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黏液蛋白糖化酵素 GALNT2 經由降低表皮生長因子接受
器的磷酸化抑制胃癌的惡性程度

Mucin glycosylating enzyme GALNT2 suppresses the
malignancy of gastric adenocarcinoma by reducing EGFR
phosphorylation

胡婉婷

Wan-Ting Hu

指導教授：賴逸儒 博士

Advisor: I-Rue Lai, MD, Ph.D.

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口試委員會審定書

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本論文係胡婉婷君 (學號 R03446005) 在國立臺灣大學醫學院解剖學暨細胞生物學研究所完成之碩 (博) 士學位論文，於民國 105 年 07 月 08 日承下列考試委員審查通過及口試及格，特此證明

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(指導教授)

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(簽名)

致謝



碩班兩年生活轉眼間就這樣過去了，從連在基本實驗技術方面都毫無基礎到現在終於完成了碩士論文，這當中有太多太多人需要感謝。首先要特別感謝我的指導教授賴逸儒老師，總是能在我們實驗遇到瓶頸時，給予我們建議，並提供新的方向讓我們思考。在生活上則像爸爸一樣會關心我們的近況，並且時常帶著實驗室的大家去放鬆紓解壓力。也謝謝實驗室的大家，心雲學姐、含貞學姐、爾言學長、信元學長和助理奕宏，在實驗上總是不藏私且有耐心教導著原本甚麼都不懂的我。做實驗之餘也還好有大家的陪伴，讓平日生活不會太過於沉悶與沉重。也謝謝黃老師實驗室的大家，時常得忍受我一天到晚去借東西和問問題。再來也要感謝我的同學們，碩一一起度過繁忙的課業，碩二也一起分享實驗好的成果抑或者在實驗撞牆時有能傾訴或討論的對象。因為有你們讓我的研究生活更加多姿多采。最後，要謝謝我的家人，總是支持我的任何決定，在背後做我最大的後盾，讓我能毫無顧慮做我想做的事情，謝謝你們。



中文摘要

背景: 胃癌在癌症相關死亡率中高居第三位，雖然其發生率隨時間有逐漸下降的趨勢，但病人的預後仍然不佳，平均五年存活率只有29%。目前研究觀察到異常的糖化作用會影響癌細胞的惡性程度。在我們之前的研究發現乙烯半乳糖胺轉移酶2(Glycosyltransferase N-acetylgalactosaminyltransferase 2, GALNT2)在胃癌病人中表現量比正常胃腺組織少，其表現降低時會透過活化肝細胞生長因子受體(MET)而增加胃癌的惡性程度。此外，在受體酪氨酸激酶磷酸化陣列試驗(RTK array)中則觀察到降低GALNT2的表現量會增加表皮生長因子受體(Epidermal Growth Factor Receptor, EGFR)的活化，但對於GALNT2是否能透過調節EGFR的磷酸化而影響胃癌的進展依然是未知。

目的: 探討GALNT2是否能透過修飾EGFR糖基構造及調節磷酸化程度進而影響胃癌的惡性程度。

材料及方法: 以細胞株實驗分析，抑制GALNT2的表現後對於AGS的細胞存活率(MTT試驗)、轉移 (transwell migration assay) 及侵襲行為(matrigel invasion assay) 的影響。利用Vicia villosa agglutinin (VVA) pull down assay觀察EGFR的糖化作用。利用臨床胃癌檢體的免疫組織化學染色分析，pEGFR和GALNT2 表現與預後的相關性。

結果: 抑制GALNT2會增加EGFR和Akt磷酸化但減少EGFR的糖化作用。此外EGFR及Akt的抑制劑可以有效減少因抑制GALNT2而增加的轉移及侵襲能力，但細胞存活率在控制組及抑制GALNT2組別間不管是否有加入EGFR抑制劑都無顯著差異。臨床檢體中，44% (31/70)的病中人有表現pEGFR，且其與GALNT2表現量呈現正相關，但和其餘臨床病理特徵與預後沒有太大相關性。

結論: 在研究中觀察到GALNT2可以透過修飾EGFR醮化作用及減少其磷酸化和下游訊息傳遞而抑制胃癌細胞的惡性程度。

關鍵字: 乙稀半乳糖胺轉移酶、表皮生長因子受體、O-醮基化、胃癌。



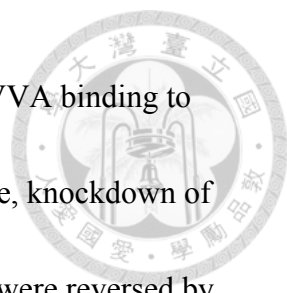
Abstract



Background: Gastric cancer is the third leading cause of cancer-related deaths worldwide. Despite a steady decline in gastric cancer incidence and mortality, the overall 5-year survival rate of patients with gastric cancer is about 29%. Aberrant glycosylation affects the tumorigenesis and progression of cancers. In our previous study, we found that down-regulation of GALNT2 enhanced malignancy of gastric cancer as a result of increasing MET phosphorylation and affected activation of epidermal growth factor receptor (EGFR). Nevertheless, it remains unknown whether GALNT2 could regulate the malignancy through modifying EGFR phosphorylation.

Aims: To investigate whether GALNT2 could modify the malignant characteristics in gastric cancer by affecting EGFR phosphorylation and glycosylation.

Materials and methods: Effects of GALNT2 knockdown on cell viability (MTT assay), migration (transwell migration assay) and invasion (matrigel invasion assay) of gastric cancer cell line (AGS) were analyzed. The Vicia villosa agglutinin (VVA) pull down assay was conducted to detect O-glycosylation of EGFR. Immunohistochemistry was performed to study the correlation of p-EGFR expression with GALNT2 and prognosis.



Results: Knockdown of GALNT2 in AGS cells decreased the VVA binding to EGFR, but increased phosphorylation of EGFR and Akt. Furthermore, knockdown of GALNT2 enhanced the migration and invasion of AGS cells, which were reversed by treated with EGFR inhibitor (gefitinib) or Akt inhibitor (MK2206). However, there was no difference on cell viability between siC and siGALNT2-transfected groups, treated with either DMSO or gefitinib. Clinically, p-EGFR was over- expressed in 44% (31/70) of gastric cancer tissues. p-EGFR was positively correlated with GALNT2 but not associated with clinical outcomes.

Conclusions: Our *in vitro* studies indicate that GALNT2 may suppress the malignancy of gastric cancer by modifying glycosylation of EGFR and reducing activation of EGFR-Akt pathway.

Keyword: Glycosyltransferase N-acetylgalactosaminyltransferase 2 (GALNT2), Epidermal growth factor receptor (EGFR), O-glycosylation, gastric adenocarcinoma

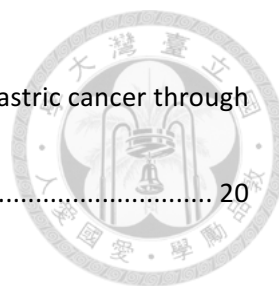
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I. Introduction

1-1. Gastric cancer

Gastric cancer is the fourth most common cancer and the third leading cause of cancer-related deaths worldwide, with an estimated 951,600 new gastric cancer cases and 723,100 deaths occurred in 2012.[1,2]. Despite a steady decline in incidence and mortality of gastric cancer have been observed in past decades, the prognosis in gastric cancer remains poor, because high proportion of people are diagnosed at late stage. In gastric cancer, poor prognosis is associated with late TNM stage. The overall 5-year relative survival rate of gastric cancer patients with stage IV in the United States was only about 4%.

Treatments of gastric cancer are according to stage and patients' general health. The major treatment is surgery, but when patients can't undergo surgery or recurrence happens after surgery, it needs other treatments, including chemotherapy, radiotherapy and target therapy.[3]



1-2. Mucin-type glycosylation

Introduction

Glycosylation is the most common post-translational modification of proteins. It refers to the process that attaches glycans to proteins, lipids, or other organic molecules. There are two major types of glycosylation: N-linked and O-linked glycosylation. The most common type of O-glycosylation is mucin-type O-glycosylation, which forms the GalNAc1-O-Serine/threonine linkage. This process is initiated by a large family of polypeptide GalNAc transferases (GALNT), consisting of at least 20 members in humans, namely GALNT1 to 20.[4]

Role of glycosylation in cancers

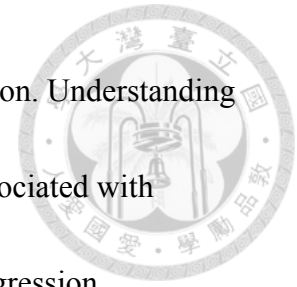
Glycobiology has become a focus of research in cancer biology.[5] Aberrant glycosylation may be owing to under-/overexpression of glycosyltransferases or mislocalization of glycosyltransferases. Glycans have been found to participate in numerous fundamental biological processes involved in cancer, such as inflammation, immune surveillance, cell–cell adhesion, cell–matrix interaction, inter- and intracellular signaling, and cellular metabolism. [6]

Common feature of tumors is the overexpression of truncated O-glycans, such as the



disaccharide Thomsen–Friedenreich antigen (T antigen) and the monosaccharide GalNAc (also known as Tn) and their sialylated forms (ST and STn).[7] Dysregulation of GALNTs has been found in many cancers and plays a critical role in cancer development. For instance, GALNT3 expression significantly correlated with shorter progression-free survival (PFS) intervals in epithelial ovarian cancer (EOC) patients with advanced disease.[8] GALNT6 is upregulated in breast cancer and might contribute to mammary carcinogenesis through aberrant glycosylation and stabilization of MUC1[9] In addition, GalNT14 is overexpressed in colorectal carcinoma and pancreatic cancer and is associated with altered sensitivity to TRAIL-induced apoptosis through modulation of the O-glycosylation of death receptors on these tumor cells. It has been reported that GALNT3, GALNT6 and GALNT10 were biomarkers associated with lymph node metastasis[10], venous invasion[11] and poor differentiation of gastric cancer[12] respectively. In vitro studies have shown that knockdown of GALNT2 increases cell proliferation and invasion in gastric cancer[13] and hepatocellular carcinoma.[14], but decreases migration and invasion of oral squamous cell carcinoma.[15] In our previous study, we found that low GALNT2 expression correlated with increased tumor depth, lymph node metastasis, and TNM stage and shorter disease-free survival and downregulation of GALNT2 enhances

malignancy of gastric cancer through increasing MET phosphorylation. Understanding the mechanisms and consequences of variations in glycosylation associated with neoplastic disease will provide important insight into neoplastic progression




1-3. Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a 170 kDa receptor containing approximately 20% of carbohydrate of its molecular mass and is heavily N-glycosylated.

