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Molecular imaging of epidermal growth factor receptor kinase activity

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ABSTRACT

Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is commonly altered in different tumor types, leading to abnormally regulated kinase activity and excessive activation of downstream signaling cascades, including cell proliferation, differentiation, and migration. To investigate the EGFR signaling events in real time and in living cells and animals, here we describe a multidomain chimeric reporter whose bioluminescence can be used as a surrogate for EGFR kinase activity. This luciferase-based reporter was developed in squamous cell carcinoma cells (UMSCC1) to generate a cancer therapy model for imaging EGFR. The reporter is designed to act as a phosphorylated substrate of EGFR and reconstitutes luciferase activity when it is not phosphorylated, thereby providing a robust indication of EGFR inhibition. We validated the reporter in vitro and demonstrated that its activity could be differentially modulated by EGFR tyrosine kinase inhibition with erlotonib or receptor activation with epidermal growth factor. Further experiments in vivo demonstrated quantitative and dynamic monitoring of EGFR tyrosine kinase activity in xenograft. Results obtained from these studies provide unique insight into pharmacokinetics and pharmacodynamics of agents that modulate EGFR activity, revealing the usefulness of this reporter in evaluating drug availability and cell targeting in both living cells and mouse models.

The emerging fields of genomics and proteomics have led to a better comprehension of the pathophysiology of cancer and the identification of novel signaling pathways. These pathways offer novel targets that have led to the development of lead molecules designed to inhibit the signaling derived from these pathways. However, this knowledge has provided considerable challenges for translating in vitro findings to in vivo cancer biology. Molecular imaging technologies have the potential to address these scientific challenges and to bridge the gap between in vitro drug discovery and in vivo target inhibition [1].

The epidermal growth factor receptor (EGFR)[1] is an established therapeutic target in oncology. Nearly 90% of all head and neck squamous cell carcinoma (HNSCC) cases exhibit an enhanced expression of EGFR that leads to constitutive activation of the receptor and, therefore, correlates with tumor proliferation, metastasis, and resistance to conventional therapy [2]. Furthermore, the overexpression of EGFR has also been detected in many epithelial malignancies, including cancers of bladder, breast, lung, brain, stomach, prostate, ovary, and pancreas [3].

EGFR, a 170-kDa transmembrane glycoprotein, belongs to the Erb/HER family of transmembrane receptor tyrosine kinases and is composed of an extracellular ligand binding domain (621 amino acids) and an intracellular protein tyrosine kinase domain (542 amino acids) connected by a small transmembrane-anchoring region (23 amino acids) [4]. The binding of a ligand, such as epidermal growth factor (EGF), causes the EGFR to dimerize with itself or with another member of the ErbB family of receptors, leading to receptor-linked tyrosine activation and the activation of downstream signaling cascades, including cell proliferation, differentiation, migration, and adhesion. Studies by Lamaze and Schmid demonstrated the importance of kinase substrates for the efficient activation of EGFR signaling, and the epidermal growth factor substrate 15 (EPS15) is required for the efficient activation of EGFR [5]. EPS15 has been identified as one downstream signaling protein [6] that can be phosphorylated on Tyr850 residue following EGFR activation [7]. The EGFR signaling pathway is considered to be a key

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[1] *Abbreviations used:* EGFR, epidermal growth factor receptor; HNSCC, head and neck squamous cell carcinoma; EGF, epidermal growth factor; EPS15, epidermal growth factor substrate 15; EKR, EGFR kinase reporter; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; siRNA, small interfering RNA; BLI, bioluminescence imaging; DMSO, dimethyl sulfoxide; HGF/SF, hepatocyte growth factor/scatter factor; TEV, tobacco etch virus.

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determinant of biological aggressiveness of tumors and a major target for novel anticancer therapies. Inhibition of this receptor with small molecule tyrosine kinase inhibitors or receptor-specific antibodies has proven to be an effective therapeutic strategy in multiple cancer sites [8].

Although EGFR activity can be measured using standard molecular biology techniques in vitro, assessing EGFR inhibition in real time in animals has not been feasible previously. We reasoned that developing a method to image EGFR activity in animals would be a valuable tool to further understand the biology of in vivo EGFR inhibition. To this end, we constructed the EGFR kinase reporter (EKR), a multidomain chimeric protein that coordinately regulates luciferase activity based on both the concept of luciferase complementation [9] and reversible phosphorylation of the relatively specific EPS15 tyrosine phosphorylation site [10,11]. We demonstrate that the EKR, but not the phenylalanine mutated control vector, is activated by micromolar concentrations of erlotonib and results in bioluminescence in living cells, providing a molecular reporter that we use to quantify in vitro EGFR activity as well as in vivo inhibition of EGFR by erlotonib.

Materials and methods

Antibodies and chemicals

Rabbit polyclonal antibodies to phospho-EGFR (Y845), Met (pY-pYpY1230/1234/1235), GAPDH, and mouse polyclonal Met antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies to EGFR and firefly luciferase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Chemicon (Millipore, Billerica, MA, USA), respectively. Mouse monoclonal antibodies to p-Tyr were purchased from Zymed (Carlsbad, CA, USA). SU11274, an inhibitor of c-Met, was purchased from Sigma–Aldrich (St. Louis, MO, USA). EGFs and luciferin were purchased from Invitrogen (Carlsbad, CA, USA) and Biosynth (Naperville, IL, USA), respectively. Erlotinib was gifted by Genentech (San Francisco, CA, USA).

