Determination of responders to chemotherapy

Field of the invention

The present invention relates to a method of determining whether a biological sample comprising human lung cancer cells is sensitive to a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent by determining the overexpression of a phosphorylated AKT protein and/ or a phosphorylated MAPK protein in the biological sample. The invention is also related to methods for deriving a candidate agent or for selecting a composition for inhibiting the progression of lung cancer in a patient wherein a phosphorylated Akt protein and/ or a phosphorylated MAPK protein is used.

Background of the Invention

EGFR, encoded by the erbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer as well as glioblastomas. The Epidermal Growth Factor Receptor (EGFR), a 170-kD glycoprotein, is composed of an N-terminus extracellular domain, a hydrophobic transmembrane domain, and a C-terminus intracellular region containing the kinase domain. EGFR ligand–induced dimerization activates the intrinsic RTK domain (an Src homology domain 1, SH1), resulting in autophosphorylation on six specific EGFR tyrosine residues in the noncatalytic tail of the cytoplasmic domain.

The cellular effects of EGFR activation in a cancer cell include increased proliferation, promotion of cell motility, adhesion, invasion, angiogenesis, and enhanced cell survival by inhibition of apoptosis. Activated EGFR induces tumor cell proliferation through stimulation of the mitogen-activated protein kinase (MAPK) cascade. Upon ligand binding to the EGFR, the SOS guanine nucleotide exchange factor is recruited to the plasma membrane via the Grb2 adaptor protein, which stimulates the exchange of GTP for GDP on the small G-protein Ras, subsequently activating the MAPK cascade consisting of Raf, MEK, and ERK. Activated ERKs (pMAPK, pERK1/2) in turn phosphorylate and activate transcription factors such as ELK-1 or c-Myc, promoting cell growth.

Multiple growth factor pathways contribute to the progression and survival of NSCLC cells through activation of multiple kinases. The EGFR enhances cancer cell survival also by signaling through the phosphatidylinositol-3-kinase (PI3K)/Akt pathway and the STAT pathway. Akt is stimulated also by other growth factors, including insulin growth factor–1, basic fibroblast growth factor, and interleukins 3 and 6. The three isoforms of Akt 1–3 are all phosphorylated (pAKT) in a similar fashion at residues T308 in the activation domain and S473 in the COOH-terminal domain.

Erlotinib (Tarceva® is a potent epidermal growth factor receptor (HER1/EGFR) tyrosine-kinase inhibitor (TKI) that provides survival benefit to patients with non-small-cell lung cancer (NSCLC) who have failed previous chemotherapy when used as a single agent (WO 01/34574). The efficacy of Tarceva®was studied in various trials. Its chemical name is N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine.

The TALENT trial was a placebo-controlled phase III study in first-line NSCLC patients who received gemcitabine and cisplatin (this concurrent chemoradiotherapy was the non-US standard of care) in combination with erlotinib (Tarceva® at 150mg/day or placebo with. The primary endpoint was survival duration, with secondary endpoints of time to progression, response rate; duration of response; pharmacokinetic and pharmacodynamic parameters, and quality of life. HER1/EGFR and HER2 expression rates were also assessed. A standard safety analysis was done. The overall outcome of the TALENT trial was negative. For the primary and secondary endpoints there was no demonstrable benefit for erlotinib (Tarceva® plus chemotherapy (gemcitabine and cisplatin) compared with gemcitabine and cisplatin alone (Gatzemeier, U., et al., Proc Am Soc Clin Oncol 23 (2004) 617 (Abstract 7010)). Identical results were seen in the US-based TRIBUTE study, with erlotinib plus carboplatin and paclitaxel (Herbst, R.S., et al., J Clin Oncol (2004) ASCO Annual Meeting Proceedings. Post-Meeting Edition; 22 (July 15 Suppl.) (Abstract 7011)). A randomised, placebo-controlled phase III study of single-agent erlotinib as second- or third-line therapy for non-small-cell lung cancer (NSCLC) (BR.21; NCIC/OSIP) found a statistically significant improvement in survival with erlotinib (6.7 months) compared with placebo (4.7 months).

Various studies are related to the investigation of biomarkers in non-small cell lung cancer and their relation to certain EGFR inhibitor drugs. Han, et al., Int J Cancer

113 (2005) 109-115 investigate 65 patients with Gefitinib (Iressa™, EGFR TKI) monotherapy. They analyse EGFR downstream molecules as response predictive markers for gefitinib in chemotherapy-resistant non-small cell lung cancer. Cappuzzo, F. et al., JNCI 96 (2004) 1133-1141 investigate 106 patients with Gefitinib (Iressa; EGFR TKI) monotherapy. They investigate Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer and find that patients with P-Akt-positive tumors who received gefitinib benefited more from the therapy that patients with P-Akt-negative tumors. Vicent, S. et al., Br J Cancer 90 (2004) 1047-1052 investigate 111 NSCLC patients. They find that pERK is activated in non-small-cell lung cancer and associated with advanced tumors. Han, S.W. et al., J Clin Oncol 23 (2005) 2493-2501 investigate 90 patients with Gefitinib (EGFR TKI) monotherapy. They analyse the predictive and prognostic impact of Epidermal Growth Factor Receptor Mutation in Non-Small-Cell lung cancer patients treated with gefitinib. Mukohara, T. et al., Lung Cancer 41 (2003) 123-130 investigate 60 patients, 20 patients per stage who either underwent neoadjuvant chemotherapy or radiation. The EGFR expression correlates with pERK and pAkt expression. The sample size is too low as mentioned by the authors themselves. Raben, D. et al., Int J Radiation Oncology Biol. Phys 59 (2004) 27-38 investigate targeted therapies for non-small-cell lung cancer. Ono, M. et al., Mol Cancer Ther 3 (2004) 465-472 assay 9 NSCLC cell lines and treated with gefitinib. Hirsch, F.R. et al., Curr Opin Oncol 17 (2005) 118-122 review the phosphorylation status of Akt and MAPK as potential marker for gefitinib resistance. Meert, et al., Clinical Cancer Research 9 (2003) 2316-2326 investigate NSCLC cell lines in aspect of EGFR inhibitor activity. Neither EGFR nor Her2 expression levels correlate with sensitivity to EGFR inhibitors. Brognard, J. et al., Cell Death and Differentiation 9 (2002) 893-904 analysed 19 NSCLC cell lines were analysed whereby 17 exhibited phosphorylation of Erk1/2 and constitutive activity. David, O. et al., Clinical Cancer Research 10 (2004) 6865-6871 disclose that overexpression of pAkt is an independent prognostic factor in NSCLC. Kakiuchi, S. et al., Human Molecular Genetics 13 (2004) 3029-3043 investigate a genome wide cDNA microarray of 33 NSCLC patients. All were given gefitinib in a monotherapeutical setting. No evidence was found for correlation between Akt/pAkt expression level, EGFR gene status or pEGFR staining and gefitinib response. Kim, R.H. et al., Cancer Cell 7 (2005) 263-273 disclosed that DJ-1 expression, an oncogene, equals pAkt level. Balsara, B.R. et al., Carcinogenesis 25 (2004) 2053-2059 investigate 110 NSCLC

