

Cisplatin-induced non-canonical endocytosis of EGFR via p38 phosphorylation of the C-terminal region containing Ser-1015 in non-small cell lung cancer cells

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Abstract. The aberrant activation of receptor tyrosine kinases (RTKs) is associated with tumor initiation in various types of human cancer, including non-small cell lung cancers (NSCLCs). Tyrosine kinase-independent non-canonical RTK regulation has also been investigated in tumor malignant alterations, including cellular stress responses. It was recently reported that the phosphorylation of epidermal growth factor receptor (EGFR) at C-terminal Ser-1015 serves a critical role in growth factor and cytokine signaling. In the present study, the role of non-canonical EGFR regulation has been investigated in NSCLC cells treated with cisplatin, a common chemotherapeutic agent. Cisplatin-induced p38 activation triggered the Ser-1015 phosphorylation of EGFR, with similar kinetics to previously reported Ser-1047 phosphorylation, in a tyrosine kinase-independent manner. In addition, phosphorylation around Ser-1015 triggered endocytosis of a dimer deficient mutant of EGFR. The non-canonical endocytosis of EGFR monomers was primarily controlled by the region around Ser-1015 only; however, Ser-1047 on internalized EGFR was equally phosphorylated. The results of the present study provide mechanistic evidence for the cisplatin-induced non-canonical regulation of EGFR.

Introduction

Receptor tyrosine kinases (RTKs) serve pivotal roles in tumor initiation and malignant progression in various types of human cancers. Epidermal growth factor receptor (EGFR) is one of the most characterized RTK and is overexpressed

in approximately 50% of non-small cell lung cancers (NSCLCs) (1-4). Activating EGFR mutations, typically the exon 19 deletion or L858R point mutation, are also frequently observed. EGFR tyrosine kinase inhibitors (TKIs) contribute to the treatment of lung cancer patients harboring EGFR mutations in the clinical setting (5-7).

EGFR becomes activated by the asymmetric homo-dimerization of intracellular TK domains and subsequent tyrosine autophosphorylation (8,9). In contrast to canonical activation, it has become evident that the TK-independent non-canonical serine/threonine phosphorylation of EGFR plays a key role in the regulation of EGFR activity. Non-canonical EGFR regulation is triggered by various conditions, including inflammatory cytokines, ultraviolet radiation, and DNA-damaging agents (10-14). The treatment of cells with these stimuli induces the clathrin-mediated endocytosis (CME) of EGFR, which is triggered by the activation of p38, in a TK-independent manner. It has recently been reported that the TK-independent functions of EGFR contribute to the initiation of autophagy and prevention of TNF- α -induced apoptosis (10,15,16). Therefore, a more complete understanding of the TK-independent functions of EGFR under cellular stress conditions is needed in the field of EGFR biology and EGFR-targeting therapeutics.

Treatments with cytotoxic platinum-containing agents, including cisplatin and carboplatin, are currently standard chemotherapy for NSCLC patients (12,17). In the present study, we characterized the cisplatin-induced non-canonical phosphorylation and endocytosis of EGFR by focusing on two major p38 target regions, Ser-1015 and Ser-1047, in lung cancer cells.

Materials and methods

Antibodies and reagents. Phospho-specific antibodies against p38 (Thr-180/Tyr-182) and EGFR (Tyr-1068) were purchased from Cell Signaling Technology. Monoclonal antibodies against phospho-EGFR (Ser-1047) (clone 1H9) and EGFR (clone LA1) were obtained from Abcam (Cambridge, UK) and EMD Millipore (Billerica, MA, USA), respectively. Antibodies against EGFR (1005) and β -actin (C-11) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). A phospho-EGFR (Ser-1015) rabbit monoclonal antibody was generated using the

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rabbit-immunospot array assay on a chip (ISAAC) system (18). Recombinant human EGF and TNF- α were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Cisplatin (CDDP), gefitinib, and a Phos-tag ligand were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). SB203580, trametinib, and PD153035 were purchased from Merck KGaA (Darmstadt, Germany). All chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was less than 0.1%.

