Effect of sialylation on EGFR phosphorylation and resistance to tyrosine kinase inhibition

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Epidermal growth factor receptor (EGFR) is a heavily glycosylated transmembrane receptor tyrosine kinase. Upon EGF-binding, EGFR undergoes conformational changes to dimerize, resulting in kinase activation and autophosphorylation and downstream signaling. Tyrosine kinase inhibitors (TKIs) have been used to treat lung cancer by inhibiting EGFR phosphorylation. Previously, we demonstrated that EGFR sialylation suppresses its dimerization and phosphorylation. In this report, we further investigated the effect of sialylation on the phosphorylation profile of EGFR in TKI-sensitive and TKI-resistant cells. Sialylation was induced in cancer progression to inhibit the association of EGFR with EGF and the subsequent autophosphorylation. In the absence of EGF the TKI-resistant EGFR mutant (L858R/T790M) had a higher degree of sialylation and phosphorylation at Y1068, Y1086, and Y1173 than the TKI-sensitive EGFR. In addition, although sialylation in the TKI-resistant mutants suppresses EGFR tyrosine phosphorylation, with the most significant effect on the Y1173 site, the sialylation effect is not strong enough to stop cancer progression by inhibiting the phosphorylation of these three sites. These findings were supported further by the observation that the L858R/T790M EGFR mutant, when treated with sialidase or sialyltransferase inhibitor, showed an increase in tyrosine phosphorylation, and the sensitivity of the corresponding resistant lung cancer cells to gefitinib was reduced by desialylation and was enhanced by sialylation.

Epidermal growth factor receptor (EGFR), one of the most studied receptor tyrosine kinases, is a drug target for cancer therapy, because its kinase activity correlates with tumorigenicity (1). Under normal conditions, EGFR forms dimers upon ligand binding and induces kinase activation (2–6). The conformational change of EGFR from tethered to extended form induced by ligand binding involves the exposure of the interface, followed by dimerization, activation, and autophosphorylation (7). The phosphorylation code of EGFR determines the propensity of the downstream signaling network to regulate cell proliferation, survival, migration, and angiogenesis (8, 9).

In a significant fraction of patients with nonsmall cell lung cancer (NSCLC), especially patients in Asia and those with the adenocarcinoma subtype, mutations in the kinase domain of EGFR cause constitutive activation and have been identified as an important factor in EGFR dysregulation (10, 11). Particularly, mutation from leucine to arginine at position 858 (L858R) and, less significantly, deletion of exon 19 that eliminates four amino acids (LREA) account for ∼90% of the mutations involved in the constitutive activation of EGFR. These mutations are commonly found in patients with increased sensitivity to EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib (12–14). However, most patients with such mutations show resistance within months after TKI therapy, and >50% of them develop a second EGFR mutation, T790M, which confers TKI resistance by increasing the affinity for ATP and decreasing the affinity for TKIs (15–17).

Studies have demonstrated that the glycans on EGFR participate in the regulation of EGFR function. The number of N-glycans and the degree of branching can regulate the cell-surface expression of EGFR in response to N-acetyl-D-glucosamine (GlcNAc) supplementation (18). In addition, studies with site-directed mutagenesis indicate that the glycans on Asn420 and 579 prevent EGFR from ligand-independent dimerization (19–21), and knocking down/fucosylation enzyme responsible for the core fucosylation, attenuates EGFR phosphorylation and EGFR binding (22, 23). Moreover, our previous study revealed that sialylation and fucosylation suppress EGFR dimerization, autophosphorylation, and EGF-induced lung cancer cell invasion (24).

Here, we investigated the effect of sialylation on EGFR dimerization to understand how extracellular sialylation influences intracellular phosphorylation in both wild-type and mutant EGFR. Our biochemical data demonstrated that sialylation could suppress EGFR dimerization by attenuating its association with EGF, and sialylation could significantly and selectively suppress tyrosine phosphorylation and affect the levels of phosphoserine and phosphothreonine on EGFR. In EGFR mutants, especially L858R/T790M, sialylation was observed to have a selective effect on EGFR phosphorylation, and inhibition of sialylation resulted in increased phosphorylation and resistance to gefitinib in this TKI-resistant lung cancer cell line. Further study of these findings should provide a better understanding of the role of sialylation in EGFR phosphorylation and resistance to TKIs.

Significance

This report reveals the influence of sialylation on the activation of epidermal growth factor receptor (EGFR) and sensitivity to tyrosine kinase inhibitors (TKIs) against EGFR phosphorylation. By utilizing biochemical approaches, we demonstrated that EGFR sialylation suppresses EGFR phosphorylation by inhibiting EGF binding and EGFR dimerization. In the TKI-resistant lung cancer cell line with L858R/T790M mutations on EGFR, the levels of phosphorylation at Y1068, Y1086, and Y1173 are upregulated, and sialylation can partially suppress the phosphorylation of EGFR at these sites and enhance EGFR sensitivity to TKI. These findings suggest that sialylation has an important role in tumorigenesis and sensitivity to TKIs by modulating EGFR phosphorylation and the associated signaling network and provide insights for therapeutic intervention.


The authors declare no conflict of interest.

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EGFR-mediated phosphorylation and disease progression affected by glycosylation and lead to the development of a new therapeutic strategy.

