(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 November 2009 (26.11.2009)

PCT

(10) International Publication Number
WO 2009/140973 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/DK2009/050116

(22) International Filing Date:
20 May 2009 (20.05.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

PA 2008 00696	20 May 2008 (20.05.2008)	DK
61/054,944	21 May 2008 (21.05.2008)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) Title: A COMBINED METHOD FOR PREDICTING THE RESPONSE TO AN ANTI-CANCER THERAPY

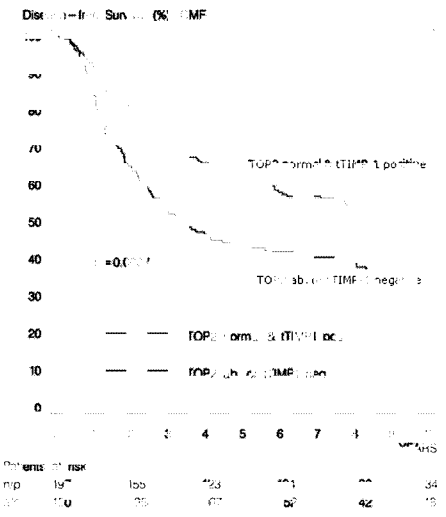

Fig. 3B

(57) Abstract: The invention provides methods for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, wherein the methods comprise the steps of determining TIMP-I DNA aberration/TIMP-1 protein aberration in combination with determining DNA aberration in TOP2A/HER2 amplicon on chromosome 17q21 including TOP2A and HER2 or aberrations of TOP2A and ErbB2 protein expression. Further provided are methods of treating cancer by using said topoisomerase IIα inhibitor therapy. The invention also comprises a kit for the application of the methods for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer.

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A COMBINED METHOD FOR PREDICTING THE RESPONSE TO AN ANTI-CANCER THERAPY

Technical field of the invention

The present invention relates to the field of anti-cancer therapy. In particular the present invention relates to a method for predicting the response to various types of anti-cancer therapies. In particular the present invention relates to improvement in therapy of individuals suffering from cancer.

Background of the invention

Tissue Inhibitor of Metalloprotease-1 (TIMP-1)

Tissue Inhibitor of Metalloprotease-1 (TIMP-1) is one out a family of four endogenous inhibitors of matrix metalloproteases (MMPs) and the gene is located on the x-chromosome. TIMP-1 is a 25 kDa protein which binds most MMPs with a 1:1 stochiometry. TIMP-1 is present in various tissues and body fluids and is stored in α-granules of platelets and released upon activation. While the main function of TIMP-1 is supposed to be MMP inhibition, some alternative functions of TIMP-1 have been described, e.g. inhibition of apoptosis and regulation of cell growth and angiogenesis. In addition, some studies have suggested that TIMP-1 may also play a role in the early processes leading to the malignant phenotype.

The present inventors have described that measurement of plasma TIMP-1 gives high specificity and high sensitivity in the detection of early stage colorectal cancer. In addition, the present inventor has shown that measurement of plasma TIMP-1 levels in preoperative or postoperative samples yields strong and stage independent prognostic information in patients with early stage colorectal cancer. By measuring TIMP-1 protein in primary breast cancer tissue the inventors of the present invention and others have shown that high tumour tissue total TIMP-1 levels are associated with shorter patient survival.

A role for TIMP-1 in the regulation of apoptosis has been reported and two possible ways for this to happen have been suggested. Both of these support the idea that TIMP-1 inhibits apoptosis.

First, proteolytic degradation of the extracellular matrix leads to loss of differentiation and to apoptosis in mammary epithelial cells both *in vitro* and *in vivo*. This indicates that the integrity of the extracellular matrix and the protection of cell-matrix interactions are crucial factors in assuring survival of mammary epithelium. Through the inhibition of MMPs, TIMP-1 is capable of inhibiting degradation of extracellular matrix, thereby possibly inhibiting apoptosis. By crossing mice that over-expressed MMP-3 in the mammary gland with TIMP-1 transgenic mice, Alexander and co-workers demonstrated such apoptosis-inhibitory effect of TIMP-1 observing that apoptosis of the mammary epithelium induced by MMP-3 was reduced by TIMP-1. The mere disintegration of the basement membrane could be responsible for apoptosis induced by proteolytic activity but it has also been speculated that integrin-mediated signalling plays a part.

Second, an apoptosis-inhibitory effect of TIMP-1 that occurs independently of MMP-inhibition has also been demonstrated. In human breast epithelial cells, an ability of endogenous TIMP-1 to inhibit apoptosis induced by abolition of cell adhesion has been demonstrated. This indicates that TIMP-1 is capable of rescuing cells from apoptosis without stabilising extracellular matrix and cell-matrix interactions. The independence of MMP-inhibition in inhibiting apoptosis is supported by the fact that reduced and alkylated TIMP-1, which has lost all MMP-inhibitory effect, still effectively inhibits apoptosis in Burkitt's lymphoma cell lines. The mechanism for this apoptosis-inhibitory effect is not known at present, but different suggestions have been made regarding signalling pathways possibly regulated by TIMP-1. Over-expression of TIMP-1 in human breast epithelial cells is associated with more efficient activation and constitutive activity of focal adhesion kinase (FAK) – a kinase that is normally involved in signalling cell survival. Also, up-regulation of TIMP-1 protein expression in Burkitt's lymphoma cells increased the expression of the anti-apoptotic protein Bcl-X_L. It was speculated that the modulation of cell signalling is mediated via interaction of TIMP-1 with a cell surface receptor as the anti-apoptotic effect of TIMP-1 in Burkitt's lymphoma cells was abolished by the neutralisation of secreted TIMP-1 by monoclonal antibodies. This view is further supported by a study that demonstrates binding of TIMP-1 to CD63 located on the surface of breast epithelial cells.

Accordingly, TIMP-1 appears to be capable of inhibiting apoptosis via two different mechanisms. Through inhibition of MMPs, TIMP-1 stabilises extracellular matrix and cell-matrix interactions thereby inhibiting apoptosis induced by disintegration of the extracellular matrix. However, TIMP-1 also inhibits apoptosis via a mechanism that is not dependent of its ability to inhibit proteolytic degradation of the extracellular matrix. This latter mechanism may be mediated by the interaction of TIMP-1 with a receptor on the cell surface regulating intracellular signalling pathways involved in apoptosis.

Two clinical studies by the inventors have suggested predictive value of TIMP-1 protein measurements (Schrohl et al., 2006 and Sorensen et al. 2007). In the study by Schrohl et al, TIMP-1 protein was measured in breast cancer extracts using ELISA. The authors describe that high TIMP-1 protein levels are associated with lack of response to chemotherapy in patients with metastatic breast cancer. In the study by Sorensen et al., the authors describe the predictive value of plasma TIMP-1 protein levels determined by ELISA. The results of this study shows that patients with metastatic colorectal cancer and high plasma TIMP-1 levels have a decreased objective response rate and a decreased survival following treatment with irinotecan based chemotherapy as compared to patients with low TIMP-1 protein levels in plasma. These two studies are in line with preclinical data generated by the inventor showing increased sensitivity to chemotherapy in cancer cells made deficient for the TIMP-1 gene (Davidsen et al. 2006).

Topoisomerase IIα

The *TOP2A* gene is located on chromosome 17q21, in the same amplicon as *HER2*, where it codes for the enzyme topoisomerase IIα. This enzyme is involved in the regulation of DNA topology and is important for the integrity of the genetic material during transcription, replication and recombination processes. During these processes topoisomerase IIα catalyzes the breakage and reunion of double stranded DNA. The expression of the topoisomerase IIα is cell cycle dependent with markedly higher levels in exponentially growing than in quiescent cell lines. It has been shown that the amount of the enzyme correlates with cell proliferation The predominant genetic mechanism for oncogene activation is through amplification of genes that leads to protein over-expression and provides the

tumor with selective growth advantages. Amplification of the *TOP2A* gene has been reported in 7-14% of patients with breast cancers and deletions with a similar frequency. In comparison, the *HER2* oncogene is amplified in 20-30% of the breast cancer patients (Harris et al. 2002).

Topoisomerase IIα is the pharmacological target of anthracyclines and several studies have shown that *TOP2A* gene aberrations, especially amplification, are predictive to the response to anthracycline based chemotherapy in patients with primary breast cancer (Park et al. 2003, Press et al. 2005, Tanner et al. 2005, Knoop et al. 2005). Fewer data are available with respect to patients with *TOP2A* deletions but a better treatment outcome for this group of patients has been observed as well. However, analysing for *TOP2A* amplifications or deletions will only identify approximately 20% of the breast cancer patient population as being anthracycline sensitive. This number should be seen in the context of the estimated 50% of high-risk breast cancer patients having benefit from adjuvant anthracyclines.

In one study a significant association between *TOP2A* amplification and topoisomerase IIα protein was found. Over-expression of topoisomerase IIα protein was present in 93% of the cases with amplification of *TOP2A*. However, the other way around, only 20% of cases with over- expression had amplification. Other studies have failed to show a similar correlation (Petit et al. 2004, Mueller et al. 2004, Durbecq et al. 2004).

Jørgensen et al. discloses a review of the pharmadiagnostic possibilities with respect to therapy selection in breast cancer including the predictive value of *TOP2A* and *HER-2* gene aberrations. The review states that a number of clinical studies have shown that patients who have tumours with *TOP2A* gene aberrations, especially amplifications, experience a significantly better effect from anthracycline-based chemotherapy that patients with normal *TOP2A* gene status. WO 2007/112746 discloses a method for performing a prognostic evaluation for high-risk breast cancer patients using *TOP2A* gene aberrations. The method for performing the prognostic evaluation comprises the steps of determining the status of an aberration of the *TOP2A* gene and estimating the probability of either recurrence-free survival or of overall survival of the patient at a later time based

upon a predefined Hazard Ratio or a pre-determined Kaplan-Meier plot corresponding to the determined status. It is well known that the term prognosis covers the fate of the disease in an untreated patient and prognostic evaluation is thus not the same as predictive evaluation, the latter term covering the likelihood of a patient to benefit from a specific treatment.

Summary of the invention

Thus, as it appears from the above, there is a need in the art for additional predictive markers that can identify additional patients that will benefit from anthracycline treatment.

Thus, an object of the present invention relates to improvement of patient selection for treatment with a topoisomerase IIα inhibitor therapy such as a topoisomerase IIα inhibitor therapy comprising an anthracycline.

In particular, it is an object of the present invention to provide a method that solves the above mentioned problems of the prior art with identifying a relevant proportion of breast cancer patients in whom topoisomerase IIα inhibitor therapy will have a high likelihood of being effective.

Thus, one aspect of the invention relates to a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a. determining in a sample obtained from said individual, the absence of TIMP-1 protein in tumour cells comprised in said sample or presence of a TIMP-1 DNA aberration in the tumour cells of said sample, and

b. determining the presence of any chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 or aberrant protein expression of a gene comprised in said amplicon

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 is present and/or the protein expression of the gene comprised in said amplicon is aberrant in said tumour cells and/or if the tumour cells are absent of TIMP-1 protein and/or if said tumour cells comprise said *TIMP-1* DNA aberration on either or both of the alleles of the *TIMP-1* gene, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no chromosomal DNA aberration in the *TOP2A/HER2* amplicon is present or no protein encoded by any gene comprised in said amplicon is aberrantly expressed in the tumour cells and if TIMP-1 protein is present in the tumour cells and/or if neither of the *TIMP-1* alleles comprise said *TIMP-1* DNA aberration.

A second aspect relates to a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a. determining in a sample obtained from said individual, the absence of TIMP-1 protein in tumour cells comprised in said sample, and

b. determining the presence of any *TOP2A* DNA aberration in the tumour cells of said sample

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a *TOP2A* DNA aberration is present and/or if the tumour cells are absent of TIMP-1 protein, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no *TOP2A* DNA aberration is present and if TIMP-1 protein is present in the tumour cells.

In one embodiment the cancer is selected from the group consisting of breast cancer, sarcomas, ovarian cancer, and lung cancer. In a preferred embodiment the cancer is breast cancer.

Another aspect of the present invention relates to a method of treating cancer in an individual comprising

a. predicting the response to an topoisomerase IIα inhibitor therapy according to any of the preceeding claims, and

b. selecting a topoisomerase IIα inhibitor therapy to which said individual has a high likelihood of responding to,

c. subjecting to said individual said topoisomerase IIα inhibitor therapy.

In one embodiment, the topoisomerase IIα inhibitor used in said method of treatment is an anthracyclines such as 4-Epirubricin, which in a further embodiment is used in combination with cyclophosphamide and 5-fluorouracil or a taxane.

Yet another aspect of the present invention is to provide a kit for predicting the response to a topoisomerase IIα inhibitor therapy comprising

a. reagents suitable for the determination of a chromosomal DNA aberration in the *TOP2A/HER2* amplicon such *TOP2A* or *HER2* DNA aberrations in a biological sample, and

b. reagents suitable for the determination of a *TIMP-1* DNA aberration or determining the level of a TIMP-1 protein in a biological sample.

Detailed description of the invention

It is well known that measurement of *TOP2A* DNA aberrations in beast cancer cells can predict benefit from adjuvant anthracycline containing chemotherapeutic drug regimes (Knoop et al JCO 2005). However, since only approximately 20% of primary breast cancer patients will display *TOP2A* DNA aberrations in their tumor

cells, this method only allows for identification of 20% of the breast cancer population who have an increased likelihood of benefit from adjuvant anthracyline treatment. This number should bee seen in the light of approximately 50% of primary breast cancer patient knowing to benefit from anthracycline treatment.

A number of other potential predictive markers have been combined with the *TOP2A* DNA aberration measurements, e.g. *HER2*, but no additive effect between these two biomarkers has been seen with regard to disease free survival or overall survival in the identified subgroup (Knoop et al., JCO 2005). Thus, at present, there is no biomarker (DNA, mRNA or protein) that has shown superior prediction to benefit from anthracycline treatment when combined with *TOP2A* DNA aberrations than *TOP2A* DNA aberration measurements alone. This is further supported by O'Malley et al. 2009 that shows that combining *TOP2A* and *HER* DNA measurements does not improve the predictive value above what can be obtained by each of the two markers.

It has previously been reported that high tumour extract protein levels of TIMP-1 protein in primary tumours derived from patients with metastatic breast cancer is associated with decreased likelihood of obtaining an objective response to chemotherapy (both anthracycline containing and not anthracycline containing drug combinations). This likelihood decreases with increasing expression of TIMP-1.The TIMP-1 protein was measured by ELISA (Schrohl et al., Clin Cancer Res 2006).

Sørensen et al. Clin Cancer Res 2007 pertains to the effect of chemotherapy on patients having metastatic colorectal cancer. In this study TIMP-1 is combined with CEA – the study shows that the combination with CEA does not provide any additive effect.

The inventors disclose for the first time that lack of TIMP-1 immunoreactivity in breast cancer cells is associated with likelihood of benefit from adjuvant anthracyline treatment but not non-anthracycline containing chemotherapy. In a retrospective study including 649 patients with high risk breast cancer, the inventors show that patients who's tumor cells lack TIMP-1 immunoreactivity are those who benefit the most from adjuvant anthracycline treatment as compared

with patients who's tumor cells lack TIMP-1 immunoreactivity and who receive adjuvant treatment with a non-anthracycline containing chemotherapy regimen (CMF) or patients who's tumor cells show TIMP-1 immunoreactivity and who receive adjuvant therapy with either anthracycline or non-anthracycline containing chemotherapy.

Thus, the present invention allows for the identification of high risk breast cancer patients with a high likelihood of benefit from adjuvant anthracycline treatment: Lack of TIMP-1 immunoreactivity in the breast cancer cells identifies app 20% of the patients who have a high likelihood of benefit from adjuvant anthracycline treatment. In practical terms, by TIMP-1 immunohistochemistry, it will be possible to identify app 20% of the patients scheduled for adjuvant treatment who will have a high likelihood of benefit from the treatment. On other hand, TIMP-1 immunohistochemistry also allows for the identification of app 80% of the patients who are scheduled for adjuvant anthracycline containing treatment who would do equally well by treatment with the much less toxic CMF. Alternatively, these 80% of the patients could be treated with any other active drug than anthracyclines, used in adjuvant treatment of breast cancer e.g. taxanes, Methotrexate, Cyclophosphamide, 5 Flourouracil and gemcitabine (Example 1).

The inventors report for the first time that the combination of TIMP-1 breast cancer cell immunoreactivity measurements and *TOP2A* DNA aberration measurements in the same tumor cells yields additive predictive value, i.e. each of the two tests identify approximately 20% of patients having a high likelihood of obtaining benefit from adjuvant anthracyline containing chemotherapy, and since there is only 4% overlap between the two patient populations, the effect of the combined assay is additive.

Thus, the present invention allows for the identification of almost double as many breast cancer patients with a high likelihood of benefit from adjuvant anthracycline treatment: *TOP2A* DNA aberration measurements identifies app 20% and lack of TIMP-1 immunoreactivity assay identifies app 20% of the patients who have a high likelihood of benefit from adjuvant anthracycline treatment. In practical terms, by the combined assay, it will be possible to identify app 40% of the patients scheduled for adjuvant treatment who will have a high

likelihood of benefit from the treatment. On other hand, the combined assay also allows for the identification of app 60% of the patients who are scheduled for adjuvant anthracycline containing treatment who would do equally well by treatment with the much less toxic CMF. Alternatively, these 60% of the patients could be treated with any other active drug than anthracyclines, used in adjuvant treatment of breast cancer e.g. taxanes, Methotrexate, Cyclophosphamide, 5 Flourouracil and gemcitabine (Example 3).

The present inventors recently found *TIMP-1* gene aberrations (deletions and amplifications) in breast cancer cells.

The present application discloses a study of *TOP2A* gene aberrations and TIMP-1 protein tumor cell content in 641 breast cancer patients who were randomized to receive adjuvant treatment with either Cyclophosfamide, Methotrexate and 5-fluorouracil (CMF) or Cyclophosfamide, 4-Epirubricin and 5-Fluorouracil (CEF). Endpoint was disease free survival (DFS). As previously reported on this patient cohort (Knoop et al), *TOP2A* aberrations were predictive for benefit (increased DFS) from CEF but not from CMF. When performing TIMP-1 immunohistochemistry using the VT7 anti TIMP-1 monoclonal antibody the inventor found that approximately 80% of the patients showed TIMP-1 immunoreactivity in the tumor cells. The remaining 20% of the tumors were absent of TIMP-1 tumor cell immunoreactivity. When performing statistical survival analyses, it was found that lack of TIMP-1 immunoreactivity in the tumor cells was significantly associated to the end-point: DFS, with a longer DFS of the patients. In contrast, no differences in DFS in relation to TIMP-1 immunoreactivity were observed in patients receiving CMF.

When combining the results of the *TOP2A* and TIMP-1 analyses, it was seen that these two biomarkers were additive in predicting response to CEF while no effect of the combination of these two biomarkers were observed in the CMF treated patients. The additive effect was based on the fact that there was only a very little overlap between the patients having *TOP2A* gene aberrations and patients lacking TIMP-1 immunoreactivity in their tumor cells (4% overlap). Since the two groups were almost identical in size, the combination of these two biomarkers doubled

the number of patients that could be predicted as CEF responders without loosing power of the predictive value of each of the biomarkers.

This means that by the use of the combined *TOP2A* and TIMP-1 test, patients who will benefit the most from adjuvant anthracycline treatment can be identified. On the other hand, the combined test can also be used to identify the approximately 60% of patients who would do equally well by receiving a non-anthracycline containing chemotherapy regimens or perhaps even better by receiving another drug combination., e.g. combinations including taxanes. This invention should be seen in the light of lack of additive effect when combining *TOP2A* with *HER2* (Knoop et al 2005) and lack of additive effect when combing TIMP with CEA in colorectal cancer drug prediction (Sørensen et al., 2007)

In order to extend the available methods for performing prediction of therapy effectiveness for breast cancer patients, beyond what is presently available in the art, novel methods for performing such prediction are herein disclosed, wherein the prediction is based upon the determined status of *TOP2A* gene aberrations (wherein the term "status" refers to the presence or absence of an aberration and, if an aberration is present, the type — amplification or deletion — of the aberration) or TOP2A protein together with determination of TIMP-1 protein or *TIMP-1* DNA aberrations in the tumor cells. Embodiments in accordance with the invention may comprise the steps of determining the status of an aberration of the *TOP2A* gene together with the TIMP-1 gene or protein status in a breast cancer tissue sample taken from a patient; and based on the results of such testing one can estimate for the individual patient the likelihood of obtaining benefit from anthracycline containing chemotherapy as compared to non-anthracycline containing chemotherapy.

For example, patients with *TOP2A* aberrations and/or absence of TIMP-1 immunoreactivity in the cancer cells should be offered chemotherapy containing anthracyclines, while the remaining patients will do equally well receiving anthracyclines or non-anthracyclines. Based on the severe toxicity of anthracyclines, it would be correct to offer the latter patients a non-anthracycline containing chemotherapy regimen.