Cell culture. PC-9 and RPC-9 cells were kind gifts from Dr Kiura (Okayama University, Okayama, Japan). A549, PC-9, and RPC-9 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in 5% CO₂. HeLa and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in 5% CO₂.

Immunoblotting. Whole cell lysates were prepared as described previously (19). Cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P nylon membrane (EMD Millipore). The membrane was treated with Block Ace (Dainippon Pharmaceutical Co., Ltd., Suita, Japan) and probed with the primary antibodies described above. Antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, mouse, or goat immunoglobulin G (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and visualized with an enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA). Some antibody reactions were performed in Can Get Signal solution (Toyobo Life Science, Osaka, Japan).

Zn²⁺-Phos-tag SDS-PAGE. Cell lysates were prepared with RIPA buffer [50 mM Tris-HCl (pH7.4), 0.15 M NaCl, 0.25% sodium deoxycholate, 1% NP-40, 1 mM EDTA, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin]. Each sample was mixed with a half volume of SDS-PAGE sample buffer (195 mM Tris-HCl (pH 6.8), 3% SDS, 15% 2-mercaptoethanol, 30% glycerol, and 0.1% bromophenol blue), and heated at 95°C for 5 min. The procedures for Zn²⁺-Phos-tag SDS-PAGE were described previously (20). In brief, the acrylamide pendant phos-tag ligand and two equivalents of ZnCl₂ were added to the separating gel before polymerization. The running buffer consisted of 100 mM Tris and 100 mM MOPS containing 0.1% SDS and 5 mM sodium bisulfite. After electrophoresis, the gel was washed twice with a solution containing 25 mM Tris, 192 mM glycine, 10% methanol, and 1 mM EDTA for 20 min and then once with a solution containing 25 mM Tris, 192 mM glycine, and 10% methanol for 20 min. Gel transfer, blocking, the antibody reaction, and detection were conducted according to the normal immunoblotting protocol described above.

Transfection of plasmid DNAs. HeLa and 293 cells were transfected using Lipofectamine 2000 and 3000, respectively (Thermo Fisher Scientific, Inc., Waltham, MA, USA), in accordance with the manufacturer's instructions. The

pEGFP-N expression vector for the dimer-deficient EGFR mutant (dd-EGFR) with DCR1/I682Q/V924R mutations and its S1015A/T1017A/S1018A (R1m) and S1046A/S1047A (R2m) mutants were generated by PCR with KOD FX Neo polymerase or KOD-Plus-Neo polymerase (Toyobo Life Science).

RNA interference. A small interfering RNA (siRNA) against p38 α (target sequence; 5'-GCAUUAACAACCAGACAGUUGA UAUU-3') was synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan). Negative control siRNA was purchased from Thermo Fisher Scientific, Inc. A549 cells were transfected with siRNAs at a final concentration of 50 nM using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.). Cells were used for experiments 72 h post-transfection.

Immunofluorescence. Cells were seeded on coverslip glass (Thermo Fisher Scientific, Inc.). Two days after seeding, cells were incubated with inhibitors and ligands or transfected with plasmid DNAs. Cells were rinsed in cold PBS and fixed in 4% paraformaldehyde (PFA) at room temperature for 15 min or in methanol at -20°C for 10 min. After PFA fixation, cells were permeabilized in PBS containing 0.5% Triton X-100 and washed by PBS. Cells were probed for 40 min with primary antibodies and then washed and incubated with isotype-specific secondary antibodies conjugated to Alexa Fluor (nvtrogen; Thermo Fisher Scientific, Inc.) for 30 min. These antibodies were diluted in PBS containing 0.5% BSA. Microscopy was performed using a LSM 700 confocal microscope (Zeiss, Oberkochen, Germany).