**Results**

**Preparation of Soluble EGFR and Its Desialylated Form from 293F Cells for Dimerization and EGF-Binding Studies.** To study the effect of sialylation on EGFR activation, the extracellular domain of EGFR was overexpressed in 293F cells and was affinity purified for biochemical assays. The desialylated soluble EGFR (sEGFR) was prepared by sialidase treatment to remove the α2,3-, α2,6-, and α2,8-linked sialic acid residues before affinity purification. The removal of sialic acids on each glycosylation site was monitored by mass spectrometry through matching with the calculated masses of both tryptic peptide fragments and glycans and by the appearance of fragmented glycans in MS/MS spectra (24). The results showed that all the sialic acid residues of sEGFR were removed except (perhaps because steric hindrance) those at Asn328 (Fig. S1).

To investigate the effect of sialylation on EGFR dimerization, multiangle laser light scattering (MALLS) was used to determine the kinetics of EGFR dimerization. Various concentrations of sEGFR with or without sialidase treatment were preincubated with EGF in a 1:1.1 molar ratio and then were analyzed by MALLS. The average molecular mass (MM) at each concentration was calculated and converted into the dimerization ratio.

Dimerization of sEGFR was induced in an EGF dose-dependent manner (Fig. 1A). Compared with sEGFR without sialidase treatment, the desialylated sEGFR showed a higher degree of dimerization, especially in the slope phase of the fitting curve. Statistical analysis with one-site specific binding showed that the dissociation constant ($K_d$) for EGF-induced dimerization of sEGFR is 0.943 μM, which is consistent with previous studies (the $K_d$ of EGFR dimerization ranges from 0.6–3.8 μM with different approaches) (2, 25, 26). Moreover, the $K_d$ value for EGF-induced dimerization of desialylated sEGFR was 0.561 μM, twofold higher than that for sEGFR. These data confirmed the suppression effect of EGFR sialylation on its dimerization. To dissect the impact of sialylation on dimerization further, the dissociation rate of EGFR dimers was measured. Samples were prepared in a saturated dimerization concentration (11 μM) and then were injected into MALLS to monitor the changes in average MM upon diffusion in the buffer system. The change of monomer–dimer stoichiometry was analyzed. As shown in Fig. 1B, the best-fitting curves showed that the dissociation rate of the desialylated sEGFR dimer (0.01586/s) is similar to the sialylated sEGFR dimer (0.01418/s), suggesting that sialylation on EGFR mainly regulates the rate of association, not its dissociation. Because previous studies indicated that the lack of glycans on specific glycosylation sites could induce ligand-independent EGFR dimerization (20, 21), we next examined whether desialylation could induce ligand-independent dimerization using MALLS analysis. The MM of sEGFR with or without desialylation in different concentrations was measured; no significant change was observed (Fig. S2), indicating that desialylation of EGFR does not induce spontaneous dimerization.

We next investigated whether sialylation could modulate EGFR–EGF interaction, because other studies have suggested that glycosylation could affect such interaction (23, 27). Surface plasmon resonance (SPR) was used to measure the binding of sEGFR (with or without desialylation) to EGF, which was immobilized onto CM5 Biacore biosensor chips. The kinetic parameters, including the $K_a$ and the association and dissociation rate constants ($K_{off}$ and $K_{on}$) of both sEGFR and desialylated sEGFR, were examined by the antibodies that recognize site-specific tyrosine phosphorylation profiles of flEGFR and desialylated flEGFR (28). In addition, the $K_d$ for EGF binding to sEGFR measured here is different from that previously reported (175 ± 5.8 nM) (2), perhaps because of the differences in glycosylation (complex type from human cells vs. high-mannose type from insect cells).

