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(54) Title: EGF RECEPTOR STATUS FOR DISEASE TREATMENT

(57) Abstract: This invention relates, e.g., to a method for predicting the response of a subject having a disease or condition mediated by EGFR (e.g., a cancer of epithelial origin, such as NSCLC) to an EGFR kinase inhibitor (e.g., Iressa® (gefitinib) and/or Tarceva® (erlotinib)). The method comprises measuring the amount of phosphorylation at residues Y1068 and T1148 in EGFR in a sample from the subject, wherein a significantly elevated level of phosphorylation at the two residues compared to a baseline value indicates that the subject is likely to be responsive to an agent that inhibits the kinase activity of EGFR. Other sites of phosphorylation that can be employed are also disclosed, including other residues in EGFR, as well as sites in other proteins of the EGFR signaling cascade.

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EGF RECEPTOR STATUS FOR DISEASE TREATMENT

This application claims the benefit of the filing dates of U.S. provisional applications 60/780,832, filed March 10, 2006 and 60/781,369, filed March 13, 2006, both of which are
5 incorporated by reference herein in their entireties.

BACKGROUND INFORMATION

Growth factor receptor tyrosine kinases play a role in the etiology and progression of a variety of disorders or diseases, including, *e.g.*, human malignancies. These biological receptors
10 are anchored by means of a transmembrane domain in the membranes of cells that express them. An extracellular domain binds to a growth factor. The binding of the growth factor to the extracellular domain results in a signal being transmitted to the intracellular kinase domain. The transduction of this signal contributes to a variety of pleiotrophic responses, which are responsible, *e.g.*, for the induction of DNA synthesis, altered gene expression, cell growth,
15 proliferation and differentiation, etc.

The epidermal growth factor receptor (EGF receptor or EGFR), also known as c-erbB1/Her 1, and the product of the neu oncogene (also known as c-erbB2/Her 2) are members of the EFG receptor super family, which belongs to the large family of receptor tyrosine kinases. The EGF receptor is a transmembrane glycoprotein which has a molecular weight of 170,000
20 and is found on many epithelial cell types. It is activated by at least three ligands, EGF, TGF- α (transforming growth factor alpha) and amphiregulin. Both epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) have been demonstrated to bind to EGF receptor and to lead to cellular proliferation and tumor growth. In contrast to several families of growth factors, which induce receptor dimerization by virtue of their dimeric nature (*e.g.* PDGF) monomeric
25 growth factors, such as EGF, contain two binding sites for their receptors and, therefore, can cross-link two neighboring EGF receptors. Receptor dimerization is essential for stimulating the intrinsic catalytic activity and for the autophosphorylation of growth factor receptors. Receptor protein tyrosine kinases (PTKs) are able to undergo both homo- and heterodimerization.

The EGF receptor is composed of 1,186 amino acids which are divided into an
30 extracellular portion of 621 residues and a cytoplasmic portion of 542 residues connected by a single hydrophobic transmembrane segment of 23 residues. The external portion of the EGF receptor can be subdivided into four domains. Domain III, residues 333 to 460, which is flanked

by two cysteine domains contains the EGF binding site of the receptor. The binding of EGF to domain III leads to the initiation of pleiotropic responses, such as those noted above. The responses are a result of three dimensional changes in the receptor leading to autophosphorylation of specific residues on the cytoplasmic domain of the receptor. These phosphorylation sites serve as docking sites for proteins that interact with the receptor and transduce downstream signals to carry out the pleiotropic responses.

Clinical studies indicate that both EGF receptor and c-erbB2 are overexpressed in certain types of tumors of epithelial origin, *e.g.*, glioblastomas (including glioblastoma multiforme), as well as cancers of the lung (adenocarcinomas, including bronchoalveolar carcinoma (BAC) and non-small cell lung cancer (NSCLC)), breast, ovary, stomach, pancreas, bladder, colon, colorectal, kidney, head and neck. The amplification and/or overexpression of the EGF receptors on the membranes of tumor cells is associated with a poor prognosis.

Such observations have stimulated investigations directed to inhibiting the function of human EGF receptors or c-erbB2 as therapeutic approaches to treat cancer. For example, anti-EGF receptor antibodies as well as anti-Her 2 antibodies have shown fruitful results in human cancer therapy. One example of such a treatment is the commercial product, the humanized monoclonal antibody 4D5 (hMAb 4D5, HERCEPTINTM). Other treatment modalities that are currently being tested clinically include tyrosine kinase inhibitors, such as Iressa® (gefitinib) and Tarceva® (erlotinib).

However, not all subjects respond to EGFR therapy. As a consequence, patients may be exposed to the deleterious side-effects associated with EGFR-targeted therapy without its benefit. It would be desirable to have a method for stratifying patients, especially for distinguishing responders from non-responders, to identify a class of subjects who will benefit from EGFR-targeted therapy. Among the responders it is known that a subset of the responders have a specific mutation(s) in the EGFR. There is a need to rapidly identify patients who harbor the mutation(s) and are likely to respond, as well as those patients who do not have the mutation(s) but are sensitive to the therapy because they have an active EGF signal pathway driven by a non-mutation mechanism.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows a heat map, illustrating unsupervised Bayesian clustering analysis. 20 patients with NSCLC (listed on the Y axis) are shown, tested for the presence of phosphorylated residues

of 20 protein endpoints (X axis). Represented is hierarchical clustering, as a Ward Dendrogram. For all patients the EGFR mutation status was determined. Indicated with dotted lines are the finding that, for two patients having the EGFR mutation L858R (identified with an asterisk), there are low levels of tyrosine phosphorylation at the EGFR Y1173 site, and high levels at the EGFR Y1068 and 1148 sites as well as elevated phosphorylation of tyrosine 992 and 845. The double phosphorylation at the Y1068 and Y1148 residues is only seen in association with the mutant and is not seen for any of the other 18 patients tested. 9 of the 20 tumors contain EGFR phosphorylation on a variety of sites other than Y1068 and Y1148 but do not have the mutation.

Figure 2 shows a heat map, illustrating unsupervised Bayesian clustering analysis, representing a time course of phosphorylation after EGF stimulation, at indicated residues in specific proteins for four cell lines (*in vitro* cell culture data). Represented is hierarchical clustering, as a Ward Dendrogram. The cell lines, listed for different time points on the Y axis, are: A549 WT (wild type); H1975 (point mutation EGFR L858R); H1650 (deletion mutation EGFR Del746-750); and H23 (K-RAF). The time points are 1 min, 2 min, 15 min, 30 min, or 60 min (as indicated). Each cell line is tested for the presence of 14 phosphorylated residues (X axis), as indicated. The two arrows show that, for the H1975 cell line (having the EGFR point mutation L858R), there is a high level of phosphorylation at the EGFR Y1068 and 1148 sites. The double phosphorylation at the latter two sites is not seen for any of the other 3 cell lines tested.

Figure 3 shows the amount of phosphorylation of particular protein endpoints as a function of time, after stimulation by EGF, for three different cell lines: wild type; H1975, having the point mutation EGFR L858R; and H1650, having the deletion mutation EGFR Del746-750. The protein endpoints measured in the figures are: **Fig. 3A**, AKT ser473; **Fig. 3B**, ERK T202/Y204; **Fig. 3C**, EGFR Y1148; **Fig. 3D**, EGFR Y1066; **Fig. 3E**, EGFR Y1173; **Fig. 3F**, EGFR Y1045; **Fig. 3G**, SMAD Y465; **Fig. 3H**, MEK ser217/221.

DESCRIPTION OF THE INVENTION

The present invention relates, *e.g.*, to a method for determining whether a subject having an EGFR-mediated disease or disorder, such as a cancer of epithelial origin, is susceptible (amenable, responsive) to EGFR-targeted therapy (*e.g.* to the administration of an agent that inhibits EGFR kinase activity). Such an inhibitory agent can target EGFR at any of a variety of

sites. For example, it can target the extracellular portion of the receptor and thereby inhibit the signal transduction cascade; it can target the intracellular kinase domain; etc. The inhibitory agent can include any of a variety of antibodies and small molecules inhibitors.

Annually, approximately 150,000 people develop lung cancer in the U.S. About 10-19% of patients suffering from NSCLC harbor a genetic mutation (L858R) near the ATP-binding pocket, in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR). These patients exhibit a positive therapeutic response to EGFR kinase inhibitors such as Iressa® (gefitinib) and Tarceva® (erlotinib).

The inventors show herein that samples from patients which harbor the L858R mutation, or tissue culture cells which contain that mutation, are hyper-phosphorylated at two residues of EGFR, Y1068 and Y1148. The mutant receptor is also associated with augmented phosphorylation on the Y845, Y992, and Y1045 residues. This qualitative and quantitative phosphorylation expression pattern was not observed in samples taken from the other 18 NSCLC patients studied; these other patients harbored different mutations, or no mutations, in EGFR. Nine of the patients who did not have the mutation exhibited phosphorylation of the EGFR on a variety of other sites, but not in the qualitative or quantitative pattern of the known mutants. The presence of these phosphorylated residues, then, serves as a marker for the presence of the L858R mutation, or a similar mutation, and can thus serve as an indication that a subject is likely to react favorably to EGFR therapy (*e.g.*, to an inhibitor of EGFR kinase activity). In general, about 98-99% of patients harboring the L585R mutation are responsive to treatment with gefitinib or erlotinib; so a demonstration that a subject has the aforementioned phosphorylated residues provides an indication that the subject is likely to have a 98-99% chance of reacting favorably to an EGFR kinase inhibitor. Hyper-phosphorylation of additional residues in EGFR, as well as of residues in proteins that lie further downstream in the EGFR tyrosine kinase signal transduction pathway, is also disclosed herein. The detection of these additional hyper-phosphorylations can provide further evidence that a subject is likely to be responsive to EGFR therapy (*e.g.*, to an inhibitor of EGFR kinase activity).

A method of the invention can supplement or replace methods involving the identification of the presence of the L858R mutation by sequencing of the EGF receptor extracted from, *e.g.*, a patient's tumor. This new method for determining an individual patient's drug sensitivity prior to treatment, optionally in conjunction with genomic analysis, can serve as the basis for individualized targeted therapy.

Advantages of a method of the invention include that the method is rapid and inexpensive. For example, a method of the invention is faster, less time consuming, and less expensive than methods which require identification of the presence of the mutation by sequencing of the EGF receptor extracted from a patient's tumor. Another advantage is that a method of the invention can provide functional information, which is not revealed, *e.g.*, by genomic sequencing.