The presently presented methods thus rely on the surprising discovery that it is possible to almost double the predictive value of *TOP2A* determinations in breast cancer patients by adding the analysis of TIMP-1 tumor cell immune reactivity in the breast cancer cells.

The invention is based on a method for predicting whether a cancer patient will benefit from an anti-cancer therapy, where the efficiency of said anti-cancer therapy depends on tumour tissue *TOP2A gene* aberrations in the tumor cells combined with absence of TIMP-1 immunoreactivity in the cancer cells, the method comprising determining whether cells from tumour tissue in the patient have *TOP2A* gene aberrations or lack TIMP-1 immunoreactivity, and establishing that the patient most likely will benefit from a specific anti-cancer therapy if *TOP2A* DNA aberrations or lack of TIMP-1 immunoreactivity is observed.

In the present application the anti-cancer therapy preferably refers to a topoisomerase II inhibitor therapy.

The prediction method of the invention preferably comprises that the determination of whether cells from tumour tissues in the patient have *TOP2A* gene aberrations and/or lack TIMP-1 immunoreactivity is performed by measuring on a sample selected from the group consisting of a tumour tissue sample, a blood sample, a plasma sample, a serum sample, a urine sample, a faeces sample, a saliva sample, and a sample of serous liquid from the thoracic and abdominal cavity. The method of measuring is conveniently performed by means of DNA level measurement, mRNA level measurement such as in situ hybridization, Northern blotting, QRT-PCR, and differential display, and protein level measurement, such as Western blotting, immunohistochemistry, immunocytochemisty, ELISA, and RIA.

One can perform a retrospective/prospective clinical trial, in order to establish the threshold level for TIMP-1 protein so as to determine resistance/sensitivity to topoisomerase IIα inhibitor treatment of the individual patient.

Retrospectively, stored tumour tissue or blood or urine, or saliva or any other body fluid is obtained from patients who have experienced recurrence of their

cancer disease and of whom it is known how they responded to the particular anti-cancer treatment. In the case of tumour tissue extracts, the tissue is homogenized and the level of TIMP-1 protein is measured in each individual patient sample. In the case of body fluids, the sample may be diluted and subsequently, the concentration of TIMP-1 protein is determined by one of the methods discussed herein. In the case of formalin fixed paraffin embedded tumor tissue, conventional immunohistochemistry can be performed either on the primary tumor or on tissue obtained from metastatic lesions.

Accordingly, one aspect of the present invention relates to a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a) determining in a sample obtained from said individual, the absence of TIMP-1 protein in tumour cells comprised in said sample or presence of a *TIMP-1* DNA aberration in the tumour cells of said sample, and

b) determining the presence of any chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 or aberrant protein expression of a gene comprised in said amplicon

c) classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 is present and/or the protein expression of the gene comprised in said amplicon is aberrant in said tumour cells and/or if the tumour cells are absent of TIMP-1 protein and/or if said tumour cells comprise said *TIMP-1* DNA aberration on either or both of the alleles of the *TIMP-1* gene, and

d) classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no chromosomal DNA aberration in the *TOP2A/HER2* amplicon is present or no protein encoded by any gene comprised in said amplicon is aberrantly expressed in the tumour cells and if TIMP-1 protein is present in the tumour cells and/or if neither of the TIMP-1 alleles comprise said *TIMP-1* DNA aberration.

The *TOP2A/HER2* amplicon on chromosome 17q21 referred to above comprises the *TOP2A* and *HER2* genes.

Thus, one embodiment according to the invention, concerns predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, wherein the chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 is a *TOP2A* DNA aberration, and the protein expression of the gene comprised in said amplicon is topoisomerase IIa expression.

Another embodiment according to the invention concerns predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, wherein the chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 is a *HER2* DNA aberration, and the protein expression of the gene comprised in said amplicon is ErbB2 expression.

In a preferred embodiment said method comprising the steps of:

a. determining in a sample obtained from said individual, the absence of TIMP-1 protein in tumour cells comprised in said sample, and

b. determining the presence of any *TOP2A* DNA aberration in the tumour cells of said sample

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a *TOP2A* DNA aberration is present and/or if the tumour cells are absent of TIMP-1 protein, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no *TOP2A* DNA aberration is present and if TIMP-1 protein is present in the tumour cells.

One embodiment of the present invention is a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a. determining in a sample obtained from said individual, the absence of TIMP-1 protein in tumour cells comprised in said sample, and

b. determining the presence of any *HER2* DNA aberration in the tumour cells of said sample

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a *HER2* DNA aberration is present and/or if the tumour cells are absent of TIMP-1 protein, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no HER2 DNA aberration is present and if TIMP-1 protein is present in the tumour cells.

One embodiment of the present invention is a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a. determining in a sample obtained from said individual, the presence of a *TIMP-1* DNA aberration in the tumour cells of said sample, and

b. determining the presence of any *TOP2A* DNA aberration in the tumour cells of said sample

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a *TOP2A* DNA aberration is present and/or if said tumour cells comprise said *TIMP-1* DNA aberration on either or both of the alleles of the *TIMP-1* gene, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no *TOP2A* DNA aberration is present and if neither of the *TIMP-1* alleles comprise said *TIMP-1* DNA aberration.

Another embodiment of the present invention is a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a. determining in a sample obtained from said individual, the presence of a *TIMP-1* DNA aberration in the tumour cells of said sample, and

b. determining the presence of any *HER2* DNA aberration in the tumour cells of said sample

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a *HER2* DNA aberration is present and/or if said tumour cells comprise said *TIMP-1* DNA aberration on either or both of the alleles of the *TIMP-1* gene, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no *HER2* DNA aberration is present and if neither of the *TIMP-1* alleles comprise said *TIMP-1* DNA aberration.

The *TOP2A* and *HER2* genes are both located in the *TOP2A/HER2* amplicon on chromosome 17q21 while the *TIMP-1* gene is located on chromosome X.

The methods provide a means of identifying, without reducing the hazard ratio, almost twice the number of cancer patients compared to conventional methods who have a high likelihood of benefiting from an anti-cancer therapy such as CEF treatment.

In one embodiment according to the invention, the sample comprising the biomarkers (HER2, TOP2A and TIMP-1) is selected from the group consisting of a tumour tissue sample, a blood sample, a plasma sample, a serum sample, a urine sample, a faeces sample, a saliva sample, and a sample of serous liquid from the thoracic or abdominal cavity and a combination hereof.

One embodiment of the invention relates to a method for predicting the response to an anti-cancer therapy in an individual having a cancer selected from the group consisting of breast cancer, sarcomas, ovarian cancer and lung cancer.

In one embodiment the sarcomas may be soft tissue sarcomas.

In another embodiment the lung cancer may be non small cell lung cancer.

In one preferred embodiment the present invention pertains to a method for predicting the response to an anti-cancer therapy in an individual having a breast cancer.

Methods of measuring DNA aberrations

Aberrations relating to DNA aberrations may be determined by means of DNA measurement such as but not limited to *in situ* hybridization, a PCR method, differential display, DNA-dot-blotting, Southern blotting or combinations hereof.

Thus in one embodiment, the level of DNA gene aberration is determined by means of DNA measurement such as but not limited to *in situ* hybridization, a PCR method, differential display, DNA-dot-blotting, Southern blotting or combinations hereof.

In a preferred embodiment, said in situ hybridization is determined by means of FISH (Fluorescent In-Situ Hybridization).

In yet a preferred embodiment, DNA aberrations are determined by FISH comprising the use of a probe mixture comprising labeled DNA probes targeted at a portion of the *TOP2A* gene region, and/or the *HER2* gene region, and/or a portion of the *TIMP-1* gene region and a probe mixture comprising fluorescein-labelled probes targeted at the centromeric region of chromosome 17 and the X chromosome, respectively.

Aberrations relating to protein expression aberrations may be determined by means of Western blotting, Immunohistochemistry, ELISA, or RIA.

Thus in one embodiment, aberrant protein expression is determined by means of protein level measurement such as Western blotting, Immunohistochemistry, Immunocytology, ELISA, and RIA.

DNA aberrations and/or aberrant protein expression may also be reflected in the level of RNA such as mRNA transcripts of the gene in questions for example aberrant splicing of the primary transcript resulting in non-functional transcripts.

Thus a DNA aberration resulting in a RNA aberration may be determined by means of RNA such as mRNA measurement such as but not limited to Northern blotting, RNA dot and a quantitative PCR method.

Thus in one embodiment, the DNA aberration or a protein expression in the tumour cells correlate with aberrant mRNA levels in the tumour cells of said sample.

DNA aberrations

DNA aberrations refer to any DNA aberrations within a chromosome including specific regions of a chromosome such as an amplicon, and any DNA aberrations within a gene or region of a gene. DNA aberrations comprise DNA amplification, DNA deletion, gene point mutation, and translocation, epigenetic modifications of DNA such as DNA methylation, and combinations hereof. DNA aberrations comprise any DNA aberration resulting in downstream aberrant transcription of said DNA or protein expression of a protein encoded by said DNA. DNA aberrations in the meaning of deletion or amplification refer to deletion or amplification or entire gene or a part of said gene. Epigenetic aberrations may lead to silencing of the gene in question and is reflected in absence of the protein encoded by said gene or at least aberrant protein expression.

Thus, one embodiment relates to a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, comprising the determination of *TOP2A* gene aberration, wherein said gene aberration is selected from the group consisting of *TOP2A* DNA amplification, *TOP2A* DNA deletion, *TOP2A* gene point mutation, and *TOP2A* DNA translocation, epigenetic

modifications of the *TOP2A* DNA such as DNA methylation, and combinations hereof.

In a particular embodiment, the *TOP2A* DNA aberration or the increase in topoisomerase IIα protein in the tumour cells correlate with aberrant TOP2A mRNA levels in the tumour cells of said sample.

A further embodiment relates to a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, comprising the determination of *HER2* gene aberration, wherein the *HER2* gene aberration is selected from the group consisting of *HER2* gene amplification, *HER2* DNA deletion, *HER2* gene point mutations and *HER2* DNA translocations, epigenetic modifications of the *HER2* DNA such as DNA methylation, and combinations hereof.

In a particular embodiment, the *HER2* DNA aberration or an increase in ErbB2 protein in the tumour cells correlate with aberrant *HER2* mRNA levels in the tumour cells of said sample.

A further embodiment relates to a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, comprising the determination of *TIMP-1* gene aberration, wherein the tumour cells comprise at least one *TIMP-1* DNA aberration resulting in lack of TIMP-1 protein expression selected from the list consisting of a deletion of one of the *TIMP-1* alleles, a deletion of both of the *TIMP-1* alleles, a partial deletion of one of the *TIMP-1* alleles, a partial deletion of both of the *TIMP-1* alleles, *TIMP-1* DNA point mutations, *TIMP-1* DNA inversion, *TIMP-1* DNA translocation, epigenetic modifications of the *TIMP-1* DNA such as DNA methylation, and combinations hereof.

In a particular embodiment, the any *TIMP-1* DNA aberration or absence of TIMP-1 protein in the tumour cells correlate with aberrant TIMP-1 mRNA levels in the tumour cells of said sample such as absence of TIMP-1 mRNA in said sample.

In the present context the term "absence of TIMP-1 protein" is to be understood as total lack of TIMP-1 immunoreactivity in the cancer cells and/or the tumor tissue stromal cells. It should be stated however, that patients with weak TIMP-1 immunoreactivy in their cancer cells and/or the tumor tissue stromal cells have more benefit from anthracyclines than patients with stronger TIMP-1 immunoreactivity in their cancer cells and/or the tumor tissue stromal cells, while these patients with weak TIMP-1 immunoreactivity have less benefit from anthracycline treatment than patients with total absence of TIMP-1 immunoreactivity in their cancer cells and/or the tumor tissue stromal cells. Evaluation of TIMP-1 immunoreactivity (number of positive cells and/or intensity) can be evaluated by simple microscopy but can also be objectively estimated by a digitized analyser.

The cells are classified as 0, +1, +2 and +3. 0 is to be understood as the cancer cells and/or the tumor tissue stromal cells absent in TIMP-1 immunoreactivity, +1 is to be understood as the cancer cells and/or the tumor tissue stromal cells having week TIMP-1 immunoreactivity. +2 is to be understood as the cancer cells and/or the tumor tissue stromal cells having TIMP-1 immunoreactivity. +3 is to be understood as the cancer cells and/or the tumor tissue stromal cells having strong TIMP-1 immunoreactivity.

The method of classifying and differentiating TIMP-1 immunoreactivity is in an embodiment of the invention objectively evaluated. The evaluation is based on the number of TIMP-1 immunoreactive cells (cancer and/or tumor tissue stromal cells) and/or the intensity of the immunoreactivity. Evaluation of TIMP-1 immunoreactivity (number of positive cells and/or intensity) can be evaluated by simple microscopy but can also be objectively estimated by a digitized analyser.

Thus, in a preferred embodiment of the present invention cancer cells and/or tumor tissue stromal cells are absent in TIMP-1 if the immunoreactivity is below +1, such as below +0.9, e.g. below +0.8, such as below +0.7, e.g. below 0.6, such as below 0.5, e.g. below 0.4, such as below 0.3, e.g. below 0.2, such as below 0.1.

Thus, in a preferred embodiment of the present invention cancer cells and/or tumor tissue stromal cells are absent in TIMP-1 if the immunoreactivity is 0.

Thus, in a preferred embodiment of the present invention a patient is likely to benefit from anthracyclines (e.g. topoisomerase IIα) if the level of TIMP-1 immunoreactivity is below +2, such as below +1.9, e.g. below +1.8, such as below +1.7, e.g. below 1.6, such as below 1.5, e.g. below 1.4, such as below 1.3, e.g. below 1.2, such as below +1, such as below +0.9, e.g. below +0.8, such as below +0.7, e.g. below 0.6, such as below 0.5, e.g. below 0.4, such as below 0.3, e.g. below 0.2, such as below 0.1. Preferably in the range from 0 - +2, e.g. in the range from 0.1 – +1,5, such as in the range from +0.5 - +1.2, e.g. in the range from 0 – +0.5, such as in the range from 0 - +1.

In a preferred embodiment a patient is likely to benefit from anthracyclines (e.g. topoisomerase IIα inhibitor) if the level of TIMP-1 immunoreactivity is below +1, such as below +0.9, e.g. below +0.8, such as below +0.7, e.g. below 0.6, such as below 0.5, e.g. below 0.4, such as below 0.3, e.g. below 0.2, such as below 0.1.

In a preferred embodiment a patient is likely to benefit from anthracyclines (e.g. topoisomerase IIα inhibitor) if the level of TIMP-1 protein is 0.

It is to be understood that TIMP-1 immunoreactivity resembles the amount of TIMP-1 protein present in the cancer cell and/or the tumor tissue stromal cell.

In another embodiment the *TIMP-1* gene is more than 1.1 fold amplified relative to a reference sample, such as more than 1.2 fold, e.g. more than 1.3 fold, such more than 1.4 fold, e.g. more than 1.5 fold, such as more than 1.6 fold, e.g. more than 1.7 fold, such as more than 1.8 fold, e.g. more than 1.9 fold, such as more than, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a reference sample. In an embodiment of the present invention the *TIMP-1* gene is between 1.1 - 2.0 amplified relative to a reference sample, such

as in the range from 1.2 – 1.9, e.g. in the range from 1.3 – 1.8, such as in the range from 1.4 – 1.7, e.g. in the range from 1.5 – 1.7, such as in the range from 1.7 – 1.9, e.g. in the range from 1.8 – 1.9 amplified relative to a reference sample.

In another embodiment the *TOP2A* gene is more than 1.1 fold amplified relative to a reference sample, such as more than 1.2 fold, e.g. more than 1.3 fold, such more than 1.4 fold, e.g. more than 1.5 fold, such as more than 1.6 fold, e.g. more than 1.7 fold, such as more than 1.8 fold, e.g. more than 1.9 fold, such as more than, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a reference sample. In an embodiment of the present invention the *TOP2A* gene is between 1.1 - 2.0 amplified relative to a reference sample, such as in the range from 1.2 – 1.9, e.g. in the range from 1.3 – 1.8, such as in the range from 1.4 – 1.7, e.g. in the range from 1.5 – 1.7, such as in the range from 1.7 – 1.9, e.g. in the range from 1.8 – 1.9 amplified relative to a reference sample.

In another embodiment the *HER2* gene is more than 1.1 fold amplified relative to a reference sample, such as more than 1.2 fold, e.g. more than 1.3 fold, such more than 1.4 fold, e.g. more than 1.5 fold, such as more than 1.6 fold, e.g. more than 1.7 fold, such as more than 1.8 fold, e.g. more than 1.9 fold, such as more than, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a reference sample. In an embodiment of the present invention the *HER2* gene is between 1.1 - 2.0 amplified relative to a reference sample, such as in the range from 1.2 – 1.9, e.g. in the range from 1.3 – 1.8, such as in the range from 1.4 – 1.7, e.g. in the range from 1.5 – 1.7, such as in the range from 1.7 – 1.9, e.g. in the range from 1.8 – 1.9 amplified relative to a reference sample.

Aberrant protein expression

Aberrant protein expression refers to any aberration in the protein expression such as the level of said protein, absence of said protein, dysfunctions in terms of functionality for example a mutation causing a non-functional protein, dysfunctions in terms of cellular localisation of said protein.

Absence usually refers to the absence of detectable protein in a sample or in tumour cells of said sample.

In one embodiment, the aberrant protein expression is determined as fold over a reference level of a control sample. In another embodiment, the aberrant protein expression is determined as fold under a reference level.

In a second embodiment relating to aberrant topoisomerase IIα protein expression, topoisomerase IIα protein is more than 2 fold over-expressed relative to a reference sample, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a reference sample.

In a second embodiment relating to aberrant ErbB2 protein expression, ErbB2 protein is more than 2 fold over-expressed relative to a control sample, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a control sample.

In a preferred embodiment of the present invention is a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, wherein the tumour cells are absent of TIMP-1 protein.

Reference

The "reference" refers to any suitable reference such as corresponding measurements on a pool of corresponding biological sample from a non-cancer individual or to non-malignant cells in a tumor, e.g. tumor tissue stromal cells.

A method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, wherein a reference obtained from a population is used to determine the level of DNA aberration or protein expression.

Said reference may be used to set the baseline of a signal such as TIMP-1, ErbB2, or topoisomerase IIα immunoreactivy in a sample in order to determine whether TIMP-1, ErbB2, or topoisomerase IIα protein is aberrantly expressed in a sample such as a sample applied to an ELISA assay.

In a particular embodiment, the reference is used to set a baseline/cut-off value for determining the presence or absence of TIMP-1 protein in a sample such as determining the presence or absence of TIMP-1 protein by means of Western blotting, Immunohistochemistry, ELISA, flow cytometry, or RIA.

In one embodiment the reference is selected from the group consisting of intra-sample, inter-sample and internal reference.

One example of a method according to the invention comprising the determination of DNA aberrations of a gene in question, wherein a reference is included targeting to the same chromosome. Thus for a DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21, such a DNA aberration in the *TOP2A* gene or *HER2* gene, a reference targeting the centromeric of region of chromosome 17 may be used to determine whether an allele of the gene in question has been deleted or amplified.

Accordingly one embodiment concerns a method for predicting the response to a topoisomerase IIα inhibitor therapy according to the invention, wherein a DNA aberration is determined by means of in situ hybridization such as FISH (Fluoroscent In-Situ Hybridization).

In another embodiment, said DNA aberration is determined as the average ratio to an internal reference sequence comprised in said sample. In one embodiment said internal reference is diploid non-malignant cells comprised in the samples for a tumor tissue sample. In a preferred embodiment of the present invention the tumour tissue sample is tumor tissue stromal cells.

In a further embodiment, the reference is the signal of a labelled probe such as a fluoroscein-labelled or Texas Red-5- labelled targeted at the centromeric region of chromosome 17 and/or the X chromosome. In a particular embodiment, the probe is a peptide nucleotide acid (PNA) based probe. This type of reference is suitable for FISH applications such as a FISH assays for determining a DNA aberration in *TOP2A/HER2* amplicon on chromosome 17q21, for example a DNA aberration in the *TOP2A* gene or *HER2* gene. In another embodiment, a similar type of reference is used in FISH assays for determining a DNA aberration in the *TIMP-1* gene.

The DNA aberration may be determined as the average ratio to a reference sequence comprised in said sample.

Thus, in one embodiment the DNA aberration is determined as the average ratio to an internal reference sequence comprised in said sample.

In one embodiment, the internal reference sequence is located on the centromeric region of chromosome 17.

In a particular embodiment, the internal reference sequence is chromosome X α-satellite (Cen X).

DNA aberrations such as DNA gene allele deletions or gene amplifications may be determined using ratios of the signal corresponding to binding of the gene specific probe versus the signal corresponding to binding of centromeric region probe of the reference probe.

Accordingly, in one embodiment, the tumour cells of the sample comprise a *TIMP-1* gene deletion if the average ratio of *TIMP-1*/ Cen X is below 0.8, and normal if

the said ratio is above 0.8 and below 2.0. In an embodiment of the present invention the average ratio of *TIMP-1*/ Cen X is below 0.7, e.g. below 0.6, such as below 0.5, e.g. below 0.4, such as below 0.3, e.g. below 0.2, such as below 0.1, e.g. in the range from 0.1 – 0.8, such as in the range from 0.2 – 0.7, e.g. in the range from 0.3 – 0.6, such as in the range from 0.4 – 0.5 and normal if the said ratio is above 0.8 and below 2.0.