EGFR is consisting of an extracellular ligand binding domain (domains I-IV), a transmembrane region, an intracellular domain with tyrosine kinase activity, and a tail containing tyrosine residues, required for downstream signaling. Ligand binding brings two receptor monomers together and allows for the dimerization and subsequent activation of the kinase domain. EGFR activation leads to receptor phosphorylation and initiates diverse downstream signaling pathways including the RAS/RAF/MAP kinase and PI3K/Akt/mTOR signaling networks, which play a vital role in several critical cellular processes including proliferation, motility, and invasion.[16]

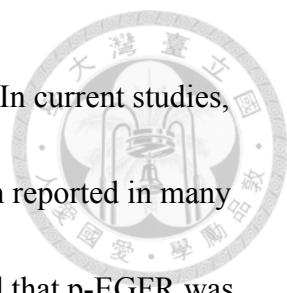
EGFR and cancers

Dysregulation of EGFR has been observed in variety of cancer, including breast cancer, colorectal cancer and non-small cell lung carcinoma (NSCLC), etc. In NSCLC,



overexpression of EGFR or mutations in intracellular EGFR have been observed in 43-89% of cases.[17] In breast cancer, EGFR overexpression is associated with large tumor size, poor differentiation, and poor clinical outcomes. Many therapeutic agents targeting EGFR have been under clinical trial.[18-20] Studies observed that EGFR-positive rate was 14-44% in gastric cancer. However, the correlation between EGFR and clinic-pathological characteristics was controversial. EGFR-positive was correlated with advanced TNM stage, lymph node metastasis, vascular invasion and shorter progression-free-survival (PFS).[21-25], but Fuse et al. found there was no correlation between EGFR expression and overall survival rate. [26] In vitro experiments showed that EGFR activation and its downstream signaling PI3K-Akt pathway were required in gastric cancer migration, and treated with EGFR inhibitor or PI3K inhibitor could suppress the migration ability.[27] In addition, knockdown of EGFR suppressed cell growth, invasion and induced cell apoptosis and cell cycle arrest in gastric cancer through Akt pathway.[28]

Aside from studying expression of EGFR in cancer and its role in cancer progression, many studies have focused on investigating correlation between phosphorylated-EGFR(p-EGFR) and clinical outcomes, because EGFR is a receptor



tyrosine kinase, it becomes activated only when it's phosphorylated. In current studies, the prognostic significance of p-EGFR on clinical outcomes has been reported in many cancers, such as breast cancer, NSCLC. [29-31] Zhang et al. reported that p-EGFR was detected in 83.3% of gastric cancer and it correlated with T stage.[32] The prognostic impact of p-EGFR remains unknown, so it needs further studies to confirm. These findings would be beneficial for predicting prognosis or providing new target for treatment.

II. Aims

In our previous study, the RTK array showed that GALNT2 knockdown increased phosphorylation of EGFR in AGS cells. This study aims to investigate whether GALNT2 could modify the malignant characteristics, including proliferation, migration and invasion in gastric cancer cells by affecting EGFR phosphorylation and glycosylation. In addition, the correlation of p-EGFR expression with GALNT2 and clinical outcomes are investigated.



III. Materials and methods

3-1. Cell line

Human gastric cancer cell line AGS was kindly provided by Min-Chuan Huang (National Taiwan University, Taiwan) in 2010. These cells were grown in 10-cm culture plates and maintained in RPMI medium with 10% fetal bovine serum, 2% sodium bicarbonate, 2 mM L-Glutamine, and 1% penicillin, 1% streptomycin, and 1% amphotericin at 37°C with 5% CO₂ in a 95% humidified atmosphere.

3-2. Western blot analysis

Preparation of cell lysates

Total cell lysates from cultured AGS cells were prepared as below. The culture plate was washed with PBS to remove the residual medium, and the cells were lysed with 10X RIPA buffer diluted with ddH₂O (1:9). Then, cells were scraped from culture plate and incubated on ice for 15 minutes. Next, extracts were centrifuged at 14,000 rpm for 10 minutes at 4°C. Finally, supernatant was removed to new eppendorf for use.



Bradford assay

The Bradford assay was used to determine the concentration of proteins. 1 μ l of standard protein (BSA) of known concentration (3, 10, 15, 20 μ g/ml) and tested proteins were mixed with 199 μ l 5X Bradford diluted with ddH₂O (1:4). Samples were incubated at room temperature for 5 minutes and the absorbance was measured at 630nm. The concentration of each sample was calculated by comparing the absorbance with the calibration curve.

Sample preparation

Protein samples were mixed with 5X sample buffer (1:4) (5% SDS, 20% glycerol, 0.004% bromphenol blue, 125mM Tris-HCl, 10% β -mercaptoethanol, pH 8.0) and boiled at 100 $^{\circ}$ C for 5 minutes.

Electrophoresis and electro-transfer

Equal amount (30 μ g) of extracted protein was loaded into each well and ran at 120V for about 2 hours. The transfer sandwich was assembled as follows: sponge, filter papers, gel, PVDF membrane (Millipore, 0.2 μ m), filter papers. Then the transfer sandwich was relocated to the transfer apparatus filled with transfer buffer (25Mm Tris,

192mM glycine, 20% methanol.). Protein samples were transferred to PVDF membrane at 90V for 90 minutes.



Blocking, antibody incubation and detection

The membrane was incubated with 5% BSA in TBST (50mM Tris, 150mM NaCl, 0.05% Tween 20, pH 7.5) at room temperature for 1 hours followed by incubated with primary antibody against GALNT2 (1:1000, Sigma), EGFR (1:1000, Cell signaling technology), phospho-EGFR (Try1068) (1:1000, Cell signaling technology), Akt (1:1000, abcam), phospho-Akt (1:1000, abcam), ERK (1:1000, Cell signaling technology), phospho-ERK (1:1000, abcam) and GAPDH (1:1000, Novus Biologicals) diluted in 5%BSA/TBST overnight at 4°C. Next day, the membrane was washed 3 times with TBST, and then incubated with mouse IgG antibody (HRP) (1:1000, GeneTex) and rabbit IgG antibody (HRP) (1:1000, GeneTex) at RT for 1 hour. The membrane was washed 3 times with TBST and developed with Luminata Crescendo Western HRP Substrate (Millipore).



3-3. Immunohistochemistry

Paraffin sectioning

Paraffin-embedded tissues were sectioned at a thickness of 5 μm . Tissue sections were stretched in 36.5°C water bath and mounted on slides coated with 3-Aminopropyltriethoxysilane (Sigma). The slides were then placed to dry at 40°C

De-paraffinization

The slides were incubated at 60°C for 30 min. Tissues were deparaffinized in 2 changes of xylene, 10 minutes each, and then rehydrated in 2 changes of 100% ethanol for 3 minutes each, 90%, 80% and 70% ethanol for 3 minutes respectively. After the slides were immersed in the above sequence, they were washed twice with PBS for 5 minutes each.

Antigen retrieval

The slides were incubated in sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) and heated using Microwave Vacuum Histoprocessor RHS-1 (Milestone) with program GPR100C (20 slides – 250mL). Then the slides were cooling by running tap water for 20 minutes.



Staining

After antigen retrieval, the slides were washed 3 times in TBS solution then incubated with 0.3% H₂O₂ for 10 minutes to block endogenous peroxidase activity followed by PBS wash for 3 times. Then applying 5% (w/v) BSA/TBS to block nonspecific binding for 1 hour. The sections were then incubated with anti-GALNT2 polyclonal antibody (1:200, Sigma) and phospho-EGF Receptor (Tyr1068) (1H12) Mouse mAb (1:250, Cell signaling technology) diluted with 5% BSA/TBS overnight at 4°C. Signals were detected employing UltraVision Quanto Detection System HRP (Thermo) and visualized by DAB quanto (Thermo). All sections were counterstained with hematoxylin for 30 seconds and rinsed in running tap water for 2 minutes.

Dehydration and mounting

The tissue slides were dehydrated through 70%, 80% ,90% and 100% ethanol for 30 seconds respectively, and then were immersed in 2 changes of xylene, 30 seconds each. After the slides were dehydrated in the above sequence, they were mounted with Micromount Mounting Medium (Leica).




3-4. IHC evaluation

Two pathologists independently quantified staining. Every tumor was given a score according to the intensity of staining (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) and percentage of stained cells (0% = 0, 1–10% = 1, 11–50% = 2, 50–80% = 3, >80%=4). The score gives a range of 0–12 as the product of multiplication between stained cells percentage score (0–4) and staining intensity score (0–3). Scoring was performed for four random distinct fields per slide, and then 4 scores were averaged. IHC score between 4–12 was defined as phospho-EGFR positive and score between 0–4 was defined as phospho-EGFR negative .

3-5. Real-time reverse transcription PCR (RT-PCR)

RNA extraction

The total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies). The isolation procedure was as below. The culture plates were washed three times with PBS and cells were extracted with 1ml Trizol Reagent. Then, cells were scraped from culture plates and incubated at room temperature for 15 minutes. Next, the homogenized samples were mixed with 0.2ml of chloroform and were incubated at RT for 3 minutes. Then the homogenized samples were centrifuged at 12,000g for 15 minutes at 4°C and



the aqueous phase was placed into a new eppendorf. The RNA was precipitated by adding 0.5ml isopropanol to the aqueous phase and the mixtures were incubated at RT for 10 min followed by centrifugation at 12,000g for 10 mins at 4°C. After the supernatant was removed, the RNA pellet was wash twice with 1ml of 75% ethanol and air dried. Finally, RNA was resuspended in RNase-free water.

