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- (54) Title:** METHOD FOR ESTABLISHING AND PREDICTING RESISTANCE OR RESPONSIVENESS TO CHEMOTHERAPY USING PEPTIDE MASS PROFILE

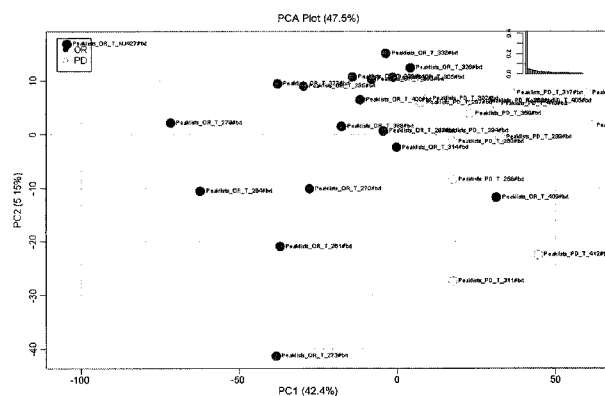


Fig. 1

- (S7) Abstract:** Title: Method for establishing and predicting resistance or responsiveness to chemotherapy using peptide mass profile Abstract Method to predict responsiveness or resistance to chemotherapy in breast cancer comprising providing an optionally processed sample from a breast cancer patient as a test sample, wherein the sample comprise peptides and/or proteins; subjecting the test sample to MALDI- FT-ICR mass spectrometry to generate mass spectra for individual peptide mass in the sample and to quantify the amount of the individual peptide mass present in the test sample; and comparing the amount of an individual peptide mass present in the test sample with the amount of a corresponding peptide mass in a reference sample, wherein the peptide masses are selected from the peptide masses as identified in table 1 and 2

Title: Method for establishing and predicting resistance or responsiveness to chemotherapy using peptide mass profile

The invention

FIELD OF THE INVENTION

The invention relates to the field of medical diagnostics, more specifically to the field of cancer diagnostics, especially breast cancer.

5

BACKGROUND OF THE INVENTION

Chemotherapy is a well-established treatment of breast cancer for patients for whom targeted therapies are not, or no longer, available. Once metastasis occurs, patients have a very poor survival, due to intrinsic or acquired resistance to drugs used. Ultimately, drug resistance of metastatic tumor cells is the main cause of death among breast cancer patients. At the moment there are no clinical markers that sufficiently predict the type of response to chemotherapeutic drugs. It is therefore of pivotal importance to identify new markers that can better predict the type of response or can act as new drugable targets for future therapy.

10
15 WO2008/085024 describes a method with which markers can be identified for a large number of diseases, thereby providing diagnostic tools for medical and veterinary diagnosis. It uses a combination of different MALDI techniques for marker detection.

20 WO 2008/133493 discloses a method for predicting the responsiveness to anti-estrogen treatment in breast cancer patients by assaying for one or more of proteins, which have found to be differentially expressed in responders and non-responders.

In March 2010 at the European Breast Cancer Conference in Spain researches found that an abnormality on chromosome 17, called CEP17, is a "highly significant indicator" that the tumor will respond to chemotherapy drugs called anthracyclines.

25 However, there is until now no protein marker for chemotherapy resistance in breast cancer. Protein markers have advantages over genetic markers. Protein markers may be used for clinical specimens that do not contain genetic

information, such as serum. In addition, genetic markers may not always give the right information, as it is the expressed product, e.g. the protein that induces a certain effect. Often it is the level of expression of proteins that indicate whether a disease has a certain outcome or not, while at the DNA level there is no difference. In fact, it has been shown that RNA expression levels poorly correlate with protein levels [1, 2].

Therefore there is still a need for peptide biomarkers that are able to identify patients who will not respond to given therapy, and to select patients for various tailored treatments. This will enable doctors to stratify patients for the most suitable, tailored, treatment option, and avoid giving them toxic drugs that pose a heavy burden on the patient without being beneficial.

Surprisingly we have found a peptide mass profile that reliably predict the response to anthracycline based combination chemotherapy in breast cancer patients.

SUMMARY OF THE INVENTION

The present invention relates to a method to predict responsiveness or resistance to chemotherapy in breast cancer comprising

- (a) providing an optionally processed sample from a breast cancer patient as a test sample, wherein the sample comprise peptides and/or proteins;
- (b) subjecting the test sample to MALDI- FT-ICR mass spectrometry to generate mass spectra for individual peptide mass in the sample and to quantify the amount of the individual peptide mass present in the test sample;
- (c) comparing the amount of an individual peptide mass present in the test sample with the amount of a corresponding peptide mass in a reference sample, wherein the peptide masses are selected from the peptide masses as identified in table 1 and 2.

DETAILED DESCRIPTION

Definitions

The term "breast cancer" as used herein refers to the erratic growth and proliferation of cells that originate in the breast tissue. A group of rapidly dividing
5 cells may form a lump or mass of extra tissue. These masses are called tumors. Tumors can either be cancerous (malignant) or non-cancerous (benign). Malignant tumors penetrate and destroy healthy body tissues. A group of cells within a tumor may also break away and spread to other parts of the body. Cells that spread from one region of the body into another are called metastases. In preferred embodiments of
10 aspects of the invention the term breast cancer refers to a malignant tumor that has developed from cells in the breast. The teachings of the present invention could be extended to chemotherapy- and anthracycline resistance of other tumor types.