In another embodiment, the tumour cells comprise *TOP2A* gene deletion if the average ratio of *TOP2A* / Cen X is below 0.8 or amplifications if the average ratio of *TOP2A* / Cen X is above 2.0, and normal if the said ratio is above 0.8 and below 2.0. In an embodiment of the present invention the average ratio of *TOP2A*/ Cen X is below 0.7, e.g. below 0.6, such as below 0.5, e.g. below 0.4, such as below 0.3, e.g. below 0.2, such as below 0.1, e.g. in the range from 0.1 – 0.8, such as in the range from 0.2 – 0.7, e.g. in the range from 0.3 – 0.6, such as in the range from 0.4 – 0.5 and normal if the said ratio is above 0.8 and below 2.0.

In third embodiment, the tumour cells comprise *HER2* gene deletion average ratio of TOP2A / Cen X is below 0.8 or amplifications if the average ratio of *HER2* / Cen X is above 2.0, and normal if the said ratio is above 0.8 and below 2.0.
In an embodiment of the present invention the average ratio of *HER2*/ Cen X is below 0.7, e.g. below 0.6, such as below 0.5, e.g. below 0.4, such as below 0.3, e.g. below 0.2, such as below 0.1, e.g. in the range from 0.1 – 0.8, such as in the range from 0.2 – 0.7, e.g. in the range from 0.3 – 0.6, such as in the range from 0.4 – 0.5 and normal if the said ratio is above 0.8 and below 2.0.

In another embodiment, a reference is used to determine the level of DNA aberration or protein expression. The said reference may be obtained from a population such as a population of non-cancer individuals, or a combined group of cancer individuals for example a group of CMF treated cancer individuals.

In yet another embodiment, said reference is a normal diploid genetic background.

For example, a suitable reference for determining the *TOP2A* DNA aberration level in the meaning *TOP2A* DNA gene amplifications, or *TOP2A* DNA gene deletions, is

the average signal from *TOP2A* DNA alleles in a corresponding biological sample from a non-cancer individual or the average signal in the non-malignant cells in said tumor sample.

In one embodiment, the determination of DNA or protein aberrations is performed on archive material from the individual, such as a paraffin block comprising tumour tissue.

The topoisomerase IIα inhibitor therapy

In one embodiment, the topoisomerase IIα inhibitor therapy comprises the administration of a composition comprising a least one topoisomerase IIα inhibitor to the individual with a cancer. In a preferred embodiment the composition used for the topoisomerase IIα inhibitor therapy comprises at least one anthracycline selected from the group consisting of 4-Epirubricin, Daunorubicin, Daunorubicin (liposomal), Doxorubicin, Doxorubicin (liposomal), Epirubicin, Idarubicin, and Mitoxantrone.

The topoisomerase IIα inhibitor may be administrated either alone or in combination with at least one other chemotherapeutic. In one embodiment according to the invention the topoisomerase IIα inhibitor therapy is CEF treatment, wherein CEF refers to Cyclophosfamide, 4-Epirubricin and 5-Fluorouracil. In yet another embodiment topoisomerase IIα inhibitor therapy is treatment with cyclophosphamide, taxanes and/or 5-fluorouracil in addition to a topoisomerase IIα inhibitor.

Any of the compounds used in the topoisomerase IIα inhibitor therapy may be administered as a prodrug. Thus, in one embodiment at least one of the drugs selected from the group consisting of cyclophosphamide, taxanes, 5-fluorouracil topoisomerase IIα inhibitor such as an anthracycline is in the form of a prodrug of said drug.

The topoisomerase IIα inhibitor therapy may be liposome encapsulated.

In one embodiment the topoisomerase IIα inhibitor therapy comprises an inducer of apoptosis or mitotic catastrophe.

In another embodiment the topoisomerase IIα inhibitor therapy is selected from the group consisting of neoadjuvant therapy, adjuvant therapy and therapy of metastatic disease.

Method of treating cancer

Another aspect of the invention relates to the treatment of cancer based on the prediction of the likelihood of responding to a topoisomerase IIα inhibitor therapy.

Said aspect concerns a method of treating cancer in an individual comprising

a. predicting the response to a topoisomerase IIα inhibitor therapy according to any of the preceeding claims,

b. selecting a topoisomerase IIα inhibitor therapy to which said individual has a high likelihood of responding to, and

c. subjecting to said individual to said topoisomerase IIα inhibitor therapy.

In one embodiment of said method of treatment, the topoisomerase IIα inhibitor is a anthracyclines selected from the group consisting of but not limited to 4-Epirubricin, Daunorubicin, Daunorubicin (liposomal), Doxorubicin, Doxorubicin (liposomal), Epirubicin, Idarubicin, and Mitoxantrone, or a combination hereof.

In a further embodiment the topoisomerase IIα inhibitor therapy is comprised in a composition further comprising cyclophosphamide and 5-fluorouracil.

In a further embodiment the topoisomerase IIα inhibitor therapy is comprised in a composition further comprising a taxane.

Kit

A third aspect of the present invention relates to a kit for predicting the response to a topoisomerase IIα inhibitor therapy comprising:

a. reagents suitable for the determination of a chromosomal DNA aberration in the *TOP2A/HER2* amplicon such as *TOP2A* or *HER2* DNA aberrations in a biological sample, and

b. reagents suitable for the determination of a *TIMP-1* DNA aberration or determining the level of a TIMP-1 protein in a biological sample.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

Hazard ratio

"Hazard ratio" (HR) refers to likelihood of obtaining benefit such as prolonged disease free survival from a treatment such as a topoisomerase IIα inhibitor therapy.

In one embodiment of the present invention HR describes the likelihood of having benefit from CEF treatment with the benefit from CMF treatment as the reference. A HR of 1 means no difference between the group receiving the treatment and the reference group. Accordingly, a HR of 0.5 means that the CEF treated patients have 50% reduced risk of experiencing a relapse as compared to CMF treated patients. Confidence intervals may be included to improve the statistic power of the evaluation.

Table 1 of Example 1 exemplifies the use of hazard ratios in order to evaluate likelihood of obtaining benefit from a treatment such as a topoisomerase IIα inhibitor therapy. The HR of the reference group (in this case CMF treated patients) is set to 1.

Accordingly, a preferred embodiment of the present inventions relates to a method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, wherein the likelihood of responding to a topoisomerase IIα inhibitor therapy is determined by means of a hazard ratio.

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

"Anti-cancer therapy" is a term used for any non-surgical therapeutic regimen that aims at curving or alleviating cancer. Examples are set forth below but anti-cancer therapy can be both chemotherapeutic and/or radiotherapeutic and/or anti-hormonal and/or biological therapy.

"A topoisomerase IIα inhibitor therapy" refers to chemotherapeutic anti-cancer therapy comprising the use of at least one topoisomerase IIα inhibitor. A topoisomerase IIα inhibitor may be administrated in combination with other chemotherapeutic drugs such as cyclophosphamide, taxanes and/or 5-fluorouracil.

"Anthracycline" refers to a group of topoisomerase IIα inhibitors 4-Epirubricin, Daunorubicin, Daunorubicin (liposomal), Doxorubicin, Doxorubicin (liposomal), Epirubicin, Idarubicin, and Mitoxantrone.

The present invention will hereinafter be described by way of the following non-limiting Figures and Examples.

Figure legends

Figure 1A shows a Kaplan Meier plot illustrating disease free survival of patients receiving adjuvant CEF. The patients were stratified according to tumor cell TIMP-1 immunoreactivity scored as + or – immunoreactivity in the cancer cells. The number of patients at risk at selected time points is given below the x-axis.

Figure 1B shows a Kaplan Meier plot illustrating disease free survival of patients receiving adjuvant CMF. The patients were stratified according to tumor cell TIMP-1 immunoreactivity scored as + or – immunoreactivity in the cancer cells. The number of patients at risk at selected time points is given below the x-axis

Figure 1C shows the Kaplan Meier curves which show the disease free survival of patients without TIMP-1 immunoreactivity in their cancer cells treated with CEF or CMF.

Figure 2A shows a Kaplan Meier plot illustrating disease free survival of patients receiving adjuvant CEF. The patients were stratified according to the presence or absence of tumor cell *TOP2A* DNA aberrations. The number of patients at risk at selected time points is given below the x-axis.

Figure 2B shows a Kaplan Meier plot illustrating disease free survival of patients receiving adjuvant CMF. The patients were stratified according to the presence or absence of tumor cell *TOP2A* DNA aberrations. The number of patients at risk at selected time points is given below the x-axis

Figure 2C shows the Kaplan Meier curves which show the disease free survival of patients with *TOP2A* DNA aberrations in their cancer cells treated with either CEF or CMF.

Figure 3A shows a Kaplan Meier plot illustrating disease free survival of patients receiving adjuvant CEF. The patients were stratified according to tumor cell TIMP-1 immunoreactivity scored as + or – immunoreactivity in the cancer cells and

presence (Ab) or absence (normal) of TOP2A DNA aberrations. The number of patients at risk at selected time points is given below the x-axis.

Figure 3B shows a Kaplan Meier plot illustrating disease free survival of patients receiving adjuvant CMF. The patients were stratified according to tumor cell TIMP-1 immunoreactivity scored as + or – immunoreactivity in the cancer cells and the presence (Ab) or absence (normal) of TOP2A DNA aberrations. The number of patients at risk at selected time points is given below the x-axis

Figure 3C shows the Kaplan Meier curves which show the disease free survival of patients without TIMP-1 immunoreactivity and/or with TOP2A DNA aberrations in their cancer cells treated with CEF or CMF.

Figure 4A and 4B
Kaplan-Meier curves for invasive disease-free survival by treatment with CMF or CEF and HT (*HER2* and TIMP-1) status (Panel 4A) and 2T (TOP2A and TIMP-1) status (Panel 4B).

Figure 5A and 5B
Forest plots illustrating hazard ratio estimates of treatment effect for invasive disease-free survival (Panel 5A) and overall survival (Panel 5B) comparison between patients with HER2 positive and HER2 negative tumors, *TOP2A* DNA aberrant and non-aberrant (normal) tumors, TIMP-1 positive and negative tumors, HT responsive and non-responsive tumors and 2T responsive and non-responsive tumors.

Figure 6A-D
This Figure shows examples of TIMP-1 immunohistochemistry. 6A: A large proportion of the epithelial cancer cells are TIMP-1 positive. 6B: Scattered and focalized TIMP-1 immunoreactivity in the epithelial cancer cells.
6C: Negative control. 6D: TIMP-1 immunoreactivity in fibroblasts but not in the epithelial cancer cells.

Figure 7A and 7B

Invasive Disease-Free Survival (IDFS) (Figure 7A) and overall survival (OS) (Figure 7B) probabilities for breast cancer patients with known TIMP-1 status. T+ and T- means patients with and without TIMP-1 immunoreactivity in their breast cancer cells, respectively. CEF and CMF refer to received adjuvant chemotherapy.

Figure 8A and 8B

Forest plots illustrating hazard ratios from multivariate models for effect of CEF with CMF as baseline in TIMP-1 subgroups and ER subgroups of patients. Figure 8A: IDFS; Figure 8B: OS

Figure 9

TIMP-1 FISH analysis showing TIMP-1 DNA amplifications in the epithelial breast cancer cells

Examples

In the present context the following aberrations are used

DBCG: Danish Breast Cancer Cooperative Group

CMF: Cyclophosphamide, Methotrexate and 5-Fluorouracil

CEF: Cyclophosphamide, 4.epi-adriamycin and 5-Fluorouracil

CAF: Cyclophosphamide, 4.epi-adriamycin and 5-Fluorouracil

TOP2A normal: No DNA aberrations found in the *TOP2A* gene

HER2 normal: No DNA aberrations found in the *HER2* gene

HT-sensitive: HER2 gene amplification or 3 plus for Her2 immunohistochemistry and TIMP-1 negative

2T-sensitive: TOP2A gene aberrations and TIMP-1 negative

TMA: Tissue Micro Arrays

ER or ER immunostaining: Immunostaining for estrogen or progesterone receptors

FISH: Fluorescence in situ hybridization

IHC: Immunohistochemistry

IDFS: Invasive Disease Free Survival

OS: Overall survival

Example 1

Lack of TIMP-1 tumour cell immunoreactivity predicts effect of adjuvant anthracycline based chemotherapy in patients (n=647) with primary breast cancer.

Methods

Patients and Methods

Briefly, DBCG (Danish Breast Cancer Cooperative Group) trial 89D was an open-labeled randomized, phase III trial comparing CEF (Cyclophosphamide, Epirubicin and Fluorouracil) against CMF (Cyclophosphamide, Methotrexate and Fluorouracil). Eligible for the 89D trial were patients with node positive (or tumor size $\geq$ 5 cm) and hormone receptor negative breast cancer, and premenopausal patients with node negative and malignancy grade II or III tumours. All patients gave informed consent to the trial. The DBCG 89D trial did not include patients with node positive, hormone receptor positive tumours. These patients were included in trials with endocrine treatment. The DBCG prepared the original protocol as well as the biomarker supplements and The Danish National Committee on Biomedical Research Ethics approved the original protocol as well as the supplements before their activation.

Pathology assessments

The pathological procedure included classification of histological type according to WHO, examination of tumour margins, invasion into skin or deep fascia, measurement of gross tumour size, number of metastatic and total number of lymph nodes identified. All invasive ductal carcinomas were graded for malignancy. All sections have subsequently been analysed centrally for ER by immunohistochemistry and these centrally obtained ER data were used in the present analyses. Tumours with $\geq$ 10% stained tumour cells were considered ER positive.

Retrospective collection of archival tumour tissue and construction of TMA's

From June 1990 to January 1998, 1224 patients were randomized in the DBCG trial 89D and 980 of these were recruited in Denmark. Archival paraffin embedded

tissue blocks from 806 Danish patients enrolled in the trial were collected between September 2001 and August 2002 from the study sites and stored centrally. Tissue Micro Arrays (TMA) were successfully constructed from 707 of 797 blocks still assessable by means of a TMA-builder from Histopathology Ltd (AH-diagnostics, Denmark). A target area was identified in the donor block on haematoxylin stained sections and two 2 mm tissue cores were transferred to the recipient TMA block. For orientation the upper corners were marked using cores of kidney tissue. For the present study, a total number of 659 tumours were available for TIMP-1 analysis. The lack of tumours (659-707) was due to their prior use in other studies resulting in no left-over tissue for the present study. Table 7 shows the flow of the patients in the study

TIMP-1 immunostaining

The mouse monoclonal antibody (clone VT7) raised against recombinant human TIMP-1 was included. The present inventions have previously validated this antibody for immunostaining. The VT7 antibody is of the IgG_1 subtype and was used in the concentration 0.25 µg/ml. In addition, an irrelevant IgG_1 monoclonal antibody (anti-TNP) raised against tri-nitro-phenol hapten was used as control. For each immunohistochemical experiment, a positive control case (human mammary carcinoma known to contain TIMP-1) was included.
Reagents used for IHC staining were obtained from Dako A/S and were used according to the manufacturer's instructions.

In brief, paraffin sections (4 µm) were dewaxed in xylene and rehydrated through a graded series of ethanol. Antigen retrieval was carried out by boiling the sections for 10 minutes in a conventional microwave oven in 10 mM citrate buffer pH 6.00 followed by 30 minutes in the hot buffer at room temperature. To block endogenous peroxidase activity, the sections were treated with 1 % hydrogen peroxide for 10 minutes. Sections were incubated with primary antibody overnight at 4ºC. The monoclonal antibodies were detected with Advance HRP (Code no K4068), and the reactions were visualized by incubating the sections with DAB+ (Code No K5007) for 5 minutes. Washes between incubations were carried out with TBS containing 0.5% Triton x-100, pH 7.6. The sections were counterstained with Mayer's haematoxylin, and all staining procedures were performed manually.

Immunostaining of tissue sections was assessed semi-quantitatively using + and – symbols as a measure of TIMP-1 immunoreactivity in the epithelial breast cancer cells. Scoring of the intensity of the signal was not included. The scoring of the tissue sections was performed blinded by two independent pathologists (GW and EB). In case of discrepancies, agreement was reached by looking at the slides together.

Statistical Methods

The immunostaining results were transferred to the DBCG secretariat for statistical analyses.

Follow-up time was quantified in terms of a Kaplan-Meier estimate of potential follow-up. IDFS (Invasive Disease-Free Survival) was the primary and OS (Overall Survival) the secondary end-point. IDFS was defined as the elapsed time from randomization until invasive breast cancer recurrence irrespective of localization, second primary invasive cancer or death attributable to any cause. OS was defined as the elapsed time from randomization until death attributable to any cause. IDFS and OS were analysed using Kaplan-Meier estimates and the log rank test. The effect of treatment regimen as well as centrally assessed TIMP-1 on IDFS and OS was quantified in terms of the hazard ratio, estimated unadjusted and adjusted using the Cox proportional hazards model. The multivariate Cox proportional hazards model was also applied to investigate interaction of treatment and TIMP-1 using the Wald test. The multivariate model included TIMP-1, menopausal status, tumour size, positive lymph nodes, histological type and grade, central ER hormone receptor status, treatment regimen and interaction terms of TIMP-1 and treatment. The proportional hazard assumptions were not fulfilled for histological type & grade and ER receptor status, and these were included in the model as stratification variables. Differences between patients with and without information about biomarkers, between treatment regimens, and correlations between TIMP-1 status and clinico-pathological variables were tested by χ^2-test excluding unknowns. P-values are two-tailed. Statistical analyses were done with the SAS 9·1 program package.

Results

The total number of tumour samples investigated was 659, among whom 12 did not receive CMF or CEF, resulting in a final number of 647 patients for subsequent analyses. 357 of these patients received CMF and 290 patients received CEF. Table 7 shows the flow of the original patients enrolled in the Danish part of DBCG 89D study and how we ended up with a total of 647 patients to be included in the final analysis. At the time of the present analyses (August 1, 2007), 308 (48%) have died and 312 (48%) have had an event corresponding to IDFS. For the patients receiving CEF 123 (42%) had died and 129 (44%) had had an event corresponding to IDFS. Among CMF treated patients, 185 (52%) had died and 183 (51%) had had an IDFS event. The median potential follow-up time with respect to IDFS was 9.8 years and 13.8 years with respect to OS.

Table 5 shows the base-line characteristics of the intention to treat population. As can be seen, patients included in the present study had significantly larger tumours ($p<0.0001$) and significantly higher grade of malignancy ($p=0.02$) than the remaining patients. No significant differences were found for the other classical base-line characteristics. When dividing the 647 patients into the two treatment groups (CMF vs. CEF) no differences in base-line characteristics were observed, indicating that although approximately one third of the patients were lost for the present study, the included patients had retained a balanced distribution.

75% of the tumour samples showed positive TIMP-1 immunoreactivity. The pattern of immunoreactivity ranged from almost all epithelial cancer cells displaying TIMP-1 immunoreactivity (Figure 6A) through scattered and focalized TIMP-1 immunoreactivity (Figure 6B) (TIMP-1 positive) to total absence of TIMP-1 tumour cell immunoreactivity (not shown). In some tumours, distinct tumor tissue stromal cell TIMP-1 immunoreactivity was observed, but if these tumours were devoid of epithelial cancer cell TIMP- immunoreactivity, they were counted as TIMP-1 negatives (Figure 6D). Figure 6C is a negative control.

Table 6 shows the base-line characteristics between patients having TIMP-1 positive and patients having TIMP-1 negative tumour cells. Patients with TIMP-1 positive tumour cells had significantly more tumour positive axillary lymph nodes ($p=0.02$) and significantly more ER positive tumours ($p=0.04$). Among the TIMP-1

negative tumors (n=160), the majority were ER-negative (n=107). However, among the TIMP-1 positive tumors (n=487) there was also a large proportion being ER-negative (n=294). This shows that even though TIMP-1 negativity primarily is found among ER-negative tumors, TIMP-1 is not a general surrogate for ER. No other differences in base-line characteristics between TIMP-1 negative/positive patients could be demonstrated.

The multivariate analysis (adjusted) included treatment arm, menopausal status, tumour size, number of positive axillary lymph nodes, histological type and malignancy grading, ER centrally measured and TIMP-1 tumour cell immunoreactivity. As stated above, the proportional hazard assumptions were not fulfilled for histological type & grade and ER receptor status, and these were therefore included in the multivariate model as stratification variables.
The present inventors first analysed the effect on IDFS and OS of CEF versus CMF in the 647 patients included in the present study. Thus, TIMP-1 immunoreactivity in the cancer cells was not taken into consideration. Patients who received CEF had a superior IDFS (adjusted HR: 0.78 (95%CI: 0.62-0.98; p= 0.03) and superior OS (adjusted HR=0.77 (95% CI: 0.61-0.97; p=0.03) when compared with patients receiving CMF (not shown). These figures are not different from those of the original study (IDFS: HR=0.76 and OS: HR=0.73) (Ejlertsen et al. 2007), suggesting that the studied subgroup is representative of the whole study group.