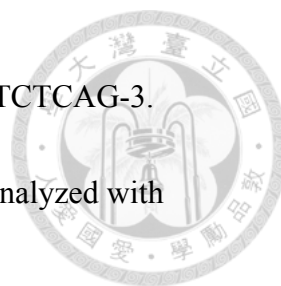
Reverse transcription

The cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kits (Applied bio-system). 2µg RNA was mixed with RT master mix (2µl of 10X RT Buffer, 0.8µl of 25X dNTP Mix (100mM), 2µl of 10X RT Random Primers, 1µl of MultiScribe™ Reverse Transcriptase, 1µl of RNase Inhibitor, 3.2µl of Nuclease-free H₂O.) and then DEPC water was added to bring the total volume to 20µl.

The thermal cycler condition was programmed as followed: 25°C/10 min, 37°C/120 min, 85°C/5 min, and 4°C/∞, and then reverse transcription was performed.

Real-time PCR

The cDNA was subjected to real-time PCR using quantitative PCR System Mx3000P (Stratagene). Primers for GALNT2 were 5-AAGGAGAAGTCGGTGA AGCA-3 and 5-TTGAGCGTGA ACTTCCACTG-3. Primers for GAPDH were



5-ACAGTCAGCCGCATCTTCTT-3 and 5-GACAAGCTTCCCGTTCTCAG-3.

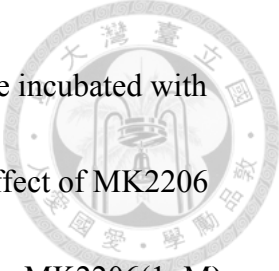
Relative quantity of mRNA expression normalized to GAPDH was analyzed with MxPro Software (Stratagene).

3-6. SiRNA knockdown of GALNT2 expression

In transient knockdown experiments, a siRNA oligonucleotides against GALNT2 (5-CAGCAGGGAACU AACUGCCUCGACA-3 and 5-UGUCGAGGCAGUUAGUU CCCUCGUG)) and a non-targeting siRNA control were synthesized by Invitrogen. The AGS cells (2×10^5 cells) were transfected with siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) with the final concentration of 10nM for 24hours. The step-by-step procedure is listed as below. Serum-free RPMI-1640 (500 μ l) containing siRNA was mixed with 500 μ l serum-free RPMI-1640 containing 10 μ l Lipofectamine RNAiMAX Reagent. The resultant mixture was kept at room temperature for 20 min, and then was added into the plates. The plates were incubated at 37°C for 24 h.

3-7. MTT assay

The cell viability was assessed by measuring the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) to the dark blue formazan product. AGS cells were seeded at a density of 2×10^3 cells/200 μ l per well.



For assessing the effect of gefitinib (ApexBio Technology), cells were incubated with 10% FBS containing DMSO (0.1%, Sigma) or gefitinib(1 μ M). The effect of MK2206 (AdooQ BioScience) was assessed by adding DMSO (0.1%, Sigma) or MK2206(1 μ M) to the 96-wells. Then MTT solution was added to each well and incubated at 37°C for 3 hours. The solution was carefully removed followed by addition of DMSO. Absorbance of sample was measured at 570 nm at day1, 2, 3, 4 and 5. Results were expressed as percentage of absorbance compared to the control cells.

3-8. Transwell migration assay

The transfected cells (3×10^4) were re-suspended in 200 μ l of serum-free RPMI containing gefitinib and EGF(50ng/ml, Sigma) or MK2206 and added to the upper chamber with an 8- μ m pore size membrane (Corning) for assessing effect of gefitinib and MK2206 on cell migration. 700 μ l of RPMI with 10%FBS was added to lower chamber as chemo-attractant. After 24 hours, the non-migrating cells on the upper surface of the membrane were removed by scrubbing with a cotton-tipped swab, and the invaded cells on the lower surface of the membrane were fixed with 100% methanol and then stained with 0.5% crystal violet (Sigma). The number of migrated cells per field



was counted under a phase contrast microscope. Four random fields were examined and analyzed at 100x magnification.

3-9. Matrigel invasion assay

Procedures were the same as trans-well migration assay, except for the upper chamber with an 8- μ m pore size membrane (Corning) being coated with Corning Matrigel Matrix diluted with serum-free RPMI (1:4) at 37°C overnight the day before experiment.

3-10. VVA lectin pull down assay

Cell lysates (0.5 mg) were incubated with 30 μ l *Vicia Villosa* Lectin (VVA)-conjugated agarose beads (Vector Laboratories) at 4 °C overnight. The lectin/glycoprotein complexes were collected by centrifugation (10,000 rpm, 1min) and washed twice with PBS. Glycoproteins were released from the complexes by boiling in 5 μ l of 5x sample buffer for 5 minutes. The precipitated proteins were resolved by SDS-PAGE, then immunoblotted to detect EGFR. EGFR of total lysates were served as internal control.

3-11. Statistic analyses

Data were represented as the mean \pm SD. Statistical analyses were performed using Prism6. The Student *t* test and 2-way ANOVA followed by a Bonferroni post hoc test

were used to compare differences between experimental groups. Chi-square was used to analyze correlation between pairs of categorical variables. The Kaplan-Meier log-rank test was performed to estimate probabilities of progression-free survival. All statistical tests were 2-sided, and $P < 0.05$ was considered statistically significant.





IV. Results

4-1. Knockdown of GALNT2 increased epidermal growth factor receptor (EGFR) phosphorylation and decreased EGFR glycosylation.

To investigate the effect of GALNT2-knockdown on EGFR phosphorylation, AGS cells were transfected with siGALNT2 or non-targeting siRNA control (SiC) for 24 hours, and then were starved for 6 hours followed by EGF stimulation for 10 minutes. The expression of p-EGFR in the treated cells was detected by Western blot analysis. Efficiency of GALNT2 knockdown was confirmed by western blot analysis and q-PCR. (Fig. 1A-C). Without EGF, there was no difference between SiC and siGALNT2-transfected group ($p=0.2073$). In the presence of EGF, p-EGFR was significantly increased in siGALNT2-transfected compared to siC group. ($p=0.0257$) (Fig. 2A & B)

Since GALNT2 is a mucin glycosylating enzyme, we hypothesized that it could modify O-glycosylation of EGFR. Thus, VVA lectin pull down was used to detect Tn antigen (GalNAc-o-Ser/Thr) expression of siC and siGALNT2 group. As shown in Fig. 2, knockdown of GALNT2 reduced VVA binding to EGFR ($p=0.0093$). Here, EGFR of total lysate served as internal control.

4-2. Knockdown of GALNT2 enhanced the malignant phenotypes of gastric cancer through increasing EGFR phosphorylation *in-vitro*.



Knockdown of GALNT2 could enhance cell viability, migration and invasion abilities in previous studies.[33] Here, we reported that knockdown of GALNT2 increased EGF-induced activation of EGFR. As a result, we investigated whether GALNT2 knockdown enhanced malignant phenotypes, including cell viability, migration and invasion by affecting EGFR phosphorylation. SiC and siGALNT2-transfected groups were starved for 6 hours and then treated with 1 μ M gefitinib or DMSO.

As shown in Fig. 4, there were no differences during day1-5 on cell viability between SiC and siGALNT2-transfected group, either treated with DMSO or gefitinib group. The number of migrated cells of siGALNT2-transfected group were 2.8 fold higher than that of siC group, the addition of gefitinib markedly suppressed the migration of siGALNT2-transfected cells (Fig. 5). Furthermore, the invasion abilities of siGALNT2-transfected cells which was markedly enhanced by knockdown of GALNT2 were suppressed when treated with gefitinib. (Fig. 6)

4-3. Knockdown of GALNT2 enhanced the malignant phenotypes of gastric cancer through increasing EGFR-Akt pathway *in-vitro*.



Upon phosphorylation of EGFR, diverse downstream signaling pathways initiated, which play a vital role in several critical cellular processes including proliferation, motility, and invasion. Previous studies showed that EGFR-ERK and EGFR-Akt signaling pathways were associated with migration and invasion in gastric cancer. To explore signalings involved in gastric cancer progression mediated by GALNT2-EGFR pathway, two target proteins, Akt and ERK, were selected to evaluate EGFR phosphorylation on their activation. As shown in Fig. 7A&B, gefitinib treatment significantly decreased phospho-Akt but not affected expression of total Akt. On the other hands, total ERK and phospho-ERK were unchanged upon gefitinib treatment. (Fig. 7A&C)

Next, we further verified the role of Akt activation in malignant phenotypes in gastric cancer. SiC and siGALNT2-transfected groups were treated with 1 μ M MK2206 to inhibit Akt activation or DMSO as control. As shown in Fig. 8, there were no differences during day1-4 on cell viability between SiC and siGALNT2-transfected group, but cell viability was lower in groups treated with 1 μ M MK2206 at day 4.