**Effect of Sialylation on the Autophosphorylation of EGFR Expressed in 293T Cells and EGFR Wild-Type CL1-0 and CL1-5 Cancer Cells.** The results from kinetics studies revealed that sialylation on EGFR could negatively regulate ligand-induced EGFR dimerization, which is critical for EGFR activation and autophosphorylation. To investigate the sialylation effect further, recombinant full-length EGFR (pI8EGFR) was transiently expressed in 293T cells and purified for the phosphorylation assay in vitro, and the phosphorylation profiles of pI8EGFR and desialylated pI8EGFR were examined by the antibodies that recognize site-specific tyrosine phosphorylation. As shown in Fig. 2, the desialylated pI8EGFR exhibited higher levels of phosphorylation on Y992, Y1068, Y1086, and Y1173 and a slight increase (<0.5-fold) in Y1148 phosphorylation. This result suggested that sialylation or desialylation of EGFR might have a selective effect on the phosphorylation of specific tyrosine residues, and, as shown previously, sialylation of EGFR suppresses its autophosphorylation through inhibition of EGFR dimerization. Interestingly, without EGF stimulation, sialylation still suppressed EGFR tyrosine phosphorylation, especially on Y1086 and Y1173.
In addition to tyrosine phosphorylation, the phosphorylation of serine or threonine residues on EGFR is known to modulate EGFR signaling. To understand further how sialylation regulates EGFR phosphorylation, we performed mass spectrometry analysis to investigate the phosphorylation pattern of EGFR comprehensively. We have developed a label-free quantification strategy that combines highly efficient protein enrichment, immobilized metal affinity chromatography (IMAC), and high-resolution mass spectrometry to characterize EGFR phosphopeptides. The EGFR proteins from two cancer cell lines, CL1-0 (mild), CL1-5 (aggressive), and sialidase-treated CL1-5, in starved or EGF-stimulated condition, were immunoprecipitated by covalently immobilized anti-EGFR mAb. The EGFR derived from these two cell lines was eluted in an acidic condition and subjected to phosphopeptide enrichment by IMAC following trypsin digestion. The phosphopeptides then were identified and quantified by mass spectrometry (Table S1). The quantification of phosphopeptides was verified further by sequential window acquisition of theoretical mass spectra (SWATH) (Fig. S3). Sixteen phosphosites were identified: three phosphotyrosines, eight phosphoserines, and five phosphothreonines. Some phosphosites showed different EGFR responsiveness in CL1-0 and CL1-5 cells (Fig. S4). For example, pY1148 and pY1173 were induced by EGF only in CL1-0 but not in CL1-5 cells; the phosphorylation of two threonine residues (pT701 and pT969) and four serine residues (pS696, pS967, pS971, and pS1142) was suppressed dramatically by EGF treatment in CL1-5 in comparison with CL1-0 cells. Removal of sialic acid residues by sialidase (Fig. SSB) altered the responsiveness of EGFR-induced phosphorylation to similar degrees in CL1-5 and CL1-0 cells, indicating that cell-surface sialylation is specifically involved in regulating EGFR phosphorylation. To link phosphorylation and sialylation, the relative change in identified phosphosites after the removal of cellular sialic acid was calculated, with positive and negative changes representing the suppression and enhancement effects of sialylation on phosphorylation, respectively. As shown in Fig. 3A, under EGF stimulation, sialylation of EGFR suppressed the phosphorylation on Y1173 but had no significant effect on Y1086 and Y1148. Surprisingly, sialylation also site-specifically regulated EGFR serine/threonine phosphorylation (Fig. 3B and C). Four phosphoserine sites (pS696, pS967, pS971, and pS1040) and one phosphothreonine site (pT701) were suppressed by sialylation in an EGF-dependent manner; in particular, phosphorylation on pS1040 was increased by around 75-fold when desialylated. On the contrary, phosphorylation on pS671 and pS1142 was enhanced by sialylation, and phosphorylation on T654 was reduced dramatically (~30-fold) in the desialylated condition.

Sialylation also had a regulatory effect on EGFR phosphorylation without EGF stimulation, and desialylation reduced the phosphorylation of Y1148 and Y1173 (Fig. 3A). Desialylation had a negative impact on the phosphorylation of serines and threonines when there was no EGF stimulation (Fig. 3B and C). Given these observations, we conclude that cellular sialylation may regulate EGFR phosphorylation by modulating the activity of other kinases responsible for EGFR phosphorylation in addition to suppressing EGFR autophosphorylation directly.

Effect of EGFR Sialylation on the Autophosphorylation of EGFR from TKI-Sensitive and -Resistant Mutants. Previous in vitro studies have shown that dimerization of the kinase domain is essential to maintain the activity of the oncogenic mutants of EGFR such as the TKI-sensitive mutant L858R (29–31). Moreover, EGF is capable of promoting the phosphorylation of EGFR mutants in many cell-based experiments. These observations collectively indicate that dimerization of EGFR is involved in the constitutive activation of EGFR mutants. Because it has been demonstrated that sialylation suppressed EGFR dimerization, we next investigated the impact of EGFR sialylation on the phosphorylation of EGFR mutants. First, an in vitro phosphorylation assay was performed to analyze the change in tyrosine phosphorylation on the flEGFR L858R and flEGFR L858R/T790M (TKI-resistant) mutants when treated with sialidase (Fig. 4 and Figs. S5 and S6). As shown in Fig. 4A, sialylation was less effective in regulating the phosphorylation of EGFR L858R, but its effect of sialylation on the TKI-resistant mutant L858R/T790M was significant. All phosphotyrosines with or without EGF stimulation were suppressed by sialylation, with most significant effect on Y1173 under EGF treatment (Fig. 4B).

To examine the effect of sialylation on the phosphorylation of EGFR mutants at the cellular level, the TKI-resistant cell line H1975 with L858R/T790M mutations on the EGFR was treated with a sialyltransferase inhibitor (STI) (32) or sialidase to reduce surface sialylation, and the level of three phosphotyrosines, pY1068, pY1086, and pY1173, which showed a high degree of

Fig. 2. Phosphorylation profiling of EGFR. (A) Purified flEGFR and desialylated flEGFR were treated with or without EGF at two concentrations of ATP (0.02 and 0.2 μM). The level of phosphorylation was analyzed by site-specific anti-EGFR phosphotyrosine antibodies (n = 3). (B) Semiquantitative results for the phosphorylation level of flEGFR incubated with 0.2 μM ATP. Relative fold change of phosphotyrosines between flEGFR and desialylated flEGFR was calculated. Error bars represent SD values. P values were calculated by paired t test. *P < 0.05; **P < 0.01.