The present inventors then analysed the association between TIMP-1 cancer cell immunoreactivity and IDFS and OS for the whole included patient cohort (n=647). No significant differences were seen between TIMP-1 positive versus TIMP-1 negative patients with regard to IDFS; unadjusted HR=1.18 (95% CI: 0.91-1.54; p=0.22) and adjusted HR=0.95 (95% CI: 0.72-1.24; p=0.69). For OS the figures were: unadjusted HR=1.17 (95% CI: 0.89-1.53; p=0.25) and adjusted HR=0.97 (95% CI: 0.73-1.28; p= 0.82).

Subgroup analyses, taking the two different treatment arms and tumour cell TIMP-1 immunoreactivity into consideration, were then performed. In the CEF treated patients (n=290), individuals with TIMP-1 positive tumours had a significant shorter IDFS than patients with TIMP-1 negative tumours; unadjusted

HR=1.56 (95% CI: 1.01-2.41; p=0.047) (Figure 7A). In contrast, in the CMF treated patients (n=347), no differences in IDFS were seen between TIMP-1 positive and negative patients; unadjusted HR=0.97 (95% CI: 0.69-1.35; p=0.84) (Figure 7A). The corresponding figures for OS were: CEF: unadjusted HR=1.41 (95% CI: 0.91-2.18; p=0.13) and CMF: unadjusted HR=1.02 (95% CI: 0.72-1.43; p=0.93) (Figure 7B).

In the multivariate analyses, no significant differences were seen between TIMP-1 positive versus TIMP-1 negative patients treated with CEF with regard to IDFS: adjusted HR=1.30 (95% CI: 0.83-2.02; p=0.25) and OS: adjusted HR=1.21 (95% CI: 0.77-1.90; p=0.42. Nor were significant differences observed in patients treated with CMF; IDFS: adjusted HR=0.76 (95% CI: 0.54-1.07; p=0.12) or OS: adjusted HR=0.84 (95% CI: 0.59-1.19; p=0.32).

When comparing IDFS in CEF versus CMF treated patients in the group with TIMP-1 immunoreactive cancer cells the HR between the two treatment groups was: adjusted HR=0.88 (95% CI: 0.68-1.13; p= 0.32) (Figure 8A). The corresponding figures for OS were: adjusted HR=0.83 (95% CI: 0.64-1.08; p= 0.17) (Figure 8B). In contrast, comparing IDFS between CEF and CMF treated patients with lack of TIMP-1 cancer cell immunoreactivity showed an adjusted HR=0.51 (95% CI: 0.31-0.84; p=0.0085) (Figure 8A) and OS adjusted HR=0.58 (95% CI: 0.35-0.96; p= 0.03) (Figure 8B) in favour of patients treated with CEF. A non-reduced Cox proportional hazards model was used to test for interactions between treatment effect and TIMP-1 with respect to IDFS and OS. A non-significant TIMP-1 profile (positive or negative immunoreactivity) versus treatment (CEF or CMF) interaction was detected for IDFS (p= 0.06) (Figure 8A) and OS (p= 0.21) (Figure 8B).

Discussion

This study shows for the first time that lack of TIMP-1 cancer cell immunoreactivity is associated with a favourable effect of adjuvant epirubicin containing adjuvant therapy in primary breast cancer as compared with CMF, suggesting a predictive value of TIMP-1 immunoreactivity for anthracyclines. Compared with CMF, anthracycline based adjuvant treatment of TIMP-1 negative patients significantly reduces the risk of recurrence with 49% and mortality with 42%.

The VT7 anti-TIMP-1 monoclonal antibody was previously selected among a panel of anti-TIMP-1 antibodies for its superiority regarding immunostaining. VT7 recognizes a linear TIMP-1 epitope located between amino acid 169-174. The VT7 immunostaining was thoroughly validated with regard to sensitivity and specificity (VT7 does not bind TIMP-2, 3 or 4) and the staining conditions were optimized regarding antigen retrieval protocol, antibody concentration and time of incubation etc.. In addition, the potential influence of fixation time (24-72 hours) was tested. On each TMA, a negative control antibody of the same IgG1 subtype (anti-TNP) was used and a slide of a known TIMP-1 positive breast cancer was included in each assay run as a positive control.

Only minor differences were observed in the characteristics of the 647 patients included in present analyses compared to the 980 Danish patients included in the original 89D trial, which indicates that the present 647 patients are representative for the whole DBCG 89D Danish study cohort. The overall benefits reported in the original 89D trial was reproduced in the present subset, which further support that the 647 patients are representative for the entire cohort of Danish patients in the DBCG trial 89D.

The present inventors have previously published that murine fibro sarcoma cells derived from TIMP-1 gene-deficient mice are significantly more sensitive to etoposide (a topoisomerase II inhibitor) in vitro than wild-type murine fibro sarcoma cells expressing TIMP-1. By applying an apoptosis assay, it was demonstrated that TIMP-1 protected the fibro sarcoma cells against apoptosis. That TIMP-1 can protect against chemotherapy-induced apoptosis has also been demonstrated by others. It is at present not clear why TIMP-1 in the present study

predicts sensitivity/resistance to CEF and not to CMF. Suggestions have been made regarding the signalling pathways possibly regulated by TIMP-1. In the MCF10A breast epithelial cell line over-expression of TIMP-1 was shown to induce constitutive activation of focal adhesion kinase (FAK) through tyrosine phosphorylation. FAK has previously been shown to be upstream regulator of the phosphatidylinositol-3 kinase (PI-3 kinase) leading to regulation of the bcl-2 family members, a well-characterised signalling pathway leading to cell survival. Phophorylated FAK associates with and thereby activates the PI-3 kinase, which in turn activates the Akt-kinase. Akt phosphorylates the protein Bad, which as a result is sequestered in the cytoplasm by the capture protein 14-3-3 and can therefore no longer interact with and inhibit bcl-2 and bcl-X_L. Bcl-2 and bcl-X_L are proteins situated in the mitochondrial membrane and when activated these anti-apoptotic proteins inhibit Bax thereby preventing the release of cytochrome c from the mitochondria. This in turn prevents activation of the caspase cascade and accordingly prevents apoptosis. Thus, TIMP-1 may inhibit apoptosis by acting like a trophic factor initiating the survival pathway including FAK, PI-3 kinase, Akt and bcl-2 family members resulting in inhibition of caspase activation and thereby inhibition of apoptosis.

By testing for TIMP-1 immunoreactivity in tumour tissue obtained from patients who were enrolled in the DBCG 89D trial, the present inventors have now shown that patients who lack TIMP-1 immunoreactivity in their breast cancer cells and who are treated with anthracycline containing combination chemotherapy have a significantly better outcome than patients treated with CMF. In the multivariate analyses, patients with TIMP-1 negative tumours had a 49% reduced risk of recurrence and 42% reduced risk of death when treated with CEF rather than with CMF. These clinical results are thus yet another support for our hypothesis that the TIMP-1 protein is associated with sensitivity/resistance to anthracycline treatment. However, an independent study is awaited to confirm the significant association between TIMP-1 immunoreactivity and anthracycline sensitivity/resistance in the adjuvant setting. Moreover, we are currently comparing the TIMP-1 results with those of *HER2* and *TOP2A* gene aberration assays, both of which have been associated with sensitivity to anthracyclines.

The present inventors have previously published that the level of TIMP-1 protein in primary breast cancers carries prognostic information. It can thus be speculated whether the observed effect of TIMP-1 immunoreactivity on IDFS is prognostic or predictive. As no effect of TIMP-1 immunoreactivity was observed among CMF patients but only among CEF treated patients, the present results suggest that TIMP-1 immunoreactivity carries some predictive value and the present study is thus in line with our preclinical observations. In the prior prognostic studies, TIMP-1 protein was extracted from the whole tumour and the measured TIMP-1 protein could thus be derived from contaminating blood, from tumor tissue stromal cells, from extracellular matrix and from the cancer cells. In contrast, in the present study, only TIMP-1 protein localization in the epithelial cancer cells was included in the final analyses, which may be another reason for the differences between the present and the previous studies.

In conclusion, the present study, demonstrates for the first time that tumours being devoid of TIMP-1 protein immunoreactivity in the epithelial cancer cells are more sensitive to anthracycline treatment than to CMF treatment. Future studies will be aimed at establishing the relationship between TIMP-1 immunoreactivity, *HER2, TOP2A* and effect of anthracyclines. Moreover, the present results will be validated in an independent patient cohort.

Example 2

Clinical study of the combined predictive value of *TOP2A* and *TIMP-1* tumor cell gene aberrations and TIMP-1 tumor cell protein immunoreactivity

Methods

647 patient samples were obtained from a randomized study in which high risk breast cancer patients were randomized to adjuvant treatment with either CMF or CEF. End-point was invasive disease free survival (IDFS).

The patients samples consisted of tissue micro arrays made from the formalin fixed paraffin embedded tissue from the primary tumors of the patients. All samples had an identification number.

TOP2A gene aberrations were tested as previously described (Koop et al. 2005).

TIMP-1 gene aberrations were tested using standard FISH technology. BAC (Bacterial artificial chromosome) clone (RP11-466C12) was identified by analysis of a 400 kb area around the *TIMP-1* gene using the UCSC genome browser (http://genome.ucsc.edu). The BAC clone is covering the previously identified genes; ARAF will-type allele (*ARAF)*, human synapsin I (*SYN1)*, tissue inhibitor of metalloproteinases-1 (*TIMP-1)*, complement factor properdin *(CFP)*, *ELK1*, ubiquitously expressed transcript *(UXT)*, and *AK094108*. The clone was cultured in LB medium (Sigma Aldrich, Denmark) supplemented with 12.5 µg/mL chloramphenicol (Sigma Aldrich, Denmark) and purified according to the alkaline purification of BAC DNA (Poulsen 2004) (Poulsen TS, 2004). The clone was verified using *in silico Bam*HI digest of the DNA sequence from the UCSC and compared with a *Bam*HI endonuclease digestion of the purified BAC clone as recommended by the enzyme manufacture (Invitrogen, Denmark).

The probe BAC DNA was labeled by nick translation with Texas Red-5-dCTP (Millipore Corporation, Temecula, California, USA) as described by the manufacturer (Roche Diagnostics GmBH, Mannheim, Germany). A total of 10 ng/µL labeled DNA were used for FISH and suppression of undesired background staining derived from repetitive sequences was achieved using specific PNA oligos (Nielsen, KV et al., 2004). A fluoroscein labeled mixture of PNAs specific for the chromosome X α-satellite sequences (CenX PNA probe) was used as a reference for the copy number of chromosome X. The PNAs was supplied by Dako A/S. Figure 1 shows a schematic representation of chromosome X and the localization of the part of region Xp11 covered by the BAC DNA as well as the area of centromere X covered by the CenX PNA probe. FISH was carried out using the Histology FISH accessory kit as described by the manufacturer (K5599, Dako A/S, Denmark), with modification. The pre-treatment step was not done by use of a water-bath but performed using a microwave oven (Whirlpool, Denmark, model JT356 with 6^{th} sense). Slides were submerged in enough 1x pre-treatment buffer to completely cover the slides, treated for 10 minutes using the steam function (6^{th} sense) followed by 15 minutes at room temperature (RT), before continuing according to the protocol supplied with the Histology FISH accessory kit.

Evaluation of FISH

Hybridization signals were scored using a Leica microscope (Leica, Denmark) equipped with a 100X oil-immersion objective (numeric aperture). A dual-bandpass fluorescence filter (Chromotechnology, Brattleboro, VT) was used to visualize the FITC and Texas Red signals simultaneously. Sixty nonoverlapping interphase nuclei with intact morphology based on DAPI counterstaining were scored to determine the number of hybridization signals for each *TIMP-1* and CenX probes. Amplification of TIMP-1 was defined as an average ratio of TIMP-1 signals relative to CenX signals (= level of amplification) of 2 or more (Ratio ≥ 2). *TIMP-1* was defined as deleted if the ratio was less than 0.8 (Ratio <0.8). Normal *TIMP-1* gene/CenX ratio was therefore defined in between (0.8 ≤ Ratio < 2).

Evaluation of TIMP-1 immunoreactivity

Immunohistochemistry for the TIMP-1 protein was performed using the VT7 anti TIMP-1 monoclonal antibody (Sørensen et al. 2005) according to a previously published procedure (Sørensen et al 2005). The mouse monoclonal antibody (clone VT7, IgG_1) raised against recombinant human TIMP-1 (Moller Sorensen, et al. 2005; Sorensen, et al. 2006) was used at a concentration of 0.4 μg/ml.

All sections were evaluated by two independent pathologists who were unaware of the clinical history of the patients. Each sample was evaluated for presence or absence of tumor cell immunoreactivity and thus scores as either + or -.

All data were then transferred to the Danish Breast Cancer Cooperative Group Secretariat for statistical analyses.

Results

290 patients had received CEF and 357 had received CMF. Of these, 216/290 and 271/357 were found positive for TIMP-1 immunoreactivity and 61/290 and 78/357 had *TOP2* gene aberrations (amplifications or deletions). 24 patients had unknown *TOP2A* DNA status.

Kaplan Meier plots for disease free survival for patients stratified according to TIMP-1 tumor cell immunoreactivy is shown in Figures 1 A and B. Figure 1B shows that in the patients receiving CMF, TIMP-1 tumor cell reactivity had no impact on

DFS (p=0.84). In contrast, in patients receiving CEF, lack of tumor cell TIMP-1 immunoreactivity was associated with a significant increased DFS (p=0.047) (Figure 1A). In contrast, patients with TIMP-1 immunoreactivity in their tumor cells had a DFS comparable to patients treated with CMF (p=0.46).

As can bee seen from Figure 1A, which shows the disease free survival of patients treated with CEF, patients absent of TIMP-1 immunoreactivity in the tumor cells do significantly better with regard to disease free survival. For example, at 5 years follow up, approximately 72% of the TIMP-1 negative patients have not experienced disease recurrence while only 60% of the TIMP-1 positive patients are free of disease.

Figure 1B shows the disease free survival of patients receiving CMF and stratified according to whether the tumor cells display TIMP-1 immunoreactivity or not. There is no difference in disease free survival between the two groups.

When analysing for *TOP2A* gene aberrations, it was found (Figures 2 A and B) that in patients receiving CMF the *TOP2A* gene aberration status had no influence on DFS (Figure 2B). In contrast, in patients receiving CEF, those patients with *TOP2A* gene aberrations (amplifications or deletions) had a significant improved DFS as compared to those patients with *TOP2A* DNA aberration who received CMF (Figure 2A).

As can bee seen from Figure 2B, which shows disease free survival of patients treated with CMF, patients with *TOP2A* DNA aberrations do much worse than patients without *TOP2A* DNA aberrations. However, when looking at Figure 2A, which shows the disease free survival of patients receiving CEF and stratified for *TOP2A* DNA aberrations, it is seen that the curve (patient with *TOP2A* DNA aberrations) do better than those who received CMF (Figure 2B)

It appeared that among the patients with negative TIMP-1 immunoreactivity in their cancer cells, only 24/160 (15%) had *TOP2A* gene aberrations. We therefore analysed the combined effect of having either *TOP2A* gene aberration or lack of TIMP-1 immunoreactivity on DFS. The results showed that it was now possible to identify almost the double number of patients with a high likelihood of obtaining

benefit from CEF treatment (as compared with CMF treatment) as could be identified by *TOP2A* analyses alone and without reducing the hazard ratio. Table 1 shows the individual adjusted hazard ratios including 95% confidence intervals. All values are based on the CMF group being set to a hazard ratio of 1.

A HR of 1 means no difference between the groups. We have used the combined CMF groups as reference. Thus, the Table shows the benefit from CEF treatment compared to treatment with CMF in the subgroups.

It is seen from the Table 1 that patients with *TOP2A* DNA aberrations or TIMP-1 negativity treated with CEF have HR below 1 and that the 95% confidence intervals do not exceed 1. This means that these patients (*TOP2A* DNA aberrations and/or TIMP-1 negativity) benefit significantly more from the CEF treatment as compared with the treatment with CMF. A HR of 0.54 means that chance of benefit for the patients (*TOP2A* DNA aberrations and/or TIMP-1 negativity) is 46%. It is also seen from the Table, that the HR for *TOP2A* DNA aberrations (amplifications or deletions) and for patients who's tumor cells are absent of TIMP-1 immunoreactivy have almost similar HR. The invention is that it is not always the same patients having *TOP2A* DNA aberrations or being absent of TIMP-1 protein immunoreactivy. Then when looking at the HR for the group of patients with *TOP2A* DNA aberrations and/or absent of TIMP-1 immunoreactivity, the HR stays almost he same (0.48 (95% confidence interval: 034-069) despite the number of patients in this subgroup is almost double up of the number of patients that could be identified by *TOP2A* DNA aberrations alone. In other words, by combining *TOP2A* DNA aberration measurements with TIMP-1 protein immunoreactivy measurements, almost double as many patients that have a high likelihood of benefit from CEF is identified as compared to *TOP2A* DNA aberration measurements alone.

By the combined method it is possible to identify 43% of the patients who had more than 50% increased likelihood of obtaining benefit from CEF treatment as compared with the benefit from CMF treatment (Hazard ratio 0.48) which is approximately the double number of what can be accomplished by analysing only for *TOP2A* DNA aberrations alone.

Figures 3A and B show the Kaplan Meir curves for DFS when *TOP-2A* DNA aberrations and TIMP-1 immunoreactivity is combined.

When looking at Figure 3B, it is seen that patients with *TOP2A* DNA aberrations and/or absence of tumor cell TIMP-1 protein immunoreactivity do worse than patients without *TOP2A* DNA aberrations and with TIMP-1 protein immunoreactivity in their tumor cells when treated with CMF. However, if the patients are treated with CEF (Figure 3A), the patients with *TOP2A* DNA Aberrations and/or lack of TIMP-1 protein immunoreactivity do much better than those treated with CMF. Thus, patients with *TOP2A* DNA aberrations and/or lack of TIMP-1 protein immunoreactivity and treated with CEF do better than patients with *TOP2A* DNA aberrations and/or lack of TIMP-1 protein immunoreactivity treated with CMF.

Figure 9 shows TIMP-1 FISH analysis showing TIMP-1 DNA amplifications in epithelial breast cancer cells

Discussion

This study demonstrates that lack or reduced concentration of TIMP-1 protein and/or TOP2A gene aberrations confers sensitivity to certain types of chemotherapy.

The present study was performed on samples obtained from a large prospective study with full clinical follow up (Ejlertsen et al., Eur J Cancer 2005). Both the *TOP2A* FISH analyses and the TIMP-1 immunohistochemistry technologies used have previously been described.

The results of the present study clearly demonstrate the additive effect of combining *TOP2A* gene aberration measurements with TIMP-1 immunohistochemistry in predicting benefit (prolonged IDFS) from adjuvant treatment with CEF in primary high risk breast cancer patients while no benefit is observed in patients treated with CMF, suggesting the value of the combined test in predicting benefit from anthracycline containing chemotherapy.

Example 3

HER2, TOP2A and TIMP-1 and responsiveness to adjuvant anthracycline containing chemotherapy in high risk breast cancer patients.

Methods

The DBCG 89D trial and its biological sub-study has previously been described in detail (Ejlertsen at al. 2007 and Knoop et al. 2005). Briefly, DBCG trial 89D is an open-labeled randomized, phase III trial comparing CEF (cyclophosphamide 600 mg/m^2, epirubicin 60 mg/m^2, and fluorouracil 600 mg/m^2) against CMF (cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2, and fluorouracil 600 mg/m^2) both intravenously for nine cycles with 3 week intervals. Eligible for the 89D trial were patients' with hormone receptor negative and node positive (or tumor size > 5 cm) breast cancer, and premenopausal patients with node negative tumors provided they had malignancy grade II or III. Patients with highly hormone responsive tumors were included in DBCG trials, 89B and 89C, with synchronized eligibility criteria. The DBCG prepared the original protocol as well as the biomarker supplements and The Danish National Committee on Biomedical Research Ethics approved the original protocol as well as the supplements before their activation (V.200.1616/89, KF 12 295 003).

Central assessment of HER2, ER AND TIMP-1 immunoreactivity

Tissue microarrays (TMA) were constructed from formalin-fixed and paraffin-embedded tumor blocks by means of a TMA-builder (Histopathology Ltd, AH-diagnostics). A target area was identified in the donor block on haematoxylin stained sections and two 2 mm tissue cores were transferred to the recipient TMA block. ER immunostaining was performed at room temperature on 3 µ TMA sections with the ER1D5 (Dako) antibody and a Tech-mate 500 (Dako). ER expression was recorded as the percentage of staining tumor cells, ignoring intensity, and the results were dichotomized as positive (≥ 10% staining cells) or negative (< 10%). Expression of HER2 was measured on whole sections using the HercepTest (Dako) and scored accordingly as 0, 1+, 2+, or 3+. TIMP-1 immunostaining was performed as previously described (Sorensen et al. 2006). In brief, sections were incubated with the anti TIMP-1 mouse monoclonal antibody VT7. VT7 was detected with mouse/rabbit Envision+ (Code No K5007, DAKO A/S),

and the reaction was visualized by incubating the sections with DAB+ (Code No K5007, DAKO A/S) for 2 periods of 3 minutes. Immunostaining of tissue sections was assessed semi-quantitatively using + and - symbols as a measure of TIMP-1 immunoreactivity in the epithelial breast cancer cells. Scoring of the intensity of the signal was not included. The scoring of the tissue sections was performed blinded by two independent pathologists (GW and EB). In case of discrepancies, agreement was reached by looking at the slides together.