Besides, the number of migrated and invaded cells in siGALNT2+MK2206 group significantly decreased compared to siGALNT2 +DMSO group. (Fig.9&10)



4.4 Correlation of p-EGFR with GALNT2 and clinico-pathological parameters.


In-vitro experiments demonstrated that GALNT2 knockdown enhanced EGFR phosphorylation and was associated with malignant phenotypes in gastric cancer. However, it is still unknown about the correlation between GALNT2 and p-EGFR in clinical samples. In this study, GALNT2 and p-EGFR expression were evaluated by using immunohistochemistry staining (Fig. 11A&B). Result showed the correlation coefficients (r) between them was 0.3281, $p=0.0076$, which indicated higher expression of GALNT2 would have higher expression of p-EGFR and vice versa. (Fig. 11C)

Furthermore, we evaluated the correlation of p-EGFR with clinico-pathological parameters and progression-free survival (PFS). Of the 70 patients, 31 (44%) were p-EGFR positive, but the expression of p-EGFR was not significantly correlated with age, cell differentiation, size, lympho-vascular invasion, TNM stage. (Table. 1) In addition, there were no significant differences in progression-free survival among patients with p-EGFR positive/negative. The 5-year PFS was 50.4% for p-EGFR positive and 60.3% for p-EGFR negative group. ($p=0.2849$) (Fig. 12)

V. Discussion




Aberrant glycosylation frequently occurs in cancers and plays a critical role in cancer progression, angiogenesis and metastasis. Common feature of tumors is the overexpression of truncated O-glycans, such as the disaccharide Thomsen–Friedenreich antigen (T antigen), the monosaccharide GalNAc (also known as Tn) and their sialylated forms (ST and STn). Specifically, STn expression modulates malignant phenotypes in gastric and breast cancer cells, such as increased migration and invasion.[6,7,34] Altered expression of glycans can be attributed to abnormal expression of glycosyltransferases. In previous studies, GALNT2 altered the expression of Tn antigen on EGFR in oral squamous cell carcinoma and hepatocellular carcinoma and Tn antigen on MET in gastric cancer. [14,15] GALNT10 modified O-glycosylation of EGFR and subsequent phosphorylation of Akt in HBV-associated HCC.[35] Besides, GALNT2 could modify O-glycosylation and activation of IGF-1R, and then affect the malignant phenotypes of neuroblastoma cells.[36] However, no studies investigate about the role of GALNT2 in modifying O-glycosylation of EGFR in gastric cancer. Here, we demonstrated that knockdown of GALNT2 decreased the Tn antigen on EGFR in gastric cancer. Since alterations in glycosylation can significantly impact



overall glycoprotein charge and conformation and therefore readily alter its biological activity. For example, aberrant glycosylation of receptor tyrosine kinases (RTKs) could modulate their activities and signalings. [7] Receptor tyrosine kinase (RTK) -targeted therapeutic agents are constantly being developed and have been shown to be effective in various clinical trials. Trastuzumab, an inhibitor of ERBB2, has been approved for the treatment of gastric cancer.[37] So investigating the roles of other RTKs in gastric cancer will provide potential targets for the future treatments. In our previous study, we revealed that GALNT2 knockdown increased EGFR phosphorylation. Constitutive activation of the EGFR, which can be resulted from ligand depend or ligand independent pathway is common in cancers [38] Here, we showed that knockdown of GALNT2 enhanced EGFR phosphorylation in the presence of EGF. On the other hand, without EGF, GALNT2 didn't affect EGFR phosphorylation. This may indicate EGFR activation modulated by GALNT2 may dependent on the presence of ligand. Previous researches showed glycosylation of EGFR might affect their bind affinity to EGF [39,40]. The mechanism of how GALNT2 affects EGFR phosphorylation needs further study.

Recent studies suggested that GALNT2 regulated the malignant phenotypes by modifying EGFR glycosylation and phosphorylation in oral squamous carcinoma and



hepatocellular carcinoma.[14,15] This study demonstrated the migration and invasion abilities of AGS cells enhanced by GALNT2 would be suppressed by inhibiting EGFR phosphorylation. Subsequently, inhibition of EGFR phosphorylation decreased expression of phospho-Akt. Previous studies revealed that EGFR promoted gastric cancer migration and invasion via Akt-activation.[27,28] In this study, cell viability, migration and invasion abilities of AGS cells were significantly suppressed by inhibiting Akt phosphorylation. These findings reveal that EGFR-Akt activation plays an important role in gastric cancer progression.

In present study, cell viability during day1-5 was not affected by GALNT2 and EGFR activation but Akt activation. In contrast to our previous study, knockdown of GALNT2 enhanced cell viability.[33] This may be because this difference was significant at day 6 in previous study, but we only assessed cell viability for 5 days. Current studies about the effect of gefitinib on cell growth inhibition are controversial. Gefitinib inhibited proliferation in prostate cancer and lung cancer.[41] However, it was ineffective against most EGFR wild-type non-small cell lung cancer, but combined EGFR siRNA or Akt inhibitor showed synergistic growth inhibition. [42,43]

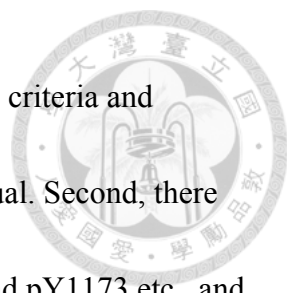
However, there is no research about correlation of GALNT2 and phospho-EGFR in clinical samples. This is the first study to investigate this relationship, but result showed

p-EGFR positively correlated with GALNT2, which was opposite to *in vitro* experiments.

The possible reasons may be as followed: first, the limitation of IHC analysis of detection of two proteins simultaneously, so two sections were used to detect expression of GALNT2 and phospho-EGFR respectively, which might result in scoring at different cells. Although, we minimize this limitation by sectioning serially, but the final slide for evaluated might not from the serial section. Immunofluorescence may serve as method to solving this problem by double labeling of different proteins in the same slide.[44,45]

Second, downregulation of GALNT2 enhances the malignancy in gastric cancer may attribute to multiple pathways, not all the patients undergo increasing EGFR phosphorylation, so it needs further investigation of subgroup analysis, and it might demonstrate clinical significance.

EGFR becomes activated upon phosphorylated, so increasing numbers of studies on correlation of p-EGFR with clinical outcomes. Patients with p-EGFR positive exhibited poorer overall survival.[30,31], but Nieto et al. showed p-EGFR didn't serve as a prognostic marker for overall survival.[29] The prognostic role of p-EGFR in gastric cancer remains unknown. In present study revealed there were no difference in clinico-pathological parameters, such as tumor size, cell differentiation, TNM stage and progression-free survival between p-EGFR positive and negative. The plausible



explanations for controversial findings are as followed. First, scoring criteria and threshold for positive and negative applied in each papers are not equal. Second, there are many phosphorylation sites of EGFR, such as pY845, pY1068 and pY1173 etc., and initiating distinct downstream signaling pathways. Recent studies detected not the same site, and it might result in different outcomes. Third, gefitinib inhibit overall phosphorylation of EGFR, not site specific, so other tyrosine sites may play a more critical role in enhancing malignancy in gastric cancer. In addition, distinct molecular drivers and tumor biology, and thus different treatment targets and predictive biomarkers are implicated in each subtype of gastric cancer, such as intestinal versus diffuse type. For example, EGFR, HER2 and MET overexpression are more prevalent in intestinal type, but loss of E-cadherin and FGFR overexpression are more common in diffuse type.[46,47] In this study, only intestinal type samples were collected for analyzing. Thus, we will collect diffuse type samples to investigate if p-EGFR serves as a prognostic marker in this subtype.

In conclusion, our findings of GALNT2 regulating malignancy by modifying O-glycosylation and phosphorylation of EGFR brings new insights into new therapeutic strategies. However, the prognostic role of pEGFR needs further investigation.



VI. Figures and table

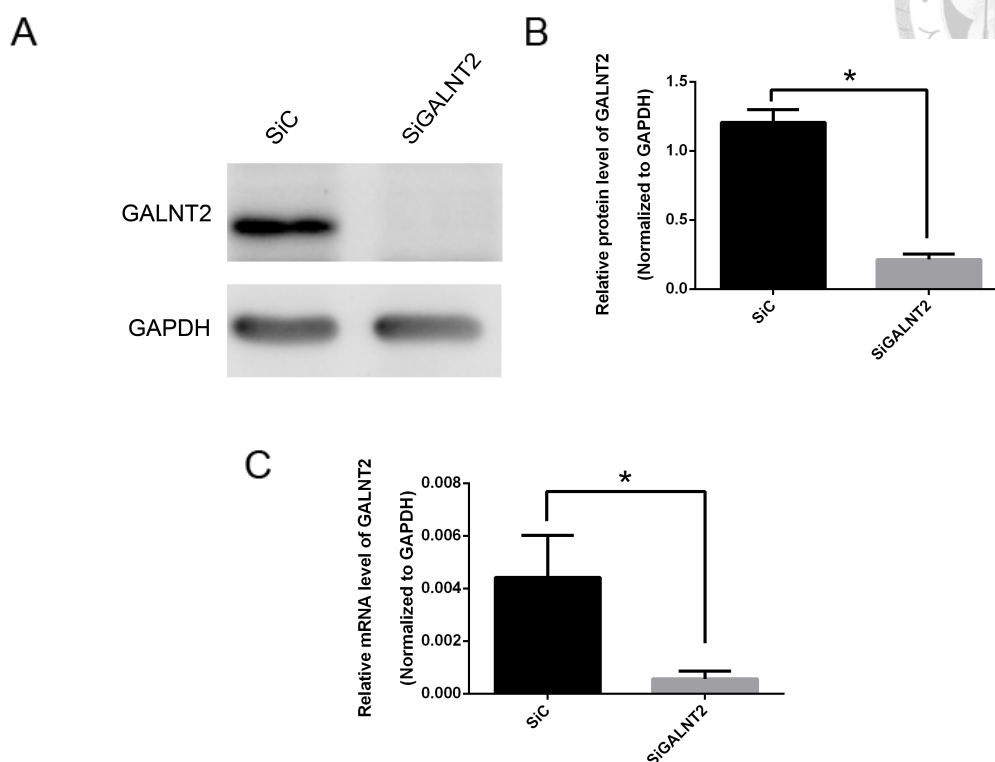
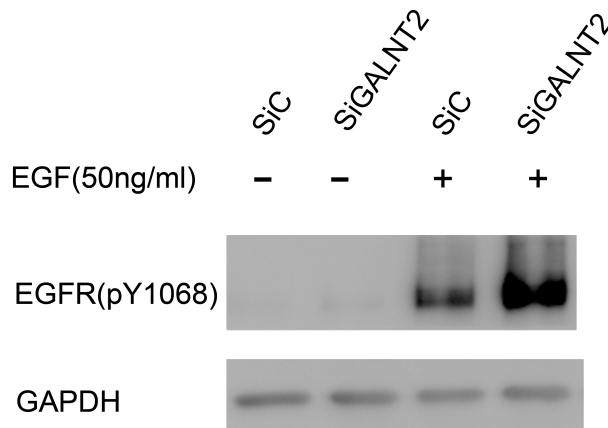


Figure 1. Efficiency of GALNT2 knockdown. (A) After transfected with 10nM non-targeting siRNA control (siC) or GALNT2 siRNA (siGALNT2) for 24 hours, cell lysates were collected and the expression of GALNT2 was confirmed by Western blot analysis. GALNT2 was significantly lower in siGALNT2 group when compared with siC group. (B) The relative protein level of GALNT2 was quantified and normalized to GAPDH. (C) The relative mRNA level of GALNT2 obtained from qPCR was quantified and normalized to GAPDH. Results are represented as mean \pm S.D. from three independent experiments. * $P < 0.05$.

