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(54) **Title**: METHODS FOR PREDICTING RESPONSE TO ANTI-CANCER THERAPY IN CANCER PATIENTS

Fig. 1 - BRCA1-associated genomic loci used to identify breast cancers with a BRCA1-deficient DNA repair system

chromosome	begin BAC	end BAC			# Bacs in region	# BACs in classifier	mid position begin	mid position end	size Mb	Aberration
1	RP4-561P1	RP11-14O19	1p35.1	1p21.3	70	18	34430455	95824367	61.4	gain
3	RP11-22E12	RP11-65J14	3q22.2	3q27.2	71	41	134528889	185893054	51.4	gain
5	RP11-7M4	RP11-20O13	5p13.2	5p13.2	52	24	37047046	93053964	56.0	loss
5	RP11-319P13	RP11-511M9	5q21.3	5q34	67	17	106806461	165898631	59.1	loss
6	RP11-15N12	RP1-153G14	6p25.2	6p22.1	27	14	3436613	27384955	23.9	gain
7	RP11-505D17	RP11-486P11	7p21.3	7p15.3	17	4	8070383	20125033	12.1	loss
7	RP11-264K23	RP11-518I12	7q31.33	7q36.3	50	12	123929280	157960428	34.0	gain
8	RP11-266D22	RP11-349C2	8q22.1	8q24.3	53	12	95352986	145710494	50.4	gain
10	RP11-29A19	RP11-566K1	10p15.3	10p14	12	7	1298380.5	10885404.5	9.6	gain
10	RP11-129O7	RP13-355A21	10p12.1	10p12.1	4	4	25183905	27998514.5	2.8	gain
12	RP11-283I3	RP11-144O23	12p13.33	12p13.2	21	11	282617.5	11040567	10.8	gain
12	RP1-97G4	RP11-478H3	12q21.2	12q23.3	32	12	77778132	106281513.5	28.5	loss
13	RP11-365J7	RP11-232K22	13q31.2	13q33.3	20	15	88049058	107819833	19.8	gain
14	RP11-262M8	RP11-226F19	14q22.1	14q24.1	21	6	52796119	69423346.5	16.6	loss
15	CTD-2033D15	RP11-154J22	15q14	15q21.1	12	6	39919724.5	48586416.5	8.7	loss
21	RP1-126N20	RP11-397E9	21q11.2	21q22.3	24	10	15761442	45935377	30.2	gain

(57) **Abstract**: Methods for optimizing the therapeutic efficacy of anti-cancer therapy by detecting phenotypic genetic traits using comparative genomic hybridization are disclosed.

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METHODS FOR PREDICTING RESPONSE TO ANTI-CANCER THERAPY IN CANCER PATIENTS

Cross-Reference to Related Applications

5 [001] This PCT application claims priority to United States provisional patent application No. 61/384,499, filed September 20, 2010 and entitled, Methods for Predicting Response to Anti-Cancer Therapy in Cancer Patients, the contents of which are incorporated herein by reference, in their entirety.

Field

10 [002] Methods provided by the present disclosure relate to optimizing the therapeutic efficacy of anti-cancer therapy by detecting phenotypic genetic traits using comparative genomic hybridization.

Background

[003] Breast cancer is the most frequently occurring cancer among women in the 15 western world. It is a heterogeneous cancer disease, consisting of several subtypes. Molecular biology has greatly enhanced our understanding of the heterogeneity of breast cancer, but few molecular tumor features are actually used in the clinic to guide the choice of a systemic treatment strategy.

[004] Neoadjuvant systemic therapy, or administration of therapeutic agents prior to 20 a main treatment, has become a widely used treatment strategy for patients with early, or locally advanced, breast cancer. Despite its early and late toxicities, this treatment strategy reduces the risk of breast cancer relapse and mortality by approximately half.

[005] In spite of these advantages, a disadvantage to the use of neoadjuvant systemic therapy is the lack of predictive tests to individualize the choice of certain 25 combinations of drugs for an individual breast cancer patient to ensure maximal benefit with minimal toxicity. For example, for highly toxic adjuvant treatment regimens, such as high dose alkylating chemotherapy with hematopoietic stem-cell rescue, the survival benefit when compared with standard chemotherapy increases by approximately 10% for patients with 10 or more positive axillary lymph nodes. It would thus be advantageous to be able to target 30 those 10% of patients who would benefit from high dose alkylating chemotherapy. However, no such predictive test presently exists. Because of the relatively high toxicity and the low level of efficacy in unselected breast cancer patients, alkylating agents are not commonly used in the treatment of breast cancer, with the exception of cyclophosphamide.

[006] Alkylating chemotherapy and platinating agents work by causing interstrand 35 DNA crosslinking, which cause DNA double strand breaks. In normal cells, these double strand breaks are repaired by a process called homologous recombination. If this process is unavailable or impaired, a situation referred to as "homologous recombination deficiency"

exists and alternative, error-prone DNA repair mechanisms take over, leading to genomic instability. The breast cancer genes BRCA1 and BRCA2 are involved in normal homologous recombination and tumors of patients carrying germ-line inactivating mutations in one or both of these genes show homologous recombination deficiency. BRCA1 and BRCA2 can also be inactivated in sporadic cancers as well, a phenomenon sometimes referred to as BRCA-likeness. Emerging preclinical evidence shows that breast cancers with a defective DNA repair system, such as a mutation in the BRCA1or BRCA2 genes, may be extremely sensitive to DNA damaging agents, such as platinum compounds and bifunctional alkylating agents. It therefore appears that patients with breast cancers harboring a defective DNA repair system may specifically benefit from high dose alkylating chemotherapy, an intensive DNA double strand break (DSB)-inducing regimen.

[007] Tumors with homologous recombination deficiency have been shown to be particularly sensitive to DNA crosslinking agents, such as alkylators and platinum drugs or platinating agents. Both classes of drugs are employed in advanced breast cancer. The novel poly(ADP-ribose) polymerase inhibitors (PARP inhibitors) are specifically effective in homologous recombination deficient tumors as well, and have shown impressive activity in clinical studies recently. Unfortunately, no clinical tests exist which can reliably determine homologous recombination deficiency in tumor biopsies.

Summary

[008] Therefore, methods of optimizing the therapeutic efficacy of anti-cancer therapies by identifying patients who would benefit from one or more anti-cancer therapies, including, without limitation, DNA double strand break-inducing regimens such as high dose alkylating chemotherapy, by reliably determining homologous recombination deficiency in tumor biopsies, and by identifying patients with breast cancers harboring a defective DNA repair system, are useful. In various aspects, the DNA double strand break-inducing regimens can be intensive direct DNA double strand break-inducing regimens, intensive indirect DNA double strand break-inducing regimens, moderate direct DNA double strand break-inducing regimens, moderate indirect DNA double strand break-inducing regimens, weak direct DNA double strand break-inducing regimens, weak indirect DNA double strand break-inducing regimens, and/or combinations thereof.

[009] The present disclosure is based on the discovery that certain chromosomal copy number aberrations in tumor cells allow tumors to be classified as either BRCA1-associated tumors, or sporadic tumors. The classification of a tumor in this manner allows for the prospective prediction of responsiveness of the patient from which the tumor was removed to anti-cancer therapy.

[010] In a first aspect, methods for using a BRCA1 aCGH classifier to detect genomic copy number variations in a test sample, as compared to a reference sample, in

the genomic loci 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22 are disclosed. The methods comprise detecting genomic copy number variations in a test sample in at least one, or a plurality, of the genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22, wherein a variation in copy number at any one or more of the genomic loci, as compared to the number of copies per cell of DNA from a reference sample, classifies the cell sample as from a BRCA1-associated tumor, and wherein such classification can be used to predict an individual subject's response to anti-cancer therapy. In some embodiments, the genomic copy number variations are detected at all 16 genomic loci. In some embodiments, the genomic copy number variations are detected at a number of genomic loci selected from greater than 1, greater than 2, greater than 3, greater than 4, greater than 5, greater than 6, greater than 7, greater than 8, greater than 9, greater than 10, greater than 11, greater than 12, greater than 13, greater than 14 and greater than 15. In some embodiments, the genomic copy number variations are detected at a number of genomic loci selected from less than 16, less than 15, less than 14, less than 13, less than 12, less than 11, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, less than 3, and less than 2.

Brief Description of the Drawings

[011] Those skilled in the art will understand that the drawings, described herein, are for illustration purposes only. The drawings are not intended to limit the scope of the present disclosure.

[012] **Fig. 1** depicts BRCA1-associated genomic loci used to identify breast cancers with homologous recombination deficiency due to a defect in the BRCA1 pathway.

[013] **Fig. 2** depicts exemplary BAC clones that may be used to detect, or to generate probes to detect, copy number aberrations in the genomic loci of **Fig. 1**.

[014] **Fig. 3** depicts relevant patient data and the protocols used for array comparative genomic hybridization in Example 1.

[015] **Fig. 4** depicts the mutation analysis for Example 1; the investigators screened for the most common mutations reported in Dutch families known to carry pathogenic germline BRCA1 or BRCA2 mutations.

[016] **Fig. 5** is a flow diagram of patients from the MBC-series of Example 1. Flow of patients through the study, including number of patients in each stage, is depicted. Reasons for dropout are listed. * = These two patients did not confer to the selection criteria and were classified as stage IIIc according to American Joint Committee on Cancer (AJCC) Staging Manual 2002. † = This patient did not confer to the selection criteria: she had a ductal carcinoma in the right breast with one positive lymph node (ER-, PR+) for which she

had a mastectomy followed by 6 cycles of CMF. Three years later ductal carcinoma in her left breast was detected and she had a lumpectomy (diameter 0.9cm), lymph node dissection and radiotherapy. Eight years later she had a recurrence of the ductal carcinoma in her left breast for which she had a mastectomy (diameter 2cm, irradical resection, ER-, PR-). Ten months later a metastasis in the left adrenal gland was discovered which was surgically extracted (ER-,PR-). Three months later lung, liver, bone and soft tissue metastases developed for which she was treated with bifunctional alkylating chemotherapy. Review of the histology showed morphologic resemblance and an identical cell type of the adrenal gland metastasis and the most recent tumor in the left breast. DNA was extracted from the most recent breast cancer tumor of the left breast. Abbreviations: FEC = 5-fluorouracil, epirubicin, cyclophosphamide.

[017] **Fig. 6** depicts the univariate Cox proportional-hazard regression analysis of the risk of tumor progression after HD chemotherapy in MBC series patients with a univariate HR for progression of 0.31 (95%CI: 0.14-0.66).

[018] **Fig. 7** depicts the univariate Cox proportional-hazard regression analysis of the risk of tumor progression after HD chemotherapy in MBC series patients, wherein adjustment for potential confounders did not substantially modify the HR.

[019] **Fig. 8** depicts the types of mutations found to be present in the MBC series patients.

[020] **Fig. 9** is a flow diagram of patients from the stage-III series. Flow of patients through the study including number of patients in each stage. Reasons for dropout are listed. Abbreviations: ER, estrogen-receptor; aCGH, array comparative genomic hybridization.

[021] **Fig. 10** depicts characteristics and treatments of 81 Stage-III series patients, which did not differ from ER-low, HER2-negative patients.

[022] **Fig. 11** depicts univariate Cox proportional-hazard regression analysis of the risk of recurrence in the Stage-III patients.

[023] **Fig. 12** depicts the association of BRCA1-classification with outcome after HD-chemotherapy and conventional chemotherapy in the stage-III series. Kaplan Meier survival curves according to BRCA1-classification. A) Recurrence Free Survival (RFS) of BRCA1-like patients who had been randomized between HD-chemotherapy or conventional chemotherapy. B) Recurrence Free Survival (RFS) of Sporadic-like patients who had been randomized between HD-chemotherapy or conventional chemotherapy.

[024] **Fig. 13** depicts performance of different cut-offs of the BRCA1-probability score using a BAC classifier comprising 427 BAC clones, as disclosed herein, to identify patients with a progression free survival of more than 24 months. A. Positive predictive values and negative predictive values at different cut-offs. B. Receiver operating curve (ROC). Red circle corresponds to cut-off chosen for further analysis.

[025] **Fig. 14** depicts Kaplan-Meier curves for progression free survival by BRCA1-like and Sporadic-like classification in the MBC-series. All patients. p-value represents logrank test of equal survival.

[026] **Fig. 15** depicts BRCA1 gene expression versus methylation status ($p<0.001$) in TN tumors.

[027] **Fig. 16** depicts BRCA1-like aCGH pattern ($p=0.285$) in TN tumors.

Detailed Description

Definitions

[028] "Anti-cancer therapy" means any one, or a plurality, of therapies and/or drugs used to treat cancer, or any combinations thereof, including a) homologous recombination deficiency-targeted drugs and/or treatments; and b) drugs or treatments that directly or indirectly cause double strand DNA breaks. This definition includes, without limitation, high dose platinum-based alkylating chemotherapy, platinum compounds, thiotepa, cyclophosphamide, iphosphamide, nitrosureas, nitrogen mustard derivatives, mitomycins, epipodophyllotoxins, camptothecins, anthracyclines, poly(ADP-ribose) polymerase (PARP) inhibitors, ionizing radiation, ABT-888, olaparib (AZT-2281), gemcitabine, CEP-9722, AG014699, AG014699 with Temozolomide, and BSI-201.

[029] "Array" refers to an arrangement, on a substrate surface, of one or a plurality of nucleic acid probes (as defined herein) of predetermined identity. In various embodiments, the sequences of the nucleic acid probes are known. In general, an array comprises a plurality of target elements, each target element comprising one or more nucleic acid probes immobilized on one or more solid surfaces, to which sample nucleic acids can be hybridized. In various embodiments, each individual probe is immobilized to a designated, discrete location (*i.e.*, a defined location or assigned position) on the substrate surface. In various embodiments, each nucleic acid probe is immobilized to a discrete location on an array and each has a sequence that is either specific to, or characteristic of, a particular genomic locus. A nucleic acid probe is specific to, or characteristic of, a genomic locus when it contains a nucleic acid sequence that is unique to that genomic locus. Such a probe preferentially hybridizes to a nucleic acid made from that genomic locus, and not to nucleic acids made from other genomic loci.

[030] The nucleic acid probes can contain sequence(s) from specific genes or clones. In various embodiments, at least some of the nucleic acid probes contain sequences from any one or more of the specific genomic regions recited in Figure 1. In various embodiments, at least some of the nucleic acid probes contain sequences of known, reference genes or clones. In various embodiments, the nucleic acid probes in a single array contain both sequences from any one or more of the specific genomic regions recited in Figure 1 and sequences of known, reference genes or clones.

[031] The probes may be arranged on the substrate in a single density, or in varying densities. The density of each of the probes can be varied to accommodate certain factors such as, for example, the nature of the test sample, the nature of a label used during hybridization, the type of substrate used, and the like. Each probe may comprise a mixture 5 of nucleic acids of varying lengths and, thus, varying sequences. For example, a single probe may contain more than one copy of a cloned nucleic acid, and each copy may be broken into fragments of different lengths. Each length will thus have a different sequence.

[032] The length, sequence and complexity of the nucleic acid probes may be varied. In various embodiments, the length, sequence and complexity are varied to provide 10 optimum hybridization and signal production for a given hybridization procedure, and to provide the required resolution among different genes or genomic locations.

[033] "BRCA1-associated tumor" means a tumor having cells containing a mutation of the BRCA1 locus or a homologous recombination pathway deficiency that directly or indirectly alters BRCA1 activity or function.

15 [034] "CGH" or "Comparative Genomic Hybridization" refers generally to molecular-cytogenetic techniques for the analysis of copy number changes, gains and/or losses, in the DNA content of a given subject's DNA. CGH can be used to identify chromosomal alterations, such as unbalanced chromosomal changes, in any number of cells including, for example, cancer cells. In various embodiments, CGH is utilized to detect one or more 20 chromosomal amplifications and/or deletions of regions between a test sample and a reference sample.

[035] "Chromosomal locus" refers to a specific, defined portion of a chromosome.

[036] "Genome" refers to all nucleic acid sequences, coding and non-coding, present in each cell type of a subject. The term also includes all naturally occurring or 25 induced variations of these sequences that may be present in a mutant or disease variant of any cell type, including, for example, tumor cells. Genomic DNA and genomic nucleic acids are thus nucleic acids isolated from a nucleus of one or more cells, and include nucleic acids derived from, isolated from, amplified from, or cloned from genomic DNA, as well as synthetic versions of all or any part of a genome.

30 [037] For example, the human genome consists of approximately 3.0×10^9 base pairs of DNA organized into 46 distinct chromosomes. The genome of a normal human diploid somatic cell consists of 22 pairs of autosomes (chromosomes 1 to 22) and either chromosomes X and Y (male) or a pair of X chromosomes (female) for a total of 46 chromosomes. A genome of a cancer cell may contain variable numbers of each 35 chromosome in addition to deletions, rearrangements and amplification of any sub-chromosomal region or DNA sequence.

[038] "Genomic locus" refers to a specific, defined portion of a genome.

[039] "HBOC tumors" refers to tumors present in a patient or a group of patients with a high risk for BRCA1-associated breast cancer (patients from Hereditary Breast and Ovarian Cancer families) but who display a negative screen result for BRCA1 and/or BRCA2 mutations. Such patients have a family history that include at least two breast cancer cases
5 and one ovarian cancer case.

[040] "Hybridization" refers to the binding of two single stranded nucleic acids via complementary base pairing. Extensive guides to the hybridization of nucleic acids can be found in: Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes Part I, Ch. 2, "Overview of principles of hybridization
10 and the strategy of nucleic acid probe assays" (1993), Elsevier, N.Y.; and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (3rd ed.) Vol. 1-3 (2001), Cold Spring Harbor Laboratory, Cold Spring Harbor Press, N.Y. The phrases "hybridizing specifically to", "specific hybridization", and "selectively hybridize to", refer to the preferential binding, duplexing, or hybridizing of a nucleic acid molecule to a particular probe under stringent
15 conditions. The term "stringent conditions" refers to hybridization conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent, or not at all, to other sequences in a mixed population (*e.g.*, a DNA preparation from a tissue biopsy). "Stringent hybridization" and "stringent hybridization wash conditions" are sequence-dependent and are different under different environmental parameters.

20 [041] Generally, highly stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (Tm) for a specific sequence at a defined ionic strength and pH. The Tm is the temperature at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the Tm for a particular probe. Often, a high stringency wash is preceded by a low stringency
25 wash to remove background probe signal. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on an array is 42° C using standard hybridization solutions, with the hybridization being carried out overnight. An example of highly stringent wash conditions is a 0.15 M NaCl wash at 72° C for 15 minutes. An example of stringent wash conditions is a wash in
30 0.2X Standard Saline Citrate (SSC) buffer at 65° C for 15 minutes. An example of a medium stringency wash for a duplex of, for example, more than 100 nucleotides, is 1X SSC at 45° C for 15 minutes. An example of a low stringency wash for a duplex of, for example, more than 100 nucleotides, is 4X to 6X SSC at 40° C for 15 minutes.

[042] "Micro-array" refers to an array that is miniaturized so as to require
35 microscopic examination for visual evaluation. In various embodiments, the arrays used in the methods of the present disclosure can be micro-arrays.

[043] "Nucleic acid" refers to a deoxyribonucleotide or ribonucleotide in either single- or double-stranded form and includes all nucleic acids comprising naturally occurring nucleotide bases as well as nucleic acids containing any and/or all analogues of natural nucleotides. This term also includes nucleic acid analogues that are metabolized in a
5 manner similar to naturally occurring nucleotides, but at rates that are improved for the purposes desired. This term also encompasses nucleic-acid-like structures with synthetic backbone analogues including, without limitation, phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, and peptide
10 nucleic acids (PNAs) (*see, e.g.*: "Oligonucleotides and Analogues, a Practical Approach," edited by F. Eckstein, IRL Press at Oxford University Press (1991); "Antisense Strategies," Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) J. Med. Chem. 36:1923-1937; and "Antisense Research and Applications" (1993, CRC Press)). PNAs contain non-ionic backbones, such as N-(2-
15 aminoethyl) glycine units. Phosphorothioate linkages are described in: WO 97/03211; WO 96/39154; and Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197. Other synthetic backbones encompassed by this term include methyl-phosphonate linkages or alternating methyl-phosphonate and phosphodiester linkages (Strauss-Soukup (1997) Biochemistry 36: 8692-8698), and benzyl-phosphonate linkages (Samstag (1996) Antisense Nucleic Acid
20 Drug Dev 6: 153-156).