Results

Cisplatin induces the non-canonical phosphorylation of EGFR. We previously reported that cisplatin induces the non-canonical phosphorylation of EGFR at Thr-669 in the juxtamembrane domain and Ser-1046/1047 in the C-terminal region via the p38 and ERK pathways, respectively (11). We and others have also suggested the importance of another p38 target, Ser-1015 in the endocytosis of EGFR (13); however, this has not been fully characterized under physiological conditions. Therefore, we generated a phospho-specific monoclonal antibody against Ser-1015. We demonstrated that cisplatin induced Ser-1015 phosphorylation with similar dynamics to previously reported Ser-1047 phosphorylation in A549 (wild type EGFR), PC-9 (exon 19 deletion), RPC-9 (erlotinib-resistant PC-9 cells harboring the T790M secondary mutation), and HeLa cells (Fig. 1A). TNF- α also triggered Ser-1015 phosphorylation (Fig. 1B). In addition, a stimulation with EGF weakly induced Ser-1015 phosphorylation in a TK-dependent manner; however, potent phosphorylation by cisplatin was independent of TK activity in A549 cells (Fig. 1C). This correlated with p38 activation (Fig. 1C). Similarly, gefitinib, an EGFR-TKI, did not inhibit the cisplatin-induced phosphorylation of Ser-1015 and Ser-1047, but completely suppressed the constitutive phosphorylation of Tyr-1068, a major autophosphorylation site, in PC-9 cells (Fig. 1D). Collectively, these results demonstrated that Ser-1015 is a target site for the cisplatin-induced signaling pathway in lung cancer cells.

p38 triggers the phosphorylation of EGFR in cells treated with cisplatin. In order to elucidate the ratio of phosphorylated EGFR upon the cisplatin stimulation, we employed immunoblotting

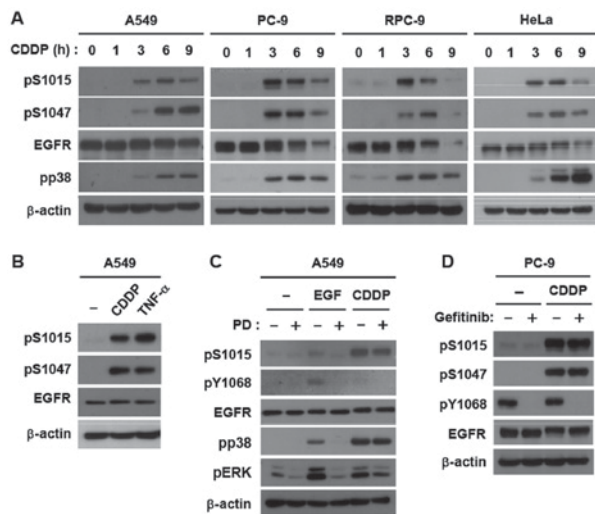


Figure 1. CDDP-induced non-canonical serine phosphorylation of EGFR in various cell lines. (A) A549, PC-9, RPC-9, and HeLa cells were treated with 100 μ M CDDP for the indicated times. (B) A549 cells were treated with 100 μ M CDDP for 6 h or 20 ng/ml tumor necrosis factor- α for 10 min. (C) A549 cells were pretreated with 1 μ M PD153035 for 30 min, followed by a 6-h treatment with 100 μ M CDDP or 10-min treatment with EGF (100 ng/ml). (D) PC-9 cells were pretreated with 1 μ M gefitinib for 30 min, and then stimulated with 100 μ M CDDP for 6 h. Whole cell lysates were analyzed by immunoblotting with selective antibodies. CDDP, cisplatin; EGFR, epidermal growth factor receptor; PD, PD153035 (EGFR inhibitor); EGF, epidermal growth factor.

