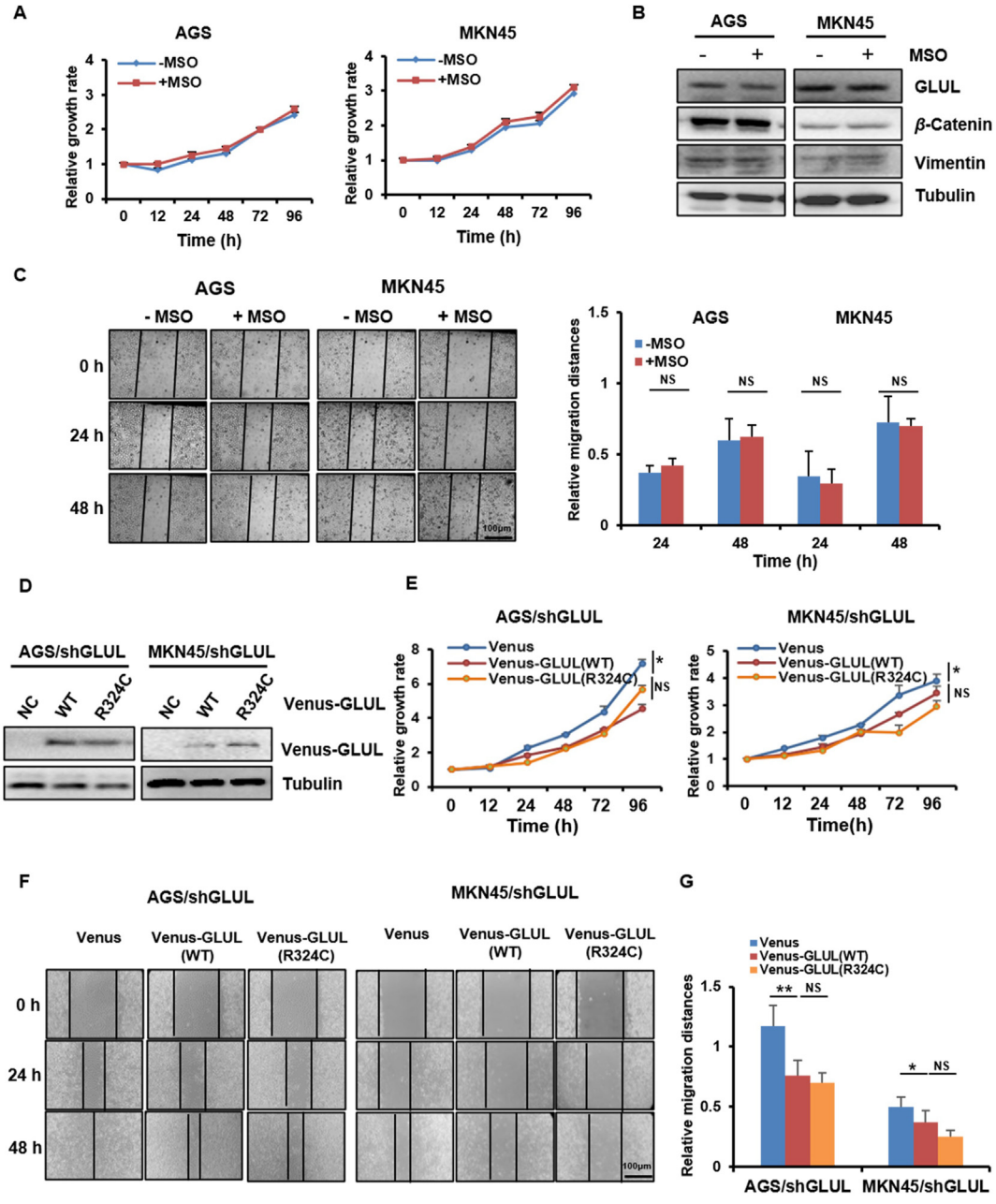

Figure 4 Inhibition of the proliferation and migration of gastric cancer is independent of GLUL enzyme activity. (A) Cell proliferations of the indicated cells treated with or without l-methionine sulfoximine (MSO, 5 mmol/L) were determined with MTT assay. Data are presented as mean ± SD. (B) Western blot analysis of the indicated protein expression in the indicated cells treated with or without MSO (5 mmol/L, 48 h). (C) The representative images and quantification of the indicated cell migration treated with or without MSO (5 mmol/L, 48 h) were determined with wound healing assay. (D) Western blot analysis of GLUL expression in the indicated cells. (E) Cell proliferations of the indicated cells were determined with MTT assay. Data are presented as mean ± SD. (F) The representative images and (G) quantification of the indicated cells migration were determined with wound healing assay. $^{*}P < 0.05$ and $^{**}P < 0.01$, and NS, no statistical significance.