Another advantage of a method of the invention is that not only is the phosphorylation state of EGFR determined, but information is provided about the phosphorylation state of sites on other proteins that are part of linked downstream signal pathways that emanate specifically from EGFR activation. This analysis can signify appropriate combination therapy. Evaluating the combination of specific phosphorylation sites provides direct functional evidence that the receptor has changed its three dimensional shape, dimerized, and has undergone autophosphorylation on the cytoplasmic region of the receptor. Such phosphorylation provides sites of interaction for downstream signaling pathways that drive the growth, survival, differentiation and motility of cells. In a method of the present invention, one can quantitatively measure the level of phosphorylation on a series of residues of, *e.g.*, the EGFR in the context of the native tissue microenvironment. In one embodiment, tissue biopsies are microdissected and the level of phosphorylation of a series of residues on the EGFR and downstream pathways are measured. This information can be used to make a decision about the susceptibility of the biopsied tissue lesion to therapy using an EGF pathway inhibitor. The existence of phosphorylation on the EGFR is transient and only occurs if the receptor is engaged in signaling. Thus, measurement of the phosphorylation sites provides functional information not obtainable by genomic or transcriptomic measurement of the receptor.

One aspect of the invention is a method for predicting the response of a subject suffering from (having) a disease or disorder mediated by EGFR (*e.g.*, a condition such as a cancer of epithelial origin) to EGFR-targeted therapy (*e.g.*, to treatment with an inhibitor of EGFR, such as an inhibitor of EGFR kinase activity). The method comprises measuring the amount of phosphorylation at residues Y1068 and T1148 in EGFR in a sample from the subject (*e.g.*, a sample containing a tissue or cell from the subject, such as a tumor biopsy). An elevated level of phosphorylation at these two residues (*e.g.* a significantly elevated level) compared to a baseline

value indicates that the subject is likely to be responsive to EGFR therapy (*e.g.* to treatment with an agent that inhibits the kinase activity of EGFR).

The method may further comprise measuring the amount of phosphorylation at residues Y845, Y992 and/or Y1045, in EGFR in the sample. An elevated level (*e.g.* a significantly
5 elevated level) of phosphorylation at one or more of Y845, Y992 and/or Y1045, compared to a baseline value, may further indicate that the subject is likely to be responsive to EGFR therapy (*e.g.* to treatment with an agent that inhibits the kinase activity of EGFR).

The method may further comprise measuring the amount of phosphorylation at residues in one or more members of a downstream EGFR signaling pathway. In theory, activated EGFR
10 could activate a wide variety of downstream signaling pathways. However, the studies reported herein identify a few specific pathways that are activated in conjunction with activation of EGFR in a subject harboring an L858R mutant or other mutations within the EGFR that render the patient susceptible to EGFR-targeted therapy, and suggest that other potential downstream pathways are not activated. The finding of hyperphosphorylated residues in certain proteins
15 indicates that the signaling pathways associated with those proteins are activated. For example, an elevated level of phosphorylation at residue ser473 and/or S308 of AKT, residue T202/Y204 of ERK, residue Y317 of SHC, residue T24 of FKHR, residue S65 of 4ebp1, residue S21/9 of GSK3 α/β , residue Y1248 of Her2, residue S1177 of enos, residue Y527 and/or Y416 of Src, residue ser 70 of Bcl-2, residue S2481 of mTOR, residue Y716 of PDGFR β , residue S1108 of
20 eIF4g, and/or residue Y376 of Fak suggests, respectively, that the following signaling pathways are activated: AKT, ERK, SHC, FKHR, 4ebp1, GSK3 α/β , Her2, enos, Src, Bcl-2, mTOR, PDGFR β , eIF4g, and/or Fak. Other targets include IGFR, apoptosis (Bcl-2), or MAPK.

An elevated level of phosphorylation of one or more of the identified residues in proteins of associated downstream pathways, compared to a baseline value, further indicates that the
25 subject is likely to be responsive to EGFR therapy (*e.g.* to treatment with an agent that inhibits the kinase activity of EGFR).

An elevated level of phosphorylation at one or more of the identified residues in proteins of associated downstream pathways, compared to a baseline value, may also indicate that the subject is likely to be responsive to treatment with an inhibitor (*e.g.* a kinase inhibitor) of the
30 protein(s) which contain the hyperphosphorylated residue, or other members of that pathway. Further, the subject may be responsive to treatment with a combination of an EGFR inhibitor and an inhibitor of a member of one of the identified downstream pathways.

Without wishing to be bound by any particular mechanism, it is suggested that the activity of the receptor (*e.g.* EGFR) concomitant with the linked downstream signaling proteins indicates that the entire EGF associated pathway is active and in use in the cancer cells to be treated. It is expected that the measurement of activation of constituents of the entire pathway is more predictive of a therapeutic response than any single analyte by itself. Since the entire pathway is active, this indicates that it is more likely that the cancer cell is being driven by this pathway, and thus would be effectively treated by blocking multiple nodes along this pathway.

A variety of mutations within the EGFR are known to confer therapeutic response to an EGFR targeted inhibitor. In a method of the invention, the sample may contain a mutation in EGFR that is associated with the response of a disease or disorder to an EGFR kinase inhibitor. Examples include the point mutant, L858R, and comparable mutations, *e.g.* a) exon 19 deletions, in which 17 different variants have been identified, b) exon 20 point mutations, c) exon 18 point mutations, d) other point mutations in exon 21 in addition to the L858R mutation, and e) other mutations in the EGF binding domain. See, *e.g.*, a review of such mutations Sharma *et al.* (2007) *Nature Review Cancer* 7, 169-181. One or more of the aforementioned EGFR mutations were present in most cases of non-small cell lung cancer, identified by their dramatic response to tyrosine kinase inhibitors. Comparable mutations that would be expected to generate the same phosphorylation pattern that L858R does and to render a subject harboring the mutation sensitive to the same inhibitors include alterations of the Leu 854 residue of EGFR to an amino acid other than Arg. Other point mutations in exon 21 that are associated with the activation of the EGFR receptor include N826S, A839T, K846R, L861Q and G863D. Other comparable mutations include mutations in the functional moiety in which L858 is located, such as mutations in the tyrosine kinase moiety of the EGFR, or in or near the ATP-binding pocket of EGFR.

Other mutations that would be expected to produce characteristic phosphorylation patterns and drug sensitivity comparable to those identified for the L858R mutant are mutations in proteins that interact with the EGFR, and that alter the conformation of the EGFR to cause it to autophosphorylate residues in the cytoplasmic domain, thereby causing the receptor to activate downstream signal pathways in a manner that contributes to a disease condition.

The disease or disorder may be, *e.g.*, a cancer of epithelial origin, such as lung cancer (*e.g.* NSCLC).

In one embodiment of the invention, the method for predicting the response of a subject to an inhibitor of EGFR kinase activity further comprises identifying whether the subject harbors a mutation in EGFR, such as the L858R mutation. Methods for determining the presence of such a mutation in a sample from a subject are routine and conventional and include, *e.g.*, probing a protein sample with an antibody that recognizes the altered protein, and sequencing a nucleic acid encoding the EGFR from a nucleic acid containing sample. When assaying for the presence of an altered EGFR protein with an antibody, one generally measures the amount of reactivity of an antibody specific for the protein resulting from the mutation of interest (*e.g.* L858R EGFR) in a sample from the subject, wherein an increased reactivity compared to a baseline value (such as the level of reactivity with a non-mutated EGFR) indicates that the subject is likely to be responsive to treatment with an EGFR kinase inhibitor.

Another aspect of the invention is a method for treating a subject suffering from a condition mediated by EGFR, such as a cancer of epithelial origin, comprising (1) measuring the amount of phosphorylation at EGFR residues Y1068 and T1148 in a sample from the subject and, optionally, at one or more of the other phosphorylation sites discussed herein and, if the levels of phosphorylation compared to a baseline value suggest that the subject is likely to be responsive to EGFR therapy (*e.g.* to treatment with an agent that inhibits the kinase activity of EGFR), (2) administering EGFR treatment to the subject (*e.g.*, administering an effective amount of an EGFR inhibitor, such as an inhibitor of EGFR kinase activity).

As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, "an" EGFR inhibitor, as used above, includes 2, 3, 4, 5 or more such inhibitors.

Among the EGFR inhibitors (inhibitors specific for EGFR) that can be administered are, *e.g.*, BIBX 1382; Cetuximab (Erbix); CI-1033 (Canertinib); EKB-569; EMD 55900; EMD 72000; Erlotinib (OSI-774; Tarceva); Gefitinib (ZD1839; Iressa); GW-2016; hR3; ICR-62; Lapatinib (GW-572016); Lavendustin A; Lavendustin B; Monoclonal Antibody E7.6.3; Panitumumab (ABX-EGF); PD 153035; PD-168393; PKI166; RG-13022; RG-14620; TheraCim hR3; Tyrophostins; Tyrphostin AG 490; Tyrophostin AG 494; Tyrphostin AG 825; Tyrphostin AG 1478; Tyrphostin 1; Tyrphostin 23 (RG-50810); Tyrphostin 25 (RG-50875); Tyrphostin 46; Tyrphostin 47 (RG-50864, AG-213); Tyrphostin 51; ZD-6474; or a derivative thereof; or a combination of two or more of those agents.

In embodiments of the invention, the subject is human. In one embodiment, the subject is a human patient suffering from NSCLC, and the EGFR kinase inhibitor is Iressa® (gefitinib) and Tarceva® (erlotinib).

A treatment method of the invention may also comprise administering an effective amount of one or more inhibitors of one or more proteins that are activated in subjects harboring an L858R (or comparable) mutation. Among the downstream proteins that can be inhibited, and exemplary inhibitors of those proteins, are, for example:

Akt inhibitors:

1L-6-hydroxymethyl-chiro-inositol 2(R)-2-O-methyl-3-O-octadecylcarbonate
CI-1033

IGF1-R inhibitors:

Compound 1 (OSI Pharmaceuticals)
CP-751,871
INSM-18
AG 1024
NVP-AEW541

EGFR Inhibitors:

Cetuximab
Erlotinib
Panitumumab

EGFR Tyrosine kinase inhibitors:

Gefitinib

BCIL-2 inhibitor:

Oblimersen

BCR-Abl and/or c-KIT inhibitor:

Imatinib mesylate

Estrogen inhibitor:

Tamoxifen

MAP Kinase inhibitor:

CC-5013
DC980598
Wortmannin

MEK/ERK inhibitor:

U0126
PD98059
FR180204

mTOR inhibitors:

Rapamycin

CCI-779 (Temsirolimus)

AP23573

5 RAD-001 (Everolimus)

PI3Kinase inhibitors:

LY294002

10 VEGFR inhibitor:
Bevacizumab

PDGF inhibitor:

AG 1296

15 Imatinib mesylate

TGFBeta inhibitor:

Pyrazole compounds

20 Apoptosis Effectors

Proteosome inhibitor:

Bortezomib

Monoclonal antibodies

25 Alemtuzumab - binds to CD52

Gemtuzumab ozogamicin – binds to CD33

Rituxumab – binds to CD20

30 In another embodiment of the invention, a subject is administered a combination of one or more EGFR inhibitors and one or more of the inhibitors of the associated downstream proteins.

