Breast cancers with a \textit{BRCA1}-like DNA copy number profile recur less often than expected after high-dose alkylating chemotherapy

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\textbf{Running title}
\textit{BRCA1}-like profile predicts high dose alkylating chemotherapy

\textbf{Keywords}
Predictive biomarker, \textit{BRCA1}, alkylating chemotherapy, breast cancer

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Statement of translational Relevance
The identification of biomarker-treatment combinations that enable selection of patients is of importance to tailor treatment to the patient’s tumor characteristics. The study investigated the combination of BRCA1-like signature with DNA double strand break inducing chemotherapy, which in preclinical work has been identified as an effective biomarker-treatment combination. The findings can be used separately or
combined: \textit{BRCA1}-like signature selected patients with a targetable deficiency in homologous recombination DNA repair due to BRCA1 inactivation, so it can be tested to predict benefit of other drugs targeting this defect. High dose double strand break inducing chemotherapy may be particularly suited for patients with a BRCA1 deficiency so this treatment could be tested with other markers that identify such a defect. The combination of \textit{BRCA1}-like signature and DNA double strand break inducing chemotherapy is particularly translatable because preclinical and clinical observations replicated, the drugs are approved and clinical experience is present.

\textbf{Abstract}

\textit{Purpose}

Breast cancers in carriers of inactivating mutations of the \textit{BRCA1} gene carry a specific DNA copy number signature (‘\textit{BRCA1}-like’). This signature is shared with cancers that inactivate \textit{BRCA1} through other mechanisms. Since \textit{BRCA1} is important in repair of DNA double strand breaks through error-free homologous recombination, patients with a \textit{BRCA1}-like tumor may benefit from high dose alkylating (HD) chemotherapy which induces DNA double strand breaks.

\textit{Experimental Design}

We investigated a single institution cohort of high risk patients that received tandem HD chemotherapy schedule comprising ifosfamide, epirubicin and carboplatin or conventional chemotherapy. We classified copy number profiles to be \textit{BRCA1}-like or
Results:

*BRCA1*-like status associated with high grade and triple negative breast cancers. *BRCA1*-like cases benefitted from the HD compared to a conventional regimen on disease free survival: (DFS, hazard ratio (HR) of 0.05, 95% confidence interval (CI): 0.01-0.38, p=0.003); Distant Disease Free Survival: (DDFS, HR of 0.06, 95%CI: 0.01-0.43, p=0.01); and overall survival (OS, HR of 0.15, 95%CI: 0.03-0.83, p=0.03) after correction for prognostic factors. No such benefit was observed in the non-*BRCA1*-like cases on DFS (HR: 0.74, 95%CI: 0.38-1.46, p = 0.39), DDFS (HR=0.79, 95%CI =0.41-1.52, p=0.47) and OS (HR: 0.93, 95%CI: 0.52-1.64, p=0.79). The p-values for interaction were 0.01 (DFS), 0.01 (DDFS) and 0.045 (OS).

Conclusions:

*BRCA1*-like tumors recurred significantly less often after HD than conventional chemotherapy. *BRCA1*-like copy number profile classification may be a predictive marker for HD alkylating chemotherapy.