patients with TMA pAkt expression. No significant difference in survival exists between pAkt negativity and positivity. Hirami, Y. et al., Cancer Letters 214 (2004) 157-164 investigate the relation of epidermal growth factor receptor, pAkt and hypoxia-inducible factor-1alpha in non-small cell lung cancers. Lee, S.H. et al., APMIS 110 (2002) 587-592 analyses 43 LN metastasis of NSCLC patients. Akt activation in NSCLC plays a role in tumor development rather than progression. Engelman, J.A. et al., Proc. Natl. Acad. Sci. USA 102 (2004) 3788-3793 analyse erbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. David, O., J Cell Mol Med 5 (2001) 430-433 discussess the role of Akt and PTEN as new diagnostics markers in lung cancer. Mantha, A. et al., Clin. Cancer Res. 11 (2005) 2398-2407 investigate the targeting of the mevalonate pathway which inhibits the function of the epidermal growth factor receptor.

Prognostic markers associated with EGFR positive cancer are investigated in WO 2004/046386. Gene expression markers for response to EGFR inhibitor drugs are disclosed by US 2004/0157255. Biomarkers and methods for determining sensitivity to epidermal growth factor receptor modulators are disclosed in WO 2004/063709. WO 01/00245 describes humanized anti-ErbB2 antibodies and methods for treating cancer with anti-ErbB2 antibodies, such as humanized anti-erbB2 antibodies.

Summary of the Invention

There is still a need to provide methods for determing the sensitivity to EGFR inhibitor therapy, in particular combination therapies of an EGFR inhibitor with a chemotherapeutic agent.

Therefore, in an embodiment of the invention, a method is provided of determining whether a biological sample comprising human lung cancer cells is sensitive to a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent, the method comprising determining the overexpression of a phosphorylated AKT protein and/ or a phosphorylated MAPK protein in the biological sample whereby the overexpression of the phosphorylated AKT protein and/ or the phosphorylated MAPK protein is an indication that the biological sample comprising human lung cancer cells is sensitive to a combination of a epidermal growth factor receptor inhibitor and a chemotherapeutic agent.

In another embodiment of the invention, an antibody that binds to the phosphorylated AKT protein or an antibody that binds to the phosphorylated MAPK protein is used for determining whether a biological sample comprising human lung cancer cells is sensitive to a combination of a epidermal growth factor inhibitor and a chemotherapeutic agent.

In another embodiment of the invention, a method of selecting a composition for inhibiting the progression of lung cancer in a patient is provided, the method comprising:

a) separately exposing aliquots of a biological sample comprising lung cancer cells that are sensitive to a combination of an EGFR inhibitor and a chemotherapeutic agent from the patient in the presence of a plurality of test compositions;

b) comparing the level of expression of a phosphorylated Akt protein and/ or a phosphorylated MAPK protein in the aliquots of the biological sample contacted with the test compositions and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in an aliquot of the biological sample not contacted with the test compositions,

c) selecting one of the test compositions which alters the level of expression of the the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot containing that test composition, relative to the aliquot not contacted with the test composition wherein an at least 10 % difference between the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the test composition and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the test composition is an indication for the selection of the test composition.

In yet another embodiment of the invention, a method of deriving a candidate agent is provided, said method comprising:

a) contacting an aliquot of a biological sample containing lung cancer cells that are sensitive to an EGFR inhibitor and a chemotherapeutic agent with the candidate agent,

(b) determining the level of expression of a phosphorylated Akt protein and/ or a phosphorylated MAPK protein in the aliquot of the biological sample

contacted with the candidate agent and determining the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in an aliquot of the biological sample not contacted with the candidate agent,

(c) observing the effect of the candidate agent by comparing the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the candidate agent,

(d) deriving said agent from said observed effect, wherein an at least 10 % difference between the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the candidate agent is an indication of an effect of the candidate agent.

In another embodiment of the invention, a candidate agent derived by the method according to the invention is provided and a pharmaceutical preparation comprising an agent according the invention.

In still another embodiment of the invention an agent according to the invention is used for the preparation of a composition for the inhibition of progression of lung cancer.

In yet another embodiment of the invention, a method of producing a drug comprising the steps of the method of the invention and

(i) synthesizing the candidate agent identified in step (c) or an analog or derivative thereof in an amount sufficient to provide said drug in a therapeutically effective amount to a subject; and/or

(ii) combining the drug candidate the candidate agent identified in step (c) or an analog or derivative thereof with a pharmaceutically acceptable carrier.

In another embodiment of the invention an Akt protein, a MAPK protein, a phosphorylated Akt protein, a phosphorylated MAPK protein, an antibody selectively binding to a phosphorylated Akt protein or a phosphorylated MAPK

protein is used for deriving a candidate agent or for selecting a composition for inhibiting the progression of lung cancer in a patient.

In still another embodiment of the invention, a kit comprising an antibody against phosphorylated MAPK and/ or phosphorylated Akt protein is provided.

The term "biological sample" shall generally mean any biological sample obtained from an individual, body fluid, cell line, tissue culture, or other source. Body fluids are e.g. lymph, sera, plasma, urine, semen, synovial fluid and spinal fluid. According to the invention, the biological sample comprises lung cancer cells and non-lung cancer cells (other cells). Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art.

The term "level of expression" or "expression level" generally refers to the amount of an amino acid product or protein in the sample, preferably the amount of a phosphorylated amino acid product or phosphorylated protein in the sample according to the invention. "Expression" refers to the process by which a gene coded information is converted into the structures present and operating in the cell including their phosphorylation according to the invention. As used herein, "expressed genes" include those that are transcribed into mRNA and then translated into protein and post translationally modified e.g. phosphorylated. Just for the sake of completeness, this term shall also include the expressed genes that are transcribed into RNA but not translated into a protein (for example, transfer and ribosomal RNAs). The terms "overexpression" and "underexpression" refer to an upward or a downward deviation respectively in levels of expression as compared to the baseline expression level in a sample used as a control. "Overexpression" is therefore also "increased expression" and "underexpression" is "decreased expression".