TOP2A and *HER2* FISH

TOP2A and *HER2* copy number was visualized by FISH (*TOP2A* pharmDX and *HER2* pharmDX, DAKO A/S). At least 60 gene signals were scored and all signals were scored if a nucleus was included. The centromere 17 signals were in addition scored in the same nuclei's, and the ratio of gene to centromere 17 was calculated. Tumors were scored as *TOP2A*/*HER2* deleted, normal or amplified according to a ratio of < 0.8, 0.8-1.9 and > 2.0.

Statistical methods

Follow-up time was quantified in terms of a Kaplan-Meier estimate of potential follow-up. Invasive Disease-Free Survival (IDFS) was the primary end-point and was defined as the time elapsed from randomization until invasive breast cancer recurrence irrespective of localization, invasive breast cancer involving the same or the contralateral breast, second primary non-breast invasive cancer or death attributable to any cause. Overall survival (OS), the secondary end-point, was defined as the elapsed time from randomization until death attributable to any cause. IDFS and OS were analyzed using Kaplan-Meier estimates and the logrank test. The effect of TIMP-1 in combination with HER2 or *TOP2A* biomarker status on IDFS and OS was quantified in terms of the hazard ratio, estimated unadjusted using the Cox proportional hazards model. The Cox proportional hazards model was also applied for multivariate analysis, based on the model developed previously for the same patient material. The multivariate model included TIMP-1, *TOP2A*, HER2, ER, tumor size, positive lymph nodes, histologic type and grade, menopausal status, and treatment with CMF or CEF. The Cox proportional hazards model on IDFS and OS was adjusted according to the results of the goodness-of-fit procedures, and ER hormone receptor status as well as histological type and

grade were included as stratification variables. Interaction between biomarkers (HT, 2T, TIMP-1, *TOP2A*, and HER2) and treatment regimens (CMF or CEF) were investigated in separate models, and the Wald Test was applied.

Differences between patients with and without information about biomarkers, between treatment regimens, and correlations between HT (HER2 positive and/or lack of TIMP-1 immunoreactivity) or 2T biomarker status and clinical and pathological variables including HER2-status were tested (excluded unknowns) by χ2-test. P-values are two-tailed. Tumors were classified as HT responsive if HER2 positive and/or lack of TIMP-1 immunoreactivity and otherwise HT non-responsive. Tumors were classified as 2T responsive if they had TOP2A aberrations and/or lack of TIMP-1 immunoreactivity and otherwise 2T non-responsive. Statistical analyses were done with the SAS 9·1 program package.

The DBCG was responsible for study design and coordination, tissue collection, biomarker analysis, data collection, analysis, and reporting. The ER1D5 antibody, HercepTest, HER2 phamDX and *TOP2A* phamDX kits and technical assistance were provided free of charge by DAKO A/S (Glostrup, Denmark).

Results

The DBCG 89D trial recruited 1224 patients between June 1990 and January 1998. Median estimated potential follow-up was 9.8 years for IDFS and 13.8 years for OS. In 2001, the DBCG completed the retrospective collection of formalin-fixed, paraffin-embedded primary breast tumor tissue blocks that were available from 821 (84%) of the 980 participants enrolled in Denmark and the construction of TMA was successful in 708 patients (72%). A total of 623 patients were accessible for HER2, *TOP2A* and TIMP-1 analyses. The assessable 623 patients differed significantly from the 357 non-assessable ($p<0.05$) with regard to menopausal status, tumor size, malignancy grade, and ER status. Number of positive lymph nodes and histological type showed no significant differences between assessable and non-assessable patients. The treatment effect was similar, with a hazard ratio favoring CEF for IDFS (adjusted hazard ratio, 0.80 (95% confidence interval (CI), 0.63 to 1.01; P= 0.06) and OS (adjusted hazard ratio, 0.79; 95% CI, 0.62 to 1.00; P=0.05) to the effect observed in the original study (IDFS: hazard ratio 0.76 and OS: hazard ratio 0.73) (Ejlertsen et al. 2007).

Among the accessible 623 patients 188 (30%) had a HER2 positive, 139 (22%) a *TOP2A* abnormal and 154 (25%) a TIMP-1 negative tumor. A *TOP2A* aberration was only detected in 33 (8%) of the 435 HER2 negative patients (Table 2). In contrast, TIMP-1 immunoreactivity was detected in 123 (28%) of the HER negative and in 130 (27%) of the 484 *TOP2A* normal patients. Table 2 shows the baseline characteristics according to 2T status for the 623 patients for whom HER2, *TOP2A* and TIMP-1 was successful performed.

Integrating TIMP-1 with TOP2A or HER2

By means of HER2 and TIMP-1 311 (50%) patients were classified as HT anthracycline responsive, e.g. had a HER2 positive, a TIMP-1 negative or a HER2 positive and TIMP-1 negative tumour profile. Patients with a HT responsive profile significantly more often ($P<0.05$) were postmenopausal, and had positive lymph nodes, tumors larger than 2 cm and ER negative tumours. Patients who had a HT responsive profile had a similar IDFS (hazard ratio, 1.22; 95% CI, 0.97 to 1.52; $P=0.09$) and inferior OS (hazard ratio, 1.33; 95% CI, 1.06 to 1.67; $P=0.01$) compared to those whose tumors were HT non-responsive. Adjustment for menopausal status, tumor size, number of positive lymph nodes, histologic type and grade, ER and *TOP2A* status, and treatment in a multivariate analysis changed the hazard ratio for IDFS (hazard ratio, 1.03; 95% CI, 0.80 to 1.33; $P=0.81$) and OS (hazard ratio, 1.05; 95% CI, 0.81 to 1.36; $P=0.73$).

With the integrated use of *TOP2A* and TIMP-1 269 (43%) patients were classified as 2T anthracycline responsive, e.g. had a *TOP2A* aberration and/or lacked TIMP-1 immunoreactivity (Table 2). A 2T responsive profile was associated with ER negativity, HER2 positivity and larger tumor size (all $P<0.01$). Patients with a 2T responsive profile had a decreased IDFS (hazard ratio, 1.26; 95% CI, 1.01 to 1.58; $P=0.04$) and OS (hazard ratio, 1.34; 95% CI, 1.07 to 1.69; $P=0.01$) as compared to those with a 2T non-responsive profile. Adjustment in a multivariate analysis for menopausal status, tumor size, number of positive lymph nodes, histologic type and grade, ER expression and HER2 status, and treatment changed the hazard ratio for IDFS (1.19; 95% CI, 0.93 to 1.51; $P=0.71$) and OS (1.18; 95% CI, 0.927 to 1.51; $P=0.18$).

Heterogeneity of treatment according to single biomarkers and profiles

In the multivariate Cox regression analysis we further examined heterogeneity of treatment effect according to HER2 status, *TOP2A* status, TIMP-1 immunoreactivity, HT profile or 2T profile. There was no statistically significant interaction showing improved IDFS and OS with CEF compared with CMF for HER2 and TIMP-1. As was previously reported a significant interaction between *TOP2A* status and treatment effect was observed for IDFS (P=0.004) and OS (P=0.03).

If treated with CEF, patients with tumors classified as HT responsive (HER2 positive or TIMP-1 negative) had a borderline significant improvement in IDFS (Figure 4A, Table 4) and a statistically significant improvement in OS. By contrast, no significant benefit from CEF as compared to CMF was observed among patients with a HT non-responsive profile. A more favorable IDFS and OS with the use of CEF in patients with a HT responsive profile was sustained after adjustment for nodal status, tumor size, histology, grade, ER status, *TOP2A* status, HER2 status, TIMP-1 expression and menopausal status (P values = 0.036 and 0.047, respectively; Figure 5).

Among patients with a 2T responsive profile CEF significantly improved IDFS and OS compared with CMF (Figure 4B, Table 4), as opposed to 2T non-responsive patients. A multivariate analysis adjusting for patient and tumor characteristics confirmed that patients with a 2T responsive profile benefited from CEF compared to CMF regarding both IDFS (Figure 5A) and OS (Figure 5B). A non-significant trend for a more favorable outcome with the use of CMF existed by contrast, in patients with a 2T non-responsive profile (Figure 5). There was a highly statistically significant interaction between the 2T profile and treatment effect were the 269 (43%) patients with a 2T responsive (*TOP2A* aberration or TIMP-1 negative) profile experienced a more favorable outcome with the use of CEF compared to CMF regarding IDFS (Wald test, P<0.0001) and OS (Wald test, P=0.004) (Figure 5).

Discussion

In general it has been acknowledged, that the selection of therapies should whenever possible be directed against specific targets within the tumor of each individual breast cancer patient. The addition of chemotherapy is however often

required and chemotherapy has been considered less target specific. Despite the demonstration of their superiority in the adjuvant setting the mechanism of action of anthracyclines is still not fully elucidated. Among the proposed mechanisms, interaction with topoisomerase II-a and induction of apoptosis however seems to occur at clinically relevant anthracycline concentrations.

The present inventors engaged in the development of a combined *TOP2A* and TIMP-1 profile and have previously examined their predictive properties individually within the DBCG 89D trial.

In the present study, among 188 patients with HER2 positive tumors 106 (56%) had abnormal *TOP2A* status, compared to 8% (33 of 435) with HER2 negative tumors. As a large number of patients with *TOP2A* abnormal tumors are contained within the HER2 positive population it was not feasible to combine these two markers. By integration of *TOP2A* and TIMP-1 in the 2T profile 43% of the patients were classified as anthracycline responsive compared to 22% using *TOP2A* and 25% using TIMP-1 alone. For the 43% of patients with a 2T responsive profile the use of CEF was associated with a relative reduction in IDFS events of 52% and a 46% relative reduction in mortality.

In contrast, a non-significant benefit from CMF was seen in the remaining 57% patients with a 2T non-responsive profile. The magnitude of difference among patients with a 2T responsive and non-responsive profile and the accuracy of these estimates are high enough to emphasize a clinical important difference. The finding of a highly statistically significant interaction between treatment and the 2T profile supports this statement. The 4% who had a *TOP2A* and a TIMP-1 responsive profile did not seem to have a different outcome.

HER2 is the most frequent used biomarker regarding sensitivity to anthracyclins, and the majority of *TOP2A* aberrations are observed among HER2 positive tumors. For comparison the present inventors combined HER2 and TIMP-1, and classified patients as HT anthracyclin responsive if the tumor lacked TIMP-1 immunoreactivity and/or were HER2 positive.

The benefit from CEF as compared to CMF was substantially larger in the 50% of patients with a HT responsive profile, and this heterogeneity was confirmed by a

statistically significant interaction between the HT profile and treatment. The present inventors did not find evidence for a differential treatment effect according to TIMP-1 or HER2 as single markers, which emphasis the power of integrating biomarkers.

In conclusion, the combined analysis of the 2T profile based on both *TOP2A* and TIMP-1 show that in combination these two biomarkers identify the greater part, if not nearly all patients who benefits significantly from substituting methotrexate in CMF with epirubicin. The 2T profile separates out a larger anthracycline responsive subgroup than HER2, *TOP2A* and TIMP-1 do individually.

Tables

Table 1

	Hazard ratio	95% confidence intervals
TOP2A DNA deletion	0.53	0.28-1.0
TOP2A DNA amplification	0.38	0.2-0.72
TIMP-1 lack of immunoreactivity in tumor cells in patients without TOP2A gene aberrations	0.54	0.31-0.93
TOP2A DNA aberrations or lack of TIMP-1 immunoreactivity in the cancer cells	0.48	0.34-0.69
Lack of TOP2A DNA aberrations or positive TIMP-1 immunoreactivity in the cancer cells	1.19	0.87-1.61

Table 2

Distribution of TIMP-1 Immunoreactivity According to HER2 and *TOP2A* Status.

	TOP2A abnormal				*TOP2A* normal				Total
	HER2		HER2		HER2		HER2		
TIMP-1	positive		negative		positive		negative		
	N	%	N	%	N	%	N	%	
Positive	89	14	26	4	68	11	286	46	469
Negative	17	3	7	1	14	2	116	19	154
Total	106	17	33	5	82	13	402	65	623

Table 3

Baseline Characteristics According to 2T Profile					
Characteristic	Responsive profile (N=269)		Non-responsive profile (N=354)		P Value
	No.	(%)	No.	(%)	
Menopausal status					P=0.0497
Premenopausal	174	65	255	72	
Postmenopausal	95	35	99	28	
Local-regional therapy					P=0.04
Breast conserving	36	13	70	20	
Mastectomy	233	87	284	80	
Estrogen receptor status					P=0.004
Positive	69	26	128	36	
Negative	184	68	203	57	
Unknown	16	6	23	7	
HER2 status					P<0.0001
Positive	120	45	68	19	
Negative	149	55	286	81	
Positive nodes			Positive nodes		
None	89	33		None	89
1-3	86	32		1-3	86
> 3	94	35		> 3	94
Tumor size, millimeters			Tumor size, millimeters		

	0-20	88	33		0-20	88
	21-50	152	57		21-50	152
	> 50	28	10		> 50	28
	Unknown	1	0		Unknown	1
Malignancy grade				Malignancy grade		
	Grade I	14	5		Grade I	14
	Grade II	124	46		Grade II	124
	Grade III	113	42		Grade III	113
	Unknown	1	0		Unknown	1
	Non-ductal	17	6		Non-ductal	17
Treatment				Treatment		
	CMF	150	56		CMF	150
	CEF	119	44		CEF	119

Table 4

Unadjusted hazard ratio estimates of treatment effect for IDFS and OS in HT and 2T Responsive and Non-responsive tumors.

	IDFS			OS		
	HR	(95% CI)	P	HR	(95% CI)	P
HT profile						
Responsive	0.73	(0.53-1.00)	0.05	0.69	(0.50-0.95)	0.02
Non-responsive	0.98	(0.71-1.37)	0.92	0.92	(0.66-1.29)	0.64
2T profile						
Responsive	0.59	(0.42-0.83)	0.003	0.63	(0.45-0.88)	0.007
Non-responsive	1.12	(0.83-1.53)	0.46	0.95	(0.69-1.30)	0.74

Table 5

Base-Line Characteristics of the Danish Intention to Treat Population (n=980)		
	Excluded N=333 (34%)	Included N=647 (66%)
	No. (%)	No. (%)
Age at enrolment		
≤ 39 Years	65 20	99 15
40-49 Years	165 50	316 49
50-59 Years	57 17	149 23
60-69 Years	46 14	83 13
Menopausal status		
Premenopausal	246 74	450 70
Postmenopausal	87 26	197 30
Nodal status		
Negative	121 36	233 36
1 – 3 positive	122 37	206 32
≥ 4 positive	90 27	208 32
Tumour size *		
0 – 20 mm	179 55	253 39
21 – 50 mm	130 40	336 52
> 50 mm	19 6	56 9
Unknown	5 2	2 0
Histologic type		

Infiltrating ductal carcinoma	313	94	602	93
Other carcinomas	17	5	44	7
Unknown	3	1	1	0
Malignancy grade (ductal carcinomas only) **				
Grade I	27	9	43	7
Grade II	177	57	298	50
Grade III	104	33	259	43
Unknown	5	2	2	0
Estrogen-receptor status				
Positive	7	2	199	31
Negative	26	8	401	62
Unknown	300	90	47	7
Hormone-receptor status				
ER or PgR positive	88	26	167	26
ER and PgR negative	201	60	431	67
Unknown	44	13	49	8
Chemotherapy				
CMF	158	47	357	55
CEF	157	47	290	45
None	18	5	0	0

p<0.000.1; ** p=0.02

Table 6

Base-Line Characteristics in relation to TIMP-1		
	TIMP1 neg. (N=160)	TIMP1 pos. (N=487)
	No. (%)	No. (%)
Age at enrolment		
≤ 39 Years	26 (16)	73 (15)
40-49 Years	78 (49)	238 (49)
50-59 Years	36 (23)	113 (23)
60-69 Years	20 (13)	63 (13)
Menopausal status		
Premenopausal	118 (74)	332 (68)
Postmenopausal	42 (26)	155 (32)
Nodal status		
Negative	72 (45)	161 (33)
1 – 3 positive	44 (28)	162 (33)
≥ 4 positive	44 (28)	164 (34)
Tumour size		
0 – 20 mm	62 (39)	191 (39)
21 – 50 mm	81 (51)	255 (52)
> 50 mm	16 (10)	40 (8)
Unknown	1 (1)	1 (0)
Histologic type		
Infiltrating ductal carcinoma	146 (91)	456 (94)

Other carcinomas	14 (9)	31 (6)
Malignancy grade (ductal carcinomas only)		
Grade I	9 (6)	34 (7)
Grade II	66 (45)	232 (51)
Grade III	70 (48)	189 (41)
Unknown	1 (1)	1 (0)
Estrogen-receptor status		
Positive	38 (24)	161 (33)
Negative	107 (67)	294 (60)
Unknown	15 (9)	32 (7)
Hormone-receptor status		
ER or PgR positive	36 (23)	131 (27)
ER and PgR negative	115 (72)	316 (65)
Unknown	9 (6)	40 (8)
Chemotherapy		
CMF	86 (54)	271 (56)
CEF	74 (46)	216 (44)

Table 7 - Diagram showing the patient flow

	CMF	CEF
Cumulative allocation	500	480
Cross-over, self-selected CMF	+18	-18
Cross-over, self-selected CEF	-4	+4
Withdraw consent to chemotherapy	-5	-13
TIMP-1 unknown*	-152	-163
Included in the analyses	357	290

*Archival tissue not available, tissue unsuited for TMA, tissue lost after TMA or TIMP-1 not assessable.

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Claims

1. A method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a. determining in a sample obtained from said individual, the absence of TIMP-1 protein in tumour cells comprised in said sample or presence of a *TIMP-1* DNA aberration in the tumour cells of said sample, and

b. determining the presence of any chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 or aberrant protein expression of a gene comprised in said amplicon

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 is present and/or the protein expression of the gene comprised in said amplicon is aberrant in said tumour cells and/or the tumour cells are absent of TIMP-1 protein and/or if said tumour cells comprise said *TIMP-1* DNA aberration on either or both of the alleles of the *TIMP-1* gene, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no chromosomal DNA aberration in the *TOP2A/HER2* amplicon is present or no protein encoded by any gene comprised in said amplicon is aberrantly expressed in the tumour cells and if TIMP-1 protein is present in the tumour cells and/or if neither of the *TIMP-1* alleles comprise said *TIMP-1* DNA aberration.

2. A method according to claim 1, wherein the chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 is a *TOP2A* DNA aberration, and the protein expression of the gene comprised in said amplicon is topoisomerase IIa expression.

3. A method according to claim 1, wherein the chromosomal DNA aberration in the *TOP2A/HER2* amplicon on chromosome 17q21 is a *HER2* DNA aberration, and

the protein expression of the gene comprised in said amplicon is ErbB2 expression.

4. A method for predicting the response to a topoisomerase IIα inhibitor therapy in an individual having cancer, said method comprising the steps of:

a. determining in a sample obtained from said individual, the absence of TIMP-1 protein in tumour cells comprised in said sample, and

b. determining the presence of any *TOP2A* DNA aberration in the tumour cells of said sample

c. classifying the individual as having a high likelihood of responding to a topoisomerase IIα inhibitor therapy if a *TOP2A* DNA aberration is present and/or if the tumour cells are absent of TIMP-1 protein, and

d. classifying the individual as having a low likelihood of responding to a topoisomerase IIα inhibitor therapy if no *TOP2A* DNA aberration is present and if TIMP-1 protein is present in the tumour cells.

5. The method according to any of claims 1, wherein a reference obtained from a population is used to determine the level of DNA aberration or protein expression.

6. The method according to claim 5, wherein said reference is a normal diploid genetic background found in the tumor tissue stromal cells.

7. The method according to claim 2, wherein the *TOP2A* gene aberration is selected from the group consisting of *TOP2A* DNA amplification, *TOP2A* DNA deletion, *TOP2A* gene point mutation, and *TOP2A* DNA translocation, epigenetic modifications of the *TOP2A* DNA such as DNA methylation, and combinations hereof.

8. The method according to claim 2, wherein topoisomerase IIα protein is more than 2 fold over-expressed relative to a reference sample, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more

than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a reference sample.

9. The method according to claim 2, wherein *TOP2A* gene is more than 2 fold amplified relative to a reference sample, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a reference sample.

10. The method according to claim 2, wherein the *TOP2A* DNA aberration or the increase in topoisomerase IIα protein in the tumour cells correlate with aberrant *TOP2A* mRNA levels in the tumour cells of said sample.