3.5. GLUL affects N-Cadherin and β-Catenin stability by alerting their ubiquitination

To investigate the effect of GLUL on N-Cadherin and β-Catenin, the proteasome inhibitor MG132 was used to treat AGS and MKN45 cells with or without GLUL knockdown. As shown in Fig. 6A, MG132 could reverse the decrease of N-Cadherin and promote the increase of β-Catenin in GLUL knockdown cells. To address whether GLUL directly affects the protein stability of N-Cadherin and β-Catenin, we compared the half-life of endogenous N-Cadherin and β-Catenin proteins in AGS and MKN45 cells with or without GLUL knockdown. After treatment with the protein synthesis inhibitor cycloheximide (CHX) at the indicated time points, the half-life values of N-Cadherin and β-Catenin were shorter and longer respectively in GLUL knockdown cells than those in the scramble control cells (Fig. 6B−E). These results indicate that GLUL can

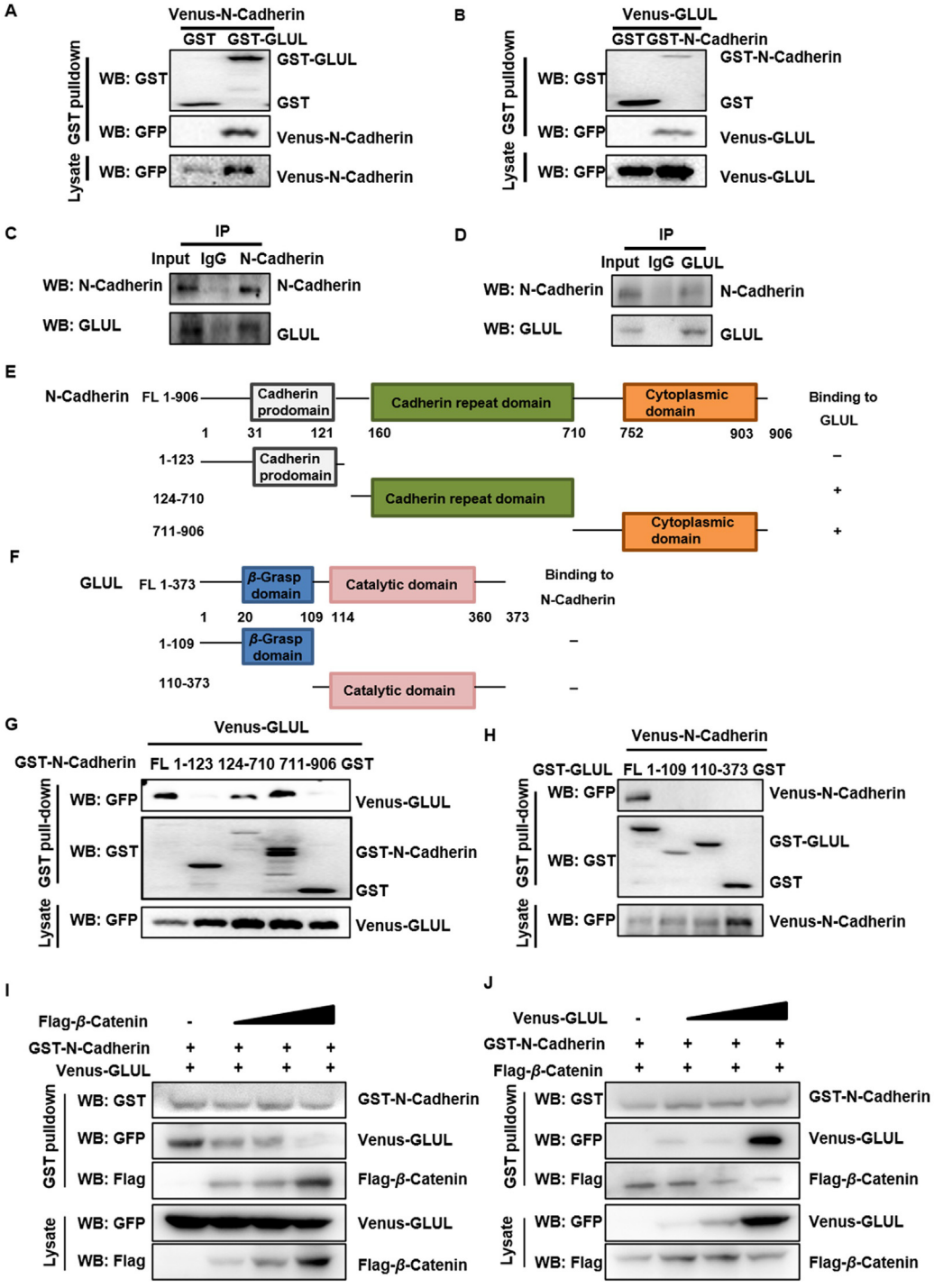

Figure 5 GLUL competes with β-Catenin to bind to N-Cadherin. (A, B) Exogenous GLUL interacts with N-Cadherin. (C, D) Endogenous GLUL interacts with N-Cadherin. Endogenous GLUL and N-Cadherin were immunoprecipitated (IP) from AGS cells and detected reciprocally. (E, F) The interacting regions of GLUL with N-Cadherin were mapped. (G) N-Cadherin truncation mutants interact with GLUL. (H) GLUL truncation mutants interact with N-Cadherin. (I, J) GLUL and β-Catenin competitively interact with N-Cadherin. HEK293T cells were co-transfected with the indicated vectors for 48 h. GST pull-down and Western blot were performed.

