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Method for predicting the response to a treatment

Field of the invention

The invention is related to a method of predicting the response to a treatment with a HER dimerization inhibitor in a patient comprising the steps of assessing a marker gene or a combination of marker genes selected from the group consisting of an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene or a combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene in a biological sample from the patient and predicting the response to the treatment with the HER dimerization inhibitor in the patient by evaluating the results of the first step. Further uses and methods wherein these markers are used are disclosed.

Background of the Invention

The human epidermal growth factor receptor (ErbB or HER) family comprises four members (HER1–4) that, through the activation of a complex signal cascade, are important mediators of cell growth, survival and differentiation. At least 11 different gene products from the epidermal growth factor (EGF) superfamily bind to three of these receptors, EGFR (also called ErbB1 or HER1), HER3 (ErbB3) and HER4 (ErbB4). Although no ligand has been identified that binds and activates HER2 (ErbB2 or neu), the prevailing understanding is that HER2 is a co-receptor that acts in concert with other HER receptors to amplify and in some cases initiate receptor-ligand signaling. Dimerization with the same receptor type (homodimerization) or another member of the HER family (heterodimerisation) is essential for their activity. HER2 is the preferred dimerization partner for other HER family members. The role of the HER family in many epithelial tumor types is well documented and has led to the rational development of novel cancer agents directed specifically to HER receptors. The recombinant humanized anti-HER2 monoclonal antibody (MAb) trastuzumab is a standard of care in patients with HER2-positive metastatic breast cancer (MBC). Overexpression/amplification of the HER2 protein/gene, which occurs in 20–30% of breast cancer cases, is a prerequisite for treatment with trastuzumab.

Pertuzumab (Omnitarg™; formerly 2C4) is the first of a new class of agents known as HER dimerization inhibitors (HDIs). Pertuzumab binds to HER2 at its

dimerization domain, thereby inhibiting its ability to form active dimer receptor complexes and thus blocking the downstream signal cascade that ultimately results in cell growth and division. Pertuzumab is a fully humanized recombinant monoclonal antibody directed against the extracellular domain of HER2. Binding of Pertuzumab to the HER2 on human epithelial cells prevents HER2 from forming complexes with other members of the HER family (including EGFR, HER3, HER4) and probably also HER2 homodimerization. By blocking complex formation, Pertuzumab prevents the growth-stimulatory effects and cell survival signals activated by ligands of HER1, HER3 and HER4 (e.g. EGF, TGFα, amphiregulin, and the heregulins). Other names for Pertuzumab are 2C4 or Omnitarg™. Pertuzumab is a fully humanized recombinant monoclonal antibody based on the human IgG1(κ) framework sequences. The structure of Pertuzumab consists of two heavy chains (449 residues) and two light chains (214 residues). Compared to Trastuzumab (Herceptin®), Pertuzumab has 12 amino acid differences in the light chain and 29 amino acid differences in the IgG1 heavy chain.

WO 2004/092353 and WO 2004/091384 present investigations that the formation of heterodimers of Her2 with other receptors should be linked to the effectiveness or suitability of Pertuzumab.

Zabrecky, J.R. et al., J. Biol. Chem. 266 (1991) 1716-1720 disclose that the release of the extracellular domain of Her2 may have implications in oncogenesis and its detection could be useful as a cancer diagnostic. Colomer, R. et al., Clin. Cancer Res. 6 (2000) 2356-2362 disclose circulating Her2 extracellular domain and resistance to chemotherapy in advanced breast cancer. The prognostic and predictive values of the extracellular domain of Her2 is reviewed by Hait, W.N., Clin. Cancer Res. 7 (2001) 2601-2604.

Summary of the Invention

There is still a need to provide further methods for determining the progression of disease in a cancer patient treated with a HER dimerisation inhibitor.

Therefore, in an embodiment of the invention, a method of predicting the response to a treatment with a HER dimerization inhibitor in a patient is provided comprising the steps of

a) assessing in a biological sample from the patient

- a marker gene or a combination of marker genes selected from the group consisting of an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene or a
- combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene, and

b) predicting the response to the treatment with the HER dimerization inhibitor in the patient by evaluating the results of step a).

In another embodiment of the invention, a probe that hybridizes with the epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the epidermal growth factor, transforming growth factor alpha or HER2 marker protein is used for predicting the response to treatment with a HER dimerization inhibitor in a patient or a probe that hybridizes with the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker protein is used for selecting a composition for inhibiting the progression of disease in a patient.

In still another embodiment of the invention, a kit is provided comprising a probe that anneals with the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker protein.

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

a) separately exposing aliquots of a biological sample from a cancer patient in the presence of a plurality of test compositions;

b) comparing the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene in the aliquots of the biological sample contacted with the test compositions and the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin,

epidermal growth factor, transforming growth factor alpha and HER2 marker gene 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 marker gene or the combination of marker genes 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 marker gene or the combination of marker genes in the aliquot of the biological sample contacted with the test composition and the level of expression of the corresponding marker gene or the combination of marker genes 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 from a cancer patient with the candidate agent and determining the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene in the aliquot,

b) determining the level of expression of a corresponding marker gene or of a corresponding combination of marker genes 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 marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene or corresponding combination of marker genes 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 marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene or combination of marker genes in the aliquot of the biological sample not contacted with the candidate agent is an indication of an effect of the candidate agent.

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

In yet another embodiment of the invention, an agent according to the invention is provided for the preparation of a composition for the treatment of cancer.

In still another embodiment of the invention, a method of producing a drug is provided 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 yet another embodiment of the invention, a marker protein or a marker polynucleotide selected from the group consisting of a amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker protein or marker polynucleotide is used for deriving a candidate agent or for selecting a composition for inhibiting the progression of a disease in a patient.

In another embodiment of the invention, a HER dimerization inhibitor is used for the manufacture of a medicament for treating a human cancer patient characterized in that said treating or treatment includes assessing in a biological sample from the patient

- a marker gene or a combination of marker genes selected from the group consisting an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene or a
- combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. s to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

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. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art. If the term "sample" is used alone, it shall still mean that the "sample" is a "biological sample", i.e. the terms are used interchangeably.

The terms "response to treatment with a HER dimerisation inhibitor" or "response of a patient to treatment with a HER dimerisation inhibitor" refer to the clinical benefit imparted to a patient suffering from a disease or condition (such as cancer) from or as a result of the treatment with the HER dimerisation inhibitor. A clincial benefit includes a complete remission, a partial remission, a stable disease (without progression), progression-free survival, disease free survival, improvement in the time-to-progression (of the disease), improvement in the time-to-death, or improvement in the overall survival time of the patient from or as a result of the treatment with the HER dimerisation inhibitor. There are criteria for determining a response to therapy and those criteria allow comparisons of the efficacy to alternative treatments (Slapak and Kufe, Principles of Cancer Therapy, in Harrison's Principles of Internal Medicine, 13 th edition, eds. Isselbacher et al., McGraw-Hill, Inc., 1994). For example, a complete response or complete remission of cancer is the disappearance of all detectable malignant disease. A partial response or partical remission of cancer may be, for example, an approximately 50 percent decrease in the product of the greatest perpendicular diameters of one or more lesions or where there is not an increase in the size of any lesion or the appearance of new lesions.

As used herein, the term "progression of cancer" includes and may refer to metastasis; a recurrence of cancer, or an at least approximately 25 percent increase in the product of the greatest perpendicular diameter of one lesion or the appearance of new lesions. The progression of cancer, preferably breast cancer, is "inhibited" if recurrence or metastasis of the cancer is reduced, slowed, delayed, or prevented.

The term "Time To Progression/death (TTP)" is a synonym for "Progression-Free Survival (PFS)". This describes a clinical endpoint frequently used in oncology trials. Here the measurement for each patient equals the time elapsed from onset of the treatment of a patient in a trial (as defined in the trial protocol) until the detection of a malignancy progression (as defined in the trial protocol) or the

occurrence of any fatality (whatever was first). If the observation of the patient was stopped (e.g. at study end) after a period and no event was observed, then this observation time t is called "censored".

The term "Time To Death (TTD)" is a synonym for "Overall Survival (OS)". This describes a clinical endpoint frequently used in oncology trials. Here the measurement for each patient equals the time elapsed from onset of the treatment of a patient in a trial (as defined in the protocol) until the occurrence of any fatality. If the observation of the patient was stopped (e.g. at study end) after a period t and the patient survived to this time, then this observation time t is called "censored".

The term "covariate" has the following meaning. The clinical endpoints are frequently considered in regression models, where the endpoint represent the dependent variable and the biomarkers represent the main or target independent variables (regressors). If additional variables from the clinical data pool are considered these are denoted as (clinical) covariates. The term "clinical covariate" here is used to describe all clicical information about the patient, which are in general available at baseline. These clinical covariates comprise demographic information like sex, age etc., other anamnestic information, concomitant diseases, concomitant therapies, result of physical examinations, common laboratory parameters obtained, known properties of the target tumor, information quantifying the extent of malignant disease, clinical performance scores like ECOG or Karnofsky index, clinical disease staging, timing and result of pretreatments and disease history as well as all similar information, which may be associated with the clinical prognosis.

The term "raw or unadjusted analysis" refers herein to regression analyses, where over the considered biomarkers no additional clinical covariates were used in the regression model, neither as independent factors nor as stratifying covariate.

The term "adjusted by covariates" refers to regression analyses, where over the considered biomarkers additional clinical covariates were used in the regression model, either as independent factors or as stratifying covariate.

The term "univariate" refers herein to regression models or graphical approaches where as independent variable only one of the target biomarkers is part of the model. These univariate models can be considered with and without additional clinical covariates.

The term "multivariate" refers herein to regression models or graphical approaches where as independent variables more than one of the target biomarkers are part of the model. These multivariate models can be considered with and without additional clinical covariates.