A



B

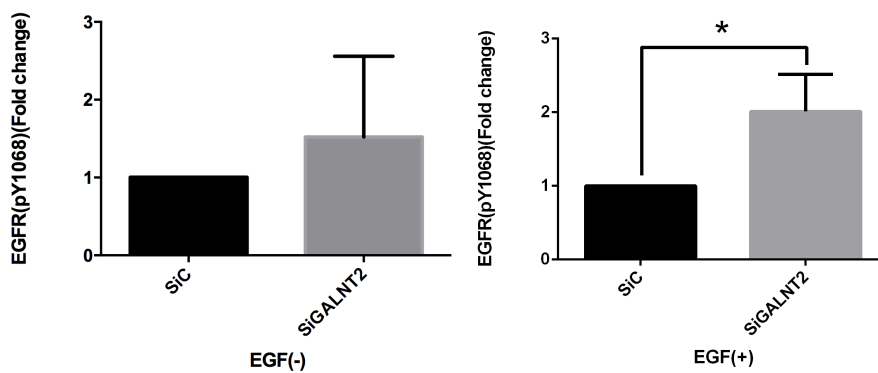
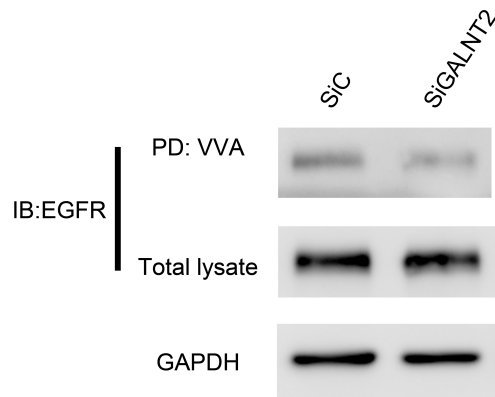


Figure 2. Effect of GALNT2 knockdown on EGFR phosphorylation. (A) After transfection for 24 hours, cells were starved for 6 hours followed by stimulated with DMSO or EGF (50ng/ml) for 10 min and then cell lysates were collected for Western blot analysis. Without EGF, there was no difference between groups. However, in the presence of EGF, phospho-EGFR significantly increased compared with siC group. (B) The expression of phospho-EGFR was quantified and normalized to GAPDH. Results are represented as mean \pm S.D. from three independent experiments. *P < 0.05.

A



B

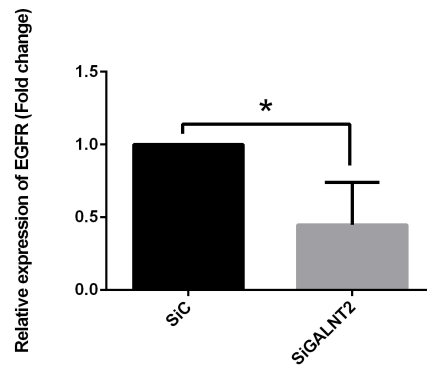


Figure 3. Effect of GALNT2 knockdown on EGFR glycosylation. (A) After 24h-transfection, lysates were pulled down by Vicia Villosa Lectin (VVA)-conjugated agarose beads. Total lysates and pulled-down lysates were immunoblotted (IB) with anti-EGFR antibody. **(B)** VVA-binding EGFR was quantified and normalized to total EGFR. GALNT2 knockdown decreased VVA-binding to EGFR. This result indicates that GALNT2 modifies O-glycosylation of EGFR. The results are represented as mean \pm S.D. from three independent experiments. *, $P < 0.05$.

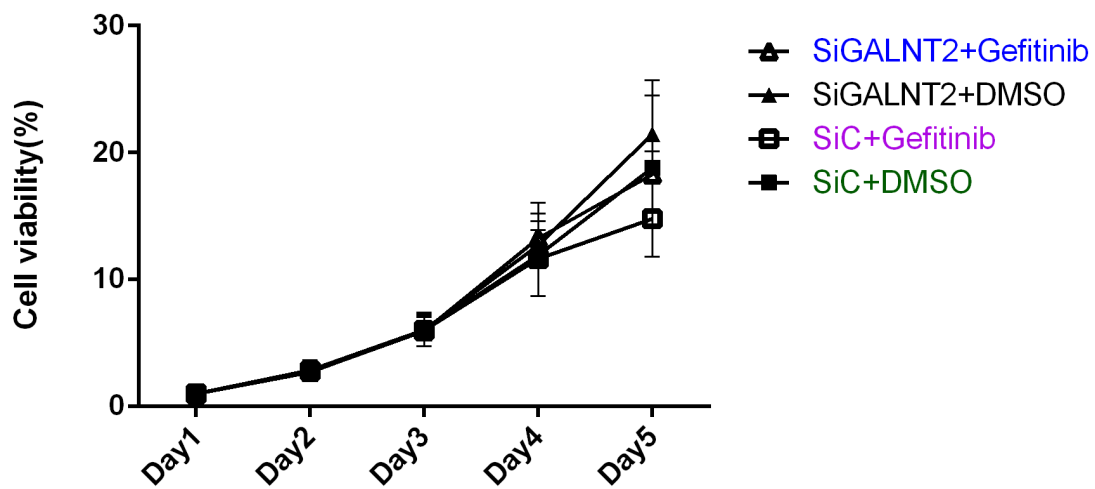


Figure 4. GALNT2 knockdown or gefitinib did not affect cell viability. After 24h-transfection, cells were incubated with 200 μ l RPMI medium+ 10% FBS treated with either DMSO or 1 μ M gefitinib. Absorbance of sample was measured at day1, 2, 3, 4 and 5. There were no differences during day1-5 between siGALNT2 and siC group treated with DMSO or gefitinib. The results are represented as mean \pm S.D. from three independent experiments. *, P < 0.05.

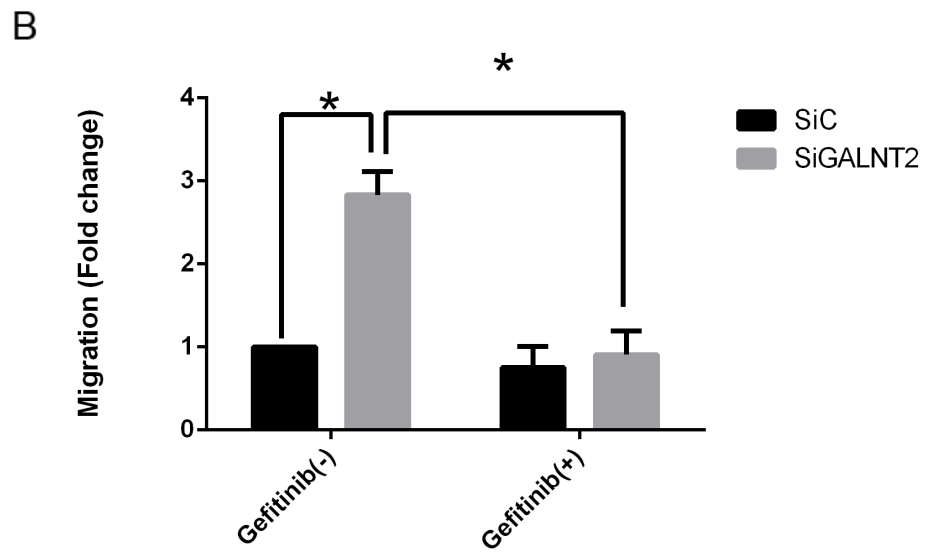
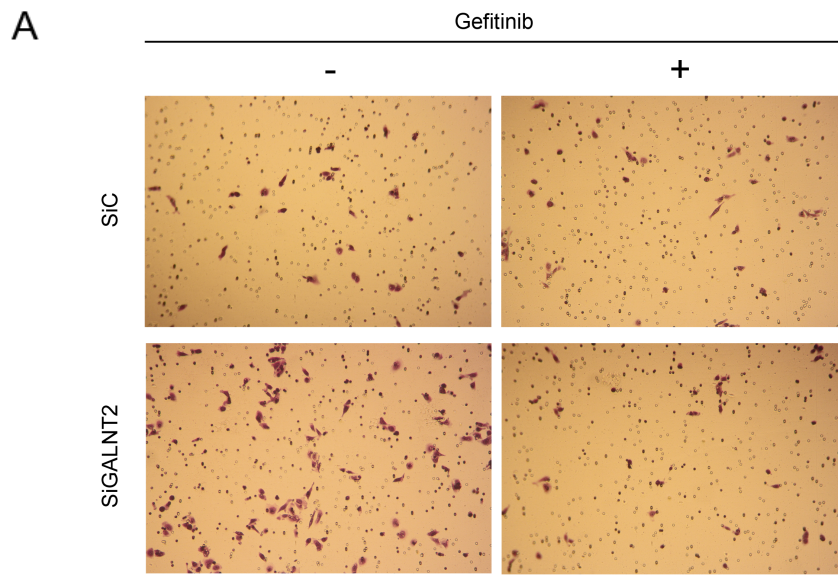


Figure 5. GALNT2 knockdown enhanced cell migration through increasing

EGFR phosphorylation. (A) After 24h-transfection, cells (3×10^4) were re-suspended

in 200 μ l of serum-free RPMI containing EGF(50ng/ml) and treated with/ without 1 μ M

gefitinib. The migrated cells were counted after 24h. (A) GALNT2-knockdown

increased the number of migrated cells compared with siC group, but the addition of

gefitinib would abolish this phenomenon. (B) The number of migrated cells was

calculated at 4 random fields per experiment. ($\times 100$) and expressed as fold change of

siC without gefitinib treatment. Results are represented as mean \pm S.D. from three

independent experiments. *P < 0.05.