increase the stability of N-Cadherin and decrease the stability of β-Catenin.

To examine whether GLUL alerts the stability of N-Cadherin and β-Catenin by affecting their ubiquitination, we co-transfected HA-Ub and Venus-N-Cadherin or Flag-β-Catenin with or without GST-GLUL into HEK293T cells and performed GFP or Flag immunoprecipitation assays. The results showed that GLUL decreased N-Cadherin ubiquitylation and increased β-Catenin ubiquitylation (Fig. 6F and G). These data suggest that GLUL affects N-Cadherin and β-Catenin stability by alerting their ubiquitination.

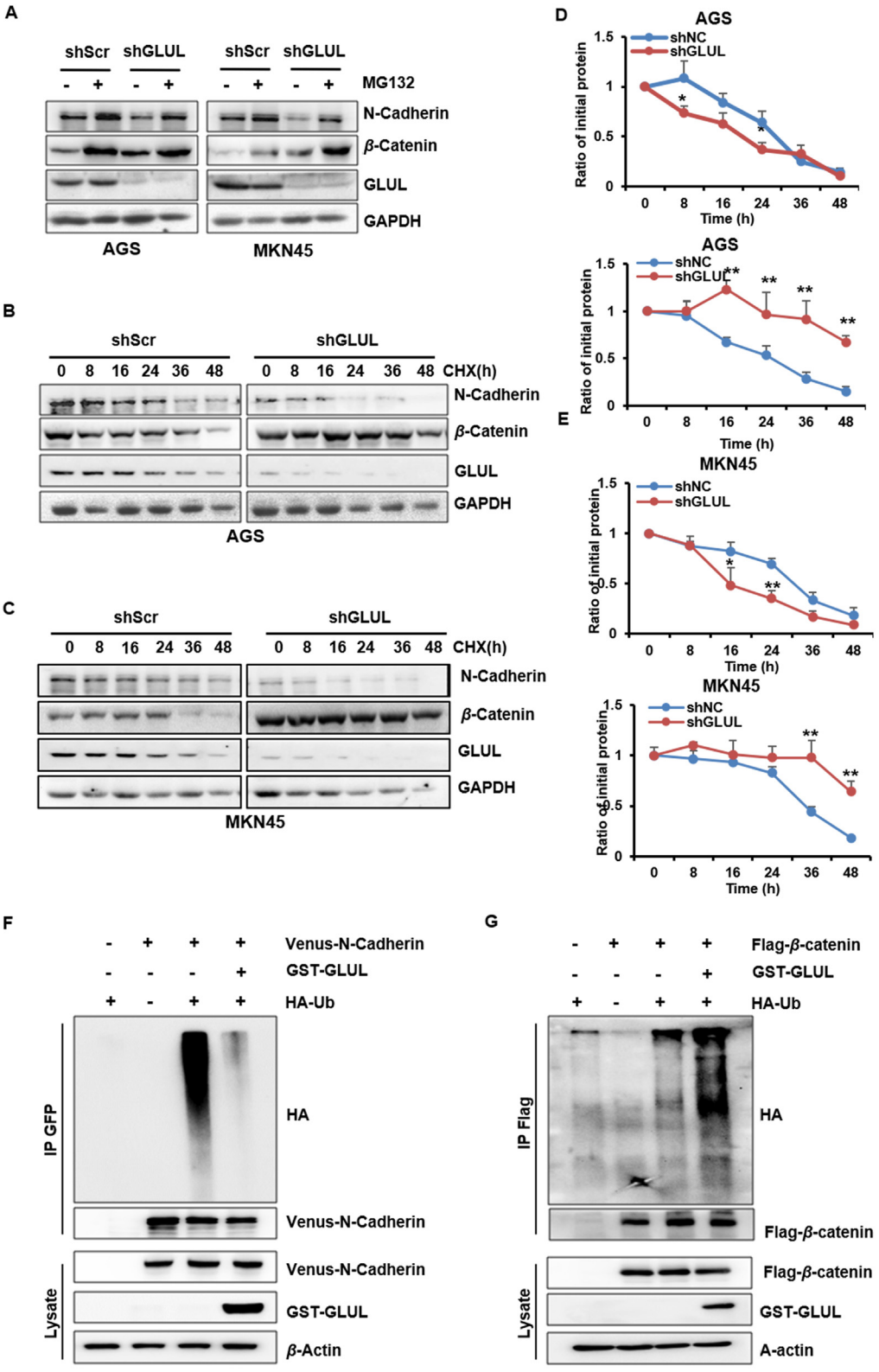

Figure 6 GLUL affects N-Cadherin and β-Catenin stability by alerting their ubiquitination. (A) Western blot analysis of indicated protein expression in the indicated cells that were treated with or without MG132 (20 μmol/L, 4 h). (B, C) The indicated cells were treated with cycloheximide (CHX, 100 μg/mL), and collected at the indicated times for Western blot. (D, E) The quantification of N-Cadherin and β-Catenin levels relative to GAPDH is shown. Data are presented as mean ± SD. (F) GLUL inhibits N-Cadherin ubiquitination. (G) GLUL promotes β-Catenin ubiquitination. HEK293T cells were co-transfected with the indicated vectors for 48 h. Immunoprecipitation with anti-GFP or anti-Flag antibodies and Western blot were performed. $^{*}P < 0.05$ and $^{**}P < 0.01$.

3.6. *GLUL protein expression is correlated with that of N-Cadherin and the independent prognostic factor in gastric cancer*

To analyze the correlation of GLUL, N-Cadherin and β-Catenin in gastric cancer, we further detected the protein expressions of N-Cadherin and β-Catenin in gastric cancer patient tumor tissues by immunohistochemistry assay. The protein expressions of N-Cadherin in human gastric cancer tissues (T) were lower than those of paired