11. The method according to claim 3, wherein the *HER2* gene aberration is selected from the group consisting of *HER2* gene amplification, *HER2* DNA deletion, *HER2* gene point mutations and *HER2* DNA translocations, epigenetic modifications of the *HER2* DNA such as DNA methylation, and combinations hereof.

12. The method according to claim 3, wherein ErbB2 protein is more than 2 fold over-expressed relative to a control sample, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a control sample.

13. The method according to claim 3, wherein *HER2* gene is more than 2 fold amplified relative to a control sample, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more

than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a control sample.

14. The method according to claim 1, wherein *TIMP-1* gene is more than 2 fold amplified relative to a control sample, such as more than 3 fold, for example more than 4 fold, such as more than 5 fold, for example more than 6 fold, such as more than 7 fold, for example more than 8 fold, such as more than 9 fold, for example more than 10 fold, such as more than 15 fold, for example more than 20 fold, such as more than 30 fold, for example more than 40 fold, such as more than 50 fold, for example more than 100 fold of a control sample.

15. The method according to claim 3, wherein the any *HER2* DNA aberration or an increase in ErbB2 protein in the tumour cells correlate with aberrant HER2 mRNA levels in the tumour cells of said sample.

16. The method according to any of the preceding claims, wherein the tumour cells comprise at least one *TIMP-1* DNA aberration selected from the list consisting of a deletion of one of the *TIMP-1* alleles, a deletion of both of the *TIMP-1* alleles, a partial deletion of one of the *TIMP-1* alleles, a partial deletion of both of the *TIMP-1* alleles, *TIMP-1* DNA point mutations, *TIMP-1* DNA inversion, *TIMP-1* DNA translocation, epigenetic modifications of the *TIMP-1* DNA such as DNA methylation, and combinations hereof.

17. The method according to any of the preceding claims, wherein the tumour cells are absent of TIMP-1 protein.

18. The method according to claims 1 wherein the level of DNA gene aberration is determined by means of DNA measurement such as but not limited to in situ hybridization, a PCR method, differential display, DNA-dot-blotting, Southern blotting or combinations hereof.

19. The method according to claim 18, wherein the *in situ* hybridization is determined by means of FISH (Fluoroscent In-Situ Hybridization)

20. The method according to claim 19, wherein FISH comprises employing a probe mixture comprising labeled DNA probes targeted at a portion of the *TOP2A* gene region, or the *HER2* gene region, or a portion of the *TIMP-1* gene region and a probe mixture comprising fluoroscein-labelled probes targeted at the centromeric region of chromosome 17 and the X chromosome, respectively.

21. The method according to claims 19, wherein said DNA aberration is determined as the average ratio to an internal reference sequence comprised in said sample.

22. The method according to claims 21, wherein the internal reference sequence is chromosome X α-satellite (Cen X).

23. The method according to claim 22, wherein the tumour cells comprise a *TIMP-1* gene deletion if the average ratio of *TIMP-1*/ Cen X is below 0.8, and normal if the said ratio is above 0.8 and below 2.0.

24. The method according to claim 22, wherein the tumour cells comprise *TOP2A* gene deletion if the average ratio of *TOP2A* / Cen X is below 0.8 or amplifications if the average ratio of *TOP2A* / Cen X is above 2.0, and normal if the said ratio is above 0.8 and below 2.0.

25. The method according to claim 22, wherein the tumour cells comprise *HER2* gene deletion average ratio of *HER2* / Cen X is below 0.8 or amplifications if the average ratio of *HER2* / Cen X is above 2.0, and normal if the said ratio is above 0.8 and below 2.0.

26. The method according to any of the preceding claims, wherein the level of gene expression is determined by means of mRNA measurement such as but not limited to Northern blotting, RNA dot and a quantitative PCR method.

27. The method according to any of the preceding claims, wherein aberrant protein expression is determined by means of protein level measurement such as Western blotting, Immunohistochemistry, immunocytochemistry, ELISA, and RIA.

28. The method according to any of the preceding claims, wherein the determination of DNA or protein aberrations is performed on archive material from the individual, such as a paraffin block comprising tumour tissue.

29. The method according any of the preceding claims, wherein the cancer is selected from the group consisting of breast cancer, sarcomas, ovarian cancer, and non small cell lung cancer

30. The method according any of the preceding claims, wherein said sample is selected from the group consisting of a tumour tissue sample, a blood sample, a plasma sample, a serum sample, a urine sample, a faeces sample, a saliva sample, and a sample of serous liquid from the thoracic or abdominal cavity and a combination hereof.

31. The method according to claim 1, wherein the topoisomerase IIα inhibitor therapy comprises an inducer of apoptosis or mitotic catastrophe.

32. The method according to any of claims 1 and 31, wherein the topoisomerase IIα therapy is selected from the group consisting of neoadjuvant therapy, adjuvant therapy and therapy of metastatic disease

33. The method according to any of claims 1 and 31-32, wherein the topoisomerase IIα inhibitor is an anthracycline.

34. The method according to claim 33, wherein the anthracycline is selected from the group consisting of but not limited to 4-Epirubricin, Daunorubicin, Daunorubicin (liposomal), Doxorubicin, Doxorubicin (liposomal), Epirubicin, Idarubicin, and Mitoxantrone, or a combination hereof.

35. The method according to claim 34, wherein the topoisomerase IIα inhibitor is comprised in a composition further comprising cyclophosphamide, taxanes and/or 5-fluorouracil.

36. The method according to claim 35, wherein at least one of cyclophosphamide, taxanes and/or 5-fluorouracil is in the form of a prodrug.

37. The method according to any of claims 1-32, wherein the topoisomerase IIα inhibitor therapy is, 4-Epirubricin.

38. The method according to any of the preceding claims, wherein the likelihood of responding to a topoisomerase IIα inhibitor therapy is determined by means of a hazard ratio.

39. A method of treating cancer in an individual comprising

a. predicting the response to an topoisomerase IIα inhibitor therapy according to any of the preceeding claims, and

b. selecting a topoisomerase IIα inhibitor therapy to which said individual has a high likelihood of responding to,

c. subjecting to said individual to said topoisomerase IIα inhibitor therapy.

40. A method according to claim 39, wherein the topoisomerase IIα inhibitor is a anthracyclines selected from the group consisting of but not limited to 4-Epirubricin, Daunorubicin, Daunorubicin (liposomal), Doxorubicin, Doxorubicin (liposomal), Epirubicin, Idarubicin, and Mitoxantrone, or a combination hereof.

41. The method according to claim 40, wherein the topoisomerase IIα inhibitor therapy is comprised in a composition further comprising cyclophosphamide and 5-fluorouracil and/or a taxane.

42. A kit for predicting the response to a topoisomerase IIα inhibitor therapy comprising

a. reagents suitable for the determination of a chromosomal DNA aberration in the *TOP2A/HER2* amplicon such *TOP2A* or *HER2* DNA aberrations in a biological sample, and

b. reagents suitable for the determination of a *TIMP-1* DNA aberration or determining the level of a TIMP-1 protein in a biological sample.

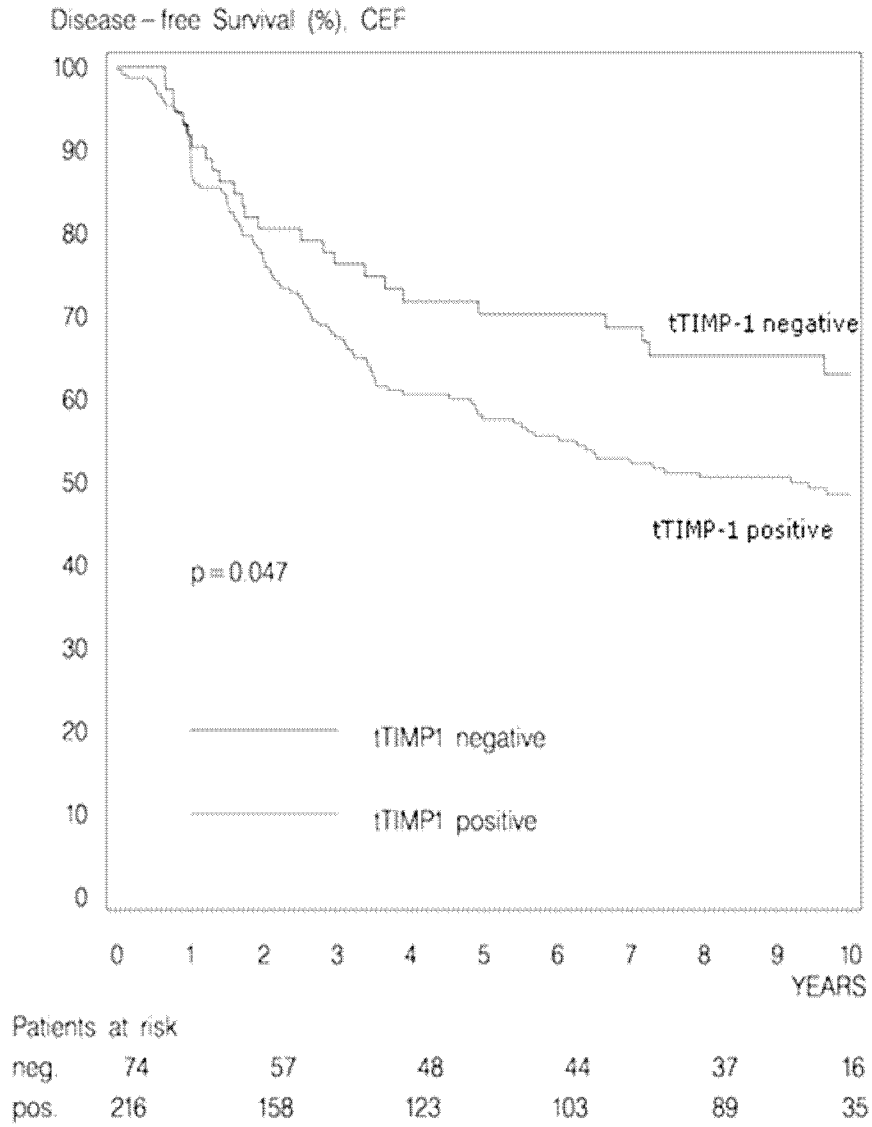

Disease – free Survival (%), CEF
100
90
80
70
60
50
40
30
20
10
0
tTIMP-1 negative
tTIMP-1 positive
p = 0.047
tTIMP1 negative
tTIMP1 positive
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
neg. 74 57 48 44 37 16
pos. 216 158 123 103 89 35

Fig. 1A

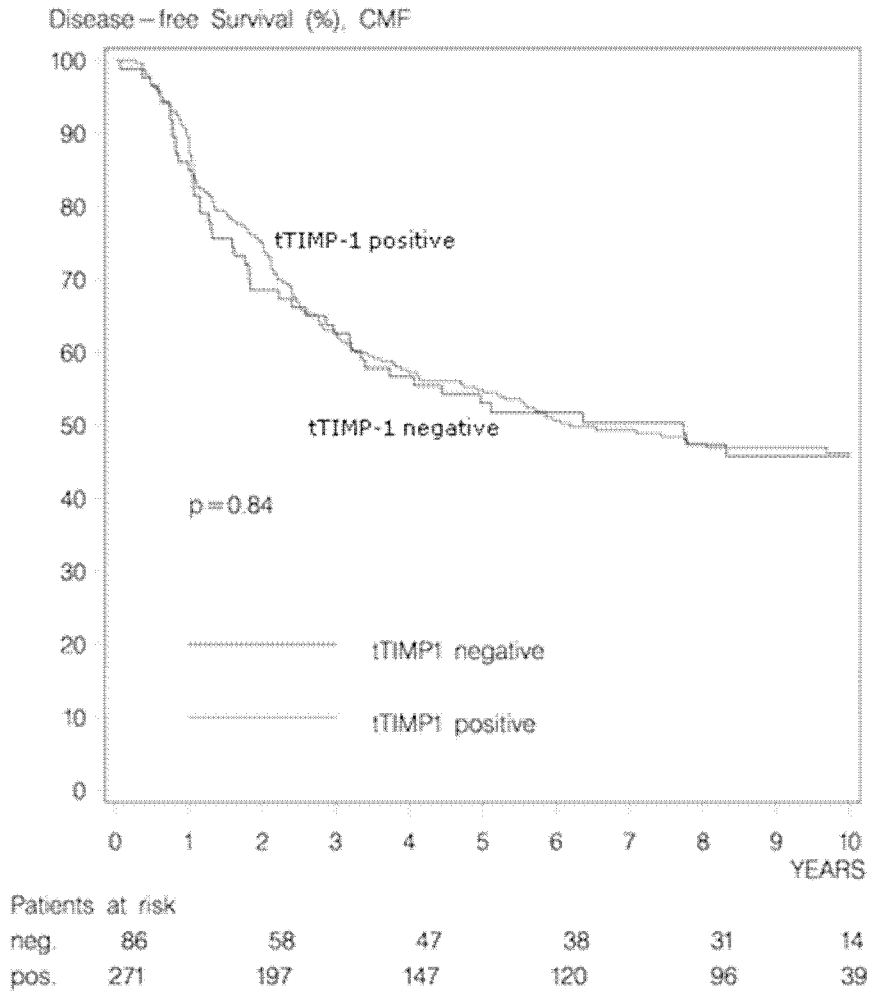

Disease – free Survival (%), CMF
100
90
80
70
60
50
40
30
20
10
0
tTIMP-1 positive
tTIMP-1 negative
p = 0.84
tTIMP1 negative
tTIMP1 positive
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
neg. 86 58 47 38 31 14
pos. 271 197 147 120 96 39

Fig. 1B

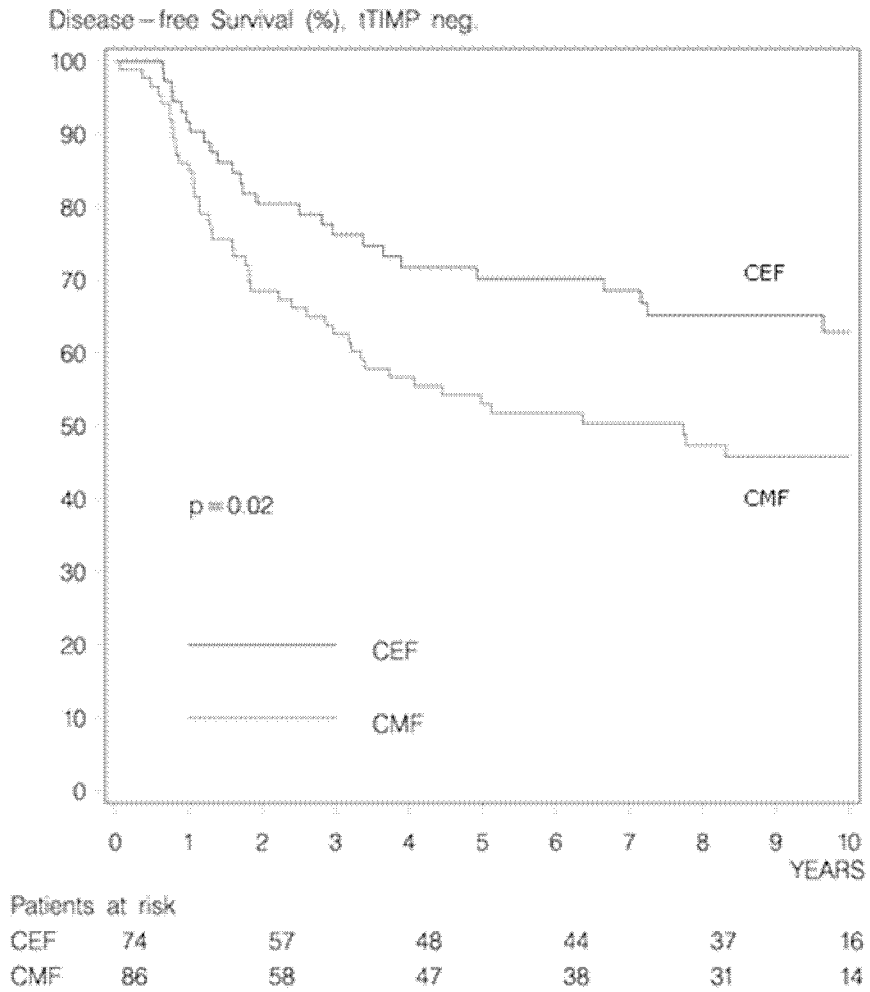

Disease – free Survival (%), tTIMP neg.
100
90
80
70
60
50
40
30
20
10
0
CEF
CMF
p = 0.02
CEF
CMF
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
CEF 74 57 48 44 37 16
CMF 86 58 47 38 31 14

Fig. 1C

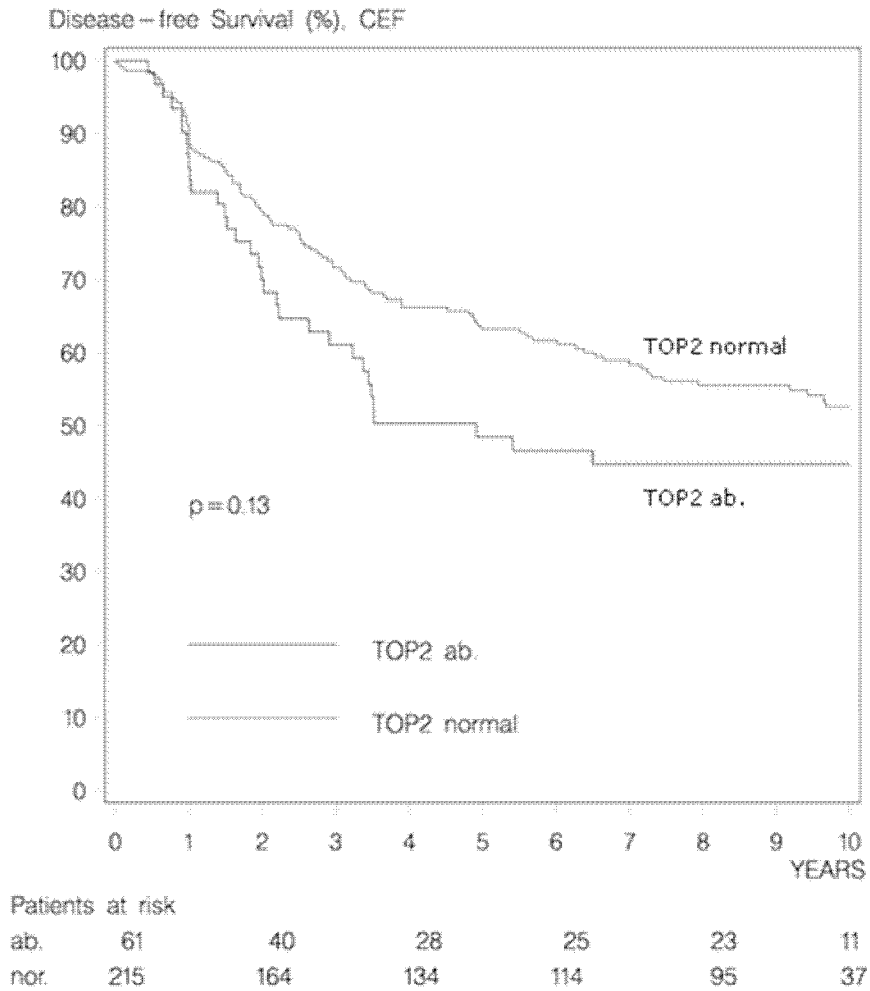

Disease – free Survival (%), CEF
100
90
80
70
60
50
40
30
20
10
0
TOP2 normal
TOP2 ab.
p = 0.13
TOP2 ab.
TOP2 normal
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
ab. 61 40 28 25 23 11
nor. 215 164 134 114 95 37

