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# Sialylation of epidermal growth factor receptor regulates receptor activity and chemosensitivity to gefitinib in colon cancer cells

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ABSTRACT

β-Galactoside α2,6-sialyltransferase (ST6Gal-I) has been shown to catalyze α2,6 sialylation of N-glycan, an action that is highly correlated with colon cancer progression and metastasis. We have recently demonstrated that ST6Gal-I-induced α2,6 sialylation is critical for adhesion and migration of colon cancer cells. Increase of α2,6 sialylation also contributes to radioresistance of colon cancer. A number of studies have focused on the involvement of sialylation in tumorigenesis, but the mechanism underlying ST6Gal-I-induced cancer progression and the identity of enzyme substrates has received scant research attention. To provide further support for the relevance of ST6Gal-I in the malignancy of colon cancer, we prepared and characterized a ST6Gal-I-knockdown SW480 colorectal carcinoma cell line. We found that inhibition of ST6Gal-I expression increased cell proliferation and tumor growth *in vitro* and *in vivo*. An examination of the effect of sialylation on epidermal growth factor receptor (EGFR) activity and downstream signaling, which are highly correlated with cell proliferation, showed that the loss of ST6Gal-I augmented EGF-induced EGFR phosphorylation and activation of extracellular signal-regulated kinase (ERK) in colon cancer cells. Moreover, ST6Gal-I induced sialylation of both wild type and mutant EGFR. These studies provide the first demonstration that ST6Gal-I induces EGFR sialylation in human colon cancer cell lines. Importantly, the anticancer effect of the EGFR kinase inhibitor, gefitinib, was increased in ST6Gal-I-deficient colon cancer cells. In contrast, overexpression of ST6Gal I decreased the cytotoxic effect of gefitinib. These results suggest that sialylation of the EGFR affects EGF-mediated cell growth and induces chemoresistance to gefitinib in colon cancer cells.



## 1. Introduction

Asparagine (N)-linked glycosylation is highly regulated process that produces a large and diverse repertoire of cellular glycans that are mostly attached to proteins [1]. Abnormal glycosylation is known to be associated with cancer malignancy [2]. Among the sugars found on the cell surface are sialic acids, which exist as terminal monosaccharide attached to cell surface glycan chains. The variety of sialic acid decorations on the cell surface governs many biological processes, including cell recognition, cell adhesion, receptor activation, and signal transduction [3]. Studies performed over the last decade have focused on the involvement of sialylation in the progression of cancer [2,3], but the actual function of sialylation in tumorigenesis has received much less research attention.

The presentation of sialic acids in cell membranes is a general phenomenon, one that reflects a process of end-capping of N-glycan by sialic acids catalyzed by numerous sialyltransferases. Among the glycosyltransferase important in adding sialic acid residues to N-linked oligosaccharides is ST6Gal-I (β galactoside α2,6-sialyltransferase, CMP-NeuAc: Galβ (1,4) GlcNAc: α2,6-sialyltransferase) [4]. Recent studies and clinical reports have emphasized the importance of ST6Gal-I in colon cancer progression and metastasis. ST6Gal-I is highly up-regulated in colon adenocarcinomas and its expression is positively associated with colon cancer cell migration and invasion [5–8]. Specifically, patients with metastasizing tumors have high levels of ST6Gal-I, and the levels of ST6Gal-I are correlated with the progression of colorectal carcinomas and cancer [7,9–12].

Glycoproteins may have many sites of N-glycan addition, and each site can potentially be modified by numerous different N-glycan structures [1]. Recently, the epidermal growth factor

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receptor (EGFR) was identified as one of the sialylated glycoproteins in human lung cancer [13]. The EGFR is a 170-kDa glycoprotein with an extracellular ligand-binding domain and an intracellular region that possesses tyrosine kinase activity. EGFR activation on cancer cells is highly correlated with cell proliferation, differentiation, cell survival, drug and radiation sensitivity, and angiogenesis [14]. High levels of EGFR expression have been associated with diminished overall survival in colon cancer patients [15]. Because its activity is correlated with tumor progression, the EGFR has been the target of anticancer drug development efforts [16]. In fact, EGFR-targeted therapy is one of the most fundamental strategies applied in cancer patients, and novel anticancer drugs targeting members of the EGFR family have been tested against a variety of human cancers [16,17]. One class of drugs used to target the EGFR is tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, which are able to reduce tumor growth and metastasis in a range of human cancer cell lines and human tumor xenografts. In a clinical setting, gefitinib treatment has been approved for various types of cancer [14,16,17].

It has been shown that sialylation and fucosylation are capable of regulating EGFR activity [13,18,19]. In addition, removal of sialic acids by sialidase can activate the EGFR [20]. Thus, understanding the regulation of EGFR glycosylation may provide novel insights into cancer biology and suggest possible therapeutic strategies. However, the identity of sialyltransferases responsible for sialylation of the EGFR and the effects of sialyltransferase-induced EGFR sialylation on the sensitivity of EGFR-targeting drugs in colon cancer are largely unknown.

Previously, we have demonstrated that ST6Gal-I induces adhesion and migration, and promotes radioresistance and protection from apoptosis in colon cancer cells [21–23]. However, the possible biological role of ST6Gal-I in this context and the identity of its substrates have not been clearly established. To provide further support for the relevance of ST6Gal-I in the malignancy of colon cancer, we prepared and characterized a ST6Gal-I-knockdown SW480 colorectal carcinoma cell line. We found that ST6Gal-I down-regulation increased cell proliferation and tumor growth *in vitro* and *in vivo*. Given that EGFR activity and downstream signaling are highly correlated with cell proliferation, we investigated sialylation of the EGFR and studied its effects on EGF-mediated signaling events and the sensitivity of human colon cancer cells to the EGFR-targeting anticancer drug, gefitinib. We found that loss of ST6Gal-I augmented EGF-induced EGFR phosphorylation and activation of extracellular signal-regulated kinase (ERK) in SW480 and HT-29 cell lines. Using wild-type and ST6Gal-I-knockdown SW480 cells as well as SW480 cell lines stably overexpressing ST6Gal-I, we showed that ST6Gal-I induced sialylation of EGFR and further demonstrated that the anticancer effect of gefitinib was increased in ST6Gal-I-deficient SW480 colon cancer cells. We further examined the sialylation of EGFR and their effect on gefitinib sensitivity in HT-29, HCT116 and SW48 cell lines. Knockdown of ST6 Gal-I in HT-29 and HCT116 increased the cell-death effect of gefitinib. In contrast, sialylation of EGFR in SW48 cells reduced the anticancer activity of gefitinib. Collectively, these results suggest that sialylation of the EGFR affects EGFR-mediated cell growth and sensitivity to the EGFR inhibitor, gefitinib, in human colon cancer cells. In addition, we suggest that EGFR sialylation level, together with EGFR expression level and the presence of EGFR mutations, may be a reliable biomarker for anti-EGFR therapy.