adjacent normal tissues (N), and negative N-Cadherin expression was present in 62 (63.92%) out of 97 tumor tissues (Fig. 7A). The protein expression of β-Catenin in human gastric cancer tissues (T) was higher than those of paired adjacent normal tissues (N), and positive β-Catenin staining was present in 61 (62.89%) out of 97 tumor tissues (Fig. 7B). The expression of N-Cadherin was positively correlated with the expression of GLUL and negatively correlated with the expression of β-Catenin, and there was no correlation between the expressions of GLUL and β-Catenin (Fig. 7C–E). Furthermore, statistical analysis showed that N-Cadherin expression was associated with TNM stage and tumor diameter longitudinal but not with age, gender, Lauren type, T stage, N stage, lymph node metastasis, differentiation and vessel carcinoma embolus (Table S1). β-Catenin expression was only associated with differentiation but not with age, gender, Lauren type, T stage, N stage, TNM stage, lymph node metastasis, tumor diameter longitudinal and vessel carcinoma embolus (Table S1). Kaplan–Meier analysis revealed that there were no significant differences between N-Cadherin or β-Catenin expression and patient overall survival or disease-free survival (Supporting information Fig. S4). Additionally, univariate Cox regression analysis indicated that GLUL expression, T stage, N stage, TNM stage and lymph node metastasis were the independent prognostic factors for overall survival (Table S2). Collectively, these data suggest that GLUL protein expression is correlated with that of N-Cadherin and the independent prognostic factor in gastric cancer.

4. Discussion

In the current study, we have found that the expression level of GLUL is significantly lower in gastric cancer tissues compared with adjacent normal tissues, and correlated with N stage and