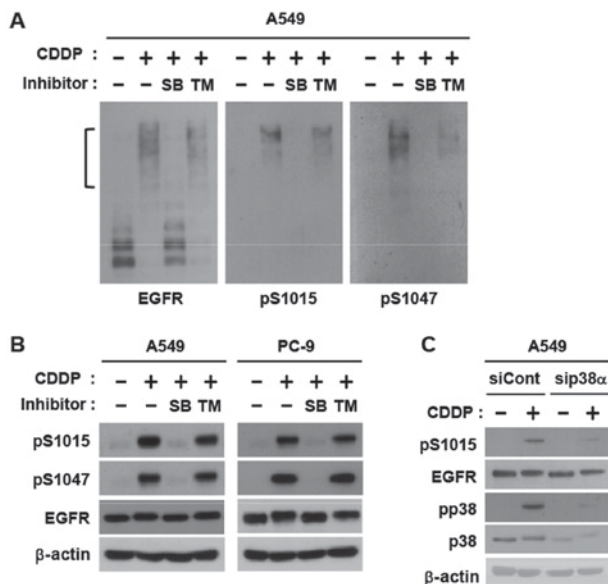


Figure 2. p38 participates in the serine phosphorylation of EGFR under the CDDP treatment. (A) A549 cells were pretreated for 30 min with 10 μ M SB or 30 nM TM, followed by the 100 μ M CDDP treatment for 6 h. Whole cell lysates were separated by Zn^{2+} -Phos-tag SDS-PAGE followed by immunoblotting with the indicated antibodies. (B) A549 and PC-9 cells were pretreated with 10 μ M SB or 30 nM TM, followed by the 100 μ M CDDP treatment for 6 h. (C) A549 cells were transfected with siRNA against p38 α or control siRNA, followed by the 100 μ M CDDP treatment for 6 h. Whole cell lysates were analyzed by immunoblotting with selective antibodies. CDDP, cisplatin; TM, trametinib; SB, SB203580; si, short interfering.

using a Phos-tag gel, which detects phosphorylated proteins as shifted bands (21,22). Cisplatin caused band shifts in all EGFR proteins expressed, indicating high phosphorylating activity (Fig. 2A). Phospho-specific antibodies against Ser-1015 and

Ser-1047 detected the only shifted EGFR band. SB203580, a p38 inhibitor, strongly abolished the band shift, which correlated with the disappearance of phospho-specific bands (Fig. 2A). A western blot analysis using normal SDS-PAGE clearly demonstrated that the phosphorylation of these two serines was completely inhibited by SB203580, but not by trametinib, a MEK inhibitor (Fig. 2B). RNAi-mediated knockdown of p38 α , a major isoform, abrogated phosphorylation of Ser-1015 (Fig. 2C). These results indicate that severe DNA-damaging conditions with cisplatin control most EGFR proteins via the phosphorylation of two p38 target regions.

Cisplatin-induced EGFR endocytosis via Ser-1015 phosphorylation. We recently demonstrated that TNF- α -induced endocytosis occurred on inactive monomeric EGFR in a TK-independent manner using a dimer-deficient EGFR mutant (dd-EGFR) lacking extra- and intracellular dimerization sequences (9,18,23,24). We detected the cisplatin-induced phosphorylation of dd-EGFR at Ser-1015 and Ser-1047 (Fig. 3A). An analysis using alanine substitution mutants of dd-EGFR at Ser-1015/Thr-1017/Ser-1018 (region 1 mutant; dd-R1m) or Ser-1046/Ser-1047 (dd-R2m) clarified the selectivity of antibodies to the corresponding phosphorylation sites (Fig. 3A). Moreover, an immunofluorescence analysis of GFP-tagged dd-EGFR demonstrated that cisplatin augmented the endocytosis of EGFR monomers, shown as green dotted particles in the cytoplasm (Fig. 3B). The cisplatin-induced endocytosis of dd-R1m, but not dd-R2m (Fig. 3B) was reduced, indicating that the p38 phosphorylation of region 1 containing Ser-1015 triggered non-canonical EGFR endocytosis under DNA-damaging conditions.