## 2. Materials and methods

### 2.1. Cell culture, transfection, and treatment

SW480 (ATCC®CCL-228) and SW48 (ATCC®CCL-231) cells were grown in Dulbecco's Modified Eagle Medium supplemented with heat-inactivated 10% fetal bovine serum (FBS) and antibiotics. Lovo (ATCC®CCL-229) were grown in F-12K medium. HT-29 (ATCC®HTB-38) and HCT116 (ATCC®CCL-247) were grown in McCoy's 5a medium. A predesigned small interfering RNA (siRNA) for ST6Gal-I was purchased from Dharmacon (Lafayette, CO, USA). The shRNA against ST6Gal-I was purchased from Sigma (St. Louis, MO, USA). Cells were transfected with ST6Gal-I plasmids [21,22] using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). A pooled population of clones stably expressing shRNA was generated by puromycin selection. Gefitinib was provided by AstraZeneca (Wilmington, DE, USA).

### 2.2. Three-dimensional cell culture and TUNEL assay

A dermal equivalent was prepared by mixing human dermal fibroblasts ($5 \times 10^4$ cells/ml) from foreskin with a type I collagen (Col I) gel matrix, reconstituted according to the manufacturer's specifications (Nitta Gelatin Inc., Tokyo, Japan) and plating 250 μl of the mixture onto 12-mm polycarbonate filter chambers (3.0 μm Millicell; Millipore, Billerica, MA, USA). SW480 cells were seeded at a density of $1 \times 10^5$ cells per dermal equivalent and cultured in growth medium, first in a submerged state for 7 days and then in an air–liquid interface state for 7 days. Three-dimensional (3D) cultures were fixed in Carnoy's solution (ethanol:chloroform:acetic acid, 6:3:1) for 30 min at 4 °C. Fixed samples were embedded in paraffin and sectioned (3 μm). For the detection of apoptosis, sections were deparaffinized in a xylene-ethanol gradient, rinsed in phosphate-buffered saline (PBS), and incubated for 5 min with 3% (v/v) $H_2O_2$ in methanol at room temperature. TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assays (ApopTag®peroxidase *In situ* Apoptosis detection kit, Chemicon, Temecula, CA, USA) were performed according to the manufacturer's protocol. Sections were dehydrated, mounted with coverslips using Synthetic Mountant (Shandon, Cheshire, UK), and observed under a light microscope (Olympus BX-FLA instrument, Olympus, PA, USA).

### 2.3. Immunoblotting, lectin affinity assay, and immunoprecipitation

Proteins were immunodetected using the following commercial antibodies: anti-integrin β1 (BD Transduction laboratories, San Jose, CA, USA); anti-phospho-EGFR$^{Y1046}$, anti-phospho-ERK, anti-ERK, anti-HA (Cell Signaling Technology, Danvers, MA, USA); anti-Flag (Sigma, St. Louis, MO, USA); anti-ST6Gal-I (IBL, Japan); anti-EGFR, anti-actin, anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and anti-phosphotyrosine (4G10®; Upstate Biotechnology, Lake Placid, NY, USA). For detection of sialylated proteins, cell lysates were incubated with a biotinylated form of the lectin, *Sambucus nigra* agglutinin (SNA; Vector Laboratories, Burlingame, CA, USA), and protein–lectin complexes were precipitated with avidin-coated protein A-agarose (Sigma, St. Louis, MO, USA). α2,6-Sialylated EGFR was detected using a lectin affinity assay by immunoprecipitating lysates with an anti-EGFR antibody, and then subjecting immunoprecipitates to immunoblotting using biotinylated SNA and horseradish peroxidase-conjugated streptavidin. α2,3 sialylation was similarly detected using a biotinylated form of the lectin, MAA (*Maackia amurensis* agglutinin, Vector Laboratories, Burlingame, CA, USA).

### 2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with Trisol® reagents (Gibco BRL, Rockville, MD, USA) and reverse transcribed using Omniscript transcriptase (Qiagen, Hilden, Germany). PCR amplifications were performed using the following primer pairs: ST6Gal-I, 5′-AAA AAC CTT ATC CCT AGG CTG C-3′ (sense) and 5′-TGG TAG TTT TTG TGC

CCA CA-3′ (antisense); GAPDH, 5′-CAT GGA GAA GGC TGG GGC TCA TTT-3′ (sense) and 5′-CGC CAG TAG AGG CAG GGA TGA TGT-3′ (antisense). Thermocycling conditions were 95 °C for 5 min, followed by 30 cycles of 60 °C for 30 s (annealing) and 72 °C for 30 s (extension) [21].

### 2.5. Cell viability assay

The growth response to EGF stimulation was evaluated using the tetrazolium dye-based MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Cells were seeded at a density of $2 \times 10^3$ cells/well in 96-well plates under reduced-serum conditions. Twenty-four hours later, cells were stimulated with EGF (R&D Systems, Minneapolis, MN, USA) at 20 ng/ml. After incubating cells for 48–72 h at 37 °C, the MTT solution was added to each well and plates were incubated for 3 h at 37 °C. The medium in each well was then discarded, and 200 μl of dimethylsulfoxide was added to each well. The optical density of the resulting solution was measured at 570 nm in a microplate reader. For growth curve experiments, cells were seeded at a density of $2 \times 10^3$ cells/well in 96-well plates in the presence of 10% FBS or under reduced-serum conditions in the presence of 20 ng/ml of EGF. Cell proliferation was estimated by measuring the absorbance at 570 nm at 24 h intervals up to 72 h. Determination of the half maximal inhibitory concentration ($IC_{50}$) was also performed using MTT assay. Exponentially growing cells were plated in 96-well plate at a density of $2 \times 10^3$ cells/well and 20 μl of gefitinib solution at various concentrations was added. After incubation for 48 h at 37 °C, 20 μl of MTT solution (5 mg/ml) was added to each well and the plates were incubated further for 3 h at 37 °C. The optical density was measured at 570 nm. $IC_{50}$ was defined as the drug concentration yielding a fraction of affected (no survival) cells = 0.5, compared with of vehicle-treated control. Each value represents the mean ± SD of at least three determinations.

### 2.6. Flow cytometry and immunofluorescence

For the detection of α2,6 sialylation, cells were detached with trypsin/EDTA at the indicated times and stained with fluorescein isothiocyanate (FITC)-conjugated SNA (FITC-SNA; Vector Laboratories, Burlingame, CA, USA). After staining, fluorescence intensity was analyzed by fluorescence-activated cell sorting (FACS), and fluorescent images were photographed under a fluorescence microscope. For detection of cell surface EGFR, cells were harvested after treatment with 10 ng/ml of EGF for 10, 30, and 60 min. Cells were then incubated with Phycoerythrin (PE)-conjugated EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min. After washing three times with PBS, cell surface EGFR was analyzed by flow cytometry. Each value represents the mean ± SD of three determinations.