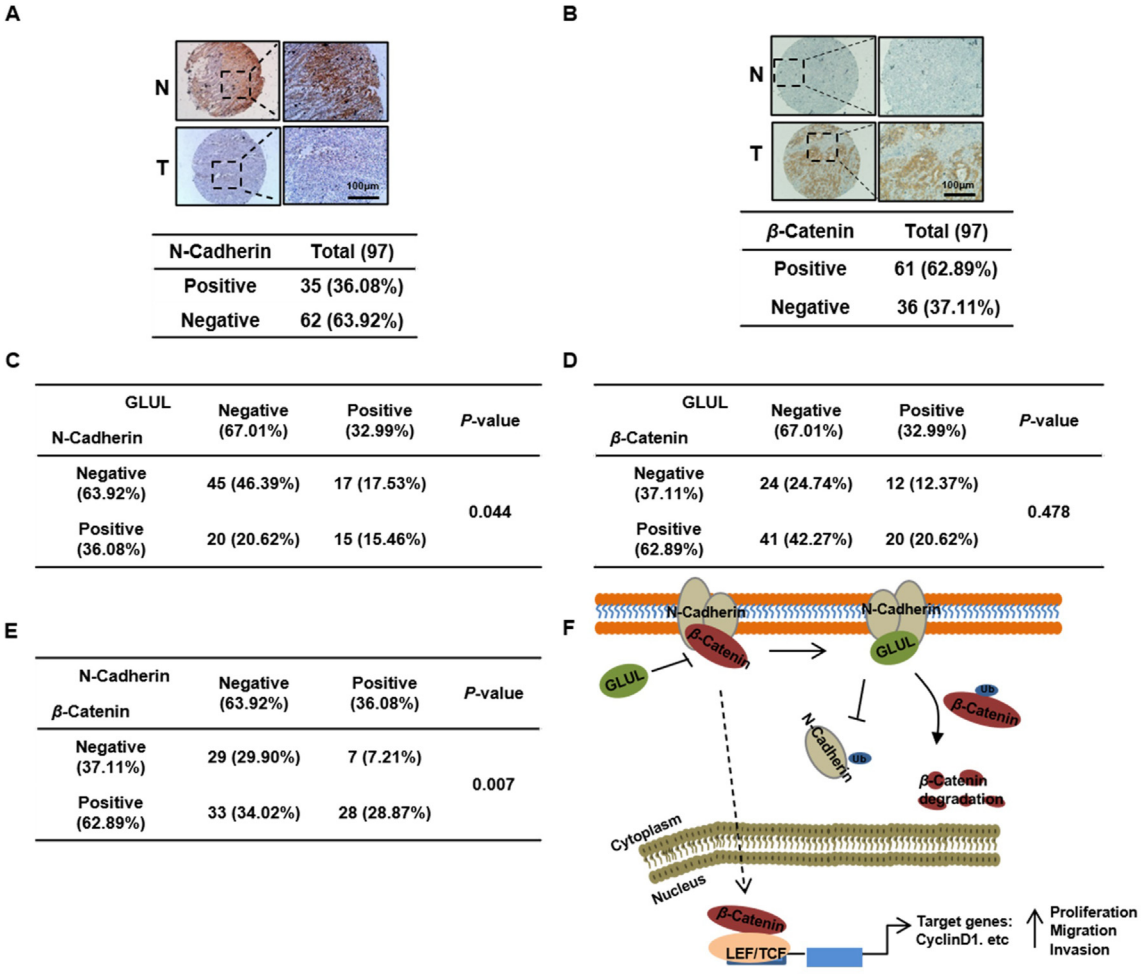

Figure 7 GLUL protein expression is correlated with that of N-Cadherin and the independent prognostic factor in gastric cancer. (A, B) N-Cadherin and β-Catenin expressions in 97 pairs of human adjacent normal tissues (N) and gastric cancer (T) tissues were examined with immunohistochemistry assay. (C)–(E) Correlation among GLUL, N-Cadherin and β-Catenin protein levels in human gastric cancer tissues. (F) Schematic of GLUL inhibiting gastric cancer progress.

TNM stage. Kaplan−Meier analysis indicates that low GLUL expression predicts poor survival for gastric cancer patients. Recent studies have revealed that the expression of GLUL is regulated both at the mRNA and protein levels. For instance, Yes-associated protein 1 elevates GLUL expression by directly binding to GLUL promoter, and knockdown of YAP1 reduces GLUL expression and inhibits liver cancer cell growth[25]. Besides, GATA binding protein 3 can upregulate GLUL expression through direct binding to GLUL promoter and promote the synthesis of glutamine for the growth of breast cancer cells[18]. The forkhead box O also transcriptionally enhances GLUL expression and glutamine production, resulting in the inhibition of mTOR and increased autophagy[22]. Moreover, thymine-DNA glycosylase causes the GLUL promoter to enhance GLUL expression, and c-Myc indirectly upregulates GLUL transcription by binding to the E boxes of TDG to increase TDG expression[26]. Additionally, GLUL protein is acetylated by histone acetyltransferases p300/CBP, and acetylated GLUL is ubiquitinated by E3 ubiquitin ligase cereblon to degrade in the proteasome. This degradation of GLUL is promoted by valosin-containing protein p97[32]. Furthermore, ubiquitin-specific peptidase 15 can antagonize cereblon-mediated ubiquitylation of GLUL to inhibit proteasomal degradation[33]. Another E3 ubiquitin ligase zinc and ring finger 1 can also ubiquitinate GLUL to facilitate proteasomal degradation[34]. However, the reasons for the low GLUL expression in gastric cancer, possibly regulated by the above factors, need to be investigated in the future.

Apart from its canonical glutamine-synthesis function, GLUL harbors non-canonical functions by palmitoylation ras homolog family member J to maintain its membrane localization and activation[35]. GLUL abolishment resulted in a gain of function phenotype with induced drug resistance in lung cancer, in which GLUL ablation suppressed cell apoptosis and promoted cell colony formation[36]. Additionally, MSO alone barely affected cell proliferation, colony formation, as well as xenograft tumor growth[7]. And combination treatment with MSO and BenSer, an inhibitor of glutamine transporter-ASCT2, showed potent antitumor activity in gastric cancer[7]. Similarly, our data presented that GLUL harbored another non-canonical function. Our findings have shown that knockdown of GLUL promotes the growth, migration, invasion and metastasis of gastric cancer cells *in vitro* and *in vivo*, and *vice versa*, which is independent of its enzyme activity.