"Nucleotides" are "nucleosides" that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those "nucleosides" that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. A "nucleotide" is the "monomeric unit" of an "oligonucleotide", more generally denoted herein as an "oligomeric compound", or a "polynucleotide", more generally denoted as a "polymeric compound". Another general expression therefor is desoxyribonucleic acid (DNA) and ribonucleic acid (RNA). As used herein the term "polynucleotide" is synonymous with "nucleic acid."

The term „probe" refers to synthetically or biologically produced nucleic acids (DNA or RNA) which, by design or selection, contain specific nucleotide sequences that allow them to hybridize under defined predetermined stringencies specifically (i.e., preferentially) to "nucleic acids". A „probe" can be identified as a „capture probe" meaning that it "captures" the nucleic acid so that it can be separated from undesirable materials which might obscure its detection. Once separation is accomplished, detection of the captured "target nucleic acid" can be achieved using a suitable procedure. „Capture probes" are often already attached to a solid phase. According to the present invention, the term hybridization under "stringent conditions" is given the same meaning as in Sambrook et al. (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), paragraph 1.101-1.104). Preferably, a "stringent hybridization" is the case when a hybridization signal is still detectable after washing for 1 h with 1xSSC and 0.1% SDS at 50° C, preferably at 55° C, more preferably at 62° C, and most preferably at 68° C, and more preferably for 1 hour with 0.2xSSC and 0.1% SDS at 50° ., preferably at 55° C, more preferably at 62°, and most preferably at 68° C. The composition of the SSC buffer is described in Sambrook et al. (Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)).

A "transcribed polynucleotide" is a polynucleotide (e.g an RNA, a cDNA, or an analog of one of an RNA or cDNA) which is complementary to or homologous with all or a portion of a mature RNA made by transcription of a gene, such as the marker gene of the invention, and normal post-transcriptional processing (e.g.

splicing), if any, of the transcript. The term "cDNA" is an abbreviation for complementary DNA , the single-stranded or double-stranded DNA copy of a mRNA. The term "mRNA" is an abbreviation of mRNA the RNA that serves as a template for protein synthesis.

The term "marker gene" is meant to include a gene which is useful according to this invention for determining the progression of cancer in a patient, particularly in a breast cancer patient. It could be also termed cancer, preferably breast cancer, marker gene. The term "marker polynucleotide" is meant to include nucleotide transcript (hnRNA or mRNA) encoded by a marker gene according to the invention, or cDNA derived from the nucleotide transcript, or a segment of said transcript or cDNA.

The term "marker protein" or "marker polypeptide" is meant to include protein or polypeptide encoded by a marker gene according to the invention, or a polypeptide or protein fragment comprising said marker protein.

The term "gene product" is meant to include marker polynucleotide and marker protein encoded by the referenced gene.

The expression of a marker gene "significantly" differs from the level of expression of the marker gene in a reference sample if the level of expression of the marker gene in a sample from the patient differs from the level in a sample from the reference subject by an amount greater than the standard error of the assay employed to assess expression, and preferably at least 10 %, and more preferably 25%, 50 %, 75 %, 100 %, 125 %, 150 %, 175 %, 200 %, 300 %, 400 %, 500 % or 1,000 % of that amount. Alternatively, expression of the marker gene in the patient can be considered "significantly" lower than the level of expression in a control subject if the level of expression in a sample from the patient is lower than the level in a sample from the control subject by an amount greater than the standard error of the assay employed to assess expression, and preferably at least 10%, and more preferably 25%, 50 %, 75 %, 100 %, 125 %, 150 %, 175 %, 200 %, 300 %, 400 %, 500 % or 1,000 % that amount.

A marker polynucleotide or a marker protein "corresponds to" another marker polynucleotide or marker protein if it is related therero, particularly preferred it is identical thereto.

The terms "level of expression" and "expression level" are used interchangeably and generally refer to the amount of a polynucleotide or an amino acid product or protein in the sample. "Expression" generally refers to the process by which a gene coded information is converted into the structures present and operating in the cell, therefore according to the invention the term "expression" of a (marker) gene includes transcription into a polynucleotide, translation into a protein and even posttranslational modification of the protein. Fragments of the transcribed polynucleotide, the translated protein or the postranslationally modified protein shall also be regarded as expressed whether they originate e.g. from a transcript generated by alternative splicing, a degraded transcript or from a posttranslational processing of the protein, e.g. by proteolysis. As used herein, "expressed genes" include those that are transcribed into a polynucleotide as mRNA and then translated into a protein. 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 "amphiregulin" relates to a gene that encodes a protein and to the protein itself that is a member of the epidermal growth factor family. It is an autocrine growth factor as well as a mitogen for astrocytes, Schwann cells, and fibroblasts. It is related to epidermal growth factor (EGF) and transforming growth factor alpha (TGF-alpha). This protein interacts with the EGF/TGF-alpha receptor to promote the growth of normal epithelial cells and inhibits the growth of certain aggressive carcinoma cell lines. According to the invention, the amino acid sequence of amphiregulin is the amino acid sequence according to SEQ ID NO: 1. According to the invention, the nucleic acid sequence of the "amphiregulin" cDNA is the nucleic acid sequence according to SEQ ID NO: 5 which is accessible at GenBank with the accession number NM_001657.

The term "transforming growth factor alpha" relates to a gene that encodes a protein and to the protein itself that is a member of the family of transforming growth factors (TGFs). Those are biologically active polypeptides that reversibly confer the transformed phenotype on cultured cells. "Transforming growth factor-alpha" shows about 40% sequence homology with epidermal growth factor and competes with EGF for binding to the EGF receptor, stimulating its

phosphorylation and producing a mitogenic response. According to the invention, the amino acid sequence of "Transforming growth factor-alpha" is the amino acid sequence according to SEQ ID NO: 3. According to the invention, the nucleic acid sequence of the "transforming growth factor-alpha" cDNA is the nucleic acid sequence according to SEQ ID NO: 7 which is accessible at GenBank with the accession number NM_003236.

The term "epidermal growth factor" relates to a gene that encodes a protein and to the protein itself that is a member of the family of growth factors. "Epidermal growth factor (EGF)" has a profound effect on the differentiation of specific cells in vivo and is a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin. The EGF precursor is believed to exist as a membrane-bound molecule which is proteolytically cleaved to generate the 53-amino acid peptide hormone that stimulates cells to divide. According to the invention, the amino acid sequence of "Epidermal growth factor" is the amino acid sequence according to SEQ ID NO: 2. According to the invention, the nucleic acid sequence of the "Epidermal growth factor (EGF)" cDNA is the nucleic acid sequence according to SEQ ID NO: 6 which is accessible at GenBank with the accession number NM_001963. The "Epidermal Growth Factor Receptor" abbreviated as 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. The mRNA has different variants translated into different receptor proteins. According to the invention, the amino acid sequence of the "Epidermal growth factor receptor" is the amino acid sequence according to SEQ ID NO: 11 (transcript variant 1; GenBank accession number NM_005228), SEQ ID NO: 12 (transcript variant 2; GenBank accession number NM_201282), SEQ ID NO: 13 (transcript variant 3; GenBank accession number NM_201283), or SEQ ID NO: 14 (transcript variant 4; GenBank accession number NM_201284). 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. 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.

The terms "human neu", "c-erbB-2", "erbB2", "erbB-2", "HER-2/neu", "Her2" and "HER-2" are used interchangeably herein. The term "Her2" relates to a gene that encodes a protein and to the protein itself that is a member of the family of the epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. This protein has no ligand binding domain of its own and therefore cannot bind growth factors. However, it does bind tightly to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and enhancing kinase-mediated activation of downstream signalling pathways, such as those involving mitogen-activated protein kinase and phosphatidylinositol-3 kinase. Allelic variations at amino acid positions 654 and 655 of isoform a (positions 624 and 625 of isoform b) have been reported, with the most common allele, Ile654/Ile655 being preferred according to the invention. Amplification and/or overexpression of this gene has been reported in numerous cancers, including breast and ovarian tumors. Alternative splicing results in several additional transcript variants, some encoding different isoforms and others that have not been fully characterized. According to the invention, the amino acid sequence of Her2 is the amino acid sequence according to SEQ ID NO: 4. According to the invention, the nucleic acid sequence of the "Her2" cDNA is the nucleic acid sequence according to SEQ ID NO: 8 which is accessible at GenBank with the accession number NM_004448.2.

The "extracellular domain of Her2" or "shed extracellular domain of Her2" is a glycoprotein of between 97 and 115 kDa which corresponds substantially to the extracellular domain of the human Her2 gene product. It can be referred to as p105 (Zabrecky, J.R. et al., J. Biol. Chem. 266 (1991) 1716-1720; US 5,401,638; US 5,604,107). The quantitation and detection of the extracellular domain of Her2 is described in US 5,401,638 and US 5,604,107.

The term "Her3" stands for another member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. This membrane-bound protein has not an active kinase domain. The protein can bind ligands but not transmit a signal into the cell. It forms heterodimers with other EGF receptor family members which do have kinase activity which leads to cell proliferation or differentiation. Amplification of this gene and/or overexpression of its protein is found in numerous cancers. According to the invention, the amino acid sequence of the "Her3" cDNA is the amino acid sequence according to SEQ ID NO: 9 which

is accessible at GenBank from the translation of the nucleic acid sequence of Her3 with the accession number NM_001005915.According to the invention, the nucleic acid sequence of the "Her3" cDNA is the nucleic acid sequence according to SEQ ID NO: 10 which is accessible at GenBank with the accession number NM_001005915.

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 construed 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) 495-497, or may be made by recombinant DNA methods (see, e.g., US 4,816,567). "Antibody fragments" comprise a portion of an intact antibody.