### 2.7. Tumor xenograft

SW480 cells ($2 \times 10^6$) were resuspended in 100 μl PBS and injected subcutaneously into the flanks of athymic nude mice (Balb/C, Charles River, Japan). Tumors were measured every 5 days with precision calipers, and animals were sacrificed 40 days after injection. Primary tumor volumes were calculated using the formula, $V = \text{length} \times (\text{width})^2/2$. All animal experiments were conducted according to the guidelines for the ethical use of animals of our institution under an approved protocol (KIRAMS 2011–12).

### 2.8. Statistical analysis

Data are expressed as means ± standard deviations (SDs). Statistical significance was determined using Student's *t*-test for comparisons between two means. The null hypothesis was rejected in cases where *p*-values were $<0.05$.

## 3. Results

### 3.1. Effect of ST6Gal-I expression status on the growth of SW480 human primary colorectal carcinoma cells

In our previous study, we established an ST6Gal-I-overexpressing clone of SW480 human colon cancer cells, an early stage colon adenocarcinoma (Duke stage B) cell line that has initially low enzymatic activity of ST6Gal-I, to evaluate the role of ST6Gal-I-induced sialylation in regulating colon cancer development [21–23]. We demonstrated that ST6Gal-I induced cell adhesion and migration, and promoted radioresistance of colon cancer cells. To provide further evidence for the relevance of ST6Gal-I in the process of malignancy, we prepared and characterized ST6Gal-I-knockdown SW480 colorectal carcinoma cell lines. Endogenous ST6Gal-I was stably knocked down by infecting SW480 cells with lentiviral shRNA constructs against ST6Gal-I followed by selection with puromycin, creating SW480-sh ST6Gal-I cells. Down-regulation of ST6Gal-I was confirmed by RT-PCR analyses (Fig. 1A). Consistent with this, the level of α2,6 sialylation, demonstrated by detection of FITC-labeled SNA by immunofluorescence microscopy, was much lower in ST6Gal-I-knockdown cells than in control cells (SW480-shv). In contrast, ST6Gal-I-overexpressing cells (SW480-ST6Gal-I #6) were stained strongly by FITC-labeled SNA compared to SW480-shv controls (Fig. 1B). The total level of α2,6 sialylation was also confirmed by flow cytometry (Fig. 1C). Importantly, we found that ST6Gal-I affected cell proliferation and growth *in vitro* and *in vivo*. As shown in Fig. 2A, cell numbers were significantly higher for SW480-sh ST6Gal-I clones than for the vector-transfected control (SW480-shv control) cell line. Cell numbers increased only slightly for the ST6Gal-I-overexpressing cell line during the course of the experiment. To test the ability of ST6Gal-I to regulate tumor growth *in vivo*, we performed xenograft experiments using ST6Gal-I-deficient (SW480-sh ST6Gal-I) and overexpressing (SW480-ST6Gal-I #6) stable cell lines. Each cell line was subcutaneously injected into athymic nude mice, and tumor volume was examined every 5 days. Critically, tumor growth was extremely minimal in mice that received the SW480 control cell line (SW480-shv). Whereas tumors developed from ST6Gal-I-overexpressing cell lines showed a slight increase compared with control tumors, tumor growth in mice injected with the ST6Gal-I-deficient cell line (SW480-sh ST6Gal-I) was dramatically increased (Fig. 2B). Taken together, these data strongly link ST6Gal-I with the regulation of colon cancer cell proliferation and tumor growth.

### 3.2. Association of ST6Gal-I knockdown with increased EGF-induced EGFR phosphorylation, downstream ERK activation, and EGFR internalization

EGFR activation in cancer cells is highly relevant to cell growth, cell survival, drug and radiation sensitivity, and metastasis [16]. High levels of EGFR expression have been associated with diminished overall survival in colon cancer patients [14,17]. Accordingly, to determine whether ST6Gal-I might regulate cell proliferation and tumor growth through effects on EGF-induced EGFR activation, we next compared EGFR phosphorylation upon EGF stimulation in cells transiently transfected with siRNA against ST6Gal-I (si-ST6Gal-I) or control siRNA (si-control) in SW480 and HT-29 cells. After knocking down ST6Gal-I expression in both of SW480 and HT-29 cell lines, we treated cells with EGF (10 ng/ml) for 5 min, and then examined cells for EGFR tyrosine phosphorylation. Immunoprecipitation of cell extracts with an anti-EGFR

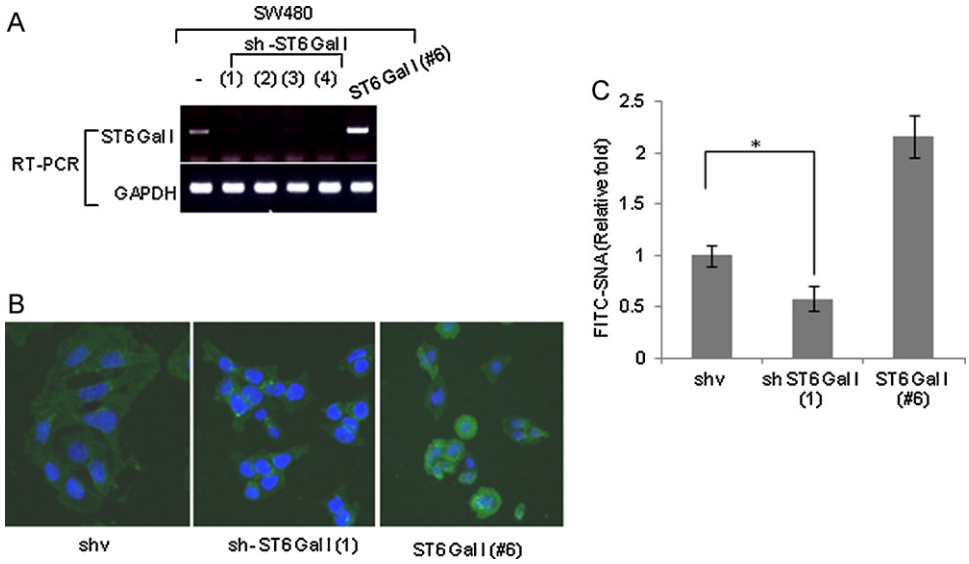


**Fig. 1.** The decrease of α2, 6 sialylation by inhibition of ST6Gal I expression. (A) SW480 cells were transduced with a lentivirus containing either an shRNA sequence against ST6Gal-I (SW480-shST6Gal-I) or an empty vector (SW480-shv). Clones stably expressing shRNA were selected by treatment with puromycin. Cells stably overexpressing ST6Gal-I (SW480-ST6Gal-I #6) were previously established [21–23]. Knockdown of ST6Gal-I was analyzed by RT-PCR. (B and C) Cells were stained with FITC-SNA, and then assessed for α2, 6 sialylation of the cell surface by fluorescence microscopy (B) and flow cytometry (C).