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal

antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be constructed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler, G. et al., Nature 256 (1975) 495, or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). "Antibody fragments" comprise a portion of an intact antibody

An antibody "which binds" an antigen of interest according to the invention, i.e., the phosphorylated MAPK or the phosphorylated pAKT protein, is one capable of binding that antigen with sufficient affinity such that the antibody is useful in detecting the presence of the antigen. The antibody according to the invention is one which binds phosphorylated MAPK or phosphorylated pAKT protein, it will usually preferentially bind phosphorylated MAPK or phosphorylated pAKT protein as opposed to the non-phosphorylated MAPK or the non-phosphorylated pAKT protein or does not significantly cross-react with non-phosphorylated MAPK or non-phosphorylated pAKT protein. In such embodiments, the extent of binding of the antibody to the non-phosphorylated proteins will be less than 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA). In other words, it will specifically bind phosphorylated MAPK or phosphorylated pAKT protein and does not specifically bind or does not all bind non-phosphorylated MAPK or non-phosphorylated pAKT protein

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN^{TM}), alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards

such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, poffiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti- metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6- azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti- adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2- ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2', 2''-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g., paclitaxel (TAXOL®, Bristol- Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE®, Rhone- Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT- 11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition, are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen,

raloxifene, aromatase inhibiting 4(5)-imidazoles, 4- hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti- androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above. The "chemotherapeutic agent" itself can be a combination of chemical compounds useful in the treatment of cancer combination as mentioned above, i.e. the combination may be gemcitabine/ cis-platin, but also e.g. cis-platin/ paclitaxel, cis-platin/ docetaxel, cis-platin/ vinorelbine, gemcitabine/ carboplatin, or carboplatin/ docetaxel.

The term "EGFR inhibitor" refers to a therapeutic agent that binds to EGFR and, optionally, inhibits EGFR activation. Examples of such agents include antibodies and small molecules that bind to EGFR. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, US 4,943, 533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBUTIX®) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); antibodies that bind type 11 mutant EGFR (US 5,212,290); humanized and chimeric antibodies that bind EGFR as described in US 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF (see WO 98/50433, Abgenix). The anti-EGFR antibody may be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, e.g., EP 0 659 439 A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 or Gefitinib (IRESSA™; Astra Zeneca), CP-358774 (Tarceva®; Genentech/OSI) and AG1478, AG1571 (SU 5271; Sugen). Particularly preferred in this application are EGFR tyrosine kinase inhibitors, particularly small molecule EGFR tyrosine kinase inhibitors as e.g. Tarceva®. A "small molecule" can be e.g. a peptide or a peptidomimetics with a molecular weight less than about 10,000 grams per mole, preferably less than about 5,000 grams per mole. Preferably, a "small molecule" is compound, i.e. an organic or inorganic compound, with a molecular weight less than about 5,000 grams per mole, preferably less than about 1,000 grams per mole, more preferably less than 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. Therefore, in a preferred embodiment of the invention, the EGFR inhibitor is a EGFR tyrosine kinase inhibitor that is a compound with a molecular weight less than about 5,000 grams per mole, preferably less than about 1,000 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such a compound. In other words, the

EGFR inhibitor is a compound that inhibts EGFR tyrosine kinase activity and that has a molecular weight less than about 5,000 grams per mole, preferably less than about 1,000 grams per mole, more preferably less than 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such a compound.

"Gemcitabine" is the chemotherapeutic agent 2',2'-difluorodeoxycytidine (dFdC) which is a pyrimidine analogue of deoxycytidine in which the deoxyribose moiety contains two fluorine atoms at the 2'-position (see Heinemann, V. et al., Cancer Res 48 (1988) 4024). It is commerciably available as Gemzar® from Eli Lilly and Company, Indianapolis, Indiana, USA.

"Cis-platin" as used throughout this application is the chemotherapeutic agent cis-diamminedichloroplatinum (see US 5,562,925) commercially available as Platinol® from Bristol-Myers Squibb Company, New York, NY, USA. "Cis-platin" is a heavy metal complex containing a central atom of platinum surrounded by two chloride atoms and two ammonia molecules in the cis position.

According to the invention, the expression that "a biological sample comprising human lung cancer cells is sensitive to a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent" shall mean that the biological sample comprising human lung cancer cells is sensitive to a treatment with a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent in contrast to a treatment with an epidermal growth factor receptor inhibitor alone. "Sensitive" can also be understood as "reacting to" or "showing a reaction to", particularly such a reaction that is of benefit to a lung cancer patient. Thereby, it can be determined whether a lung cancer patient is sensitive to to a treatment with a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent in contrast to a treatment with an epidermal growth factor receptor inhibitor alone. This means that the patient will benefit from such a treatment.

A "MAPK" protein is a member of a highly conserved cytosolic serine/threonine protein kinase family known as mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs). This protein family has several subgroups. ERKs are activated and tyrosine- or threonine-phosphorylated in response to a wide variety of extracellular signals, including osmotic stress, heat shock, pro-inflammatory cytokines , hormones, and mitogens. The term "MAPK protein" as used in this invention preferably refers to a member of the MAPK

protein family comprising or preferably consisting of MAPK1 and MAPK3. The amino acid sequences of MAPK1 (ERK2) are SEQ ID NO: 1) and MAPK3 (ERK1) (SEQ ID NO: 2). These amino amino acid sequences are encoded by the mRNA sequences, i.e. cDNA sequences SEQ ID NO: 3 and 4 for MAPK1 ans SEQ ID NO: 5 for MAPK 3. The primary phosphorylation sites in MAPK1 are Thr185 and Tyr185 and the primary phosphorylation in MAPK3 are Thr202 and Tyr204. These phosphorylation sites are also recognized by the antibody used in the present invention, i.e. preferably the polyclonal antibody serum against the phosphorylated forms of MAPK1 and MAPK3.

The term "Akt" protein refers to a protein of the Akt/PKB subfamily of second-messenger regulated serine/threonine protein kinases which has three members termed Akt1/PKBalpha, Akt2/PKBbeta (Staal, S.P., Proc. Natl. Acad. Sci. USA 84 (1987) 5034-5037) and Akt3/PKBgamma (Nakatani, K. et al., Biochem. Biophys. Res. Comm. 257 (1999) 906-910; US 6,881,555) respectively. The isoforms are homologous and are activated by phosphorylation in response to phosphatidylinositol 3'-OH kinase (PI3K) signaling. The PI3K/Akt/PKB pathway appears to be important for regulating cell survival/cell death (Dudek, H. et al., Science 275 (1997) 661-665) also in tumorigenesis. The term "Akt protein" as used in this invention preferably refers to a member of the Akt protein family comprising or preferably consisting of Akt1, Akt2 and Akt3. Phosphorylation of Akt1/PKBα occurs on two sites Thr^{308} and on Ser^{473} (Meier, R., et al., J. Biol. Chem. 272 (1997) 30491-30497). Equivalent phosphorylation sites occur in Akt2/PKBbeta (Thr^{309} and Ser^{474}) and Akt3/PKBgamma (Thr^{305} and Ser^{472}). The term "phosphorylated Akt" protein refers to a phosphorylated "Akt" protein, preferably phosphorylated at the sites described above. The term "MAPK protein" as used in this invention preferably refers to a member of the MAPK protein family comprising or preferably consisting of MAPK1 and MAPK3. Akt 1 is also known as human RAC-alpha serine/threonine-protein kinase (EC 2.7.1.37) (RAC-PK-alpha), Protein kinase B (PKB) (C-AKT) and the amino acid sequence of Akt 1 is SEQ ID NO: 6. AKT2 is also known as human RAC-beta serine/threonine-protein kinase (EC 2.7.1.37) (RAC-PK-beta), Protein kinase Akt-2 or Protein kinase B, beta (PKB beta) and the amino acid sequence of Akt 2 is SEQ ID NO: 7. AKT3 is also known as human RAC-gamma serine/threonine-protein kinase (EC 2.7.1.37) (RAC-PK-gamma), protein kinase Akt-3 or Protein kinase B, gamma (PKB gamma) (STK-2) and the amino acid sequence of Akt 3 is SEQ ID NO: 8.