Cisplatin-induced EGFR endocytosis in lung cancer cells. Internalized endogenous EGFR was stained with pS1015, pS1047, and total EGFR antibodies in NSCLC cell lines. As expected, pS1015-EGFR co-localized with pS1047-EGFR in A549 and PC-9 cells (Fig. 4A). The staining patterns of pS1015-EGFR and total EGFR also overlapped (Fig. 4B). Moreover, p38 inhibition, but not MEK inhibition abolished cisplatin-induced EGFR endocytosis and Ser-1015 phosphorylation (Fig. 4B). Similar immunofluorescence staining was observed in cisplatin-treated RPC-9 cells (our unpublished data). These results indicate that although only Ser-1015 is involved in the internalization process, Ser-1015 and Ser-1047 were both simultaneously phosphorylated on internalized EGFR proteins via p38 activation.

Discussion

A phosphoproteomic analysis identified more than 30 Ser/Thr phosphorylation sites in the intracellular domain of EGFR (25). Thr-669 in the juxtamembrane domain is the most characterized site, is phosphorylated by ERK, and is involved in the negative feedback regulation of tyrosine kinase activity (26). Ser-1046 and Ser-1047, target sites of the p38 pathway, are considered to regulate receptor desensitization via the internalization of EGFR (12,14,27). Commercially available phospho-specific antibodies to these Ser/Thr sites have been widely used in analyses of these sites. Another important p38 target Ser-1015 is suggested to be involved in receptor endocytosis (13,18); however,

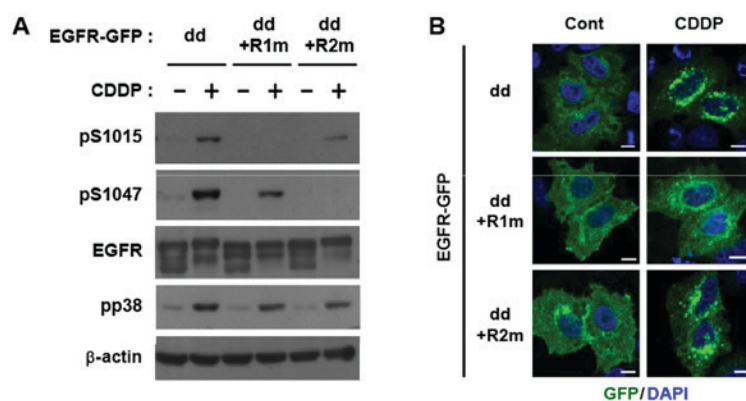


Figure 3. Phosphorylation and endocytosis of the dimer-deficient EGFR mutant. (A) 293 cells were transiently transfected with GFP-tagged dd-EGFR, dd+R1m and dd+R2m mutants, and cells were then treated with 100 μ M CDDP for 8 h. Whole cell lysates were analyzed by immunoblotting with the indicated antibodies. (B) HeLa cells were transiently transfected with GFP-tagged dd-EGFR (dd) and its mutants, dd+R1m and dd+R2m, and were then treated with 100 μ M CDDP for 6 h. The cellular localization of dd-EGFR was assessed by the immunofluorescence of GFP. Scale bar, 10 μ m. EGFR, epidermal growth factor receptor; CDDP, cisplatin; GFP, green fluorescent protein; dd, dimer-deficient.