Fig. 3. Identification of EGFR phosphorylation in the lung cancer cell line CL1-5. The intensities of identified phosphopeptides containing phosphotyrosines (A), phosphothreonines (B), and phosphoserines (C) are shown. The EGFR phosphopeptides derived from EGF-treated or untreated cells were identified by mass spectrometry, and the intensity of phosphopeptides was quantified based on a label-free strategy and normalized with the sum of intensity of the three most abundant EGFR peptides. The relative fold change of each sample was calculated by dividing the intensity of normalized EGFR phosphopeptides from sialidase-treated cells by the intensity of normalized EGFR phosphopeptides of untreated cells. The positive (fold change >0) or negative (fold change <0) effect of desialylation on EGFR phosphorylation is indicated (n = 4). Error bars represent SD values.
suppression by sialylation (>0.5-fold), was examined (Fig. 4C and Figs. S5C and S7A). Generally, as consistent with the results in the in vitro phosphorylation assay, in the presence or absence of EGF stimulation, the level of phosphorylation on these tyrosine residues, except for Y1086 in the absence of EGF, was elevated upon attenuation of cellular sialylation, with a more significant effect on Y1173 phosphorylation.

Effect of Sialylation on Gefitinib Sensitivity in Gefitinib-Resistant Cancer Cells. Based on the inhibitory effect of sialylation on the phosphorylation of EGFR mutant L858R/T790M, we hypothesized that sialylation also might influence the responsiveness of cells toward TKI by attenuating the overall signaling output of EGFR. To address this possibility, the sensitivity toward gefitinib in the presence of STI was measured in three TKI-resistant cell lines, H1975 (EGFR L858R/T790M), CL68 (EGFR Del19/T790M), and CL97 (EGFR G719A/T790M). H1975 cells treated with STI showed a significantly higher resistance to gefitinib under concentrations ranging from 15–30 μM (Fig. 5A), which was consistent with the results in our previous study (33). Therefore the effect of sialylation on autophosphorylation would not be expected to be as prominent in the L858R or L858R/T790M EGFR mutant as in the wild-type EGFR. However, in our in vitro and in vivo studies we observed site-specific suppression that without EGF stimulation the levels of phosphorylation at Y1068, Y1086, and Y1173 were up-regulated in the TKI-resistant cells harboring the T790M mutation, but in the presence of EGF only the phosphorylation at Y1086 remained significantly higher than that of the TKI-sensitive cells (Fig. 5C and Fig. S7C). However, we could not observe a good correlation between EGFR sialylation and gefitinib sensitivity in all of the cell lines examined, indicating that the suppression effect of sialylation on EGFR phosphorylation is insufficient to combat tumorigenesis.

Discussion

The activation of EGFR depends on intermolecular dimerization between two kinase domains and is triggered by dimerization of the two extracellular domains. Because sialylation attenuates the dimerization of EGFR extracellular domain, it is not surprising that all the EGFR autophosphorylation sites are down-regulated when EGFR is highly sialylated. A study suggested that the elevated kinase activity of the EGFR L858R mutant is caused primarily by the suppression of the intrinsic disorder of the kinase domain that thus facilitates the kinase domain dimerization (31). A more recent study based on the crystal structures showed that neither the L858R nor the L858R/T790M mutant was in the constitutively active conformation, but the dynamic nature of these mutants led to a greater activity even in their monomeric forms (33). Therefore the effect of sialylation on autophosphorylation would not be expected to be as prominent in the L858R or L858R/T790M EGFR mutant as in the wild-type EGFR. However, in our in vitro and in vivo studies we observed site-specific suppression...
of pY1173 by sialylation, especially under EGF stimulation, in the L858R/T790M mutant. It has been reported that the rates of autophosphorylation in the wild-type EGFR and EGFR L858R mutant are different, suggesting that different EGFR kinases (wild-type or mutants) have different preferences for phosphorylation sites (34). Although the mechanism remains unknown, we speculate that sialylation changes the phosphorylation propensity toward Y1173 in EGFR L858R/T790M. This notion is supported by the observation that the phosphorylation of Y1173 is more dependent on EGF-induced dimerization than are the other phosphosites (Fig. S3); therefore, sialylation suppressed the phosphorylation of Y1173 more significantly. In addition, sialylation also was reported to induce a conformational alteration of other glycoproteins, including MUC1 (35).

EGFR signaling is a complicated network regulated by its phosphorylation. According to the PhosphoSitePlus database (36), more than 50 EGFR phosphophores have been determined by mass spectrometry and other methods. Phosphorylation on each site has a distinct function in regulating the downstream signaling, the kinase activity, and receptor internalization. In addition to tyrosine phosphorylation, many serine and threonine residues are known to be phosphorylated in EGFR, indicating the complex nature of the EGFR signaling network. In this study, we found that sialylation of EGFR regulates the phosphorylation of EGFR, including tyrosine and serine/threonine phosphorylation, in lung cancer cells. Although the precise effect of sialylation on phosphorylation is not well understood, it also may affect other intermolecular interactions, as reported in other related studies. For example, GM3, the ganglioside containing the sialylactose epitope, has been reported to interact with EGFR and inhibit its kinase activity in a model supplemented with the GM3 glycolipid, and treatment with neuraminidase can rescue the auto-phosphorylation of EGFR (37). In addition, galectin-3 also can regulate the cellular trafficking and the level of surface EGFR through binding to the gangliosides on EGFR, and the binding can be blocked by sialylation on EGFR (38, 39).

