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Breast Cancer

DNA Repair Gene Patterns as Prognostic and Predictive Factors in Molecular Breast Cancer Subtypes

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ABSTRACT

DNA repair pathways can enable tumor cells to survive DNA damage induced by chemotherapy and thus provide prognostic and/or predictive value. We evaluated Affymetrix gene expression profiles for 145 DNA repair genes in untreated breast cancer (BC) patients ($n = 684$) and BC patients treated with regimens containing neoadjuvant taxane/anthracycline ($n = 294$) or anthracycline ($n = 210$). We independently assessed estrogen receptor (ER)-positive/HER2-negative, HER2-positive, and ER-negative/HER2-negative subgroups for differential expression, bimodal distribution, and the prognostic and predictive value of DNA repair gene expression. Twenty-two genes were consistently overexpressed in ER-negative tumors, and five genes were overexpressed in ER-positive tumors, but no differences in expression were associated with HER2 status. In ER-positive/HER2-negative tumors, the expression of nine genes (*BUB1, FANCI, MNAT1, PARP2, PCNA, POLQ, RPA3, TOP2A,* and *UBE2V2*) was associated with poor prognosis, and the expression of one gene (*ATM*) was associated with good prognosis. Furthermore, the prognostic value of specific genes did not correlate with proliferation. A few genes were associated with chemotherapy response in BC subtypes and treatment-specific manner. In ER-negative/HER2-negative tumors, the *MSH2*, *MSH6,* and *FAN1* (previously *MTMR15*) genes were associated with pathological complete response and residual invasive cancer in taxane/anthracycline-treated patients. Conversely, *PMS2* expression was associated with residual invasive cancer in treatments using anthracycline as a single agent. In HER2-positive tumors, *TOP2A* was associated with patient response to anthracyclines but not to taxane/anthracycline regimens. In genes expressed in a bimodal fashion, *RECQL4* was significantly associated with clinical outcome. In vitro studies showed that defects in RECQL4 impair homologous recombination, sensitizing BC cells to DNA-damaging agents. ***The Oncologist*** 2013;18:1063–1073

Implications for Practice: The identification of molecular mechanisms and biomarkers of sensitivity to chemotherapy in breast cancer is still controversial. In this context, the cellular DNA repair machinery is expected to play an important role in response to different types of chemotherapy. The differential expression of many DNA repair genes between ER-positive and ER-negative breast tumors could contribute to the different clinical behavior of these two breast cancer subtypes. We demonstrated that specific DNA repair genes are prognostic and predictive of chemotherapy response in a molecular subtype and treatment specific manner suggesting their contribution in the risk of tumor recurrence and response to chemotherapy. Such prognostic and predictive value warrant further exploration in clinical trials for optimizing treatment tailoring to improve patient outcome. Furthermore, defects of RECQL4 impair homologous recombination and sensitize breast cancer cells to DNA damaging agents, e.g., PARP inhibitors and platinum agents, which allows for selecting patients who more likely will benefit from these agents.

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Introduction

Breast cancer (BC) represents a molecularly and clinically heterogeneous disease comprising different molecular subtypes harboring different genetic alteration patterns [1–5]. Distinct gene pathways and biological processes are associated with prognosis and chemotherapy sensitivity in different BC subtypes [6–8]. DNA repair pathways maintain genomic integrity and are among the targets most frequently altered in cancer [9, 10]. The DNA repair machinery allows normal cells to repair DNA damage or induce apoptosis and cell cycle arrest if repair is not possible [11]. Anticancer drugs are highly influenced by the cellular DNA repair capacity, and specific alterations in DNA repair pathways have been reported to be associated with differences in patient response to chemotherapy in several cancers [12]. Drugs that inhibit a single specific DNA repair pathway in these tumors could prove useful as single-agent therapies, harboring the advantages of selectively targeting tumor cells and having fewer negative side effects. Several BC subtypes such as triple-negative BC (TNBC) are characterized by a higher degree of genomic instability because of defects in the DNA repair machinery [13, 14]. Moreover, TNBCs possessing *BRCA* mutations or having *BRCA*ness features and therefore higher genomic instability have been shown to respond better to DNA-damaging agents such as platinum-containing compounds; to PARP inhibitors; and, in some cases, to chemotherapy. The role of DNA repair pathways in BC subtypes, however, has not been well established.