[044] "Probe" or "nucleic acid probe" refer to one or more nucleic acid fragments whose specific hybridization to a sample can be detected. In various embodiments, probes are arranged on a substrate surface in an array. The probe may be unlabelled, or it may contain one or more labels so that its binding to a nucleic acid can be detected. In various
25 embodiments, a probe can be produced from any source of nucleic acids from one or more particular, pre-selected portions of a chromosome including, without limitation, one or more clones, an isolated whole chromosome, an isolated chromosome fragment, or a collection of polymerase chain reaction (PCR) amplification products.

[045] In some embodiments, the probe may be a member of an array of nucleic
30 acids as described in WO 96/17958. Techniques capable of producing high density arrays can also be used for this purpose (*see, e.g.*, Fodor (1991) Science 767-773; Johnston (1998) Curr. Biol. 8: RI 71 -RI 74; Schummer (1997) Biotechniques 23: 1087-1092; Kern (1997) Biotechniques 23: 120-124; and U.S. Patent No. 5,143,854).

[046] The sequence of the probes can be varied. In various embodiments, the
35 probe sequence can be varied to produce probes that are substantially identical to the probes disclosed herein, but that retain the ability to hybridize specifically to the same targets or samples as the probe from which they were derived.

[047] "Reference sample" refers to nucleic acids comprising sequences whose quantity or degree of representation, copy number, and/or sequence identity are known. Such nucleic acids serve as a reference to which one or more test samples are compared.

[048] "Sample" refers to a material, or mixture of materials, containing one or more components of interest. Samples include, but are not limited to, material obtained from an organism and may be directly obtained from a source, such as from a biopsy or from a tumor, or indirectly obtained such as after culturing and/or processing.

[049] "Test sample" refers to nucleic acids comprising sequences whose quantity or degree of representation, copy number, and/or sequence identity are unknown. In various embodiments, the present disclosure is directed to the detection of the quantity or degree of representation, copy number, and/or sequence identity of one or more test samples.

[050] Reference is now made in detail to certain embodiments of arrays and methods. The disclosed embodiments are not intended to be limiting of the claims. To the contrary, the claims are intended to cover all alternatives, modifications, and equivalents.

Arrays, Micro-Arrays and Probes

[051] In various aspects, the present disclosure relates to the determination of copy number changes in the DNA content of a given test sample, as compared to one or more reference samples. In some embodiments, the copy number changes comprise gains or increases in the DNA content of a test sample. In some embodiments, the copy number changes comprise losses or decreases in the DNA content of a test sample. In some embodiments, the copy number changes comprise both gains or increases and losses or decreases in the DNA content of a test sample.

[052] Copy number changes can be determined by hybridizations that are performed on a solid support. For example, probes that selectively hybridize to specific chromosomal regions can be spotted onto a surface. In various aspects, the spots of probes are placed in an ordered pattern, or array, and the pattern is recorded to facilitate correlation of results. Once an array is generated, one or more test samples can be hybridized to the array. In various aspects, arrays comprise a plurality of nucleic acid probes immobilized to discrete spots (*i.e.*, defined locations or assigned positions) on a substrate surface.

[053] Thus, in several aspects, copy number changes of genomic loci are analyzed in an array-based approach. In some embodiments, copy number changes of genomic loci are analyzed using comparative genomic hybridization. In some embodiments, copy number changes of genomic loci are analyzed using array-based comparative genomic hybridization.

[054] Any of a variety of arrays may be used. A number of arrays are commercially available for use from Vysis Corporation (Downers Grove, Ill), Spectral Genomics Inc.

(Houston, TX), and Affymetrix Inc. (Santa Clara, CA). Arrays can also be custom made for one or more hybridizations.

[055] Methods of making and using arrays are well known in the art (*see, e.g.*, Kern *et al.*, Biotechniques (1997), 23:120-124; Schummer *et al.*, Biotechniques (1997), 23:1087-1092; Solinas-Toldo *et al.*, Genes, Chromosomes & Cancer (1997), 20: 399-407; Johnston, Curr. Biol. (1998), 8: R171-R174; Bowtell, Nature Gen. (1999), Supp. 21:25-32; Watson *et al.*, Biol. Psychiatry (1999), 45: 533-543; Freeman *et al.*, Biotechniques (2000), 29: 1042-1046 and 1048-1055; Lockhart *et al.*, Nature (2000), 405: 827-836; Cuzin, Transfus. Clin. Biol. (2001), 8:291-296; Zarrinkar *et al.*, Genome Res. (2001), 11: 1256-1261; Gabig *et al.*, Acta Biochim. Pol. (2001), 48: 615-622; and Cheung *et al.*, Nature (2001), 40: 953-958; *see also, e.g.*, U.S. Patent Nos. 5,143,854; 5,434,049; 5,556,752; 5,632,957; 5,700,637; 5,744,305; 5,770,456; 5,800,992; 5,807,522; 5,830,645; 5,856,174; 5,959,098; 5,965,452; 6,013,440; 6,022,963; 6,045,996; 6,048,695; 6,054,270; 6,258,606; 6,261,776; 6,277,489; 6,277,628; 6,365,349; 6,387,626; 6,458,584; 6,503,711; 6,516,276; 6,521,465; 6,558,907; 6,562,565; 6,576,424; 6,587,579; 6,589,726; 6,594,432; 6,599,693; 6,600,031; and 6,613,893).

[056] Substrate surfaces suitable for use in the generation of an array can be made of any rigid, semi-rigid or flexible material that allows for direct or indirect attachment (*i.e.*, immobilization) of nucleic acid probes to the substrate surface. Suitable materials include, without limitation, cellulose (*see, e.g.*, U.S. Patent No. 5,068,269), cellulose acetate (*see, e.g.*, U.S. Patent No. 6,048,457), nitrocellulose, glass (*see, e.g.*, U.S. Patent No. 5,843,767), quartz and/or other crystalline substrates such as gallium arsenide, silicones (*see, e.g.*, U.S. Patent No. 6,096,817), plastics and plastic copolymers (*see, e.g.*, U.S. Patent Nos. 4,355,153; 4,652,613; and 6,024,872), membranes and gels (*see, e.g.*, U.S. Patent No. 5,795,557), and paramagnetic or supramagnetic microparticles (*see, e.g.*, U.S. Patent No. 5,939,261). When fluorescence is to be detected, arrays comprising cyclo-olefin polymers may be used (*see, e.g.*, U.S. Patent No. 6,063,338). The presence of reactive functional chemical groups (such as, for example, hydroxyl, carboxyl, and amino groups) present on the surface of the substrate material can be used to directly or indirectly attach nucleic acid probes to the substrate surface.

[057] More than one copy of each nucleic acid probe may be spotted onto an array. For example, each nucleic acid probe may be spotted onto an array once, in duplicate, in triplicate, or more, depending on the desired application. Multiple spots of the same probe allows for assessment of the reproducibility of the results obtained.

[058] Related nucleic acid probes may also be grouped together, in probe elements, on an array. For example, a single probe element may include a plurality of spots of related nucleic acid probes, which are of different lengths but that comprise substantially

the same sequence or that are derived from the sequence of a specific genomic locus. Alternatively, a single probe element may include a plurality of spots of related nucleic acid probes that are fragments of different lengths resulting from digestion of more than one copy of a cloned nucleic acid. An array may contain a plurality of probe elements and probe elements may be arranged on an array at different densities.

[059] Array-immobilized nucleic acid probes may be nucleic acids that contain sequences from genes (*e.g.*, from a genomic library) including, for example, sequences that collectively cover a substantially complete genome, or any one or more subsets of a genome. In various embodiments, the sequences of the nucleic acid probes on an array comprise those for which comparative copy number information is desired. In some embodiments, to obtain DNA sequence copy number information across an entire genome, an array comprising nucleic acid probes covering a whole genome or a substantially complete genome is used. In some embodiments, at least one relevant genomic locus has been determined and is used in an array, such that there is no need for genome-wide hybridization. In some embodiments, a plurality of relevant genomic loci have been determined and are used in an array, such that there is no need for genome-wide hybridization. In some embodiments, the array comprises a plurality of specific nucleic acid probes that originate from a discrete set of genes or genomic loci and whose copy number, in association with the type of condition or tumor is to be tested, is known. Additionally, the array may comprise nucleic acid probes that will serve as positive or negative controls. In some embodiments, the array comprises a plurality of nucleic acid sequences derived from karyotypically normal genomes.

[060] The probes may be generated by any number of known techniques (*see, e.g.*, Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes Part I, Ch. 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays" (1993), Elsevier, N.Y.; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (3rd ed.) Vol. 1-3 (2001), Cold Spring Harbor Laboratory, Cold Spring Harbor Press, N.Y.; Innis (Ed.) "PCR Strategies" (1995), Academic Press: New York, N.Y.; and Ausubel (Ed.), "Short Protocols in Molecular Biology" 5th Ed. (2002), John Wiley & Sons). Nucleic acid probes may be obtained and manipulated by cloning into various vehicles. They may be screened and re-cloned or amplified from any source of genomic DNA.

[061] Nucleic acid probes may also be obtained and manipulated by cloning into vehicles including, for example, recombinant viruses, cosmids, or plasmids. Nucleic acid probes may also be synthesized *in vitro* by chemical techniques (*see, e.g.*, Nucleic Acids Res. (1997), 25: 3440-3444; Blommers *et al.*, Biochemistry (1994), 33: 7886-7896; and Frenkel *et al.*, Free Radic. Biol. Med. (1995), 19: 373-380). Probes may vary in size from

synthetic oligonucleotide probes and/or PCR-type amplification primers of a few base pairs in length to artificial chromosomes of more than 1 megabases in length. In various embodiments, probes comprise at least 10, at least 12, at least 15, at least 18, at least 20, at least 22, at least 30, at least 50 or at least 100 contiguous nucleotides of a sequence present in a BAC clone set forth in **Fig. 2**. In some embodiments, probes comprise a sequence that is unique in a genome. In some embodiments, probes comprise a sequence that is unique in the human genome.

[062] Probes may be obtained from any number of commercial sources. For instance, several P1 clones are available from the DuPont P1 library (*see, e.g.*, Shepard *et al.*, Proc. Natl. Acad. Sci. USA (1994), 92: 2629), and available commercially from Incyte Corporation (Wilmington, DE). Various libraries spanning entire chromosomes are available commercially from Clontech Laboratories, Inc. (Mountain View, CA), or from the Los Alamos National Laboratory (Los Alamos, CA). In various aspects, the present disclosure relates to the use of the human 3600 BAC/PAC genomic clone set, covering the full human genome at 1 Mb spacing, obtained from the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK).

[063] In some embodiments, the nucleic acid probes are derived from mammalian artificial chromosomes (MACs) and/or human artificial chromosomes (HACs), which can contain inserts from about 5 to 400 kilobases (kb) (*see, e.g.*, Roush, Science (1997), 276: 38-39; Rosenfeld, Nat. Genet. (1997), 15: 333-335; Ascenzioni *et al.*, Cancer Lett. (1997), 118: 135-142; Kuroiwa *et al.*, Nat. Biotechnol. (2000), 18: 1086-1090; Meija *et al.*, Am. J. Hum. Genet. (2001), 69: 315-326; and Auriche *et al.*, EMBO Rep. (2001), 2: 102-107).

[064] In some embodiments, the nucleic acid probes are derived from satellite artificial chromosomes or satellite DNA-based artificial chromosomes (SATACs). SATACs can be produced by inducing *de novo* chromosome formation in cells of varying mammalian species (*see, e.g.*, Warburton *et al.*, Nature (1997), 386: 553-555; Csonka *et al.*, J. Cell. Sci. (2000), 113: 3207-3216; and Hadlaczky, Curr. Opin. Mol. Ther. (2001), 3: 125-132).

[065] In some embodiments, the nucleic acid probes are derived from yeast artificial chromosomes (YACs), 0.2-1 megabses in size. YACs have been used for many years for the stable propagation of genomic fragments of up to one million base pairs in size (*see, e.g.*, Feingold *et al.*, Proc. Natl. Acad. Sci. USA (1990), 87:8637-8641; Adam *et al.*, Plant J. (1997), 11: 1349-1358; Tucker *et al.*, Gene (1997), 199: 25-30; and Zeschnigk *et al.*, Nucleic Acids Res. (1999), 27: E30).

[066] In some embodiments, the nucleic acid probes are derived from bacterial artificial chromosomes (BACs) up to 300 kb in size. BACs are based on the E. coli F factor plasmid system and are typically easy to manipulate and purify in microgram quantities (*see, e.g.*, Asakawa *et al.*, Gene (1997), 191: 69-79; and Cao *et al.*, Genome Res. (1999), 9: 763-774).

[067] In some embodiments, the nucleic acid probes are derived from P1 artificial chromosomes (PACs), about 70-100 kb in size. PACs are bacteriophage P1-derived vectors (*see, e.g.*, Ioannou *et al.*, Nature Genet. (1994), 6: 84-89; Boren *et al.*, Genome Res. (1996), 6: 1123-1130; Nothwang *et al.*, Genomics (1997), 41: 370-378; Reid *et al.*, Genomics
5 (1997), 43: 366-375; and Woon *et al.*, Genomics (1998), 50: 306-316).

[068] In some embodiments, the array comprises a series of separate wells or chambers on the substrate surface, into which probes may be immobilized as described herein. The probes can be immobilized in the separate wells or chambers and hybridization can take place within the wells or chambers. In various embodiments, the arrays can be
10 selected from chips, microfluidic chips, microtiter plates, Petri dishes, and centrifuge tubes. Robotic equipment has been developed for these types of arrays that permit automated delivery of reagents into the separate wells or chambers which allow the amount of the reagents used per hybridization to be sharply reduced. Examples of chip and microfluidic chip techniques can be found, for example, in U.S. Patent No. 5,800,690; Orchid, "Running
15 on Parallel Lines" New Scientist (1997); McCormick *et al.*, Anal. Chem. (1997), 69:2626-30; and Turgeon, "The Lab of the Future on CD-ROM?" Medical Laboratory Management Report. December 1997, p. 1.

BRCA1 Arrays

[069] An array comparative genomic hybridization (aCGH) profile that distinguishes
20 BRCA1-mutated breast cancers from sporadic breast cancers has been identified and is disclosed herein. In various aspects, the present disclosure relates to the use of a BRCA1 array comprising this unique BRCA1 aCGH profile to identify breast cancers with a homologous recombination deficiency due to a defect in BRCA1 or in the homologous recombination pathway which results in a BRCA1-like phenotype, and to thus identify
25 patients, from whom the cancers have been excised, who will be highly sensitive to certain anti-cancer therapy. Therefore, in various aspects, the present disclosure relates to the use of a BRCA1 array comprising this BRCA1 aCGH profile to prospectively optimize the therapeutic efficacy of anti-cancer therapy in an individual subject by detecting phenotypic genetic traits associated with deficiencies in the BRCA1 gene or in the homologous
30 recombination pathway which results in a BRCA1-like phenotype.

[070] In various embodiments, a BRCA1 array comprising a BRCA1 aCGH profile for identifying individual subjects who will experience a therapeutic benefit from anti-cancer therapy is provided. In some embodiments, a BRCA1 array is used to detect BRCA1-associated genomic copy number variations in a test sample, as compared to a reference
35 sample, at one, or a plurality, of the genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22. In some embodiments, a BRCA1 array is used to

detect an increase in genomic copy numbers in a test sample, as compared to a reference sample, in any one, or a plurality, of the genomic loci selected from 1p35-21, 3q22-27, 6p25-22, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 13q31-33, and 21q11-22. In some embodiments, a BRCA1 array is used to detect a decrease in genomic copy numbers in a test sample, as compared to a reference sample, in any one, or a plurality, of the genomic loci selected from 5p13, 5q21-34, 7p21-15, 12q21-23, 14q22-24 and 15q14-21. In each of the aforementioned embodiments, detection of BRCA1-associated genomic copy number variations classifies the test sample as from a BRCA1-associated tumor and classifies the subject from whom the test sample was excised as an individual who will experience a therapeutic benefit from anti-cancer therapy.

[071] The genomic loci may be detected individually, or in any combination of two or more loci. In some embodiments, a BRCA1 array is used that is capable of detecting BRCA1-associated genomic copy number variations in all 16 of the above-listed chromosomal loci. In some embodiments, a BRCA1 array is used that is capable of detecting BRCA1-associated genomic copy number variations in a number of genomic loci selected from greater than 1, greater than 2, greater than 3, greater than 4, greater than 5, greater than 6, greater than 7, greater than 8, greater than 9, greater than 10, greater than 11, greater than 12, greater than 13, greater than 14 and greater than 15. In some embodiments, a BRCA1 array is used that is capable of detecting BRCA1-associated genomic copy number variations in a number of genomic loci selected from less than 16, less than 15, less than 14, less than 13, less than 12, less than 11, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, less than 3, and less than 2. In some embodiments, a BRCA1 array is used that is capable of detecting BRCA1-associated genomic copy number variations in all 16 of the BRCA1-associated genomic loci set forth in **Fig. 1**. In each of the aforementioned embodiments, detection of BRCA1-associated genomic copy number variations classifies the test sample as from a BRCA1-associated tumor and classifies the subject from whom the test sample was excised as an individual who will experience a therapeutic benefit from anti-cancer therapy.

[072] The BRCA1 arrays comprise at least one probe. In various embodiments, the BRCA1 arrays comprise a plurality of probes. In some embodiments, the BRCA1 arrays comprise a plurality of probes, wherein the probes comprise nucleic acid sequences derived from BAC clones. The BRCA1-associated genomic loci set forth in **Fig. 1** are bounded by the BAC probes set forth in **Fig 2**. In some embodiments, arrays capable of detecting BRCA1-associated genomic copy number variations comprise at least one, or a plurality, of probes derived from the BAC clones of **Fig. 2**. The BAC clones set forth in **Fig. 2** are not intended to be limiting in any way, and other probes within the BRCA1-associated genomic loci of **Fig. 1** can also be used in the BRCA1 arrays. In some embodiments, arrays capable

of detecting BRCA1-associated genomic copy number variations comprise all 371 of the BAC clones of **Fig. 2**. In some embodiments, arrays capable of detecting BRCA1-associated genomic copy number variations comprise a number of BAC clones of **Fig. 2** selected from greater than 1, greater than 10, greater than 20, greater than 25, greater than 50, greater than 75, greater than 100, greater than 125, greater than 150, greater than 175, greater than 200, greater than 225, greater than 250, greater than 275, greater than 300, greater than 325 and greater than 350. In some embodiments, arrays capable of detecting BRCA1-associated genomic copy number variations comprise a number of BAC clones of **Fig. 2** selected from less than 371, less than 350, less than 325, less than 300, less than 275, less than 250, less than 225, less than 200, less than 175, less than 150, less than 125, less than 100, less than 75, less than 50, less than 25, less than 20, and less than 10.

[073] In some embodiments, a BRCA1 array capable of detecting BRCA1-associated genomic copy number variations comprises at least one, or a plurality, of probes that independently hybridize to at least one, or a plurality, of the genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22. In these embodiments, the probes are as defined above and/or may be obtained in methods as described above.

[074] In some embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations comprise at least one, or a plurality, of probes, wherein the probes comprise at least one, or a plurality, of the distinct BAC clones of **Fig. 2**. In some embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations comprise at least one, or a plurality, of probes, wherein the probes comprise at least one, or a plurality, of the BAC clones of **Fig. 2**, and wherein the probes specifically hybridize to at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14 or at least 15 of the genomic loci set forth in **Fig. 1**. In some embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations comprise a plurality of probes, wherein the nucleic acid sequences of the probes are unique to the genomic loci set forth in **Fig. 1**. In some embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations comprise a plurality of probes, wherein the probes comprise a plurality of BAC clones specific to all of the genomic loci set forth in **Fig. 1**. In some embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations comprise at least one, or a plurality, of probes, wherein the probes comprise at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75, at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, at least 300, at least 325 or at least 350 of the distinct BAC clones of **Fig. 2**.

[075] In various embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations that comprise at least one, or a plurality, of probes, and/or that comprise at least one, or a plurality, of distinct BAC clones, allow for the individual analysis of at least one, or a plurality, of distinct genomic loci. Therefore, in some embodiments, the probes, and/or the distinct BAC clones, capable of detecting BRCA1-associated genomic copy number variations are arranged on the BRCA1 arrays in a positionally-addressable manner.

[076] In various embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations comprise at least one, or a plurality, of distinct BAC clones, wherein the distinct BAC clones represent at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14 or at least 15 of the genomic loci set forth in **Fig. 1**. In various embodiments, BRCA1 arrays capable of detecting BRCA1-associated genomic copy number variations comprise at least one, or a plurality, of distinct BAC clones, wherein the distinct BAC clones represent all 16 of the genomic loci set forth in **Fig. 1**.