Keywords

Predictive biomarker, *BRCA1*, alkylating chemotherapy, breast cancer

Introduction
Inactivating germline mutations in the *BRCA1* gene confer a high risk of developing breast cancer. Although these germline mutations are shown to be rare in the general population (1,2), it is thought that a considerable subgroup of breast cancers has characteristics similar to *BRCA1* germline mutated breast cancer but without harboring a *BRCA1* mutation, so called BRCAness (3). *BRCA1* is important in error-free DNA double strand break (DSB) repair through the homologous recombination pathway (4,5). In the absence of functional homologous recombination, DNA repair is mostly performed by error-prone non-homologous end-joining (NHEJ). This in turn leads to genomic instability (6,7). Genomic instability can be measured with DNA copy number profiles. It has been established that DNA copy number (CN) profiles of breast cancers that developed in *BRCA1* mutation carriers have a specific signature (8,9). To detect these tumors classifiers have been trained that estimate the probability that a copy number profile is similar to a *BRCA1* mutated breast cancer (‘*BRCA1*-like’) (10). In addition this classification can also identify tumors that have a defect in *BRCA1* due to other causes than mutation, for example hypermethylation of the gene promoter(11,12). This means that the biomarker (*BRCA1*-like signature) captures aspects of the broader concept BRCAness. Preclinical mechanistic studies indicate that deficiencies in this pathway strongly sensitize tumors to drugs inducing DNA damage through DNA double strand breaks, such as platinum compounds and bifunctional alkylators (3,13–16). In general, these drugs are currently not standard therapy in breast cancer. High dose regimens contain such agents. However, in the general population these regimens did not confer a sufficient large survival advantage in the adjuvant or metastatic setting that could
justify its toxicity profile. In addition, no known prognostic indicators could identify a subgroup of patients that benefitted substantially from this therapy. Young patients, however seemed to have a better recurrence free survival than older patients (17,18). We therefore investigated in a randomized clinical trial whether patients that classified as BRCA1-like based on their DNA CN profiles, form a subgroup of patients that benefit substantially from DSB inducing high dose (HD) chemotherapy (19). The HD regimen in these previous studies contained 4 cycles of 5-fluorouracil, epirubicin and cyclophosphamide (FEC: 500/90/500 mg/m$^2$) followed by 1 cycle of carboplatin (1600 mg/m$^2$), thiotepa (480 mg/m$^2$) and cyclophosphamide (6g/m$^2$). Patients in the control arm received 5 cycles of FEC (500/90/500 mg/m$^2$) only. We found that patients with a BRCA1-like profile had an approximately 6-fold lower hazard for an event for DFS, RFS and OS (20,21)

In this study we investigated in an independent single institution cohort whether BRCA1-like patients also benefitted from a tandem HD regimen (22).

Methods

Patients

The patients in this study consist of a cohort of patients that has been treated with adjuvant HD therapy or conventional chemotherapy. High risk breast cancer patients in the HD cohort were enrolled in a single-arm study that was conducted between 1992 and 1997 that evaluated safety and efficacy of the regimen. High risk was defined as...
stage II or III primary breast cancer with more than 10 tumor positive lymph nodes, premenopausal patients with 3-9 tumor-positive lymph nodes, or hormone receptor negative disease. Conventionally treated patients from the same time period were identified by the cancer registry of the University of Heidelberg Gynecology and Obstetrics department. Conventionally treated patients were matched for nodal ratio, tumor size and hormone receptor and HER2 status (22). We collected all available archival tissue from the archives of the Institute of Pathology Heidelberg. The HD regimen consisted of 2 cycles induction chemotherapy (total dose: 7500mg/m$^2$ ifosfamide, 120 mg/m$^2$ epirubicin q 3 weeks), followed by 2 cycles of 12 g/m$^2$ ifosfamide, 900 mg/m$^2$ carboplatin and 180 mg/m$^2$ epirubicin q 4 to 6 weeks, with autologous stem cell transplantation. Following chemotherapy, hormone receptor positive premenopausal patients received gosereline as adjuvant endocrine therapy for two years and hormone receptor positive postmenopausal patients received tamoxifen for five years. The conventional regimen consisted of 500mg/m$^2$ cyclophosphamide, 40mg/m$^2$ methotrexate, 600 mg/m$^2$ 5-fluorouracil (CMF) or 60/90 mg/m$^2$ epirubicin, 600 mg/m$^2$ cyclophosphamide (EC) or 600 mg/m$^2$ 5-fluorouracil, 60 mg/m$^2$ epirubicin, 600 mg/m$^2$ cyclophosphamide (FEC) chemotherapy. The Ethical Committee of the University of Heidelberg approved this study. Patients enrolled in the trials supplied written informed consent.