The term "predict" as used herein refers to identifying or forecasting whether a breast tumor will exhibit resistance or develop resistance against
15 chemotherapy.

The term "resistance" as used herein with reference to resistance to treatment or therapy refers to the fact that the normal therapeutic efficacy of the drug is not attained and that, for instance, a tumor continues to grow. Preferably, a tumor is classified as being resistant if the patient from whom the tumor was derived
20 is classified as being a non-responder according to, for instance, the criteria used or set forth by the European Organisation for Research and Treatment of Cancer (EORTC) or the Union International Contre le Cancer (UICC).

The term "reference sample" as used herein refers to a control sample that is a sample from a tumor which is resistant or that is a sample from a tumor that is
25 not resistant to chemotherapy or that is a sample from a normal breast tissue, and which is used for comparison purpose with a test sample, that is, in order to classify the test sample – or more specifically the levels of certain proteins or peptides therein, as being indicative of a resistant or responsive tumor. In a preferred embodiment of a method according to the invention, the control sample is from a resistant tumor. The
30 level of a protein or peptide of a control sample as described herein or an expression profile of proteins or peptides as described herein is compared to the level or profile in a suspected tumor sample. If there is no significant difference between the levels or

profiles, then the suspected tumor sample is determined as having the same indication as that control sample. A control sample may be from a single individual or from multiple individuals.

The term "reference profile" as used herein refers to a collection of
5 expression levels for a multitude of given proteins or peptides, which expression levels in combination and/or relative to each other provide specific information which can be used for the purpose of comparison with other profiles. The collection in aspects of this invention is indicative for a resistant or responsive phenotype of the tumor and can be used as a reference for comparison with the test profile. Preferably, such reference
10 profile is based on samples of one or more non-resistant breast tumors. Alternatively, the reference profile may be based on samples of one or more resistant breast tumors. The skilled person is well aware of methods for comparing data collections such as test and reference profiles of gene expression data, and such methods can suitably be used in aspects of the present invention.

15 The term "chemotherapy-resistant tumor" as used herein refers to a tumor, including the individual cells therein, that is or becomes refractory to treatment by a chemotherapy or anthracycline based therapy such as FEC (= fluorouracil, epirubicine, cyclophosphamide), or FAC (= fluorouracil, doxorubicine, cyclophosphamide). In specific embodiments, the chemotherapy-resistant tumor
20 becomes resistant to chemo treatment after initiation of the treatment and may occur during the treatment. In further specific embodiments, the resistance to chemotherapy manifests at about 2-24 months while the patient is receiving chemotherapy. In de novo resistance, the patient does not respond to initial therapy. Acquired resistance is where the patient develops metastatic disease during therapy.

25 Acquired resistance to chemotherapy is well-known in the art. In particular, breast cancer patients while undergoing treatment with chemotherapy have recurrence of the disease. In specific embodiments, the disease metastasizes during therapy with anthracycline based chemotherapy, which results in resistant metastases.

30 The term "chemotherapy or anthracycline-sensitive tumor" as used herein refers to a tumor, including the individual cells therein, that is treatable with chemotherapy or anthracycline. In specific embodiments, the chemotherapy or anthracycline -sensitive tumor remains sensitive during the treatment. In further

specific embodiments, the chemotherapy or anthracycline -sensitive tumor is still sensitive up to at least about seven to ten years. Preferably, a tumor is classified as being sensitive if the patient from whom the tumor was derived is classified as being a responder as can be classified using EORTC or UICC criteria. Preferably, the
5 responder is classified using EORTC or UICC criteria.

The term "different" as used herein with reference to the comparison of expression profiles or expression levels refers to a degree of difference which is statistically significantly increased or decreased compared to a reference or a control. The term "significantly" or "statistically significant" refers to statistical significance
10 and generally means that values differ two standard deviations (SD). In preferred embodiments, the difference is classified as statistically significant if the expression level is at least a 20 percent increased or decreased compared to expression level of the same expression product in control individuals. Preferably, the increase or decrease is at least 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200 or 250 percent. Most
15 preferably, the increase or decrease is at least 100 percent (herein also referred to as "one fold").

The term "level" as used herein refers to the measurable absolute level or a measurable relative level compared to the level of another protein. If the expression level is determined of a protein from more than one individual (as a control), usually
20 the median or mean expression level of these individuals is used for comparison.

Some of the most common chemotherapy combinations used for breast cancer are CMF (cyclophosphamide, methotrexate and fluorouracil), FEC (epirubicin, cyclophosphamide and fluorouracil), FAC (fluorouracil, doxorubicine, cyclophosphamide), FEC-T (epirubicin, cyclophosphamide, fluorouracil and taxotere),
25 E-CMF (epirubicin, followed by CMF), AC (doxorubicin (adriamycin) and cyclophosphamide), MMM (methotrexate, mitozantrone and mitomycin), MM (methotrexate and mitozantrone).

Drug resistance of metastatic tumor cells is the main cause of death among breast cancer patients. By comparing the mass spectra of tissues from patients with
30 either breast cancer resistant to chemotherapy or breast cancer responsive to chemotherapy, the inventors have found that there are significant differences in the peptide masses profile. They found a list of peptide masses that were predominantly present in tissue from patients with breast cancer resistant to chemotherapy and

found another list of peptide masses that were predominantly present in tissue from patients with breast cancer responsive to chemotherapy. The combination of peptide masses provide an unique profile that may be able to determine between a patient responsive to chemotherapy and a patient resistant to chemotherapy.