Array Comparative Genomic Hybridization

[077] In various aspects, the present disclosure relates to the analysis of tumor cell samples by array-based comparative genomic hybridization. Array comparative genomic hybridization (aCGH) is a technique that is used to detect genomic copy number variations at a higher level of resolution than chromosome-based comparative genomic hybridization. In aCGH, nucleic acids from a test sample and nucleic acids from a reference sample are labelled differentially. The test sample and the reference sample are then and hybridized to an array comprising a plurality of probes. The ratio of the signal intensity of the test sample to that of the reference sample is then calculated, to measure the copy number changes for a particular location in the genome. The difference in the signal ratio determines whether the total copy numbers of the nucleic acids in the test sample are increased or decreased as compared to the reference sample. The test sample and the reference sample may be hybridized to the array separately or they may be mixed together and hybridized simultaneously. Exemplary methods of performing aCGH can be found, for example, in U.S. Patent Nos. 5,635,351; 5,665,549; 5,721,098; 5,830,645; 5,856,097; 5,965,362; 5,976,790; 6,159,685; 6,197,501; and 6,335,167; European Patent Nos. EP 1 134 293 and EP 1 026 260; van Beers *et al.*, Brit. J. Cancer (2006), 20; Joosse *et al.*, BMC Cancer (2007), 7:43; Pinkel *et al.*, Nat. Genet. (1998), 20: 207-211; Pollack *et al.*, Nat. Genet. (1999), 23: 41-46; and Cooper, Breast Cancer Res. (2001), 3: 158-175.

[078] Samples that are labelled differentially are labelled such that one of the two samples is labelled with a first detectable agent and the other of the two samples is labelled with a second detectable agent, wherein the first detectable agent and the second

detectable agent produce distinguishable signals. Detectable agents that produce distinguishable signals can include, for example, matched pairs of fluorescent dyes.

[079] In some embodiments, the methods of the present disclosure comprise analyzing at least one test sample of tumor DNA from a subject by array-based comparative genomic hybridization to obtain information relating to the copy number aberrations present in the sample(s), if any; based on the information obtained, classifying the tumor as a BRCA1-associated tumor or a sporadic tumor; and, based on the classification, optimizing the therapeutic efficacy of anti-cancer therapy for the subject by predicting the subject's prospective response to anti-cancer therapy.

[080] Information relating to the copy number aberrations present in a sample can include, for example, a gain of genetic material at one or more genomic loci, a loss of genetic material at one or more genomic loci, chromosomal abnormalities at one or more genomic loci, and genome copy number changes at one or more genomic loci. This information is obtained by analyzing the difference in signal intensity between the test sample and a reference sample at one or more genomic loci. The analysis can be performed using any of a variety of methods, means and variations thereof for carrying out array-based comparative genomic hybridization.

[081] In various embodiments, the reference sample is a nucleic acid sample that is representative of a normal, non-diseased state, for example a non-tumor/non-cancer cell, and contains a normal amount of copy numbers of the complement of the genomic loci being tested. The reference sample may be derived from a genomic nucleic acid sample from a normal and/or healthy individual or from a pool of such individuals. In various embodiments, the reference sample does not comprise any tumor or cancerous nucleic acids. In some embodiments, the reference sample is derived from a pool of female subjects. In some embodiments, the reference sample comprises pooled genomic DNA isolated from tissue samples (*e.g.* lymphocytes) from a plurality (*e.g.* at least 4-10) of healthy female subjects. In some embodiments, the reference sample comprises an artificially-generated population of nucleic acids designed to approximate the copy number level from each tested genomic region, or fragments of each tested genomic region. In some embodiments, the reference sample is derived from normal, non-cancerous cell lines or from cell line samples.

[082] Test samples may be obtained from a biological source comprising tumor cells, and reference samples may be obtained from a biological source comprising normal reference cells, by any suitable method of nucleic acid isolation and/or extraction. In various aspects, the test sample and the reference sample are DNA. Methods of DNA extraction are well known in the art. A classical DNA isolation protocol is based on extraction using organic solvents, such as a mixture of phenol and chloroform, followed by precipitation with ethanol (*see, e.g.*, Sambrook *et al.*, *supra*). Other methods include salting out DNA extraction,

trimethylammonium bromide salt extraction, and guanidinium thiocyanate extraction. Additionally, there are numerous DNA extraction kits that are commercially available from, for example, BD Biosciences Clontech (Palo Alto, CA), Epicentre Technologies (Madison, WI), Gentra Systems, Inc. (Minneapolis, MN), MicroProbe Corp. (Bothell, WA), Organon
5 Teknika (Durham, NC), and Qiagen Inc. (Valencia, CA).

[083] The test samples and the reference samples may be differentially labelled with any detectable agents or moieties. In various embodiments, the detectable agents or moieties are selected such that they generate signals that can be readily measured and such that the intensity of the signals is proportional to the amount of labelled nucleic acids
10 present in the sample. In various embodiments, the detectable agents or moieties are selected such that they generate localized signals, thereby allowing resolution of the signals from each spot on an array.

[084] Methods for labeling nucleic acids are well-known in the art. For exemplary reviews of labeling protocols, label detection techniques and recent developments in the
15 field, see: Kricka, Ann. Clin. Biochem. (2002), 39: 114-129; van Gijlswijk *et al.*, Expert Rev. Mol. Diagn. (2001), 1: 81-91; and Joos *et al.*, J. Biotechnol. (1994), 35: 135-153. Standard nucleic acid labeling methods include: incorporation of radioactive agents, direct attachment of fluorescent dyes or of enzymes, chemical modification of nucleic acids to make them detectable immunochemically or by other affinity reactions, and enzyme-mediated labeling
20 methods including, without limitation, random priming, nick translation, PCR and tailing with terminal transferase. Other suitable labeling methods include psoralen-biotin, photoreactive azido derivatives, and DNA alkylating agents. In various embodiments, test sample and reference sample nucleic acids are labelled by Universal Linkage System, which is based on the reaction of monoreactive cisplatin derivatives with the N7 position of guanine moieties in
25 DNA (*see, e.g.*, Heetebrij *et al.*, Cytogenet. Cell. Genet. (1999), 87: 47-52).

[085] Any of a wide variety of detectable agents or moieties can be used to label test and/or reference samples. Suitable detectable agents or moieties include, but are not limited to: various ligands; radionuclides such as, for example, ^{32}P, ^{35}S, ^{3}H, ^{14}C, ^{125}I, ^{131}I, and others; fluorescent dyes; chemiluminescent agents such as, for example, acridinium esters,
30 stabilized dioxetanes, and others; microparticles such as, for example, quantum dots, nanocrystals, phosphors and others; enzymes such as, for example, those used in an ELISA, horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase and others; colorimetric labels such as, for example, dyes, colloidal gold and others; magnetic labels such as, for example, Dynabeads™; and biotin, dioxigenin or other haptens and
35 proteins for which antisera or monoclonal antibodies are available.

[086] In some embodiments, the test samples and the reference samples are labelled with fluorescent dyes. Suitable fluorescent dyes include, without limitation, Cy-3,

Cy-5, Texas red, FITC, Spectrum Red, Spectrum Green, phycoerythrin, rhodamine, and fluorescein, as well as equivalents, analogues and/or derivatives thereof. In some embodiments, the fluorescent dyes selected display a high molar absorption coefficient, high fluorescence quantum yield, and photostability. In some embodiments, the fluorescent dyes exhibit absorption and emission wavelengths in the visible spectrum (i.e., between 400nm and 750nm) rather than in the ultraviolet range of the spectrum (i.e., lower than 400nm). In some embodiments, the fluorescent dyes are Cy-3 (3-N,N'-diethyltetramethylindo-dicarbocyanine) and Cy-5 (5-N,N'-diethyltetramethylindo-dicarbocyanine). Cy-3 and Cy-5 form a matched pair of fluorescent labels that are compatible with most fluorescence detection systems for array-based instruments. In some embodiments, the fluorescent dyes are Spectrum Red and Spectrum Green.

[087] A key component of aCGH is the hybridization of a test sample and a reference sample to an array. Exemplary hybridization and wash protocols are described, for example, in Sambrook *et al.* (2001), *supra*; Tijssen (1993), *supra*; and Anderson (Ed.), "Nucleic Acid Hybridization" (1999), Springer Verlag: New York, N.Y. In some embodiments, the hybridization protocols used for aCGH are those of Pinkel *et al.*, Nature Genetics (1998), 20:207-211. In some embodiments, the hybridization protocols used for aCGH are those of Kallioniemi, Proc. Natl. Acad. Sci. USA (1992), 89:5321-5325.

[088] Methods of optimizing hybridization conditions are well known in the art (*see, e.g.*, Tijssen, (1993), *supra*). To create competitive hybridization conditions, the array may be contacted simultaneously with differentially labelled nucleic acid fragments of the test sample and the reference sample. This may be done by, for example, mixing the labelled test sample and the labelled reference sample together to form a hybridization mixture, and contacting the array with the mixture.

[089] The specificity of hybridization may be enhanced by inhibiting repetitive sequences. In some embodiments, repetitive sequences (*e.g.*, Alu sequences, L1 sequences, satellite sequences, MRE sequences, simple homo-nucleotide tracts, and/or simple oligonucleotide tracts) present in the nucleic acids of the test sample, reference sample and/or probes are either removed, or their hybridization capacity is disabled. Removing repetitive sequences or disabling their hybridization capacity can be accomplished using any of a variety of well-known methods. These methods include, but are not limited to, removing repetitive sequences by hybridization to specific nucleic acid sequences immobilized to a solid support (*see, e.g.*, Brison *et al.*, Mol. Cell. Biol. (1982), 2: 578- 587); suppressing the production of repetitive sequences by PCR amplification using adequately designed PCR primers; inhibiting the hybridization capacity of highly repeated sequences by self-reassociation (*see, e.g.*, Britten *et al.*, Methods of Enzymology (1974), 29: 363-418); or removing repetitive sequences using hydroxyapatite which is commercially

available from a number of sources including, for example, Bio-Rad Laboratories, Richmond, VA. In some embodiments, the hybridization capacity of highly repeated sequences in a test sample and/or in a reference sample is competitively inhibited by including, in the hybridization mixture, unlabelled blocking nucleic acids. The unlabelled blocking nucleic acids are therefore mixed with the hybridization mixture, and thus with a test sample and a reference sample, before the mixture is contacted with an array. The unlabelled blocking nucleic acids act as a competitor for the highly repeated sequences and bind to them before the hybridization mixture is contacted with an array. Therefore, the unlabelled blocking nucleic acids prevent labelled repetitive sequences from binding to any highly repetitive sequences of the nucleic acid probes, thus decreasing the amount of background signal present in a given hybridization. In some embodiments, the unlabelled blocking nucleic acids are Human Cot-1 DNA. Human Cot-1 DNA is commercially available from a number of sources including, for example, Gibco/BRL Life Technologies (Gaithersburg, MD).

[090] Once hybridization is complete, the ratio of the signal intensity of the test sample as compared to the signal intensity of the reference sample is calculated. This calculation quantifies the amount of copy number aberrations present in the genomic DNA of the test sample, if any. In some embodiments, this calculation is carried out quantitatively or semi-quantitatively. In several aspects, it is not necessary to determine the exact copy number aberrations present in the genomic loci tested, as detection of an aberration, *i.e.* a gain or loss of genetic material, from the copy number in normal, non-cancerous genomic DNA is indicative of the presence of a disease state and is thus sufficient. Therefore, in several embodiments the quantification of the amount of copy number aberrations present in the genomic DNA of a test sample comprises an estimation of the copy number aberrations, as a semi-quantitative or relative measure usually suffices to predict the presence of a disease state and thus prospectively direct the determination of therapy for a subject.

[091] Quantitative techniques may be used to determine the copy number aberrations per cell present in a test sample. Several quantitative and semi-quantitative techniques to determine copy number aberrations exist including, for example, semi-quantitative PCR analysis or quantitative real-time PCR. The Polymerase Chain Reaction (PCR) *per se* is not a quantitative technique, however PCR-based methods have been developed that are quantitative or semi-quantitative in that they give a reasonable estimate of original copy numbers, within certain limits. Examples of such PCR techniques include, for example, quantitative PCR and quantitative real-time PCR (also known as RT-PCR, RQ-PCR, QRT-PCR or RTQ-PCR). In addition, many techniques exist that give estimates of relative copy numbers, as calculated relative to a reference. Such techniques include many array-based techniques. Absolute copy number estimates may be obtained by *in situ*

hybridization techniques such as, for example, fluorescence *in situ* hybridization or chromogenic *in situ* hybridization.

[092] Fluorescence *in situ* hybridization permits the analysis of copy numbers of individual genomic locations and can be used to study copy numbers of individual genetic
5 loci or particular regions on a chromosome (*see, e.g.*, Pinkel et al., Proc. Natl. Acad. Sci. U.S.A. (1988), 85, 9138-42). Comparative genomic hybridization can also be used to probe for copy number changes of chromosomal regions (*see, e.g.*, Kallioniemi *et al.*, Science (1992), 258: 818-21; and Houldsworth *et al.*, Am. J. Pathol. (1994), 145: 1253-60).

[093] Copy numbers of genomic locations may also be determined using
10 quantitative PCR techniques such as real-time PCR (*see, e.g.*, Suzuki *et al.*, Cancer Res. (2000), 60:5405-9). For example, quantitative microsatellite analysis can be performed for rapid measurement of relative DNA sequence copy numbers. In quantitative microsatellite analysis, the copy numbers of a test sample relative to a reference sample is assessed using quantitative, real-time PCR amplification of loci carrying simple sequence repeats.
15 Simple sequence repeats are used because of the large numbers that have been precisely mapped in numerous organisms. Exemplary protocols for quantitative PCR are provided in Innis *et al.*, PCR Protocols, A Guide to Methods and Applications (1990), Academic Press, Inc. N.Y. Semi-quantitative techniques that may be used to determine specific DNA copy numbers include, for example, multiplex ligation-dependent probe amplification (*see, e.g.*,
20 Schouten *et al.* Nucleic Acids Res. (2002), 30(12):e57; and Sellner *et al.*, Human Mutation (2004), 23(5):413-419) and multiplex amplification and probe hybridization (*see, e.g.*, Sellner *et al.* (2004), *supra*).

BRCA1 Array Comparative Genomic Hybridization

[094] In various aspects, the present disclosure relates to the use of a BRCA1
25 aCGH classifier capable of identifying BRCA1-associated tumors in predicting an individual subject's response to anti-cancer therapy. In various aspects, a BRCA1 aCGH classifier capable of identifying BRCA1-associated tumors is set forth on a BRCA1 array as described herein.

[095] Using the methods described above, in various aspects, a BRCA1 aCGH
30 classifier is capable of detecting genomic copy number variations in a test sample, as compared to a reference sample, in at least one, or a plurality, of the genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22. Using the methods described above, in various aspects, a BRCA1 aCGH classifier is capable of detecting
35 genomic copy number variations in a test sample, as compared to a reference sample, in at least one, or a plurality, of the genomic loci selected from 1p35.1-21.3, 3q22.2-27.2, 5p13.2, 5q21.3-34, 6p25.2-22.1, 7p21.3-15.3, 7q31.33-36.3, 8q22.1-24.3, 10p15.3-14, 10p12.1,

12p13.33-13.2, 12q21.2-23.3, 13q31.2-33.3, 14q22.1-24.1, 15q14-21.1 and 21q11.2-22.3. Using the methods described above, in various aspects, a BRCA1 aCGH classifier is capable of detecting genomic copy number variations in a test sample, as compared to a reference sample, in at least one, or a plurality, of the genomic loci set forth in **Fig. 1**.

5 [096] Using the methods described above, in various aspects, a BRCA1 aCGH classifier is capable of detecting genomic copy number variations in a test sample, using at least one, or a plurality, of probes that independently hybridize to at least one genomic locus set forth in **Fig. 1**. Using the methods described above, in various aspects, a BRCA1 aCGH classifier is capable of detecting genomic copy number variations in a test sample, as
10 compared to a reference sample, using at least one, or a plurality, of the distinct BAC clones set forth in **Fig. 2**.

Therapeutic Uses

[097] In various aspects, the BRCA1 classifiers can be used to predict an individual subject's response to anti-cancer therapy.

15 [098] Using the methods described above, in various aspects, the BRCA1 classifiers are capable of determining whether an individual metastatic breast cancer patient, in continuous complete remission after high dose alkylating chemotherapy, has a BRCA1-associated tumor. Using the methods described above, in various aspects, the BRCA1 classifiers are capable of determining whether a metastatic breast cancer patient with a
20 BRCA1-associated tumor has a significantly higher complete remission rate. The BRCA1 classifiers are therefore capable of predicting response to anti-cancer therapy in an individual patient. Using the methods described above, in various aspects, the BRCA1 classifiers are capable of predicting improved outcome after platinum-based high dose alkylating chemotherapy by identifying breast cancer patients specifically benefiting from
25 HD-chemotherapy within ER-low and HER2-negative stage-III breast cancer.

[099] The BRCA1 classifiers can be used as pre-selection tools, to prospectively detect subjects with a high risk of carrying a BRCA1-mutation and/or a BRCA1-associated tumor. Additionally, the BRCA1 classifiers can be used as predictive tests to identify breast cancer patients likely to benefit from anti-cancer therapy.

30 [0100] The BRCA1 classifiers can also be used to detect a BRCA1 profile in ER+ luminal sporadic tumors. It is therefore believed that the BRCA1 classifiers and the second series BRCA1 classifiers can also be used as predictive tests to identify breast cancer patients having ER+ luminal sporadic tumors who are likely to benefit from anti-cancer therapy.

35 [0101] For the first time, in this disclosure clinical evidence has been provided to show that patients with so-called "triple negative" sporadic tumors who also display a BRCA1 profile, as determined by the BRCA1 classifiers, are more sensitive and respond better to

high dose alkylating chemotherapy containing carboplatin, thiotepa, and cyclophosphamide (see the following Examples). Therefore, the use of the BRCA1 classifiers can be used to prospectively predict how an individual subject will respond to anti-cancer therapy. Until the present disclosure, no such test had been available.

5 [0102] As shown in the following Examples, the BRCA1 classifiers have been applied, via aCGH, to search for "BRCA1-like" patterns in metastatic tumors. Those patterns, where found, have been related to the treatment results of anti-cancer therapy. What was discovered was that all of the long-term survivors of stage IV breast cancer had tumors that displayed the BRCA1-like patterns discoverable by the BRCA1 classifiers. It is 10 also shown that triple-negative tumors that displayed the BRCA1-like patterns benefited markedly from high-dose alkylating therapy in the adjuvant setting, while the triple-negative tumors displaying sporadic-like patterns did not.

[0103] The examples provide evidence of a relation between the BRCA1-like pattern, detectable by the BRCA1 classifiers, and better treatment response to anti-cancer therapy. 15 The examples also provide evidence that BRCA1 inactivation in triple negative tumors, which can be obtained by the use of the BRCA1 classifiers, may identify patients that respond better to alkylating agents.

[0104] The BRCA1 classifiers can be used in a clinical setting to detect the presence or absence of homologous recombination deficiency in ER-low, HER2-negative stage-III 20 breast cancer patients. The examples disclose a comparison of the rates of cancer recurrence in patients treated according to the BRCA1-classifiers (i.e. patients with a BRCA1-like tumor: HD-chemotherapy, others: conventional chemotherapy) with the rates of cancer recurrence in patients treated with conventional chemotherapy (substitute of current clinical practice) resulted in a multivariate HR of 0.47 (95% CI 0.23-0.91). As shown in the 25 Examples, recurrence rates for ER-low, HER2-negative stage-III breast cancers can be cut in half by utilizing the BRCA1 classifiers to tailor chemotherapy treatment.

[0105] In further aspects, the present disclosure relates to kits for use in the diagnostic applications described above. The kits can comprise any or all of the reagents to perform the methods described herein. The kits can comprise one or more of the BRCA1 30 classifiers. In the diagnostic applications such kits may include any or all of the following: assay reagents, buffers, nucleic acids such hybridization probes and/or primers that specifically bind to at least one of the genomic locations described herein, as well as arrays comprising such nucleic acids. In addition, the kits may include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. While 35 the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to

electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

Examples

5 [0106] The following examples describe in detail the therapeutic efficacy of chemotherapy by detecting phenotypic genetic traits using comparative genomic hybridization. It will be apparent to those skilled in the art that many modifications, both to materials and methods, may be practiced without departing from the scope of the disclosure.

Example 1

10 [0107] The present inventors have developed a BRCA1-classifier (**Fig. 2**) to identify tumors of metastatic breast cancer (MBC) patients (n=39) with a long progression-free survival after treatment with high dose platinum-based alkylating chemotherapy (HD-chemotherapy). This classifier was prospectively validated in estrogen-receptor low, HER2-negative tumors of stage-III breast cancer patients (n=77), who had been randomized 15 between adjuvant HD-chemotherapy and conventional chemotherapy. Additionally, the concordance between the BRCA1-classifier and BRCA1-mutations in the MBC tumors was assessed.