Detailed Description of the Invention

Conventional techniques of molecular biology and nucleic acid chemistry, which are within the skill of the art, are explained in the literature. See, for example, Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989; Gait, M.J. (ed.), Oligonucleotide Synthesis - A Practical Approach, IRL Press, 1984; Hames, B.D., and Higgins, S.J. (eds.), Nucleic Acid Hybridisation - A Practical Approach, IRL Press, 1985; and a series, Methods in Enzymology, Academic Press, Inc., all of which are incorporated herein by reference. All patents, patent applications, and publications mentioned herein, both supra and infra, are incorporated herein by reference.

In an embodiment of the invention, a method is provided of determining whether a biological sample comprising human lung cancer cells is sensitive to a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent, the method comprising determining the overexpression of a phosphorylated AKT protein and/ or a phosphorylated MAPK protein in the biological sample whereby the overexpression of the phosphorylated AKT protein and/ or the phosphorylated MAPK protein is an indication that the biological sample comprising human lung cancer cells is sensitive to a combination of a epidermal growth factor receptor inhibitor and a chemotherapeutic agent.

Preferably, in the method according to the invention, the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the biological sample is determined by

a) determining the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the biological sample,

b) determining the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in a biological sample comprising human lung cancer cells that are not sensitive a combination of a epidermal growth factor inhibitor and a chemotherapeutic agent,

c) determining the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) thereby determining the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein.

Preferably, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) is at least 10 %. More preferably, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) is at least 25 %. In another embodiment, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) is at least 50 %, 75 %, 100 %, 125 %, 150 %, 175 %, 200 %, 300 %, 400 %, 500 % or 1,000 %. The difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) can be up to 10,000 or 50,000 %. The difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) is preferably between 10 % to 10,000 %, more preferably 25 % to 10,000 %, 50 % to 10,000 %, 100 % to 10,000 %, even more preferably 25 % to 5,000 %, 50 % to 5,000 %, 100 % to 5,000 %.

In a preferred embodiment of the invention, the biological sample is a primary lung tumor or a metastasis (regional or distant) which can be obtained e.g. by lung biopsy or from other organs by way of biopsy. A metastasis can also be a distant metastasis e.g. from the liver or lymph node. It has to be noted that such distant metastasis also contain lung cancer cells as the metastases originate from the lung.

In another preferred embodiment the cancer is another cancer than lung cancer as pancreatic cancer. However, other cancers with solid tumours are also feasible such as ovarian, colorectal, head and neck, renal cell carcinoma, glioma and gastrointestinal cancers, particularly stomach cancer.

In another preferred embodiment the EGFR inhibitor is a EGFR tyrosine kinase inhibitors, particularly small molecule EGFR tyrosine kinase inhibitors as e.g. Tarceva®. Therefore in other words, in a particularly preferred embodiment of the invention, the EGFR inhibitor is erlotinib or N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine.

In yet another preferred embodiment of the invention, the chemotherapeutic agent is gemcitabine and/or cis-platin.

In another preferred embodiment of the invention, the overexpression of the phosphorylated Akt protein or of the phosphorylated MAPK protein is determined using a reagent which specifically binds the phosphorylated protein and preferably not specifically or not at all to the non-phosphorylated protein. Preferably an

antibody, an antibody derivative, or an antibody fragment specifically binds to the phosphorylated AKT protein or the phosphorylated MAPK protein and preferably not specifically or not at all to the non-phosphorylated AKT protein or the non-phosphorylated MAPK protein.

In yet another preferred embodiment of the invention, the phosphorylated AKT protein is phosphorylated at an amino acid position corresponding to amino acid position 473 of the Akt1 protein or the MAPK protein is phosphorylated at amino acid positions corresponding to amino acid positions 202 and 204 of MAPK1. Preferably, the amino acid sequence of the MAPK protein is the amino acid sequence SEQ ID NO: 1 or 2 and the amino acid sequence of the AKT protein is the amino acid sequence SEQ ID NO: 6, 7 or 8.

There are many different types of immunoassays which may be used in the method of the present invention, e.g. enzyme linked immunoabsorbent assay (ELISA), fluorescent immunosorbent assay (FIA), chemical linked immunosorbent assay (CLIA), radioimmuno assay (RIA), and immunoblotting. For a review of the different immunoassays which may be used, see: Lottspeich and Zorbas (eds.), Bioanalytik, 1st edition 1998, Spektrum Akademischer Verlag, Heidelberg, Berlin, Germany. Therefore, in yet another preferred embodiment of the invention, the expression level is determined using a method selected from the group consisting of proteomics, flow cytometry, immunocytochemistry, immunohistochemistry and enzyme-linked immunosorbent assay.

In a preferred embodiment of the invention, the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein is determined by

a) immunohistochemically staining the biological sample,

b) assigning a grade selected from the numbers 1, 2, 3 and 4 for the level of expression of the phosphorylated AKT protein and/ or the phosphorylated MAPK protein upon visual inspection of the staining of the cells in the biological sample whereby the highest detectable grade for the level of expression is assigned,

c) determining the percentage of cells with the highest detectable grade in the immunohistochemically stained biological sample,

d) multiplying the assigned grade with the percentage of cells with the highest detectable grade in the immunohistochemically stained biological sample and with the number 100, and

e) determining overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the biological sample when the result of the multiplication in step d) is above 100.