- 5 In a first aspect the present invention is directed to a method to predict responsiveness or resistance to chemotherapy in breast cancer comprising
- (a) providing an optionally processed sample from a breast cancer patient as a test sample, wherein the sample comprise peptides and/or proteins;
 - (b) subjecting the test sample to MALDI- FT-ICR mass spectrometry to
 - 10 generate mass spectra for individual peptide mass in the sample and to quantify the amount of the individual peptide mass present in the test sample;
 - (c) comparing the amount of an individual peptide mass present in the test sample with the amount of a corresponding peptide mass in a reference sample, wherein the peptide masses are selected from the peptide masses as identified in table
 - 15 1 and 2

Peptides masses listed in table 1 indicate a breast tumor responsive to chemotherapy. In contrast peptide masses listed in table 2 indicate breast tumor resistant to chemotherapy.

- 20 In a preferred embodiment the reference sample is optionally processed sample from a breast cancer patient being responsive to chemotherapy or from a breast cancer patient being resistant to chemotherapy. Suitably the reference sample is a pooled sample of more than one breast cancer patient, suitably more than 10, more suitably more than 50, and even more than 100 cancer patients. In a preferred embodiment the reference sample is a combined pooled sample from more than two
- 25 breast cancer patients, both from a patient responsive and from a patient resistant to chemotherapy. By comparing the peptide mass profile of a tissue of a patient with breast cancer, still unknown whether the cancer is responsive to chemotherapy or resistant, to the list of peptide masses of the present invention, a doctor can see whether the patient will be responsive to chemotherapy or not.

- 30 In another preferred embodiment the optionally processed sample is a body tissue sample processed by subjecting the sample to laser capture microdissection to provide collections of microdissected cells, the collections preferably amounting to about 200- 3,000 cells. Preferably the optionally processed samples are body tissue

samples, body fluid samples, or collections of microdissected cells processed by
subjection to protein digestion, preferably using trypsin, to provide processed samples
comprising peptide fragments from the proteins in the samples. Suitably the body
tissue is selected from the group consisting tissues of breast cancer tumor, breast
5 cancer tumor stroma, and lymph node. Also so suitably the body fluid is selected from
the group consisting of blood, serum, cerebrospinal fluid (CSF), urine, saliva and
nipple aspirate. Preferred samples are body fluid samples, preferably a body fluid
comprising about 0.05- 5 mg/ml of protein, and wherein in step (b) an amount of 1-10
10 μ l of optionally processed body fluid is subjected to MALDI- FT-ICR mass
spectrometry. Another suitable test sample may be circulating tumor cells that are
collected from blood or other fluids. Body fluids and circulating tumor cells are more
easily obtained from a patient than tissue sample that often need local anaesthetic to
obtain. Preferably the individual masses are in a mass range of 800 to 4,000 Da.

In preferred embodiment of the present invention the individual peptide
15 masses present in the test sample are selected from the group consisting of peptide
masses as identified in table 1 or 2 with a p value lower than 0.03, preferably lower
than 0.01.

In another preferred embodiment it is determined whether a breast cancer
may be responsive to chemotherapy. For this the amount of the individual peptide
20 mass present in the test sample is determined. If the amount of the individual peptide
mass selected from the group consisting of peptide masses as identified in table 1 is
higher than the amount of peptide mass having a corresponding mass in the reference
sample, it indicates a tumor responsive for chemotherapy. A preferred reference
sample for determining responsiveness to chemotherapy is a reference sample that
25 comprises one or more samples from one or more breast cancer patients being
resistant to chemotherapy or a reference sample that comprises samples from both
breast cancer patients resistant to chemotherapy and from breast cancer patients that
are responsive to chemotherapy. The advantage of the present invention is that only
the individual peptide masses as indicated in table 1, preferably the peptide masses
30 with a p value lower than 0.03, more preferably lower than 0.01, are measured. This
saves time, and provides a more reliable method for determining whether a patient is
responsive to chemotherapy or not.

In yet another preferred embodiment of the present invention it is determined whether a breast cancer is resistant to chemotherapy. For this the amount of the individual peptide mass present in the test sample is determined. If the amount of the individual peptide mass selected from the group consisting of peptide masses as identified in table 2 is higher than the amount of a peptide masses having a corresponding mass in the reference sample, this indicates a tumor resistant for chemotherapy. To determine chemotherapy resistance a preferred reference sample comprises one or more samples from one or more breast cancer patients being responsive to chemotherapy, or a reference sample that comprises samples from both breast cancer patients resistant to chemotherapy and from breast cancer patients that are responsive to chemotherapy is used. The advantage of the present invention is that only the individual peptide masses as indicated in table 2, preferably the peptide masses with a p value lower than 0.03, more preferably lower than 0.01, are measured. This saves time, and provides a more reliable method for determining whether a patient is responsive to chemotherapy or not.