[0108] The new classifier scored 16/39 tumors as BRCA1-like in the MBC-series (of which 2 harbored a BRCA1-mutation). In the adjuvant validation series, patients with 20 BRCA1-like tumors (39/77=51%) benefited more from HD-chemotherapy than those with Sporadic-like tumors (38/77=49%) (test for interaction p=0.026). HD-chemotherapy strongly decreased the risk of recurrence (HR=0.15, p=0.001; 5-year recurrence free survival (RFS) 78% versus 29%), while RFS in the Sporadic-like group was not improved by HD-chemotherapy.

25 [0109] Based on these results, it is apparent that the benefit of intensive alkylator-based chemotherapy for the treatment of BRCA1-like tumors may outweigh the side-effects of this regimen. Furthermore, this BRCA1-classifier may represent an effective test to identify BRCAness in breast cancers and may therefore predict effectiveness of other HRD-targeting agents such as poly(ADP-ribose)polymerase(PARP)-inhibitors.

30 [0110] It has been suggested that Comparative Genomic Hybridization (CGH) can be useful in identifying the genomic instability inherent to HRD tumors by visualizing the copy number aberrations (CNAs)[8]. In the Netherlands Cancer Institute, a conditional knockout mouse model for BRCA1 breast tumors has been generated[18]. Usin this model, mouse mammary tumors lacking BRCA1 were shown to be extremely sensitive to cisplatin[7]. 35 Furthermore, these tumors displayed striking genomic instability measured by the extent of CNAs using CGH[18]. These findings support the use of this model to discern tumors with HRD as has been suggested by Turner et al[8]. For this study, a BRCA1 CGH classifier,

designed to identify human BRCA1-mutated breast cancers from sporadic breast cancers was constructed[19;20]. This classifier was translated to an array based platform (aCGH) and consisted of the characteristic CNAs of breast cancers from a patient series of known BRCA1 germ-line carriers.

5 [0111] For purposes of this study, it was hypothesized that these characteristic CNAs would not only be present in tumors with a BRCA1-mutation, but also in tumors with a wider range of molecular defects in the BRCA1-pathway. If true, this BRCA1-classifier would be capable of predicting sensitivity to DSB-inducing agents, such as alkylating agents and the new PARP-inhibitors, in breast cancer patients. To test this hypothesis, patients were 10 studied who had been treated with one of the few regimens in which only alkylating agents were used: high dose platinum-based alkylating chemotherapy (HD-chemotherapy). It was demonstrated that this classifier was capable of selectively predicting improved outcome after HD-chemotherapy in estrogen receptor (ER)-low, HER2-negative stage III breast cancer patients who participated in a randomized trial of adjuvant HD-chemotherapy versus 15 conventional chemotherapy.

Methods

[0112] To determine whether the BRCA1-classifier predicts benefit from HD-chemotherapy, two patient series were studied. First, patients with metastatic breast cancer (MBC) who had received HD-chemotherapy (5-fluorouracil, epirubicin, cyclophosphamide 20 (FEC) as induction followed by high dose cyclophosphamide, thiotepa and carboplatin (CTC) with autologous stem cell support) were studied. Since the aim of this study was different from the aim for which the classifier was initially developed, a new cut-off of the BRCA1-probability score of the BRCA1-classifier in this patient series was determined. To validate the cut-off and determine whether the BRCA1-classifier was a predictive marker, stage III 25 breast cancer patients were studied in the adjuvant setting who had been randomized to either conventional or HD-chemotherapy (CTC) with autologous stem cell support. All trials described herein were approved by the Institutional Review board of the Netherlands Cancer Institute. This study was designed following the REMARK guidelines (Appendix I)[22].

Patient Selection first series (MBC series)

30 [0113] Patients were included from three pilot studies carried out at the Netherlands Cancer Institute between 1993 and 2004 (one patient was included in 1989 with the setup of the trial)[23-25]. Inclusion criteria have been published previously[23-25].

[0114] Patients were eligible when their formalin-fixed paraffin-embedded (FFPE) primary tumor tissue contained more than 60% of tumor cells and when they had received at 35 least one course of CTC. Exclusion criteria consisted of progressive disease on induction chemotherapy (FEC), as these patients did not proceed to HD-chemotherapy; treatment-related death; contralateral breast cancer; stage IIIc[26] breast cancer.

Patient selection second series (stage-III series)

[0115] Patients of the second series were selected from a large randomized controlled multicentre trial performed in the Netherlands between 1993 and 1999. Inclusion criteria have been published previously[27]. Eligible patients were randomized between either conventional chemotherapy (five courses FEC), or HD-chemotherapy which was identical except that instead of the fifth course of FEC, a course of CTC was given. Based on previous experience that BRCA1-like tumors virtually always have a low ER and negative HER2 expression and comprise about 30-50% of all ER-low, HER2-negative tumors, patients with tumors with a low ER expression (< 25%) and a HER2-negative status in this randomized trial were studied. Cases were only included when their FFPE primary tumor tissue was available and contained more than 60% of tumor cells.

Comparative Genomic Hybridization and mutation analyses

[0116] Genomic DNA was extracted from all FFPE primary tumors as previously described[28]. Of seven patients only lymph node tissue, removed at first diagnosis containing primary tumor tissue, was available. Tumor DNA and reference DNA were labeled and hybridized as published previously and as disclosed herein[29]. The data discussed in this Example have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE12127.

[0117] A BRCA1-classifier (**Fig. 2**) was constructed and refined for two purposes; 1) to use as a pre-selection tool to detect subjects with a high risk of carrying a BRCA1-mutation, which resulted in a slightly modified version[30]; and 2) to use as a predictive test to identify breast cancer patients likely to benefit from DSB-inducing agents. For the latter, the original classifier was used as described herein. BRCA1 class detection was performed on each individual aCGH tumor profile using the BRCA1-classifier (**Fig. 2**), resulting in a BRCA1-probability score ranging from 0 to 1. All protocols used for aCGH are described in **Fig. 3**.

[0118] For mutation analysis a method developed especially for DNA isolated from FFPE material was utilized. The most common mutations reported in Dutch families known to carry pathogenic germline BRCA1 or BRCA2 mutations were screened. The analysis included 37 distinct BRCA1 mutations accounting for 749 of 1166 BRCA1 families (~64%) and 40 distinct BRCA2 mutations accounting for 264 of 520 BRCA2 families (~51%) in the Netherlands (**Fig. 4**).

Histopathology

[0119] Two pathologists reviewed all tumors and scored whole H&E-slides for tumor percentages. ER, HER2 and progesterone receptor status was determined by immunohistochemistry (IHC) as described before[27;32]. Pronase was used as pretreatment for EGFR (EGFR Ab-10 clone 111.6; 1:200; Neomarkers; EGFR clone 31G7, 1:400; Zymed)

and the standard procedure for CK 5/6 (clone D5/16 B4, M7237, 1:200, Dako). CK5 and EGFR were considered positive if any (weak or strong) staining of tumor cells was observed. Tumors were classified as basal-like according to the Nielsen basal-like breast cancer IHC definition, as published previously[33].

5 **Statistical analysis**

[0120] The cut-off of the BRCA1-probability score on the MBC series was determined to obtain the highest positive predictive value for response (defined as a progression free survival (PFS) longer than 24 months, the median overall survival of MBC patients) and validated in the stage-III series.

10 [0121] Differences between groups of interest were tested using Fisher's exact tests and exact Chi-square test for trend. Patients with missing values for a variable were excluded from analyses involving that variable. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. Hazard ratios (HR) were calculated using Cox proportional hazards regression.

15 [0122] In the MBC series, complete remission after CTC-treatment was defined as disappearance of all evaluable tumor mass assessed by physical examination and imaging studies. PFS was defined as the time from the first CTC-course to the appearance of the first progression of disease (based on clinical signs and symptoms, substantiated with imaging and/or biochemical analyses and/or cytology/histology), or death, whichever occurred earlier.
20 Patients who did not experience a progression were censored at the end of follow-up. Because of the small sample size, potential confounders were not added at once but one at a time to a model including the BRCA1-classifier.

[0123] In the stage-III series, recurrence free survival (RFS) was calculated from randomization to the appearance of a local or regional recurrence, metastases or to death
25 from any cause[27]. All other events were censored. Overall survival (OS) was time from randomization to death from any cause, or end of follow-up. Patients alive at their last follow-up visit at the time of analysis were censored at that time. All treatment comparisons were based on patients who completed their assigned treatment (per-protocol analysis). The effect of HD-chemotherapy versus conventional chemotherapy on RFS was assessed,
30 expressed as hazard ratio (HR), differed by BRCA1-like status based on multivariate proportional hazards regression with an interaction term, adjusting for potential confounders.

[0124] All calculations were performed using the statistical package SPSS 15.0 and SAS 9.1 (for Windows, respectively SAS Institute Inc., Cary, NC, USA).

Results

35 **MBC series**

[0125] Based on aCGH-profiles of 39 patients (**Fig. 5**), tumors with a BRCA1-probability score >0.63 (**Fig. 13**) were considered to be BRCA1-like (N=16, 41%) and others

as Sporadic-like (N=23, 59%). Compared with Sporadic-like tumors, BRCA1-like tumors were more often HER2-receptor negative (p=0.06), ER-negative (p=0.02), and basal-like (p<0.001) (Table 1).

Table 1. Patient characteristics by profile of the MBC-series

Variable	Patients with Sporadic-like tumors		Patients with BRCA1-like tumors		p-value
	n	%	n	%	
Total	23	100	16	100	
Age at CTC*					
Mean (years)	46.5		40.0		0.122
Range (years)	23.0 - 59.5		32.6 – 51.0		
≤ 40 years	7	30.4	8	50.0	0.318
> 40years	16	69.6	8	50.0	
Metastatic disease*					
≤ 2 sites of metastases	12	52.2	10	62.5	0.743
> 2 sites of metastases	11	47.8	6	37.5	
Histological grade†					
Grade 1 and 2	9	39.1	4	25.0	0.495
Grade 3	14	60.9	12	75.0	
HER2 receptor†					
Negative	15	65.2	15	93.8	0.056
Positive	8	34.8	1	6.3	
Estrogen receptor status†					
Negative	11	47.8	14	87.5	0.017
Positive	12	52.2	2	12.5	
Progesterone receptor status†					
Negative	11	47.8	12	75.0	0.240
Positive	6	26.1	2	12.5	
Unknown	6	26.1	2	12.5	
CK 5/6 status†					
Negative	22	95.7	8	50.0	0.001
Positive	1	4.3	8	50.0	
EGFR status†					
Negative	19	82.6	9	56.2	0.024
Positive	2	8.7	7	43.8	
Unknown	2	8.7	0	0.0	
Nielsen basal-like breast cancer definition †					
Negative	22	95.7	7	43.8	<0.001
Positive	1	4.3	9	56.2	
Prior Chemotherapy‡					
No	13	56.5	14	87.5	0.076
Yes	10	43.5	2	12.5	
Prior Radiotherapy					
No	5	21.7	5	31.3	0.711
Yes	18	78.3	11	68.8	
Number of CTC courses					
< 3 courses	9	39.1	3	18.8	0.291
3 courses	14	60.9	13	81.3	
CTC Response					
All other responses	14	60.9	3	18.8	0.020
Complete Remission	9	39.1	13	81.3	

* at start first CTC treatment. † Of primary tumor, except for two patients of whom only the lymph node metastasis tissue of the primary tumor was available. ‡ Prior chemotherapy, in all cases consisted of cyclophosphamide, methotrexate and fluoruracil (CMF) in the adjuvant setting, except one case who received five courses of adjuvant $FE_{90}C$. Missing data excluded from analysis; p-value calculated using the Fisher exact test. *Abbreviations*: CI, confidence interval; IHC, immunohistochemistry; CTC, carboplatin-thiotepa-cyclophosphamide.

[0126] BRCA1-like patients had a significantly better response to CTC-treatment, defined by achievement of complete remission (p=0.02), and significantly longer PFS (**Fig. 14**, p=0.001), with a univariate HR for progression of 0.31 (95%CI: 0.14-0.66, **Fig. 6**). Adjustment for potential confounders did not substantially modify the HR (**Fig. 7**).

MBC series and mutation analysis

[0127] Two BRCA1-mutated tumors, both of which had a BRCA1-like tumor were identified. Additionally, two BRCA2-mutated tumors were identified, one of which had a BRCA1-like tumor (**Fig. 8**). Mutations were not necessarily germ-line mutations since DNA derived from the tumors was tested. In fact, three of the four BRCA-mutated patients identified in this analysis had been tested by a familial cancer clinic and were known mutation carriers. The familial cancer clinic had tested one additional patient of this study, who was found to be wild type BRCA1/2 in both analyses. For one patient, all DNA was used for aCGH, and mutation analyses could not be performed.

Stage-III series

[0128] **Fig. 9** summarizes the flow of patients through the study including the number of patients in each stage. Reasons for dropout are listed. Tumor aCGH profiles could be obtained for 81 patients. Characteristics and treatments of these 81 patients did not differ from those of the ER-low, HER2-negative patients not in the current analysis (**Fig. 10**). Four of these 81 patients were not treated according to protocol and were excluded from further analysis.

[0129] Of the 77 patients, 39 tumors (51%) were scored as BRCA1-like. Patient characteristics did not differ by treatment arm within the patients with BRCA1- or Sporadic-like tumors (Table 2). Patients with BRCA1-like tumors were generally younger, and their tumors were more often poorly differentiated and progesterone receptor negative. Tumor size according to TNM classification, number of positive lymph nodes and treatment were significantly associated with RFS (**Fig. 11**) and therefore included in multivariate analyses as potential confounders.

Table 2. Patient characteristics distributed by treatment arm per BRCA1-classification of the stage-III series

Variable	Total		Patients with Sporadic-like tumors					Patients with BRCA1-like tumors				
			Conventional Chemotherapy		High Dose chemotherapy		p-val	Conventional Chemotherapy		High Dose chemotherapy		p-val
	n	%	n	%	n	%		n	%	n	%	
Total	77	1	21	53.8	17	44.7		21	53.8	18	46.2	
Age in												
≤ 35 years	14	1	3	14.3	2	11.8		5	23.8	4	22.2	
35 - 40 years	16	2	2	9.5	4	23.5	0.82 0†	5	23.8	5	27.8	0.71 5†
41 - 45 years	11	1	2	9.5	2	11.8		4	19.0	3	16.7	
46 - 50 years	22	2	9	42.9	3	17.6		7	33.3	3	16.7	
> 50 years	14	1	5	23.8	6	35.3		0	0.0	3	16.7	
Type of												
Mastectomy	57	7	17	81.0	14	82.4	1.00 0*	14	66.7	12	66.7	1.00 0*
Breast	20	2	4	19.0	3	17.6		7	33.3	6	33.3	
Tumor												
T1	17	2	2	9.5	3	17.6		6	28.6	6	33.3	
T2	45	5	13	61.9	10	58.8	0.60 5†	11	52.4	11	61.1	0.45 8†
T3	15	1	6	28.6	4	23.5		4	19.0	1	5.6	
No. of positive												
4-9	48	6	14	66.7	9	52.9	0.50 9*	14	66.7	11	61.1	0.75 0*
≥ 10	29	3	7	33.3	8	47.1		7	33.3	7	38.9	
Histologic												
I	4	5.	1	4.8	3	17.6		0	0.0	0	0.0	
II	16	2	9	42.9	3	17.6	1.00 0†	3	14.3	1	5.6	0.62 6*
III	51	6	9	42.9	10	58.8		18	85.7	14	77.8	
Not determined	6	7.	2	9.5	1	5.9		0	0.0	3	16.7	
Estrogen												
0 % positive	65	8	14	66.7	15	88.2		19	90.5	17	94.4	
10 % positive	6	7.	4	19.0	1	5.9	0.24 9†	1	4.8	0	0.0	1.00 0†
20 % positive	2	2.	1	4.8	0	0.0		1	4.8	0	0.0	
25 % positive	4	5.	2	9.5	1	5.9		0	0.0	1	5.6	
Progesterone												
Negative	69	8	16	76.2	15	88.2	0.42 7*	21	100.0	17	94.4	0.46 2*
Positive (≥10%)	8	1	5	23.8	2	11.8		0	0.0	1	5.6	
P53 status												
Negative	43	5	11	52.4	11	64.7	0.52 1*	11	52.4	10	55.6	1.00 0*
Positive (≥10%)	34	4	10	47.6	6	35.3		10	47.6	8	44.4	

Missing values not included in the statistical analyses. p-value calculated using: * Fisher exact test; † Exact Chi-square test for Trend.

[0130] The beneficial effect of HD-chemotherapy differed significantly between patients with BRCA1-like tumors and those with Sporadic-like ones (test for interaction p=0.03). Among patients with BRCA1-like tumors, the risk of recurrence was almost 7-fold decreased after HD-chemotherapy compared to conventional chemotherapy (multivariate HR 0.15, 95%CI 0.05-0.46, p=0.001, **Fig. 12**, Table 3), while in patients with Sporadic-like tumors no significant difference was observed (multivariate HR 0.74, 95%CI 0.31-1.77, p=0.50, **Fig. 12**, Table 3).

Table 3. Multivariate Cox proportional-hazard analysis of the risk of recurrence (RFS) in the stage-III series

Variable	No. Events	Hazard Ratio	95% CI	p-value
Lymph Nodes				
4 - 9 LN positive	22	1.00		
≥ 10 LN positive	19	2.14	1.11 - 4.13	0.023
p T-stage				
1 or 2	30	1.00		
3	11	1.94	0.93 - 4.04	0.079
aCGH classifier				
Sporadic-like tumor	22	1.00		
BRCA1-like tumor	19	2.27	1.06 - 4.88	0.035
BRCA1-like tumor				
Conventional chemotherapy	15	1.00		
High Dose chemotherapy	4	0.15*	0.05 - 0.46	0.001
Sporadic-like tumor				
Conventional chemotherapy	14	1.00		
High Dose chemotherapy	8	0.74*	0.31 - 1.77	0.498

* Homogeneity of both hazard ratios was rejected based on an interaction term with p=0.026.

5 [0131] Similar trends were observed for overall survival (data not shown, test for interaction p=0.09), in which patients with BRCA1-like tumors benefited significantly from HD-chemotherapy (HR 0.22, 95%CI 0.07-0.66) while patients with Sporadic-like tumors appeared not to benefit (HR 0.75, 95%CI 0.29-1.90).

[0132] The aim of this study was to investigate whether an aCGH classifier (**Fig. 2**),
10 initially constructed to identify BRCA1-mutated tumors, was capable of predicting response to DSB-inducing agents, such as high dose platinum-based alkylating chemotherapy. Remarkably, with this classification it was found that MBC patients who were in continuous complete remission (55 to147 months) after high dose alkylating chemotherapy all had a BRCA1-like tumor. Furthermore, BRCA1-like MBC patients had a significantly higher
15 complete remission rate suggesting this classifier was predictive of drug response. To validate the BRCA1-classifier and prove that it indeed predicted for response to HD chemotherapy, the classifier was applied to tumor DNA of stage-III breast cancer patients selected from a large trial in which patients had been randomized between conventional adjuvant chemotherapy of that time and a HD-chemotherapy regimen similar to the one used
20 in MBC patients. It was found that the BRCA1-classifier predicted for improved outcome after platinum-based high dose alkylating chemotherapy by identifying breast cancer patients specifically benefiting from HD-chemotherapy within ER-low and HER2-negative stage-III breast cancer patients.

[0133] In the MBC series 41% (16/39) and in the stage-III series 51% (39/77) of the tumors were BRCA1-like, suggesting that the classifier identified not only BRCA1 mutation carriers but also tumors with potentially other defects in the BRCA1-pathway. To further substantiate this, mutation analysis was performed on material of the MBC series. Four patients (4/38; 11%) were identified with a mutation in BRCA1 or BRCA2 in their primary tumor. This is comparable to the reported frequency (9-12%) of BRCA1 and BRCA2 mutations in non-Dutch European breast cancer patients younger than 45 years[35-37]. Only three of the mutation carriers were scored as BRCA1-like (3/16, 19%), suggesting that the BRCA1-classifier also reflects other defects in the BRCA1-pathway.

[0134] A statistically significant benefit from adjuvant HD-chemotherapy with a 5-year RFS of 78% was observed in BRCA1-like patients, but not among Sporadic-like patients; this difference was statistically significant. The 5-year RFS observed in all conventionally treated stage-III patients of 38% is comparable to disease free survival rates of ER-, HER2-negative breast cancer patients treated with similar anthracycline-based regimens[41;42]. The 5-year RFS of HD-chemotherapy remains impressive when put into perspective of current clinical practice, with 5-year disease free survival rates of 64-67% after taxane containing chemotherapy[41;42]; especially when taking into account that those rates were observed in patients with earlier breast cancer stages than solely stage III[41;42].