antibody following by immunoblotting with the anti-phosphotyrosine antibody (4G10), showed that EGFR tyrosine phosphorylation was increased in si-ST6Gal-I-treated cells compared to si-control-treated cells. Consistent with this, si-ST6Gal-I-treated cells showed higher levels of phospho-EGFR detected by a specific anti-phospho-EGFR$^{Y1068}$ antibody (Fig. 3A). To determine whether ST6Gal-I also regulates EGFR-mediated intracellular signaling, we examined the phosphorylation status of the downstream EGFR signaling molecules, ERK1/2. EGF-induced ERK1/2 phosphorylation levels were greatly enhanced by ST6Gal-I knockdown in both of SW480 and HT-29 cell lines (Fig. 3A). Next, we reconfirmed EGF-induced tyrosine phosphorylation of EGFR and downstream ERK1/2 activation in stable ST6Gal-I-knockdown cells (SW480-sh ST6Gal-I) and ST6Gal-I-overexpressing cells (SW480-ST6Gal-I-#6). As shown in Fig. 3B, EGF-induced tyrosine phosphorylation of EGFR and activation of ERK1/2 were augmented by the loss of ST6Gal-I expression. In contrast to the case of ST6Gal-I depletion, overexpression of ST6Gal-I reduced EGFR tyrosine phosphorylation and activation of ERK1/2 in SW480 and SW48 (ST6Gal I$^{-/-}$) cells (Fig. 3B and C).

In growth curve experiments, SW480-sh ST6Gal-I clones showed markedly increased proliferative activity in the presence of EGF stimulation (Fig. 3D). It is generally thought that the initial steps leading to EGFR activation involve ligand-induced conformational changes of the extracellular domain, followed by receptor dimer formation and internalization into the cell [16]. To understand how EGF-induced EGFR activation was accelerated by ST6Gal-I depletion, we next analyzed the amount of cell surface EGFR upon EGF stimulation in SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I, and SW480-shv controls. Consistent with the observed increase in EGF-induced EGFR phosphorylation, rapid decrease of cell membranous EGFR was shown in ST6Gal-I-knockdown cell lines (Fig. 3E). SW620 cells were used as negative control of EGFR. These results suggest that EGFR phosphorylation and activation of ERK in ST6Gal-I-depleted cells is due to increased EGFR internalization. On the basis of these findings, we suggest that knockdown of ST6Gal-I and subsequent loss of α2,6 sialylation accelerates EGFR phosphorylation, internalization of receptor, and increases cellular growth in response to EGF in human colon cancer cells.

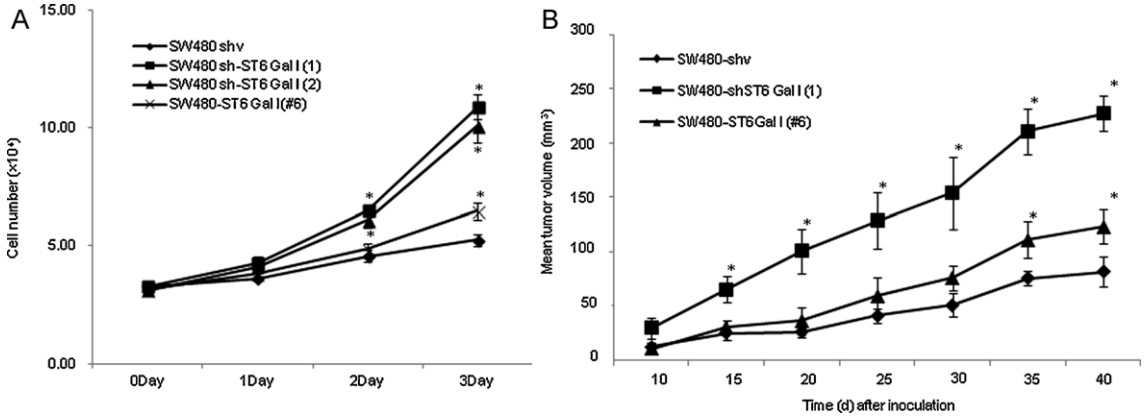


**Fig. 2.** The effect of ST6Gal-I knockdown on SW480 colon cancer cell growth. (A) SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I (SW480-ST6Gal-I #6), and SW480-shv (controls) cells were seeded in 24-well plates and counted daily. The graph is an average of three separate experiments, each of which was done in triplicate. *$p < 0.05$ *versus* SW480-shv control cells. (B) SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I, and vector control (SW480-vector) cells were resuspended in PBS at $2 \times 10^6$ cells/100 μl and injected subcutaneously into the flanks of athymic nude mice. Tumor volumes were measured every 5 days using precision calipers. Animals were sacrificed 40 days after injection, at which time tumors were collected.