Suitably in practice, a tissue from a breast cancer patient unknown whether to respond or being resistant to chemotherapy will be compared to two reference samples, one comprising tissue from breast cancer patients responsive to chemotherapy, one comprising tissue from breast cancer patients resistant to chemotherapy. In a most preferred embodiment, the reference sample is a pooled combined sample from more than two patients, and from breast cancer patients responsive to chemotherapy, and from breast cancer patients resistant to chemotherapy. In addition, suitably the peptide masses being indicative for responsiveness (peptide masses identified in table 1, preferably with a p value lower than 0.03, more preferably lower than 0.01), together with the peptide masses being indicative for resistance (peptide masses identified in table 2, preferably with a p value lower than 0.03, more preferably lower than 0.01) are measured. This will give a differential peptide mass profile that will indicate whether a patient may be responsive to chemotherapy (having more peptide masses corresponding to peptide masses in table 1) or resistant to chemotherapy (having more peptide masses corresponding to peptide masses identified in table 2).

Any suitable method to determine the amount of individual peptide mass may be used. Preferred methods are selected from the group consisting of mass spectrometry, peptide array, immuno-histochemical assay, ELISA, Protein array, Western Blot, and immunoaffinity chromatography.

5 In a preferred embodiment the amount of the individual peptide mass in the test sample is at least 20% higher than the amount of the corresponding peptide mass in the reference sample. Preferably at least 10 of the individual peptide mass are present in a higher amount in the test sample than the corresponding peptide mass in the reference sample. Suitably, at least 2, more suitably at least 5, most suitably at
10 least 10, or even at least 15 peptide masses selected from the group consisting of peptide masses from table 1 and 2 with a p value lower than 0.03 are present in an higher amount in the test sample than the corresponding peptide mass in the reference sample. Most suitably, at least 2, more suitably at least 5, most suitably at least 10, peptide masses selected from the group consisting of peptide masses from
15 table 1 and 2 with a p value lower than 0.01 are present in an higher amount in the test sample than the corresponding peptide mass in the reference sample.

The present invention is suitable to detect responsiveness or resistance to chemotherapy in breast cancer. More suitably the chemotherapy is anthracycline based preferably selected from the group consisting of FEC, FAC.

20 Yet another aspect of the invention is directed to a method to identify a compound that target breast cancer tumor that is resistant to chemotherapy comprising the steps

- a) providing a test sample from a breast cancer patient that is resistant to chemotherapy
- 25 b) subjecting the test sample to MALDI- FT-ICR mass spectrometry to generate mass spectra for individual peptide masses in the test sample and to quantify the amount of the individual peptide masses present in the test sample;
- c) contact the test sample with a compound to provide a treated sample
- d) subjecting the treated sample to MALDI- FT-ICR mass spectrometry to
30 generate mass spectra for individual peptide masses in the treated sample and to quantify the amount of the individual peptide masses present in the treated sample;
- e) comparing the amount of an individual peptide masses present in the treated sample with the amount of a corresponding peptide mass in the test sample,

wherein the peptide masses are selected from the peptide masses as identified in table 1, preferably with a p value lower than 0.03, more preferably lower than 0.01

- f) identifying the compound wherein the treated sample has at least 2, more preferably at least 5, most preferably at least 10 peptide masses selected from the group consisting of peptide masses as identified in table 1, preferably with a p value lower than 0.03, more preferably lower than 0.01.

The peptide mass profile as identified in table 1 indicates tumor being responsive to chemotherapy. This information may be used to identify compounds that may be able to treat the breast tumor that is resistant to chemotherapy.

- Exposing a tumor sample resistant to chemotherapy to a compound, determining the peptide profile of the treated sample may identify the compound that causes especially the peptide masses in table 1 to be enhanced. This compound may be a promising compound to treat breast cancer patients that are resistant to chemotherapy.

METHODS

Experimental procedures

Reagents

PEN membrane (1 mm) covered glass slides and PALM caps were purchased from
5 PALM laboratories (Carl Zeiss MicroImaging, GmbH, Munich, Germany), PCR clean
LoBind 0.5ml tubes were from Eppendorf AG (Hamburg, Germany), trypsin gold
mass spectrometry grade was obtained from Promega (Promega Benelux B.V., Leiden,
Netherlands), RapiGest SF surfactant and LC glass vials were from Waters
Corporation (Milford, MA, USA), MTP AnchorChip™ 600/384 T F target plate, 2,5-
10 dihydroxybenzoic acid (DHB), and peptide calibration standard (containing
angiotensin I and II, substance P, bombesin, rennin substrate, ACTH clip 1-17, ACTH
clip 18-39, and somatostatin 28) were obtained from Bruker (Bruker Daltonik GmbH,
Bremen, Germany), HPLC grade water and acetonitrile (ACN) were purchased from
Fluka analytical (Sigma-Aldrich Corporation, St. Louis, MO, USA), and trifluoroacetic
15 acid (TFA) was purchased from Thermo Fischer Scientific Inc. (Rockford, IL, USA).

Patients and tumor tissues

For the discovery phase of this study, we have used 50 snap frozen primary breast
tumor tissues present in our N₂ bio bank, of which long-term clinical follow-up was
available. Tissues were selected from patients that received anthracyclin-based
20 chemotherapy; 13 FEC (5-fluorouracil + epirubicin + cyclofosfamide) / 37 FAC (5-
fluorouracil + doxorubicin [Adriamycin®] + cyclofosfamide). Of these patients, 22
showed objective response (OR), 5 stable disease (SD) >6 months, 21 progressive
disease (PD), and 5 SD <6 months upon treatment. Furthermore, 31 tumors were
estrogen receptor (ER) negative, and 19 were ER positive, as assessed by ligand
25 binding assay or enzyme-linked immuno sorbent assay (≥ 10 fmol/mg cytosolic
protein). Clinical response was defined by standards of the International Union
against Cancer criteria of tumor response [3].