We have further found that GLUL competes with β-Catenin to bind to N-Cadherin, increases the stability of N-Cadherin and decreases the stability of β-Catenin by alerting their ubiquitination. Moreover, our results have presented that there are lower N-cadherin and higher β-Catenin expression levels in gastric cancer tissues compared with adjacent normal tissues. GLUL protein expression is correlated with that of N-Cadherin and is the independent prognostic factor in gastric cancer. The complexes of N-Cadherin and β-Catenin play a key role in mediating cell adhesion that is involved in the regulation of migration, invasion and metastasis[37]. β-Catenin proteins are controlled by the multiprotein destruction complex which includes adenomatous polyposis coli, Axin and the kinases glycogen synthase kinase 3 and casein kinase 1[38]. β-Catenin is recruited into this multiprotein destruction complex through binding APC or Axin and sequentially phosphorylated by CK1 and GSK3β, which generates a binding site for the E3 ubiquitin ligase β-transducin repeat-containing protein and β-Catenin is ubiquitinated and destroyed[38,39]. N-Cadherin can stabilize β-Catenin by competing with APC or Axin to bind to β-Catenin[38,40], therefore the binding of GLUL to N-Cadherin not only stabilizes N-Cadherin but also releases β-Catenin for degradation. However, since GLUL is not an E3 ubiquitin ligase, it cannot directly mediate the ubiquitination and degradation of N-cadherin. Although E3 ubiquitin ligase Fbxo45 blocks calcium-sensitive proteolysis of N-cadherin[30], the ubiquitination and degradation of N-cadherin need to be further investigated.

The relationship between GLUL and β-Catenin has been well-studied in liver cancer. β-Catenin is a GLUL upstream regulator, and the promoter of GLUL can be activated by β-Catenin[41]. Therefore, the expression of GLUL is increased by activation of β-Catenin, and the expressions of GLUL and β-Catenin show a positive correlation in human liver cancer tissues[41]. Moreover, activating β-Catenin mutations are usually found in GLUL-positive liver cancer tissues[42], cause metabolic changes and autophagy to alert the sorafenib sensitivity of liver cancer cells[43], and activate the mechanistic target of rapamycin complex 1 to promote liver cancer cell proliferation by upregulating GLUL expression[44]. In this study, we have demonstrated that GLUL is also a β-Catenin upstream regulator, and GLUL can decrease the expression and stability of β-Catenin protein by affecting its ubiquitination in gastric cancer, suggesting that there is a regulation feedback loop between GLUL and β-Catenin.

5. Conclusions

Our study has demonstrated that GLUL inhibits the proliferation, migration, invasion and metastasis of gastric cancer, which is independent of its enzyme activity. GLUL competes with β-Catenin to bind to N-Cadherin, affecting the stability of N-Cadherin and β-Catenin by alerting their ubiquitination (Fig. 7F). Moreover, GLUL protein expression is correlated with that of N-Cadherin and the independent prognostic factor in gastric cancer. These findings reveal that GLUL stabilizes N-Cadherin by antagonizing β-Catenin to inhibit the progress of gastric cancer.

Acknowledgments

This work was supported by funds from the National Key Research and Development Program of China No. 2017YFA0505104 (Zhi Shi), the National Natural Science Foundation of China Nos. 81772540 and 82272996 (Zhi Shi), the Science and Technology Program of Guangdong No. 2019A050510023 (Zhi Shi, China) and the Science and Technology Program of Guangzhou No. 20220-6010081 (Zhi Shi, China).

Author contributions

Qiwei Jiang, Xingyuan Shi, Yang Yang, Zihao Xing, Zhenjie He and Shengte Wang performed the experiments and prepared the manuscript. Yong Li, Songwang Cai, Yubin Su, Meiwan Chen and Zhesheng Chen provided some material support. Qiwei Jiang, Yong Li, Songwang Cai, Xingyuan Shi and Zhi Shi conceived the conception of the study and designed the experiments. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2023.11.008.