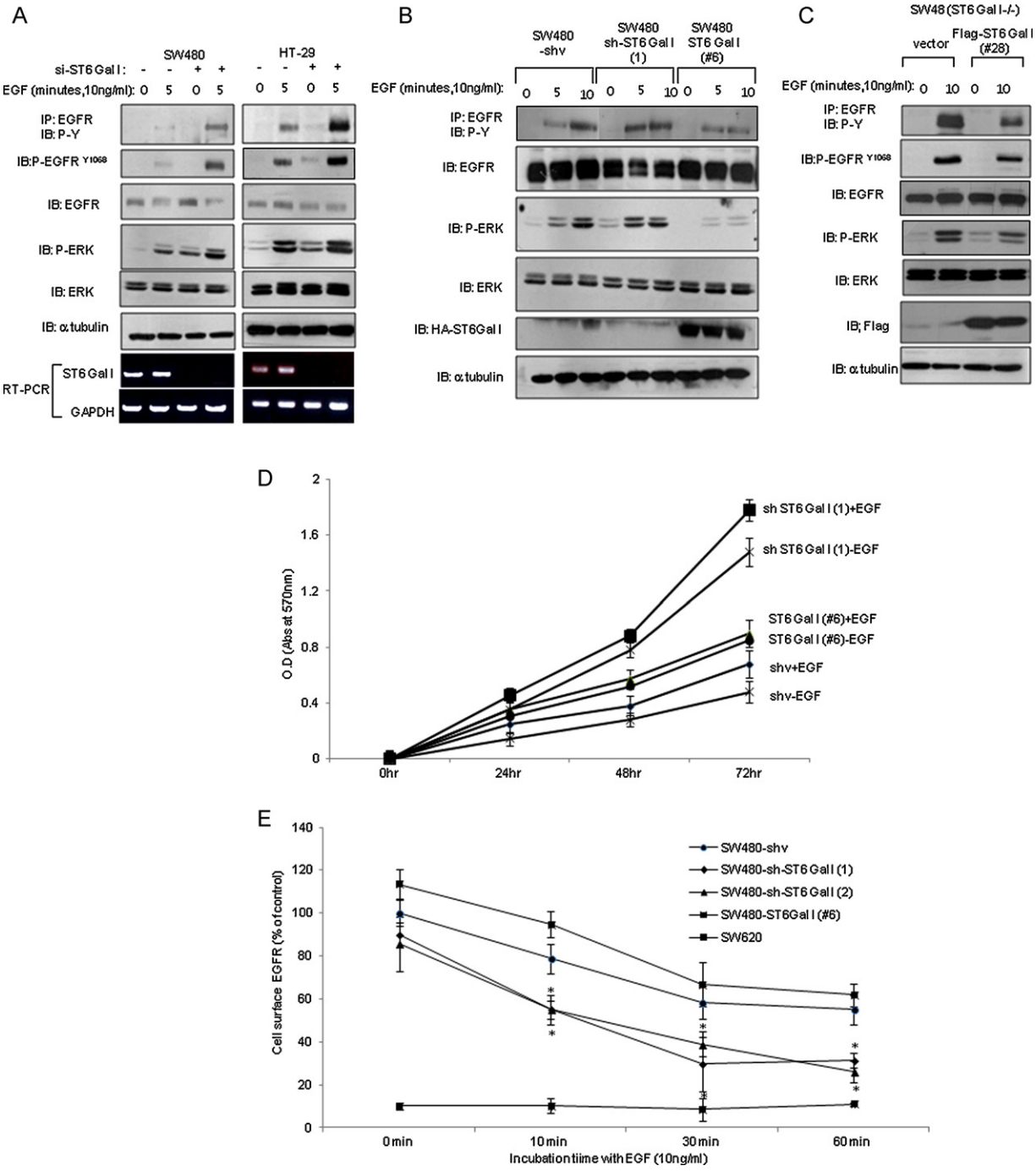


**Fig. 3.** Increased EGF-mediated phosphorylation of EGFR and subsequent activation of ERK in ST6Gal-I-knockdown colon cancer cells. (A) SW480 and HT-29 cells were transfected with siRNA against ST6Gal-I, and then treated with EGF (10 ng/ml) for 5 min under reduced-serum conditions. Cell lysates were immunoprecipitated with an anti-EGFR antibody and immunoblotted using an anti-phospho-tyrosine antibody to detect tyrosine phosphorylated EGFR. Phospho-EGFR$^{Y1046}$ and phospho-ERK were also detected by immunoblotting with antibodies specific for phospho-EGFR$^{Y1046}$ and phospho-ERK respectively. Knockdown of ST6Gal-I was confirmed by RT-PCR. (B) SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I, and SW480-shv control cells were treated with 10 ng/ml EGF in reduced-serum culture media, and then EGF-induced EGFR phosphorylation and downstream ERK phosphorylation were determined by immunoblotting. (C) SW48 cells were stably transfected with Flag-tagged ST6 Gal-I plasmid, and then treated with EGF (10 ng/ml) for 10 min. Cell lysates were immunoprecipitated with an anti-EGFR antibody and immunoblotted using an anti-phospho-tyrosine antibody. Phospho-EGFR$^{Y1046}$and phospho-ERK were detected by immunoblotting. (D) Cell growth experiments in SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I, and SW480-shv control cells. Cells were cultured in the absence or presence of EGF (10 ng/ml) under reduced-serum conditions. Cell growth was analyzed by MTT assay as described in Section 2. OD value, 570 nm. (E) SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I, and SW480-shv control cells were treated with EGF. After EGF treatment, cells were labeled with phycoerythrin-EGFR, and then internalization of cell surface EGFRs was analyzed by flow cytometry.

### 3.3. α2,6 sialylation of EGFR by ST6Gal-I in human colon cancer cells

N-glycoslyation sites have been identified in the extracellular domain of EGFR [20,24–27]. To confirm N-linked glycosylation in EGFR, we performed enzymatic deglycosylation using PNGase F, which removes almost all N-linked oligosaccharides. Following PNGase F digestion, EGFR migrated to a lower molecular-weight position on SDS polyacrylamide gels (Fig. 4A), indicating that EGFR is highly modified by N-glycosylation. Integrin β1, used as a positive control for the deglycosylation reaction, also exhibited band shifting.