This study was approved by the Medical Ethics Committee of the Erasmus MC
Rotterdam, The Netherlands (MEC 02.953), and was performed in accordance to the
30 Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands.

Where ever possible we adhered to the Reporting Recommendations for Tumor Marker Prognostic Studies REMARK [4].

Laser Capture Microdissection (LCM)

5 LCM was performed on 10 μm tissue cryosections that were fixed in ice-cold 70% ethanol and stained with hematoxylin as previously described [5]. Laser microdissection and pressure catapulting (LMPC) was performed directly after staining. Tumor epithelial and stromal cells were separately collected, using a P.A.L.M. LMPC device, type P-MB (Carl Zeiss MicroImaging, GmbH, Munich,
10 Germany). From each cryosection, an area of $\sim 400,000 \mu\text{m}^2$ that corresponds to $\sim 4,000$ cells (area x slide thickness / $1000 \mu\text{m}^3$ cell volume) was collected in P.A.L.M. tube caps containing 10 μl of 0.1% RapiGest, and then spun down into 0.5 ml Eppendorf Protein LoBind tubes. Collected cells were stored at -80°C until further processing. Since we used small numbers of microdissected cells in this study, protein
15 concentration was typically below the detection limit of any protein assay. Hence, protein concentration for samples undergoing MS analysis was estimated based on microdissected tissue area and extrapolations from protein assays performed on whole tissue lysates (i.e., $\sim 4,000$ cells corresponds to ~ 400 ng of total protein) as described previously [6].

20 Sample preparation

Microdissected cells were lysed by sonication directly in RapiGest solution in a cup horn sonifier bath, using an Ultrasonic Disruptor Sonifier II (Model W-250/W-450, Bransons Ultrasonics, Danbury, CT, USA) for 1 min at 60% amplitude. Proteins were subsequently equilibrated for 2 min at 37°C , and denatured at 99°C for 5 min, and
25 processed for overnight trypsin digestion at a 1:20 v/v ratio, as previously described [5] and according to the instructions of the RapiGest manufacturer. Peptides were lyophilized and stored in -80°C until further analysis. Prior to FTICR MS analysis, samples were reconstituted in 6 μl 50% ACN, 0.1% TFA. In order to fully cleave RapiGest in the samples, 0.6 μl 500mM HCl was added, shortly mixed, incubated at
30 37°C for 45 minutes, and centrifuged for 10 minutes at 10,600g to pellet any contaminating particulate material.

MALDI-FTICR MS

For MALDI-FTICR MS analysis, a matrix solution was prepared from 10 mg/ml DHB in 0.1% TFA in MilliQ water. The matrix solution was vortexed for 1 min prior to use. 0.5 µl DHB matrix was spotted on an AnchorChip™ target plate, after which 0.5 µl prepared sample was added and mixed on the spot. Peptide calibration standard was spotted on distinct calibration positions together with DHB matrix. Samples were spotted in duplicate and left on the AnchorChip™ for drying prior to MALDI-FTICR analysis on the Apex IV Qe instrument with a 9.6 tesla magnet equipped with a 20 Hz nitrogen laser (Bruker Daltonik). For each measurement, 100 scans of 10 shots each at 60% laser power were accumulated, and spectra were acquired in the mass range of 800-4,000 m/z using XMass software v7.0.8 (Bruker). MALDI-FTICR data were acquired at 512K and further processed with a Gaussian filter and two zero fillings. For each sample, mass spectra were recorded from duplicate spots and accumulated in to one final spectrum. Prior to sample analysis, external calibration was performed on the peptide calibration standard using a quadratic equation. In addition, a post-acquisition internal calibration step was performed in DataAnalysis software, version 3.3, build 139 (Bruker) using auto-lytic trypsin MH+ peptide masses (842.50940, 1045.56370, 2211.10400 m/z), and ubiquitous keratin (1179.60100 m/z), actin (1198.70545, 1790.89186 m/z) and histone MH+ peptide masses (976.44824, 1515.74913, 2343.16486, 3183.61423 m/z), so that accuracy of <1ppm could be acquired. Spectra in which less than 5 calibration peaks were present, were excluded from further data analysis because of poor quality, which was the case for 1 OR tumor, 1 PD tumor, 4 OR stroma, and 3 PD stroma spectra.

Data analysis

All isotopic peaks with a signal-to-noise ratio >4 were annotated using DataAnalysis software package, version 3.3, build 139 (Bruker Daltonik). Peak lists were saved in general text format and imported into the home made script in the R-program, Peptrix v2.4.1. The Peptrix package was used to compare mass spectra for identification of differentially abundant peaks by label free quantitation using MS peak intensity. A matrix file was generated indicating presence, absence, and intensities of peaks in different samples. Peak masses present in less than 4 samples were omitted from the matrix. Within Peptrix, a univariate Wilcoxon-Mann-Whitney

rank sum test was performed for pair wise comparisons between tumor and stromal cells and between OR and PD patients. Peaks were considered to be differentially abundant if p-values were <0.05. Differentially abundant peak masses (annotated as doubly and triply charged peptide masses) were subjected to targeted MS/MS analysis
5 for peptide identification purposes.