An antibody "which binds" an antigen of interest according to the invention 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 human Her2 and does not (significantly) cross-react with other proteins. In such embodiments, the extent of binding of the antibody to other proteins will be less than 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

Dimerization - the pairing of receptors - is essential to the signaling activity of all HER receptors. According to the invention, the term "Her dimerisation inhibitor" or preferably "Her2 heterodimerisation inhibitor" refers to a therapeutic agent that binds to Her2 and inhibits Her2 heterodimerisation. These are preferably antibodies, preferably monoclonal antibodies, more preferably humanized antibodies that bind to Her2 and inhibit Her2 heterodimerisation. Examples of antibodies that bind HER2 include 4D5, 7C2, 7F3 or 2C4 as well as humanized variants thereof, including huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 as described in Table 3 of U.S. Pat. No. 5,821,337; and humanized 2C4 mutant numbers 560, 561, 562, 568, 569, 570, 571, 574, or 56869 as described in WO 01/00245. 7C2 and 7F3 and humanized variants thereof are described in WO 98/17797. This term shall not apply to monoclonal antibodies as tratuzumab sold under the name Herceptin™ as the mechanism of action is different and as this antibody does not inhibit Her dimerisation.

Preferred throughout the application is the “antibody 2C4”, in particular the humanized variant thereof (WO 01/00245; produced by the hybridoma cell line deposited with the American Type Culture Collection, Manassass, VA, USA under ATCC HB-12697), which binds to a region in the extracellular domain of Her2 (e.g., any one or more residues in the region from about residue 22 to about residue 584 of Her2, inclusive). The "epitope 2C4" is the region in the extracellular domain of ErbB2 to which the antibody 2C4 binds. The expression "monoclonal antibody 2C4" refers to an antibody that has antigen binding residues of, or derived from, the murine 2C4 antibody of the Examples in WO 01/00245. For example, the monoclonal antibody 2C4 may be murine monoclonal antibody 2C4 or a variant thereof, such as humanized antibody 2C4, possessing antigen binding amino acid residues of murine monoclonal antibody 2C4. Examples of humanized 2C4 antibodies are provided in Example 3 of WO 01/00245. Unless indicated otherwise, the expression "rhuMAb 2C4" when used herein refers to an antibody comprising the variable light (VL) and variable heavy (VH) sequences of SEQ ID Nos. 3 and 4 of WO 01/00245, respectively, fused to human light and heavy IgG1 (non-A allotype) constant region sequences optionally expressed by a Chinese Hamster Ovary (CHO) cell. Preferred embodiments of WO 01/00245 are preferred herein as well. The humanized antibody 2C4 is also called pertuzumab (Omnitarg™).

A “kit” is any manufacture (e.g a package or container) comprising at least one reagent, e.g a probe, for specifically detecting a marker gene or protein of the

invention. The manufacture is preferably promoted, distributed, or sold as a unit for performing the methods of the present invention.

The verbs "determine" and "assess" shall have the same meaning and are used interchangeably throughout the application.

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 Limited, 1984; Hames, B.D. and Higgins, S.J. (eds.), Nucleic acid hybridisation - a practical approach, IRL Press Limited, 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 of predicting the response to a treatment with a HER dimerization inhibitor in a patient comprising the steps of

a) assessing in a biological sample from the patient
   - - a marker gene or a combination of marker genes selected from the group consisting of an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene or a
   - - combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene, and
b) predicting the response to the treatment with the HER dimerization inhibitor in the patient by evaluating the results of step a).

Preferably, in the method according to the invention, the step a) of assessing the marker gene or the combination of marker genes comprises

a1) assessing the level of expression of the marker gene or the combination of marker genes,
a2) determining whether the level of expression assessed in step a1) is above or below a threshold value.

The threshold value is preferably a value expressed in mass/ volume for blood serum or blood plasma or mass/ mass for tumor tissue. It can be measured by methods known to the expert skilled in the art and also disclosed by this invention.

In a preferred embodiment of the invention, if the threshold value is above or below the threshold value, the response to the treatment can be determined. With regard to the use of a single marker gene according to the invention, the situation is as follows for metastatic breast cancer patients. This may however depend on the indication but can be determined based upon the disclosure of this invention. With regard to the transforming growth factor alpha marker gene alone, low expression levels are favorable for progression free survival (time to death or progression) and overall survival (time to death) when treatment with a HER dimerization inhibitor is considered for Her2 low expressing metastatic breast cancer patient, i.e. a low expression level of transforming growth factor alpha marker gene predicts a good response in these patients to a treatment with a HER dimerization inhibitor (see Fig. 7). This is the case both for raw and adjusted for clinical covariates. Therefore, it is preferred that the level of expression assessed in step a1) of the transforming growth factor alpha marker gene is below a threshold value for predicting a good response to a treatment with a HER dimerization inhibitor in Her2 low expressing metastatic breast cancer patients. This is also the case for the Her2 marker gene alone in these patients, particularly for the soluble Her2 extracellular domain, and the epidermal growth factor marker gene alone in these patients.

With regard to the amphiregulin marker gene alone, in a raw analysis low expression levels are favorable for progression free survival (time to death or progression) and overall survival (time to death) when treatment with a HER dimerization inhibitor is considered inHer2 low expressing metastatic breast cancer patients, i.e. a low expression level of the amphiregulin marker gene predicts a good response to a treatment with a HER dimerization inhibitor in these patients. This is the case both for raw and adjusted for clinical covariates. Analyses adjusted for clinical covariates indicate that high expression levels are favorable for progression free survival (time to death or progression) and overall survival (time to death) after treatment with a HER dimerization inhibitor in these patients.

Since the marker genes, in particular in serum, may be used in multiple-marker prediction models potentially including other clinical covariates, the direction of a beneficial effect of a single marker gene within such models cannot be determined

in a simple way, and may contradict the direction found in univariate analyses, i.e. the situation as described for the use of a single marker gene.

More preferably, in the the method according to the invention, the threshold value is determined before step a1) of the method of the invention by

1) assessing the level of expression of the marker gene or the combination of marker genes in a plurality of biological samples from patients before treatment with the HER dimerization inhibitor,
2) treating the patients with the HER dimerization inhibitor,
3) correlating the response of the patients treated with the HER dimerization inhibitor to the level of expression of the marker gene or the combination of marker genes determined in step a) thereby determining the threshold value.

The threshold value is preferably a value expressed in mass/ volume for blood serum or blood plasma or mass/ mass for tumor tissue.

The present invention also considers mutants or variants of the marker genes according to the present invention and used in the methods according to the invention. In those mutants or variants the native sequence of the marker gene is changed by substitutions, deletions or insertions. "Native sequence" refers to an amino acid or nucleic acid sequence which is identical to a wild-type or native form of a marker gene or protein.

The present invention also considers mutants or variants of the proteins according to the present invention and used in the methods according to the invention. "Mutant amino acid sequence," "mutant protein" or "mutant polypeptide" refers to a polypeptide having an amino acid sequence which varies from a native sequence or is encoded by a nucleotide sequence intentionally made variant from a native sequence. "Mutant protein," "variant protein" or "mutein" means a protein comprising a mutant amino acid sequence and includes polypeptides which differ from the amino acid sequence of the native protein according to the invention due to amino acid deletions, substitutions, or both.

The present invention also considers a method of predicting the response to a treatment with a combination of a HER dimerization inhibitor and another substance or agent as a chemotherapeutic agent or a therapeutic antibody used for treating cancer. The chemotherapeutic agent may be e.g. gemcitabine (Gemzar®; chemical name: 2',2'-difluorodeoxycytidine (dFdC)), carboplatin (diammine-

(cyclobutane-1,1-dicarboxylato(2-)-O,O')-platinum), or paclitaxel (Taxol®, chemical name: β-(benzoylamino)-α-hydroxy-,6,12b-bis(acetyloxy)-12-(benzoyloxy)-2a,3,4,4a,5,6,9,10,11,12,12a,12b-dodecahydro-4,11-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-7,11-methano-1H-cyclodeca(3,4)benz(1,2-b)oxet-9-yl ester,(2aR-(2a-α,4-β,4a-β,6-β, 9-α(α-R*,β-S*),11-α,12-α,12a-α, 2b-α))-benzenepropanoic acid).

In a preferred embodiment of the invention, the biological sample is blood serum, blood plasma or tumor tissue. Tumor tissue may be formalin-fixed paraffin embedded tumor tissue or fresh frozen tumor tissue.

In another preferred embodiment of the invention, the HER dimerisation inhibitor inhibits heterodimerisation of HER2 with EGFR or HER3 or Her4. Preferably, the HER dimerisation inhibitor is an antibody, preferably the antibody 2C4. Preferred throughout the application is the "antibody 2C4", in particular the humanized variant thereof (WO 01/00245; produced by the hybridoma cell line deposited with the American Type Culture Collection, Manassass, VA, USA under ATCC HB-12697), which binds to a region in the extracellular domain of Her2 (e.g. , any one or more residues in the region from about residue 22 to about residue 584 of Her2, inclusive). Examples of humanized 2C4 antibodies are provided in Example 3 of WO 01/00245. The humanized antibody 2C4 is also called Omnitarg™ or pertuzumab.

In still another preferred embodiment of the invention, the patient is a cancer patient, preferably a breast cancer, ovarian cancer, lung cancer or prostate cancer patient. The breast cancer patient is preferably a metastatic breast cancer patient or a HER2 low expressing breast or metastatic breast cancer patient, or a Her2 high expressing breast or metastatic breast cancer patient. The ovarian cancer patient is preferably a metastatic ovarian cancer patient. The lung cancer patient is preferably a non-small cell lung cancer (NSCLC) patient

It is preferred that two, three or all four marker genes, marker polynucleotides or marker proteins are used in combination, i.e. used in all disclosed embodiments of the invention or methods, uses or kits according to the invention. Preferably, the level of expression of

- a combination of the the epidermal growth factor, the transforming growth factor alpha or the HER2 marker gene, marker protein or marker polynucleotide,

- a combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene,
- a combination of the epidermal growth factor and the transforming growth factor alpha or the HER2 marker gene, marker protein or marker polynucleotide, or
- a combination of the transforming growth factor alpha and the HER2 marker gene, protein or polynucleotide is assessed.