*DNA isolation and array CGH*
We collected archival FFPE tissue and selected tumor blocks that contained a region with 60% tumor cells. Tumor DNA isolation and Array Comparative Genomic Hybridization was performed as previously reported (23). One ug of DNA was labeled using the Enzo Genomic Labeling Kit (Enzo Life Sciences, Farmingdale, NY) according to manufacturer’s instruction. The labeled DNA was then hybridized to a Nimblegen 135k whole genome array containing 134,937 in situ synthesized oligonucleotides (Roche NimbleGen, Madison, WI) according to the manufacturer’s protocol. Slides of the arrays were scanned using a G2505C microarray scanner (Agilent Technologies). Images were analysed using Nimblescan version 2.5.26 feature extraction software (Roche Nimblegen, Madison, USA). Oligonucleotides were mapped according to the human genome build NCBI36.

**BRCA1-like copy number aberration classification**

Raw image files were processed using Nimblescan software and the DNAcopy algorithm. Unaveraged DNAcopy files were used as input for the BRCA1-like classifier as previously described (i.e. no training was performed, just application as described) (24). The BRCA1-like classifier is a nearest shrunken centroids classifier (25) that was trained to distinguish copy number profiles of BRCA1-mutated tumors from tumors without a BRCA1 mutation. Visualization of characteristic regions of copy number alteration (10), and the actual locations and centroids of the classifier have been described before (20). This classifier assigns a class label to a copy number profile, either BRCA1-like or non-BRCA1-like, based on the posterior probability of being non-BRCA1-like (probability: 0).
or being BRCA1-like (probability: 1). The cutoff for optimal chemotherapy prediction was 0.63, as determined on a training cohort (metastatic breast cancer series), and validated on a randomized trial of high dose chemotherapy vs. conventional chemotherapy (20). We used these settings to investigate the current cohort. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (26) and are accessible through GEO Series accession number GSE50407 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50407).

Statistics

Calculations were performed in R v. 3.0.1 (27). Categorical clinical characteristics were compared with Fisher exact (2x2 tables) or Chi-square tests (larger than 2x2 tables). Survival was evaluated according to BRCA1-like status by Kaplan-Meier curves and log rank statistics. Hazard ratios were estimated with a Cox Proportional Hazards model adjusting for relevant prognostic factors. To assess whether BRCA1-like status is a predictive marker a test for interaction with chemotherapy regimen was performed (28). A description of this study according to the REporting recommendations for tumor MARKer prognostic studies (REMARK) guidelines is provided in supplemental table 1. Survival was calculated from start date of chemotherapy until date of event or date of last follow-up. Events were defined as any relapse or death (DFS), any relapse other than in the ipsi-lateral breast, chest wall, axillary or supraclavicular lymph nodes or death (DDFS) or death (OS). We assessed both DFS and DDFS to investigate whether a
treatment benefit would be due to decreased systemic disease events, ie. the adjuvant treatment is successful in its aim to kill occult micro-metastases.

Results

We collected the archival material for 82 % of the patients that were treated at the University of Heidelberg. A flow diagram of patients included is shown in Figure 1.

We generated copy number profiles for 117 of these patients. The clinical characteristics for these patients are shown in Table 1. The treatment cohorts were not significantly different, except that patients treated with HD chemotherapy were more likely to have higher lymph node stage (non-BRCA1-like p<0.001; BRCA1-like p=0.05). BRCA1-like tumors have a higher N stage (p=0.01), higher grade (p=0.03), and are more often ER negative (p=0.01) and triple negative (p<0.001). A trend for fewer events in the conventionally treated non-BRCA1-like patients compared to high dose treated non-BRCA1-like patients is present for DFS (p=0.05) and DDFS (p=0.07). Concluding, BRCA1-like patients treated with HD chemotherapy have more unfavorable prognostic factors than the other patients in this study.