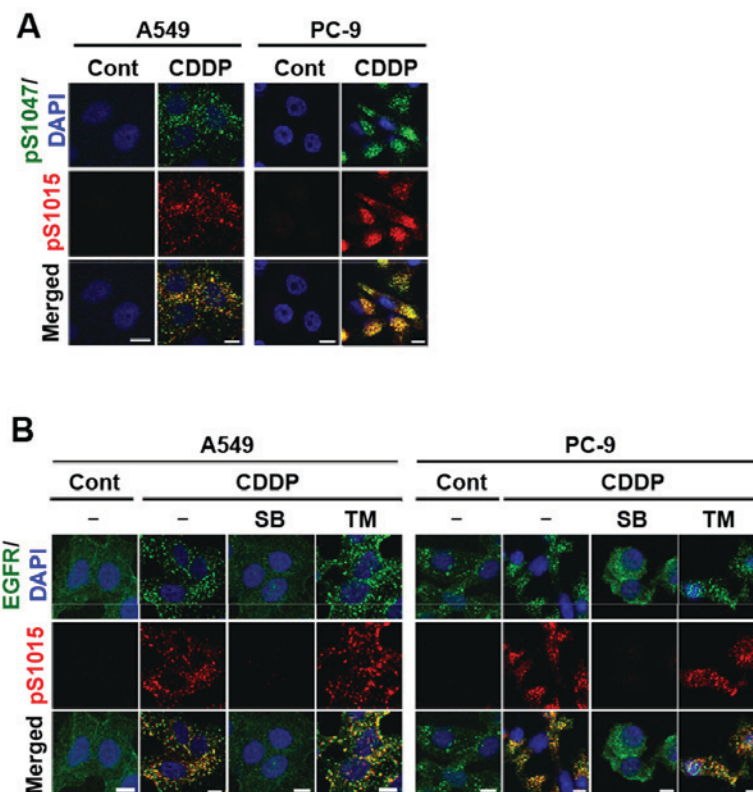


Figure 4. CDDP-triggered endocytic trafficking of serine phosphorylated EGFR. (A) A549 and PC-9 cells were treated with 100 μ M CDDP for 6 h. (B) A549 and PC-9 cells were pretreated with 10 μ M SB or 30 nM TM, followed by the 100 μ M CDDP treatment for 6 h. In all data, the localization of total EGFR and phospho-serine EGFR was detected by a confocal microscopy analysis. Scale bar, 10 μ m. Cont, control; CDDP, cisplatin; SB, SB203580; TM, trametinib; EGFR, epidermal growth factor receptor.

its physiological characterization has not yet been conducted due to the lack of a high-quality phospho-specific antibody. In the present study, we demonstrated that Ser-1015 was phosphorylated with similar dynamics to Ser-1047 phosphorylation using a newly generated antibody. Nevertheless, only Ser-1015 was essential for the non-canonical endocytosis of wild type EGFR as well as EGFR harboring primary and secondary mutations. Taken together with previous findings on Ser-1046/1047, cooperative regulation with Ser-1015 needs to be reconsidered and the original unknown functions of Ser-1046/1047 elucidated.

Evidence for the physiological functions of TK-inactive EGFR has been increasing. For example, lysosomal-associated protein transmembrane 4B (LAPTM4B) binds to inactive EGFR on endosomes, which participates in the initiation of autophagy (16,28). Furthermore, cellular stress conditions with p38 activation have been shown to affect autophagy (15). Distinct from activation with ligands, EGFR phosphorylated by p38 is sorted to lipid lysobisphosphatidic acid (LBPA)-rich perinuclear MVBs upon a UVC or cisplatin stimulation, demonstrating that canonical and non-canonical

EGFR endocytic trafficking is involved in different cellular responses (29). It is essential to investigate the role of the MAPK-dependent non-canonical regulation of EGFR in the initiation of autophagy in cisplatin-treated cancer cells.

p38-induced EGFR endocytosis is known to be dependent on clathrin (12,30). In NSCLCs, clathrin light chain isoform b (CLCb) is up-regulated, and this is associated with a poor patient prognosis, particularly in the advanced stages of tumors. This type of CME entirely depends on dynamin-1 (Dyn1) and increases the activation of EGFR downstream pathways, particularly Akt (31,32). However, the role of CLC in EGFR trafficking has yet to be characterized; therefore, it is of interest to investigate whether the CLCb/Dyn1 machinery is involved in the p38-mediated endocytosis of Ser/Thr-phosphorylated EGFR in NSCLC cells, and the findings obtained will contribute to our understanding of new functions of non-canonical EGFR in tumor malignant alterations.