Another aspect of the invention is a method for treating a subject suffering from a disease or disorder (*e.g.*, a condition mediated by EGFR, such as a cancer of epithelial origin), comprising administering to the subject an effective amount of an EGFR inhibitor if a sample
35 from the subject is shown to harbor a significantly elevated level of phosphorylation at residues Y1068 and T1148 in EGFR, compared to a baseline value. Another embodiment comprises further administering an effective amount of an inhibitor of one or more of the members of the downstream EGFR signal transduction pathways noted elsewhere herein.

Another aspect of the invention is in a method for treating a subject suffering from a
40 condition mediated by EGFR, such as a cancer of epithelial origin, the improvement comprising determining that a sample from the subject harbors a significantly elevated level of

phosphorylation at EGFR residues Y1068 and T1148, compared to a baseline, and then administering an effective amount of an EGFR inhibitor.

Another aspect of the invention is a kit for predicting the response of a subject suffering from a condition mediated by EGFR, such as a cancer of epithelial origin, comprising means for measuring the amount of phosphorylation at EGFR residues Y1068 and T1148. A kit of the invention may further comprise means for measuring the amount of phosphorylation at one of the other EGFR residues discussed herein and/or at one or more of the other residues of associated pathways that are identified herein. The components of the kit may, optionally, be packaged in one or more containers.

Another aspect of the invention is a pharmaceutical composition comprising: (a) an effective amount of an inhibitor of EGFR; (b) an effective amount of an inhibitor of one of more of the associated pathways identified herein; and (c) a pharmaceutically acceptable carrier.

Another aspect of the invention is a method for identifying new markers that can predict the response of a subject having an EGFR-mediated disease or disorder to treatment with an EGFR inhibitor. The method comprises determining amino acid residues in a member of the EGFR signaling pathway that are over- or under-phosphorylated, compared to a baseline value. For example, EGFR residues that are preferentially phosphorylated in samples from subjects that are responsive to an EGFR inhibitor (or cells or cell lines derived from such subjects), but not in subjects that are resistant to such treatment, can serve as markers to identify subjects who would be good candidates for treatment with an EGFR inhibitor.

Another aspect of the invention is a method comprising (a) obtaining a tissue sample; (b) obtaining data regarding the levels of phosphorylation of one or more of EGFR Y845, Y992, or Y1045; AKT S473 or T308, ERK T202/Y204, SHC Y317, FKHR T24, 4ebp1 S65, GSK3 a/b S21/9, Her2 Y1248, enos S1177, Src Y527 and/or Y416, mTOR S2481, PDGFRb Y716, eIF4g S1108, Bcl-2 S70 or Fak Y376 in the sample; and (c) providing a report of those phosphorylation levels.

The present invention provides methods for selecting subjects having a disease or disorder for treatment with an epidermal growth factor receptor (EGFR) or EGFR pathway inhibitor, comprising: determining the presence or amount of a phosphorylated Y1068 and/or Y1148 residue in EGFR which is present in a sample obtained from a subject. The amino acid residue numbering of EGFR and members of the EGFR signaling pathway are well-known and

can be determined routinely, or can be downloaded from various known databases. See, *e.g.*, the world wide web site ncbi.nlm.nih.gov. The numbering of EGFR amino acid residues is in accordance with the known EGFR sequences (*e.g.*, Ullrich *et al.* (1984) *Nature* 309, 418-425. Subjects in whom the presence of these phosphorylated residues have been detected (*i.e.*, in the
5 samples derived from) are candidates for EGFR therapy.

A "subject," as used herein, includes any animal that has an EGFR-mediated condition, *e.g.* a cancer of epithelial origin, such as NSCLC. Suitable subjects (patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients, are included.

10 Suitable "samples" contain tissues or cells from the subject which comprise EGFR, *e.g.*, biopsy samples (such as a tumor biopsy), primary tissue, metastatic tissue, blood, serum, body fluids, low-molecular weight fraction, etc.

A "baseline value," as used herein, refers to the level of phosphorylation at a given amino acid residue of a protein (*e.g.*, on the amino acid side chain) in a normal (*e.g.*, non-
15 cancerous) subject, or in a subject (*e.g.*, a subject having a cancer, such as NSCLC) that lacks the EGFR L858R mutation. Generally, the baseline value is determined by preparing a reference standard derived from tissue culture cells that lack the EGFR L858R mutation. An increase in the amount of phosphorylation of a protein at a particular amino acid residue compared to a baseline value can reflect, *e.g.*, an increased frequency of phosphorylation at the amino acid
20 residue.

The level of phosphorylation of a given amino acid residue can be measured qualitatively or quantitatively. The amount (quantity) of phosphorylation at a given residue may be higher or lower than is observed at other residues (*e.g.*, other tyrosine residues which are also phosphorylated), or at the same residue in a control sample (a baseline value). A residue may be
25 hyper-phosphorylated (phosphorylated at a significantly increased level compared to a baseline value). In addition to hyperphosphorylation as a detection threshold, the presence or absence of phosphorylation at the 1068 and/or 1148 residues can also be utilized. Alternatively, a qualitative scale (such as a scale of 1 to 5) can be used.

A "significantly elevated" level of phosphorylation (compared to a baseline value) is a
30 level whose difference from the baseline value is statistically significant, using statistical methods that are appropriate and well-known in the art, generally with a probability value of less than five percent chance of the change being due to random variation. For example, the

phosphorylation of EGFR residues Y845, Y992 and Y1045 in a subject harboring the EGFR L858R mutation may range from about 2-fold to 10-fold higher (e.g. 5-fold higher), or more, than the level observed in a subject harboring wild type EGFR.

Methods for measuring the level of phosphorylation at an amino acid residue are conventional and routine. In general, the measurement relies on the existence of sets of antibodies that are specific for either the non-phosphorylated or the phosphorylated forms of a particular amino acid residue of interest in the context of a protein of interest (such as EGFR). Such antibodies are commercially available or can be generated routinely, using conventional procedures. In one embodiment, a synthetic peptide comprising an amino acid of interest from a protein of interest (either in the non-phosphorylated or phosphorylated form) is used as an antigen to prepare a suitable antibody. The antibody can be polyclonal or monoclonal. Antibodies are selected and verified to detect only the phosphorylated version of the protein but not the non-phosphorylated version of the native or denatured protein, and vice-versa.

Such antibodies can be used in a variety of ways. For example, one can prepare whole cell lysates from patient samples and spot them in an array format onto a suitable substrate, such as nitrocellulose strips or glass slides. Preferably, the proteins in the samples are denatured before spotting. In general, the cells are spotted at serial dilutions, such as two-fold serial dilutions, to provide a wide dynamic range. Suitable controls, such as positive controls or controls for base line values, can be included. Each array is then probed with a suitable detectable antibody, as described above, to determine and/or to quantitate which amino acid residue(s) in the various proteins of interest are phosphorylated. Methods for immunoprecipitation are conventional. For a further discussion of this method of reverse phase protein lysate microarrays (RPMA), see, e.g., Nishizuka *et al.* (2003) *Proc. Natl. Acad. Sci.* 100,14229-14239.

Other suitable assays employing such antibodies to assess the level and/or degree of phosphorylation at a residue of interest include, e.g., Western blots, ELISA assays, immunoprecipitation, mass spectroscopy, and other conventional assays. Suitable methods include those that can detect the phosphoprotein in a very small sample (e.g. about 200 cells). Alternatively, methods can be used that are suitable for a large sample size (e.g. about 20,000-25,000 cells).

Assays to measure the presence and/or amount of phosphorylated residues can be readily adapted to high throughput formats, e.g. using robotics, if desired.

The inventive method can further comprise determining the activation state of the EGFR. That is, the presence or absence of an activating mutation, such as L858R, can be determined in a patient sample. For example, a subject having both an activating mutation such as L858R and hyper-phosphorylation at the EGFR residues Y1068 and/or Y1148 can be determined in accordance with the present invention to be a responder, and thus selected for EGFR treatment.

As noted above, in addition to measuring the amount of phosphorylation of the noted residues in EGFR, one can further determine the phosphorylation status of other members of an associated EGFR-signaling pathway, *e.g.*, residue S473 and/or T308 of AKT, residue T202/Y204 of ERK, residue Y317 of SHC, residue T24 of FKHR, residue S65 of 4ebp1, residue S21/9 of GSK3 α/β , residue Y1248 of Her2, residue S1177 of enos, residue Y527 and/or Y416 of Src, residue S2481 of mTOR, residue Y716 of PDGFR β , residue S1108 of eIF4g, residue S70 of Bcl-2, and/or residue Y376 of Fak. Members of the EGFR pathway include, but are not limited to, Shc, Grb2, Gab1, etc. For a review of members of the pathway, see Oda *et al.* (2005) *Molecular Systems Biology* (article number 2005.0010).

Any of a variety of diseases or disorders (*e.g.*, EGFR-mediated conditions) can be subjected to a diagnostic method of the invention and/or treated in accordance with the present invention. In particular, samples from a variety of cancers of epithelial origin can be tested, including both primary and metastatic disease, including, *e.g.*, glioblastomas (including glioblastoma multiforme), as well as cancers of the lung (adenocarcinomas, including bronchoalveolar carcinoma (BAC), squamous cell lung cancer, non-small cell lung cancer (NSCLC), etc.), breast, ovary, stomach, pancreas, bladder, colon, colorectal, kidney, head and neck, or melanoma. Subsets of patients having these conditions have been shown to respond to EGFR kinase inhibitors and to harbor mutations that result in hyper-phosphorylation of the residues as noted herein.

Other conditions that are mediated by EGFR can also be subjected to a method of the invention. These conditions include, *e.g.*, hyperproliferative conditions, such as cancers, pre-cancerous conditions, metabolic disorders (*e.g.*, diabetes), skin disorders or diseases, cardiovascular disease, hyperproliferative cell diseases or disorders, psoriasis, obesity, inflammatory airway disease, asthma, COPD, and neurological disorders. Furthermore, the overexpression of EGFR on synovial fibroblast cells has been identified as being involved in inflammatory arthritis (*e.g.*, rheumatoid arthritis, erythematous-associated arthritis, and psoriatic arthritis), and patients suffering from those conditions can also be assayed by a method

of the invention. Other conditions that can be subjected to a method of the invention include inflammatory airway disease, asthma or COPD; or a neurological disorder.

One aspect of the invention is a method for treating a subject suffering from a disease or disorder mediated by EGFR, such as a cancer of epithelial origin, comprising (1) measuring the amount of phosphorylation at EGFR residues Y1068 and T1148 in a sample from the subject and, optionally, at one or more of the other phosphorylation sites discussed herein and, if the levels of phosphorylation compared to a baseline value suggest that the subject is likely to be responsive to EGFR therapy (*e.g.* to treatment with an agent that inhibits the kinase activity of EGFR), (2) administering EGFR treatment to the subject (*e.g.*, administering an effective amount of an EGFR inhibitor, such as an inhibitor of EGFR kinase activity).