The median follow-up for DFS and DDFS were respectively 2.4 and 2.5 years. The median follow-up for OS was 8.5 years. Kaplan-Meier curves for non-BRCA1-like patients and
BRCA1-like patients comparing the outcome of conventional and HD chemotherapy on Disease Free Survival (DFS), Distant Disease Free Survival (DDFS) and Overall Survival (OS) are shown in Fig 2.

When adjusted for the potential confounding factors estrogen receptor status, HER2 status, size, number of tumor positive lymph nodes and grade, BRCA1-like patients had a better DFS (HR: 0.05, 95%CI: 0.01-0.38, p=0.003); DDFS (HR: 0.06, 95%CI: 0.01-0.43, p=0.01) and OS (HR: 0.15, 95% CI: 0.03-0.83, p=0.03) with adjuvant HD chemotherapy than with conventional chemotherapy. No such benefit was observed in the non-BRCA1-like cases for DFS (HR: 0.74, 95%CI: 0.38-1.46, p = 0.39), DDFS (HR: 0.79, 95%CI: 0.41-1.52, p=0.47) and OS (HR: 0.93, 95%CI: 0.52-1.64, p=0.79), The p-values for interaction were 0.01 (DFS), 0.01 (DDFS) and 0.045 (OS) (Table 2). No evidence against proportionality of treatment-specific hazards was observed among BRCA1-like and non-BRCA1-like patients.
Discussion

We investigated a single institution cohort treated with high dose and conventional chemotherapy to assess whether BRCA1-like patients benefit from a tandem HD alkylating chemotherapy regimen (20). Patients with a BRCA1-like breast cancer significantly recurred less after the tandem HD alkylating chemotherapy regimen. BRCA1-like status may therefore serve as a predictive marker for tandem HD chemotherapy with iphosphamide, epirubicin and carboplatin.

High dose alkylating regimens in breast cancer are not used anymore after it has been shown that this regimen does not confer a survival benefit (17,18). However, these regimens contain potent inducers of DNA double strand breaks which are predicted beneficial in patients with tumors that do not have a functional homologous recombination, due to a defect in BRCA1 (4,14–16,29). To identify these patients we used a BRCA1-like classifier which assigns patients that share specific DNA copy number aberrations with BRCA1-mutated tumors to the BRCA1-like class (8–10). The tumors assigned to be BRCA1-like comprise BRCA1 mutated and BRCA1 wildtype tumors that have another mechanism to inactivate BRCA1 function, for example BRCA1 promoter hypermethylation (11,12,20). Previously we investigated this classifier in patients that were randomized to receive either 5 cycles of conventional adjuvant 5-FU, epirubicin and cyclophosphamide (FEC) or 4 cycles of conventional FEC followed by 1 cycle of the
HD regimen carboplatin (1600 mg/m2), thiothepa (480 mg/m2) and cyclophosphamide (6 g/m2) (19). In this study, patients with a BRCA1-like copy number profile in their tumor derived a substantial benefit from another HD regimen compared to conventional chemotherapy. Patients with a non-BRCA1-like tumor that received HD chemotherapy did not benefit at all compared to conventional FEC. Although the HD regimen in the current study (2 cycles induction + 2 cycles of 12 g/m2 ifosfamide, 900 mg/m2 carboplatin and 180 mg/m2 epirubicin) is different from our previous study, we obtained similar results. Our data suggests that patients with a BRCA1-like tumor benefit from an HD alkylating regimen, while patients with a non-BRCA1-like tumor do not. Given the differences in drugs and doses, it may be that BRCA1-like tumors, generally are more sensitive to the dose or DNA crosslinking properties, rather than a specific agent.