Fig. 2A

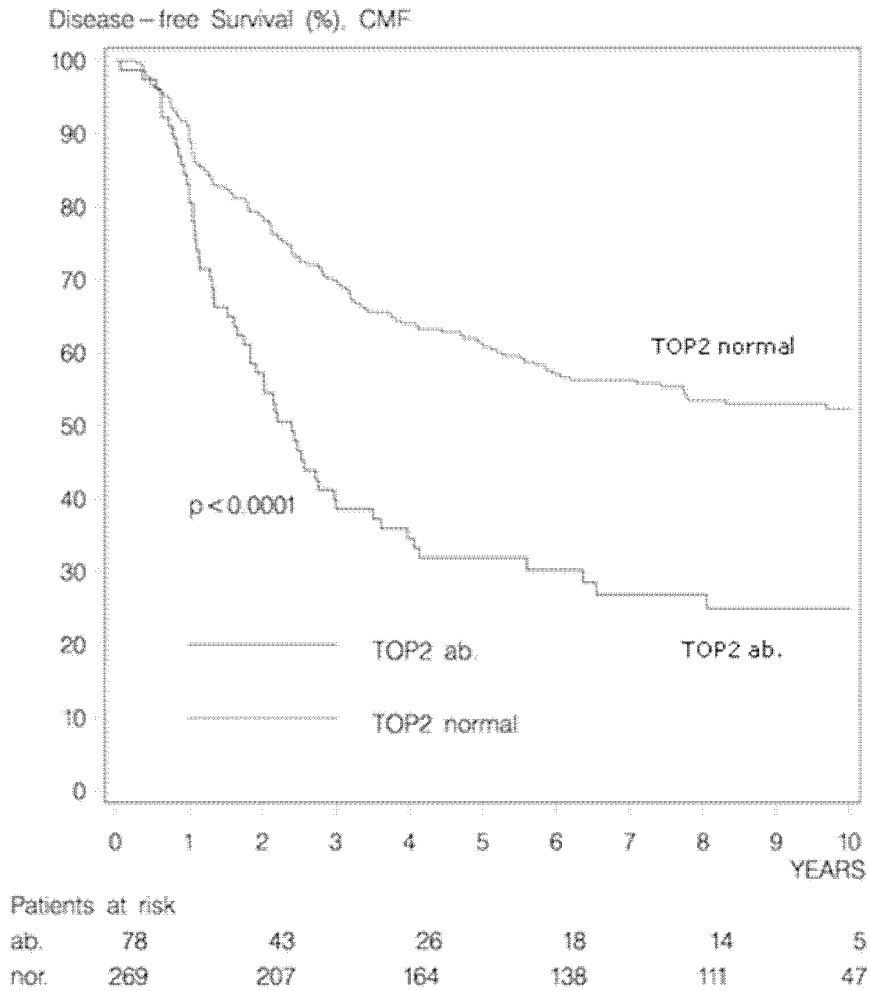

Disease – free Survival (%), CMF
100
90
80
70
60
50
40
30
20
10
0
TOP2 normal
p < 0.0001
TOP2 ab.
TOP2 ab.
TOP2 normal
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
ab. 78 43 26 18 14 5
nor. 269 207 164 138 111 47

Fig. 2B

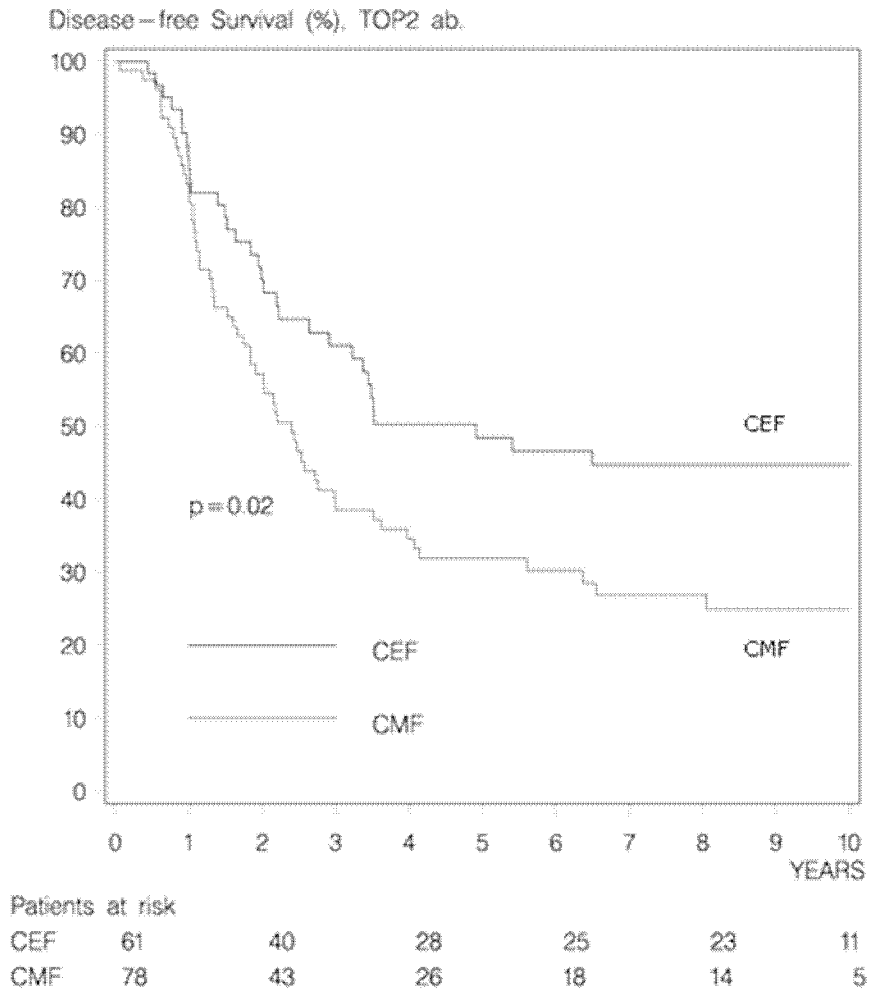

Disease-free Survival (%), TOP2 ab.
100
90
80
70
60
50
40
30
20
10
0
p=0.02
CEF
CMF
CEF
CMF
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
CEF 61 40 28 25 23 11
CMF 78 43 26 18 14 5

Fig. 2C

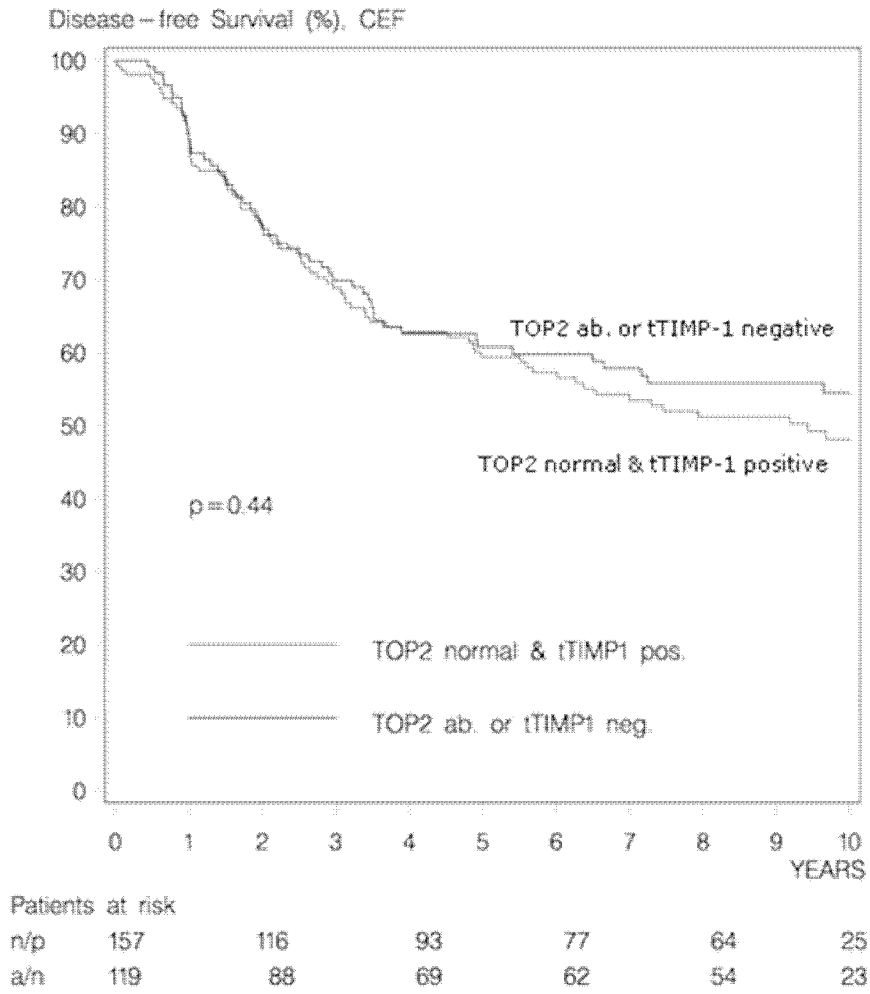

Disease – free Survival (%), CEF
100
90
80
70
60
50
40
30
20
10
0
TOP2 ab. or tTIMP-1 negative
TOP2 normal & tTIMP-1 positive
p = 0.44
TOP2 normal & tTIMP1 pos.
TOP2 ab. or tTIMP1 neg.
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
n/p 157 116 93 77 64 25
a/n 119 88 69 62 54 23

Fig. 3A

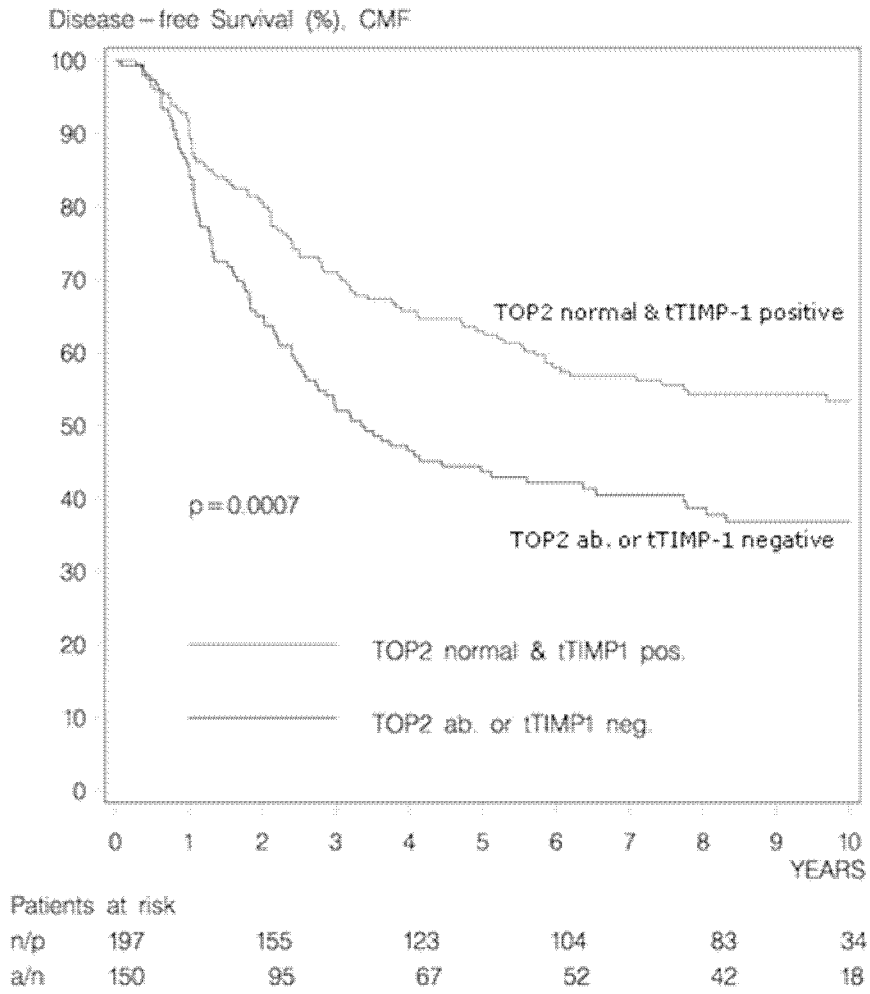

Disease – free Survival (%), CMF
100
90
80
70
60
50
40
30
20
10
0
TOP2 normal & tTIMP-1 positive
p = 0.0007
TOP2 ab. or tTIMP-1 negative
TOP2 normal & tTIMP1 pos.
TOP2 ab. or tTIMP1 neg.
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
n/p 197 155 123 104 83 34
a/n 150 95 67 52 42 18

Fig. 3B

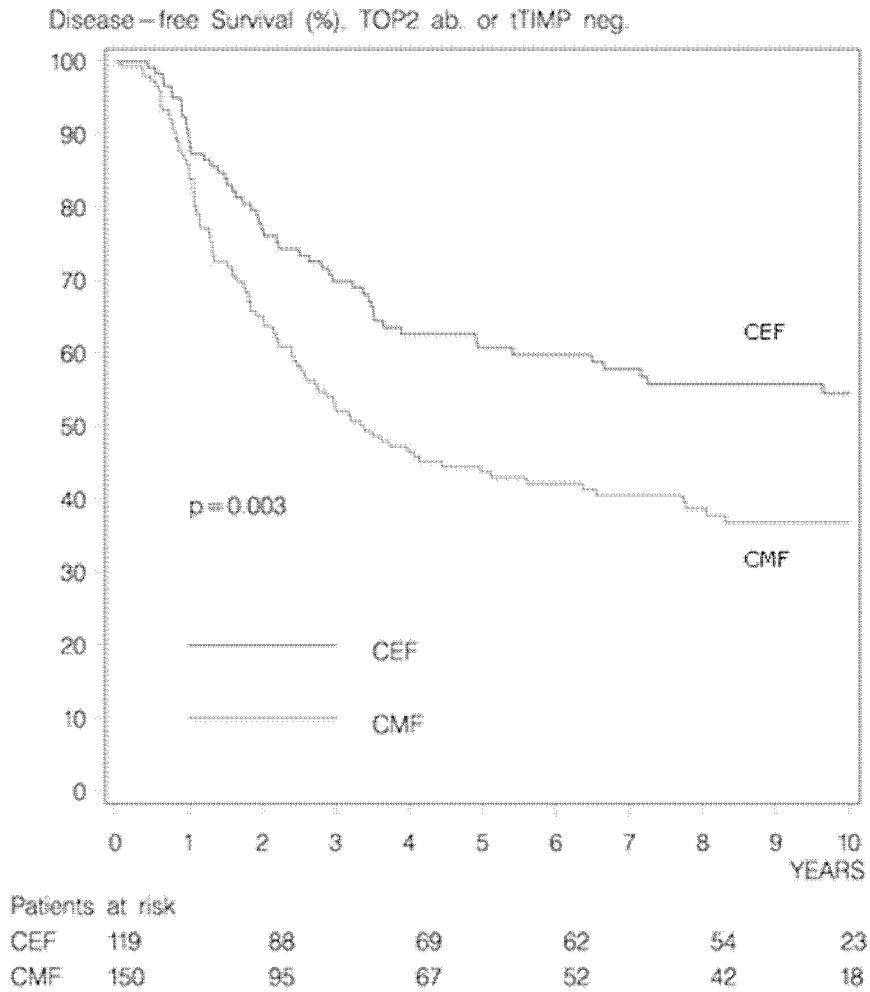

Disease-free Survival (%), TOP2 ab. or tTIMP neg.
100
90
80
70
60
50
40
30
20
10
0
CEF
CMF
p=0.003
CEF
CMF
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
CEF 119 88 69 62 54 23
CMF 150 95 67 52 42 18

Fig. 3C

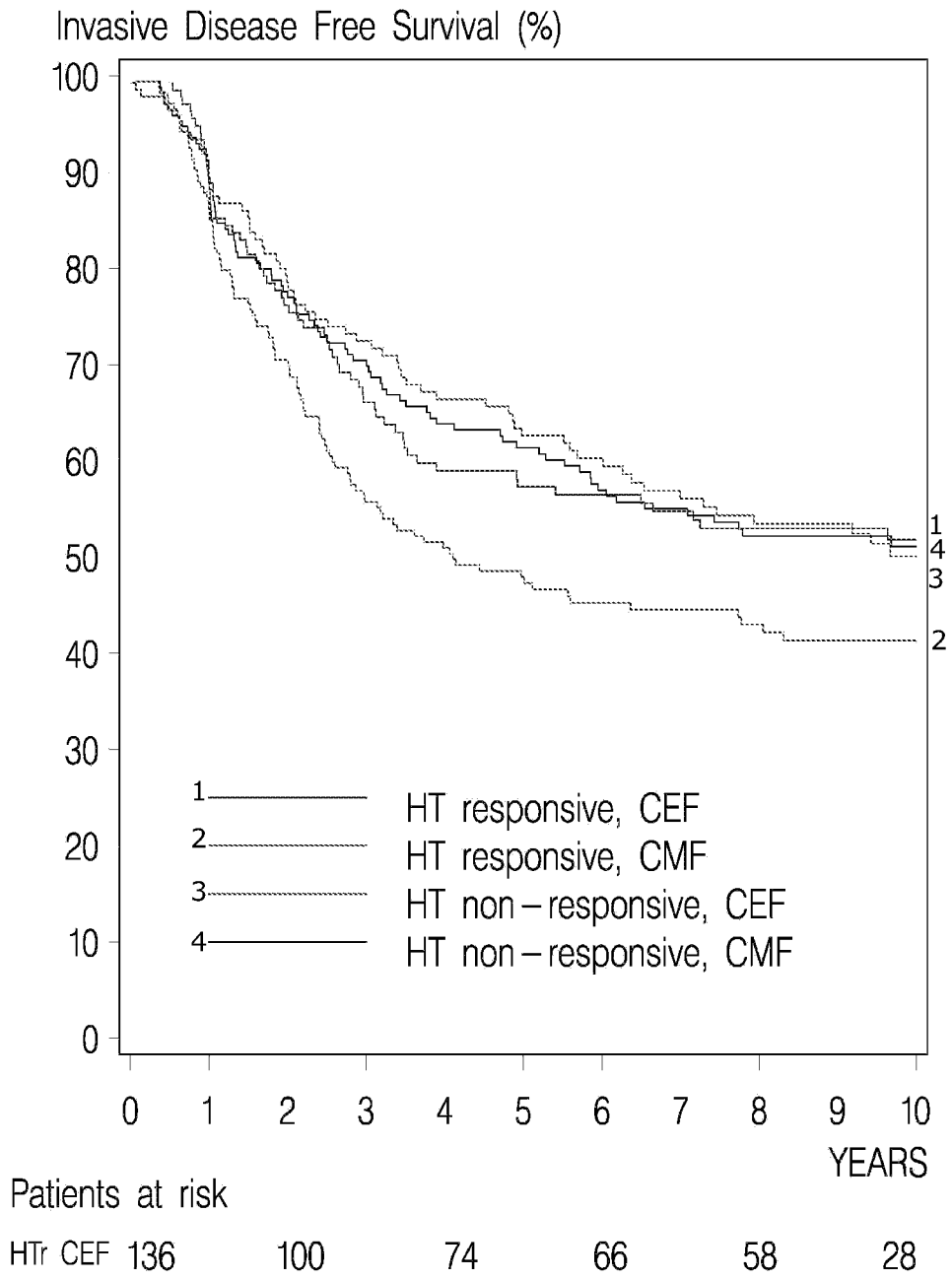

Invasive Disease Free Survival (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
HT responsive, CEF
HT responsive, CMF
HT non – responsive, CEF
HT non – responsive, CMF
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
HTr CEF 136 100 74 66 58 28

Fig. 4A

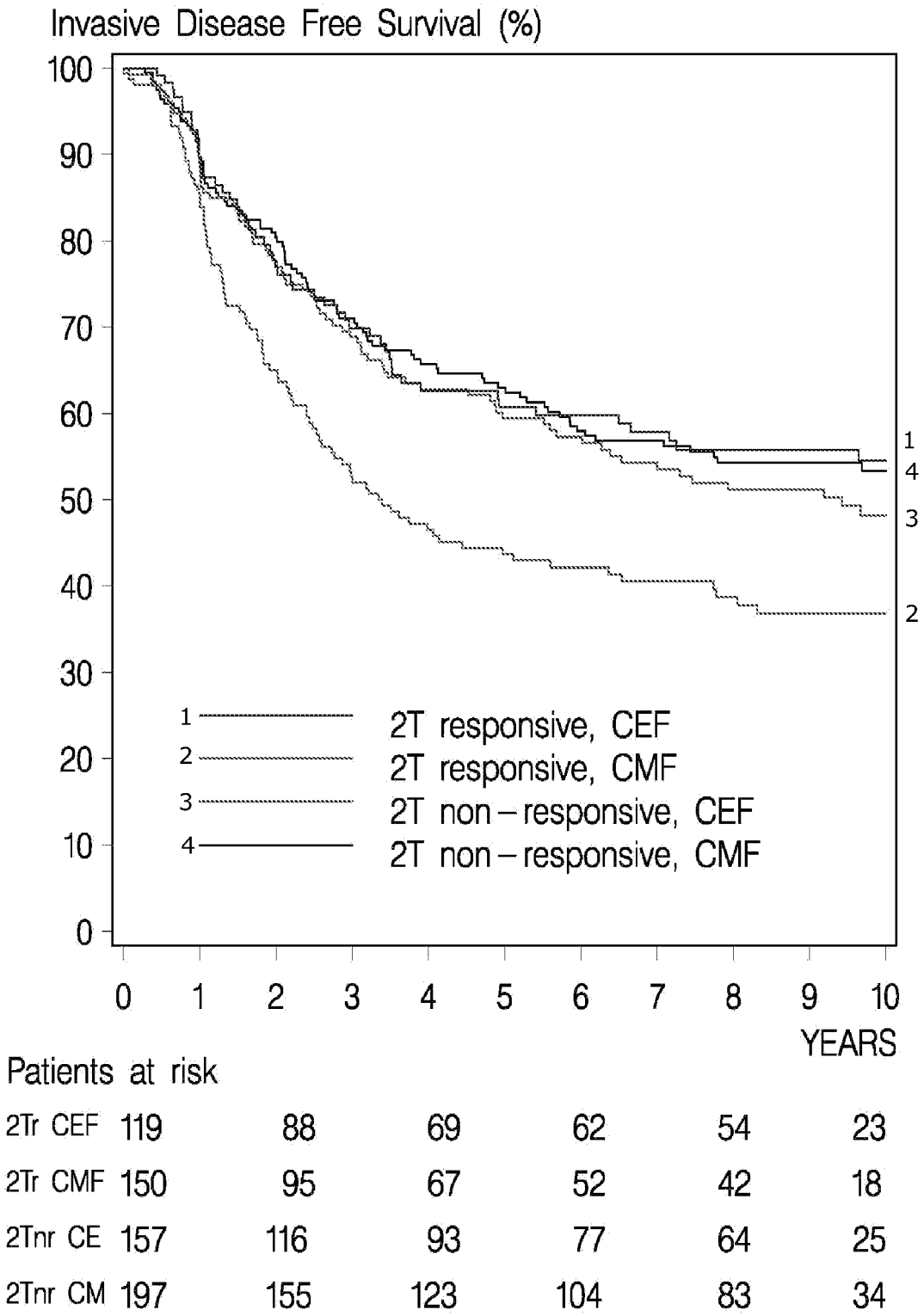

Invasive Disease Free Survival (%)
100
90
80
70
60
50
40
30
20
10
0
1
4
3
2
1 2T responsive, CEF
2 2T responsive, CMF
3 2T non – responsive, CEF
4 2T non – responsive, CMF
0 1 2 3 4 5 6 7 8 9 10
YEARS
Patients at risk
2Tr CEF 119 88 69 62 54 23
2Tr CMF 150 95 67 52 42 18
2Tnr CE 157 116 93 77 64 25
2Tnr CM 197 155 123 104 83 34