Studies have shown that distinct EGFR downstream signaling can be initiated by different patterns of EGFR phosphorylation. Therefore investigating the phosphorylation profiles of EGFR is important for understanding the regulation of cellular functions. Because the up-regulation of phosphorytyrosines was observed in lung cancer cells with EGFR L858R/T790M mutation, the relationship between EGFR genotype and its phosphorylation patterns and the contribution of EGFR phosphorylation in TKI resistance should be further studied. It has been shown that specific EGFR downstream signaling pathways can be elicited by phosphorylation on specific sites. For example, the phosphorylation of Y1068 can recruit GAB-1 or growth factor receptor-bound protein 2 (Grb-2) to activate survival signals (40), whereas the phosphorylation of Y1173 is responsible for eliciting the activation of ERK via inhibition of the SH2 domain-containing transforming protein (SCH) and Grb2 (41, 42). Furthermore, the up-regulation of pY1173 also has been reported in patients who have NSCLC with EGFR mutation, and Akt, MAPK, and Stat3 signaling is higher in pY1173-positive patients (43). It also has been shown that patients with stage IIIb and IV NSCLC with positive pY1173 staining have a shorter superior progression-free survival rate than patients with negative pY1173 staining (44). These data suggest that site-specific phosphorylation of EGFR plays an important role in the maintenance of TKI resistance and that targeting these selective EGFR phosphorylations could be a future direction for drug discovery. Because sialylation modulates the phosphorylation of EGFR, it is possible that sialylation can regulate TKI sensitivity in cells (Fig. 6). Similarly, glycosylation with the bisecting GlcNAc on N-glycans inhibits mammary tumor progression (45). Our preliminary data also revealed that a sialic acid-containing glycolipid, SSEA4, is up-regulated in the TKI-resistant mutants of lung cancer cell lines, compared with the cells with wild-type EGFR (Fig. S8). All these observations suggest a new strategy for lung cancer therapy, possibly using a combination approach (46).

In summary, this study shows the complexity of EGFR sialylation and phosphorylation. Compared with the TKI-sensitive lung cancer-cell mutant L858R, the TKI-resistant lung cancer-cell mutant L858R/T790M has a higher degree of phosphorylation at Y1086 with EGF stimulation and also has higher phosphorylation at Y1068, Y1086, and Y1173 without EGF stimulation. Although sialylation is induced to suppress the phosphorylation of EGFR, the effect of suppression is not strong enough to inhibit the downstream signaling of cancer progression. Development of new-generation TKIs to inhibit the phosphorylation of these sites could overcome the problem of drug resistance.

Materials and Methods

Cell Lines. The A549 (wild-type), H3255 (L858R), and H1975 (L858R/T790M) cell lines were obtained from ATCC; the PC9 (exon 19 deletion, Del19) cell line was obtained from RIKEN BioResource Center. C1-0 and C1-5 (both wild-type) cell lines were as described previously (47), and CL68 (Del19/T790M), CL83 (wild-type), and CL97 (G719A/T790M) cell lines were established from patients who provided informed consent and with the approval of the institutional review board (National Taiwan University Hospital Research Ethics Committee). Among these cell lines, H3255 and PC9 are gefitinib sensitive, and H1975, CL68 and CL97 are gefitinib resistant.

Determination of MM by MALLS Measurement. MALLS measurements were made with a system composed of a multilang light scattering photometer (DAWN HELEOS II; Wyatt Technology), a differential refractive index detector (Optilab T-Rex; Wyatt Technology), and a generic UV- absorbance detector equilibrated with 50 mM sodium phosphate buffer at a flow rate of 0.07 mL/min. Samples (0.2 mL) were manually applied to the sample injector conjugated with 0.1 μm Anotop filter (Whatman), and data collection and processing were performed by ASTRA software (Wyatt Technology). The differential refractive index increment (shd) of sEGFR was estimated by the saccharide-protein conjugation method (protein: 0.185; saccharide: 0.147). MM was calculated according to the scattered light intensity, and protein concentration was measured by UV absorbance within a 0.2-min interval of the signal peak. To measure EGF-induced EGFR dimerization, sEGFR was pre-incubated with EGF in a molar ratio of 1:1.1 for 30 min at 37°C. The percentage of dimerization was calculated by the following formula: dimerization ratio = (observed MM − monomer MM)/(dimer MM − monomer MM).

In Vitro EGFR Phosphorylation Assay. The EGFR protein was purified from 293T cells transiently overexpressing FLAG-tagged wild-type or mutant EGFR.

TKI sensitive (L858R) TKI resistant (L858R/T790M)
Plasmid DNA-transfected 293T cells were lysed with lysis buffer [20 mM Tris (pH 7.4), 400 mM NaCl, 10% (vol/vol) glycerol, 1 mM EDTA, 0.5 mM DTT, 0.2% Triton X-100] and were pretreated with phosphatase (Promega) at 37 °C for 30 min to remove the phosphorylation in cellular proteins. The phosphatase-treated cell lysates were incubated further with sialidase (α2,3/6/8-sialidase; Roche) at 4 °C overnight. The FLAG-tagged wild-type or mutant EGFR then were purified with anti-FLAG M2 agarose (Sigma-Aldrich) and were eluted with 3× FLAG peptide (Sigma-Aldrich) in elution buffer (20 mM Tris (pH 7.4), 400 mM NaCl, 10% (vol/vol) glycerol, 1 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100, 0.1 mg/mL 2,3/6/8-sialidase; 2× FLAG peptide). Plasmid DNA-transfected 293T cells were lysed with lysis buffer [20 mM Tris (pH 7.4), 400 mM NaCl, 2 mM MnCl2, 1 mM Tris(2-carboxyethyl)phosphine, 0.1 mg/mL BSA] for 5 min at room temperature, followed by the addition of ATP and further incubation at room temperature for 10 min. Reactions were stopped by the addition of 4× protein sample buffer (Life Technologies) containing 5% (vol/vol) of 2-mercapto ethanol. Samples were separated by SDS-PAGE and subjected to immunoblotting with antibodies specific for EGFR phosphosites (Cell Signaling).