In this study, we sought to assess the differential expression, bimodal distribution, and prognostic and predictive role of DNA repair genes in individual BC molecular subtypes including estrogen receptor (ER)-positive/HER2-negative, ER-negative/HER2-negative, and HER2-positive cancers. A large series of publicly available gene expression datasets was collected. The predictive value of DNA repair gene expression was assessed in BC patients treated with regimens containing neoadjuvant taxane/anthracycline or anthracycline. We performed gene set analyses by grouping DNA repair genes according to biological pathways.

We also selected one relevant DNA repair gene that arose as a result of our in silico analysis for (a) *in vitro* assessment of the biological functions of this gene within the DNA repair pathways and (b) exploration of the potential mechanisms of the cell response to DNA-damaging chemotherapy in BC.

Materials and Methods

Patient Cohorts

Clinical characteristics of the patients included in the various datasets used in this study are presented in supplemental online Table 1. Response to chemotherapy was dichotomized as pathological complete response (pCR) or residual invasive cancer (RD). To further validate the differential expression of DNA repair-related genes in BC subtypes, we examined a BC cell line dataset that included 51 cell lines [15].

Gene Expression Normalization, Molecular Subtype Definition, and Molecular Markers

All gene expression data were generated with Affymetrix U133A and U133 Plus2 gene chips (Affymetrix, Santa Clara, CA, http://www.affymetrix.com) and normalized using the MAS5 algorithm (http://www.bioconductor.org) with the mean expression centered to 600 and $\log_2$-transformed. ER and HER2 status was defined as previously reported [16]. To take into account the scaling factor existing between the U133A and U133 Plus2 platforms [17], slightly different cutoff values were adopted (ESR1 >10.6 as ER-positive and ERBB2 >13.04 as HER2-positive in U133 Plus2 chips). Molecular subgroups were defined according to ER and HER2 status. A proliferation marker (mitosis kinase score) was used to assess the independence of the prognostic value for the DNA repair genes relative to proliferation. The proliferation marker was calculated as previously reported [18].

Identification of Differentially Expressed DNA Repair Pathway Genes

Based on a literature review and on reported DNA repair pathways assembled from the PubMed and Gene Ontology databases, we selected the most functionally relevant annotated DNA repair genes. We identified a set of 145 DNA repair-related genes with documented roles in several DNA repair pathways.

We assessed differential expression of DNA repair-related genes between ER-positive and ER-negative tumors (after adjusting for HER2 status) using the Student's *t* test. To control for the potentially confounding effects of HER2 status in this comparison, the randomized block design included in BRB-Array Tools (National Cancer Institute, Division of Cancer Treatment and Research, Biometric Research Branch, Bethesda, MD, http://linus.nci.nih.gov/BRB-Array-Tools.html) was used. The randomized block design fits two linear models to the expression data for each gene. The full model included the ER class variable and the blocking variable (i.e., HER2), whereas the reduced model included only the blocking variable. Likelihood ratio statistics were used to test the significance of the differences between ER-negative and ER-positive tumors. A similar analysis was performed for HER2-positive and HER2-negative tumors using ER status as a blocking variable.

Statistical analyses were performed using BRB-Array Tools v 3.7.0 Patch_1 and R software (R Foundation, Vienna, Austria, http://www.r-project.org). All statistical tests were two-sided. To provide more robust and reproducible findings, the strategy adopted to identify differentially expressed prognostic and predictive DNA repair genes was used on different datasets as independent discovery cohorts rather than combining them in a larger series. This approach allowed us to claim significant findings based not only on statistical significance but also on consistency across independent discovery cohorts, similar to other studies [7, 16, 18]. Consistent observations are more likely to represent true associations.

Distant metastasis-free survival (DMFS), defined as the time from diagnosis to distant metastasis, was the primary endpoint used to assess the prognostic value of DNA repair gene expression. DMFS was available in all prognostic datasets. To make the results comparable across the datasets, survival was censored at 10 years. Univariate Cox regression analysis was used to correlate genes as continuous variables with DMFS in various clinical subgroups. To identify differentially expressed genes between cases based on pCR and RD, we performed unequal variance *t* tests on each probe set.