In another embodiment of the invention, a method is provided of determining whether a lung cancer patient benefits from a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent, the method comprising determining the overexpression of a phosphorylated AKT protein and/ or a phosphorylated MAPK protein in a sample from the patient whereby the overexpression of the phosphorylated AKT protein and/ or the phosphorylated MAPK protein is an indication that the patient benefits from a combination of a epidermal growth factor receptor inhibitor and a chemotherapeutic agent. All other preferred embodiments described above equally apply to this embodiment. Preferably, in the method according to the invention, the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the sample from the patient is determined by

a) determining the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the sample from the patient,
b) determining the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in a sample from a lung cancer patient who does not benefit from a combination of a epidermal growth factor inhibitor and a chemotherapeutic agent,
c) determining the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) thereby determining the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein. The term "benefit" means that the patient does not have a benefit from a treatment with combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent in contrast to a treatment with an epidermal growth factor receptor inhibitor alone.

In another preferred embodiment of the invention, an antibody that binds to the phosphorylated AKT protein or an antibody that binds to the phosphorylated MAPK protein is used for determining whether a biological sample comprising human lung cancer cells is sensitive to a combination of a epidermal growth factor inhibitor and a chemotherapeutic agent.

In still another embodiment of the invention, a method of selecting a composition for inhibiting the progression of lung cancer in a patient is provided, the method comprising:

a) separately exposing aliquots of a biological sample comprising lung cancer cells that are sensitive to a combination of an EGFR inhibitor and a chemotherapeutic agent from the patient in the presence of a plurality of test compositions;

b) comparing the level of expression of a phosphorylated Akt protein and/ or a phosphorylated MAPK protein in the aliquots of the biological sample contacted with the test compositions and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in an aliquot of the biological sample not contacted with the test compositions,

c) selecting one of the test compositions which alters the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot containing that test composition, relative to the aliquot not contacted with the test composition wherein an at least 10 % difference between the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the test composition and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the test composition is an indication for the selection of the test composition.

Preferably, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in step c) is at least 25 %. More preferably, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in step c) is at least 50 %. In another embodiment, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in step c) is at least 75 %, 100 %, 125 %, 150 %, 175 %, 200 %, 300 %, 400 %, 500 % or 1,000 %. The difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step c) can be up to 10,000 or 50,000 %. The difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step c) is preferably between 10 % to 10,000 %, more preferably 25 % to 10,000 %, 50 % to 10,000 %, 100 % to 10,000 %, even more preferably 25 % to 5,000 %, 50 % to 5,000 %, 100 % to 5,000 %.

In yet another embodiment of the invention, a method of deriving a candidate agent is provided, said method comprising:

a) contacting an aliquot of a biological sample containing lung cancer cells that are sensitive to an EGFR inhibitor and a chemotherapeutic agent with the candidate agent,

(b) determining the level of expression of a phosphorylated Akt protein and/ or a phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and determining the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in an aliquot of the biological sample not contacted with the candidate agent,

(c) observing the effect of the candidate agent by comparing the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the candidate agent,

(d) deriving said agent from said observed effect, wherein an at least 10 % difference between the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the candidate agent is an indication of an effect of the candidate agent.

Preferably, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in step d) is at least 25 %. More preferably, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in step d) is at least 50 %. In another embodiment, the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in step d) is at least 75 %, 100 %, 125 %, 150 %, 175 %, 200 %, 300 %, 400 %, 500 % or 1,000 %. The difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step d) can be up to 10,000 or 50,000 %. The difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step d) is preferably between 10 % to 10,000 %, more

preferably 25 % to 10,000 %, 50 % to 10,000 %, 100 % to 10,000 %, even more preferably 25 % to 5,000 %, 50 % to 5,000 %, 100 % to 5,000 %.

In a preferred embodiment said candidate agent is a candidate inhibitory agent or a candidate enhancing agent.

In another embodiment of the invention a candidate agent derived by the method according to the invention is provided.

In yet another embodiment a pharmaceutical preparation comprising an agent according to the invention is provided.

In still another embodiment an agent according to the invention is used for the preparation of a composition for the inhibition of progression of lung cancer.

In yet another embodiment of the invention, a method of producing a drug comprising the steps of the method of the inventioj is provided and

(i) synthesizing the candidate agent identified in step (c) or an analog or derivative thereof in an amount sufficient to provide said drug in a therapeutically effective amount to a subject; and/or
(ii) combining the drug candidate the candidate agent identified in step (c) or an analog or derivative thereof with a pharmaceutically acceptable carrier.

In still another embodiment an Akt protein, a MAPK protein, a phosphorylated Akt protein, a phosphorylated MAPK protein, an antibody selectively binding to a phosphorylated Akt protein or a phosphorylated MAPK protein is used for deriving a candidate agent or for selecting a composition for inhibiting the progression of lung cancer in a patient.

In another embodiment of the invention, a kit is contemplated comprising an antibody against phosphorylated MAPK and/ or phosphorylated Akt protein. Such kits known in the art further comprise plastics ware which can be used during the amplification procedure as e.g. microtitre plates in the 96 or 384 well format or just ordinary reaction tubes manufactured e.g. by Eppendorf, Hamburg, Germany and all other reagents for carrying out the method according to the invention, preferably an immunoassay, e.g. enzyme linked immunoabsorbent assay (ELISA), fluorescent immunosorbent assay (FIA), chemical linked immunosorbent assay (CLIA), radioimmuno assay (RIA), and immunoblotting. For a review of the

different immunoassays and reagents which may be used, see: Lottspeich and Zorbas (eds.), Bioanalytik, 1st edition 1998, Spektrum Akademischer Verlag, Heidelberg, Berlin, Germany.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figures 1 Kaplan–Meier curves for time to death (OS) analysis among all patients randomly assigned to Erlotinib/Gemcitabine/Cisplatin (A) or Placebo/Gemcitabine/Cisplatin treatment (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 2 Kaplan–Meier curves for time to death (OS) analysis among all patients with biomarker data, randomly assigned to Erlotinib/Gemcitabine/Cisplatin (A) or Placebo/Gemcitabine/-Cisplatin treatment (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 3 Kaplan–Meier curves for time to progression/death (PFS) analysis among all patients randomly assigned to Erlotinib/Gemcitabine/Cisplatin (A) or Placebo/Gemcitabine/-Cisplatin treatment (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 4 Kaplan–Meier curves for time to progression/death (PFS) analysis among all patients with biomarker data, randomly assigned to Erlotinib/Gemcitabine/Cisplatin (A) or Placebo/-Gemcitabine/Cisplatin treatment (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 5 Kaplan–Meier curves for time to death (OS) analysis among all biomarker patients treated with Placebo/Gemcitabine/Cisplatin comparing patients with pMAPK H-Score $\geq$ 100 (H) with patients with pMAPK H-Score < 100 (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 6 Kaplan–Meier curves for time to death (OS) analysis among all biomarker patients with pMAPK H-Score < 100 comparing Erlotinib/Gemcitabine/Cisplatin (A) with Placebo/Gemcitabine/-Cisplatin treatment (circles indicate censored observation time, when the observation was terminated before the event occurred).