Plasmid construction

The EKR reporter was generated in the mammalian expression vector pEF. Construction of the EKR luciferase reporter was based on the split luciferase design of Luker and coworkers [9]. The N-terminal domain (N-Luc) was polymerase chain reaction (PCR) amplified using primers that generated a product comprising a *Sal*I restriction site followed by a Kozak consensus sequence and a *Not*I restriction site at the 3′ end. The C-terminal firefly luciferase domain (C-Luc) was amplified using primers that produce a 5′ *Xba*I site followed by the EPS15 substrate sequence (corresponding to amino acids 843–858) flanked by the linker GSHSGSGKP on each side, with a 3′ *Eco*RI restriction site after the termination codon. The SH2 domain was amplified from the mouse p52 Shc domain with insertion of a 5′ *Not*I site and a 3′ *Xba*I site for cloning. The EKR–Mut reporter was constructed by mutagenesis of the EPS15 tyrosine phosphorylation site (Y850) to alanine using the QuikChange kit (Stratagene, La Jolla, CA, USA). All plasmids were verified by DNA sequencing.

Cell culture and transfection

The HNSCC cell line, UMSCC1, was grown in RPMI-1640 medium (Invitrogen). Complete medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 100 U/ml penicillin/streptomycin. Cell cultures were maintained in a humidified incubator at 37 °C and 5% CO_2. To construct stable cell lines, the EKR reporter plasmids (wild type and mutant) were stably transfected into UMSCC1 cells using Fugene (Roche Diagnostics, Indianapolis, IN, USA) and stable clones were selected with 500 μg/ml G418 (Invitrogen). Resulting clones were isolated and cultured for further analysis by Western blot for determination of expression levels of the recombinant protein.

Western blots and immunoprecipitation

UMSCC1–EKR cells in culture dishes were collected and centrifuged at 1800*g* for 5 min and 4 °C. Cell pellets were washed twice with cold phosphate-buffered saline (PBS) and then lysed with a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50 mM NaF, and 1 mM Na_3VO_4 and supplemented with a Complete Protease Inhibitor mixture (Roche Diagnostics). Cells in lysis buffer were rocked at 4 °C for 30 min. The lysates were then cleared by centrifugation. Protein content was determined by a detergent-compatible protein assay kit from Bio-Rad (Hercules, CA, USA). Lysates with equal amounts of protein were separated by Laemmli SDS–PAGE (polyacrylamide gel electrophoresis), and the levels of protein expression were detected by Western blot analysis with anti-EGFR (1:1000), p-EGFR (1:1000), Met (1:500), p-Met (1:1000), luciferase (1:5000), p-Tyr (1:1000), and GAPDH (1:1000) using the ECL (enhanced chemiluminescence) Western Blotting System (GE Healthcare, Piscataway, NJ, USA). For immunoprecipitation, cell supernatant extracts (2000 μg) were incubated with the luciferase antibody for 1 h. Immune complexes were captured using protein G-Sepharose (GE Healthcare) and washed three times using RIPA buffer. The resulting pellet was boiled for 5 min in sample buffer (0.125 M Tris–HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue) and resolved by SDS–PAGE.

Small interfering RNA transfections

UMSCC1–EKR–WT cells were plated onto 6-well plates at a density of 2.5×10^5 cells/ml and incubated for 24 h in culture medium. The cells were then transfected with small interfering RNAs (siRNAs) specific to EGFR (J-003114-11 or J-003114-11 from Dharmacon, Lafayette, CO, USA) or nontargeting siRNA (D-001210-01, Dharmacon). siRNA transfection was carried out using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Cells were analyzed 68 h later for bioluminescence imaging (BLI) followed by Western blot.

In vitro and in vivo bioluminescence imaging

Live cell BLI was performed using the IVIS imaging system (Caliper Life Sciences, Hopkinton, MA, USA). D-Luciferin (100 μg/ml final concentration) was added to the growth medium prior to imaging, and photon counts were acquired after 10 min of incubation at 37 °C. An exposure time of 1 min was used for acquisition. Living Image 3.0 software was used for data analysis (Caliper Life Sciences). For in vivo experiments, UMSSC1–EKR cells (1.0×10^6) were inoculated into the flanks of *nu*/*nu* CD-1 nude mice (Charles River Laboratories, Wilmington, DE, USA) and grown for 10 days. When tumors reached approximately 100 mm^3 in volume, mice were randomized into two groups and treatment was initiated. Erlotinib was prepared in 1% Tween 80 and delivered at a dose of 100 mg/kg by oral gavage. Control animals were treated with vehicle only. For in vivo bioluminescence, mice were anesthetized using a 2% isofluorane/air mixture and injected with a single dose of 100 mg/kg D-luciferin in PBS intraperitoneally. Image acquisition was initiated 5 min following injection of luciferin. Serial biolumi-