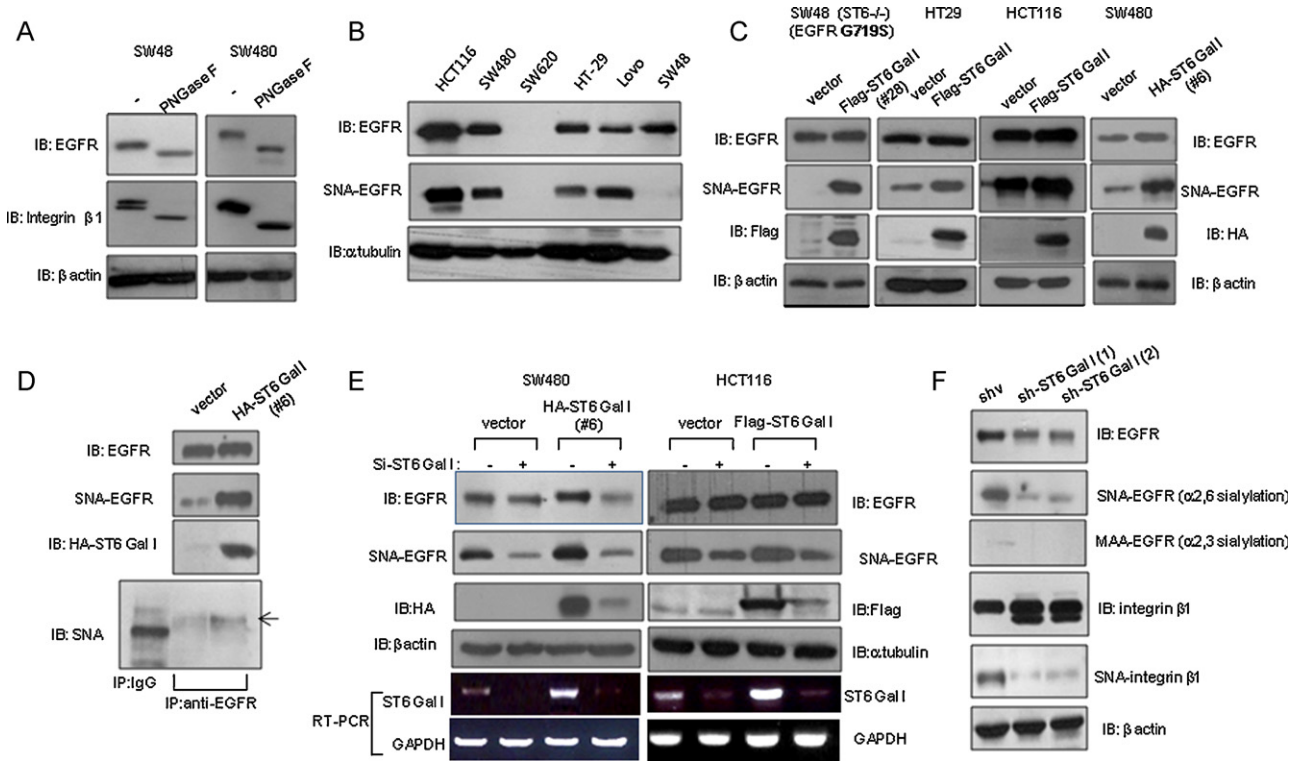


**Fig. 4.** Sialylation of both wild type and mutant EGFR by ST6Gal-I in human colon cancer cells. (A) SW48 (ST6Gal-I$^{-/-}$) and SW480 human colorectal carcinoma cells were harvested and digested with PNGase. After the deglycosylation reaction, lysates were examined by immunoblotting. (B) Human colon cancer cell lines including HCT116, SW480, SW620, HT-29, Lovo, and SW48 were harvested and then sialylation of EGFR was determined by SNA lectin affinity assay. (C) ST6Gal I-1 was overexpressed in SW480, SW48, HCT116, and HT-29 colon cancer cell lines. Cell lysates from each cell lines were harvested and the levels of EGFR and ST6Gal-I protein were assessed by immunoblotting. Sialylation of EGFR was assayed by SNA lectin affinity assay, as described in Section 2. (D) EGFR and ST6Gal-I levels, and EGFR sialylation were determined in SW480 control cells (SW480-vector) and a previously established clone of SW480 cells stably expressing ST6Gal-I (SW480-ST6Gal-I #6). (E) SW480 and HCT116 cells over expressing ST6Gal-I and vector-control cells were treated with siRNA against ST6Gal-I, and then subjected to RT-PCR and immunoblotting to detect the level of ST6Gal-I expression. Sialylation of EGFR was assayed by SNA lectin affinity assay. (F) Cell lysates from SW480-shv and SW480-sh ST6Gal-I stable clones were harvested and the expression of EGFR and integrin β1 was analyzed. Lectin affinity assays were performed on the same lysates to detect EGFR and integrin β1 sialylation. SNA and MAA were used for the detection of α2, 6 and α2, 3 sialylation, respectively.

Most EGFR studies have focused on EGFR amplification, activating mutations, and the development of EGFR-TKIs [14,16,17], whereas regulation of EGFR activity *via* posttranslational modification such as glycosylation has garnered considerably less research attention. Although glycosylation, specifically fucosylation and sialylation, has been previously shown to modulate EGFR activity [13,18,19], no studies have addressed the relevance of EGFR sialylation in the context of colon cancers that highly express ST6Gal-I. Therefore, we sought to evaluate the role of ST6Gal-I in sialylation of EGFR and assess its impact on colon cancer progression *via* regulation of EGFR activity. We observed that α2,6 sialylation of EGFR was detected in human colon cancer cell lines including HCT116, SW480, HT-29, and Lovo (Fig. 4B). In the case of SW620 (EGFR-negative cell line) and SW48 (ST6Gal I-negative cell line) cells, there was no level of sialylated EGFR. Importantly, it raises the question about α2,6 sialylation of wild type and mutant EGFR and their effect on EGFR tyrosine kinase signaling cascades. Therefore, to elucidate the effect of ST6Gal-I activity in colon cancer cells, we stably transfected SW48 human colon cancer cells, which lack ST6Gal-I expression and also harbors mutant EGFR (G719S) [28], with ST6Gal-I. As shown in Fig. 4C, SW48 cells overexpressing ST6Gal-I showed increased sialylation of EGFR, measured by lectin affinity assay using biotinylated SNA (see Section 2). Similar results were obtained using ST6Gal-I-overexpressing SW480, HCT116, and HT-29, which are human colon cancer cells that express wild type EGFR (Fig. 4C) [29]. EGFR sialylation were also confirmed in SW480 control cells (SW480-vector) and a previously established clone of SW480 cells stably expressing ST6Gal-I (SW480-ST6Gal-I #6) by immunoprecipitation of EGFR and lectin blotting using biotinylated SNA and avidin-horseradish peroxidase (HRP) (Fig. 4D).

To confirm the ST6Gal-I-induced sialylation of EGFR, we treated ST6Gal-I-overexpressing cells and vector control cells with siRNA against ST6Gal-I. siRNA-mediated knockdown of ST6Gal-I (si-ST6Gal I) reduced EGFR affinity for SNA in SW480 and HCT116 cell lines (Fig. 4E). We also found that stable knockdown of ST6Gal-I decreased the level of EGFR sialylation (Fig. 4F). Interestingly, we found no evidence for α2,3 sialylation of EGFR in lectin (MAA) affinity assays. Collectively, these findings suggest that ST6Gal-I induces α2,6 sialylation of EGFR in human colorectal carcinoma cells.

### *3.4. Increased cytotoxic efficacy of gefitinib with loss of EGFR α2,6-sialylation*

Finally, we tested the effect of ST6Gal-I expression status on the anticancer efficacy of the EGFR kinase inhibitor, gefitinib [30]. To examine the half maximal inhibitory concentration ($IC_{50}$) of gefitinib in our experimental conditions, we performed the cell viability assay. The $IC_{50}$ value for gefitinib in SW480-shv controls, sh ST6Gal-I stable clone and stably overexpressing ST6Gal-I were shown in Fig. 5A. Based on the results of the evaluation of growth inhibition, we treated SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I (SW480-ST6Gal-I #6), and SW480-shv controls with 10 μM gefitinib for 48 h in growth media. Cell death, analyzed by propidium iodide staining, was