In a particularly preferred embodiment of the invention, the combination of marker genes consists of:

- the transforming growth factor alpha and the HER2 marker gene,
- the transforming growth factor alpha and the epidermal growth factor marker gene, or
- the amphiregulin, the epidermal growth factor, the transforming growth factor alpha and the HER2 marker gene.

In another particularly preferred embodiment of the invention, the combination of the marker genes consists of:

- the epidermal growth factor and the HER2 marker gene,
- the amphiregulin and the epidermal growth factor marker gene,
- the amphiregulin and the transforming growth factor alpha marker gene,
- the amphiregulin and the HER2 marker gene,
- the amphiregulin, the epidermal growth factor and the transforming growth factor alpha marker gene,
- the amphiregulin, the epidermal growth factor and HER2 marker gene,
- the amphiregulin, the transforming growth factor alpha and the HER2 marker gene, or
- the epidermal growth factor, the transforming growth factor alpha and the Her 2 marker gene.

In a preferred embodiment of the invention, the level of expression of the marker gene or the combination of marker genes in the sample is assessed by detecting the level of expression of a marker protein or a fragment thereof or a combination of marker proteins or fragments thereof encoded by the marker gene or the combination of marker genes. Preferably, the level of expression of the marker

protein or the fragment thereof or the combination of marker proteins or the fragments thereof is detected using a reagent which specifically binds with the marker protein or the fragment thereof or the combination of marker proteins or the fragments thereof. Preferably, the reagent is selected from the group consisting of an antibody, a fragment of an antibody or of an antibody derivative.

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 level of expression is determined using a method selected from the group consisting of proteomics, flow cytometry, immunocytochemistry, immunohistochemistry, enzyme-linked immunosorbent assay, multi-channel enzyme-linked immunosorbent assay, and variations of these methods. Therefore more preferably, the level of expression is determined using a method selected from the group consisting of proteomics, flow cytometry, immunocytochemistry, immunohistochemistry, enzyme-linked immunosorbent assay, multi-channel enzyme-linked immunosorbent assay, and variations of these methods.

In another preferred embodiment of the invention, the fragment of the marker protein is the extracellular domain of the Her2 marker protein. Preferably, the extracellular domain of the Her2 marker protein has a molecular mass of approximately 105.000 Dalton. "Dalton" stands for a mass unit that is equal to the weight of a hydrogen atom, or 1.657×10^{-24} grams.

In another preferred embodiment of the invention

- the amino acid sequence of the amphiregulin marker protein is the amino acid sequence SEQ ID NO: 1,
- the amino acid sequence of the epidermal growth factor marker protein is the amino acid sequence SEQ ID NO: 2,
- the amino acid sequence of the transforming growth factor alpha marker protein is the amino acid sequence SEQ ID NO: 3, or
- the amino acid sequence of the HER2 marker protein is the amino acid sequence SEQ ID NO: 4.

In another preferred embodiment of the invention, the threshold value in blood serum for

- the transforming growth factor alpha marker protein is between 2.0 and 10.0, preferably between 2.0 and 5.0 pg/ ml, more preferably about 3.5 pg/ ml,
- the epidermal growth factor marker protein is between 100 to 250 pg/ ml, preferably about 150 pg/ ml, or
- the amphiregulin marker protein is between 6 to 15 pg/ ml, preferably about 12 pg/ ml.

In still another preferred embodiment of the invention, the threshold value in blood serum for the extracellular domain of the Her2 marker protein is between 12 to 22 ng/ml, preferably about 18 ng/ml.

In yet another preferred embodiment of the invention, the level of expression of the marker gene or the combination of marker genes in the biological sample is assessed by detecting the level of expression of a transcribed marker polynucleotide encoded by the marker gene or a fragment of the transcribed marker polynucleotide or of transcribed marker polynucleotides encoded by the combination of marker genes or fragments of the transcribed marker polynucleotide. Preferably, the transcribed marker polynucleotide is a cDNA, mRNA or hnRNA or wherein the transcribed marker polynucleotides are cDNA, mRNA or hnRNA.

Preferably, the step of detecting further comprises amplifying the transcribed polynucleotide. The amplification is performed preferably with the polymerase chain reaction which specifically amplifies nucleic acids to detectable amounts. Other possible amplification reactions are the Ligase Chain Reaction (LCR; Wu D. Y. and Wallace R.B., Genomics 4 (1989) 560-569; and Barany F., Proc. Natl. Acad. Sci. USA 88 (1991)189-193); Polymerase Ligase Chain Reaction (Barany F., PCR Methods and Applic. 1 (1991) 5-16); Gap-LCR (WO 90/01069); Repair Chain Reaction (EP 0439182 A2), 3SR (Kwoh, D.Y. et al., Proc. Natl. Acad. Sci. USA 86 (1989) 1173-1177; Guatelli, J.C. et al., Proc. Natl. Acad. Sci. USA 87 (1990) 1874-1878; WO 92/08808), and NASBA (US 5,130,238). Further, there are strand displacement amplification (SDA), transciption mediated amplification (TMA), and Qβ-amplification (for a review see e.g. Whelen, A.C. and Persing, D.H., Annu. Rev. Microbiol. 50 (1996) 349-373; Abramson, R.D. and Myers T.W., Curr. Opin. Biotechnol. 4 (1993) 41-47). More preferably, the step of detecting is using the method of quantitative reverse transcriptase polymerase chain reaction.

Other suitable polynucleotide detection methods are known to the expert in the field and are described in standard textbooks as Sambrook J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989; and Ausubel, F. et al., Current Protocols in Molecular Biology, 1987, J. Wiley and Sons, NY. There may be also further purification steps before the polynucleotide detection step is carried out as e.g. a precipitation step. The detection methods may include but are not limited to the binding or intercalating of specific dyes as ethidiumbromide which intercalates into the double-stranded polynucleotides and changes their fluorescence thereafter. The purified polynucleotide may also be separated by electrophoretic methods optionally after a restriction digest and visualized thereafter. There are also probe-based assays which exploit the oligonucleotide hybridisation to specific sequences and subsequent detection of the hybrid. It is also possible to sequence the DNA after further steps known to the expert in the field. The preferred template-dependent DNA polymerase is Taq polymerase.

In yet another preferred embodiment of the invention, the level of expression of the marker gene is assessed by detecting the presence of the transcribed marker polynucleotide or the fragment thereof in a sample with a probe which anneals with the transcribed marker polynucleotide or the fragment thereof under stringent hybridization conditions or the level of expression of the combination of the marker genes in the samples is assessed by detecting the presence of transcribed marker polynucleotides or the fragments thereof in a sample with probes which anneal with the transcribed marker polynucleotides or the fragments thereof under stringent hybridization conditions. This method may be performed in a homogeneous assay system. An example for a "homogeneous" assay system is the TaqMan® system that has been detailed in US 5,210,015, US 5,804,375 and US 5,487,972. Briefly, the method is based on a double-labelled probe and the 5'-3' exonuclease activity of Taq DNA polymerase. The probe is complementary to the target sequence to be amplified by the PCR process and is located between the two PCR primers during each polymerisation cycle step. The probe has two fluorescent labels attached to it. One is a reporter dye, such as 6-carboxyfluorescein (FAM), which has its emission spectra quenched by energy transfer due to the spatial proximity of a second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). In the course of each amplification cycle, the Taq DNA polymerase in the process of elongating a primed DNA strand displaces and degrades the annealed

probe, the latter due to the intrinsic 5'-3' exonuclease activity of the polymerase. The mechanism also frees the reporter dye from the quenching activity of TAMRA. As a consequence, the fluorescent activity increases with an increase in cleavage of the probe, which is proportional to the amount of PCR product formed. Accordingly, amplified target sequence is measured detecting the intensity of released fluorescence label. Another example for "homogeneous" assay systems are provided by the formats used in the LightCycler® instrument (see e.g. US 6,174,670), some of them sometimes called "kissing probe" formats. Again, the principle is based on two interacting dyes which, however, are characterised in that the emission wavelength of a donor-dye excites an acceptor-dye by fluorescence resonance energy transfer. The COBAS® AmpliPrep instrument (Roche Diagnostics GmbH, D–68305 Mannheim, Germany) was recently introduced to expand automation by isolating target sequences using biotinylated sequence-specific capture probes along with streptavidin-coated magnetic particles (Jungkind, D., J. Clin. Virol. 20 (2001) 1-6; Stelzl, E. et al., J. Clin. Microbiol. 40 (2002) 1447-1450). It has lately been joined by an additional versatile tool, the Total Nucleic Acid Isolation (TNAI) Kit (Roche Diagnostics). This laboratory-use reagent allows the generic, not sequence-specific isolation of all nucleic acids from plasma and serum on the COBAS® AmpliPrep instrument based essentially on the method developed by Boom, R. et al., J. Clin. Microbiol. 28 (1990) 495-503.

In another preferred embodiment of the invention, the nucleic acid sequence of the amphiregulin marker polynucleotide is the nucleic acid sequence SEQ ID NO: 5,

- the nucleic acid sequence of the epidermal growth factor marker polynucleotide is the nucleic acid sequence SEQ ID NO: 6,
- the nucleic acid sequence of the transforming growth factor alpha marker polynucleotide is the nucleic acid sequence SEQ ID NO: 7, or
- the nucleic acid sequence of the HER2 marker polynucleotide is the nucleic acid sequence SEQ ID NO: 8.