[0135] The facts that the subgroup analysis performed was based on strong preclinical and clinical evidence of a molecular based concept (HRD and sensitivity to alkylating agents) and the information that the instant findings were confirmed in two independent datasets, provide substantial evidence for the BRCA1-classifier to be a predictive test for selective benefit of HD-chemotherapy. Moreover, one could envision that different cut-offs of the BRCA1-probability score could be used for different stages of breast cancer. For example, in metastatic patients who have exhausted their treatment options, it would be justified to set a low cut-off to ensure less false negative results (i.e. under-treatment).

[0136] Based on these results, the benefit of intensive alkylator-based chemotherapy for the treatment of BRCA1-like tumors may outweigh the side-effects of this regimen. Since response to platinum/ alkylating agents is a read-out of HRD, this classifier may represent a clinical test for BRCAness in this specific subgroup. This classifier may also be predictive for other agents/regimens that target HRD, e.g. PARP-inhibitors.

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Example 2

[0183] Tumors with homologous recombination deficiency (HRD), such as BRCA1 associated breast cancers, are not able to reliably repair DNA double strand breaks (DSBs), and are therefore highly sensitive to both DSB-inducing chemotherapy and PARP inhibitors. In the study presented in this Example, markers that may indicate the presence of HRD in *HER2*-negative breast cancers and related them to neoadjuvant chemotherapy response were studied. Array Comparative Genomic Hybridization (aCGH), *BRCA1* promoter methylation, *BRCA1* mRNA expression, and *EMSY* amplification were assessed in 163 HER2 negative pretreatment biopsies from patients scheduled for neoadjuvant chemotherapy. Features of BRCA1 dysfunction were frequent in triple-negative (TN) tumors: a BRCA1-like aCGH pattern, promoter methylation and reduced mRNA expression were observed in respectively 57%, 25% and 36% of the TN tumors. Abnormalities associated with BRCA1 inactivation are present in about half of the TN breast cancers, but were not predictive of chemotherapy response.

[0184] Neoadjuvant chemotherapy has become a widely used treatment strategy for patients with early or locally advanced breast cancer. It is equally effective as similar drug therapy following local treatment and it has additional advantages: breast conserving therapy is more frequently possible as a result of tumor shrinkage and the effect of the drugs on the tumor can be assessed during treatment. The complete disappearance of all tumor cells at microscopic examination (pathologic complete remission, or pCR) correlates well with overall survival [1,2] and achieving a pCR is considered an appropriate intermediate endpoint for clinical trials. Current neoadjuvant drug regimens achieve a pCR rate of 5-10% in luminal type breast cancers, and about 40% in basal-like and in HER2/neu-positive tumors [3,4].

[0185] Bifunctional alkylators and platinating agents cause interstrand DNA crosslinking, which cause DNA double strand breaks (DSBs) during DNA replication. In

normal cells, these DSBs are repaired by a process called homologous recombination. If this process is unavailable or impaired, a situation referred to as 'homologous recombination deficiency' (HRD) is present and alternative, error-prone DNA repair mechanisms take over, leading to genomic instability. The breast cancer genes *BRCA1* and *BRCA2* are essential for homologous recombination and tumors of patients carrying germ-line mutations in these genes show HRD as a result of the loss of the second, unmutated allele. *BRCA1* and *BRCA2* can be inactivated in sporadic cancers as well [5,6], a phenomenon referred to as 'BRCA-ness'. Many additional genes are involved in homologous recombination, including the Fanconi anemia genes and the *BRCA2* inactivating gene *EMSY* [7].

[0186] Tumors with HRD have been shown to be particularly sensitive to DNA crosslinking agents, such as alkylators and platinum drugs [8-10]. Both classes of drugs are employed in locally advanced breast cancer. Importantly, the novel poly (ADP-ribose) polymerase (PARP)-inhibitors are specifically effective in HRD tumors as well, and have shown impressive activity in clinical studies recently [11-13]. Unfortunately, no clinical tests exist which can reliably determine HRD in tumor biopsies. Previous studies have focused on genes that have a role in homologous recombination, such as the *BRCA1* and *-2* genes, *FANC* genes and *EMSY* [6]. It has been shown that breast cancers of *BRCA1* and *BRCA2* mutation carriers have a characteristic pattern of DNA gains and losses in an array comparative genomic hybridization (aCGH) assay [5,14-18]. In a recent study from the Netherlands Cancer Institute, a subgroup of hormone receptor negative tumors characterized by BRCA1-like aCGH pattern were shown to benefit markedly from intensive platinum-based chemotherapy [19]. Another recent report showed that a subset of TN tumors might be sensitive to the DNA DSB inducing drug cisplatin, as a result of low *BRCA1* expression levels or *BRCA1* promoter methylation [20].

[0187] In this study, the present inventors prospectively determined the frequency in which these HRD-associated features occur in untreated patients with breast cancer. The findings were correlated with response to chemotherapy that causes DNA DSBs. If HRD is indeed confirmed to be the 'Achilles heel' of certain sporadic tumors, such tests could eventually serve to individualize drug treatment.

[0188] *Patients*

[0189] Pre-treatment biopsies of primary breast tumors from 163 women with HER2 negative breast cancer were collected. All patients had received neoadjuvant treatment at the Netherlands Cancer Institute between 2004 and 2009 as part of two ongoing clinical trials, or were treated off protocol according to the standard arm of one of these studies. Both studies had been approved by the ethical committee and informed consent was obtained from all patients. For eligibility, breast carcinoma with either a primary tumor size of at least 3 cm was required, or the presence of fine needle aspiration (FNA) -proven axillary

lymph node metastases. Biopsies were taken using a 14G core needle under ultrasound guidance. After collection, specimens were snap-frozen in liquid nitrogen and stored at -70°C. Each patient had two or three biopsies taken to assure that enough tumor material was available for both diagnosis and further study.

[0190] Depending on the particular study, a treatment regimen was assigned to each patient, which consisted of one of the following: 1.) Six courses of dose-dense Doxorubicin/Cyclophosphamide (ddAC); or 2.) Six courses of Capecitabine/Docetaxel (CD); or 3.) If the therapy response was considered unfavorable by MRI evaluation after three courses, ddAC was changed to CD or vice versa. For the current study, only patients who started with ddAC (group 1 and group 3) were considered, thus all patients received at least three courses of ddAC (a DSB-inducing regimen).

[0191] *Pathology and response evaluation*

[0192] All pre-treatment biopsies were reviewed by two pathologists. ER and PR percentages were determined by immunohistochemistry (IHC), and HER2 was assessed by IHC and CISH. For some analysis ER and PR were dichotomized as percentage lower than 50% or higher (variable names: ER_50, PR_50). Pre-treatment lymph node status was assessed at pathology. The response of the primary tumor to chemotherapy was evaluated by contrast-enhanced MRI [21] after 3 courses of chemotherapy, and after completion of chemotherapy by pathologic evaluation of the resection specimen. The primary end point of both studies was a pCR, defined as the complete absence of residual invasive tumor cells seen at microscopy. If only non-invasive tumor (carcinoma *in situ*) was detected, this was considered a pCR as well. When a small number of scattered tumor cells were seen, the samples were classified as 'near pCR' (npCR). Because the aim of this study was to determine if HRD was correlated with a higher sensitivity to chemotherapy, tumors with a npCR were included in the group of complete remission for analytical purposes. Patients with larger amounts of residual tumor left were classified as non-complete responders (NR).

[0193] *Array-CGH*

[0194] Tumor DNA and reference DNA were co-hybridized using two different CyDyes to a microarray containing 3.5k BAC/PAC derived DNA segments covering the whole genome with an average spacing of 1MB and processed as described before [22]. Classification of subtypes was performed using an aCGH BRCA1 and BRCA2 classifier [5] [23]. In this Example, the same classifier used in the preceding Example (**Fig. 2**) was utilized and a BRCA1 probability score ≥ 0.63 was considered as a BRCA1-like aCGH pattern [19]. Under this cut-off a tumour was called sporadic-like. The cut-off for a BRCA2-like aCGH pattern was 0.5, as described previously [23].

[0195] *RT–PCR*

[0196] mRNA isolation and extraction were performed using RNA Bee, according to the manufacturer's protocol (Isotex, Friendswood, TX). A 5 µm section halfway through the biopsy was stained for Hematoxylin and Eosin and analyzed by a pathologist for tumor cell percentage. Only samples that contained at least 60% tumor cells were included in the
5 further analysis. RT-qPCR was performed using TaqMan Pre-designed gene expression Assay for *BRCA1* (#Hs01556193). The standard curve method was used. *GAPDH* and *B-actin* were measured for normalization purposes and the average of both gene expression values was used. The cut-off between *BRCA1* low and normal gene expression was 0.25. This cut-off was empirically determined.

10 [0197] *MLPA*

[0198] Hypermethylation of the *BRCA1* promoter was determined using a custom Methylation specific MLPA set, according to the manufacturers' protocol (MRC-Holland; ME005-custom). When the two *BRCA1* markers both showed methylation, the *BRCA1* promoter was considered to be methylated. Amplification of *EMSY* (C11orf30) was
15 determined using a custom MLPA set, containing seven different *EMSY* probes and nine reference probes (MRC Holland; X025). This EMSY MLPA set was first validated by an *EMSY* FISH assay (Dako). From the comparison of the *EMSY* FISH assay and the MLPA, it was concluded that an average of the seven probes above 1.5 corresponded to *EMSY* amplification, as detected by at least 6 copies of the probe at the FISH assay. DNA
20 fragments were analyzed on a 3730 DNA Analyzer (AB, USA). Probe sequences for both MLPA kits are available on request (info@mlpa.com). For normalization and analysis the Coffalyzer program was used (MRC-Holland).

[0199] *Statistical tests*

[0200] The Fisher's exact test was used to assess association between the
25 dichotomized HRD characteristics, pathological and clinical variables. Logistic regression was performed to adjust for the following variables: age, T-stage, N-stage, ER percentage, PR percentage. All data analyses were performed using SPSS version 17.

[0201] *Overview of samples*

[0202] The frequency of HRD characteristics was studied in pre-treatment biopsies,
30 and subsequently the findings were related to neoadjuvant chemotherapy response. A total of 60 triple negative (TN) and 103 ER+ *HER2*- tumors were studied, which all received neoadjuvant chemotherapy with doxorubicin and cyclophosphamide (AC-regimen). Table 4 shows the clinical pathological characteristics of all tumors. The majority of the tumors were T-stage 2 or 3 and lymph node positive. Most patients were treated by 6x ddAC, although
35 some switched to the DC regimen after 3 courses of AC. TN tumors had a higher percentage of responders (pCR+ npCR) than ER+ patients. Table 5 gives the frequencies of the HRD characteristics per tumor group. BRCA1-related abnormalities (aCGH BRCA1-like profile,

BRCA1 promoter methylation and low *BRCA1* mRNA expression) were predominantly observed in the TN tumors (table 5). The percentage of aberrations was not different between patients treated with 6 cycli of AC versus patients treated with 3 cycli AC followed by 3 cycli of DC (data not shown). As the pattern of characteristics and also the response
5 rates to chemotherapy are different in hormone receptor positive and negative tumors, they were analyzed separately.

Table 4 Patient and tumor characteristics

	TN		ER+	
Number of patients	60		103	
Median age (sd)	42 (11.8)		48 (8.9)	
Progesterone receptor				
Positive			70	68%
Negative	60	100%	32	31%
NA			1	1%
T-stage				
T1	3	5%	12	12%
T2	42	70%	56	54%
T3	10	17%	31	30%
T4	5	8%	4	4%
N-stage				
Node negative	23	38%	16	16%
Node positive	37	62%	87	84%
Chemotherapy				
6 x ddAC	51	85%	81	79%
3 x ddAC, 3 x DC	9	15%	22	21%
Response				
pCR	21	35%	12	12%
npCR	10	17%	12	12%
PR+ NR	27	45%	77	75%
unknown	2	3%	2	2%

(n)pCR=(near) pathological complete remission; PR + NR= partial and non response

ddAC= dose dense doxorubixin cyclophosphamide, DC= docetaxel, capecitabine

10

Table 5 Summary of HRD characteristics

	TN (n=60)	ER+ (n=103)	p-value
aCGH BRCA1 like			
BRCA1 like	34 (57%)	6 (6%)	
Sporadic like	26 (43%)	97 (94%)	**<0,001**
BRCA1expression			
low	13 (22%)	2 (2%)	
normal/high	23 (38%)	58 (56%)	**<0,001**
Not determined	24 (40%)	43 (42%)	
BRCA1 promotor methylation			
Methylated	12 (20%)	1 (1%)	

	TN (n=60)	ER+ (n=103)	p-value
Unmethylated	37 (62%)	55 (53%)	**<0,001**
Not determined	11 (18%)	47 (46%)	
EMSY Amplification			
Amplification	2 (3%)	11 (11%)	
Retention	34 (57%)	72 (70%)	0.339
Not determined	24 (40%)	20 (19%)	

* Due to limited biopsy material, methylation, gene expression and EMSY amplification were not performed on all samples.

[0203] *TN tumors and BRCA1-related abnormalities*

[0204] The BRCA1-like aCGH profile was predominantly seen in TN tumors (57% in TN vs 6% in ER+ tumors, $p<0.001$), (table 5). Other features of *BRCA1* inactivation were assessed by determination of *BRCA1* promoter methylation and the level of *BRCA1* mRNA expression. These two characteristics were again predominantly observed in TN tumors, but were less frequent than a BRCA1-like aCGH pattern: 25% of TN tumors showed *BRCA1* promoter methylation and 36% of TN tumors showed a low *BRCA1* gene expression.

[0205] The relation between the three BRCA1-related abnormalities was subsequently determined. **Figs. 15 and 16** show the relation between mRNA expression, methylation and a BRCA1-like aCGH pattern. The cut-off between low and normal *BRCA1* gene expression was empirically determined based on methylation status. It was assumed that methylated samples would have a low mRNA expression, so the cut-off was set at 0.25 (**Fig. 15**). All methylated samples therefore have, by definition, a low *BRCA1* gene expression. The median mRNA gene expression of methylated samples was 0.156 while unmethylated samples show a value of 0.398. This difference was statistically significant ($p<0.001$). The relation between the BRCA1-like aCGH pattern and *BRCA1* mRNA expression was also studied (**Fig. 16**), as low gene expression could be expected to be associated with a BRCA1-like aCGH pattern. Indeed, most BRCA1-like samples have a low expression of the *BRCA1* gene, whereas sporadic-like samples have more frequently a normal mRNA expression level. Samples with a BRCA1-like aCGH profile have a median mRNA expression of 0.226, while sporadic-like samples have a median mRNA expression value of 0.426, however, this difference was not statistically significant. From the 12 tumors with *BRCA1* promoter methylation, 8 had a BRCA1-like aCGH pattern and 4 a sporadic-like aCGH pattern.

[0206] Next, the association between BRCA1 inactivation and clinical and pathological variables and response to chemotherapy with DSB causing agents was studied. There was no difference in T-stage or N-stage between tumors with BRCA1-alterations and without (table 6). Patients with tumors showing *BRCA1* methylation were younger than those with non-methylated tumors. Treatment response on A/C was not different between tumors

with BRCA1 alterations and without these alterations: 58% vs. 48%, (p=0.47) for BRCA1-like vs. a sporadic-like aCGH profile; 55% vs. 61% (p=0.70) for methylated vs. unmethylated tumors and 54% vs. 61% (p=0.68) for low gene expression vs. normal gene expression.

5 Table 6. Clinical and pathological characteristics according to BRCA1 alterations in TN tumors.

	BRCA1-like aCGH					BRCA1 methylation					BRCA1 gene expression				
Variable	Sporadic like		BRCA1 like		P-value	Unmethylated		Methylated		P-value	normal mRNA		low mRNA		P-value
	N	%	N	%		N	%	N	%		N	%	N	%	
T_stage															
T1/2	20	77	25	74		27	73	10	83		18	78	10	77	
T3/4	6	23	9	26	0.76	10	27	2	17	0.47	5	22	3	23	0.93
N_stage															
LN neg	10	38	13	38		16	43	4	33		8	35	4	31	
LN pos	16	62	21	62	0.99	21	57	8	67	0.54	15	65	9	69	0.81
Age															
<=40	10	38	19	56		15	41	11	92		9	39	8	62	
>40	16	62	15	44	0.18	22	59	1	8	**0.002**	14	61	5	38	0.2
Response															
PR+NR	13	50	14	41		14	38	5	42		9	39	6	46	
pCR+npCR	12	46	19	56	0.47	22	59	6	50	0.7	14	61	7	54	0.68
Unknown	1	4	1	3		1	3	1	8						

[0207] In the series of patients described in this Example, the frequency of certain features associated with homologous recombination deficiency (HRD) was studied in untreated breast cancers and possible relationships with neoadjuvant treatment response
10 were explored. This study was restricted to *HER2*- negative tumors, as the focus of study was the effect of DNA double strand break (DSB) -inducing agents unperturbed by the effect of targeted therapy such as Traztuzumab. In TN tumors we found mainly BRCA1-related abnormalities.

[0208] In TN tumors, no difference in response rates was observed between patients with BRCA1-like aCGH tumors and tumors with a sporadic-like aCGH pattern. In the study presented in Example 1, the BRCA1-like aCGH pattern was shown to be associated with an important survival benefit of intensive treatment with platinum-based chemotherapy for high-
5 risk primary breast cancer [19]. It is possible that any hypersensitivity to DSB inducing agents only shows at higher doses, while the lower standard dose causes increased genomic instability rather than cell death.

[0209] In a recent report by *Kriege et al,* it was shown that BRCA2 hereditary breast cancers were more sensitive to chemotherapy with anthracyclines or CMF than sporadic
10 breast cancers [24]. For BRCA1 hereditary breast cancer, there was no significant difference in sensitivity. The authors explain the difference in outcome between *BRCA1*- and *BRCA2*-mutated tumors by different tumor characteristics, including higher grade, triple negativity and a higher incidence of *p53* mutations. The finding presented in this Example, that aberrations in BRCA1 are characteristic for TN tumors, is in line with this. *BRCA1*-mutated
15 tumors are usually basal like or triple negative.

[0210] In conclusion, in TN tumors, BRCA-ness occurred in about half of all cases, but did not predict a better treatment response to standard dose chemotherapy with AC. It is certainly possible that conventional doses of cisplatin or carboplatin would be highly effective in this subgroup, as suggested in the literature [20].

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[0239] Finally, it should be noted that there are alternative ways of implementing the embodiments disclosed herein. Accordingly, the present embodiments are to be considered as illustrative and not restrictive. Furthermore, the claims are not to be limited to the details given herein, and are entitled their full scope and equivalents thereof.

CLAIMS

What is claimed is:

1. A method for optimizing the therapeutic efficacy of anti-cancer therapy in a patient, comprising:

obtaining a cell sample from the patient;

detecting the copy numbers of genomic DNA in the patient's cell sample in at least 3 genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

wherein a variation in the copy numbers in the patient's cell sample classifies the cell sample as from a BRCA1-associated tumor and indicates that the patient will benefit from anti-cancer therapy.

2. A method for optimizing the therapeutic efficacy of anti-cancer therapy in a patient, comprising:

obtaining a cell sample from the patient;

detecting the copy numbers of genomic DNA in the patient's cell sample in any one of the genomic loci selected from 1p35-21, 3q22-27, 6p25-22, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 13q31-33, and 21q11-22; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

wherein an increase in the copy numbers in the patient's cell sample classifies the cell sample as from a BRCA1-associated tumor and indicates that the patient will benefit from anti-cancer therapy.

3. A method for optimizing the therapeutic efficacy of anti-cancer therapy in a patient, comprising:

obtaining a cell sample from the patient;

detecting the copy numbers of genomic DNA in the patient's cell sample in any one of the genomic loci selected from 5p13, 5q21-34, 7p21-15, 12q21-23, 14q22-24 and 15q14-21; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

wherein a decrease in the copy numbers in the patient's cell sample classifies the cell sample as from a BRCA1-associated tumor and indicates that the patient will benefit from anti-cancer therapy.

4. A method for optimizing the therapeutic efficacy of anti-cancer therapy in a patient, comprising:

obtaining a cell sample from the patient;

using a BRCA1 array to detect the copy numbers of genomic DNA in the
5 patient's cell sample, wherein the array comprises at least three of the BAC probes of **Fig. 2**; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

wherein a variation in the copy numbers in the patient's cell sample classifies
10 the cell sample as from a BRCA1-associated tumor and indicates that the patient will benefit from anti-cancer therapy.