Figure 7 Kaplan–Meier curves for time to death (OS) analysis among all biomarker patients with pMAPK H-Score < 100 and pMAPK H-Score ≥ 100 comparing Erlotinib/Gemcitabine/Cisplatin (LA, HA) with Placebo/Gemcitabine/Cisplatin (H) treatment, respectively (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 8 Kaplan–Meier curves for time to progression/death (PFS) analysis among all biomarker patients with pMAPK H-Score < 100 and pMAPK H-Score ≥ 100 comparing Erlotinib/Gemcitabine/-Cisplatin (LA, HA) with Placebo/Gemcitabine/Cisplatin (H) treatment, respectively (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 9 Kaplan–Meier curves for time to death (OS) analysis among all Placebo/Gemcitabine/Cisplatin treated biomarker patients comparing pAKT1 H-Score < 300 (L) and pAKT1 H-Score ≥ 300 (H) (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 10 Kaplan–Meier curves for time to death (OS) analysis among all Erlotinib/Gemcitabine/Cisplatin treated biomarker patients comparing pAKT1 H-Score < 300 (L) and pAKT1 H-Score ≥ 300 (H) (circles indicate censored observation times, when the observation was terminated before the event occurred).

Figure 11 Kaplan–Meier curves for time to death (OS) analysis among all biomarker patients with pAKT1 H-Score < 300 and pAKT1 H-Score ≥ 300 comparing Erlotinib/Gemcitabine/Cisplatin (LA, HA) with Placebo/Gemcitabine/Cisplatin (H) treatment, respectively (circles indicate censored observation times, when the observation was terminated before the event occurred).

Example 1

Biomarker analysis on tumor tissue samples

The objective of the exploratory tumor biomarker analyses for the clinical study was the identification of those markers or combinations of markers which predict best for positive or negative clinical outcome of Tarceva® treatment. As the clinical results of the study did not allow to generate hypotheses about how to select patient populations that derive more benefit from the treatment with Tarceva®, special emphasis was on the identification of markers that discriminate between patients (subgroups) that specifically benefit in the Tarceva® combination vs. the chemotherapy-alone control arm. Additionally, the identification of markers that discriminate between patients (subgroup) that have a detrimental effect from the specific combination with Tarceva® vs. the chemotherapy-alone control arm was investigated .

The aim of this study was to analyze tumor-specific biomarker related to the EGFR signaling pathway e.g. EGFR, HER2, pAKT, and pMAPK.

Biomarker data was correlated with clinical data (overall and therapy-specific analysis).

Material and methods:

Clinical samples:

Biomarker analyses were performed on a sample subset of 141 patients, for which formalin-fixed paraffin embedded (FFPE) tissue blocks from initial diagnosis had been received.

Antibodies for IHC testing:

Antibody	Target protein	Slide pretreatment	Dilution
Abcam ab8932 (available from Abcam, Cambridge, United Kingdom)	pAKT (antibody against phosphorylated Ser 473 of Akt)	pressure cooker 120°C/5min, citrate buffer pH9	1:450
Zymed 36-8800 (available from Zytomed GmbH, Berlin, Germany or Invitrogen, Carlsbad, CA, USA)	pMAPK (ERK1+2 Thr202/Tyr204)	pressure cooker 120°C/5min, citrate buffer pH6	1 : 150

pMAPK IHC protocol:

1. Cut about 3-4 um thick sections from the Paraffin-Array- blocks.
2. Mount sections on glass slides and let them dry over night.
3. Deparaffinize the slides in xylene followed by descending ethanol series:
   - xylene over night
   - xylene 2 x 10 minutes
   - abs.Ethanol 2 x 10 min.
   - 96% Ethanol 2 x 5 min.
   - 80% Ethanol 1 x 5 min.
   - 70% Ethanol 2 x 5 min.
   - PBS Buffer 10 min. (change buffer once or twice)
4. Antigene retrival / sample pretreatment
   Pressure cooker 5 minutes at 120°C in 1x citrate buffer pH 6 (Biocyc GmbH, order number 400300692). Wash in TBS/PBS (1:10) buffer for 5 minutes
5. Peroxidase blocking
   - Slides are incubated in 3% H_2O_2 for 10 minutes.
   - Wash 2 x 5 minutes in TBS/PBS buffer.
6. Antibody Incubation
   - Put the slides in normal serum (1.5%) diluted in Tris-Buffer or a other blocking solution.
   - Put the primary antibody (Zymed Rabbit anti phospho-ERK1+2 cat no. 36-8800) diluted 1:150 on the slides and incubate it in a humified chamber at 30°C for 2 hours.
   - Wash 2 x 5 minutes in TBS/PBS buffer.

- Put Envision Polymer HRP (Dako) on the slides and incubate in a humified chamber at 30°C for 30 minutes.
- Wash 2 x 5 minutes in TBS/PBS buffer.

7. Detection
   - Wash the slides with 0.05 M Tris buffer pH 7.6 for 20 minutes
   - Cover slides for 5 minutes with DAB-Chromogen (Liquid DAB Dako code no. K3467) and incubate it for 5-10 minutes.
   - Wash the slides with demin. water for 5 minutes to stop the color reaction.
   - Counterstain with hematoxyline (Harris Hämatoxylin HTX 31000, Medite GmbH)
   - Rinse with water
   - Differentiate in HCL-Ethanol
   - „blue" for 5 min in water
   - Ascending ethanol series
   - xylene
   - cover

pAKT IHC protocol:

Protocol as for pMAPK, except

- Compare 4.: Sample pretreatment in citrate buffer pH 9 (Dako code no. S2367)
- Compare 6.: Primary antibody (abcam AKT phospho S473) diluted 1:450 (compare 6.)

IHC data reporting:

One pathologist evaluated all immunostainings. The nuclear staining intensity (pAKT, pMAPK) was estimated by visual inspection in a four step scale (0, 1, 2, 3). In addition to nuclear staining intensity, the percentage of positive cells, and the reason for analysis failure (i.e. lack of tumor cells in the tissue spot or lack of the tissue spot on the TMA slide) was recorded.

Exploratory statistical analysis:

The statistical analyses of the biomarker data aimed at exploring the potential to predict clinical benefit and/or toxicities, by each marker separately and/or by suitable combinations.

According to experience many biomarkers show a skewed statistical distribution across patients and within patient. Frequently there is also some biochemical background of this skewness, in that the variation process has a multiplicative structure. Skewed distributions present with problems when linear statistical approaches (e.g. regression) are to be used. When used as covariates in statistical models the skewness as well can obscure the results. Therefore suitable transformations need to be found which transform these measurements into distributions with an approximate Gaussian shape. Typical choices in the biomarker area are transformations of the form log(x+c). These transformations do not change the order of the values, such that non-parametric analyses based on ranks or cut-offs remain unchanged by the transformation. Such transformations are also a prerequisite when linear multivariate approaches like e.g. Discriminant Analysis and Principle Component Analysis are to be used.