Any agent that is capable of modulating an epidermal growth factor receptor or a member of its pathway, particularly an agent that inhibits EGFR kinase activity, can be utilized in accordance with the present invention. Suitable agents include, without limitation, EGF receptor antibodies, EGF receptor tyrosine kinase inhibitors, and EGF derivatives (including, *e.g.*, selective and non-selective inhibitors), *e.g.*, an agent selected from: BIBX 1382; Cetuximab (Erbix); CI-1033 (Canertinib); EKB-569; EMD 55900; EMD 72000; Erlotinib (OSI-774; Tarceva); Gefitinib (ZD1839; Iressa); GW-2016; hR3; ICR-62; Lapatinib (GW-572016); Lavendustin A; Lavendustin B; Monoclonal Antibody E7.6.3; Panitumumab (ABX-EGF); PD 153035; PD-168393; PKI166; RG-13022; RG-14620; TheraCim hR3; Tyrophostins; Tyrphostin AG 490; Tyrphostin AG 494; Tyrphostin AG 825; Tyrphostin AG 1478; Tyrphostin 1; Tyrphostin 23 (RG-50810); Tyrphostin 25 (RG-50875); Tyrphostin 46; Tyrphostin 47 (RG-50864, AG-213); Tyrphostin 51; ZD-6474; derivatives thereof. Additional EGFR agents are known, *e.g.*, as disclosed in WO9220642; WO9519774; WO9519970; US 5,866,572; US 6,355,678; US 6,864,286; US 5,747,498; US 6,476,040; 5,770,599; Rewcastle *et al.* (1995) *J.Med.Chem.* 38, 3482-3487; Fry *et al.* (1994) *Science* 265, 1093-1095; Buchdunger *et al.* (1994) *Proc. Natl. Acad. Sci. USA*, 91, 2334-2338; Trinks *et al.* (1994) *J.Med. Chem.* 37, 1015-1027; Bridges *et al.* (1995) *Bioorganic & Medicinal Chemistry* 3, 1651-1656, 1995; Ward *et al.* (1994) *Biochem.Pharmacology* 48, 659-666. Combinations of these agents, with each other or with conventional agents, such as chemotherapeutic agents, can be used.

Although compounds may be characterized as being "EGFR inhibitors," "EGFR kinase inhibitors," or "inhibitors of the EGFR pathway," the present invention is not limited to the mechanism by which such agents achieve therapeutic efficacy. For example, a patient subset

selected in accordance with the present invention may respond to EGFR treatment, although the mechanism of action may not be related to, or completely related to, modulation of the EGFR pathway.

The Examples identify a number of downstream proteins in the EGFR tyrosine kinase cascade that are hyper-phosphorylated at particular residues in subjects suffering from NSCLC and having an L858R mutation (and/or in cells in culture having the mutation). These proteins, then, are active (activated, in use) in those subjects, and thus can serve as targets for treatment. Among the targets that have been identified by the present inventors are: AKT, ERK, SHC, FKHR, 4ebp1, GSK3 a/b, Her2, enos, Src, mTOR, PDGFRb, eIF4g, Bcl-2 and/or Fak. Therefore, in one embodiment of the invention, subjects having a cancer of epithelial cell origin, such as NSCLC, and harboring an EGFR L858R mutation, as shown by a diagnostic method of the invention, are treated not only with an effective amount of an inhibitor of EGFR kinase, but also with an effective amount of an inhibitor of one or more of the downstream proteins noted above. Suitable inhibitors of these proteins are well-known, and are discussed elsewhere herein. In one embodiment, a subject is treated with one or more inhibitors of these downstream proteins, but is not treated with an EGFR inhibitor.

Accordingly, one aspect of the invention is a pharmaceutical composition comprising:

- (a) an effective amount of an inhibitor of EGFR kinase (including, but not limited to the inhibitors listed elsewhere herein);

- (b) an effective amount of an inhibitor of at least one of the proteins AKT, ERK, SHC, FKHR, 4ebp1, GSK3 a/b, Her2, enos, Src, mTOR, PDGFRb, eIF4g, Bcl-2 and/or Fak. The inhibitor(s) can be selected from the inhibitors of these proteins that are noted above, or others; and
- (c) a pharmaceutically acceptable carrier.

Such a pharmaceutical composition can be administered to a subject having a condition mediated by EGFR, such as a cancer of epithelial origin, wherein the subject is determined by a method of the invention to be likely to be responsive to an EGFR kinase inhibitor.

The inhibitors discussed herein can be formulated into various compositions, *e.g.*, pharmaceutical compositions, for use in therapeutic treatment methods. The pharmaceutical compositions can be assembled as a kit. Generally, a pharmaceutical composition of the invention comprises an anticancer-effective amount of the inhibitor. An "anticancer effective amount," as used herein, is an amount that is sufficient to effect at least a therapeutic response in

the individual over a reasonable time frame. For example, it can ameliorate, at least to a detectable degree, the symptoms of a cancer, or can inhibit the growth of a tumor, etc.

The composition can comprise a carrier, such as a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art. For a discussion of pharmaceutically acceptable carriers and other components of pharmaceutical compositions, see, *e.g.*, Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Company, 1990.

One skilled in the art will appreciate that the particular formulation will depend, in part, upon the particular inhibitory agent of the invention, or other chemotherapeutic agent, that is employed, and the chosen route of administration. Accordingly, there is a wide variety of suitable formulations of compositions of the present invention. Formulations suitable for oral, parenteral, aerosol, transdermal, topical, or other forms of administration will be evident to the skilled worker.

One skilled in the art can easily determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired anti-cancer effective amount or effective concentration of the agent in the individual patient. One skilled in the art also can readily determine and use an appropriate indicator of the "effective concentration" of the compounds of the present invention by a direct or indirect analysis of appropriate patient samples (*e.g.*, blood and/or tissues).

The dose of an inhibitory agent of the invention, or composition thereof, administered to an animal, particularly a human, in the context of the present invention should be sufficient to effect at least a therapeutic response in the individual over a reasonable time frame (an anti-cancer effective amount). The exact amount of the dose will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity or mechanism of any disorder being treated, the particular agent or vehicle used, its mode of administration and the like. The dose used to achieve a desired anticancer concentration *in vivo* will be determined by the potency of the particular inhibitory agent employed, the pharmacodynamics associated with the agent in the host, the severity of the disease state of

infected individuals, as well as, in the case of systemic administration, the body weight and age of the individual. The size of the dose also will be determined by the existence of any adverse side effects that may accompany the particular inhibitory agent, or composition thereof, employed. It is generally desirable, whenever possible, to keep adverse side effects to a minimum.

When given in combined therapy (*e.g.* an EGFR kinase inhibitor in conjunction with one or more inhibitors of downstream proteins in the EGFR signaling pathway), the inhibitors can be given at the same time, or the dosing can be staggered as desired. The two or more drugs can also can be combined in a composition. Doses of each can be less when used in combination than when either is used alone.

Another aspect of the invention is a method for monitoring the effectiveness of a treatment of an EGFR-mediated condition, such as a cancer of epithelial origin, with an EGFR kinase inhibitor. It is expected that phosphorylation at the residues discussed herein will diminish as a result of effective treatment. A method of the invention can be used to monitor such a decrease in phosphorylation.

Another aspect of the invention is a kit useful for any of the methods disclosed herein. For example, the kit can be useful for predicting the response of a subject suffering from a disease or disorder mediated by EGFR, such as a cancer of epithelial origin, comprising means (*e.g.*, reagents) for measuring the amount of phosphorylation at EGFR residues Y1068 and T1148. A kit of the invention may further comprise means for measuring the amount of phosphorylation at one of more of the further EGFR residues as noted elsewhere herein and/or of associate downstream markers as noted elsewhere herein. The means can comprise, *e.g.*, antibodies that are specific for particular unphosphorylated or phosphorylated isoforms of EGFR or of the diagnostic downstream tyrosine kinases that are identified herein. Furthermore, the kit may comprise means (*e.g.*, reagents or devices) for preparing a sample (*e.g.*, for collecting a tissue and/or excising a sample from the tissue); for spotting test samples on a suitable surface, such as nitrocellulose strips; for performing immuno-quantitation (*e.g.*, labeled antibodies, or reagents for labeling antibodies); instructions for performing a method of the invention; etc. The components of the kit may, optionally, be packaged in one or more containers.

In another embodiment, the kit is suitable for therapeutic treatment of a cancer in a subject. Such a kit can comprise combinations of inhibitors (*e.g.* in the form of a pharmaceutical composition) as discussed elsewhere herein. Among other uses, kits of the invention can be in

experimental applications (*e.g.*, to identify a phosphorylation pattern that is predictive of the response of a subject to a therapeutic agent). A skilled worker will recognize components of kits suitable for carrying out any of the methods of the invention.

Optionally, a kit of the invention comprises include suitable buffers; one or more
5 containers or packaging material; and/or instructions for performing the method. The reagents of the kit may be in containers in which the reagents are stable, *e.g.*, in lyophilized form or stabilized liquids. The reagents may also be in single use form, *e.g.*, in single dosage form.

Another aspect of the invention is a method for identifying markers that can predict the response of a subject having an EGFR-mediated disease or disorder to treatment with an EGFR
10 inhibitor. The method comprises determining amino acid residues in a member of the EGFR signaling pathway (*e.g.*, EGFR and/or the downstream members of the signaling cascade) that are over- or under-phosphorylated, compared to a baseline value. For example, EGFR residues that are preferentially phosphorylated in samples from subjects that are responsive to an EGFR inhibitor (or cells or cell lines derived from such subjects), but not in subjects that are resistant
15 to such treatment, can serve as markers to identify subjects who would be good candidates for treatment with an EGFR inhibitor, such as a kinase inhibitor. The identification of one or more such residues (a pattern of phosphorylation) can serve as a way to identify mutations that are correlated with responsiveness to an EGFR inhibitor of interest, without having to necessarily sequence a sample from the subject to identify the mutation. A variety of mutations of EGFR
20 can be detected in such a method. The mutations include, *e.g.*, point mutations, truncations, deletions, insertions, substitutions, translations, duplications, etc. at any position in EGFR (*e.g.*, in exons 18, 19, 20 or 21, or others).

It is noted that EGFR and the downstream tyrosine kinases in the EGFR cascade can be activated by a variety of factors in addition to mutations such as EGFR L858R; and that, once
25 activated, the proteins can drive the cancer phenotype of the cells. Such factors include, *e.g.*, interaction with receptors that can "cross-talk" with EGFR, co-factors, auto-ligands or the like. Therefore, the identification of a particular phosphorylation pattern in a subject can serve as an indication that the subject is likely to be responsive to agents that inhibit EGFR kinase, even if the subject does not actually harbor an activating mutation such as EGFR L858R.