5. The method of claim 4, wherein the array comprises at least 10 of the BAC probes of **Fig. 2**.

6. The method of claim 4, wherein the BRCA1 array comprises at least 50 of the BAC probes of **Fig. 2**.

7. The method of claim 4, wherein the BRCA1 array comprises at least 100 of the BAC probes of **Fig. 2**.

8. The method of claim 4, wherein the BRCA1 array comprises the BAC probes of **Fig. 2**.

9. A method for optimizing the therapeutic efficacy of anti-cancer therapy in a patient, comprising:

obtaining a cell sample from the patient;

detecting the copy numbers of genomic DNA in the patient's cell sample in
5 any three of the genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

10 wherein a variation in the copy numbers in the patient's cell sample classifies the cell sample as from a BRCA1-associated tumor and indicates that the patient will benefit from intensive alkylator-based chemotherapy.

10. A BRCA1 array comprising at least three of the BAC probes of **Fig. 2**.

11. A BRCA1 array comprising the BAC probes of **Fig. 2**.

12. A BRCA1 array capable of detecting the copy numbers of genomic DNA in at least three of the genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15, 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22.

13. A BRCA1 array capable of detecting the copy numbers of genomic DNA in at least three of the genomic loci selected from 1p35.1-21.3, 3q22.2-27.2, 5p13.2, 5q21.3-34, 6p25.2-22.1, 7p21.3-15.3, 7q31.33-36.3, 8q22.1-24.3, 10p15.3-14, 10p12.1, 12p13.33-13.2, 12q21.2-23.3, 13q31.2-33.3, 14q22.1-24.1, 15q14-21.1 and 21q11.2-22.3.

14. A method of assessing anti-cancer therapies for breast cancer, comprising:

obtaining a cell sample from the patient;

detecting the copy numbers of genomic DNA in the patient's cell sample in at least 3 genomic loci selected from 1p35-21, 3q22-27, 5p13, 5q21-34, 6p25-22, 7p21-15,
5 7q31-36, 8q22-24, 10p15-14, 10p12, 12p13, 12q21-23, 13q31-33, 14q22-24, 15q14-21 and 21q11-22; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

wherein a variation in the copy numbers in the patient's cell sample classifies
10 the cell sample as from a BRCA1-associated tumor and indicates that the patient will benefit from the anti-cancer therapies.

15. A method of assessing anti-cancer therapies for breast cancer, comprising:

obtaining a cell sample from the patient;

detecting the copy numbers of genomic DNA in the patient's cell sample in any one of the genomic loci selected from 1p35-21, 3q22-27, 6p25-22, 7q31-36, 8q22-24,
5 10p15-14, 10p12, 12p13, 13q31-33, and 21q11-22; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

wherein an increase in the copy numbers in the patient's cell sample classifies the cell sample as from a BRCA1-associated tumor and indicates that the patient
10 will benefit from the anti-cancer therapies.

16. A method of assessing anti-cancer therapies for breast cancer, comprising:

obtaining a cell sample from the patient;

detecting the copy numbers of genomic DNA in the patient's cell sample in any one of the genomic loci selected from 5p13, 5q21-34, 7p21-15, 12q21-23, 14q22-24 and
5 15q14-21; and

comparing the copy numbers in the patient's cell sample to corresponding copy numbers in a non-cancerous cell sample;

wherein a decrease in the copy numbers in the patient's cell sample classifies the cell sample as from a BRCA1-associated tumor and indicates that the patient will benefit
10 from the anti-cancer therapies.

Fig. 1 - BRCA1-associated genomic loci used to identify breast cancers with a BRCA1-deficient DNA repair system

chromosome	begin BAC	end BAC			# Bacs in region	# BACs in classifier	mid position begin	mid position end	size Mb	Aberration
1	RP4-561P1	RP11-14O19	1p35.1	1p21.3	70	18	34430455	95824367	61.4	gain
3	RP11-22E12	RP11-65J14	3q22.2	3q27.2	71	41	134528889	185893054	51.4	gain
5	RP11-7M4	RP11-20O13	5p13.2	5p13.2	52	24	37047046	93053964	56.0	loss
5	RP11-319P13	RP11-511M9	5q21.3	5q34	67	17	106806461	165898631	59.1	loss
6	RP11-15N12	RP1-153G14	6p25.2	6p22.1	27	14	3436613	27384955	23.9	gain
7	RP11-505D17	RP11-486P11	7p21.3	7p15.3	17	4	8070383	20125033	12.1	loss
7	RP11-264K23	RP11-518I12	7q31.33	7q36.3	50	12	123929280	157960428	34.0	gain
8	RP11-266D22	RP11-349C2	8q22.1	8q24.3	53	12	95352986	145710494	50.4	gain
10	RP11-29A19	RP11-566K1	10p15.3	10p14	12	7	1298380.5	10885404.5	9.6	gain
10	RP11-129O7	RP13-355A21	10p12.1	10p12.1	4	4	25183905	27998514.5	2.8	gain
12	RP11-283I3	RP11-144O23	12p13.33	12p13.2	21	11	282617.5	11040567	10.8	gain
12	RP1-97G4	RP11-478H3	12q21.2	12q23.3	32	12	77778132	106281513.5	28.5	loss
13	RP11-365J7	RP11-232K22	13q31.2	13q33.3	20	15	88049058	107819833	19.8	gain
14	RP11-262M8	RP11-226F19	14q22.1	14q24.1	21	6	52796119	69423346.5	16.6	loss
15	CTD-2033D15	RP11-154J22	15q14	15q21.1	12	6	39919724.5	48586416.5	8.7	loss
21	RP1-126N20	RP11-397E9	21q11.2	21q22.3	24	10	15761442	45935377	30.2	gain

Clone	chromosome
RP4-539L13	1p36.22
RP4-561P1	1p35.1
RP11-327P22	1p34.3
RP4-739H11	1p34.2
RP11-342M1	1p34.2
RP11-182I23	1p34.1
RP11-420M12	1p34.1
RP11-330M19	1p33
RP11-428D12	1p33
RP11-243A18	1p32.3
RP11-20F20	1p32.2
RP4-662P1	1p31.3
RP4-700A9	1p31.3
RP5-1033K19	1p31.3
RP11-250D8	1p31.1
RP5-1043L3	1p22.3
RP11-427B20	1p22.2
RP4-621B10	1p22.1
RP11-14O19	1p21.3
RP5-1077K16	1p13.3
RP11-98D18	1q21.3
RP11-216N14	1q21.3
RP11-180L13	1q23.3
RP1-97P20	1q24.2
RP4-799G3	1q42.13
GS-160-H23	1q44
RP11-132H1	2p24.3
RP11-247H16	2p24.3
RP11-258F20	2p22.3
RP11-30C22	2p16.1
RP11-440P5	2p16.1
RP11-549H5	2q11.2
RP11-368A17	2q13
RP11-62P16	2q23.3
RP11-176L20	2q31.1
RP11-38H6	2q32.1
RP11-1021H16	2q32.2
RP11-140C4	2q33.3
RP11-105N14	2q34
RP11-423F9	2q35
RP11-378A13	2q35
RP11-86O17	2q36.3
RP11-457P23	2q36.3
RP11-3B7	3p21.31
RP11-447A21	3p21.1
RP11-484I19	3q12.3
RP11-115B22	3q13.11
RP11-324H4	3q13.31
RP11-129J11	3q22.1
RP11-22E12	3q22.2
RP11-91O5	3q22.2
RP11-269A14	3q22.3
RP11-80p20	3q22.3
RP11-349D24	3q23
RP11-349D24	3q23
RP11-89E16	3q23
RP11-231L11	3q23
RP11-235I18	3q23
RP11-160a13	3q23
RP11-160A13	3q23
RP11-165M11	3q24
RP11-21M4	3q24
RP11-345O2	3q24
RP11-145F16	3q25.1
RP11-251C9	3q25.1
RP11-3F11	3q25.1
RP11-64O13	3q25.1
RP11-362A9	3q25.2
RP11-240G5	3q25.2
RP11-223L18	3q25.2
RP11-117L15	3q25.31
RP11-111F10	3q25.31
RP11-209h21	3q25.33
RP11-209H21	3q25.33
RP11-209H21	3q25.33
RP11-67F24	3q26.1
RP11-203L15	3q26.1
RP11-395F21	3q26.2
RP11-816J6	3q26.2
RP11-362K14	3q26.2
RP11-163H6	3q26.31
RP11-569P10	3q26.31
RP11-477P16	3q26.31
RP11-477P16	3q26.31
RP11-91K9	3q26.32
RP11-682A21	3q26.32
RP11-420J11	3q26.33
RP11-510K16	3q26.33
RP11-416O18	3q26.33
RP11-65J14	3q27.2
RP11-11O12	4p16.3
RP11-324I10	4p16.2
RP11-565I3	4p16.1
RP11-390C19	4p15.1
RP11-365H22	4q12
RP11-148K14	4q12
RP11-355L4	4q12
RP11-100N20	4q21.21
RP11-19C20	4q21.3
RP11-502A23	4q22.1
RP11-438P8	4q24
RP11-510D4	4q26
RP11-6C14	4q32.1
RP11-218F10	4q32.3
RP11-148L24	4q34.1
CTD-2324F15	5p15.31
CTD-2267H19	5p13.2
RP11-15A14	5p13.1
RP11-92M7	5q11.2
CTD-2022G9	5q11.2
RP11-364C6	5q11.2
RP4-572A3	5q11.2
RP11-313P15	5q12.1
RP11-19B7	5q12.3
RP11-480H11	5q12.3
RP11-402F5	5q13.1
RP11-494P23	5q13.1
RP11-115I6	5q13.2
RP11-399M5	5q13.2
RP11-97L2	5q13.3
CTD-2200O3	5q13.3
RP11-30D15	5q14.1
RP11-241j12	5q14.1
RP11-356D23	5q14.2
RP11-356D23	5q14.2
RP11-463C5	5q14.2
RP11-3H15	5q14.3
RP11-12D3	5q14.3
CTD-2011L22	5q15
RP11-20O13	5q15
RP11-319P13	5q21.3
RP11-17L14	5q21.3
RP11-276O18	5q21.3
RP11-467F22	5q22.2
RP11-249M12	5q23.1
RP11-11P11	5q23.1
RP11-14L4	5q23.2
CTB-54G2	5q23.2
RP1-66P19	5q31.1
RP1-98O22	5q31.2
RP11-515C16	5q31.3
RP11-226O9	5q32
RP11-481E16	5q32
RP11-26B2	5q33.2
CTB-3C20	5q33.3
RP11-469G19	5q34
RP11-511M9	5q34
RP11-15N12	6p25.2
RP11-163I22	6p25.1
RP11-196B15	6p24.3
RP11-97A19	6p24.1
RP1-257A7	6p24.1
RP3-365E2	6p23
RP1-232K4	6p22.3
RP11-68J15	6p22.3

Fig. 2

RP11-408C8	6p22.3
RP4-625H18	6p22.3
RP3-444C7	6p22.3
RP11-176J5	6p22.2
RP11-289G11	6p22.2
RP1-153G14	6p22.1
RP3-402H5	6p12.3
RP11-472M19	6p12.1
RP11-767J14	6q12
RP11-223L24	6q14.3
RP3-429G5	6q21
RP1-230I3	6q22.31
RP11-524A17	6q22.32
RP3-460G2	6q24.1
RP11-288H12	6q25.3
GS-57-H24	6q27
RP11-505D17	7p21.3
RP5-855F16	7p21.3
RP11-512E16	7p21.2
RP11-486P11	7p15.3
RP11-174I14	7q21.13
RP11-455I9	7q21.3
RP5-1059M17	7q22.1
RP11-148A10	7q22.1
RP11-126C19	7q31.31
RP11-264K23	7q31.33
RP11-193I17	7q32.3
RP11-355K3	7q32.3
RP11-355K3	7q32.3
RP11-221G19	7q33
RP11-269N18	7q34
RP11-426J23	7q34
RP4-548K24	7q36.1
RP11-69O3	7q36.3
RP4-764O12	7q36.3
RP11-452C13	7q36.3
RP11-518I12	7q36.3
RP11-540E4	8p23.1
RP11-44K6	8p11.21
RP11-266D22	8q22.1
RP11-21E8	8q22.3
RP11-188I6	8q22.3
RP11-20P9	8q23.1
RP11-115M9	8q24.13
RP11-16G11	8q24.13
RP11-96B2	8q24.13
RP11-495D4	8q24.13
RP11-3O20	8q24.21
RP11-4C17	8q24.22
RP11-343P9	8q24.23
GS-489-D14	8q24.3
RG-41-L13	9p24.3

GS-43-N6	9p24.3
RP11-509J21	9p24.2
RP11-509D8	9p24.1
RP11-490C5	9p22.3
RP11-5P15	9p21.2
RP11-195F19	9p13.3
RP11-336N8	9q21.2
RP11-66D1	9q21.31
RP11-176L21	9q22.1
RP11-400A24	9q31.3
RP11-388N6	9q31.3
RP11-88M9	9q32
RP11-78H18	9q32
RP11-205K6	9q33.3
GS-112-N13	9q34.3
GS-306-F7	10p15.3
GS-23-B11	10p15.3
RP11-118K6	10p15.2
RP11-154P11	10p15.1
RP4-542G16	10p14
GS1-756B1	10p14
RP11-566K1	10p14
RP11-129O7	10p12.1
RP11-307B23	10p12.1
RP11-128B16	10p12.1
RP13-355A21	10p12.1
RP11-505N10	10p11.22
RP11-124O11	10q11.21
RP11-313B15	10q21.1
RP11-210G22	10q21.3
RP11-470J18	10q23.1
RP11-165M8	10q23.2
RP11-359E7	10q26.11
RP11-436O19	10q26.13
RP11-120E20	11p15.4
RP1-316D7	11p13
RP11-138N3	11q13.2
RP11-569N5	11q13.2
RP11-291N1	11q14.3
RP11-432I22	11q24.2
RP11-106O22	11q24.2
RP11-264E20	11q24.3
GS-770-G7	11q25
GS-124-K20	12p13.33
RP11-283I3	12p13.33
RP11-283I3	12p13.33
GS-496-A11	12p13.33
RP11-359B12	12p13.33
RP5-1096D14	12p13.33
RP11-543P15	12p13.32
RP11-264F23	12p13.32
RP11-319E16	12p13.32

RP11-13C13	12p13.2
RP11-144O23	12p13.2
RP3-405J10	12q13.13
RP11-548L8	12q13.2
RP11-183H16	12q13.2
RP1-97G4	12q21.2
RP11-202G24	12q21.2
RP11-26L7	12q21.2
RP11-362A1	12q21.31
RP11-87P13	12q21.31
RP11-435O22	12q21.32
RP11-239F20	12q21.33
RP11-2K12	12q22
RP11-435E3	12q22
RP11-510I5	12q23.1
RP11-210L7	12q23.2
RP11-478H3	12q23.3
RP11-162P23	12q24.12
RP1-267D11	12q24.23
RP11-18C24	12q24.31
RP11-185C18	13q14.3
RP11-521L15	13q21.33
RP11-365J7	13q31.2
RP11-478H12	13q31.2
RP11-388D4	13q31.3
RP11-319L6	13q31.3
RP11-632L2	13q31.3
RP11-74A12	13q32.1
RP11-318K19	13q32.1
RP11-235O20	13q32.1
RP11-383H17	13q32.2
RP11-442I9	13q32.3
RP11-279D17	13q32.3
RP11-118F16	13q32.3
RP11-255P5	13q33.1
RP11-562E17	13q33.2
RP11-232K22	13q33.3
RP11-310D8	13q34
RP11-245B11	13q34
RP11-144C18	14q12
RP11-356O9	14q21.1
RP11-34O18	14q21.1
RP11-332O9	14q21.1
RP11-262M8	14q22.1
RP11-60K23	14q22.2
RP11-484F16	14q22.3
RP11-66E7	14q23.3
RP11-204K16	14q24.1
RP11-226F19	14q24.1
RP11-368K8	14q24.3
RP11-368K8	14q24.3
RP11-406A9	14q31.1

Fig. 2

RP11-179O11	14q31.2
RP11-262P9	14q32.13
RP11-725G5	14q32.13
RP11-365N19	14q32.32
RP11-13O24	15q11.2
CTD-2033D15	15q14
RP11-64K12	15q15.1
RP11-380D11	15q15.1
RP11-151N17	15q15.3
RP11-519G16	15q21.1
RP11-154J22	15q21.1
RP11-105D1	15q21.2
RP11-232J12	15q21.3
RP11-266O8	15q26.1
RP11-299G20	15q26.3
RP11-152P23	16p13.2
RP11-626G11	16p12.3
RP11-489A11	16p12.2
RP11-368N21	16p11.2
RP11-105C20	16q21
RP11-296I10	16q22.1
RP11-94L15	17q12
RP11-411B10	18p11.21
RP11-411B10	18p11.21
RP11-58G13	18q12.2
RP11-71F23	18q21.1
RP11-520K18	18q21.32
RP11-45A1	18q22.3
RP11-315M18	18q23
CTD-3113P16	19p13.3
RP11-268O21	19p13.3
CTB-1025J19	19p13.2
RP11-413M10	19q13.11
RP5-852M4	20p13
RP4-741H3	20p13
RP4-600E6	20q12
RP4-796I11	20q12
RP1-126N20	21q11.2
RP11-304D2	21q21.1
RP11-15H23	21q21.2
RP11-15H6	21q21.3
RP1-245P17	21q22.11
RP1-255P7	21q22.12
RP11-98O13	21q22.13
RP5-1031P17	21q22.2
RP1-265B9	21q22.3
RP11-397E9	21q22.3
XX-91c	22q11.21
CTA-125H2	22q12.1
CTA-57G9	22q12.1
CTA-415G2	22q12.3
CTA-221H1	22q12.3

CTA-397C4	22q13.31
RP11-445O16	23p11.1
RP13-130F17	23q12
RP4-583H20	23q13.1
RP11-236O12	23q13.3
RP3-394H4	23q23
RP3-394F12	23q25
RP3-428A13	23q25

Fig. 2

Fig. 3

Specifications of REMARK recommendations

INTRODUCTION	
Marker examined	A previously reported Comparative Genomic Hybridization (CGH) classifier translated to an array platform (Supplementary Table 1) based on the characteristic copy number aberrations (CNAs) of BRCA1-mutated tumors.
Study Objectives	To investigate whether this classifier was capable of predicting response to high dose platinum-containing alkylating chemotherapy in breast cancer patients .
Hypotheses	Breast cancer cells with homologous recombination DNA-repair deficiency (HRD) have been shown to be hypersensitive to alkylating agents and platinum compounds. We have previously constructed an array Comparative Genomic Hybridization (aCGH) classifier that detects *BRCA1*-mutated tumors which are thought to harbor HRD. We assumed that this classifier would not only identify tumors with a BRCA1-mutation, but also tumors with a wider range of molecular defects in the "BRCA1-pathway". We therefore hypothesized that this BRCA1-classifier would be capable of predicting sensitivity to double strand break inducing agents, such as alkylating agents and the new PARP-inhibitors, in breast cancer patients.