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Supporting Information

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**SI Materials and Methods**

**Cell Culture.** Lung cancer cell lines were cultivated in RPMI medium 1640 (Life Technologies) supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM L-glutamine, and 1 mM sodium pyruvate at 37 °C in 5% (vol/vol) CO₂. To identify EGFR phosphorylation, CL1-0 and CL1-5 cells were cultured overnight in a Hyperflask (Corning) with a total number of 5 × 10⁶ cells and then were starved in serum-free RPMI medium for 24 h.

**Purification of FLAG-Tagged sEGFR.** By using anti-FLAG (M2) agarose (Sigma-Aldrich), recombinant sEGFR was affinity purified from 50 mL of the concentrated culture medium (~10 fold) from 293F cells transiently overexpressing FLAG-tagged sEGFR. The concentrated medium first was treated with sialidase (from Clостridium perfringens, 40 mL/mL; Roche) at 37 °C overnight in the presence of EDTA-free protease inhibitor mixture (Roche) before affinity purification. The anti-FLAG affinity column was loaded with 250 μL of agarose beads washed three times with 10 mL Tris-buffered saline [TBS, 25 mM Tris (pH 7.4), 137 mM NaCl, 2.7 mM KCl] before incubation with sEGFR-containing medium, and sEGFR was eluted with 0.1 M glycine-HCl (pH 2.5) followed by neutralization with 1:15 volume of 1 M Tris-HCl (pH 9). The eluted FLAG-tagged sEGFR was buffer-exchanged to 50 mM sodium phosphate and SPR running buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20] for MALLS and SPR assay, respectively.

**Desialylation and Detection of Sialylation of Desialylated Samples.** Sialidase (2.23/6/8-sialidase purchased from Roche; 100 mL/mL) was used to treat cell lysate (1 mg/mL) by incubation at 4 °C overnight before affinity purification. For treatment of cancer cells, 10⁶ cells were seeded in a 10-cm dish and incubated in RPMI medium 1640 supplemented with 0.1% BSA and sialidase (20 mL/mL; Roche) at 37 °C overnight. For treatment with STI, 50 μM of STI was added to the culture medium, and the cells (10⁶/mL) were treated for 3 d. To detect the level of sialylation, the glycoforms of sialidase-treated sEGFR were analyzed by mass spectrometry as described previously (1). For monitoring the removal of sialic acids on IIEGFR used for in vitro phosphorylation assays, 200 μL of sialidase-treated lysates was immunoprecipitated with 2 μg of biotinylated SNA lectin (Vector Laboratories) and 50 μL of NeutrAvidin beads (Thermo Scientific) in 0.4 mL lysis buffer, followed by anti-EGFR immunoblotting, and the relative sialylation of EGFR was quantified by the intensity of SNA-precipitated EGFR/total EGFR in cell lysate. To detect the levels of sialylation in desialylated cells, 1 μg of biotinylated SNA or Maackia amurensis lectin II (MALII) was used to stain 10⁵ cells at 4 °C for 30 min in 50 μL FACS staining/washing buffer [2% (vol/vol) FCS, 0.1% NaCl in PBS], followed by staining with Alexa Fluor 488-conjugated streptavidin (BD Biosciences) at 4 °C for 30 min. Then cells were analyzed with FACS Canto (Becton Dickinson) using FACS Diva (Becton Dickinson) and FlowJo (Tree Star) software.

**SPR Study.** The binding of sEGFR to EGF was analyzed by using a Biacore T200 (GE Healthcare). All experiments were performed at 25 °C in degassed 25 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20. EGF was immobilized on a Biacore CMS biosensor chip via amine coupling. Briefly, the Biacore CMS chip was activated with N-hydroxysuccinimide and 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride. Then EGF (200 μg/mL; Millipore) in 10 mM sodium acetate (pH 4) was flowed over the activated surface at 5 μL/min for 10 min. Free ligands were washed away, and the remaining reactive functional groups were blocked with 1 M ethanolamine-HCl. Immobilized EGF contributed a signal of 327 response units (RU). A series of concentrations of purified sEGFR, as the analyte, were flowed over the EGF-immobilized surface (and over the control surface without ligand) at 10 μL/min for 10 min using a multicycle method. The RU signal corresponding to the height of the plateau was corrected by the RU signal generated with the control surface. The kinetic parameters, including the K_d, K_on, and K_off, were calculated by Biacore T200 evaluation software. Each experiment was performed at least three times, and SD values were calculated and shown.