Our present study is limited by several factors, which are shared by other investigations of BRCA1 deficiency as a potential biomarker. In clinical studies there is to date neither agreement on different biomarkers that can be used to identify BRCA1 defects, nor any randomized evidence to demonstrate that selection of patients with a BRCA1 defect for a particular therapy results in improved treatment outcomes as has been reviewed extensively (3,30–32). Clearly our present study is limited by a small sample size and the non-randomized design relying on a balanced patient cohort treated with several chemotherapy combinations during the same time period at the same institution. Strikingly, the patients expected to have a poor prognosis, (BRCA1-like patients
associated with high N stage, high grade and ER negative/triple negative tumors) have a significantly better survival when treated with HD chemotherapy in comparison with conventional chemotherapy, while the non-BRCA1-like patients do not experience that HD treatment benefit.

Regarding the therapy regimens, patients in the control group received conventional chemotherapy consisting of either EC, CMF or EC-CMF as they were treated in the pre-taxane era. This allows for a unique view on biology but limits the conclusions on whether HD chemotherapy is better than a modern taxane-containing regimen. However, it has been suggested that BRCA1 mutated patients are relatively resistant to taxanes (33). Because we do not have an untreated control group it is not possible to accurately model a potential prognostic effect that the BRCA1-like profile status may confer. Secondly, due to the combinations of different drugs, it is impossible to dissect whether the survival benefit could be due to a particular drug. Thirdly, it could also be that the amount of DNA damage is greatly increased due to the higher dose, and that the cumulative dose is responsible for efficient killing of tumor cells.

Further research should therefore aim to overcome the limitations. First, by investigating other randomized trials between high dose and conventional chemotherapy whether BRCA1-like patients benefit from the HD regimen. Single cohorts and especially non-randomized cohorts do not reach the highest level of evidence and findings should therefore be confirmed in independent studies. (34,35). These studies can strengthen the evidence while prospective, randomized evidence is being gathered.
in (neo-) adjuvant and metastatic breast cancer (http://clinicaltrials.gov: NCT01898117, NCT01057069, NCT01646034). Furthermore, identifying the role or contribution of specific agents, dose and biomarker are important to determine the use of this information in the treatment of breast cancer. Specifically, the treatment burden associated with an HD chemotherapy regimen requires proper selection of patients. If possible, less toxic therapy should be investigated, for example an intermediate dose or only the effective drug in this regimen. Furthermore, Poly(ADP)-Ribose Polymerase 1 (PARP1) inhibitors may prove effective with a more beneficial toxicity profile (36–39).

Conclusion

Concluding, in this single institution study we observed that BRCA1-like copy number profiles identify a group of patients that recur less often than expected after receiving HD than after conventional chemotherapy treatment. BRCA1-like copy number profile status may therefore be a predictive biomarker for HD alkylating chemotherapy and could then be used to guide treatment choices to improve overall survival among patients with a poor prognosis.

Abbreviations

DNA Deoxyribonucleic acid

BRCA1 Breast Cancer 1, Early Onset

CN Copy Number

HD High Dose
DFS  Disease Free Survival
DDFS  Distant Disease Free Survival
OS   Overall Survival
FEC   5-fluorouracil, epirubicin and cyclophosphamide
CMF   cyclophosphamide, methotrexate, 5-fluorouracil
AC    epirubicin, cyclophosphamide
NHEJ  Non Homologous End Joining
FFPE  Formalin-fixed Paraffin embedded
ER    Estrogen Receptor
PR    Progesterone Receptor
HER2  Human Epidermal Growth Factor Receptor 2
N     Pathological Nodal stage
NCBI  National Center for Biotechnology Information
GEO   Gene Expression Omnibus
HR    hazard ratio
CI    confidence interval

**Competing Interests**

SC Linn is named inventor on a patent application for the *BRCA1*-like classifier. All other authors have no competing interests.
Author Contributions

Conception and design: PCS, FM, SCL

Acquisition of data: PCS, FM, AS, SA, HPS, HFE, BY

Analysis and interpretation of data: PCS, FM, MH, HFE, BY, SCL

Writing, review and/or revision of the manuscript PCS, FM, MH, SCL, with substantial contributions

