SUPPLEMENTAL MATERIALS AND METHODS

Cell culture and treatment

Human breast cancer cell lines MCF-7, ZR-75-1 and SkBr3 were obtained from American Type Culture Collection (Manassas, VA). The characteristics of these cell lines are indicated in Table 1.[1] EGFR/HER pan-inhibitor CI1033,[2] HER2 specific inhibitor CP724714[3] and EGFR/HER1 specific inhibitor AG1478[4] and Erlotinib[5] were purchased from SelleckChem (Houston, TX) and dissolved in dimethyl sulfoxide (DMSO). MCF-7 and ZR-75-1 cells were maintained in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum. SkBr3 cells were maintained in McCoy medium containing 10% fetal bovine serum.

For experiments involving IR, exponentially growing cells were exposed to IR and incubated at 37°C for the indicated time prior to analysis. For experiments involving treatment with both inhibitor and IR, cells were incubated with inhibitor for 1 h prior to IR exposure.

Antibodies and recombinant proteins

All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise indicated. These included mouse IgG for ATM (2C1) (Novus Biologicals, Littleton, CO), Cdc2 (17), Chk1 (G-4), Chk2 (B-4) (EMD Biosciences, San Jose, CA), c-Myc (9E10), phospho-ERK1/2 (E-4) and phospho-Tyr; rabbit IgG for ATM (Ab-3) (EMD Biosciences, San Jose, CA), Cdc2 (C-19), Chk1 (FL-476), Chk2 (Cell Signaling, Danvers, MA), GAPDH, HER1 (Cell Signaling), HER2 (Cell Signaling), HER3 (Cell Signaling), HER4 (Cell Signaling), phospho-HER1-Tyr1173 (53A5) (Cell Signaling), phospho-HER2-Tyr1196 (D66B7) (Cell Signaling), phospho-HER3-Tyr1289 (21D3) (Cell Signaling), phospho-HER4-Tyr984 (Cell Signaling); and goat IgG for Actin (I-19), ATR (N-19), phospho-Cdc2-Tyr15, ERK1/2 (C-14-G).

Recombinant p53 protein for ATM and ATR kinase assay was a glutathione S-transferase (GST) fusion protein containing full-length human p53 (Addgene, Cambridge,

MA). Recombinant Cdc25C protein, the substrate for Chk1 and Chk2 kinase assay, was a GST fusion protein containing residues 200-256 of human Cdc25C (kindly provided by Dr. Helen Piwnica-Worms, Washington University School of Medicine). All GST fusion proteins were purified as described previously.[6] GST was used as a control substrate in all kinase assays and was prepared according to standard procedures (GE Healthcare Bio-Sciences, Piscataway, NJ).

Immunoblotting, immunoprecipitation and kinase assay

Immunoblotting, immunoprecipitation and kinase assay were performed as described previously.[6-8] Specific protein signals on Western blots were visualized by chemiluminescence exposed to X-ray film, scanned using EPSON Perfection V330Photo scanner and analyzed using the ImageJ analytical program (NIH, Bethesda, MD).

The analyses of HER1, HER2 and HER3, for both phosphorylation and protein level, were done by Western blot analysis using the appropriate antibodies described above. For the analysis of HER4, HER4 was immunoprecipitated from cell lysate first using anti-HER4 antibody and probed for level of protein and phosphorylation using anti-HER4 and anti-phospho-Tyr antibody, respectively.

Cell cycle analysis

Fluorescence-activated cell sorting (FACS) analysis was performed on 20,000 cells using a FACS-Calibur instrument (Beckon Dickinson, Mansfield, MA), as described previously.[6]

Analysis for mitotic cells

As described previously, cells were fixed in 70% ethanol and stained with propidium iodide (PI) and anti-phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY).[9] Mitotic cells, which contain both 4N-DNA content and phospho-histone H3, were determined

using a FACSCalibur instrument (Beckon Dickinson) and analyzed by using CELLQUEST software. Each analysis was performed using 20,000 cells.

shRNA retroviral vectors and viral infection

Retroviral vectors expressing short-hairpin RNAs (shRNAs) were obtained from OriGene Technologies, Inc. (Rockville, MD). The sequences for shRNAs targeting Her2 are: 5'-CTGG CTCT CACA CTGA TAGA CACC AACC G-3', 5'-GCAG AGGA TGGA ACAC AGCG GTGT GAGA A-3', 5'-TACC GCTC ACTG CTGG AGGA CGAT GACA T-3' and 5'-TGTT GGAT GATT GACT CTGA ATGT CGGC C-3'. The sequences for shRNAs targeting Her3 are: 5'-CCAA GACC ATCT GTGC TCCT CAGT GTAA T-3', 5'-TGCT GAGA TAGT GGTG AAGG ACAA TGGC A-3', 5'-TGGC TTTG ACAG TGAT AGCA GGAT TGGT A-3' and 5'-TGAA TCGG CAAC GAGA TGGA GGTG GTCC T-3'. The sequences for shRNAs targeting Her4 are: 5'-TGGA GTAT GTCC ACGA GCAC AAGG ATAA C-3', 5'-TCAG ACTG TGGA TTCC AGTA ACAT TGAC A-3', 5'-AGGC ACTG TGCT GCCG CCTC CACC TTAC A-3' and 5'-TGTA AGGC AATG CTGC CTAC TATC AAAC T-3'. The sequence for control shRNA targeting firefly luciferase is 5'-CCCG CCTG AAGT CTCT GATT AA-3'.

Phoenix A retroviral packaging cells[10] were transfected with shRNA retroviral expressing vectors using MBS mammalian transfection kit (Stratagene, La Jolla, CA), according to manufacturer's instruction. At 48 h post transfection, medium containing amphotropic retrovirus was collected, filtered through a 0.4 μM filter and MCF-7 cells infected with retroviral vectors in the presence of 4 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Clones stably expressing shRNAs were selected in medium containing 2 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO). The levels of HER receptors in these clones were determined by Western blot analysis.

Expressing vectors and transfection

pcDNA3 vector expressing myc-tagged wild-type HER2 (HER2-wt), myc-tagged dominant negative mutant HER2 (HER2-mut) and control pcDNA3 vector were gifts from Dr. Ming-Fong Lin (University of Nebraska Medical Center, Omaha, NE).[11] HER2-wt contains a full-length HER2 cDNA and HER2-mut contains the N-terminal portion of the HER2 cDNA (amino acids 1-685), which includes the extra-cellular region, the trans-membrane region and the first 10 amino acids of the intracellular region of HER2.[11]

MCF-7 cells were transfected with the indicated vectors using Lipofactamine, according to manufacturer's instruction (Life Technologies, Grand Island, NY). The transfected cells were incubated with G418 to select stable HER2-wt and HER2-mut expressing clones. HER2-wt and HER2-mut expression in the clones were determined by Western blotting using anti-Myc antibody.

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