30 In one embodiment of the invention, a sample from a subject (patient) suffering from an EGFR-mediated condition is subjected to a diagnostic method of the invention, and it is determined whether EGFR or members of the EGFR tyrosine pathway are activated (*e.g.*, are

hyperphosphorylated at particular residues). The proteins that are identified as being activated can serve as targets for treatment, and one or more suitable inhibitors of these proteins (e.g. of their kinase activity) can be administered to the subject. In this way, the treatment can be personalized for each subject.

5 In the foregoing and in the following examples, all temperatures are set forth in uncorrected degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

EXAMPLES

Example I - The identification of a subset of phosphorylated residues on the EGF receptor that correlate with the genetic mutation status of the receptor and the susceptibility to EGF inhibitor therapy

10 We used reverse phase protein microarrays to monitor the *in vivo* EGFR phosphoprotein circuits of 20 microdissected human lung carcinoma samples and compared the phosphorylation pattern to the EGFR mutation status for each patient's tumor. We then correlated these results to
15 *in vitro* time course analysis of residue specific phosphorylation of the EGF receptor following EGF stimulation of cultured non-small cell lung cancer cell lines for the following categories of genomic alterations: EGFR point mutation L858R; EGFR deletion E746-A750; and wild type EGFR.

A. Materials and methods.

Human Tissue Microdissection

Samples: Twenty early-stage lung adenocarcinoma surgical specimens were collected and frozen at the time of surgery. All patient samples were collected and tested with informed consent. All patients had no known history of kinase inhibitor treatment prior to surgery. Patients were
25 primarily from Caucasian and African American origins.

Laser Capture Microdissection: 8um frozen sections were prepared on either glass or membrane slides. Frozen sections were fixed in 70% ethanol, stained with Mayer's Hematoxylin and Scott's Tap Water Substitute, and dehydrated in gradient ethanol, with a final clearing in xylene.
30 The slides were rapidly air dried and tumor cells were isolated by laser capture microdissection (PixcellTM and VeritasTM, Arcturus Molecular Devices; CA, USA).

Reverse Phase Protein Microarrays. The microdissected cells were subjected to lysis in 2.5% beta-mercaptoethanol in T-PER (Pierce, Rockford, IL) and 2X SDS Tris-glycine buffer (Invitrogen, Carlsbad, CA). Reverse phase protein microarrays were printed in duplicate with the whole cell protein lysates as described by Sheehan *et al.* (2005) *supra*. Briefly, the lysates
5 were printed on glass backed nitrocellulose array slides (FAST Slides Whatman, Florham Park, NJ) using a GMS 417 arrayer (Affymetrix, Santa Clara, CA) equipped with 500 µm pins, or an Aushon Biosystems 2470 arrayer equipped with 350 µm pins. Each lysate was printed in a dilution curve representing neat, 1:2, 1:4, 1:8, 1:16 and negative control dilutions. The slides were stored with desiccant (Drierite, W.A. Hammond, Xenia, OH) at -20°C prior to
10 immunostaining.

Protein Microarray Immunostaining: Immunostaining was performed on an automated slide stainer per manufacturer's instructions (Autostainer CSA kit, Dako, Carpinteria, CA). Each slide was incubated with a single primary antibody at room temperature for 30 minutes. Polyclonal
15 primary antibodies were: 14-3-3 zeta/gamma/eta, COX-2, Shc Y317 (Upstate-Millipore;NJ, USA), APC2 (LabVision;Fremont,CA), EGFR Y1173, EGFR Y1148 (Invitrogen-Biosource, Carlsbad, CA), BUB3, CyclinD1, Cyclin E (BD, Franklin Lakes, NJ), Beta Actin, 4EBP1 Thr37, BCL-2ser70, EGFR, EGFR Y845, EGFR Y992, EGFR Y1045, EGFR Y1068, EGFR L858R, FKHR Thr24, IRS-1 ser612, mTOR ser2481, ERK T202/Y204, Akt ser473, Akt Thr308,
20 SMAD2 ser465, STAT3 ser727, Src Y416, and Src Y527 (Cell Signaling Technology, Danvers, MA). The negative control slide was incubated with antibody diluent. Secondary antibody was goat anti-rabbit IgG H+L (1:5000) (Vector Labs, Burlingame, CA).

Bioinformatics method for microarray analysis. Each array was scanned, spot intensity analyzed,
25 data normalized, and a standardized, single data value was generated for each sample on the array (Image Quant v5.2, GE Healthcare, Piscataway, NJ). Spot intensity was integrated over a fixed area. Local area background intensity was calculated for each spot with the unprinted adjacent slide background. This resulted in a single data point for each sample, for comparison to every other spot on the array. The Ward method for two-way hierarchical clustering was
30 performed using JMP v5.0 (SAS Institute, Cary NC). Wilcoxon two-sample rank sum test was used to compare values between two groups. *P* values less than 0.05 were considered significant.

When we couldn't assume a normal distribution of the variables we used non-parametric methods.

Lung time course

- 5 Cell Culture: A549 cells (Wild Type Epidermal Growth Factor Receptor) (ATCC; VA, USA) were cultured in F-12K Medium (ATCC; VA, USA) supplemented with 10% fetal bovine serum (ATCC; VA, USA), in 5% CO₂. H1975 cells (L858R mutation Epidermal Growth Factor Receptor) (ATCC; VA, USA) were cultured in RPMI 1640 Medium (ATCC; VA, USA) supplemented with 10% fetal bovine serum (ATCC; VA, USA), in 5% CO₂. Approximately 5.0
10 $\times 10^4$ A549 cells were plated per well in 6 well plates. Approximately 6.5 $\times 10^4$ A549 cells were plated per well in 6 well plates. Media + 10% serum was replaced with serum free media on day 3 of the culture.

- Time course activation with EGF: The time course was initiated 24 hours after serum starvation.
15 Cells were treated with 5ng/ml, 50ng/ml, and 500 ng/ml Epidermal Growth Factor (EGF) peptide (Cell Signaling; MA, USA). An additional plate of A549 and H1975 cells were treated with 1mM pervanadate supplemented media. In addition, as an untreated control, media alone was added to the cells for each time point and treatment condition.

- The cells were incubated with EGF, or no treatment, for 0, 1, 3, 5, 7, 9, 11, 15, 30, 60, or
20 180 minutes. At each time point, the cells were washed twice with Dulbeccos phosphate buffered saline (Invitrogen) and then lysed in a 2.5% solution of 2-mercaptoethanol in T-PER (Pierce)/ 2X SDS Tris-glycine SDS buffer (Invitrogen). The lysate was immediately frozen on dry ice and stored at -80°C.

- 25 Reverse Phase Protein Microarray:

- The cell lysates were thawed on ice then heated at 100°C for 8 minutes. The lysates were centrifuged at 14,000rpm for 1 minute. A reverse phase protein microarray was prepared in 384 well microtiter plates with 20ul of lysate/well. Serial two-fold dilutions of each lysate (EGF treated, pervanadate treated, or intreated control) at the following concentrations in 2.5%
30 solution of 2-mercaptoethanol in T-PER (Pierce)/ 2X SDS Tris-glycine SDS buffer: undiluted, 1:2, 1:4 and 1:8.

The RPPM was constructed by immobilizing 30nL of lysate on a nitrocellulose substratum (FAST slide, Whatman: NJ, USA) using a robotic Aushon Biosystems 2470 arrayer. The arrays were subsequently probed with primary antibodies to a series of known phosphorylated, cleaved or total protein cell signaling proteins using a Dako Autostainer and
 5 Dako's CSA (Catalyzed Signal Amplification) chemistry. Each array was scanned, spot intensity analyzed, data normalized, and a standardized, single data value was generated for each sample on the array (Image Quant v5.2, GE Healthcare, Piscataway, NJ). Spot intensity was integrated over a fixed area. Local area background intensity was calculated for each spot with the unprinted adjacent slide background. This resulted in a single data point for each sample, for
 10 comparison to every other spot on the array.

B. Study with patient samples: Unsupervised Bayesian clustering analysis was performed on microdissected clinical samples from 20 non-small cell lung cancer (NSCLC) patients. The location of the mutation in the EGF receptor in many of the patient samples was previously
 15 reported in Yang *et al.* (2005) *Clin Cancer Res.* 11, 2106-10 and Erratum in: *Clin Cancer Res* 11, 7960-1 (2005). If a patient is not listed below, no mutation was detected, *i.e.* the patient is assumed to have wild type EGFR.

EGFR mutations in patients with Non-Small Cell Lung Cancer

20 10419 Point mutation exon 20 H773L, V774M

1186 Point mutation exon 21 L858R

11139 Point mutation exon 21 L858R

Germ-line variations of EGFR gene in patients with Non-Small Cell Lung Cancer

25 1712 intron 20, polymorphism 164,315 A→C

10403 exon 21 polymorphism 2,508 C→T

11191 exon 21 polymorphism 2,508 C→T

Only two of the patients were known to be responsive to Iressa® (gefitinib) and/or
 30 Tarceva® (erlotinib). Both of these patients harbored the EGFR L858R mutation. The L858R mutation has previously been associated with clinical response to gefitinib.

As shown in the heat map in Figure 1, 11 of the 20 microdissected clinical samples that were tested exhibited phosphorylation of one or more of the residues of EGFR. Of the tested samples, only the samples from the two patients harboring the L858R mutation exhibited hyper-phosphorylation of the two tyrosine EGFR residues, Y1068 and Y1148 (a double phosphorylation). These two patients also exhibited a baseline amount of phosphorylation at the EGFR Y1173 residue.

C. *In vitro* time courses with NSCLC and control cell lines: Phosphorylation that was induced by an EGFR ligand (EGF) was evaluated in a time course *in vitro* with the following cell lines: A549 (wild type); H1975 (EGFR L858R); and H1650 (EGFR Del746-750). Unsupervised Bayesian clustering analysis was performed. As shown in the heat map in Figure 2, EGFR tyrosine residues 992, 1045, 1068 and 1148 exhibited sustained phosphorylation. The NSCLC cell line with the same point mutation described for the patient study above (cell line H1975, point mutation L858R) exhibited a sustained and higher EGFR phosphorylation compared to the wild type at the same two sites as the patient samples (Y1068 and Y1148). By contrast, the wild type EGFR was associated with a transient phosphorylation of only one site.

We also examined the phosphorylation status of downstream pathways in the EGFR circuit, for the same cell lines. Phosphorylation of AKTser473, ERK T202/Y204, and Shc Y317 showed sustained activation after EGF stimulation in the time course assay for the point mutation cell line, whereas the wild type EGFR cell line did not. The statistically different phosphorylation sites in the mutation cell line compared to the wild type over the time course following treatment with 50ng/mL of EGF are listed in Table 1 with the associated P value.