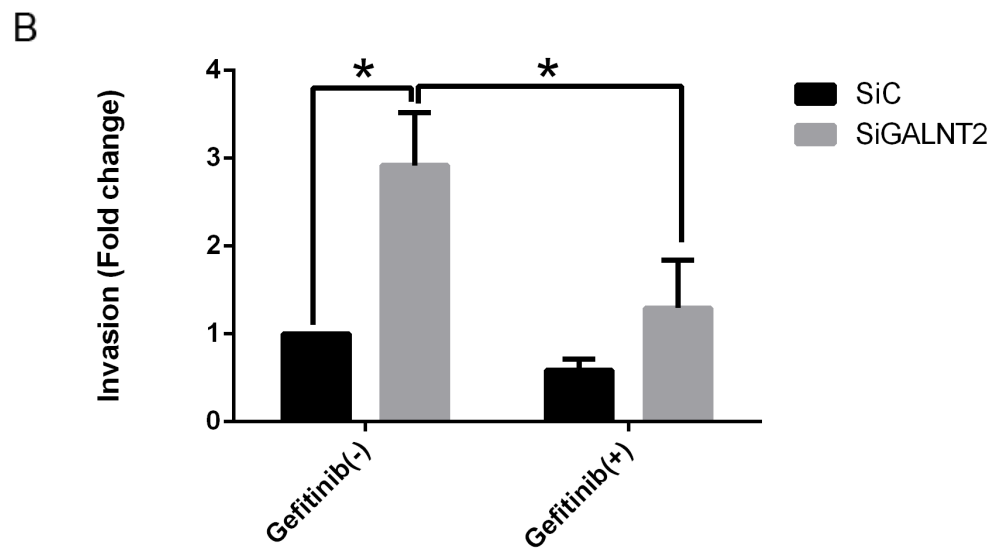
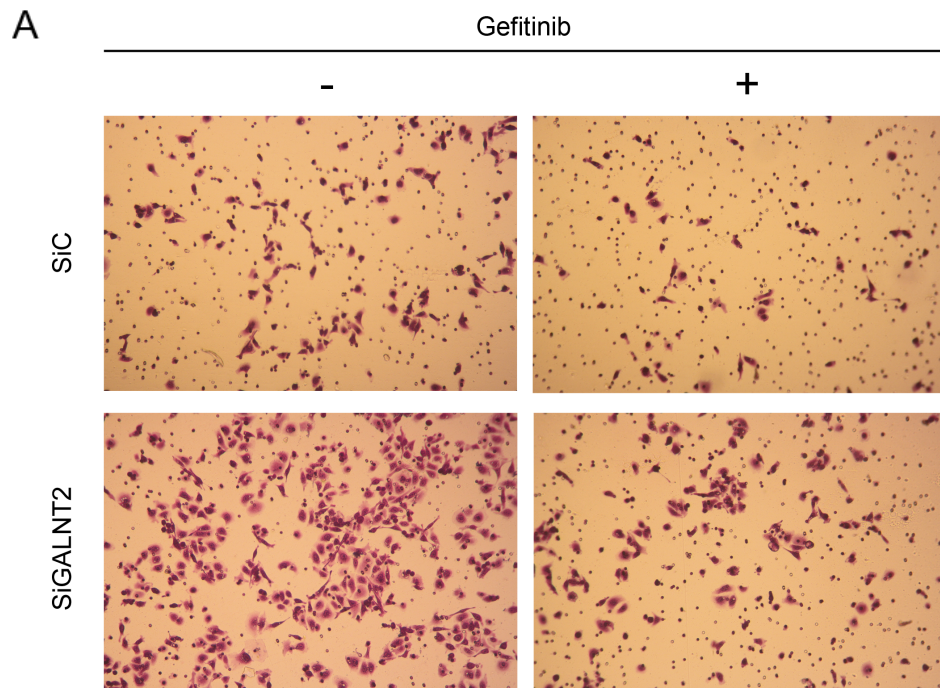
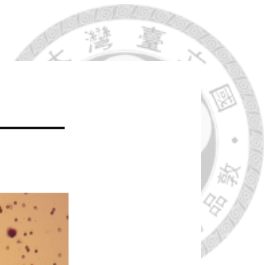


Figure 6. GALNT2 knockdown enhanced cell invasion through increasing

EGFR phosphorylation. (A) After 24h-transfection, cells (3×10^4) were re-suspended

in 200 μ l RPMI medium+ 10% FBS of serum-free RPMI containing EGF(50ng/ml) and

treated with/ without 1 μ M gefitinib. The invaded cells were counted after 24h. (A)

GALNT2-knockdown increased the number of invaded cells compared with siC group,

but the addition of gefitinib would abolish this phenomenon. (B) The number of

invaded cells was calculated at 4 random fields per experiment. ($\times 100$) and expressed

as fold change of siC without gefitinib treatment. Results are represented as mean \pm S.D.

from three independent experiments. *P < 0.05.

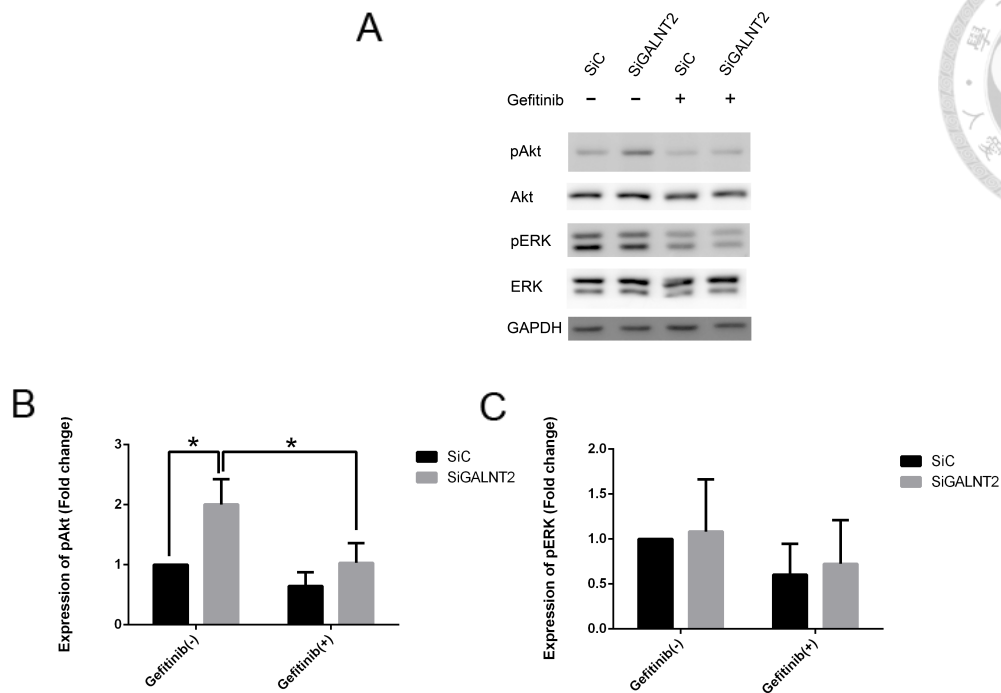


Figure 7. Effect of EGFR inhibitor (gefitinib) on Akt and ERK1/2

phosphorylation and total form. (A) After 24h-transfection, cells were starved for 6h and then treated with 1 μ M gefitinib followed by 10-min stimulation of EGF.

Subsequently, cell lysates were collected for Western blot analysis. Expression of phospho-Akt significantly increased in the siGALNT2 group compared to the siC group, and this phenomenon would be reversed by the addition of gefitinib. However, total ERK, Akt and phospho-ERK showed no difference between siC and siGALNT2 treated with/without gefitinib. (B) Expression of phospho-Akt (left panel) and phospho-ERK (right panel) were quantified and normalized to GAPDH. Results are represented as mean \pm S.D. from three independent experiments. *P < 0.05.