Collectively, our results showing the precise phosphorylation patterns of EGFR using a new phospho-specific antibody will drive new research directions that will provide a clearer understanding of the TK-independent functions of EGFR in cisplatin-treated NSCLC cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TT contributed to the experimental design, conduction of experiments, data analysis and interpretation and manuscript writing. TO and AM contributed to the antibody preparation. EO performed the cell culture experiments. HS contributed to the experimental design, data analysis and interpretation and manuscript writing. All authors approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yarden Y and Pines G: The ERBB network: At last, cancer therapy meets systems biology. *Nat Rev Cancer* 12: 553-563, 2012.
2. Casaletto JB and McClatchey AI: Spatial regulation of receptor tyrosine kinases in development and cancer. *Nat Rev Cancer* 12: 387-400, 2012.
3. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, *et al*: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 304: 1497-1500, 2004.
4. Health Quality Ontario: Epidermal growth factor receptor mutation (EGFR) testing for prediction of response to EGFR-targeting tyrosine kinase inhibitor (TKI) drugs in patients with advanced non-small-cell lung cancer: an evidence-based analysis. *Ont Health Technol Assess Ser* 10: 1-48, 2010.
5. Chung C: Tyrosine kinase inhibitors for epidermal growth factor receptor gene mutation-positive non-small cell lung cancers: An update for recent advances in therapeutics. *J Oncol Pharm Pract* 22: 461-476, 2015.
6. Bennisroune A, Gardin A, Aunis D, Cremel G and Hubert P: Tyrosine kinase receptors as attractive targets of cancer therapy. *Crit Rev Oncol Hematol* 50: 23-38, 2004.
7. Mendelsohn J and Baselga J: Epidermal growth factor receptor targeting in cancer. *Semin Oncol* 33: 369-385, 2006.
8. Lemmon MA and Schlessinger J: Cell signaling by receptor tyrosine kinases. *Cell* 141: 1117-1134, 2010.
9. Jura N, Endres NF, Engel K, Deindl S, Das R, Lamers MH, Wemmer DE, Zhang X and Kuriyan J: Mechanism for activation of the EGF receptor catalytic domain by the juxtamembrane segment. *Cell* 137: 1293-1307, 2009.
10. Nishimura M, Shin MS, Singhirunnusorn P, Suzuki S, Kawanishi M, Koizumi K, Saiki I and Sakurai H: TAK1-mediated serine/threonine phosphorylation of epidermal growth factor receptor via p38/extracellular signal-regulated kinase: NF- κ B-independent survival pathways in tumor necrosis factor alpha signaling. *Mol Cell Biol* 29: 5529-5539, 2009.
11. Refaat A, Aminullah, Zhou Y, Kawanishi M, Tomaru R, Abdelhamed S, Shin MS, Koizumi K, Yokoyama S, Saiki I and Sakurai H: Role of tyrosine kinase-independent phosphorylation of EGFR with activating mutation in cisplatin-treated lung cancer cells. *Biochem Biophys Res Commun* 458: 856-861, 2015.
12. Zwang Y and Yarden Y: p38 MAP kinase mediates stress-induced internalization of EGFR: Implications for cancer chemotherapy. *EMBO J* 25: 4195-4206, 2006.
13. Tong J, Taylor P and Moran MF: Proteomic analysis of the EGFR interactome and post-translational modifications associated with receptor endocytosis in response to EGF and stress. *Mol Cell Proteomics* 13: 1644-1658, 2014.
14. Adachi S, Natsume H, Yamauchi J, Matsushima-Nishiwaki R, Joe AK, Moriwaki H and Kozawa O: p38 MAP kinase controls EGF receptor downregulation via phosphorylation at Ser1046/1047. *Cancer Lett* 277: 108-113, 2009.
15. Tan X, Lambert PF, Rapraeger AC and Anderson RA: Stress-induced EGFR trafficking: Mechanisms, functions and therapeutic implications. *Trends Cell Biol* 26: 352-366, 2016.
16. Tan X, Thapa N, Sun Y and Anderson RA: A kinase-independent role for EGF receptor in autophagy initiation. *Cell* 160: 145-160, 2015.
17. Kelland L: The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* 7: 573-584, 2007.
18. Tanaka T, Zhou Y, Ozawa T, Okizono R, Banba A, Yamamura T, Oga E, Muraguchi A and Sakurai H: Ligand-activated epidermal growth factor receptor (EGFR) signaling governs endocytic trafficking of unliganded receptor monomers by non-canonical phosphorylation. *J Biol Chem* 293: 2288-2301, 2018.
19. Refaat A, Zhou Y, Suzuki S, Takasaki I, Koizumi K, Yamaoka S, Tabuchi Y, Saiki I and Sakurai H: Distinct roles of transforming growth factor-beta-activated kinase 1 (TAK1)-c-Rel and interferon regulatory factor 4 (IRF4) pathways in human T cell lymphotropic virus 1-transformed T helper 17 cells producing interleukin-9. *J Biol Chem* 286: 21092-21099, 2011.

