Supplementary Materials and Methods

Cell culture, treatments and reagents. A549 cells were cultured in F12K medium (GIBCO) supplemented with 10% FBS (GIBCO) and 1% pen/strep (GIBCO). HCC827 were cultured in RPMI (GIBCO) medium supplemented with 10% FBS and 1% pen/strep. NIH-3T3 cells were stably transfected with wild type EGFR or L858R EGFR mutant (MT) or with the exon 19 deletion EGFR MT (EGFR Δ747-752) (kindly provided by Dr. H. Band, University of Nebraska Medical Center) (19), and were cultured in DMEM (GIBCO) medium supplemented with 10% fetal bovine serum (FBS) and 1% pen/strep. For the treatments DMEM medium (GIBCO) was used with no FBS. EGF was added directly into the treatment medium at a final concentration of 100 ng/ml. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) cross linker agent (Thermo Scientific) was dissolved in phosphate buffer saline (PBS) and then added to the treatment medium at a final concentration of 1 mM. Tyrosine kinase inhibitor (TKI) AG1478 (Cell Signaling), Erlotinib (Tarceva) and Gefitinib (InvivoGen) were dissolved in dimethyl sulfoxide (DMSO) and then added to the treatment medium at a final concentration of 1 μM. PBS or DMSO was added at the appropriate concentration to the control-untreated cells. Cells were collected by scraping in either PBS or directly in the lysis buffer: 1% NP-40 (Igepal, from Sigma), 50 mM Tris, 10% Glycerol, 0.02% NaN_3, 150 mM NaCl, pH 7.4, containing a cocktail of phosphatase and protease inhibitors (Sigma) as well as 1 mM NaF and Na_3VO_4. Lysates were passed 5 times through a 30 gauge needle prior to centrifugation and further processing of the samples (either immuno-precipitation or immuno-blotting). All the other reagents were from Sigma, unless differently specified.

Cigarette smoke exposure. Serum-starved cells were placed in a vacuum oven with a chamber volume of 0.45 ft^3 with the temperature set at 37°C. A negative pressure was generated by a vacuum (~20 inHg) and smoke from one cigarette (University of Kentucky 2R4F) was drawn into the chamber by bleeding the chamber (a decrease of ~5 inHg over ~30s) through a short piece of tubing equipped with a valve and holding the cigarette (for some treatments, smoke from ½ cigarette was drawn into the chamber). The

chamber atmosphere was then lowered to atmospheric pressure for the remaining of the exposure time (15 or 30 min.). The detailed experimental conditions for the CS exposure were previously optimized (5).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immuno-blotting (IB). Primary Abs were incubated in 5% milk-TBST for 2 h at RT. Secondary Abs, either goat αmouse- or goat αrabbit-horseradish peroxidase (HRP) conjugated (Jackson ImmunoResearch), were incubated for 90 min. at RT at 1:10000 dilution in 5% milk-TBST. Bands were visualized by enhanced chemiluminescence (ECL, PIERCE). Extensive washes in TBST were done in between each step. When performing sequential IBs to assess the specific sites of EGFR phosphorylation, the Abs were stripped off the membranes ("Stripping Buffer" from Thermo Scientific) in between of each IB. Primary Abs used in this study for the IBs were: α2232 (αEGFR, Cell Signaling, 1:1000), αc-Src (Santa Cruz Biotech, 1:2000), αtyrosine Y416 phosphorylated (p-) c-Src (Cell Signaling, 1:2000), αp-Y20 (Santa Cruz Biotech, 1:3000), αp-Y1173 EGFR (Santa Cruz Biotech, 1:1000), and αp-Y1086, αp-Y1068, αp-Y845 EGFR, αp-ERK 1/2, αERK 1/2, αp-Akt, αAkt (Cell Signaling, 1:1000).

Legends to supplementary figures

Supplementary figure 1. CS-induced ERK and Akt activation is not inhibited by TKI. Serum-starved A549 cells were incubated (or not) with 1 μM AG1478 for 30 min and with EGF or CS, as in figure 1. Subsequently, 50 μg of proteins from the cell lysates were assessed by IB for levels of active/phosphorylated (p-) ERK 1/2 and AKT. Each treatment and experiment/IP-IB was repeated three times obtaining consistent results: the pictures shown are representative.

Supplementary figure 2. NSCLC cells harboring TKI-sensitive EGFR MT become resistant to TKI following exposure to CS. Serum-starved HCC827 cells were incubated (or not) with 1 μM gefitinib for 30 min. Then the cells were treated for 30 min. with 100 ng/ml EGF or exposed to smoke from 1 cigarette for 30 min. EGFR was IPed from the total cell lysates, separated by SDS-PAGE and

IBed for total receptor, total tyrosine phosphorylation level (p-EGFR) and specific Tyr-residue phosphorylation level (Y845, Y1068, and Y1173). Each treatment and experiment/IP-IB was repeated three times obtaining consistent results: the pictures shown are representative.

Supplementary figure 3. CS exposure eliminates the TKI-dependent inhibition of anchorage independent growth of EGFR-transformed cells. The anchorage independent growth of NIH-3T3 cells stably over-expressing the L858R EGFR MT was assessed by soft agar/agarose colony formation as described in Material and Methods. Single-suspended cells were seeded within a 0.275% agarose gel. The cells were fed daily with culture medium supplemented, or not, with 1 μM TKI (Gefitinib) and exposed (or not) to smoke from 1 cigarette for 30'. The treatments were repeated twice (at 24h and 48h after the cell seeding) and colony formation was assessed after 72h. Each treatment was reproduced three times; 10 random pictures per dish were acquired by optical microscopy and then the number of cell colonies (composed of at least 3 cells) was assessed. **A.** The graphic represents number of colonies per dish as % of the untreated (NT) cells; * = p <0.05 in respect to TKI-treated cells. **B**. Sample images of the colonies are shown.