Because we used different discovery datasets to assess the consistency of our findings, we also calculated combined *p* values to use the data more efficiently. To calculate the combined *p* value for the pooled prognostic and predictive datasets, we let *pij* denote the *p* value for gene *i* (where $i = 1$–TOT) in the prognostic or predictive dataset *j* (where $j = 1$ or 2) for disease subtype *k* (where $k = 1$ for an ER-positive tumor and 2 for an ER-negative tumor). For each gene *i*, we calculated a Fisher's combined probability *p* value over the two or three datasets with 4 or 6 *df* (i.e., two or three times the number of *p* values combined) according to the following formula, in which *ln* is the natural logarithm: $Pik = -2(ln(pi1k) + ln(pi2k))$.

We assessed bimodal index [19] to identify gene expression with bimodal distribution that could be biologically relevant. Briefly, identifying genes with bimodal expression patterns from large-scale expression profiling data is an important task. Bimodal expression patterns can result naturally from differential expression with the two modes centered on the mean expression of a gene in two distinct subgroups of samples. In the context of cancer, bimodal expression patterns can result from genomic lesions that occur in some patients but not in others.

Functional Evaluation of *RECQL4* in Cellular Response to Chemotherapy and DNA Damaging Agents

Immunohistochemistry

The formalin-fixed, paraffin embedded BC tissues (39 ER-negative/HER2-negative and 61 ER-positive/HER2-negative) were deparaffinized and rehydrated. Antigen retrieval was performed by using EnVision Flex Target Retrieval solution (Dako, Glostrup, Denmark, http://www.dako.com). Immunohistochemistry was performed with RECQL4 monoclonal antibody (Abnova, Taipei, Taiwan, http://www.abnova.com/) using an automated staining workstation (DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com) with diaminobenzidine chromogen as substrate. Slides were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Immunostaining results were scored semiquantitatively, and only nuclear staining was taken into account. The scores for immunohistochemistry were 0, no staining; 1, few nuclear-stained tumor cells (<5% of positive tumor cells); 2, 5%–20% of positive tumor cells; and 3, >20% positive tumor cells. For pairwise comparisons, the scores were collapsed to low (score 1–2) versus high (score 3) expression.

RECQL4 *Functional Studies*

RECQL4 was targeted with two distinct small interfering RNAs (siRNAs; Thermo Scientific Molecular Biology, Waltham, MA, http://www.thermoscientificbio.com/Dharmacon/), which were applied to the MDA-MB-231 BC cell line. MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, http://www.atcc.org/). We optimized the transfection conditions for MDA-MB-231 cells as previously described [18]. Gene silencing was performed in three replicates in 96-well plates using predetermined optimal transfection conditions. Plates were incubated at 37°C for 96 hours.

Results

Differentially Expressed DNA Repair Genes by Molecular Tumor Subtype

Even in HER2-positive tumors, large-scale molecular differences exist according to ER status [20]; therefore, we compared the expression of DNA repair genes between ER-positive and ER-negative tumors after adjusting for HER2 status. Then we compared the expression of DNA repair genes between HER2-positive and HER2-negative tumors after adjusting for ER status. In two discovery datasets, we identified 27 genes that were differentially expressed between ER-positive and ER-negative tumors with a *p* value ≤.01 in each dataset (Table 1, supplemental online Table 2A, and supplemental online Fig. 1) (expected false discovery rate ~0.2 gene). These differences in gene expression were validated using three independent BC datasets and a series of 51 BC cell lines (Table 1, supplemental online Table 2A). All 27 genes were confirmed as significantly differentially expressed in each dataset, supporting the consistency and reliability of our findings. Conversely, in the cell line-derived data, only 9 of 27 genes were differentially expressed ($p \leq .05$). Using the same stringent criteria, no genes were differentially expressed between HER2-positive and HER2-negative tumors in the two discovery datasets (supplemental online Table 2B).