In another embodiment of the invention, a probe that hybridizes with the epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the epidermal growth factor, transforming growth factor alpha or HER2 marker protein is used for predicting the response to treatment with a HER dimerization inhibitor in a patient or a probe that hybridizes with the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide

under stringent conditions or an antibody that binds to the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker protein is used for selecting a composition for inhibiting the progression of disease in a patient. The disease is preferably cancer and the patient is preferably a cancer patient as disclosed above.

In another embodiment of the invention, a kit comprising a probe that anneals with the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker protein is provided. 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. Preferably combinations of the probes or antibodies to the various marker polynucleotides or marker proteins are provided in the form of kit as the preferred combinations of the marker polynucleotides or marker proteins as disclosed above.

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

a) separately exposing aliquots of a biological sample from a cancer patient in the presence of a plurality of test compositions;

b) comparing the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene in the aliquots of the biological sample contacted with the test compositions and the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene 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 marker gene or the combination of marker genes in the aliquot containing that test composition relative to the aliquot not contacted with the test composition wherein a significant or an at least 10 % difference between the level of expression of the marker gene or the combination of marker genes in the aliquot of the biological sample contacted with the test composition and the level of expression of the corresponding marker gene or the combination of marker genes in the aliquot of the biological sample not contacted with the test composition is an indication for the selection of the test composition. The disease is preferably cancer and the patient is preferably a cancer patient as disclosed above.

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

a) separately exposing aliquots of a biological sample from a cancer patient in the presence of a plurality of test compositions;

b) comparing the level of expression of

- a marker gene or a combination of marker genes selected from the group consisting of the epidermal growth factor, transforming growth factor alpha and HER2 marker gene or of
- a combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene

in the aliquots of the biological sample contacted with the test compositions and the level of expression of a

- marker gene or a combination of marker genes selected from the group consisting of the epidermal growth factor, transforming growth factor alpha and HER2 marker gene or a
- combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene

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 marker gene or the combination of marker genes in the aliquot containing that test composition relative to the aliquot not contacted with the

test composition wherein a significant or an at least 10 % difference between the level of expression of the marker gene or the combination of marker genes in the aliquot of the biological sample contacted with the test composition and the level of expression of the corresponding marker gene or the combination of marker genes in the aliquot of the biological sample not contacted with the test composition is an indication for the selection of the test composition. The disease is preferably cancer and the patient is preferably a cancer patient as disclosed above.

The expression of a marker gene "significantly" differs from the level of expression of the marker gene in a reference sample if the level of expression of the marker gene in a sample from the patient differs from the level in a sample from the reference subject by an amount greater than the standard error of the assay employed to assess expression, and preferably at least 10 %, and more preferably 25%, 50 %, 75 %, 100 %, 125 %, 150 %, 175 %, 200 %, 300 %, 400 %, 500 % or 1,000 % of that amount. Alternatively, expression of the marker gene in the patient can be considered "significantly" lower than the level of expression in a reference subject if the level of expression in a sample from the patient is lower than the level in a sample from the reference subject by an amount greater than the standard error of the assay employed to assess expression, and preferably at least 10%, and more preferably 25%, 50 %, 75 %, 100 %, 125 %, 150 %, 175 %, 200 %, 300 %, 400 %, 500 % or 1,000 % that amount. The difference of the level of expression be up to 10,000 or 50,000 %. The difference of the level of expression 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 another embodiment of the invention, a method of deriving (or identifying) a candidate agent is provided said method comprising:

a) contacting an aliquot of a biological sample from a cancer patient with the candidate agent and determining the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene in the aliquot,

b) determining the level of expression of a corresponding marker gene or of a corresponding combination of marker genes 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 marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene or corresponding combination of marker genes in the aliquot of the biological sample not contacted with the candidate agent,

d) deriving (or identifying) said agent from said observed effect, wherein an at least 10 % difference between the level of expression of the marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene or combination of marker genes in the aliquot of the biological sample not contacted with the candidate agent is an indication of an effect of the candidate agent.

In still 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 from a cancer patient with the candidate agent and determining the level of expression in the aliquot of a
   - marker gene or a combination of marker genes selected from the group consisting of the epidermal growth factor, transforming growth factor alpha and HER2 marker gene or a
   - combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene,

b) determining the level of expression of a corresponding marker gene or of a corresponding combination of marker genes 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 marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene or corresponding combination of marker genes 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 marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene

or combination of marker genes 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 candidate agent is a candidate inhibitory agent. Preferably, said candidate agent is a candidate enhancing agent.

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

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

In yet another embodiment of the invention, an agent according to the invention is used for the preparation of a composition for the treatment of cancer. Preferred forms of cancer are disclosed above.

In another preferred embodiment of the invention, a method of producing a drug comprising the steps of the method according to 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, a marker protein or a marker polynucleotide selected from the group consisting of a amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker protein or marker polynucleotide is used for deriving a candidate agent or for selecting a composition for inhibiting the progression of a disease in a patient. The disease is preferably cancer and the patient is preferably a cancer patient as disclosed above.

In another embodiment of the invention, a HER dimerization inhibitor is used for the manufacture of a medicament for treating a human cancer patient characterized in that said treating or treatment includes assessing in a biological sample from the patient

- a marker gene or a combination of marker genes selected from the group consisting an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene or a
- combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene.

The manufacture of a medicament for treating a human cancer patient and particularly the formulation is described in WO 01/00245, incorporated herein by reference, particularly for the antibody 2C4.

In an preferred embodiment of the invention, in the use of the Her dimerization inhibitor for the manufacture of a medicament for treating a human cancer patient, the treatment includes assessing the marker gene or the combination of marker genes at least one time or repeatedly during treatment. Preferably, the level of expression of the marker gene or the level of expression of the combination of marker genes is assessed. Preferably, the HER dimerization inhibitor is an antibody, preferably the antibody 2C4. Preferably, the patient is a breast cancer, ovarian cancer, lung cancer or prostate cancer patient.

In all embodiments of the invention, combinations of the marker genes, marker polynucleotides or marker proteins are used as disclosed above. In all embodiments of the invention, preferred values for the difference of the level of expression determined in the respective steps are also as disclosed above.

The following examples, 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

Figure 1: Scatterplot TGF-alpha logarithmic transformation versus categorized clinical benefit

Figure 2: Scatterplot Amphiregulin logarithmic transformation versus categorized clinical benefit

Figure 3: Ordinal clinical benefit TGF-alpha

Figure 4: Ordinal clinical benefit Amphiregulin

Figure 5: Ordinal clinical benefit EGF

Figure 6: Ordinal clinical benefit HER2-ECD

Figure 7: Overview exploratory cut-points and log-rank p-values for TTP and TTD for Amphiregulin, EGF, TGF-alpha, HER2-ECD

Figure 8: TGF-alpha Kaplan Meier plot for time to progression/death based on exploratory single marker cut-point

Figure 9: TGF-alpha Kaplan Meier plot for time to death based on exploratory single marker cut-point

Figure 10: Amphiregulin Kaplan Meier plot for time to progression/death based on exploratory single marker cut-point

Figure 11: Amphiregulin Kaplan Meier plot for time to death based on exploratory single marker cut-point

Figure 12: EGF Kaplan Meier plot for time to progression/death based on exploratory single marker cut-point

Figure 13: EGF Kaplan Meier plot for time to death based on exploratory single marker cut-point

Figure 14: HER2-ECD Kaplan Meier plot for time to progression/death based on exploratory single marker cut-point

Figure 15: HER2-ECD Kaplan Meier plot for time to death based on exploratory single marker cut-point

Figure 16: As example for a combination score, further improving the separation between the greater clinical benefit/lesser clinical benefit groups in TTP: Ordinal clinical benefit HER2-ECD TGF alpha combination

Figure 17: Overview exploratory cut-points and log-rank p-values for TTP and TTD for a combination of TGF-alpha and HER2-ECD

Figure 18: HER2-ECD/TGF-alpha Kaplan Meier plot for time to progression/death based on exploratory combination marker cut-point

Figure 19: HER2-ECD/TGF-alpha Kaplan Meier plot for time to death based on exploratory combination marker cut-point

Examples

General remarks to prediction rules:

The general form of a prediction rule consists in the specification of a function of one or multiple biomarkers potentially including clinical covariates to predict response or non-response, or more generally, predict benefit or lack of benefit in terms of suitably defined clinical endpoints.

The simplest form of a prediction rule consists of an univariate model without covariates, where the prediction is determined by means of a cutoff or threshold. This can be phrased in terms of the Heaviside function for a specific cutoff c and a biomarker measurement x, where the binary prediction A or B is to be made, then

$$\text{If } H(x-c)=0 \text{ then predict A.}$$
$$\text{If } H(x-c)=1 \text{ then predict B.}$$

This is the simplest way of using univariate biomarker measurements in prediction rules. If such a simple rule is sufficient, it allows for a simple identification of the direction of the effect, i.e. whether high or low expression levels are beneficial for the patient.

The situation can be more complicated if clinical covariates need to be considered and/or if multiple biomarkers are used in multivariate prediction rules. In order to illustrate the issues here are two hypothetical examples:

Covariate adjustment (hypothetical example):
For a biomarker X it is found in a clinical trial population that high expression levels are associated with a worse prognosis (univariate analysis). A closer analysis shows that there are two tumor types in the population, one of which possess a worse prognosis than the other one and at the same time the biomarker expression for this tumor group is generally higher. A adjusted covariate analysis reveals that for each of the tumor types the relation of clinical benefit and prognosis is reversed, i.e. within the tumor types, lower expression levels are associated with better prognosis. The overall opposite effect was masked by the covariate tumor type – and the covariate adjusted analysis as part of the prediction rule reversed the direction.