References

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. *CA Cancer J Clin* 2021;**71**:7−33.
2. Liu Y, Song Z, Liu Y, Ma X, Wang W, Ke Y, et al. Identification of ferroptosis as a novel mechanism for antitumor activity of natural product derivative a2 in gastric cancer. *Acta Pharm Sin B* 2021;**11**:1513−25.
3. Safari M, Mahjub H, Esmaeili H, Abasi M, Roshanaei G. Identifying factors affecting cancer-related death in patients with adenocarcinoma gastric cancer: an analysis in the presence of competing risks. *Int J Cancer Manag* 2021;**14**:e103402.
4. Guan XM. Cancer metastases: challenges and opportunities. *Acta Pharm Sin B* 2015;**5**:402−18.
5. Fares J, Fares MY, Khachfe HH, Salhab HA, Fares Y. Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduct Target Ther* 2020;**5**:28.
6. Meirson T, Gil-Henn H, Samson AO. Invasion and metastasis: the elusive hallmark of cancer. *Oncogene* 2020;**39**:2024−6.
7. Ye J, Huang Q, Xu J, Huang J, Wang J, Zhong W, et al. Targeting of glutamine transporter ASCT2 and glutamine synthetase suppresses gastric cancer cell growth. *J Cancer Res Clin Oncol* 2018;**144**:821−33.
8. Frieg B, Gorg B, Gohlke H, Haussinger D. Glutamine synthetase as a central element in hepatic glutamine and ammonia metabolism: novel aspects. *Biol Chem* 2021;**402**:1063−72.
9. Eisenberg D, Gill HS, Pfluegl GMU, Rotstein SH. Structure−function relationships of glutamine synthetases. *Biochim Biophys Acta* 2000;**1477**:122−45.
10. Zhou Y, Eid T, Hassel B, Danbolt NC. Novel aspects of glutamine synthetase in ammonia homeostasis. *Neurochem Int* 2020;**140**:104809.
11. Sandhu MRS, Gruenbaum BF, Gruenbaum SE, Dhaher R, Deshpande K, Funaro MC, et al. Astroglial glutamine synthetase and the pathogenesis of mesial temporal lobe epilepsy. *Front Neurol* 2021;**12**:665334.
12. Kim GW, Lee DH, Jeon YH, Yoo J, Kim SY, Lee SW, et al. Glutamine synthetase as a therapeutic target for cancer treatment. *Int J Mol Sci* 2021;**22**:1701.
13. Liu P, Zheng S, Xu X. Glutamine synthetase promotes hepatocellular carcinoma invasiveness through epithelial−mesenchymal transition. *Transplantation* 2019;**103**:327−8.
14. Long J, Wang HG, Lang ZW, Wang TL, Long M, Wang BE. Expression level of glutamine synthetase is increased in hepatocellular carcinoma and liver tissue with cirrhosis and chronic hepatitis B. *Hepatol Int* 2011;**5**:698−706.
15. Yuneva MO, Fan TWM, Allen TD, Higashi RM, Ferraris DV, Tsukamoto T, et al. The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type. *Cell Metab* 2012;**15**:157−70.
16. Brunelli L, Caiola E, Marabese M, Broggini M, Pastorelli R. Capturing the metabolomic diversity of KRAS mutants in non-small-cell lung cancer cells. *Oncotarget* 2014;**5**:4722−31.
17. Wang YY, Fan SH, Lu J, Zhang ZF, Wu DM, Wu ZY, et al. GLUL promotes cell proliferation in breast cancer. *J Cell Biochem* 2017;**118**:2018−25.
18. Kung HN, Marks JR, Chi JT. Glutamine synthetase is a genetic determinant of cell type-specific glutamine independence in breast epithelia. *PLoS Genet* 2011;**7**:e1002229.
19. Choi JH, Lim KH, Park E, Kim JY, Choi YK, Baek KH. Glutamate-ammonia ligase and reduction of G0 population in PANC-1 cells. *J Cell Biochem* 2013;**114**:303−13.
20. Audard V, Cavard C, Richa H, Infante M, Couvelard A, Sauvanet A, et al. Impaired E-cadherin expression and glutamine synthetase overexpression in solid pseudopapillary neoplasm of the pancreas. *Pancreas* 2008;**36**:80−3.
21. Yang LF, Achreja A, Yeung TL, Mangala LS, Jiang DH, Han C, et al. Targeting stromal glutamine synthetase in tumors disrupts tumor microenvironment-regulated cancer cell growth. *Cell Metab* 2016;**24**:685−700.
22. van der Vos KE, Eliasson P, Proikas-Cezanne T, Vervoort SJ, van Boxtel R, Putker M, et al. Modulation of glutamine metabolism by the PI(3)K-PKB-FOXO network regulates autophagy. *Nat Cell Biol* 2012;**14**:829−37.
23. Zou J, Wang YX, Mu HJ, Xiang J, Wu W, Zhang B, et al. Down-regulation of glutamine synthetase enhances migration of rat astrocytes after *in vitro* injury. *Neurochem Int* 2011;**58**:404−13.