The basic statistics and interdependencies of the different markers were descriptively investigated. Methodological analyses comparing different measurement approaches, e.g. for IHC, were performed with regard to reliability and validity. Benefit to Tarceva® is defined by the clinical endpoints survival time (or Time to Death, TTD), PFS time (time to progression, TTP/D), objective response, best response (CR/PR/SD/PD).

The p-values emerging from these analyses are not to be interpreted in a confirmative sense; they are to be seen as a special descriptive tool in order to guide the exploration towards an efficient candidate prediction rule. Markers were evaluated on a univariate level regarding their potential for prediction (e.g. search for cut-offs) of the clinical endpoints. Further multivariate techniques (e.g. Linear Discriminant Analysis, Multiple Logistic Regression, Principal Component Analysis with Rotation, Cluster Analysis, CART methodology) were employed in order to study combinations of markers. Biomarker and response correlations with clinical covariates were investigated. Candidate groupings derived from biomarkers were checked with time to event variables (Kaplan-Meier curves, Cox proportional hazard model, logrank test).

Results:

Analysis of the TMA sample subset in comparison to the overall study population

The patient subset with samples for biomarker analysis was compared to the overall study population regarding baseline patient characteristics and clinical outcome parameters. The summary is shown in the table below.

	Main Clinical Population		Patient subset with IHC/FISH biomarker data	
	Placebo (N=582)	Tarceva® (N=580)	Placebo (N=70)	Tarceva® (N=71)
Age (years)	59.1	59.9	57.5	59.1
Male (%)	75.3	78.6	80.0	74.6
Disease stage IV (%)	67.2	64.8	74.3	80.3
Adeno carcinoma (%)	37.6	37.9	41.4	46.5
# of metastatic sites	3.7	3.7	3.4	4.0
# of affected organs	2.5	2.5	2.1	2.4
Sum longest diameter	92.8	95.4	80.1	93.5
Average symptom burden	26.5	26.7	24.3	25.3
Responders (%)	38.3 (N=418)	42.4 (N=396)	42.3 (N=52)	43.5 (N=46)
Hazard ratio TTD	1.035		1.217	
Hazard ratio TTP	0.980		1.262	

Findings:

The patient subset with biomarker data is not representative for the BO16411 study population. There are differences in hazard ratios for TTD and TTP between main population and the biomarker subgroup. Tarceva®-treated patients of the biomarker subgroup have a worse prognosis compared to the main clinical population. Several baseline covariates indicate that the biomarker subgroup – and within this subgroup particularly Tarceva®-related patients represented a more morbid case mix as compared to the main study population. KM plots (Figs. 1 to 4) should be taken into account as well.

Results of pMAPK IHC analysis

- pMAPK IHC data showed sufficient scatter to be eligible for further statistical analysis.
  For determining correlation between pMAPK expression and clincal outcome, cut-off values were established by descriptive statistical analysis: Franklin H-score was determined by combining staining intensity and percentage of stained tumor cells (pMAPK_hsco = (pMAPK_Nuclear_Staining + 1) * pMAPK_Nuclear_Pos_Cells; range: 0-400). "Positive" pMAPK staining was defined by H-scores of =/> 100, else the staining was "negative".
- Kaplan-Meier plots are shown in Figs. 5 to 8

pMAPK H-Score (Cutpoint 100) --- COX Model without covariates

Variable	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq	Hazard Ratio	95% Hazard Ratio Confidence Limits	
Time to progression/death							
trtgr only	0.27747	0.21560	1.6562	0.1981	1.320	0.865	2.014
trtgr	0.12352	0.22058	0.3136	0.5755	1.131	0.734	1.743
pMAPK (≥100)	0.90942	0.23706	14.7164	0.0001	2.483	1.560	3.951
trtgr pMAPK<100	0.49122	0.30087	2.6656	0.1025	1.634	0.906	2.947
trtgr pMAPK≥100	-0.25703	0.30706	0.7007	0.4026	0.773	0.424	1.412
Time to death							
trtgr only	0.15228	0.27818	0.2997	0.5841	1.164	0.675	2.009
trtgr	-0.02494	0.28668	0.0076	0.9307	0.975	0.556	1.711
pMAPK (≥100)	0.73352	0.29272	6.2797	0.0122	2.082	1.173	3.696
trtgr pMAPK<100	0.98292	0.45017	4.7674	0.0290	2.672	1.106	6.457
trtgr pMAPK≥100	-0.70524	0.36205	3.7943	0.0514	0.494	0.243	1.004

Findings:

- For patients treated with chemotherapy/placebo "positive" pMAPK expression is associated with worse prognosis (TTD: HR 4.882, p=0.0001), while „negative" pAKT expression appears to be associated with longer survival
- Trend: „Positive pMAPK" patients might benefit from the chemotherapy/ Tarceva® combo (HR 0.500, p: 0.0516) (----)

Results of pAKT IHC analysis

- pAKT IHC data showed sufficient scatter to be eligible for further statistical analysis
- For determining correlation between pAKT expression and clincal outcome, cut-off values were established by descriptive statistical analysis: Franklin H-score was determined by combining nuclear staining intensity and percentage of stained tumor cells (pAKT_hsco = (pAKT_Nuclear_Staining + 1) * pAKT_Nuclear_Pos_Cells; range: 0-400). "Positive" pAKT staining was defined by H-scores of =/> 300, else the staining was "negative".
- Kaplan-Meier plots are shown in Figs. 9 to 11

Correlation of IHC and clinical data

pAKT1 H-Score (Cutpoint 300) --- COX Model without covariates

Variable		Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq	Hazard Ratio	95% Hazard Ratio Confidence Limits	
Time to progression/death								
trtgr	only	0.23796	0.20820	1.3064	0.2530	1.269	0.844	1.908
trtgr		0.19809	0.20973	0.8921	0.3449	1.219	0.808	1.839
pAKT1 (≥300)		0.66669	0.23379	8.1320	0.0043	1.948	1.232	3.080
trtgr	pAKT1<300	0.12333	0.37441	0.1085	0.7419	1.131	0.543	2.356
trtgr	pAKT1≥300	0.23300	0.25463	0.8373	0.3602	1.262	0.766	2.079
Time to death								
trtgr	only	0.14794	0.27012	0.3000	0.5839	1.159	0.683	1.969
trtgr		0.10118	0.27209	0.1383	0.7100	1.106	0.649	1.886
pAKT1 (≥300)		0.39280	0.29959	1.7191	0.1898	1.481	0.823	2.664
trtgr	pAKT1<300	0.69986	0.50449	1.9244	0.1654	2.013	0.749	5.412
trtgr	pAKT1≥300	-0.14277	0.32063	0.1983	0.6561	0.867	0.462	1.625

Findings:

- For patients treated with chemotherapy/placebo "positive" pAKt expression is associated with worse prognosis (TTD HR 2.258, p=0.0573), while „negative" pAKT expression appears to be associated with longer survival
- A similar difference was not found for patients treated with chemotherapy/ Tarceva® combo.