LC-MS/MS

Identification of differential peptide masses was performed by nLC-MS/MS analysis on an Orbitrap (Thermo Fischer Scientific, Germany) XL mass spectrometer, using
10 an inclusion list of target peptide masses. Tryptic digests of whole tumor tissue lysates were subjected to nLC separation, using a 15 cm x 75 µm inner diameter C18 reversed phase column. Peptides were eluted from the column by the following binary gradient: from 100% solvent A (0.1% formic acid in water) to 75% solvent A/ 25% solvent B (80% acetonitrile and 0.08% formic acid in water) in 120 minutes, followed
15 by 25-50% solvent B for a further 60 minutes, with a column flow rate of 300 nL/min. A data dependent acquisition was performed in the high resolution Orbitrap with a survey scan from 400-1800 Th. Based on this survey scan, up to 5 ions corresponding to the masses in the inclusion list were fragmented if present. If no target ions were present, the 5 most intensive ions were selected for collision-activated dissociation
20 fragmentation, after which these masses were excluded for further MS/MS analysis for 3 minutes.

Protein identification and quantitation

Bioworks 3.2 software package (Thermo Fischer Scientific, Germany) was used for
25 peak picking and MS/MS identification, and corresponding SEQUEST features, using HUPO criteria, were obtained from the UniProt and SwissProt databases. The number of allowed missed cleavages was set to 1, mass tolerance for precursor ions was 10 ppm, and for fragment ions 0.5 Da. The cut-off for matching identified peptides with theoretical mass was 2 ppm.
30 Precursor MS scan peptide mass intensities were used for peptide and protein quantitation.

Results

Primary breast cancer tissues from patients that received anthracycline-based chemotherapy in advanced stage of disease were subjected to global proteome analysis. For the identification of proteins that associate with chemotherapy resistance, we compared proteomes of tissues from patients responsive (OR + SD >6 months) to therapy with tissues from therapy-resistant (PD + SD <6 months) patients. Patient characteristics are summarized in Table 1. Tumor tissues were subjected to LCM, and, whenever possible, ~4000 tumor and surrounding stromal cells were collected from each tissue. Tumor cells were procured from 20 different OR, 10 SD, and 16 PD tissues, and stromal cells were collected from 18 OR, 10 SD, and 18 different PD tissues. Tryptic digests were prepared and analyzed in duplicate by MALDI-FTICR MS.

Comparative proteomics of breast cancer tissues

Mass spectra of sufficient quality were obtained for 19 OR tumor, 14 OR stroma, 15 PD tumor, 15 PD stroma, and all SD samples.... Using Peptrix software, OR and PD tumor, OR and PD stroma, and all tumor versus all stroma mass spectra were compared based on peak intensity and peak count. All detected peaks in all mass spectra were populated into a matrix file, which was then used for further statistical analysis. In total, 3,948 peaks were detected in all tumor samples, taking all isotopic peaks into account. In stromal samples, a total of 4,715 peaks were detected. Wilcoxon-Mann-Whitney rank sum analysis revealed the presence of 135 differential peak masses ($p < 0.05$) between OR and PD tumor cells, of which 28 peak masses had a p -value < 0.01 (supplemental table S1). Furthermore, 231 differential peaks were observed between OR and PD stromal cells ($p < 0.05$), of which 17 had a p -value < 0.01 . Comparison of tumor and stromal samples revealed a very large difference in sample composition.

Identification of differentially abundant proteins

For the identification of peptide sequences corresponding to differentially abundant peak masses, a mass search was performed. Differential peak masses were matched to our list of identified proteins obtained through nLC-MS/MS analysis. From the 135 differential peak masses between OR and PD tumor cells, 70 were matched with

peptide sequences, representing 65 different proteins (see tables 1-4). 11 differential stromal peak masses were matched to peptides and their corresponding proteins. Based on this putative peptide profile, chemotherapy responding breast cancer patients can be distinguished from chemotherapy resistant patients (fig 1). In a principal components analysis, it was shown that chemotherapy-resistant breast cancer patients (light dots) can be separated from chemotherapy responsive breast cancer patients (dark dots).

Figure 1. Principal Components Analysis of breast cancer patients. Groups can be separated from each other based on the differentiating peptide profile.

Table 1: peptide masses that are present in OR:PD positive patients

Peptide mass Da	p-value	OR:PD	Tissue
2305.072724	9.80E-04	+	stroma
2063.929124	0.002111308	+	stroma
3085.446824	0.002507105	+	stroma
2009.058124	0.002714834	+	tumor
1834.914924	0.003742871	+	tumor
1800.868124	0.005462092	+	stroma
2220.950824	0.005804654	+	stroma
2347.075324	0.005946875	+	stroma
1325.626924	0.006096424	+	stroma
1697.815724	0.006096424	+	stroma
2265.126724	0.006096424	+	stroma
2306.073824	0.006096424	+	stroma
1240.552924	0.006501702	+	stroma
794.515324	0.007192917	+	stroma
842.425424	0.007192917	+	stroma
2462.088524	0.007215417	+	stroma
3341.762124	0.007955847	+	tumor
1514.729524	0.008980671	+	stroma
1703.789324	0.008980671	+	stroma
1969.067724	0.009611013	+	tumor
2182.005724	0.009882035	+	stroma
2643.261824	0.009882035	+	stroma
3330.552924	0.01426209	+	tumor
3634.960924	0.01426209	+	tumor
1848.806724	0.01607209	+	tumor
2722.284024	0.01798863	+	tumor
1038.006024	0.0202379	+	tumor
3637.950724	0.02531842	+	tumor
1803.933324	0.0255138	+	tumor