**Tip-Based pH/Acid Controlled IMAC.** EGF tryptic peptides were desalted by self-made reversed-phase StageTips with poly(styrene-divinylbenzene) copolymer (SDB-XC) (2) as the reversed-phase sorbent. Phosphopeptides were purified through tip-based IMAC procedures constructed in house as previously described (3). All purification steps for buffer exchange and sample loading involved manipulation via centrifugation. Briefly, Ni²⁺ ions were removed with 50 mM EDTA in 1 M NaCl. Then the tip was activated with 100 mM FeCl₃ and equilibrated with loading buffer [6% (vol/vol) acetic acid at pH 3.0] before sample loading. Tryptic peptides were reconstituted in loading buffer and loaded onto the IMAC tip. After successive washes with 6% (vol/vol) acetic acid, 25% (vol/vol) acetonitrile, and 6% (vol/vol) acetic acid, the bound peptides were eluted with 200 mM NH₄H₂PO₄. The eluted phosphopeptides and unbound peptides in flow-through were desalted using reversed-phase StageTips.

**LC-MS/MS Analysis.** The TripleTOF 5600 system (AB SCIEX) was equipped with a nanoACQUITY UPLC (Waters). Three micrometers of ReproSil-Pur C18-APQ particles (Dr. Maisch) were packed into a 15-cm self-pulled column with a 100-μm inner diameter and 7-μm opening to prepare an analytical column using “stone-arch” frit (4). The LC system consisted of water with 0.1% formic acid (buffer A) and acetonitrile with 0.1% formic acid (buffer B). Peptides were separated through a gradient of up to 80% (vol/vol) buffer B over 120 min at the flow rate of 500 nL/min. Data were acquired using an ion spray voltage of 2.5 kV, curtain gas at 20 psi, nebulizer gas at 15 psi, and an interface heater temperature of 150 °C. For information-dependent acquisition, the MS survey scan range was m/z 300–1,500, and data were acquired for 250 ms. The top 10 precursor ions were selected based on exceeding a threshold of 100 cps in each MS survey scan, and 10 MS/MS scans were performed for 200 ms each. The collision energy was adjusted automatically by the rolling CID function of Analyst TF 1.5. To minimize repeated scans, dynamic exclusion was set at 6 s, and the precursor then was removed from the exclusion list.

**Data Processing and Protein Identification.** The raw MS/MS data were processed using the AB SCIEX MS Data Converter and analyzed using Mascot (Matrix Science; version 2.3) against the UniProt database (version 57.8, Homo sapiens, 20,329 sequences) with the following constraints: allowing for tryptic peptides with up to two missed cleavage sites, a fragment ion mass tolerance of 0.1 Da, and a parent ion tolerance of 20 ppm. For unlabelled phosphopeptides, phosphorylation (S, T, Y) and oxidation (M) were
selected as variable modifications. The identification false-discovery rate was evaluated by search against a randomized decoy database created by Mascot at the peptide spectrum match (PSM) level. In addition, only PSMs with \( P \) value less than 0.05 were accepted.

**Quantitative Analysis by IDEAL-Q.** Quantitative analysis of phosphopeptide was performed by IDEAL-Q software as previously described (5, 6). Briefly, the raw data files were converted into files of mzML format by using the AB SCIEX MS Data Converter. IDEAL-Q performs quantitation analysis using spectral data in mzXML or mzML format and Mascot search results in XML format. IDEAL-Q was used to process sequentially all peptides in each LCMS/MS run, both identified and unidentified, to quantify as many peptides as possible.

IDEAL-Q first predicted the retention time of identified peptides in its current run and then determined the peak cluster based on the predicted retention time. Therefore, the unidentified peptides could be detected and aligned according to these peak clusters with a similar peptide predicted retention time. Therefore, the unidentified peptides were predicted the retention time of identified peptides in its current run and then determined the peak cluster based on the predicted retention time. Therefore, the unidentified peptides could be detected and aligned according to these peak clusters with a similar peptide predicted retention time. Therefore, the unidentified peptides could be detected and aligned according to these peak clusters.

To quantify the peptide abundance of these identified and assigned unidentified peptide peaks, extracted ion chromatography areas of each peptide were calculated. Fold change of each peptide was determined further between different samples.

**SWATH-MS Measurement.** SWATH-MS measurements were acquired by the TripleTOF 5600 system. The instrument was specifically tuned to allow a quadrupole resolution of 25 Da per mass selection. Using an isolation width of 26 Da (25 Da of optimal ion transmission efficiency + 1 Da for the window overlap), a set of 26 overlapping windows was constructed covering the mass range from 350 to 1,000 Da. The collision energy for each window was determined based on the appropriate collision energy for a 2+ ion centered upon the window with a spread of ± 15 eV. An accumulation time of 100 ms was used for each fragment ion scan, and total cycle time was about 2.7 s (2.7 s total for stepping through the 26 isolation windows + 0.05 s for the optional survey scan). The targeting quantitation analysis of SWATH was implemented by Skyline (7). The isolation scheme in Skyline for MS1 and MS/MS filtering was set up as “TOF mass analyzer.” The resolution setting for MS1 and MS/MS was 20,000 and 10,000, respectively. For retention filtering, only scans within 5 min of MS/MS IDs were used. To quantify the targeting peptide abundance, the three most abundant transition ions were used for quantification.