Some of the results of this time course are plotted in Figure 3. The cell line having the L858R point mutation exhibited a sustained increase in phosphorylation after EGF stimulation at residues Y1148 of EGFR (Fig. 3C), Y1068 of EGFR (Fig. 3D), and Y1045 of EGFR (Fig. 3F), and at residue ser473 of the downstream gene AKI (Fig. 3A). Furthermore, the cell line reached a sustained activation and reached a lower steady state (compared to the initial value at time=0) of residue Y1173 of EGFR (Fig. 3E) and residue ser217/221 of the downstream gene MEK (Fig. 3H).

Without wishing to be bound by any particular mechanism, it is suggested that the pattern of phosphorylation associated with EGF stimulation of EGFR L858R cell lines - the sustained phosphorylation of EGFR tyrosine residues Y992, Y1045, Y1068 and Y1148 - is

associated with EGFR docking proteins in the MAPK/ERK signaling pro-survival pathway. This is consistent with sustained activation of AKT. Furthermore, again without wishing to be bound by any particular mechanism, it is suggested that the sustained phosphorylation of EGFR Y1045 may be a mechanism whereby the cell limits growth and proliferation signals via receptor degradation.

This Example indicates that the measurement of specific phosphorylation patterns of kinase inhibitor targets such as EGFR can serve as the basis for rational treatment design and prognostic assays. The observed phosphorylated receptor patterns may indicate receptor mutation status and correlate with drug sensitivity for individualized therapy.

Example II - The identification of additional phosphorylated residues on the EGF receptor that correlate with the genetic mutation status of the receptor and the susceptibility to EGF inhibitor therapy

Additional time course studies in cell cultures were carried out, using the cell lines having the L858R mutation described in Example I. The studies were performed essentially as described in Example I, but many more data points (more replicates) were obtained, allowing for the generation of highly statistically significant data. 21 experiments were carried out for each of the cell lines, and a series of time points were taken, ranging from 1 min to 3 hours following stimulation with EGF at 0.5 ng/ml, 50 ng/ml and 500 ng/ml. The results of this analysis are summarized in Table 1.

Table 1 - Phosphorylated residues on the EGFR and on downstream proteins that are statistically significantly different in the L858R mutant compared to the EGFR wild type.

Phos Resid	P (T test)	P (Rank Sum Test)	Result
EGFR Y1173		0.0001	different
EGFR Y1148	0.0093		different
EGFR Y992	0.0001		different
FKHR T24		0.0001	different
4ebp1 S65	0.0444		different
GSK3 a/b			
S21/9	0.0063		different
EGFR Y1068	0.0003		different
AKT T308		0.0102	different
Her2 Y1248		0.0001	different
SHC Y317		0.0001	different
enos S1177	0.0213		different
Src Y527	0.0059		different
mTor S2481	0.0004		different

PDGFRb Y716			0.0001	different
elF4g S1108	0.0059			different
Fak Y376	0.0313			different
AKT S473				different
Creb S133			0.1062	Not different
IRS1 S612		0.3286		Not different
Stat1 Y701	0.9307			Not different
Braf S445		0.1722		Not different
Bad S112			0.0559	Not different
Src Y416			0.0602	Not different

Example III - Further studies with patient samples

An additional set of lung cancer samples were analyzed according to the methods described in Example 1. This additional set included 4 samples with the L858R mutation, 2 with an exon 19 deletion, 4 wild type EGFR, and one replicate L858R mutation from the original sample set.

Results: All of the EGFR mutation cases exhibited phosphorylation of one or more sites on the EGF receptor. All of the L858R mutation cases reacted with an antibody recognizing the L858R point mutation form of the protein. The following downstream protein endpoints (within the set described for example 1) appear to cluster with the L858R mutation, in keeping with the cell line data shown in Table 1: Src Y416, Shc Y317, Bcl-2 ser70, AKT ser473 and AKT Thr308, IRS-1 ser612, and 14-3-3 zeta gamma etc. As an example of internal consistency, the replicate sample showed high ranking of endpoints in a manner similar to that found for Example 1.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions and to utilize the present invention to its fullest extent. The preceding preferred specific embodiments are to be construed as merely illustrative, and not limiting of the scope of the invention in any way whatsoever. The entire disclosure of all applications, patents, and publications cited above and in the figures, including U.S. provisional applications 60/780,832, filed March 10, 2005 and 60/781,369, filed March 13, 2006, are hereby incorporated in their entirety by reference.

WE CLAIM:

1. A method for predicting the response of a subject suffering from a disease or condition mediated by EGFR to an inhibitor of EGFR kinase activity, comprising
measuring the amount of phosphorylation at residues Y1068 and T1148 in EGFR in a sample from the subject,
wherein a significantly elevated level of phosphorylation at the two residues compared to a baseline value indicates that the subject is likely to be responsive to an agent that inhibits the kinase activity of EGFR.
2. The method of claim 1, further comprising measuring the amount of phosphorylation at residues Y845, Y992 and/or Y1045 in EGFR in a sample from the subject,
wherein a significantly elevated level of phosphorylation at one or more of residues Y845, Y992 and/or Y1045 compared to a baseline value further indicates that the subject is likely to be responsive to treatment with an EGFR kinase inhibitor.
3. The method of claim 1 or 2, further comprising measuring the amount of phosphorylation at a residue in a member of the EGFR signaling pathway.
4. The method of claim 3, wherein the further phosphorylation that is measured is at residue AKT S473 and/or T308, ERK T202/Y204, SHC Y317, FKHR T24, 4ebp1 S65, GSK3 a/b S21/9, Her2 Y1248, enos S1177, Src Y527 and/or Y416, mTOR S2481, PDGFRb Y716, eIF4g S1108, Bcl-2 S70 and/or Fak Y376,
wherein a significantly elevated level of phosphorylation at one or more of residues AKT S473 and/or T308, ERK T202/Y204, SHC Y317, FKHR T24, 4ebp1 S65, GSK3 a/b S21/9, Her2 Y1248, enos S1177, Src Y527 and/or Y416, mTOR S2481, PDGFRb Y716, eIF4g S1108, Bcl-2 S70 and/or Fak Y376 compared to a baseline value further indicates that the subject likely to be responsive to treatment with an EGFR kinase inhibitor.
5. The method of claim 4, wherein a significantly elevated level of phosphorylation at one or more of residues AKT S473 and/or T308, ERK T202/Y204, SHC Y317, FKHR T24, 4ebp1 S65, GSK3 a/b S21/9, Her2 Y1248, enos S1177, Src Y527 and/or Y416, mTOR S2481, PDGFRb Y716, eIF4g S1108, Bcl-2 S70 and/or Fak Y376 compared to a baseline value further indicates

that the subject is likely to be responsive to treatment with an inhibitor of one or more of AKT, ERK, SHC, FKHR, 4ebp1, GSK3 a/b, Her2, enos, Src, mTOR, PDGFRb, eIF4g, Bcl-2 and/or Fak.

6. The method of claim 4, wherein a significantly elevated level of phosphorylation at one or more of residues AKT S473 and/or T308, ERK T202/Y204, SHC Y317, FKHR T24, 4ebp1 S65, GSK3 a/b S21/9, Her2 Y1248, enos S1177, Src Y527 and/or Y416, mTOR S2481, PDGFRb Y716, eIF4g S1108, Bcl-2 S70 and/or Fak Y376 compared to a baseline value further indicates that the subject is likely to be responsive to treatment with a combination of an inhibitor of EGFR kinase and an inhibitor of one or more of AKT, ERK, SHC, FKHR, 4ebp1, GSK3 a/b, Her2, enos, Src, mTOR, PDGFRb, eIF4g, Bcl-2 and/or Fak.

7. The method of any of claims 1-6, wherein the disease or disorder is a cancer of epithelial cell origin.

8. The method of claim 7, wherein the cancer, including both primary and metastatic disease, is a glioblastoma, a melanoma, or a cancer of the lung, breast, ovary, stomach, pancreas, bladder, head and neck, colon, colorectal, or kidney.

9. The method of claim 8, wherein the disease or disorder is non-small cell lung cancer (NSCLC).

10. The method of any of claims 1-9, wherein the disease or disorder is associated with a mutation in EGFR or in a protein that interacts with EGFR such that the mutation causes the receptor to be conformationally altered and to become engaged in an active signaling state contributing to a disease condition.

11. The method of claim 10, wherein the mutation is in a nucleic acid encoding EGFR, and is a mutation that alters L858 to L858R; a deletion in exon 19; a point mutation in exon 20; a point mutation in exon 21 other than L858R, or another mutation in a nucleic acid encoding the EGF binding domain.

12. The method of any of claims 1-11, further comprising testing the subject for an altered EGFR protein that results from one of the following mutations in a nucleic acid that encodes the EGFR: a mutation that alters L858 to L858R; a deletion in exon 19; a point mutation in exon 20; a point mutation in exon 21 other than L858R; or another mutation in the EGF binding domain.

13. The method of claim 12, wherein the altered EGFR protein that is tested for contains L858R.

14. The method of claim 13, wherein the altered EGFR protein is detected by measuring the amount of reactivity of an antibody specific for the L858R EGFR protein in a sample from the subject,

wherein an increased reactivity compared to the level of reactivity in non-mutated EGFR indicates that the subject is likely to be responsive to treatment with an EGFR kinase inhibitor.

15. The method of any of claims 1-14, wherein the subject is human.

16. A method for treating a subject suffering from a cancer of epithelial origin, comprising measuring the amount of phosphorylation at residues Y1068 and T1148 in EGFR in a sample from the subject, and,

if the levels of phosphorylation are significantly elevated compared to a baseline value, administering to the subject an effective amount of an EGFR kinase inhibitor.

17. The method of claim 16, wherein the EGFR inhibitor is BIBX 1382; Cetuximab (Erbix); CI-1033 (Canertinib); EKB-569; EMD 55900; EMD 72000; Erlotinib (OSI-774; Tarceva); Gefitinib (ZD1839; Iressa); GW-2016; hR3; ICR-62; Lapatinib (GW-572016); Lavendustin A; Lavendustin B; Monoclonal Antibody E7.6.3; Panitumumab (ABX-EGF); PD 153035; PD-168393; PKI166; RG-13022; RG-14620; TheraCim hR3; Tyrophostins; Tyrphostin AG 490; Tyrophostin AG 494; Tyrphostin AG 825; Tyrphostin AG 1478; Tyrphostin 1; Tyrphostin 23 (RG-50810); Tyrphostin 25 (RG-50875); Tyrphostin 46; Tyrphostin 47 (RG-50864, AG-213); Tyrphostin 51; ZD-6474; a derivative thereof, or a combination thereof.

18. The method of claim 16, wherein the subject is a human patient suffering from NSCLC, and the EGFR kinase inhibitor is Iressa® (gefitinib) and/or Tarceva® (erlotinib).