DNA Repair Genes as Prognostic Factors in Each Molecular Tumor Subtype

We assessed the prognostic value of DNA repair gene expression in untreated node-negative BC patients. The analysis was performed separately in each prognostic dataset based on the three molecular BC subtypes. Genes that correlated with patient outcome at a *p* value <.05 in all datasets were defined as prognostic (expected false discovery rate ~0.2 gene). We found that none of the 145 DNA repair genes analyzed showed prognostic value in HER2-positive and ER-negative/HER2-negative tumor subtypes (supplemental online Tables 3B and 3C). In ER-positive/HER2-negative tumors, we found nine genes that were significantly associated with poor prognosis and one gene (*ATM*) that was associated with good prognosis (Table 2, supplemental online Table 3A). Interestingly, some of these genes weakly correlated or did not correlate (*ATM*, *MNAT1*) with proliferation markers (supplemental online Fig. 2). We assessed the prognostic value of each of these genes in a multivariate model including a proliferation marker [18] and progesterone receptor (PGR). *TOP2A*, *POLQ,* and *MNAT1* expression retained significance after adjustment for these markers ($p = .031$, $p = .012$, $p = .0006$, respectively) (supplemental online Table 4). Similar results were obtained using the genomic grade index [21] as a proliferation marker (data not shown). These markers seem to provide prognostic information independent of proliferation.

DNA Repair Genes as Predictive Factors of Response to Chemotherapy in Each Molecular Tumor Subtype

We assessed the predictive value of DNA repair gene expression according to specific treatment regimens (anthracycline- or taxane/anthracycline-containing regimens) in each of the predictive datasets according to molecular tumor subtype (supplemental online Tables 5A and 5B). Genes with a combined *p* value <.05 and a consistent correlation with the regi-

Table 1. DNA repair genes overexpressed in ER-negative and ER-positive breast tumors