METHODS (I)	Patients
Patient series	To determine whether the BRCA1-classifier was predictive of benefit of high dose alkylating chemotherapy, we studied two patient series. 1) metastatic breast cancer patients who all had received high dose platinum-containing alkylating chemotherapy (MBC series). 2) high risk breast cancer patients in the adjuvant setting who had been randomized to conventional chemotherapy or to high dose alkylating chemotherapy (CTC) (stage-III series).
Characteristics MBC series	Patients were included from three studies carried out in the Netherlands Cancer Institute between 1993 and 2004 [23-25]. From all patients written informed consent was obtained. Treatment protocols of these three studies resembled each other closely. Inclusion criteria for these studies were: biopsy proven stage IV breast cancer, age <55 years, performance status: Zubrod Scale 0-1 and either hormone receptor negative tumors or, if positive, failure of at least one adequate hormonal therapy. In 2003, the inclusion criteria were narrowed by 3 new criteria: 1) patients with HER2-negative tumors; 2) presence of 'oligometastatic disease' (defined as patients in whom all detectable tumor localizations could either be resected or irradiated with curative intent); 3) no involvement of the central nervous system. (one patient in our current study was included after 2003).
inclusion criteria MBC series	(Supplementary Figure 1) availability of formalin-fixed paraffin-embedded (FFPE) primary tumor tissue containing more than 60% of tumor cells. Patients had to have received at least one course of platinum-containing therapy.
exclusion criteria MBC series	(Supplementary Figure 1) Failure to receive at least one course of platinum-based chemotherapy (i.e. progressive disease on the first chemotherapy regimen consisting of fluorouracil, epirubicin and cyclophosphamide ($FE_{120}C$)); treatment-related death; contralateral breast cancer; stage IIIc breast cancer and unavailability or ineligibility of primary tumor tissue.
Treatments MBC series	Patients were treated as previously described [23-25]. In short, treatment was started with 5-fluorouracil 500mg/m2 ($FE_{120}C$), epirubicin 120mg/m2, cyclophosphamide 500mg/m2. The second course of $FE_{120}C$ was used for stem cell mobilization. One patient in our study received 6 courses of adriamycine-vincristine, methotrexate-5-fluorouracil instead of $FE_{120}C$. When patients responded to $FE_{120}C$, including stable disease, the alkylating regimen consisting of a total dose of 1060 mg/m2 carboplatin, 320 mg/m2 thiotepa and 4000mg/m2 cyclophosphamide (CTC) was administered. The majority of patients received three courses of this so-called 'tiny' CTC regimen. Resection or irradiation of residual disease was performed whenever necessary and possible after the last course. In our current study five patients were included from the second study and additionally received reinfusion of autologous lymphocytes with GM-CSF after CTC and one patient received oral paclitaxel as part of the third study. Patients attended the hospital every four to eight weeks.
Characteristics Stage-III series	Patients were included from a large multicentre randomized controlled trial performed in the Netherlands between 1993 and 1999, in which patients were randomized to conventional chemotherapy of that time or to high dose alkylating chemotherapy (CTC) with autologous stem cell support [27]. For this trial patients had to have at least four axillary lymph nodes with metastases but no distant metastases (stage III disease); Eastern Cooperative Oncology Group–Zubrod performance status:0-1; Chemotherapy had to begin within six weeks after the last surgery; No other cancers were allowed except adequately treated in situ carcinoma of the cervix or basal-cell carcinoma of the skin.
inclusion criteria Stage-III series	Since BRCA1-like tumors were highly associated with ER- and HER2-negativity we selected the patients with an ER-low (≤25% expression) and HER2-negative tumor from the stage-III series (Figure 2). Patients were included when their FFPE primary tumor tissue blocks were available and contained more than 60% of tumor cells.

exclusion criteria Stage-III series	Patients were excluded from analysis if they did not complete the assigned treatment. (Figure 2)
Treatments Stage-III series	Patients had been treated as previously described [27]. In short, conventional chemotherapy consisted of 5-fluorouracil 500mg/m^2, epirubicin 90mg/m^2, cyclophosphamide 500mg/m^2 ($FE_{90}C$) every three weeks for five courses. In the HD chemotherapy group stem cells were mobilized and harvested after the third course of $FE_{90}C$. The high dose alkylating regimen consisted of 6000 mg/m^2 cyclophosphamide, 480 mg/m^2 thiotepa and 1600 mg/m^2 Carboplatin (CTC) and was administered after 4 courses of $FE_{90}C$. Both groups received radiotherapy and, in case of hormone receptor positive breast cancer, tamoxifen after chemotherapy. Of the patients randomized to the high-dose arm five patients received five courses of $FE_{90}C$ instead, one patient received only 3 courses of $FE_{90}C$ and one patient received only 2 courses of $FE_{90}C$. Of the patients randomized to the conventional arm, one patient only received three courses of $FE_{90}C$.

METHODS (II)	Assay
Specimen characteristics	DNA is used from FFPE primary tumor tissue of the patients. This DNA is extracted as published previously [28]. For the reference DNA, DNA was isolated from peripheral blood lymphocytes from six apparently healthy female individuals (extraction protocol, see GEO database under GEO record number: GSE12127). It was pooled and sonicated until its median fragment length was similar to that of tumor DNA. After isolation all samples were stored at 4°C.
Assay methods	DNA was labeled according to the manufacturers' instructions (Kreatech Biotechnology, Amsterdam, http://www.kreatech.com/) and used for aCGH (for the hybridization protocol see GSE12127). Slides were scanned with an Agilent DNA Microarray Scanner BA on the same day. Data processing included signal intensity measurement in ImaGene Software; Computation of the profiles included local background subtraction, Cy5/Cy3 ratio, 2log-transformation and subarray normalisation to its median. Bad morphology or uniformity spots were flagged in ImaGenes. When flagged spots accounted for >5% of all spots, hybridizations were excluded.
quality control procedures	To determine the quality of each CGH profile and to be able to compare experiments, we used a profile-quality score. This score is based on the median multiplied by the standard deviation of the distance between the log2 ratios and the ratios of the underlying ploidy level estimated by CGH-segmentation [29]. The logarithm of the minus square root of the median score had to be higher than 0.85 to pass the quality check. For the quality of hybridization we assessed a statistic based on the variance observed between the triplo-spotted BAC-clones, an empirically defined cut-off (on former CGH analyses experiences) of 1.0 is used in our institute. The second quality control measure was provided by the known HER2-receptor status; nine patients were found to be HER2-receptor positive on immunohistochemistry of which 7 were reconfirmed using CISH. The HER2-receptor gene is located on the aCGH platform. Of all known HER2-positive patients the lowest log2-ratio for this location on the platform was 1.22 (CISH: 6) and the highest 2.56 (CISH>10). This corresponds to respectively 5 to 12 copies of the *HER2*-gene, verifying the precision of the aCGH platform.
Reproducibility assessments	In total six tumor DNA samples were hybridized two times or more; Reproducibility was scored with a Pearson correlation coefficient. The lowest score obtained was a rho of 0.82 (highest 0.99). Furthermore, during the hybridizations reference samples were run against themselves (self - self hybridizations) to ensure that all BAC-clones reported on target and that labeling and hybridization protocol were correct. All 6 self-self hybridizations showed a straight band with log2ratios of the CGH-segmentations of zero.
Scoring protocols	The BRCA1-classifier (Supplementary Table 1) was run on all individual aCGH profiles and used a shrunken centroid algorithm (value of centroid is based on the previously built classifier, see Supplementary Table 1). A BRCA1-probability score was calculated for every aCGH profiles, i.e. patient. We trained the cut-off on the MBC series to obtain the highest positive predictive value for response (determined as a PFS longer than 24 months, the median overall survival of metastatic breast cancer patients). The cut-off was set at higher than or equal to 0.63 (Supplementary Figure 2). The cut-off of the BRCA1-score determined in the MBC series was validated in the stage-III series.
Blinding	Since the classifier was built on an independent dataset, the algorithm was blinded to the study-endpoint. To determine the cut-off the outcome (<24 months or ≥24 months) was not blinded to the researcher. In the validation series the algorithm was already available and the cut-off was set with no prior knowledge of the outcome in RFS.

METHODS (II)	Study design
Case selection	Two patient series were used: 1) patients from three phase-II studies: MBC series 2) patients from a randomized controlled trial: Stage-III series. The translational study presented here was performed retrospectively. No stratification was used in the MBC series. In the stage-III series eligible patients underwent randomization before treatment and were stratified according to age (younger than 50 years of age vs. 50 years or older), menopausal status (premenopausal vs. postmenopausal), the number of lymph-node metastases (4 to 9 nodes or 10 or more) and tumor size pT1, pT2, or pT3). For our translational study no stratification was used.

Fig. 3

Time period of cases	Patients from the MBC series were enrolled from 1993 to 2004; Patients from the stage-III series were enrolled between 1993 and 1999. For the MBC series no patients were lost to follow-up; the median follow up (i.e. overall survival) was 25.4 months. For the stage-III series the median follow-up for patients without a recurrence was respectively 8.5 and 9.8 years, for the conventional and high dose chemotherapy arm.
Clinical endpoints	For the MBC series: complete remission after CTC-treatment, defined as disappearance of all evaluable tumor mass assessed by physical examination and imaging studies; Progression free survival (PFS) was defined as the time from the first CTC-course to the appearance of the first progression of disease (based on clinical signs and symptoms, substantiated with imaging and/or biochemical analyses and/or cytology/histology), death, or end of follow-up, whichever occurred earlier. Patients who did not experience a progression were censored at the end of follow-up. For the stage-III series: recurrence free survival (RFS) was calculated from randomization to the appearance of a loco-regional recurrence, metastasis or to death from any cause. All other events were censored[27]. Overall survival (OS) was defined as the time from start of randomization to death from any cause, or end of follow-up. Patients known to be alive at the time of analysis were censored at their last follow-up visit[27].
Variables examined or considered	MBC-series: BRCA1-classifier, Estrogen-receptor (ER) status, progesterone-receptor (PR) status, HER2-receptor status, Nielsen basal-like breast cancer IHC classifier, differentiation grade, all determined on the primary tumor; Prior chemotherapy, prior radiotherapy; At the start of first CTC-treatment: age, number of metastases, site of metastases (visceral only, bone only and soft tissue only). Number of CTC-courses and local treatment after CTC. Stage-III series: age, type of surgery, tumor classification, number of positive lymph nodes, differentiation grade, ER-, PR-receptor, p53 status, treatment, BRCA1-classifier.

METHODS (III)	Statistical analysis
Statistical methods, variable selection procedures, missing data handling	Differences between groups of interest were tested using Fisher's exact tests and exact Chi-square test for trend. Patients with missing values for a variable were excluded from analyses involving that variable. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. Hazard ratios were calculated using Cox proportional hazards regression. Because of the small sample size, we could not fit a fully adjusted multivariate model for the MBC series. Alternatively, we added known prognostic factors and potential confounders defined as variables with an univariate significant analysis ($p<0.05$) or with an HR<·5 or >2, and added one potential confounder at a time to a model including the factor of interest, i.e., BRCA1-profile. To differentiate between a prognostic and a predictive marker in the stage-III series, we assessed whether the effect of high dose chemotherapy on RFS, expressed as hazard ratio (HR), differed by BRCA1-profile status based on multivariate proportional hazards regression with an interaction term, adjusting for potential confounders. For this, an per protocol analysis was done. All calculations were performed using the statistical package SPSS 15.0 and SAS 9.1 (for Windows, respectively SAS Institute Inc.,Cary, NC, USA).
Handling of marker value in analysis	Whereas the cut-off for the BRCA1-probability score was previously set on 0.8 (above the 95% reference interval) to identify patients with a BRCA1-mutation, we trained the cut-off on the MBC series to obtain the highest positive predictive value for response (determined as a PFS longer than 24 months, the median overall survival of metastatic breast cancer patients). The cut-off of the BRCA1-score determined in the MBC series was validated in the stage-III series.
Assessment additional valued of marker	To empirically quantify the benefit from treating patients with a BRCA1-like tumor with HD-chemotherapy and patients with a Sporadic-like tumor with conventional chemotherapy, we created a variable with 3 categories: 1) patients treated with conventional chemotherapy (current clinical practice), 2) patients with BRCA1-like tumors treated with HD-chemotherapy and patients with Sporadic-like tumors who were treated with conventional chemotherapy (clinical practice after implementation of our marker), 3) patients with Sporadic-like tumors who were treated with HD-chemotherapy. Patients with Sporadic-like tumors treated with conventional chemotherapy are in categories 1 and 2 and therefore the dataset was augmented by duplicating those patients. The multivariate HR for category 2 versus category 1 showed the relative survival benefit of selecting patients with a BRCA1-like tumor for HD-chemotherapy and treating patients with a Sporadic-like tumor with conventional chemotherapy versus treating all patients with conventional chemotherapy. The standard error of this HR was calculated using 2000 bootstrap samples.

RESULTS (I)	Data
Flow of patients	See Supplementary Figure 1 and Figure 2. See Supplementarty Table 7 for characteristics of the non-selected patients versus the selected patients of the stage-III series.
Characteristics	See Table 1 and Table 2.

Fig. 3

RESULTS (II)	Analysis and presentation
Relation of marker to standard prognostic variables	See Table 1 and Table 2: patient characteristics for the relation of the BRCA1-classifier to standard prognostic variables.
Univariate analyses	See Supplementary Table 3 and Supplementary Table 7 for the univariate analyses of all candidate variables listed under "Methods Study design".
Effect of marker on time-to-event outcome (Kaplan – Meier plot)	See Figure 1 and Figure 3.
Multivariable analyses	See Supplementary Table 4 and Table 3.
Additional analyses	An intriguing question is what the impact of introducing the BRCA1-classifier into clinical practice for ER-, HER2-negative stage-III breast cancer patients would be (marker-based strategy[46]). Comparing RFS of patients treated according to the BRCA1-classifier (i.e. patients with a BRCA1-like tumor: HD-chemotherapy, others: conventional chemotherapy) with RFS of patients all treated with conventional chemotherapy (substitute of current clinical practice) resulted in a multivariate median HR of 0.47 (95% CI 0.23-0.91). Consequently, recurrence rates for this particular type of breast cancer could possibly be halved by tailoring treatment according to the BRCA1-classifier

DISCUSSION	
Interpretation, limitations and implication	See Discussion section in manuscript.

Fig. 3

Fig. 4 Mutation Analysis: mutations tested.

BRCA1 mutations analyzed*	*BRCA2* mutations analyzed*
IVS21-36del510†	6503delTT
2804delAA	8289 T>A (C2689X)
5382insC	7647delTG
2312del5	9900insA
185delAG	5441del4
1411insT	6174delT
185insA	5873 C>A (S1882X)
2138delA	1538del4
3867 G>T (E1250X)	5579insA
2457 C>T (Q780X)	2041insA
3109insAA	2127insTT
3604delA	3034del4
4446 C>T (R1443X)	4677delA
5622 C>T (R1835X)	5804del4
2841 G>T (E908X)	3036del4
1406insA	6872del4
3938insG	5823delAT
2080delA	4761delA
3875del4	5805del4
2316del5	5909insA
3600del11	2001del4
3889delAG	2024del5
1438delT	3040del4
2329delC	4637del5
3939insG	4682del4
2355insG	4684del4
2809insA	4705del4
2845delA	4708insA
1438insT	4763delG
2057del8	4767ins4
2080insA	4783delC
2331del4	5466insT
2845insA	5578delAA
2846del4	6498delTA
2883del4	6505insC
3940insG	6508del7
5389del7	6819delTG
	6860del4
	6862delTGTT
	9927del4
Total: 64.2%‡	Total: 50.8%‡

* In short, frame shift and splice site mutations were detected by the Detection of Small Deletions and Insertions (DSDI) method while substitutions were detected by the Allelic Discrimination (AD) assay. We used tumor DNA isolated initially for aCGH analysis for mutation analysis. For seven samples extra DNA was isolated to complete the analysis, four of which did not contain any tumor cells and was used in the AD assay only. In total 21 genomic *BRCA1* and *BRCA2* fragments were amplified with fluorescent-labeled primers in nine multiplex PCR reactions for the DSDI-assay. One *BRCA1* fragment only amplified when the large genomic deletion around exon 22 was present [31]. PCR products were pooled, run in one lane on an ABI PRISM 3730 DNA analyzer and analyzed with Genemapper 4.0 (Applied Biosystems, Foster City, CA, U.S.A.). For AD, 5 *BRCA1* and 2 *BRCA2* fragments were amplified with primers and 'Minor Groove Binding' probes (Applied Biosystems, Warrington Cheshire, UK) and run on the ABI PRISM 7500 Fast Real-Time PCR system. Direct sequencing of suspect mutations identified by DSDI or AD was performed on the ABI PRISM 3730 DNA analyzer.

† Hogervorst F. on behalf of the Netherlands DNA diagnostic laboratories, personal communication. ‡ Percentage accounting for number of families with these specific germline mutations in relation to pathogenic germline *BRCA1* and *BRCA2* mutations of all known families.

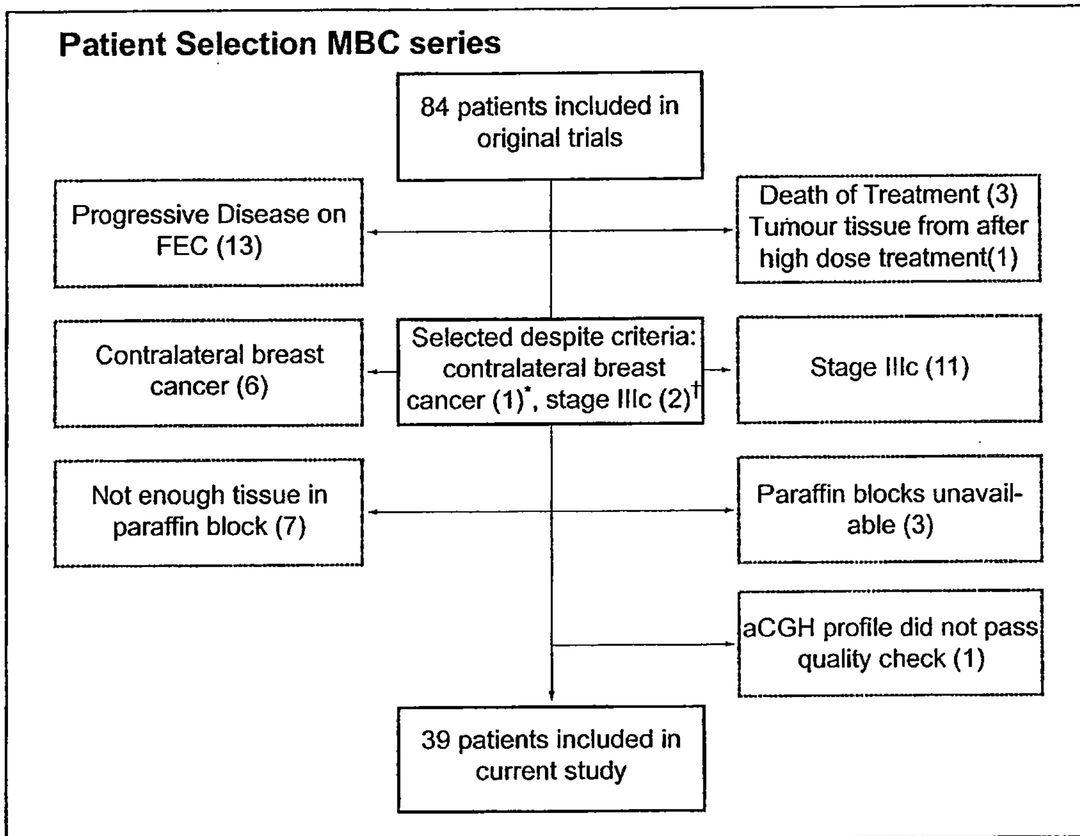


Fig. 5

Fig. 6

Univariate Cox proportional-hazard regression analysis of the risk of tumor progression (PFS) after HD-chemotherapy treatment in the MBC-series

Variable	Hazard Ratio	95% CI	p-value
aCGH classifier			
Sporadic-like tumor	1.00		
BRCA1-like tumor	0.31	0.14 - 0.66	0.002
Estrogen Receptor status†			
Negative	1.00		
Positive	1.05	0.52 - 2.13	0.885
HER2-receptor status†			
Negative	1.00		
Positive	1.74	0.80 - 3.79	0.163
Nielsen basal-like breast cancer definition†			
Negative	1.00		
Positive	0.49	0.21 - 1.14	0.099
Histological grade†			
1 (good) / 2 (intermediate)	1.00		
3 (poor)	0.98	0.47 - 2.03	0.957
Prior Chemotherapy‡			
No	1.00		
Yes	2.39	1.14 - 4.98	0.020
Prior Radiotherapy			
No	1.00		
Yes	2.81	1.13 - 7.00	0.026
Age at CTC*			
≤ 40 years	1.00		
> 40years	1.15	0.56 - 2.38	0.709
Metastatic disease*			
≤ 2 sites of metastases	1.00		
> 2 sites of metastases	2.14	1.04 - 4.37	0.038
Site of metastases*			
Other	1.00		
Soft tissue metastases only§	0.46	0.22 - 0.96	0.037
Site of metastases*			
Other	1.00		
Visceral metastases only	1.11	0.26 - 4.73	0.887
Site of metastases*			
Other	1.00		
Bone metastases only	1.73	0.51 - 5.80	0.377
Number of CTC courses			
< 3 courses	1.00		
3 courses	0.36	0.18 - 0.75	0.006
Local Treatment after CTC			
No	1.00		
Yes	0.28	0.13 - 0.58	0.001

* at start first CTC treatment.

† Of primary tumor, except for two patients of whom only the lymph node metastasis tissue of the primary tumor was available.

‡ Prior chemotherapy, in all cases consisted of cyclophosphamide, methotrexate and fluoruracil (CMF) in the adjuvant setting, except one case who received five courses of adjuvant $FE_{90}C$.

§ Soft tissue metastases consisted of locoregional disease, lymph node metastasis and skin metastasis.

Abbreviations: CI, confidence interval; CTC, carboplatin-thiotepa-cyclophosphamide.