Multivariate prediction (hypothetical example):

For a biomarker X it is found in a clinical trial population that high expression levels are slightly associated with a worse prognosis (univariate analysis). For a second biomarker Y a similar observation was made by univariate analysis. The combination of X and Y revealed that a good prognosis is seen if both biomarkers are low. This makes the rule to predict benefit if both biomarkers are below some cutoffs (AND- connection of a Heaviside prediction function). For the combination rule there is no longer a simple rule phraseable in an univariate sense. E.g. having low expression levels in X will not automatically predict a better prognosis.

These simple examples show that prediction rules with and without covariates cannot be judged on the univariate level of each biomarker. The combination of multiple biomarkers plus a potential adjustment by covariates does not allow to assign simple relationships towards single biomarkers.

Statistical methods

The statistical tasks comprise the following steps:

1. Pre-selection of candidate biomarkers
2. Pre-selection of relevant clinical prognostic covariates
3. Selection of biomarker prediction functions at an univariate level
4. Selection of biomarker prediction functions including clinical covariates at an univariate level
5. Selection of biomarker prediction functions at a multivariate level
6. Selection of biomarker prediction functions including clinical covariates at a multivariate level

The following text details the different steps:

Ad1: Pre-selection of candidate biomarkers: The statistical pre-selection of candidate biomarkers is oriented towards the strength of association with measures of clinical benefit. For this purpose the different clinical endpoints may be transformed in derived surrogate scores, as e.g. an ordinal assignment of the degree of clinical benefit or morbidity scores regarding TTP or TTD which avoid censored observations. These surrogate transformed measures can be easily used for simple correlation analysis, e.g. by the non-parametric Spearman rank correlation approach. An alternative here is to use the biomarker measurements as metric

covariates in Time-to-event regression models, as e.g. Cox proportional hazard regression. Depending on the statistical distribution of the biomarker values this step may require some pre-processing, as e.g. variance stabilizing transformations and the use of suitable scales or, alternatively, a standardization step like e.g. using percentiles instead of raw measurements. A further approach is inspection of bivariate scatter plots, e.g. by displaying the scatter of (x-axis=biomarker value, y-axis=measure of clinical benefit) on a single patient basis. Here also some non-parametric regression line as e.g. achieved by smoothing splines can be useful to visualize the association of biomarker and clinical benefit.

The goal of these different approaches is the pre-selection of biomarker candidates, which show some association with clinical benefit in at least one of the benefit measures employed, while results for other measures are not contradictory. When there are available control groups, then differences in association of biomarkers with clinical benefit in the different arms could be a sign of differential prediction which makes the biomarker eligible for further consideration.

Ad2: Pre-selection of relevant clinical prognostic covariates: The term "clinical covariate" here is used to describe all other information about the patient, which are in general available at baseline. These clinical covariates comprise demographic information like sex, age etc., other anamnestic information, concomitant diseases, concomitant therapies, result of physical examinations, common laboratory parameters obtained, known properties of the target tumor, information quantifying the extent of malignant disease, clinical performance scores like ECOG or Karnofsky index, clinical disease staging, timing and result of pretreatments and disease history as well as all similar information, which may be associated with the clinical prognosis. The statistical pre-selection of clinical covariates parallels the approaches for pre-selecting biomarkers and is as well oriented towards the strength of association with measures of clinical benefit. So in principle the same methods apply as considered under 1. In addition to statistical criteria, also criteria from clinical experience and theoretical knowledge may apply to pre-select relevant clinical covariates.

The prognosis by clinical covariates could interact with the prognosis of the biomarkers. They will be considered for refined prediction rules if necessary.

Ad3: Selection of biomarker prediction functions at an univariate level: The term "prediction function" will be used in a general sense to mean a numerical function

of a biomarker measurement which results in a number which is scaled to imply the target prediction.

A simple example is the choice of the Heaviside function for a specific cutoff c and a biomarker measurement x, where the binary prediction A or B is to be made, then

If H(x-c)=0 then predict A.
If H(x-c)=1 then predict B.

This is probably the most common way of using univariate biomarker measurements in prediction rules. The definition of a prediction function usually recurs to an existing training data set which can be used to explore the prediction possibilities. In order to achieve a suitable cutoff c from the training set different routes can be taken. First the scatterplot with smoothing spline mentioned under 1 can be used to define the cutoff. Alternatively some percentile of the distribution could be chosen, e.g. the median or a quartile. Cutoffs can also be systematically extracted by investigating all possible cutoffs according to their prediction potential with regard to the measures of clinical benefit. Then these results can be plotted to allow for an either manual selection or to employ some search algorithm for optimality. This was realized based on the endpoints TTP and TTD using a Cox model, where at each test cutoff the biomarker was used as a binary covariate. Prediction criteria were the resulting Hazard ratios. Then the results for TTP and TTD can be considered together in order to chose a cutoff which shows prediction in line with both endpoints

Another uncommon approach for choosing a prediction function can be based on a fixed parameter Cox regression model obtained from the training set with biomarker values (possibly transformed) as covariate. Then the prediction could simply depend on whether the computed Hazard ratio is smaller or greater than 1.

A further possibility is to base the decision on some likelihood ratio (or monotonic transform of it), where the target probability densities were pre-determined in the training set for separation of the prediction states. Then the biomarker would be plugged into some function of the density ratios.

Ad4: Selection of biomarker prediction functions including clinical covariates at an univariate level: Univariate here refers to using only one biomarker – with regard to clinical covariates this can be a multivariate model. This approach parallels the search without clinical covariates, only that the methods should allow

for incorporating the relevant covariate information. The scatterplot method of choosing a cutoff allows only a limited use of covariates, e.g. a binary covariate could be color coded within the plot. If the analysis relies on some regression approach then the use of covariates (also many of them at a time) is usually facilitated. The cutoff search based on the Cox model described under 3, allows for an easy incorporation of covariates and thereby leads to a covariate adjusted univariate cutoff search. The adjustment by covariates may be done as covariates in the model or via the inclusion in a stratified analysis.

Also the other choices of prediction functions allow for the incorporation of covariates.

This is straightforward for the Cox model choice as prediction function. There is the option to estimate the influence of covariates on an interaction level, which means that e.g. for different age groups different Hazard ratios apply.

For the likelihood ratio type of prediction functions, the prediction densities must be estimated including covariates. Here the methodology of multivariate pattern recognition can be used or the biomarker values can be adjusted by multiple regression on the covariates (prior to density estimation).

The CART technology (Classification And Regression Trees; Breiman L., Friedman J.H., Olshen R.A., Stone C.J., Chapman & Hall (Wadsworth, Inc.), New York, 1984) can be used for a biomarker (raw measurement level) plus clinical covariates employing a clinical benefit measure as response. This way cutoffs are searched and a decision tree type of functions will be found involving the covariates for prediction. The cutoffs and algorithms chosen by CART are frequently close to optimal and may be combined and unified by considering different clinical benefit measures.

Ad5: Selection of biomarker prediction functions at a multivariate level: When there are several biomarker candidates which maintain their prediction potential within the different univariate prediction function choices, then a further improvement may be achieved by combinations of biomarkers, i.e. considering multivariate prediction functions.

Based on the simple Heaviside function model combinations of biomarkers may be evaluated, e.g. by considering bivariate scatterplots of biomarker values where optimal cutoffs are indicated. Then a combination of biomarkers can be achieved

by combining different Heaviside function by the logical AND and OR operators in order to achieve an improved prediction.

The CART technology (Classification And Regression Trees) can be used for multiple biomarkers (raw measurement level) and a clinical benefit measure as response, in order to achieve cutoffs for biomarkers and decision tree type of functions for prediction. The cutoffs and algorithms chosen by CART are frequently close to optimal and may be combined and unified by considering different clinical benefit measures.

The Cox-regression can be employed on different levels. A first way is to incorporate the multiple biomarkers in a binary way (i.e. based on Heaviside functions with some cutoffs). The other option is to employ biomarkers in a metric way (after suitable transformations), or a mixture of the binary and metric approach. The evolving multivariate prediction function is of the Cox type as described under 3.

The multivariate likelihood ratio approach is difficult to realize but presents as well as an option for multivariate prediction functions.

Ad6: Selection of biomarker prediction functions including clinical covariates at a multivariate level: When there are relevant clinical covariates then a further improvement may be achieved by combining multiple biomarkers with multiple clinical covariates. The different prediction function choices will be evaluated with respect to the possibilities to include clinical covariates.

Based on the simple logical combinations of Heaviside functions for the biomarkers, further covariates may be included to the prediction function based on logistic regression model obtained in the training set.

The CART technology and the evolving decision trees can be easily used with additional covariates, which would include these in the prediction algorithm.

All prediction functions based on the Cox-regression can use further clinical covariates. There is the option to estimate the influence of covariates on an interaction level, which means that e.g. for different age groups different Hazard ratios apply.

The multivariate likelihood ratio approach is not directly extendible to the use of additional covariates.

Example 1

Baseline blood sera from HER2 low expressing metastatic breast cancer patients treated with Pertuzumab were assessed for levels of HER ligands and shedded HER2 (HER2 ECD), as described below.

Kits used for assessment of the serum biomarkers:

Marker	Assay	Distribution
HER2-ECD	Bayer HER-2/neu ELISA, Cat.#: EL501	DakoCytomation N.V./S.A.,Interleuvenlaan 12B, B-3001 Heverlee
Amphiregulin	DuoSet ELISA Development System Human Amphiregulin, Cat. #: DY262	R&D Systems Ltd., 19 Barton Lane, Abingdon OX14 3NB, UK
EGF	Quantikine human EGF ELISA kit, Cat. #: DEG00	R&D Systems Ltd., 19 Barton Lane, Abingdon OX14 3NB, UK
TGF-alpha	Quantikine® Human TGF-alpha Immunoassay, Cat. #: DTGA00	R&D Systems Ltd., 19 Barton Lane, Abingdon OX14 3NB, UK

Protocols:

HER2-ECD:

HER2-ECD ELISA was performed according to the recommendations of the manufacturer.