Patent Claims

1. A method of determining whether a biological sample comprising human lung cancer cells is sensitive to a combination of an epidermal growth factor receptor inhibitor and a chemotherapeutic agent, the method comprising

determining the overexpression of a phosphorylated AKT protein and/ or a phosphorylated MAPK protein in the biological sample

whereby the overexpression of the phosphorylated AKT protein and/ or the phosphorylated MAPK protein is an indication that the biological sample comprising human lung cancer cells is sensitive to a combination of a epidermal growth factor receptor inhibitor and a chemotherapeutic agent.

2. The method according to claim 1 wherein the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the biological sample is determined by

   a) determining the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the biological sample,
   b) determining the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in a biological sample comprising human lung cancer cells that are not sensitive a combination of a epidermal growth factor inhibitor and a chemotherapeutic agent,
   c) determining the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) thereby determining the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein.

3. The method according to claim 2 whereby the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) is at least 10 %.

4. The method according to any of the claims 2 to 3 whereby the difference of the level of expression of phosphorylated AKT protein and/ or phosphorylated MAPK protein determined in step a) and b) is at least 25 %.

5. The method according to any of the claims 1 to 4, wherein the biological sample is a primary lung tumor or a metastasis.

6. The method according to any of the claims 1 to 5 wherein the EGFR inhibitor is erlotinib or N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine.

7. The method according to any of the claims 1 to 6 wherein the chemotherapeutic agent is gemcitabine or cis-platin.

8. The method according to any of the claims 1 to 7, wherein the overexpression of the phosphorylated Akt protein or of the phosphorylated MAPK protein is determined using a reagent which specifically binds the phosphorylated protein.

9. The method of claim 8, wherein the antibody, the antibody derivative, or the antibody fragment specifically binds to the phosphorylated AKT protein or the phosphorylated MAPK protein.

10. The method according to any of the claims 1 to 9, wherein the phosphorylated AKT protein is phosphorylated at an amino acid position corresponding to amino acid position 473 of the Akt1 protein or the MAPK protein is phosphorylated at amino acid positions corresponding to amino acid positions 202 and 204 of MAPK1.

11. The method according to any of the claims 1 to 11 wherein the amino acid sequence of the MAPK protein is the amino acid sequence SEQ ID NO: 1 or 2 and the amino acid sequence of the AKT protein is the amino acid sequence SEQ ID NO: 6, 7 or 8.

12. The method according to any of the claims 1 to 11 wherein the expression level is determined using a method selected from the group consisting of proteomics, flow cytometry, immunocytochemistry, immunohistochemistry and enzyme-linked immunosorbent assay.

13. The method according to any of the claims 1 to 12 wherein the overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein is determined by

a) immunohistochemically staining the biological sample,

b) assigning a grade selected from the numbers 1, 2, 3 and 4 for the level of expression of the phosphorylated AKT protein and/ or the phosphorylated MAPK protein upon visual inspection of the staining of the cells in the biological sample whereby the highest detectable grade for the level of expression is assigned,

c) determining the percentage of cells with the highest detectable grade in the immunohistochemically stained biological sample,

d) multiplying the assigned grade with the percentage of cells with the highest detectable grade in the immunohistochemically stained biological sample and with the number 100, and

e) determining overexpression of phosphorylated AKT protein and/ or phosphorylated MAPK protein in the biological sample when the result of the multiplication in step d) is above 100.

14. Use of an antibody that binds to the phosphorylated AKT protein or of an antibody that binds to the phosphorylated MAPK protein for determining whether a biological sample comprising human lung cancer cells is sensitive to a combination of a epidermal growth factor inhibitor and a chemotherapeutic agent.

15. A method of selecting a composition for inhibiting the progression of lung cancer in a patient, the method comprising:

a) separately exposing aliquots of a biological sample comprising lung cancer cells that are sensitive to a combination of an EGFR inhibitor and a chemotherapeutic agent from the patient in the presence of a plurality of test compositions;

b) comparing the level of expression of a phosphorylated Akt protein and/ or a phosphorylated MAPK protein in the aliquots of the biological sample contacted with the test compositions and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in an aliquot of the biological sample not contacted with the test compositions,

c) selecting one of the test compositions which alters the level of expression of the the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot containing that test composition, relative to the aliquot not contacted with the test composition wherein an at least 10 % difference between the level of

expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the test composition and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the test composition is an indication for the selection of the test composition.

16. A method of deriving a candidate agent, said method comprising:

a) contacting an aliquot of a biological sample containing lung cancer cells that are sensitive to an EGFR inhibitor and a chemotherapeutic agent with the candidate agent,

b) determining the level of expression of a phosphorylated Akt protein and/ or a phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and determining the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in an aliquot of the biological sample not contacted with the candidate agent,

c) observing the effect of the candidate agent by comparing the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the candidate agent,

d) deriving said agent from said observed effect, wherein an at least 10 % difference between the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the phosphorylated Akt protein and/ or the phosphorylated MAPK protein in the aliquot of the biological sample not contacted with the candidate agent is an indication of an effect of the candidate agent.

17. The method according to claim 16, wherein said candidate agent is a candidate inhibitory agent.

18. The method according to claim 16, wherein said candidate agent is a candidate enhancing agent.

19. A candidate agent derived by the method according to any of the claims 16 to 18.

20. A pharmaceutical preparation comprising an agent according to claim 19.

21. Use of an agent according to claim 19 for the preparation of a composition for the inhibition of progression of lung cancer.

22. A method of producing a drug comprising the steps of the method of any one of Claims 16 to 18; and

i) synthesizing the candidate agent identified in step (c) or an analog or derivative thereof in an amount sufficient to provide said drug in a therapeutically effective amount to a subject; and/or

ii) combining the drug candidate the candidate agent identified in step (c) or an analog or derivative thereof with a pharmaceutically acceptable carrier.

23. Use of an Akt protein, a MAPK protein, a phosphorylated Akt protein, a phosphorylated MAPK protein, an antibody selectively binding to a phosphorylated Akt protein or a phosphorylated MAPK protein for deriving a candidate agent or for selecting a composition for inhibiting the progression of lung cancer in a patient.

24. A kit comprising an antibody against phosphorylated MAPK and/ or phosphorylated Akt protein.