	Discovery datasets				Validation and testing datasets							
	Stockholm		ExpO		WANG		TRANSBIG		Mainz		51 Cell lines	
Gene symbol	Parametric *p* value	Ratio ER^-/ER^+	Parametric *p* value	Ratio ER^-/ER^+	Parametric *p* value	Ratio ER^-/ER^+	Parametric *p* value	Ratio ER^-/ER^+	Parametric *p* value	Ratio ER^-/ER^+	Parametric *p* value	Ratio ER^-/ER^+
ER-negative breast tumors												
BLM	1.00E-07	1.42	1.00E-07	1.37	**1.00E-07**	1.38	**1.00E-07**	1.73	**1.00E-07**	1.64	.3672081	1.08
BUB1	1.00E-07	2.03	1.00E-07	2.13	**1.00E-07**	1.91	**1.00E-07**	2.68	**1.00E-07**	2.45	**.0008376**	1.46
CHAF1A	7.02E-05	1.31	7.30E-05	1.19	**9.50E-06**	1.23	**.0011042**	1.18	**1.00E-07**	1.4	**.042302**	1.24
EXO1	7.70E-06	1.95	1.00E-07	2.04	**1.00E-07**	1.89	**1.00E-07**	2.37	**1.00E-07**	2.09	**.0350731**	1.27
FANCA	.004415	1.51	1.00E-07	1.81	**1.00E-07**	1.77	**1.00E-07**	1.96	**2.00E-07**	2.02	.6016553	1.14
FANCI	2.30E-06	1.59	8.74E-05	1.39	**1.00E-07**	1.46	**1.00E-07**	1.58	**1.00E-07**	1.66	.8798109	0.99
FEN1	.0001172	1.49	1.00E-07	1.7	**5.25E-05**	1.27	**1.00E-07**	1.52	**2.00E-07**	1.53	.5039301	1.1
H2AFX	.0002872	1.45	4.00E-07	1.4	**1.00E-07**	1.5	**3.98E-05**	1.4	**.0004756**	1.45	.3116935	1.24
MSH2	3.60E-06	1.36	1.00E-07	1.4	**6.90E-06**	1.21	**1.00E-07**	1.51	**1.00E-07**	1.42	.4380301	1.08
MSH6	1.00E-07	1.32	2.00E-07	1.24	**1.00E-07**	1.32	**1.00E-07**	1.37	**1.00E-07**	1.44	.9221048	1
MUTYH	8.26E-05	1.25	2.08E-05	1.18	**1.00E-07**	1.27	**1.00E-07**	1.43	**5.20E-06**	1.3	**.0422797**	1.23
NEIL3	.0012021	1.43	.0001711	1.32	**.0066894**	1.18	**2.60E-06**	1.41	**5.75E-05**	1.5	.1388067	1.18
PCNA	.0001687	1.37	.000732	1.31	**3.13E-05**	1.27	**1.00E-07**	1.4	**.0003012**	1.33	.0833689	1.28
POLD1	.004031	1.33	.0002285	1.28	**3.00E-07**	1.28	**5.81E-05**	1.4	**1.30E-05**	1.34	**.0433534**	1.44
PRKDC	.0051798	1.27	6.00E-07	1.41	**3.27E-05**	1.25	**.0011284**	1.27	**.0019594**	1.24	**.024177**	1.41
RAD51	.0006344	1.58	2.00E-07	1.6	**4.00E-07**	1.56	**1.00E-07**	1.74	**.0002733**	1.63	.9504415	1.01
RAD54B	4.00E-06	1.82	4.10E-05	1.54	**.0002812**	1.35	**.0007533**	1.46	**.0033886**	1.39	.7418248	0.94
RAD54L	1.30E-06	1.95	1.00E-07	2.04	**1.00E-07**	1.69	**1.00E-07**	2.39	**1.00E-07**	2.39	**.0079323**	2.04
RNF8	1.66E-05	1.27	1.30E-06	1.24	**1.00E-07**	1.27	**1.00E-07**	1.36	**5.27E-05**	1.2	.1003565	1.17
SHFM1	6.80E-06	1.47	1.00E-07	1.39	**1.59E-05**	1.24	**4.74E-05**	1.3	**.0015339**	1.22	.5925215	0.93
TOP2A	.0014627	1.97	4.29E-05	1.97	**1.66E-05**	1.85	**5.00E-07**	2.14	**9.25E-05**	2.47	.9598749	1.01
XRCC6	1.29E-05	1.27	2.00E-06	1.18	**3.10E-06**	1.19	**1.00E-07**	1.23	**1.00E-07**	1.3	.0639947	1.18
ER-positive breast tumors												
MGMT	4.40E-06	0.74	5.50E-06	0.79	**4.10E-06**	0.81	**5.00E-06**	0.73	**4.78E-05**	0.77	.1165405	0.61
XPA	6.00E-07	0.65	1.00E-07	0.77	**1.00E-07**	0.69	**1.00E-07**	0.66	**1.00E-07**	0.58	**.0461017**	0.78
PARP3	.0018074	0.69	.0002025	0.78	**.0001061**	0.73	**1.00E-07**	0.62	**4.30E-06**	0.67	.3092348	1.37
CCNO	.0044393	0.61	9.00E-07	0.57	**1.00E-07**	0.49	**9.20E-06**	0.55	**.0063925**	0.61	.1640713	1.43
POLI	2.31E-05	0.71	2.00E-07	0.73	**1.00E-07**	0.59	**8.00E-07**	0.67	**1.00E-07**	0.57	**.0041838**	0.64

Note: DNA repair genes overexpressed in ER-negative and ER-positive breast tumors after adjustment for HER2 status; *p* value $<$1.00E-07 was the threshold at this value.
Abbreviation: ER, estrogen receptor.

Table 2. Cox proportional hazards analysis in ER-positive/HER2-negative tumors

	WANG		TRANSBIG		Mainz		
Gene symbol	Parametric *p* value	Hazard ratio	Parametric *p* value	Hazard ratio	Parametric *p* value	Hazard ratio	Combined *p* value
PCNA	.0002	2.07	.0001	4.45	.0167	2.15	9.14E-08
TOP2A	.0012	1.40	.0000	2.60	.0001	1.99	7.34E-11
MNAT1	.0137	1.72	.0132	2.71	.0008	6.32	1.95E-05
UBE2V2	.0009	2.41	.0004	3.59	.0357	2.65	2.25E-06
RPA3	.0158	1.60	.0387	2.00	.0297	2.27	1.30E-03
BUB1	.0002	1.89	.0000	3.87	.0008	2.55	3.55E-10
FANCI	.0001	1.74	.0006	2.96	.0063	2.46	7.47E-08
PARP2	.0433	2.36	.0490	2.42	.0055	4.97	8.95E-04
POLQ	.0001	2.21	.0310	1.80	.0324	2.09	1.07E-05
ATM	.0088	0.57	.0498	0.60	.0151	0.45	5.58E-04