Amphiregulin:

- Prepare all reagents (provided with the kit), standard dilutions (provided with the kit) and samples
- Provide EvenCoat Goat Anti-mouse IgG microplate strips (R&D, Cat. # CP002; not provided with the kit) in the frame. The frame is now termed ELISA plate.
- Determine of the required number of wells (number of standard dilutions + number of samples).
- Determine the plate layout.
- Add 100 µl diluted capture antibody (provided with the kit; 1:180 in PBS) to each well.
- Incubate at r.t. for 1 hour.

- Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with 400 µl Wash buffer (not provided with the kit; 0.05 % Tween- 20 in PBS was used), using a manifold dispenser, and subsequent aspiration. After the last wash, remove any remaining Wash buffer by aspirating. Invert the plate and blot it against clean paper towels.
- Add 100 µl standard dilution or diluted sample (see below) per well. Change tip after every pipetting step.
- Cover plate with the adhesive strip (provided with the kit).
- Incubate for 2 hours at r.t. on a rocking platform.
- Repeat the aspiration/wash as described previously.
- Aspirated samples and wash solutions are treated with laboratory disinfectant.
- Add 100 µl Detection Antibody (provided with the kit) diluted 1:180 in Reagent diluent (not provided with the kit; 1 % BSA (Roth; Albumin Fraction V, Cat. # T844.2) in PBS was used) per well
- Incubate for 2 hours at r.t.
- Repeat the aspiration/wash as described previously.
- Add 100 µl working dilution of the Streptavidin-HRP to each well (provided with the kit; 1:200 dilution in Reagent diluent). Cover with a new adhesive strip.
- Incubate for 20 min at r.t.
- Repeat the aspiration/wash as described previously.
- Add 100 µl Substrate Solution (R&D, Cat. # DY999; not provided with the kit) to each well.
- Incubate for 20 min at r.t. Protect from light.
- Add 50 µl Stop Solution (1.5 M H2SO4 (Schwefelsäure reinst, Merck, Cat. # 713); not provided with the kit) to each well. Mix carefully.
- Determine the optical density of each well immediately, using a microplate reader set to 450 nm.

Amphiregulin standard curve:

A 40 ng/ml amphiregulin stock solution was prepared in 1 % BSA in PBS, aliquotted and stored at -80°C. Amphiregulin solutions in 20 % BSA in PBS were not stable beyond 2 weeks and were therefore not used. From the aliquotted amphiregulin stock solution, the amphiregulin standard curve was prepared freshly in 20 % BSA in PBS prior to each experiment. The highest concentration was 1000

pg/ml (1:40 dilution of the amphiregulin stock solution). The standards provided with the ELISA kit produced a linear standard curve. Excel-based analysis of the curves allowed the determination of curve equations for every ELISA.

Amphiregulin samples:

When samples were diluted 1:1 in Reagent Diluent, all samples were within the linear range of the ELISA. Each sample was measured in duplicates. Dependent on the quality of the data, and on sufficient amounts of serum, determinations were repeated in subsequent experiments if necessary.

EGF:

- Prepare all reagents (provided with the kit), standard dilutions (provided with the kit) and samples
- Remove excess antibody-coated microtiter plate strips (provided with the kit) from the frame. The frame is now termed ELISA plate.
- Determine of the required number of wells: (Number of standard dilutions + number of samples) x 2
- Determine the plate layout.
- Add 50 µl Assay Diluent RD1 (provided with the kit) to each well
- Add 200 µl standard dilution or diluted sample (e.g. 1:20 in Calibrator Diluent RD6H) per well. Change tip after every pipetting step.
- Cover plate with the adhesive strip (provided with the kit).
- Incubate for 2 hours at r.t. on a rocking platform.
- Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with 400 µl Wash Buffer (provided with the kit), using a manifold dispenser, and subsequent aspiration. After the last wash, remove any remaining Wash buffer by aspirating. Invert the plate and blot it against clean paper towels.
- Aspirated samples and wash solutions are treated with laboratory disinfectant.
- Add 200 µl of Conjugate (provided with the kit) to each well. Cover with a new adhesive strip.
- Incubate for 2 hours at r.t.
- Repeat the aspiration/wash as described previously.
- Add 200 µl Substrate Solution (provided with the kit) to each well.
- Incubate for 20 min at r.t. Protect from light.
- Add 50 µl Stop Solution (provided with the kit) to each well. Mix carefully.

- Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

EGF standard curve:

The standards provided with the ELISA kit produced a linear standard curve. Also very small concentrations showed detectable results.

EGF samples:

A total of four assays with the samples was performed. Each sample was measured 2 - 5 times, the number of determinations being dependent on the quality of the results (mean +/- SD) and the availability of sufficient amounts of serum. When samples were diluted 1:20 in Calibrator Diluent RD6H, all samples were within the linear range of the ELISA.

TGF-alpha:

- Prepare all reagents (provided with the kit), standard dilutions (provided with the kit) and samples
- Remove excess antibody-coated microtiter plate strips (provided with the kit) from the frame. The frame is now termed ELISA plate.
- Determine of the required number of wells: (Number of standard dilutions + number of samples) x 2
- Determine the plate layout.
- Add 100 µl Assay Diluent RD1W (provided with the kit) to each well
- Add 50 µl standard dilution or sample per well. Change tip after every pipetting step.
- Cover plate with the adhesive strip (provided with the kit).
- Incubate for 2 hours at r.t. on a rocking platform.
- Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with 400 µl Wash Buffer (provided with the kit), using a manifold dispenser, and subsequent aspiration. After the last wash, remove any remaining Wash buffer by aspirating. Invert the plate and blot it against clean paper towels.
- Aspirated samples and wash solutions are treated with laboratory disinfectant.
- Add 200 µl of TGF-alpha Cojugate (provided with the kit) to each well. Cover with a new adhesive strip.
- Incubate for 2 hours at r.t.
- Repeat the aspiration/wash as described previously.

- Add 200 µl Substrate Solution (provided with the kit) to each well.
- Incubate for 30 min at r.t. Protect from light.
- Add 50 µl Stop Solution (provided with the kit) to each well. Mix carefully.
- Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

TGF-alpha standard curve:
The standards provided with the ELISA kit produced a linear standard curve. Also very small concentrations showed detectable results.

TGF-alpha samples:
A total of four assays with the samples was performed. Samples were measured in 2 – 4 independent assays.

The serum data was analyzed to identify factors the baseline serum levels of which would be associated with response to the Pertuzumab treatment. For all factors a skewed pattern of the distribution (mean, standard deviation, median, minimum, maximum) was observed. A monotonic transform was used to reduce the skewness based on the logarithm: Log(x+1). In a univariate analysis, it was explored whether suitable cut-points for the factors could be defined which would relate to the probability of response (in this example defined as clinical benefit). Here, patients with clinical benefit were defined as those who achieved a partial response (PR) or maintained stable disease for at least 6 months. Scatterplots of the factors versus the response categories were investigated. Figure 1 and Figure 2 show a plotting of the clinical response categories versus the logarithmic transformation of the serum levels of TGF-alpha and amphiregulin, respectively, to exemplify the approach.

Based on the scatterplots, cut-points were selected for the factors to define groups of patients, who have experienced greater clinical benefit. Figure 3 (TGF-alpha), Figure 4 (Amphiregulin), Figure 5 (EGF), and Figure 6 (HER2-ECD) show the clinical benefit in relation to the different factor groupings based on the exploratory cut-points calculated to the original factor units. The cut-points separate out some of the patients without clinical benefit, and hence, elevate the response rate for the group with greater clinical benefit.

Example 2

In this example the exploratory cut-points from Example 1 were used to assess the univariate effect of the factor groupings on different measures of the clinical benefit of the Pertuzumab treatment, using time to progression/or death (TTP) and time to

death (TTD) as alternative clinical endpoints. Significant effects were observed for TGF-alpha, Amphiregulin, EGF and HER2-ECD in Kaplan-Meier estimates and log-rank tests for TTP and/or TTD, as shown in an overview in Figure 7.

The Kaplan-Meier plots displaying the hazard ratio are given for TTP and TTD (highest number of events observed) in Figure 8 and Figure 9 (TGF-alpha), 10 and 11 (Amphiregulin), 12 and 13 (EGF), and 14 and 15 (HER2-ECD), showing the pronounced effect of a grouping based on these factors on the clinical outcome of the patients treated with Pertuzumab.

Example 3

In this example multivariate approaches were used to identify combinations of factors that would further improve the identification of patients with greater benefit from the Pertuzumab treatment. Results, as derived from a CART approach (Classification And Regression Trees), are reflected. The CART classification approach made it necessary to specify as the benefit group all values in clinical benefit above of 0. As variables serum levels of Her2-ECD, TGF-alpha, Amphiregulin, and EGF were employed. A combination of serum Her2-ECD and serum TGF-alpha levels were selected to give best results. From the CART results optimized cut-points for a combination of serum Her2-ECD and serum TGF-alpha levels were derived, resulting in a rule for exploratory categorization of clinical benefit in the study population – a combination of low serum Her2-ECD values and low serum TGF-alpha values capturing 2/2 PR and 2/3 SD>6 months in the study population and excluding a reasonable number of fast progressing patients. Figure 16 shows the clinical benefit in relation to the TGF-alpha / HER2-ECD combination groupings based on the exploratory combination cut-point. Figure 17 summarizes the effect of a combination of TGF-alpha and HER2-ECD on TTP and TTD. The Kaplan-Meier estimates and the hazard ratios given in Figure 18 (TTP) and Figure 19 (TTD) demonstrate the significant effect of the grouping based on a combination of these factors for on the clinical outcome of the patients treated with Pertuzumab.