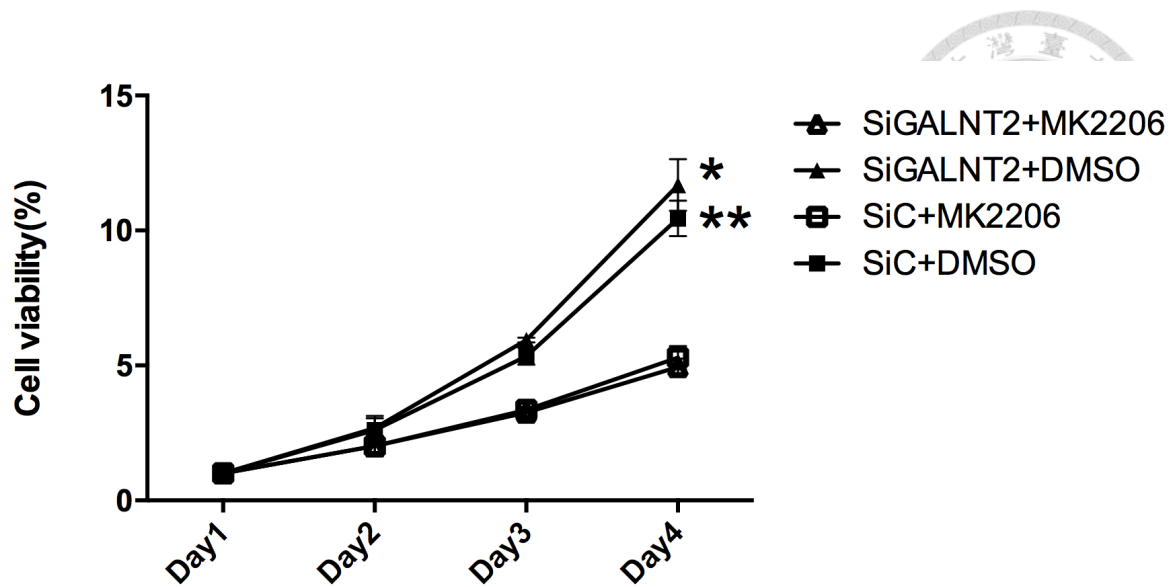


Figure 8. Inhibition of Akt phosphorylation decreased cell viability. After 24h-transfection, cells were incubated with 200 μ l RPMI medium+ 10% FBS treated with either DMSO or 1 μ M MK2206. Absorbance of sample was measured at day1, 2, 3 and 4. There were no differences between siGALNT2 and siC group. However, cell viability was significantly lower in groups treated with MK2206 than groups treated with DMSO. Results are represented as mean \pm S.D. from three independent experiments. *P < 0.05, siG2+DMSO compared to siG2+MK2206; **P < 0.05, siC+DMSO compared to siC+MK2206



A

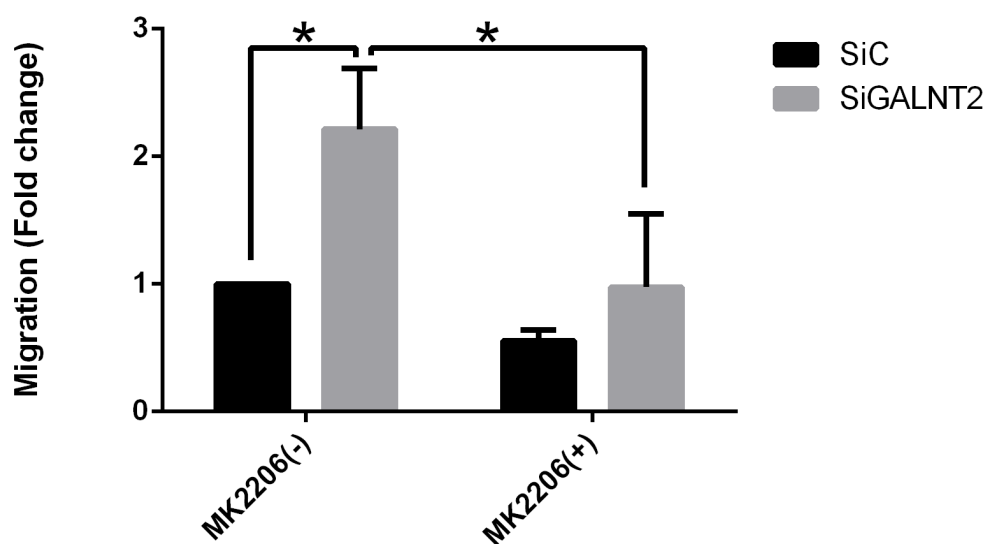
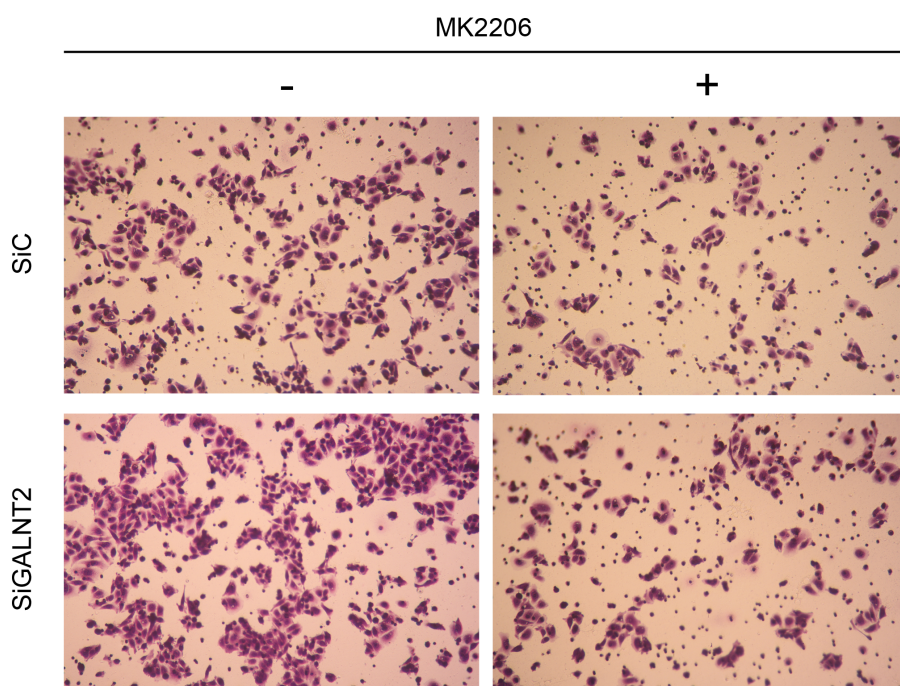


Figure 9. GALNT2 knockdown enhanced cell migration through increasing Akt

phosphorylation. (A) After 24h-transfection, cells (3×10^4) were re-suspended in 200 μ l of serum-free RPMI containing with/ without 1 μ M MK2206. The migrated cells were counted after 24h. (A) GALNT2-knockdown increased the number of migrated cells compared with siC group, but the addition of MK2206 would abolish this phenomenon. (B) The number of migrated cells was calculated at 4 random fields per experiment. ($\times 100$) and expressed as fold change of siC without MK2206 treatment. Results are represented as mean \pm S.D. from three independent experiments. *P < 0.05.

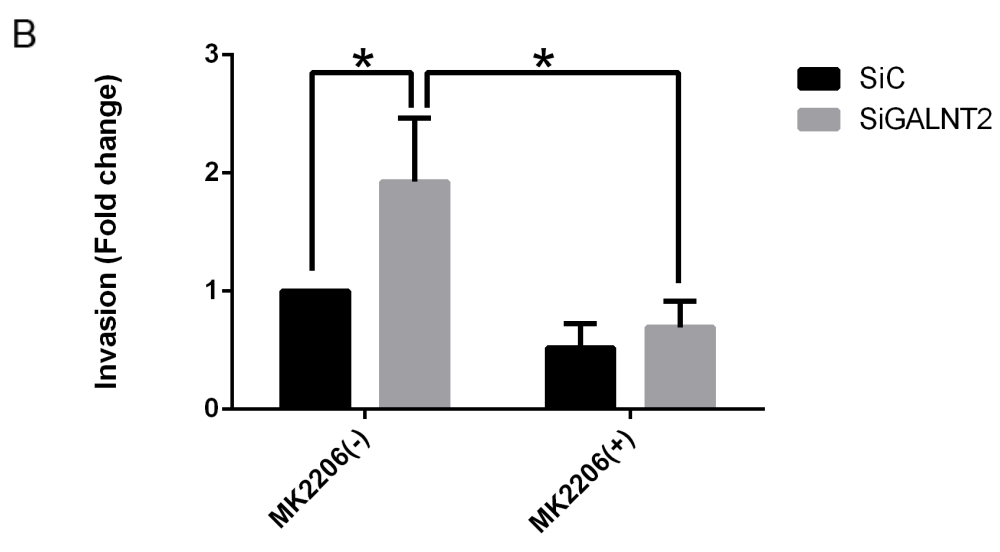
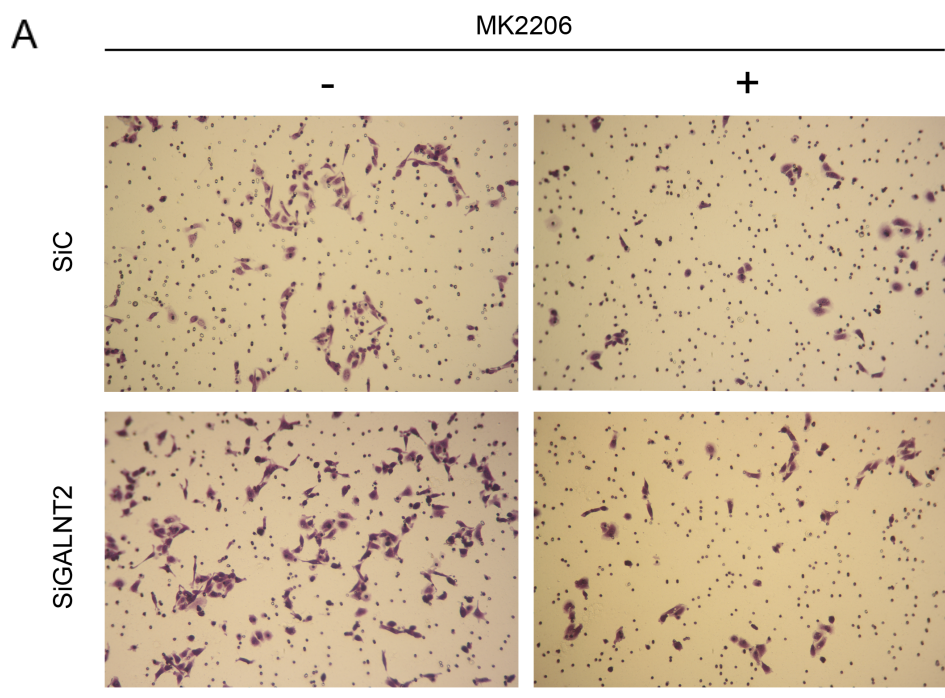


Figure 10. GALNT2 knockdown enhanced cell invasion through increasing Akt

phosphorylation. (A) After 24h-transfection, cells (3×10^4) were re-suspended in 200 μ l of serum-free RPMI containing with/ without 1 μ M MK2206. The migrated cells were counted after 24h. (A) GALNT2-knockdown increased the number of invaded cells compared with siC group, but the addition of MK2206 would abolish this phenomenon. (B) The number of invaded cells was calculated at 4 random fields per experiment. ($\times 100$) and expressed as fold change of siC without MK2206 treatment. Results are represented as mean \pm S.D. from three independent experiments. *P < 0.05.