1944.878924	0.0255138	+	tumor
2922.352824	0.02760192	+	tumor
3340.755324	0.02794024	+	tumor
3350.576624	0.02933639	+	tumor
1029.591224	0.03002501	+	tumor
976.062824	0.03010091	+	tumor
2138.049324	0.03010091	+	tumor
3076.644624	0.03010091	+	tumor
2419.249624	0.03105303	+	tumor
886.061224	0.03131214	+	tumor
1404.810024	0.03131214	+	tumor
1426.766724	0.03131214	+	tumor
1611.843424	0.03131214	+	tumor
1743.791024	0.03131214	+	tumor
1793.878324	0.03131214	+	tumor
2279.186824	0.03131214	+	tumor
2567.243124	0.03131214	+	tumor
2662.385424	0.03131214	+	tumor
2768.326324	0.03131214	+	tumor
2779.313424	0.03131214	+	tumor
2854.397024	0.03131214	+	tumor
2960.334724	0.03131214	+	tumor
2961.318724	0.03131214	+	tumor
3342.747024	0.03131214	+	tumor
3351.589024	0.03131214	+	tumor
3795.858424	0.03131214	+	tumor
954.080624	0.036303	+	tumor
2674.262824	0.036303	+	tumor
2794.297324	0.036303	+	tumor
3332.559124	0.0363532	+	tumor
2312.185924	0.04253066	+	tumor
2896.487524	0.04253066	+	tumor
3348.565024	0.04253066	+	tumor
2008.053424	0.04533272	+	tumor
2663.384224	0.04627936	+	tumor
1026.078324	0.04795378	+	tumor
2918.517924	0.04795378	+	tumor
1987.076124	0.04878663	+	tumor

Table 2: peptide masses that are present in OR:PD negative patients

Peptide mass Da	p-value	OR:PD	Tissue
1991.891624	0.000499394	-	tumor
2745.290324	0.001232227	-	tumor
1940.080824	0.001334505	-	tumor
1921.064524	0.003801999	-	tumor
1985.063324	0.004223652	-	tumor
1613.900624	0.005804654	-	stroma
1763.799724	0.006732545	-	stroma
2183.209624	0.007411747	-	tumor
2822.293524	0.007411747	-	tumor
3113.604424	0.007411747	-	tumor
2420.241624	0.008041432	-	stroma
1938.071424	0.00845795	-	tumor
2824.307324	0.008658083	-	stroma
1516.749224	0.008980671	-	stroma
3085.435724	0.008980671	-	stroma
3030.592824	0.009611013	-	tumor
993.497324	0.009882035	-	stroma
882.208824	0.01031402	-	tumor
1780.794624	0.01188119	-	tumor
2263.117124	0.01188119	-	tumor
2531.373624	0.01200892	-	tumor
842.497524	0.01888901	-	tumor
1138.500424	0.01888901	-	tumor
1640.742324	0.01888901	-	tumor
1686.973124	0.01888901	-	tumor
2368.252624	0.01888901	-	tumor
2799.343024	0.01888901	-	tumor
2904.371124	0.01888901	-	tumor
3330.643724	0.01888901	-	tumor
801.879724	0.02324039	-	tumor
1420.662424	0.02324039	-	tumor
1639.908824	0.02324039	-	tumor
1642.804324	0.02324039	-	tumor
2694.208924	0.02324039	-	tumor
2736.260824	0.02324039	-	tumor
2854.388424	0.02324039	-	tumor
3029.589024	0.02324039	-	tumor
1038.001424	0.02606067	-	tumor
1614.907424	0.02606067	-	tumor
1934.008924	0.02606067	-	tumor
1787.927524	0.03010091	-	tumor
1984.058524	0.03010091	-	tumor
2166.059224	0.03010091	-	tumor
2181.199824	0.03105303	-	tumor
2182.204524	0.03105303	-	tumor

2312.181824	0.03105303	-	tumor
2436.231424	0.03105303	-	tumor
2245.199024	0.03415649	-	tumor
1044.557424	0.036303	-	tumor
2367.252324	0.04101963	-	tumor
795.466524	0.04533272	-	tumor
1610.890724	0.04533272	-	tumor
1709.903924	0.04533272	-	tumor
1769.997824	0.04533272	-	tumor
1920.070624	0.04533272	-	tumor
2038.020624	0.04533272	-	tumor
2417.273624	0.04533272	-	tumor
2709.285624	0.04533272	-	tumor
2732.304124	0.04533272	-	tumor
2842.301024	0.04533272	-	tumor

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Claims

1. Method to predict responsiveness or resistance to chemotherapy in breast cancer comprising

(a) providing an optionally processed sample from a breast cancer patient as a test sample, wherein the sample comprise peptides and/or proteins;

5 (b) subjecting the test sample to MALDI- FT-ICR mass spectrometry to generate mass spectra for individual peptide mass in the sample and to quantify the amount of the individual peptide mass present in the test sample;

(c) comparing the amount of an individual peptide mass present in the test sample with the amount of a corresponding peptide mass in a reference sample,
10 wherein the peptide masses are selected from the peptide masses as identified in table 1 and 2

2. Method according to claim 1 wherein the reference sample is optionally processed sample from a breast cancer patient being responsive to chemotherapy or from a breast cancer patient being resistant to chemotherapy.