20. Zhou Y, Yamada N, Tanaka T, Hori T, Yokoyama S, Hayakawa Y, Yano S, Fukuoka J, Koizumi K, Saiki I and Sakurai H: Crucial roles of RSK in cell motility by catalysing serine phosphorylation of EphA2. *Nat Commun* 6: 7679, 2015.
21. Zhou Y, Tanaka T, Sugiyama N, Yokoyama S, Kawasaki Y, Sakuma T, Ishihama Y, Saiki I and Sakurai H: p38-Mediated phosphorylation of Eps15 endocytic adaptor protein. *FEBS Lett* 588: 131-137, 2014.
22. Kinoshita E and Kinoshita-Kikuta E: Improved Phos-tag SDS-PAGE under neutral pH conditions for advanced protein phosphorylation profiling. *Proteomics* 11: 319-323, 2011.
23. Wang Q, Villeneuve G and Wang Z: Control of epidermal growth factor receptor endocytosis by receptor dimerization, rather than receptor kinase activation. *EMBO Rep* 6: 942-948, 2005.
24. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Zhu HJ, Walker F, Frenkel MJ, Hoyne PA, *et al*: Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell* 110: 763-773, 2002.
25. Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V and Sullivan M: PhosphoSitePlus: A comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res* 40: D261-D270, 2012.
26. Sato K, Shin MS, Sakimura A, Zhou Y, Tanaka T, Kawanishi M, Kawasaki Y, Yokoyama S, Koizumi K, Saiki I and Sakurai H: Inverse correlation between Thr-669 and constitutive tyrosine phosphorylation in the asymmetric epidermal growth factor receptor dimer conformation. *Cancer Sci* 104: 1315-1322, 2013.
27. Countaway JL, Nairn AC and Davis RJ: Mechanism of desensitization of the epidermal growth factor receptor protein-tyrosine kinase. *J Biol Chem* 267: 1129-1140, 1992.
28. Tan X, Sun Y, Thapa N, Liao Y, Hedman AC and Anderson RA: LAPTM4B is a PtdIns(4,5)P2 effector that regulates EGFR signaling, lysosomal sorting and degradation. *EMBO J* 34: 475-490, 2015.
29. Tomas A, Vaughan SO, Burgoyne T, Sorkin A, Hartley JA, Hochhauser D and Futter CE: WASH and Tsg101/ALIX-dependent diversion of stress-internalized EGFR from the canonical endocytic pathway. *Nat Commun* 6: 7324, 2015.
30. Tomas A, Futter CE and Eden ER: EGF receptor trafficking: Consequences for signaling and cancer. *Trends Cell Biol* 24: 26-34, 2014.
31. Chen PH, Bendris N, Hsiao YJ, Reis CR, Mettlen M, Chen HY, Yu SL and Schmid SL: Crosstalk between CLCb/Dyn1-mediated adaptive clathrin-mediated endocytosis and epidermal growth factor receptor signaling increases metastasis. *Dev Cell* 40: 278-288.e5, 2017.
32. Schmid SL: Reciprocal regulation of signaling and endocytosis: Implications for the evolving cancer cell. *J Cell Biol* 216: 2623-2632, 2017.