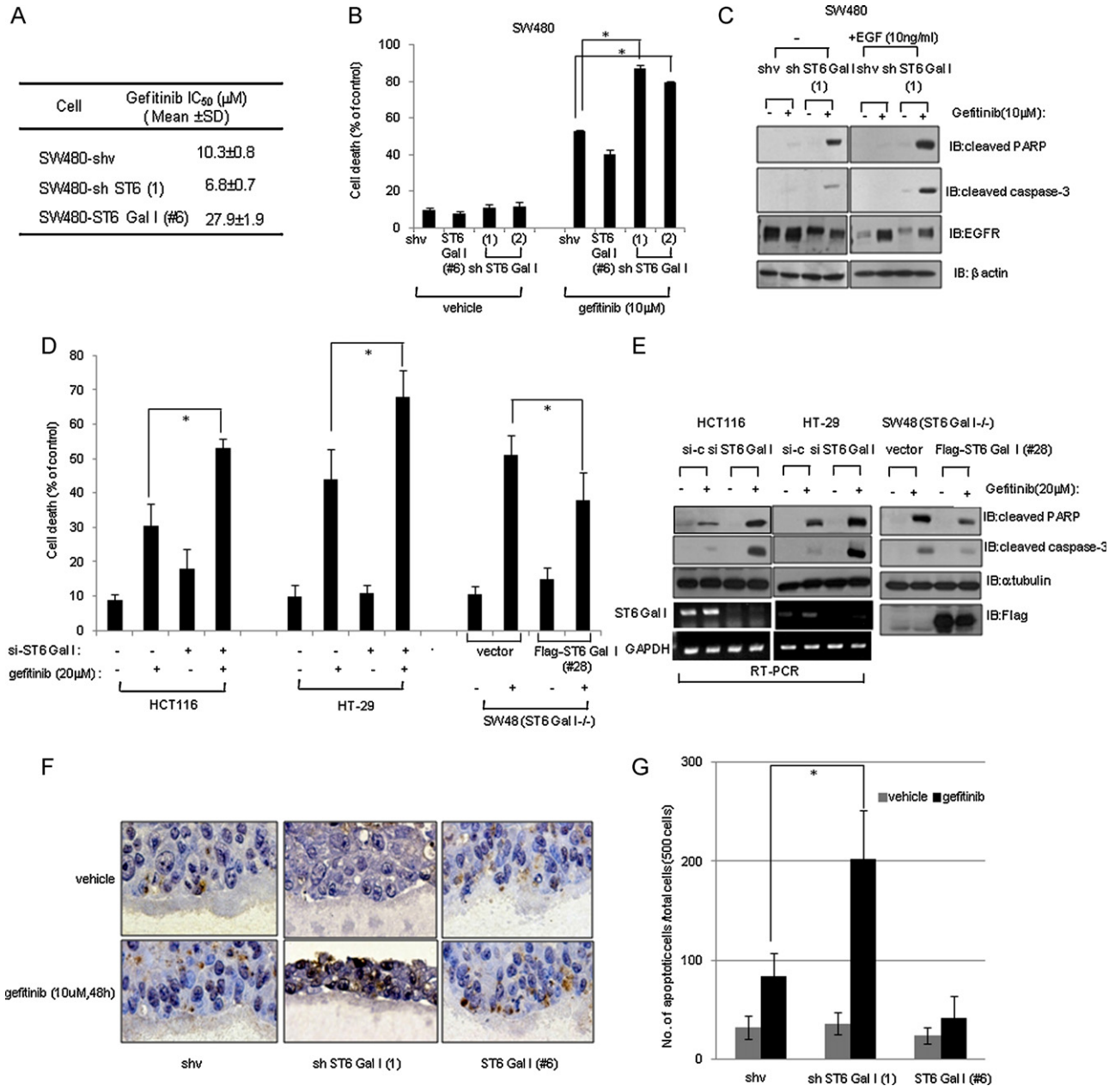


**Fig. 5.** Enhancement of gefitinib-induced cell death in ST6Gal-I-knockdown human colon cancer cells. (A) An exponentially growing cell suspension ($10^4$ cells/ml) was seed into 96 well plates. Cells were treated with increasing doses of gefitinib and cell viability was measured by MTT assay. $IC_{50}$ was calculated from three independent experiments. (B) ST6Gal-I-knockdown cells (SW480-sh ST6Gal-I-1), SW480 cells stably overexpressing ST6Gal-I (SW480-ST6Gal-I #6), and SW480 control (SW480-shv) cells were treated with gefitinib (10 μM) for 48 h in growth media, and then cell death were detected by FACs analysis after propidium iodide staining. (C) After treatment with gefitinib (10 μM, 24 h) in the presence or absence of EGF in reduced-serum culture media, cells were harvested and extracts were immunoblotted for cleaved PARP and caspase-3. (D) HT-29 and HCT116 cells were transfected with siRNA against ST6Gal-I, and then treated with gefitinib (20 μM) for 48 h in growth media, and then cell death were detected by FACs analysis after propidium iodide staining. SW48 cells were stably transfected with Flag-tagged ST6Gal I expression vector. Cells were treated with gefitinib (20 μM) for 48 h in growth media, and then cell death was detected by FACs analysis. (E) After treatment with gefitinib for 48 h, cells lysates were immunoblotted for cleaved PARP and caspase-3. (F) Gefitinib-induced apoptosis in a 3D culture system was analyzed by TUNEL assay, as described in Section 2. (G) Apoptotic cells were quantified by counting the number of TUNEL-positive cells in a total of 500 cells.

significantly increased in ST6Gal-I-depleted cells. The opposite effect was observed in ST6Gal-I-overexpressing cells (Fig. 5B). An examination of two apoptotic markers, poly-(ADP ribose) polymerase (PARP) and caspase 3, supported the conclusion that gefitinib-induced cell death was significantly increased by knockdown of ST6Gal-I, showing that PARP cleavage and caspase 3 activation were enhanced in EGF-treated ST6Gal-I-knockdown cells compared to SW480-shv control cells and, especially, ST6Gal-I-overexpressing SW480 cells (Fig. 5C). The same experiments were carried out using HT-29, HCT116 and SW48 cell lines. In order to provide the evidence of ST6Gal I activity in chemosensitivity of gefitinib in these cell lines, we first analyzed cell growth inhibition using various gefitinib concentration and determined the $IC_{50}$ value of gefitinib in HT-29 (20.1 μM), HCT116 (45.3 μM), and SW48 (19.6 μM) cell lines (data not shown). We observed that inhibition of ST6Gal I expression in HT-29 and HCT116 cells increased the gefitinib-induced cell death. Conversely, the effect of gefitinib was reduced in ST6Gal I overexpressing SW48 cells (Fig. 5D and E). Another confirmation was provided by an analysis of the apoptotic activity of gefitinib in a 3D culture system using TUNEL assays. SW480-sh ST6Gal-I stable clones, SW480 cells stably overexpressing ST6Gal-I (SW480-ST6Gal-I #6), and

SW480-shv controls were grown in a 3D culture system for 7 days, as described in Section 2, and treated with 10 μM gefitinib for 48 h. As shown in Fig. 5F and G, TUNEL-positive (apoptotic) cells were significantly increased in ST6Gal-I-knockdown cell lines. Collectively, these results suggest that gefitinib-induced apoptotic cell death is enhanced in the absence of EGFR sialylation.