**Western Blot.** Cells were lysed by lysis buffer [1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 150 mM NaCl, 100 mM sodium phosphate (pH 7.2), 1× EDTA-free protease inhibitor cocktail from Roche] and then were incubated on ice for 15 min. After incubation, the samples were centrifuged at 13,000 \( \times g \) for 15 min. The supernatants were collected, and protein concentrations were determined by bicinchoninic acid protein assay kit (Thermo Scientific). The samples were separated on 4–12% NuPAGE (Life Technologies) and transferred to PVDF membranes (Millipore). The membranes were blocked with blocking buffer [5% (wt/vol) BSA in TBS] for 1 h and then were incubated with anti-EGFR or EGFR phosphosite-specific antibodies (1:1,000 diluted in blocking buffer). After washing with 0.05% Tween 20 in TBS, the membranes were incubated with peroxidase-conjugated second antibodies, and the signal was developed by adding chemiluminescent substrates.

**Proliferation Assay.** Cell proliferation was measured with the INCUCYTE kinetics imaging system (Essen BioScience). Growth tests were conducted with 1 × 10\(^5\) cells per well in 200 \( \mu \)L of culture medium containing the indicated concentrations of gefitinib for 4 d. For the groups pretreated with STI, 50 mM STI was used to treat the cells for 3 d and maintained throughout the additional 4-d gefitinib treatment. The degree of cell confluence was calculated based on the images taken (three distinct fields for each well) every 4 h. Each condition was triplicated in the experiments.

**Statistical Analysis.** Values are expressed as mean ± SD of at least three experiments. Paired \( t \) tests were used to analyze the statistical significance of the differences, with a value of \( P < 0.05 \) considered statistically significant.

Fig. S1. Sialylation analysis of sEGFR. The glycoforms of sEGFR, with or without sialidase treatment, were analyzed by LC-MS/MS. The peak areas of identified glycopeptides with various numbers of sialic acid were quantified, and the relative percentage of each sialic acid-containing glycopeptide was calculated against the sum of peak area of all signals with the same peptide backbone (with or without glycosylation).

Fig. S2. Determination of the molecular mass of sEGFR. The molecular mass of sEGFR and desialylated sEGFR at various concentrations was measured by MALLS.
Fig. S3. Quantification of phosphopeptides by sequential window acquisition of all theoretical spectra mass spectrometry (SWATH-MS).
Fig. S4. Responsiveness of EGFR phosphorylation to EGF in lung cancer cells. The phosphorylation of immunoprecipitated EGFR from lung cancer cells CL1-0 (A), CL1-5 (B), and sialidase-treated CL1-5 (C) was identified by MS and quantified with the label-free method. The relative fold change of each phosphosite is shown. Error bars represent SD values.
**Fig. S5.** Detection of sialylation in desialylated samples. (A) Lectin pull-down experiment with SNA for detection of sialylation on fEGFR in cell lysates. (B and C) Lectin staining followed by flow cytometric analysis for detecting the sialylation of CL1-5 (MALII staining, B) and H1975 (SNA staining, C) cell lines after desialylation. The levels of sialylation are shown as geometric means of fluorescence intensity.

**Fig. S6.** In vitro phosphorylation profile of EGFR mutants. (A and B) Purified fEGFR and desialylated fEGFR with the L858R (A) or L858R/T790M (B) mutation were incubated with or without EGF at 0.02 or 0.2 μM of ATP. The level of phosphorylation was analyzed by site-specific anti-EGFR phosphotyrosine antibodies. (C) The relative fold change of phosphorylation on five tyrosine residues upon EGF stimulation. The signal intensity of tyrosine phosphorylation in 0.2 μM ATP was quantified. Error bars represent SD values.
Fig. S7. Profile of EGFR tyrosine phosphorylation in lung cancer cell lines with EGFR mutations. (A) Tyrosine phosphorylation of H1975 cells treated with STI or sialidase. (B) The expression levels of EGFR in different lung cancer cell lines. (C) Phosphorylation profiles of EGFR from seven lung cancer cell lines (H3255, PC9, A549, H1975, CL97, CL68) with or without EGF treatment. The intensity of A549-derived EGFR in immunoblots was used to normalize between different blots. Site-specific EGFR phosphorylation was analyzed by specific antibodies. S, TKI sensitive; R, TKI resistant.

Fig. S8. Expression of SSEA4 on the surface of lung cancer cells. Cell-surface expression of SSEA4 was analyzed by flow cytometry. Lung cancer cell lines with EGFR wild-type (CL83 and A549) or EGFR mutants (H1975, PC9, H3255, CL68, and CL97) were stained with FITC-conjugated anti-SSEA4 mAb. The histograms of the cells stained with anti-SSEA4 mAb and isotype control are shown in red and gray, respectively. In the right panel, the expression level of SSEA4 is presented as geometric mean of fluorescence intensity.
Table S1. EGFR phosphopeptides identified by LC-MS/MS analysis

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Phosphosites</th>
<th>Miss cleavage</th>
<th>Mascot score</th>
<th>z</th>
<th>Observed m/z</th>
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<tr>
<td>1. pTLRRLLLQER</td>
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<td>4. ELVEPlpTPGEGEPNQALLR</td>
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<td>50.67</td>
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