Table 3. Anthracycline sensitivity related to DNA repair genes

	MDACC/IGR (FA [E]C)[a]		TOP (anthracycline)			
Gene symbol	Parametric *p* value	Fold-change pCR/RD	Parametric *p* value	Fold-change pCR/RD	Combined *p* value	Association with
HER2-positive						
TOP2A	**.013**	**4.49**	**.039**	**2.46**	**.004**	**pCR**
FANCF	.089	1.89	.017	1.46	.012	pCR
CHAF1A	.577	1.14	.009	1.70	.031	pCR
ER-negative/HER2-negative						
POLG	.339	1.14	.000	1.56	3.60E-04	pCR
REV3L	.028	1.39	.085	1.24	.017	pCR
FANCI	.256	1.18	.011	1.60	.020	pCR
HERC2	.031	1.29	.120	1.23	.025	pCR
POLQ	.124	1.21	.033	1.44	.027	pCR
ERCC5	.053	1.38	.089	1.23	.030	pCR
MSH6	.014	1.37	.417	1.14	.036	pCR
PMS2	**.015**	**0.65**	**.044**	**0.79**	**.005**	**RD**
FANCC	.013	0.73	.328	0.88	.027	RD
DMC1	.011	0.58	.563	0.90	.036	RD
ER-positive/HER2-negative						
POLI	.039	0.66	—	—	—	RD
SLK	.030	1.32	—	—	—	pCR
RAD50	.014	0.65	—	—	—	RD
CETN2	.044	0.68	—	—	—	RD
APEX1	.014	0.70	—	—	—	RD
RAD51L1	.013	0.42	—	—	—	RD
GTF2H5	.000	0.55	—	—	—	RD
NEIL1	.049	0.64	—	—	—	RD
PALB2	.001	0.62	—	—	—	RD

Note: No ER-positive patients were enrolled in the TOP trial; combined *p* value <0.05. Also, genes in bold are significant ($p < 0.05$) in both datasets.
[a]Patients received chemotherapy regimen with 5-fluorouracil, doxorubicin (epirubicin), and cyclophosphamide.
Abbreviations: —, no value; ER, estrogen receptor; pCR, pathological complete response; RD, residual invasive cancer.

men are shown in Tables 3 and 4. Significant associations were defined as having a *p* value $\leq$.05 in two of the independent datasets. Despite the less stringent statistical criteria adopted relative to our assessment for prognostic value, few genes were consistently associated with pCR.

In patients receiving anthracycline-based regimens, only *TOP2A* was significantly associated with pCR in HER2-positive samples (combined $p = .004$), whereas *PMS2* was significantly associated with RD in ER-negative/HER2-negative tumors (combined $p = .005$) (Table 3). Notably, *TOP2A* expression did not show a significant association with anthracycline-containing regimens when other chemotherapeutic agents were used in combination.

In taxane/anthracycline regimens for ER-positive/HER2-negative and HER2-positive subtypes, none of the DNA repair genes was consistently associated with pCR or RD (Table 4). In ER-negative/HER2-negative tumors, however, mismatch repair (MMR) genes *MSH2* and *MSH6* were associated with a higher pCR rate (combined $p = .003$ and $p = .002$, respectively), and *FAN1* (previously *MTMR15*) gene expression was associated with RD (combined $p = .0003$) (Table 4).

RECQL4 *Impairs Homologous Recombination Sensitizing Breast Cancer Cell Lines to DNA Damaging Agents*

We hypothesized that genes with consistent bimodality among tumor subtypes could be enriched in biologically relevant genes. We defined bimodal genes (supplemental online Table 6) and assessed their prognostic and predictive value. Only *RECQL4* showed bimodal distribution, and its expression was significantly associated with clinical outcomes (Fig. 1). *RECQL4* was prognostic in ER-positive/HER2-negative tumors (supplemental online Table 3) after adjusting for proliferation and PGR expression ($p = .046$) (supplemental online Table 4). *RECQL4* was also significantly associated (ER-positive/HER2-negative, $p = .012$) or trended toward a significant association (ER-negative/HER2-negative) with patient response to taxane/anthracycline-containing regimens (supplemental online Table 5B). Moreover, RECQL4 is an important helicase involved in the regulation of several DNA repair pathways. Consequently, we assessed the involvement of RECQL4 in DNA double-strand break (DSB) mechanisms and treatment responses. Overexpression of RECQL4 in ER-negative/HER2-negative tumors (supplemental online Table 2A) was