24. Yin Y, Sun WF, Xiang J, Deng LX, Zhang B, Xie P, et al. Glutamine synthetase functions as a negative growth regulator in glioma. *J Neurooncol* 2013;**114**:59−69.
25. Cox AG, Hwang KL, Brown KK, Evason KJ, Beltz S, Tsomides A, et al. Yap reprograms glutamine metabolism to increase nucleotide biosynthesis and enable liver growth. *Nat Cell Biol* 2016;**18**:886−96.
26. Bott AJ, Peng IC, Fan YJ, Faubert B, Zhao L, Li JY, et al. Oncogenic Myc induces expression of glutamine synthetase through promoter demethylation. *Cell Metab* 2015;**22**:1068−77.
27. Tardito S, Oudin A, Ahmed SU, Fack F, Keunen O, Zheng L, et al. Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma. *Nat Cell Biol* 2015;**17**:1556−68.
28. Zhang WJ, Yang Y, Dong ZZ, Shi Z, Zhang JT. Single-nucleotide polymorphisms in a short basic motif in the ABC transporter ABCG2 disable its trafficking out of endoplasmic reticulum and reduce cell resistance to anticancer drugs. *J Biol Chem* 2019;**294**:20222−32.
29. Zhang WJ, Li Y, Wei MN, Chen Y, Qiu JG, Jiang QW, et al. Synergistic antitumor activity of regorafenib and lapatinib in preclinical models of human colorectal cancer. *Cancer Lett* 2017;**386**:100−9.
30. Chung FZ, Sahasrabuddhe AA, Ma K, Chen X, Basrur V, Lim MS, et al. Fbxo45 inhibits calcium-sensitive proteolysis of N-cadherin and promotes neuronal differentiation. *J Biol Chem* 2014;**289**:28448−59.
31. Mariotti A, Perotti A, Sessa C, Ruegg C. N-cadherin as a therapeutic target in cancer. *Expert Opin Inv Drug* 2007;**16**:451−65.
32. Nguyen TV, Li J, Lu CC, Mamrosh JL, Lu G, Cathers BE, et al. p97/VCP promotes degradation of CRBN substrate glutamine synthetase and neosubstrates. *Proc Natl Acad Sci U S A* 2017;**114**:3565−71.
33. Van Nguyen T. USP15 antagonizes CRL4(CRBN)-mediated ubiquitylation of glutamine synthetase and neosubstrates. *Proc Natl Acad Sci U S A* 2021;**118**:e2111391118.
34. Saitoh F, Araki T. Proteasomal degradation of glutamine synthetase regulates schwann cell differentiation. *J Neurosci* 2010;**30**:1204−12.
35. Eelen G, Dubois C, Cantelmo AR, Goveia J, Bruning U, DeRan M, et al. Role of glutamine synthetase in angiogenesis beyond glutamine synthesis. *Nature* 2018;**561**:63−9.
36. Muthu M, Kumar R, Syed Khaja AS, Gilthorpe JD, Persson JL, Nordstrom A. GLUL ablation can confer drug resistance to cancer cells *via* a malate-aspartate shuttle-mediated mechanism. *Cancers* 2019;**11**:1945.
37. Miyamoto Y, Sakane F, Hashimoto K. N-Cadherin-based adherens junction regulates the maintenance, proliferation, and differentiation of neural progenitor cells during development. *Cell Adhes Migr* 2015;**9**:183−92.
38. Sferrazza G, Corti M, Brusotti G, Pierimarchi P, Temporini C, Serafino A, et al. Nature-derived compounds modulating Wnt/β-catenin pathway: a preventive and therapeutic opportunity in neoplastic diseases. *Acta Pharm Sin B* 2020;**10**:1814−34.
39. Zhang B, Ma JX. Wnt pathway antagonists and angiogenesis. *Protein Cell* 2010;**1**:898−906.

40. Tseng YS, You MY, Hsu YC, Yang KC. N-Cadherin promotes cardiac regeneration by stabilizing β-catenin. *Eur Heart J* 2019;**40**:3276.
41. Cadoret A, Ovejero C, Terris B, Souil E, Levy L, Lamers WH, et al. New targets of β-catenin signaling in the liver are involved in the glutamine metabolism. *Oncogene* 2002;**21**:8293−301.
42. Dal Bello B, Rosa L, Campanini N, Tinelli C, Viera FT, D'Ambrosio G, et al. Glutamine synthetase immunostaining correlates with pathologic features of hepatocellular carcinoma and better survival after radiofrequency thermal ablation. *Clin Cancer Res* 2010;**16**:2157−66.
43. Sohn BH, Park IY, Shin JH, Yim SY, Lee JS. Glutamine synthetase mediates sorafenib sensitivity in β-catenin-active hepatocellular carcinoma cells. *Exp Mol Med* 2018;**50**:e421.
44. Michael AOA, Ko SJ, Tao JY, Moghe A, Yang H, Xu M, et al. Inhibiting glutamine-dependent mTORC1 activation ameliorates liver cancers driven by β-catenin mutations. *Cell Metab* 2019;**29**:1135−50.