19. The method of any of claims 16-18, further comprising administering to the subject an effective amount of an inhibitor of one or more of the following members of a downstream EGFR transduction pathway: AKT, ERK, SHC, FKHR, 4ebp1, GSK3 a/b, Her2, enos, Src, mTOR, PDGFRb, eIF4g, Bcl-2 and/or Fak.
20. A method for treating a subject suffering from a cancer of epithelial origin, comprising administering to the subject an effective amount of an EGFR kinase inhibitor if a sample from the subject is shown to harbor a significantly elevated level of phosphorylation at residues Y1068 and T1148 in EGFR, compared to a baseline value.
21. In a method for treating a subject suffering from a cancer of epithelial origin, the improvement comprising determining that a sample from the subject harbors a significantly elevated level of phosphorylation at residues Y1068 and T1148 in EGFR, compared to a baseline, and then administering an effective amount of an EGFR inhibitor.
22. A kit for predicting the response of a subject suffering from a cancer of epithelial origin to an inhibitor of EGFR kinase, comprising means for measuring the amount of phosphorylation at one or more of residues Y1068 and T1148 in EGFR, optionally in one or more containers.
23. The kit of claim 22, which further comprises means for measuring the amount of phosphorylation at one or more of the following residues: EGFR residues Y845, Y992, Y1045; and/or AKT S473 and/or T308, ERK T202/Y204, SHC Y317, FKHR T24, 4ebp1 S65, GSK3 a/b S21/9, Her2 Y1248, enos S1177, Src Y527 and/or Y416, mTOR S2481, PDGFRb Y716, eIF4g S1108, Bcl-2 S70 and/or Fak Y376.
24. A pharmaceutical composition, comprising
an effective amount of an inhibitor of EGFR;
an effective amount of an inhibitor of one or more of AKT, ERK, SHC, FKHR, 4ebp1, GSK3 a/b, Her2, enos, Src, mTOR, PDGFRb, eIF4g, Bcl-2 and/or Fak; and
a pharmaceutically acceptable carrier.

25. A method for identifying markers that can predict the response of a subject having an EGFR-mediated disease or disorder to an inhibitor of EGFR kinase activity, comprising

determining amino acid residues in one or more proteins from the EGFR signaling pathway that are over- or under-phosphorylated, compared to a baseline value.

26. A method comprising

obtaining a tissue sample;

obtaining data regarding the levels of phosphorylation of one or more of EGFR Y845, Y992, or Y1045; AKT S473 or T308, ERK T202/Y204, SHC Y317, FKHR T24, 4ebp1 S65, GSK3 α/β S21/9, Her2 Y1248, enos S1177, Src Y527 and/or Y416, mTOR S2481, PDGFR β Y716, eIF4g S1108, Bcl-2 S70 or Fak Y376 in the sample; and

providing a report of those phosphorylation levels.

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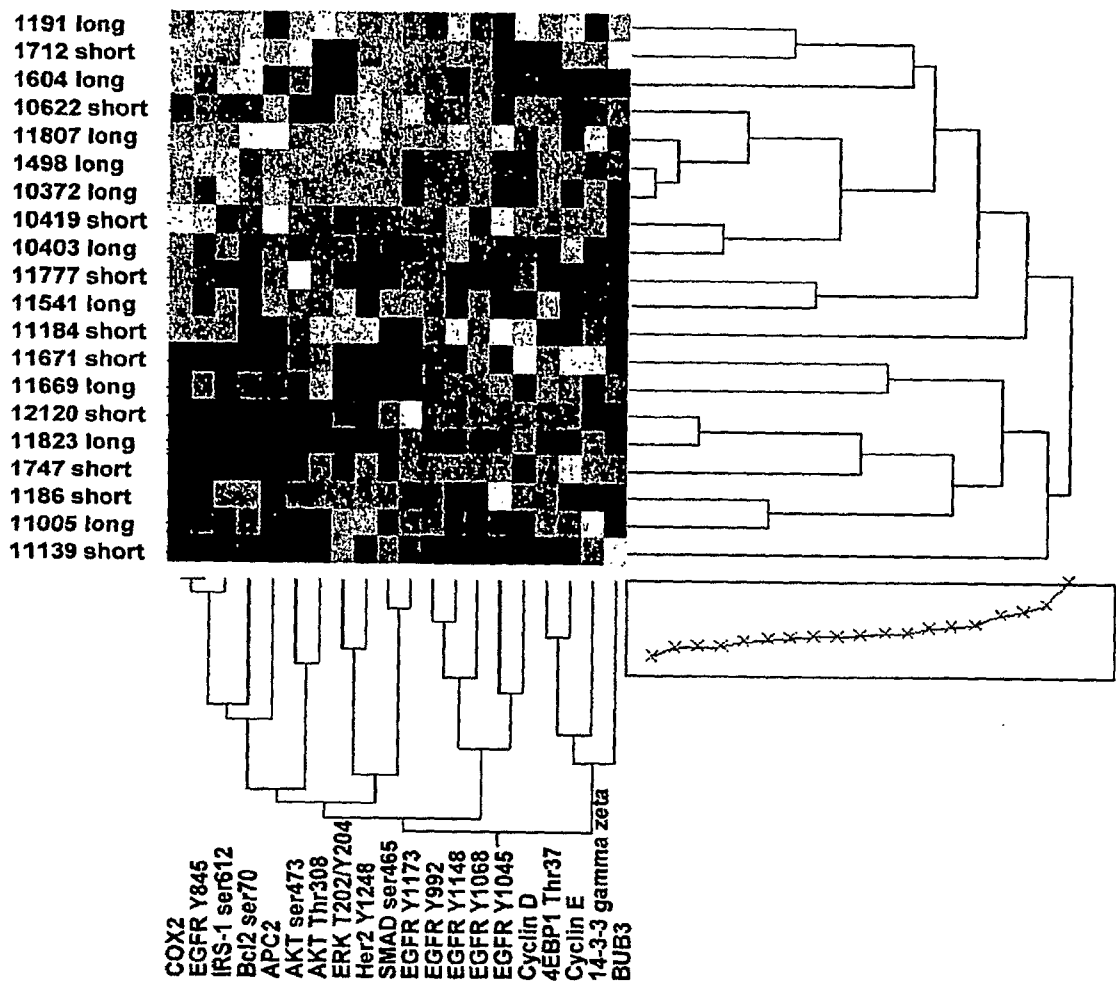


FIG. 1

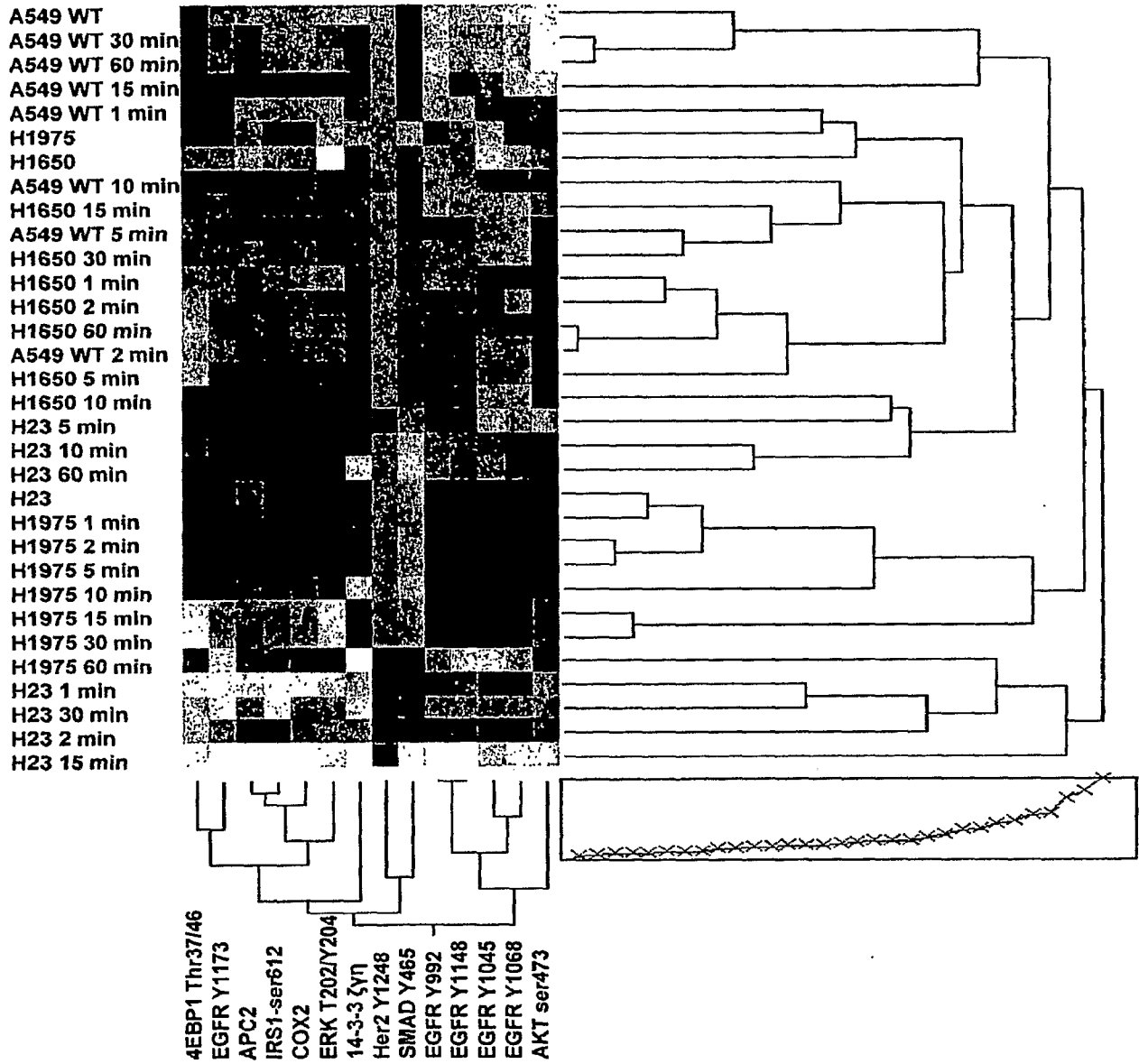


FIG. 2

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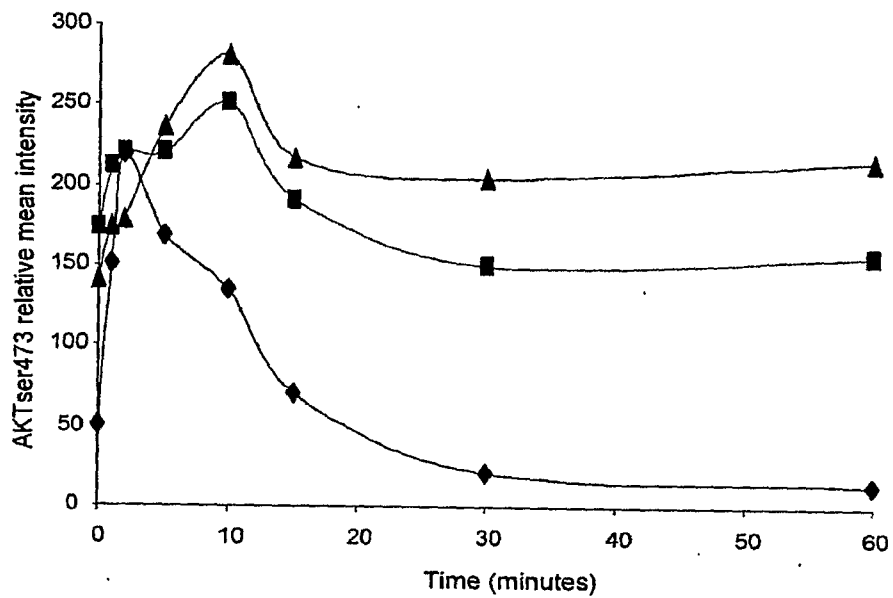