Fig. 7

Multivariate Cox proportional-hazard regression analysis of the risk of tumor progression (PFS) after HD-chemotherapy treatment in the MBC-series

Variable	No. Events	Hazard Ratio	95% CI	p-value
Estrogen Receptor Status[†]				
Negative	20	1.00	—	
Positive	13	0.36	0.15 - 0.85	0.021
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.16	0.06 - 0.41	<0.001
HER2 receptor status[†]				
Negative	24	1.00	—	
Positive	9	1.06	0.47 - 2.41	0.887
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.31	0.14 - 0.70	0.005
Nielsen basal-like breast cancer definition[†]				
Negative	26	1.00	—	
Positive	7	1.38	0.38 - 5.02	0.627
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.25	0.08 - 0.80	0.020
Metastatic disease*				
≤ 2 sites of metastases	17	1.00	—	
> 2 sites of metastases	16	2.09	1.01 - 4.33	0.048
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.31	0.14 - 0.67	0.003
Site of metastases*				
Other	21	1.00	—	
Soft tissue metastases only[§]	12	0.57	0.27 - 1.19	0.135
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.35	0.16 - 0.75	0.007

Variable	No. Events	Hazard Ratio	95% CI	p-value
Prior Radiotherapy				
No	6	1.00	—	
Yes	27	2.54	1.01 - 6.40	0.048
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.33	0.15 - 0.71	0.005
Prior Chemotherapy[‡]				
No	21	1.00	—	
Yes	12	1.49	0.68 - 3.29	0.321
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.36	0.16 - 0.82	0.015
Number of CTC courses				
< 3 courses	12	1.00	—	
3 courses	21	0.49	0.23 - 1.03	0.060
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.37	0.17 - 0.81	0.013
Local treatment after CTC				
No	18	1.00	—	
Yes	15	0.39	0.18 - 0.86	0.020
aCGH classifier				
Sporadic-like tumor	23	1.00	—	
BRCA1-like tumor	10	0.44	0.19 - 0.99	0.048

* at start first CTC treatment. † Of primary tumor, except for two patients of whom only the lymph node metastasis tissue of the primary tumor was available. ‡ Prior chemotherapy, in all cases consisted of cyclophosphamide, methotrexate and fluoruracil (CMF) in the adjuvant setting , except one case who received five courses of adjuvant $FE_{90}C$. § Soft tissue metastases consisted of locoregional disease, lymph node metastasis and skin metastasis. *Abbreviations*: CI, confidence interval; CTC, carboplatin-thiotepa-cyclophosphamide.

Fig. 8 Types of mutation present in MBC-series*

Patient	BRCA	Mutation	aCGH-profile	Estrogen receptor	PFS (months)	
4†	*BRCA2*	5441del4	BRCA1-like	positive	60.2‡	(censored)
5†	*BRCA1*	36del510	BRCA1-like	negative	36.5‡	(progression)
8†	*BRCA1*	185delAG	BRCA1-like	negative	134.6‡	(censored)
9	*BRCA2*	9900insA	sporadic-profile	negative	9.6	(progression)

* Of one patient with a Sporadic-like tumor all DNA had been used for aCGH analysis and no mutation analyses could be performed. In two BRCA1-like tumors only ~53% of the type of *BRCA1*-mutations and ~42% of the type of *BRCA2*-mutations prevalent in The Netherlands were determined due to technical difficulties. Similarly, of three Sporadic-like tumors ~58% of the type of *BRCA1*-mutations and ~36% of the type of *BRCA2*-mutations prevalent in The Netherlands could be tested instead of the intended ~64% and ~51% for respectively *BRCA1* and *BRCA2*.

† In retrospect three patients had already been tested by our familial cancer clinic and were known mutation carriers.

‡ Three of these four mutation carriers had a long PFS (>30 months) after platinum-containing chemotherapy. Interestingly, the one mutation carrier with a short PFS (9.6 months) had a mutation in the last exon (exon 27) of *BRCA2*. Deletion of this exon has been studied in a mouse model in which 67% of the mice were viable opposed to total embryonic lethality in the complete knock out [34]. This suggests partial functionality (hypomorphic allele) of the *BRCA2* protein, which might explain the unresponsiveness of this patient to platinum-based chemotherapy'

Abbreviations: aCGH: array Comparative Genomic Hybridization; PFS: progression free survival.

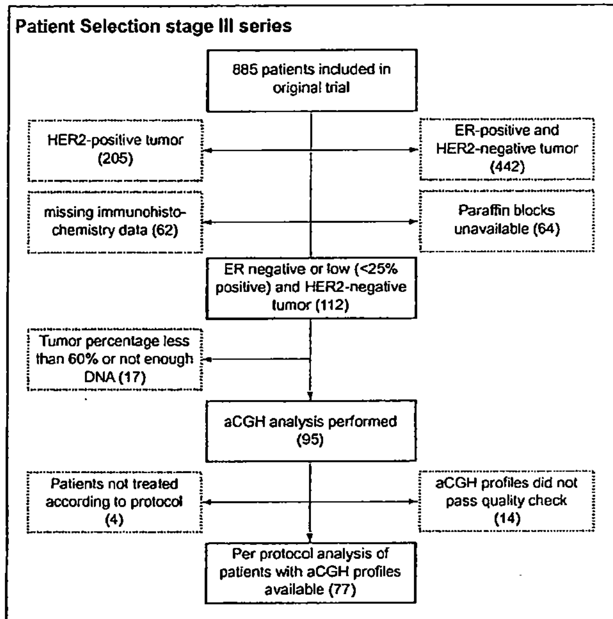


Fig. 9

Fig. 10

Distribution of clinicopathological variables between HER2-negative and ER-low patients analyzed with aCGH and HER2-negative and ER-low patients not currently in the analysis from the stage-III series

Variable	Total		In analysis with CGH classifier		not in current analysis		p-value
	n	(%)	n	(%)	n	(%)	
Total	176	100	81	46	95	54	
Treatment							
Conventional chemotherapy	88	50	42	51.8	46	48.4	0.549*
High Dose chemotherapy	79	44.9	35	43.2	44	51.1	
Not treated according to protocol	9	5.1	4	4.9	5	5.3	
Age in categories							
≤ 35 years	30	17	15	18.5	15	15.8	
35 - 40 years	35	19.9	16	19.8	19	20.0	
41 - 45 years	40	22.7	12	14.8	28	29.5	0.416†
46 - 50 years	48	27.3	23	28.4	25	26.3	
> 50 years	23	13.1	15	18.5	8	8.4	
Type of surgery							
Breast conserving therapy	45	25.6	21	25.9	24	25.3	1.000*
Mastectomy	131	74.4	60	74.1	71	74.7	
Tumor classification							
T1	44	25	20	24.7	24	25.3	
T2	106	60.2	46	56.8	60	63.1	0.324†
T3	24	13.6	15	18.5	9	9.5	
Unknown	2	1.1	0	0.0	2	2.1	
No. of positive lymph nodes							
4-9	112	63.4	51	63.0	61	64.2	0.876*
≥ 10	64	36.4	30	37.0	34	35.8	
Histologic grade							
I	13	7.4	4	5.0	9	9.5	
II	36	20.5	18	22.2	18	19.0	0.710†
III	118	67	53	65.4	65	68.4	
Not determined	9	5.1	6	7.4	3	3.1	
Estrogen receptor status							
0 % positive cells	146	83	68	84.0	78	82.1	
10 % positive cells	14	8	7	8.6	7	7.4	0.505†
20 % positive cells	2	1.1	2	2.5	0	0.0	
25 % positive cells	14	8	4	4.9	10	10.5	
Progesterone receptor status							
Negative (<10%)	153	86.9	73	90.1	80	84.2	0.488*
Positive (≥10%)	21	11	8	9.9	13	13.7	
Unknown	2	1.1	0	0	2	2.1	
P53 status							
Negative (<10%)	92	52.3	43	53.1	49	51.6	1.000*
Positive (≥10%)	82	46.6	38	46.9	44	46.3	
Unknown	2	1.1	0	0	2	2.1	

Missing values not included in the anlaysis.

p-value calculated using: * Fisher exact tes, † Exact Chi-square test for Trend.

Fig. 11

Univariate Cox proportional-hazard regression analysis of the risk of Recurrence (RFS) after randomization in the stage-III series

Variable	No. of events	Hazard Ratio	95% CI	p-value
Age				
≤ 40 years	17	1.00		
> 40 years	24	0.82	0.44 - 1.53	0.532
Type of surgery				
Breast conserving therapy	10	1.00		
Mastectomy	31	1.09	0.53 - 2.22	0.821
Tumor classification				
T1 & T2	30	1.00		
T3	11	2.40	1.120- 4.81	0.013
No. of positive lymph nodes				
4-9	22	1.00		
≥ 10	19	1.90	1.03 - 3.52	0.041
Histologic grade				
I & II	11	1.00		
III	28	1.04	0.52 - 2.09	0.911
Estrogen receptor status				
Negative (<10%)	34	1.00		
Positive (≥10%)	7	0.89	0.39 - 2.00	0.771
Progesterone receptor status				
Negative (<10%)	37	1.00		
Positive (≥10%)	4	0.71	0.25 - 1.98	0.507
P53 status				
Negative (<10%)	25	1.00		
Positive (≥10%)	16	0.77	0.41 - 1.44	0.408
Treatment				
Conventional Chemotherapy	29	1.00		
High Dose Chemotherapy	12	0.39	0.20 - 0.76	0.006
aCGH classifier				
Sporadic-like tumor	22	1.00		
BRCA1-like tumor	19	0.95	0.51 - 1.75	0.859

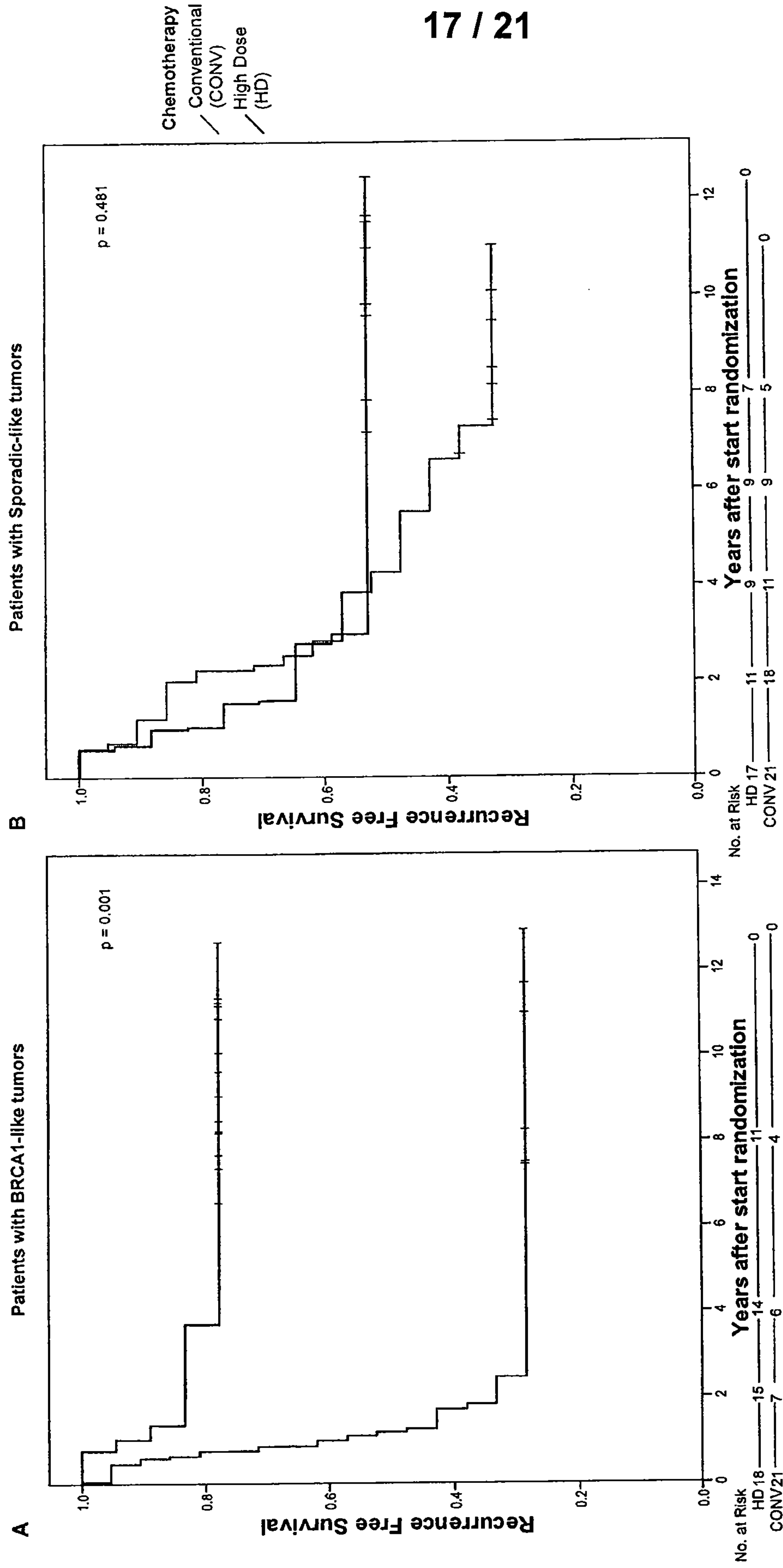
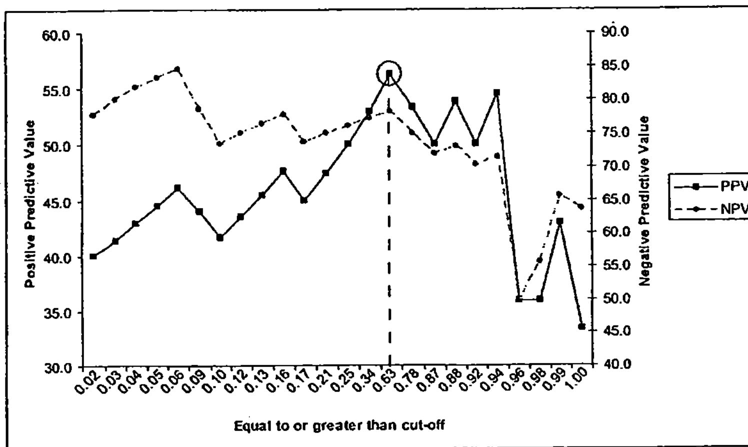


Fig. 12

A.



B.

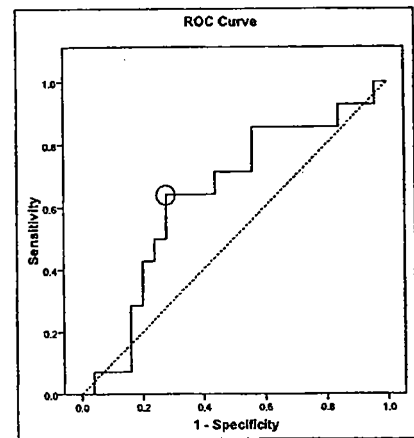


Fig. 13

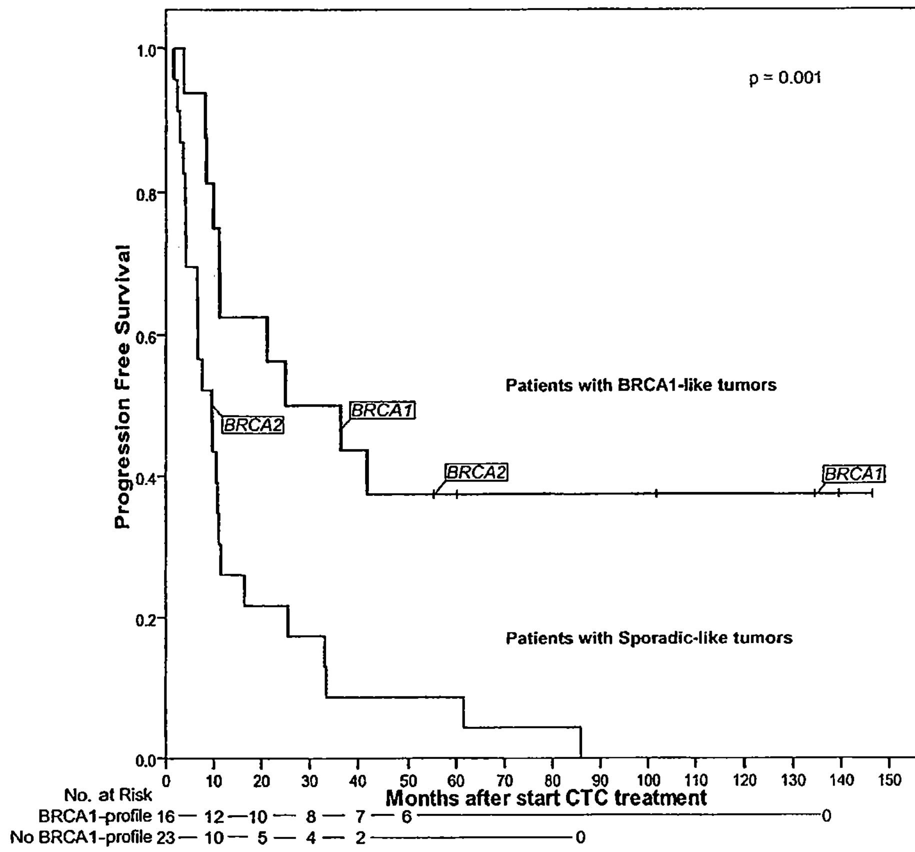


Fig. 14

BRCA1 gene expression (RT-PCR)

1.5
1.0
0.5
cut-off
0.0

p<0.001

Unmethylated Methylated

Fig. 15

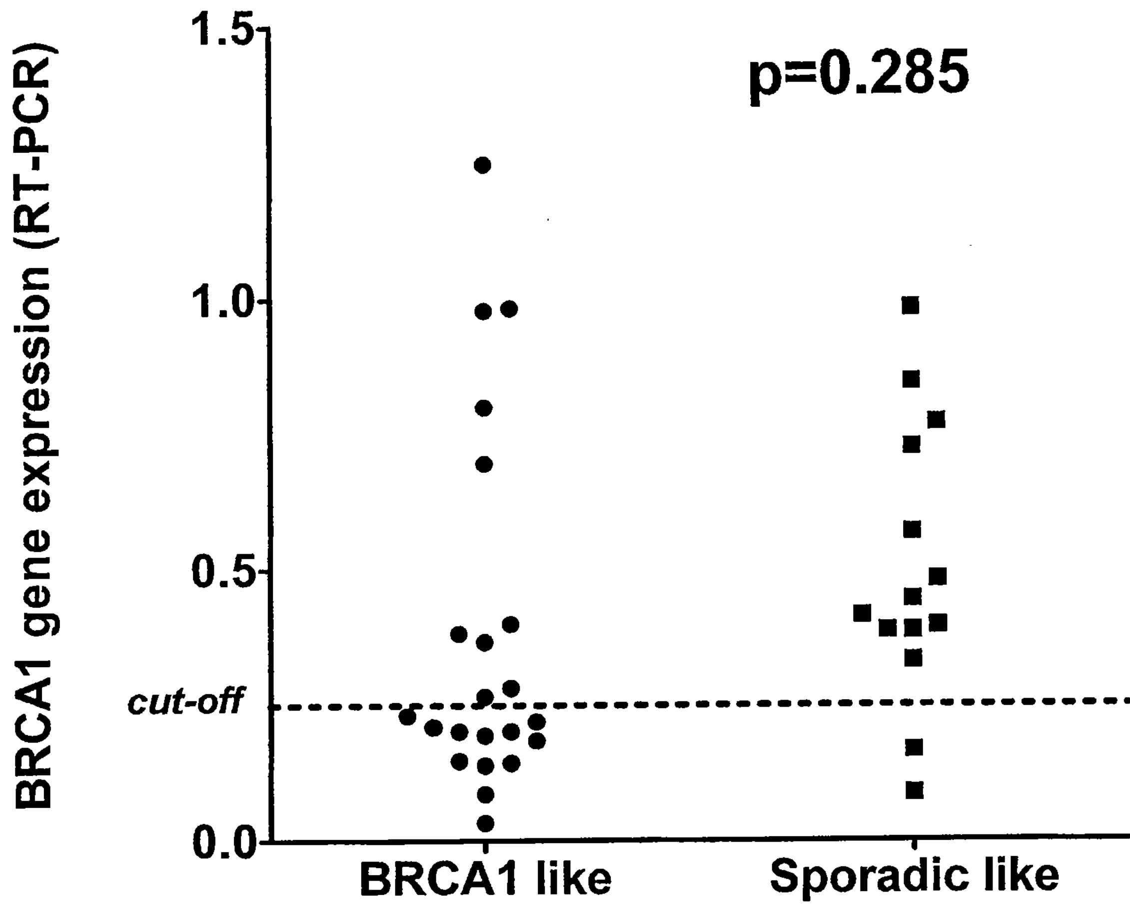


Fig. 16