Patent Claims

1. A method of predicting the response to a treatment with a HER dimerization inhibitor in a patient comprising the steps of
   a) assessing in a biological sample from the patient
      - a marker gene or a combination of marker genes selected from the group consisting of an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene or a
      - combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene and
   b) predicting the response to the treatment with the HER dimerization inhibitor in the patient by evaluating the results of step a).

2. The method according to claim 1 wherein the step a) of assessing the marker gene or the combination of marker genes comprises
   a1) assessing the level of expression of the marker gene or the combination of marker genes,
   a2) determining whether the level of expression assessed in step a1) is above or below a threshold value.

3. The method according to claim 2 wherein the threshold value is determined before step a1) of the method of claim 2 by
   1) assessing the level of expression of the marker gene or the combination of marker genes in a plurality of biological samples from patients before treatment with the HER dimerization inhibitor,
   2) treating the patients with the HER dimerization inhibitor,
   3) correlating the response of the patients treated with the HER dimerization inhibitor to the level of expression of the marker gene or the combination of marker genes determined in step a) thereby determining the threshold value.

4. The method according to any of the claims 1 to 3 wherein the biological sample is blood serum, blood plasma or tumor tissue.

5. The method according to any of the claimss 1 to 4 wherein the HER dimerisation inhibitor inhibits heterodimerisation of HER2 with EGFR or HER3.

6. The method according to claim 5 wherein the HER dimerisation inhibitor is an antibody, preferably the antibody 2C4.

7. The method according to any of the claims 1 to 6 wherein the patient is a cancer patient, preferably a breast cancer, ovarian cancer, lung cancer or prostate cancer patient.

8. The method according to any of the claims 1 to 7 wherein the combination of marker genes consists of:
   - the transforming growth factor alpha and the HER2 marker gene, or
   - the amphiregulin, the epidermal growth factor, the transforming growth factor alpha and the HER2 marker gene.

9. The method according to any of the claims 1 to 8, wherein the level of expression of the marker gene or the combination of marker genes in the sample is assessed by detecting the level of expression of a marker protein or a fragment thereof or a combination of marker proteins or fragments thereof encoded by the marker gene or the combination of marker genes.

10. The method of claim 9, wherein the level of expression of the marker protein or the fragment thereof or the combination of marker proteins or the fragments thereof is detected using a reagent which specifically binds with the marker protein or the fragment thereof or the combination of marker proteins or the fragments thereof.

11. The method of claim 10, wherein the reagent is selected from the group consisting of an antibody, a fragment of an antibody or of an antibody derivative.

12. The method according to any of the claims 9 to 11 wherein the level of expression is determined using a method selected from the group consisting of proteomics, flow cytometry, immunocytochemistry, immunohistochemistry, enzyme-linked immunosorbent assay, multi-channel enzyme-linked immunosorbent assay, and variations of these methods.

13. The method according to any of the claims 9 to 12 wherein the fragment of the marker protein is the extracellular domain of the Her 2 marker protein.

14. The method according to claim 13 wherein the extracellular domain of the Her2 marker protein has a molecular mass of approximately 105.000 Dalton.

15. The method according to any of the claims 9 to 14
wherein the amino acid sequence of the amphiregulin marker protein is the amino acid sequence SEQ ID NO: 1,
wherein the amino acid sequence of the epidermal growth factor marker protein is the amino acid sequence SEQ ID NO: 2,
wherein the amino acid sequence of the transforming growth factor alpha marker protein is the amino acid sequence SEQ ID NO: 3, or
wherein the amino acid sequence of the HER2 marker protein is the amino acid sequence SEQ ID NO: 4.

16. The method according to any of the claims 9 to 15 wherein the threshold value in blood serum for
- the transforming growth factor alpha marker protein is between 2.0 and 5.0 pg/ ml, preferably about 3.5 pg/ ml,
- the epidermal growth factor marker protein is between 100 to 250 pg/ ml, preferably about 150 pg/ ml, or
- the amphiregulin marker protein is between 6 to 15 pg/ ml, preferably about 12 pg/ ml.

17. The method according to any of the claims 13 to 15 wherein the threshold value in blood serum for the extracellular domain of the Her2 marker protein is between 12 to 22 ng/ml, preferably about 18 ng/ml.

18. The method according to any of the claims 1 to 8, wherein the level of expression the marker gene or the combination of marker genes in the biological sample is assessed by detecting the level of expression of a transcribed marker polynucleotide encoded by the marker gene or a fragment of the transcribed marker polynucleotide or of transcribed marker polynucleotides encoded by the combination of marker genes or fragments of the transcribed marker polynucleotide.

19. The method of claim 18 wherein the transcribed marker polynucleotide is a cDNA, mRNA or hnRNA or wherein the transcribed marker polynucleotides are cDNA, mRNA or hnRNA.

20. The method according to any of the claims 18 to 19, wherein the step of detecting further comprises amplifying the transcribed polynucleotide or the the transcribed polynucleotides.

21. The method according to claim 20 wherein the step of detecting is using the method of quantitative reverse transcriptase polymerase chain reaction.

22. The method according to any of the claims 18 to 21,
- wherein the level of expression of the marker gene is assessed by detecting the presence of the transcribed marker polynucleotide or the fragment thereof in a sample with a probe which anneals with the transcribed marker polynucleotide or the fragment thereof under stringent hybridization conditions or
- wherein the level of expression of the combination of the marker genes in the samples is assessed by detecting the presence of transcribed marker polynucleotides or the fragments thereof in a sample with probes which anneal with the transcribed marker polynucleotides or the fragments thereof under stringent hybridization conditions.

23. The method according to any of the claims 18 to 22
wherein the nucleic acid sequence of the amphiregulin marker polynucleotide is the nucleic acid sequence SEQ ID NO: 5,
wherein the nucleic acid sequence of the epidermal growth factor marker polynucleotide is the nucleic acid sequence SEQ ID NO: 6,
wherein the nucleic acid sequence of the transforming growth factor alpha marker polynucleotide is the nucleic acid sequence SEQ ID NO: 7, or
wherein the nucleic acid sequence of the HER2 marker polynucleotide is the nucleic acid sequence SEQ ID NO: 8.

24. Use of a probe that hybridizes with the epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the epidermal growth factor, transforming growth factor alpha or HER2 marker protein for predicting the response to treatment with a HER dimerization inhibitor in a patient or the use of a probe that hybridizes with the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the amphiregulin,

epidermal growth factor, transforming growth factor alpha or HER2 marker protein for selecting a composition for inhibiting the progression of disease in a patient.

25. A kit comprising a probe that anneals with the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker polynucleotide under stringent conditions or an antibody that binds to the amphiregulin, epidermal growth factor, transforming growth factor alpha or HER2 marker protein.

26. A method of selecting a composition for inhibiting the progression of disease in a patient, the method comprising:
    a) separately exposing aliquots of a biological sample from a cancer patient in the presence of a plurality of test compositions;
    b) comparing the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene in the aliquots of the biological sample contacted with the test compositions and the level of expression of a marker gene or a combination of marker genes selected from the group consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene 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 marker gene or the combination of marker genes 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 marker gene or the combination of marker genes in the aliquot of the biological sample contacted with the test composition and the level of expression of the corresponding marker gene or the combination of marker genes in the aliquot of the biological sample not contacted with the test composition is an indication for the selection of the test composition.

27. A method of deriving a candidate agent, said method comprising:
    a) contacting an aliquot of a biological sample from a cancer patient with the candidate agent and determining the level of expression of a marker gene or a combination of marker genes selected from the group

consisting of the amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker gene in the aliquot,

b) determining the level of expression of a corresponding marker gene or of a corresponding combination of marker genes 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 marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene or corresponding combination of marker genes 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 marker gene or combination of marker genes in the aliquot of the biological sample contacted with the candidate agent and the level of expression of the corresponding marker gene or combination of marker genes in the aliquot of the biological sample not contacted with the candidate agent is an indication of an effect of the candidate agent.

28. The method according to claim 27, wherein said candidate agent is a candidate inhibitory agent.

29. The method according to claim 27, wherein said candidate agent is a candidate enhancing agent.

30. A candidate agent derived by the method according to any of the claims 27 to 29.

31. A pharmaceutical preparation comprising an agent according to claims 30.

32. Use of an agent according to claim 30 for the preparation of a composition for the treatment of cancer.

33. A method of producing a drug comprising the steps of the method of any one of claims 27 to 29; 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.

34. Use of a marker protein or a marker polynucleotide selected from the group consisting of a amphiregulin, epidermal growth factor, transforming growth factor alpha and HER2 marker protein or marker polynucleotide for deriving a candidate agent or for selecting a composition for inhibiting the progression of a disease in a patient.

35. Use of a HER dimerization inhibitor for the manufacture of a medicament for treating a human cancer patient characterized in that said treatment includes assessing in a biological sample from the patient

- a marker gene or a combination of marker genes selected from the group consisting an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene or a
- combination of marker genes comprising an amphiregulin marker gene and a marker gene selected from an epidermal growth factor, a transforming growth factor alpha and a HER2 marker gene.

36. Use according to claim 35 wherein the treatment includes assessing the marker gene or the combination of marker genes at least one time or repeatedly during treatment.

37. Use according to any of the claims 35 to 36 wherein the level of expression of the marker gene or the level of expression of the combination of marker genes is assessed.

38. Use according to any of the claims 35 to 37 wherein the HER dimerization inhibitor is antibody, preferably the antibody 2C4.

39. The use according to any of the claims 35 to 38 wherein the patient is a breast cancer, ovarian cancer, lung cancer or prostate cancer patient.