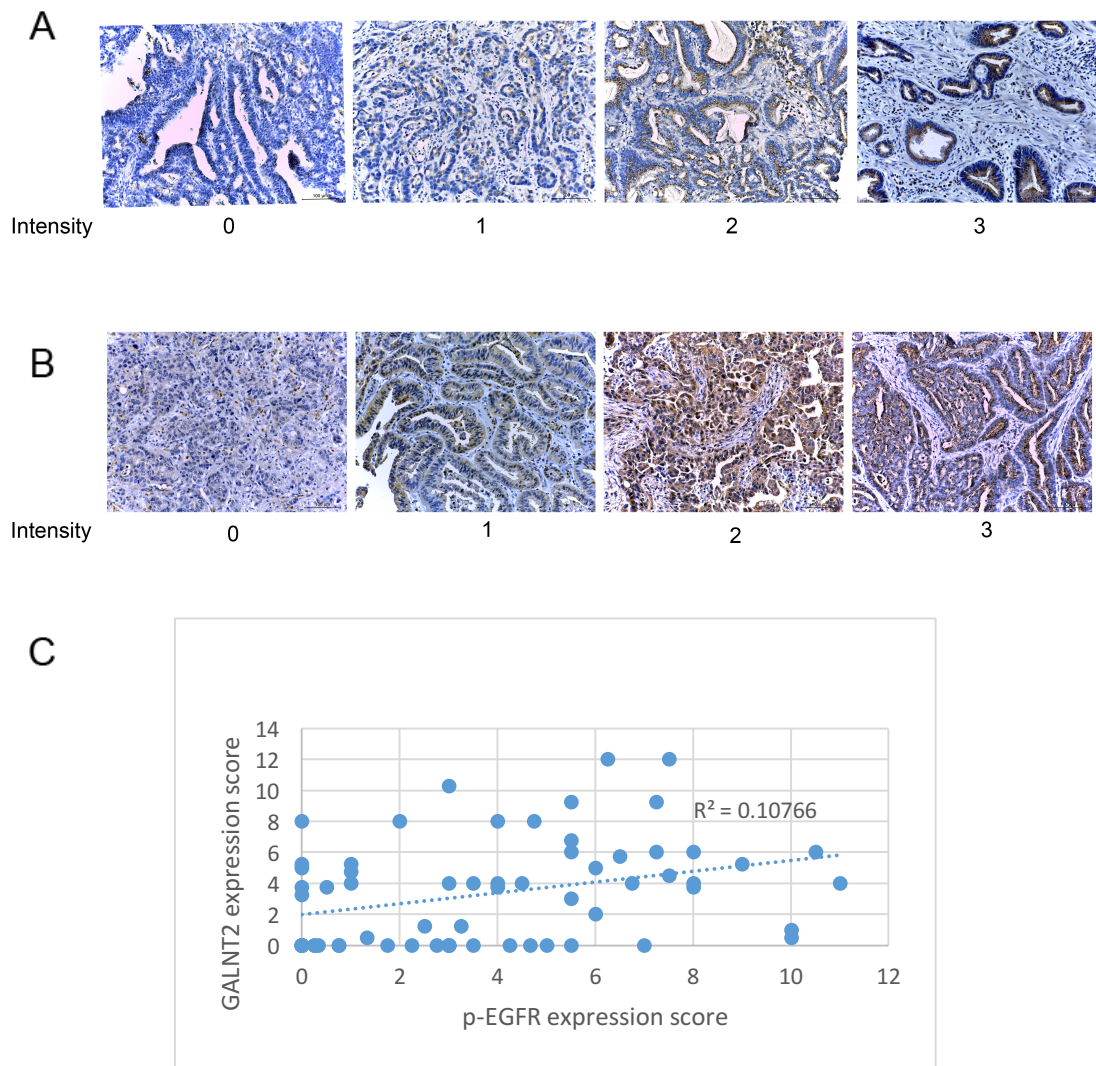


Figure 11. Correlation between GALNT2 and p-EGFR expression.

(A) Representative IHC staining of GALNT2 (B) Representative IHC staining of p-EGFR. ; No staining = 0, weak staining =1, moderate staining = 2, strong staining = 3

(C) The expression of GALNT2/p-EGFR equals IHC intensity multiplies staining percentage. p-EGFR was positively correlated with GALNT2. The Pearson correlation coefficients (r) was 0.3281, p=0.0076

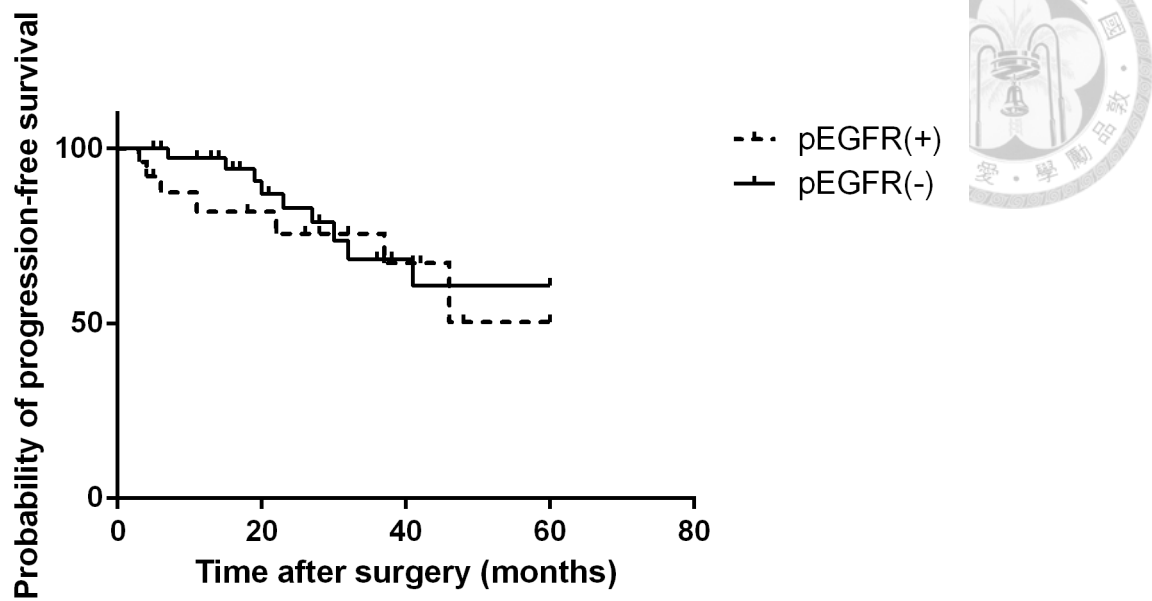


Figure 12. Kaplan-Meier survival analysis of progression-free survival in gastric cancer patients with p-EGFR(+) or p-EGFR(-). Of the 65 patients, 26 (40%) were p-EGFR positive and 39 (60%) were p-EGFR negative. The 5-year PFS was 50.4% for p-EGFR positive and 60.3% for p-EGFR negative group, and there was no difference between two groups. ($p=0.2849$)

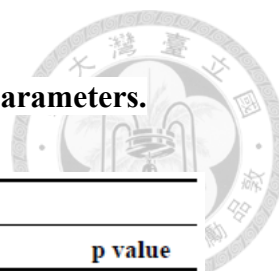


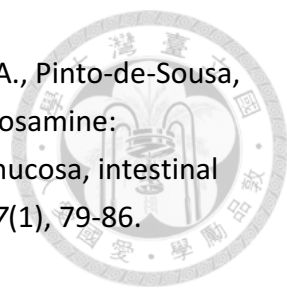
Table 1. Correlation between p-EGFR and clinico-pathological parameters.

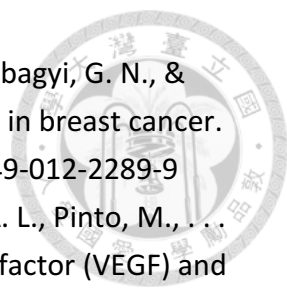
	p-EGFR			p value
	Total	Positive	Negative	
Age,mean (range)	n=70	69.4 (38-84)	71.2 (43-89)	0.5779
Differentiation	n=69			0.4099
Well-differentiation		10 (32%)	10 (26%)	
Mod-differentiation		16 (52%)	25 (66%)	
Poor-differentiation		5 (16%)	3 (8%)	
Size	n=68			0.587
< 5cm		17 (57%)	24 (63%)	
≥ 5cm		13 (43%)	14 (37%)	
Lymphovascular invasion	n=68			0.7401
Absent		13 (43%)	18 (47%)	
Present		17 (57%)	20 (53%)	
Neural invasion	n=68			0.9541
Absent		16 (53%)	20 (53%)	
Present		14 (47%)	18 (47%)	
Primary tumor	n=70			0.2403
T1		10 (32%)	11 (28%)	
T2		5 (16%)	12 (31%)	
T3		8 (26%)	12 (31%)	
T4		8 (26%)	4 (10%)	
Regional lymph node	n=70			0.7991
N0		11 (35%)	18 (46%)	
N1		8 (26%)	7 (18%)	
N2		6 (19%)	7 (18%)	
N3		6 (19%)	7 (18%)	
Distant metastasis	n=70			0.425
M0		29 (94%)	38 (97%)	
M1		2 (6%)	1 (3%)	
Stage	n=70			0.7147
I		20 (50%)	12 (40%)	
II		7 (18%)	7 (23%)	
III		12 (30%)	9 (30%)	
IV		1 (3%)	2 (7%)	




VII. References

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