Table 4. Taxane sensitivity-related DNA repair genes

	MDACC/MAQC (T/FAC)[a]		USO (FEC/TX)[b]			
Gene symbol	**Parametric *p* value**	**Fold-change pCR/RD**	**Parametric *p* value**	**Fold-change pCR/RD**	**Combined *p* value**	**Association with**
ER-negative/HER2-negative						
MSH6	**.011**	1.26	**.024**	1.62	**.002**	**pCR**
FEN1	.030	1.36	.163	1.40	.031	pCR
MSH2	**.018**	1.32	**.016**	1.40	**.003**	**pCR**
MTMR15	**.001**	0.76	**.039**	0.74	**.0003**	**RD**
CCNH	.005	0.77	.877	0.99	.028	RD
XRCC5	.008	1.22	.589	1.07	.031	pCR
CCNO	.308	0.82	.020	0.54	.038	RD
ER-positive/HER2-negative						
PCNA	.286	1.24	.001	1.58	.002	pCR
RAD54L	.244	1.48	.030	2.11	.043	pCR
BUB1	.078	1.70	.008	1.66	.005	pCR
LIG1	.113	1.26	.057	1.47	.039	pCR
MSH3	.013	0.60	.379	0.78	.030	RD
PMS2	.088	1.37	.083	1.46	.043	pCR
RECQL4	.337	1.46	.005	3.51	.012	pCR
NHEJ1	.830	0.96	.006	0.58	.033	RD
HER2-positive						
RAD51	.020	1.44	—	—	—	pCR
MRE11A	.049	2.01	—	—	—	pCR
MPG	.022	0.70	—	—	—	RD
ERCC8	.029	1.32	—	—	—	pCR
RAD50	.006	0.70	—	—	—	RD
CETN2	.015	0.71	—	—	—	RD
NTHL1	.020	0.53	—	—	—	RD
ATM	.045	1.36	—	—	—	pCR
CDK7	.023	0.71	—	—	—	RD
UIMC1	.040	1.26	—	—	—	pCR

Note: No HER2-positive patients were enrolled in the USO dataset; combined *p* value <0.05. Also, genes in bold are significant ($p < 0.05$) in both datasets.

[a]Patients received paclitaxel (weekly or every 3 weeks) followed by 5-fluorouracil, doxorubicin, and cyclophosphamide.

[b]Patients received 5-fluorouracil, eprirubicin, and cyclophosphamide followed by docetaxel.

Abbreviations: —, no value; ER, estrogen receptor; pCR, pathological complete response; RD, residual invasive cancer.

confirmed by immunohistochemical detection of RECQL4 performed on a series of BC samples including 39 cases of ER-negative/HER2-negative tumors and 61 cases of ER-positive/HER2-negative tumors ($p = .0264$) (Fig. 2A).

We examined the effect of *RECQL4* gene silencing on chemosensitivity to taxanes (1 μM and 4 μM paclitaxel) in the MDA-MB-231 cell line. Our results show that the down-regulation of *RECQL4* expression by two independent siRNAs led to an increase in taxane resistance in MDA-MB-231 cells (Fig. 2B).

We expected that *RECQL4* likely functions in homologous recombination (HR) repair, and we predicted that HR deficiencies would cause synthetic lethality on PARP inhibition; therefore, we examined whether *RECQL4* silencing could cause hypersensitivity to PARP inhibitors (AG14361 and olaparib). We found that *RECQL4* silencing resulted in decreased cellular survival after treatment with PARP inhibitors AG14361 (1 μM) (Fig. 2C) and olaparib (data not shown). Of note, *RECQL4* silencing yielded sensitivity similar to that observed when *BRCA1* is silenced (Fig. 2C). Western blot analysis was used to confirm *RECQL4* and *BRCA1* silencing in MDA-MB-231 cells (Fig. 2D). *RECQL4* silencing caused a fourfold increase in the percentage of cells staining for γH2AX foci in the absence of any added genotoxic stress agent when compared with non-targeting siRNA-transfected cells (Fig. 2E).