FIG. 3A

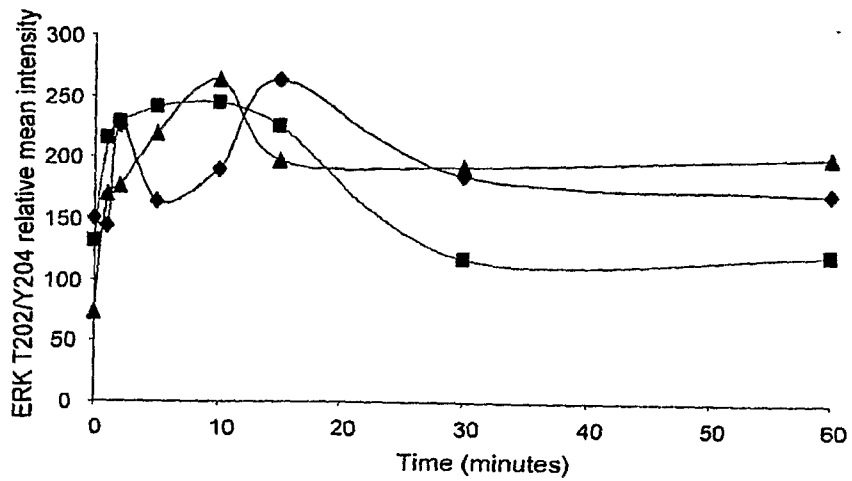
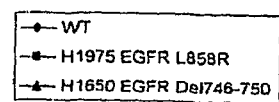


FIG. 3B



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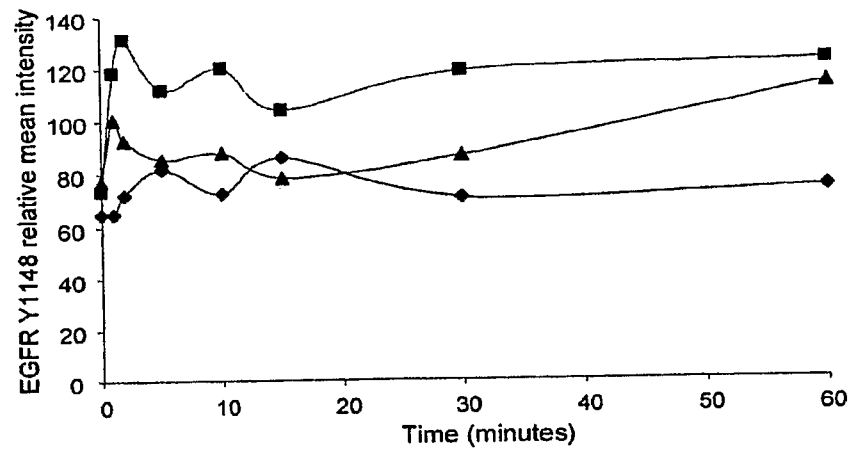


FIG. 3C

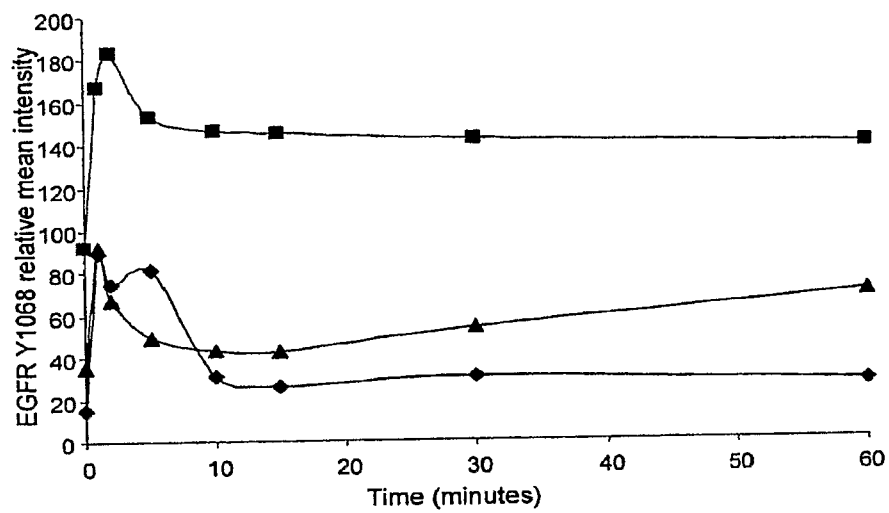


FIG. 3D

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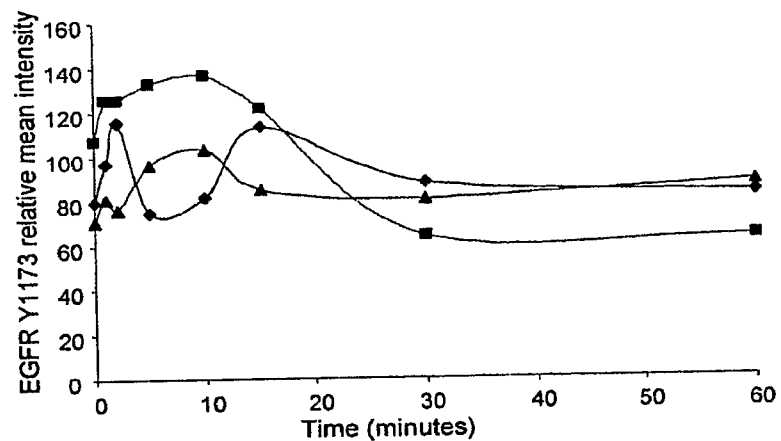


FIG. 3E

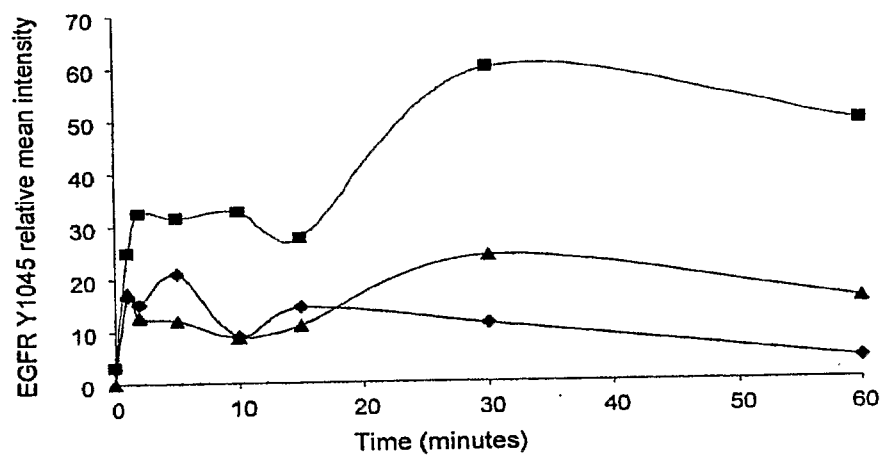


FIG. 3F

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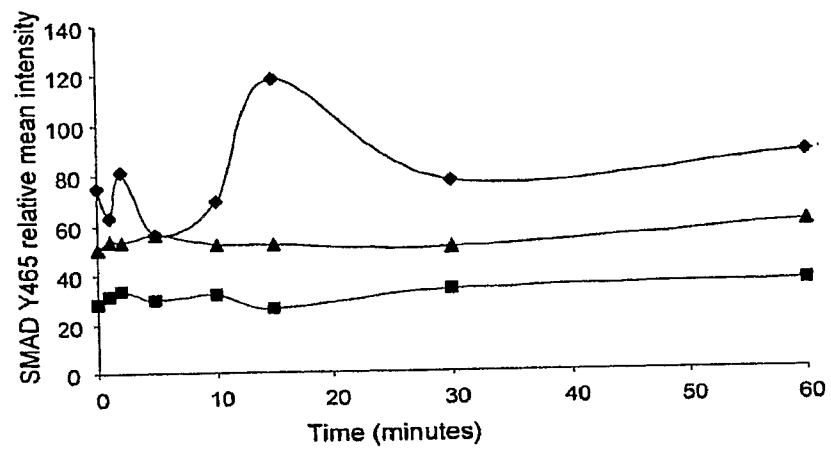


FIG. 3G

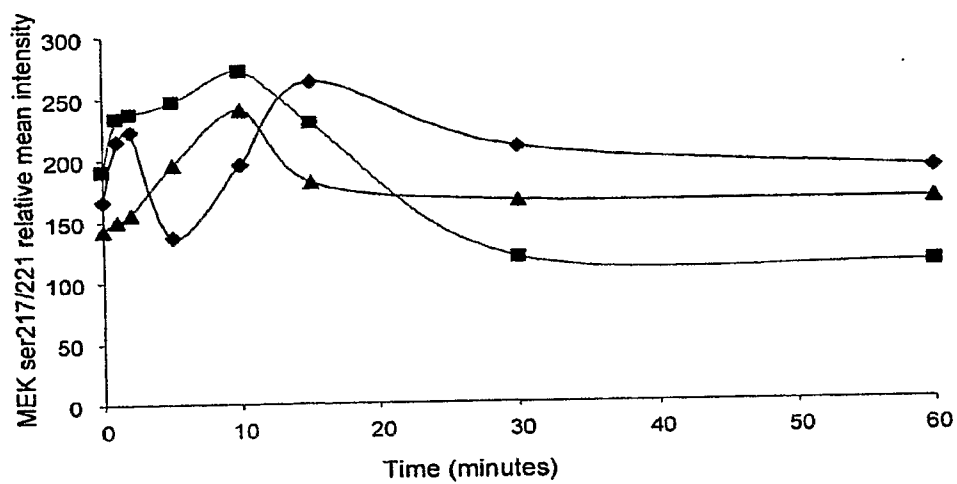


FIG. 3H