## 4. Discussion

Studies have shown that a critical event in most colon cancers is up-regulation of ST6Gal-I expression and a subsequent increase in α2,6 sialylation at the cell surface [6,7,10]. Although ST6Gal-I activity has been closely linked to cancer progression and metastasis [7,9,12,31,32], studies reported to date have yet to identify ST6Gal-I substrates among various glycoproteins or clearly establish their functional activities. Colorectal cancer (CRC) is the leading cause of death from most of cancer. Notably, more than 50% of CRC-related deaths are due to metastasis [15,16,33,34]. As numerous reports have shown, the lethality of colon cancer reflects its ability to metastasize and evade apoptosis, thereby rendering it resistant to chemotherapy and radiotherapy [33]. In this context, targeting the regulation of metastasis is one of the most important strategies for cancer therapy after surgical resection. Recently, we and others demonstrated that ST6Gal-I is highly relevant to cancer cell adhesion, migration, invasion, and protection against apoptosis, showing, for example, that α2,6 sialylation of integrin β1 increases colon cancer cell migration [21–23,35–37]. We also showed that ST6Gal-I activity induces radioresistance in colon cancer. In this regard, tumoral ST6Gal-I expression or the degree of α2,6 sialylation of the cell surface may be considered to have prognostic value in colon cancer. Several sialyltransferase inhibitors have been developed and shown to exhibit potent anti-metastatic activity *in vitro* and *in vivo* [38,39]. Evidence for functional roles of ST6Gal-I in cancer progression suggests that targeting ST6Gal-I could be an effective strategy for inhibiting cancer metastasis and blocking the recurrence of cancer in secondary organs.

In this study, ST6Gal-I-knockdown SW480 colorectal carcinoma cells exhibited much more rapid proliferation and robust tumor growth compared to SW480 control cells, which showed a substantially diminished rate of tumor growth (Fig. 2), as previously reported by other groups [40–42]. Given that a high level of EGFR expression has generally been considered essential for tumorigenesis and diminished overall survival in colon cancer patients, we examined how EGF-induced EGFR phosphorylation and downstream ERK activation was affected by changes in ST6Gal-I expression status. We found that knockdown of ST6Gal-I enhanced EGFR phosphorylation and promoted more rapid ERK activation (Fig. 3A and B), in agreement with a previous report that sialidase affects cell proliferation and EGFR regulation [43]. In contrast, overexpression of ST6Gal I reduced EGFR tyrosine phosphorylation and activation of ERK1/2 in SW480 and SW48 (ST6Gal $I^{-/-}$) cells (Fig. 3B and C). To elucidate the relationship between sialylation of the EGFR and receptor function, we investigated the amount of cell surface EGFR. Consistent with reports that α2,6 sialylation affects the internalization of CD45, PECAM, and Fas receptors [37,44–46], we found that decrease of cell surface EGFR was more rapid in ST6Gal-I-knockdown cells (Fig. 3E) as compared with shv control cells, possibly reflecting the increased affinity of EGF for unsialylated EGFRs, more dimer formation between EGFRs that contains low level of sialic acids, and finally rapid internalization into the cells.

Importantly, the EGFR has been characterized as a sialylated glycoprotein in human lung cancer [13]. Although previous reports have indicated that sialylation and fucosylation can regulate EGFR activity [13,18,19,43], no research attention has been devoted to the study of enzymes primarily involved in sialylating EGFRs. Moreover, there has no investigation of the effect of EGFR-TKIs on sialylated EGFR in cancer. Here, we tested the hypothesis that ST6Gal-I-induced sialylation of EGFR affects EGFR activity and the anticancer efficacy of gefitinib in colon cancer. Gefitinib (Iressa, ZD-1893) is an active EGFR-TKI that blocks the signal transduction pathway implicated in the proliferation and survival of cancer cells [30]. Our results strongly suggest that ST6Gal-I knockdown in SW480, HT-29, and HCT116 cells potentiates the cell death effect of gefitinib. As shown in Fig. 5, gefitinib-induced apoptosis was substantially increased by ST6Gal-I depletion, as evaluated by propidium iodide staining and cleavage of the apoptotic markers, PARP and caspase-3. However, overexpression of ST6Gal I induced chemoresistance in SW480 and SW48 cells. Whereas tumors derived from sh-ST6Gal-I cells were fast growing and attained large volumes, unfortunately, xenografted parental SW480 cells showed a very low tumor-formation rate and extremely minimal tumor growth (average tumor volume, $<100\ mm^3$) (Fig. 1E), as previously reported by other groups [40–42]. Therefore, SW480 human colon cancer cell xenograft system was deemed unsuitable as an *in vivo* model for testing drug effects on tumor formation. As an alternative to *in vivo* experiments, we utilized a 3D culture system to test the anticancer effects of gefitinib. As shown in Fig. 5F, gefitinib induced a dramatic increase in the number of TUNEL-positive (apoptotic) cells in ST6Gal-I-knockdown cells. These results imply that ST6 Gal-I affects gefitinib sensitivity in colon cancer cells.

EGFR amplification and activating mutations of the EGFR are strongly associated with epithelial malignancy, a relationship that has given rise to therapeutic applications in many cancers [16,34,47]. While no beneficial biomarkers have yet been identified for anti-EGFR therapy, the presence of activating EGFR mutations has emerged as a relevant (and perhaps the only reliable) predictor of EGFR-inhibitor sensitivity [16,48,49]. Since the discovery of the benefit of EGFR-targeted therapy in cancer patients, there has been a growing awareness of the need to understand the mechanisms operating in tumors that eventually lead to resistance to anti-EGFR drugs. In this context, it has been demonstrated that localization of EGFRs to lipid rafts alters the responsiveness of cancer cells to gefitinib [50] showing that membrane localization of the EGFR plays a functional role in EGFR-TKI resistance. This latter study highlights the importance of investigating factors that determine EGFR-TKI sensitivity in addition to studying EGFR mutations and amplification. In addition, our data showed that ST6 Gal-I induced α2,6 sialylation of both wild type EGFR [29] and mutant EGFR (G719S) [28] in colon cancer cell lines (Fig. 4C). Importantly, EGF-induced EGFR activation and gefitinib-induced cell death was affected by ST6Gal I expression regardless of the presence of EGFR tyrosine kinase mutation (Figs. 3C and 5D, E). On the basis of our results, we suggest that ST6Gal-I overexpression in colon cancer could be a cause of resistance to anti-EGFR drugs. Moreover, the sialylation status of EGFR could be a reliable predictor of the efficacy of anti-EGFR therapy.

In conclusion, we have demonstrated that ST6Gal-I induces sialylation of the EGFR in human primary colorectal carcinoma. Loss of α2,6 sialylation promoted cell proliferation and tumor growth *in vitro* and *in vivo*. In addition, knockdown of ST6Gal-I increased the EGF-induced phosphorylation of EGFR and downstream activation of ERK. Importantly, the anticancer efficacy of the EGFR-TKI, gefitinib, was significantly increased in ST6Gal-I-deficient colon cancer cells. In contrast, ST6 Gal-I overexpression reduced the cell death effect of gefitinib. Overall, our work provides support for a potential role of ST6Gal-I-mediated EGFR sialylation in cell growth and sensitivity to chemotherapeutic agents. Together with the presence of activating EGFR mutations and increases in *EGFR* gene copy number, sialylation of EGFR could

represent a reliable biomarker for anti-EGFR therapy. Future studies on the combined effect of sialyltransferase inhibitors and chemotherapeutic agents/radiotherapy are warranted.

## Conflict of interest

All of the authors disclose no conflicts of interest.

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