15 3. Method according to claim 1 or 2, wherein the optionally processed sample is a body tissue sample processed by subjecting the sample to laser capture microdissection to provide collections of microdissected cells, the collections preferably amounting to about 200- 3,000 cells.

4. Method according to any one of the preceding claims, wherein the
20 optionally processed samples are body tissue samples, body fluid samples, or collections of microdissected cells processed by subjection to protein digestion, preferably using trypsin, to provide processed samples comprising peptide fragments from the proteins in the samples.

5. Method according to any one of the preceding claims, wherein the body
25 tissue is selected from the group consisting tissues of breast cancer tumor, breast cancer tumor stroma, and lymph node.

6. Method according to any one of the preceding claims, wherein the body fluid is selected from the group consisting of blood, serum, cerebrospinal fluid (CSF), urine, saliva and nipple aspirate.

7. Method according to any one of the preceding claims, wherein the samples are body fluid samples, preferably a body fluid comprising about 0.05- 5 mg/ml of protein, and wherein in step (b) an amount of 1-10 μ l of optionally processed body fluid is subjected to MALDI- FT-ICR mass spectrometry.
- 5 8. Method according to any of the preceding claims wherein the sample is a sample comprising circulating tumor cells that are collected from blood or other fluids.
9. Method according to any one of the preceding claims, wherein in step (b) the individual masses are in a mass range of 800 to 4,000 Da.
10. Method according to any one of the preceding claims, wherein the
10 individual peptide masses present in the test sample are selected from the group consisting of peptide masses as identified in table 1 or 2 with a p value lower than 0.03, preferably lower than 0.01.
11. Method according to any one of the preceding claims, wherein the amount of
15 the individual peptide mass present in the test sample is higher than the amount of peptide mass having a corresponding mass in the reference sample indicates a tumor responsive for chemotherapy, wherein the individual peptide mass from the test sample is selected from the group consisting of peptide masses as identified in table 1 preferably with a p value lower than 0.03, more preferably lower than 0.01
12. Method according to any one of the preceding claims, wherein the amount of
20 the individual peptide masses present in the test sample is higher than the amount of a peptide masses having a corresponding mass in the reference sample indicates a tumor resistant for chemotherapy, wherein the individual peptide masses from the test sample is selected from the group consisting of peptide masses as identified in table 2, preferably with a p value lower than 0.03, more preferably lower than 0.01.
- 25 13. Method according to any of the preceding claims wherein the amount of peptide or protein is determined by a method selected from the group consisting of mass spectrometry, peptide array, immuno-histochemical assay, ELISA, Protein array, Western Blot, and immunoaffinity chromatography.
14. Method according to any one of the preceding claims wherein the amount of
30 the individual peptide mass in the test sample is at least 20% higher than the amount of the corresponding peptide mass in the reference sample, and wherein the individual peptide mass from the test sample is selected from the group consisting of

peptides as identified in table 1 and 2, preferably with a p value lower than 0.03, more preferably with a p value lower than 0.01.

15. Method according to any one of the preceding claims wherein at least 10 of the individual peptide mass are present in a higher amount in the test sample than
5 the corresponding peptide mass in the reference sample, and wherein the individual peptide mass from the test sample is selected from the group consisting of peptides as identified in table 1 and 2, preferably with a p value lower than 0.03, more preferably with a p value lower than 0.01.

16. Method according to any one of preceding claims wherein the chemotherapy
10 is anthracycline based preferably selected from the group consisting of FEC and FAC.

17. Method to identify a compound that target breast cancer tumor that is resistant to chemotherapy comprising the steps

a) providing a test sample from a breast cancer patient that is resistant to chemotherapy

15 b) subjecting the test sample to MALDI- FT-ICR mass spectrometry to generate mass spectra for individual peptide masses in the test sample and to quantify the amount of the individual peptide masses present in the test sample;

c) contact the test sample with a compound to provide a treated sample

d) subjecting the treated sample to MALDI- FT-ICR mass spectrometry to
20 generate mass spectra for individual peptide masses in the treated sample and to quantify the amount of the individual peptide masses present in the treated sample;

e) comparing the amount of an individual peptide masses present in the treated sample with the amount of a corresponding peptide mass in the test sample, wherein the peptide masses are selected from the peptide masses as identified in table
25 1, preferably with a p value lower than 0.03, more preferably lower than 0.01

f) identifying the compound wherein the treated sample has at least 2, more preferably at least 5, most preferably at least 10 peptide masses selected from the group consisting of peptide masses as identified in table 1.

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	UMAR A ET AL: "Identification of a Putative Protein Profile Associated with Tamoxifen Therapy Resistance in Breast Cancer" MOL CELL PROTEOMICS, vol. 8, no. 6, June 2009 (2009-06), pages 1278-1294, XP002587882 the whole document	1-17
X	UMAR A ET AL: "High-throughput proteomics of breast carcinoma cells: a focus on FTICR-MS" EXP REV PROTEOMICS, vol. 5, no. 3, June 2008 (2008-06), pages 445-455, XP009135020 the whole document, in particular figure 1	1-17



Further documents are listed in the continuation of Box C.



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