Administrative, technical, or material support: HFE, BY

Study supervision FM, SCL

Acknowledgments

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References


**Figure Legends**

Figure 1. Flow diagram of patients in this study.
Fig. 2 A. Kaplan-Meier curves of disease free survival of non-BRCA1-like patients treated with high dose or conventional chemotherapy. B. Kaplan-Meier curves of disease free survival of BRCA1-like patients treated with high dose (HD) or conventional chemotherapy (CONV). C. Kaplan-Meier curves of distant disease free survival of non-BRCA1-like patients treated with high dose or conventional chemotherapy. D. Kaplan-Meier curves of distant disease free survival of BRCA1-like patients treated with high dose or conventional chemotherapy. E. Kaplan-Meier curves of overall survival of non-BRCA1-like patients treated with high dose or conventional chemotherapy. F. Kaplan-Meier curves of overall survival of BRCA1-like patients treated with high dose or conventional chemotherapy. HR: hazard ratio, 95%CI: 95% confidence interval. The p-values for interaction, ie. for a difference between the HRs by BRCA1-like status without adjustment for prognostic factors were * p= 0.01; #: p = 0.02 and $: p = 0.02.

**Table Names and Legends**

**Table 1. Clinical characteristics of the cohort.**

Table 1. Patient characteristics of the cohort. ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal Growth Factor Receptor 2. P values were calculated with Chi Square test (> 2x2 tables) for trend or Fisher Exact tests (2x2 tables). * P values for comparing all non BRCA1-like patients and all BRCA1-like patients with Chi Square tests (> 2x2 tables for trend or Fisher Exact tests (2x2 tables).
Table 2. Multivariate Analysis

Cox Proportional Hazards model of disease free survival (DFS), distant disease free survival (DDFS) and overall survival (OS) adjusted for potentially confounding clinical factors. ER: estrogen receptor; HER2 human epidermal growth factor receptor 2; T tumor; Conv: conventional regimen; CI: confidence interval. Interaction tests between BRCA1-like status and chemotherapy regimen adjusted for confounders: *: p = 0.01 ; #: p = 0.01 ; $: p = 0.045.
Figure 1. Consort Diagram

High Dose Patients
N=144

Surgical therapy elsewhere
n=58

Surgical therapy at
University of Heidelberg
N=86

No archival tissue retrievable
n=16

DNA isolated for analysis
N=70

Insufficient DNA quality to perform aCGH
n=2

Patients with aCGH profile N=68

BRCA1 like
N=11

Non BRCA1-like
N=57

Conventionally treated Patients
N=91

Surgical therapy elsewhere
n=32

Surgical therapy at
University of Heidelberg
N=59

No archival tissue retrievable
n=10

DNA isolated for analysis
N=49

Patients with aCGH profile
N=49

BRCA1 like
N=5

Non BRCA1-like
N=44
Table 1. Patient Characteristics

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Table 1. Patient characteristics of the cohort. ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal Growth Factor Receptor 2.
P values were calculated with Chi Square test (> 2x2 tables) for trend or Fisher Exact tests (2x2 tables).
* P values for comparing all non BRCA1-like patients and all BRCA1 like patients with Chi Square tests (> 2x2 tables for trend or Fisher Exact tests (2x2 tables).
Table 2 Cox Proportional Hazards models for DFS, DDFS and OS

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Cox Proportional Hazards model of disease-free survival (DFS), distant disease-free survival (DDFS) and overall survival (OS) adjusted for potentially confounding clinical factors. ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; T tumor; Conv: conventional regimen; CI: confidence interval. Interaction tests between BRCA1-like status and chemotherapy regimen adjusted for confounders: *: p = 0.01 ; #: p = 0.01 , $: p = 0.045.
Breast cancers with a BRCA1-like DNA copy number profile recur less often than expected after high-dose alkylating chemotherapy

Philip C Schouten, Frederik Marme, Sebastian Aulmann, et al.

*Clin Cancer Res*  Published OnlineFirst December 5, 2014.

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