Fig. 4B

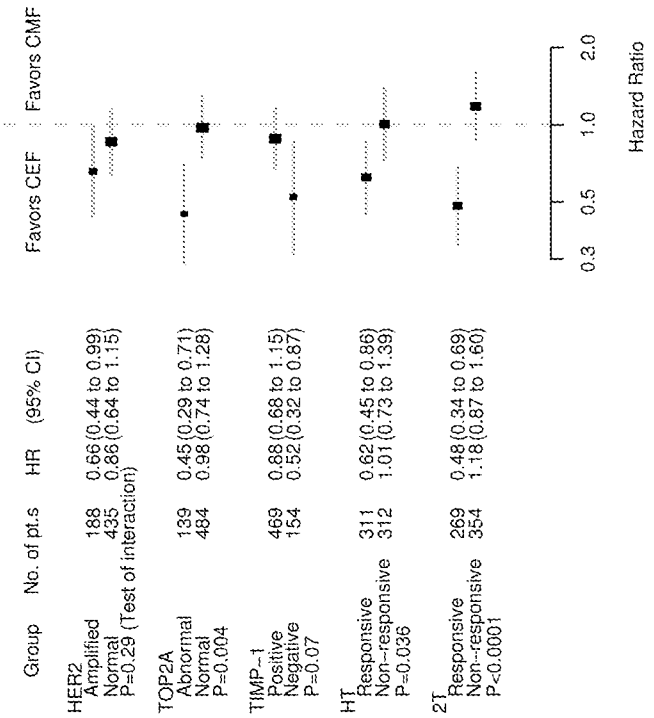

Group
No. of pt.s
HR
(95% CI)
Favors CEF
Favors CMF
HER2
Amplified 188 0.66 (0.44 to 0.99)
Normal 435 0.86 (0.64 to 1.15)
P=0.29 (Test of interaction)
TOP2A
Abnormal 139 0.45 (0.29 to 0.71)
Normal 484 0.98 (0.74 to 1.28)
P=0.004
TIMP-1
Positive 469 0.88 (0.68 to 1.15)
Negative 154 0.52 (0.32 to 0.87)
P=0.07
HT
Responsive 311 0.62 (0.45 to 0.86)
Non-responsive 312 1.01 (0.73 to 1.39)
P=0.036
2T
Responsive 269 0.48 (0.34 to 0.69)
Non-responsive 354 1.18 (0.87 to 1.60)
P<0.0001
0.3
0.5
1.0
2.0
Hazard Ratio

Fig. 5A

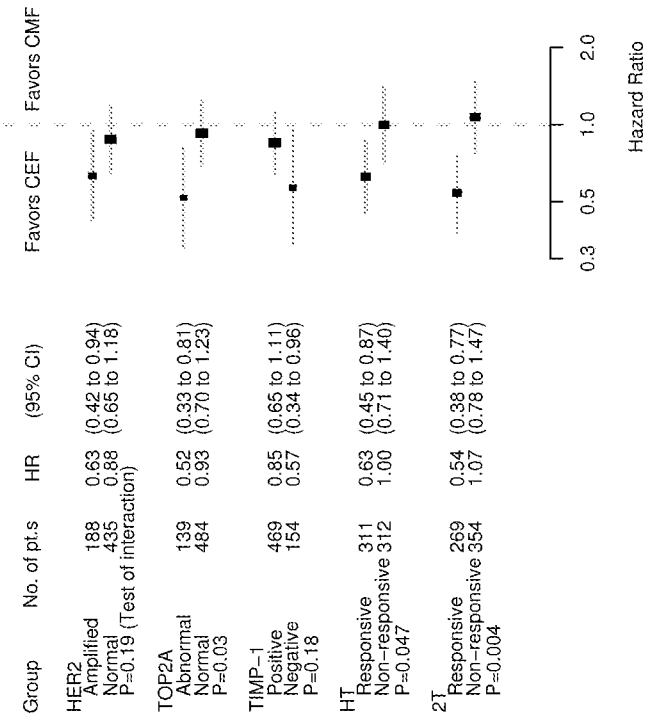

Group
No. of pt.s
HR
(95% CI)
Favors CEF
Favors CMF
HER2
Amplified 188 0.63 (0.42 to 0.94)
Normal 435 0.88 (0.65 to 1.18)
P=0.19 (Test of interaction)
TOP2A
Abnormal 139 0.52 (0.33 to 0.81)
Normal 484 0.93 (0.70 to 1.23)
P=0.03
TIMP−1
Positive 469 0.85 (0.65 to 1.11)
Negative 154 0.57 (0.34 to 0.96)
P=0.18
HT
Responsive 311 0.63 (0.45 to 0.87)
Non−responsive 312 1.00 (0.71 to 1.40)
P=0.047
2T
Responsive 269 0.54 (0.38 to 0.77)
Non−responsive 354 1.07 (0.78 to 1.47)
P=0.004
0.3
0.5
1.0
2.0
Hazard Ratio

Fig. 5B

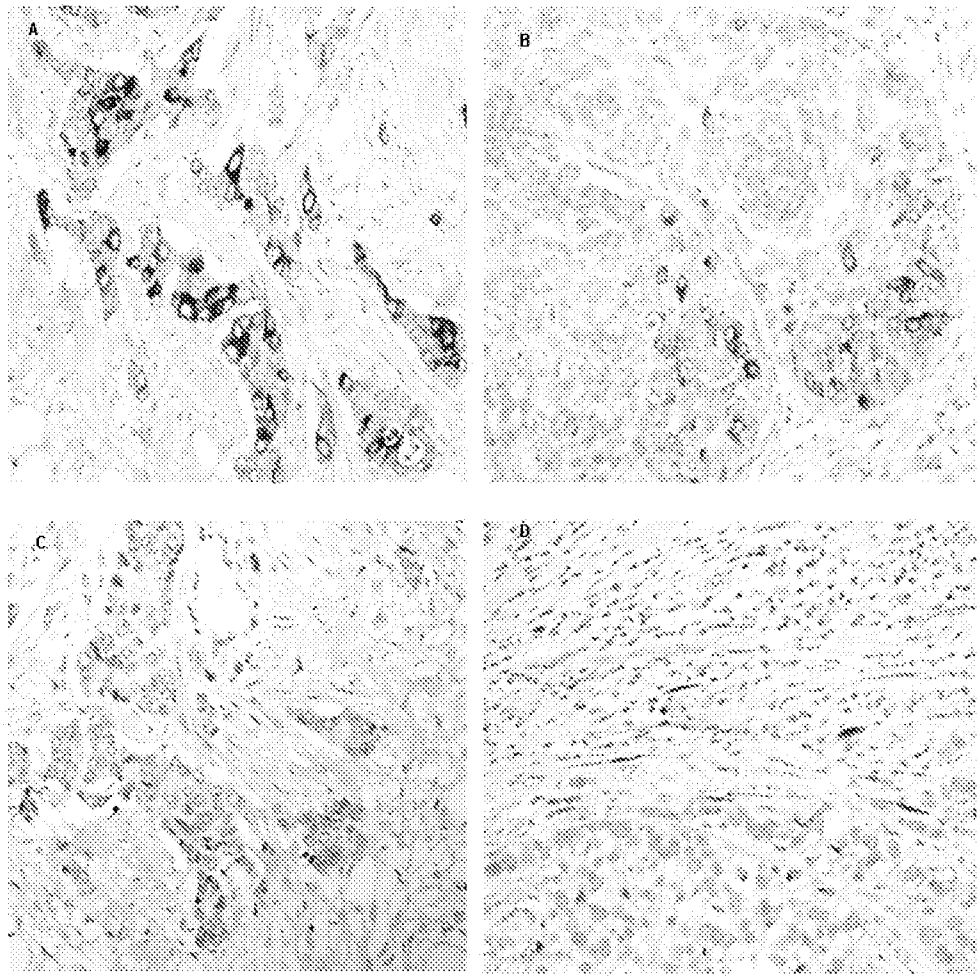
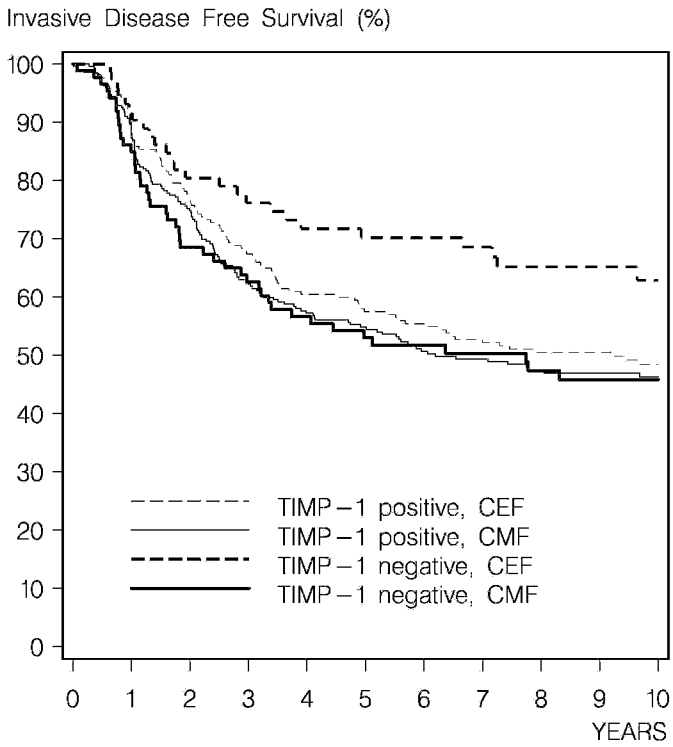
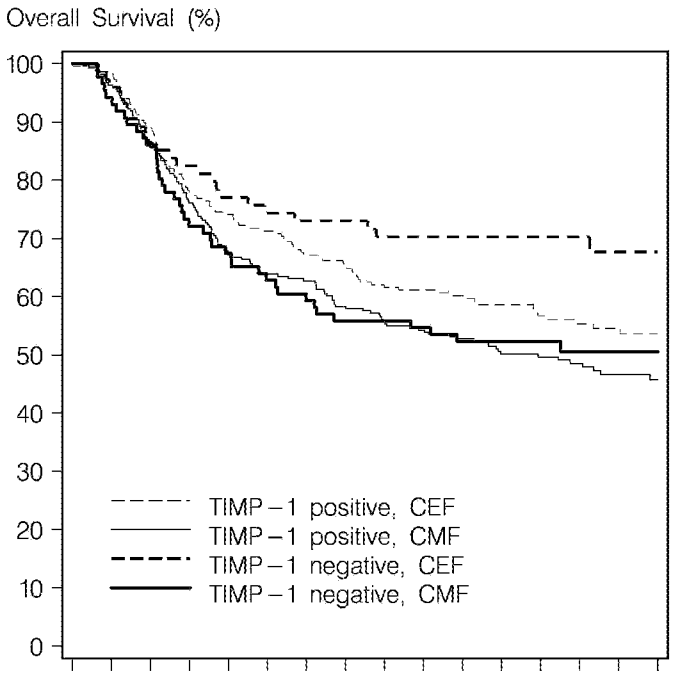


Fig. 6A-D



Patients at risk						
T+ CEF	216	158	123	103	89	35
T+ CMF	271	197	147	120	96	39
T– CEF	74	57	48	44	37	16
T– CMF	86	58	47	38	31	14

Fig. 7A



Patients at risk

T+ CEF	216	193	160	145	133	120	87	59
T+ CMF	271	232	182	170	151	131	100	62
T– CEF	74	64	57	54	52	48	34	23
T– CMF	86	74	58	51	48	41	33	20

Fig. 7B

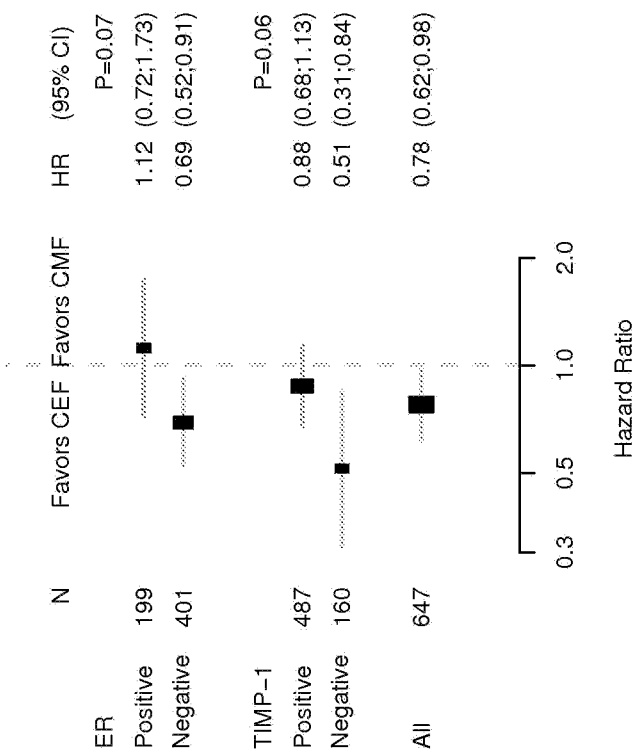

N
Favors CEF
Favors CMF
HR (95% CI)
ER
P=0.07
Positive 199 1.12 (0.72;1.73)
Negative 401 0.69 (0.52;0.91)
TIMP-1
P=0.06
Positive 487 0.88 (0.68;1.13)
Negative 160 0.51 (0.31;0.84)
All 647 0.78 (0.62;0.98)
0.3
0.5
1.0
2.0
Hazard Ratio

Fig. 8A

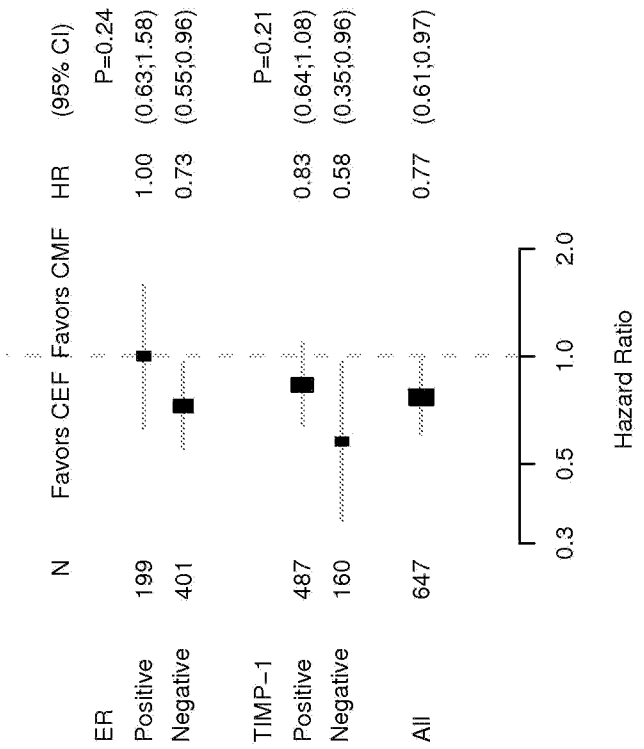

N
Favors CEF
Favors CMF
HR
(95% CI)
ER
P=0.24
Positive 199 1.00 (0.63;1.58)
Negative 401 0.73 (0.55;0.96)
TIMP-1
P=0.21
Positive 487 0.83 (0.64;1.08)
Negative 160 0.58 (0.35;0.96)
All 647 0.77 (0.61;0.97)
0.3
0.5
1.0
2.0
Hazard Ratio

Fig. 8B

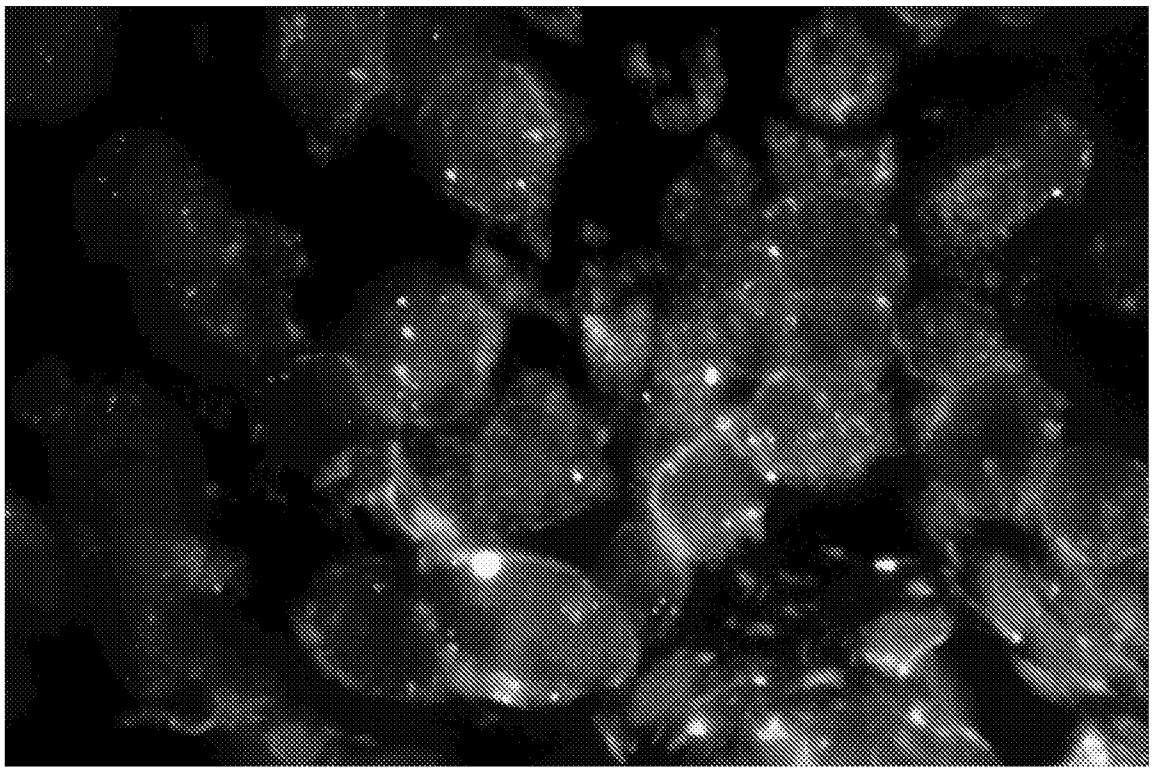


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2009/050116

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

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)
C12Q

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, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHROHL ANNE-SOFIE ET AL: "Primary tumor levels of tissue inhibitor of metalloproteinases-1 are predictive of resistance to chemotherapy in patients with metastatic breast cancer." CLINICAL CANCER RESEARCH : AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH 1 DEC 2006, vol. 12, no. 23, 1 December 2006 (2006-12-01), pages 7054-7058, XP002505722 ISSN: 1078-0432 page 7055, right-hand column, paragraph 5; figure 1; table 1 page 7058, right-hand column, lines 10,11	1-42

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 2009	24/09/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lanzrein, Markus

2

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2009/050116

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JORGENSEN J T ET AL: "Pharmacodiagnostics and targeted therapies - a rational approach for individualizing medical anticancer therapy in breast cancer" ONCOLOGIST, ALPHAMED PRESS, US, vol. 12, no. 4, 1 April 2007 (2007-04-01), pages 397-405, XP002444118 ISSN: 1083-7159 page 401, right-hand column, paragraph 1 - page 403, left-hand column, paragraph 1; figures 5-7	1-42
Y	WO 2007/112746 A (DAKO DENMARK AS [DK]; JOERGENSEN JAN TROEST [DK]; EJLERTSEN BENT [DK];) 11 October 2007 (2007-10-11) example 2	1-42

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2009/050116

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007112746 A	11-10-2007	CA 2647016 A1	11-10-2007
		EP 2002014 A1	17-12-2008