We next examined whether PARP inhibitor sensitivity and spontaneous DNA damage response activation in *RECQL4*-silenced cells was the result of defective DSB sensing and repair (Fig. 3). We performed immunofluorescence to measure the formation of RAD51 foci 8 hours after ionizing radiation, which is used as an indirect measure of HR repair (Fig. 3A). In contrast to control cells, *RECQL4*-silenced cells demonstrated complete absence of RAD51 foci in response to ionizing radiation (Fig. 3A). The reduced RAD51

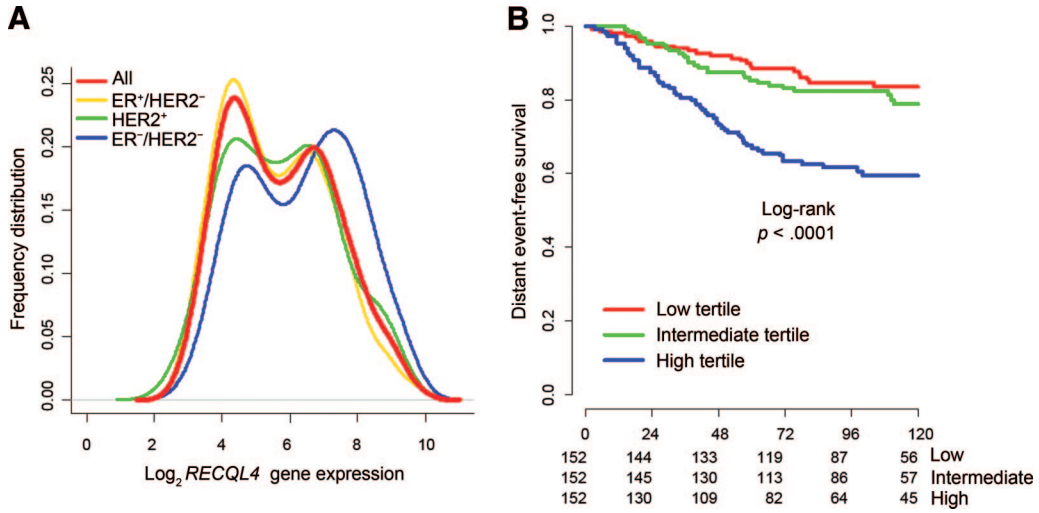


Figure 1. *RECQL4* shows bimodal distribution, and its expression is significantly associated with clinical outcomes. (A): The frequency distribution calculated by the kernel function for *RECQL4* in each molecular subtype and in the overall population is presented. (B): The Kaplan-Meier estimates for distant event-free survival are shown according to the tertiles of *RECQL4* expression in ER-positive/HER2-negative tumors.

foci in the *RECQL4*-silenced cells combined with the increased PARP inhibitor sensitivity suggested that these cells might harbor a reduced capability to repair DSBs by HR in the absence of RECQL4. Consistently, we found that *RECQL4*-silenced cells are hypersensitive to cisplatin (Fig. 3B), which induces DNA damage repair via HR-dependent pathways. In all cases, *RECQL4* silencing yielded sensitivity similar to that observed in siBRCA1 cells.

We then directly assessed HR repair in *RECQL4*-silenced cells using MDA-MB-231 cells expressing a dislocation-dependent reconstituted green fluorescent protein reporter plasmid, in which the *GFP* gene is interrupted by a single SceI restriction site [22]. Transient expression of the SceI endonuclease introduces a site-specific DSB within the reporter. Cells that are proficient in HR repair display restoration of *GFP* gene expression and increased fluorescence. Cells were analyzed by two-color flow cytometry to quantify GFP-positive cells relative to the total number of cells. We found that *RECQL4*-silenced cells have a significant defect in HR repair activity compared with control and *BRCA1*-silenced cells (Fig. 3C). Taken together, these results indicate that RECQL4 acts similar to BRCA1 and that *RECQL4* silencing contributes to chemoresistance to taxanes and hypersensitivity to DNA damaging agents; this phenomenon most likely occurs through defects in HR repair.

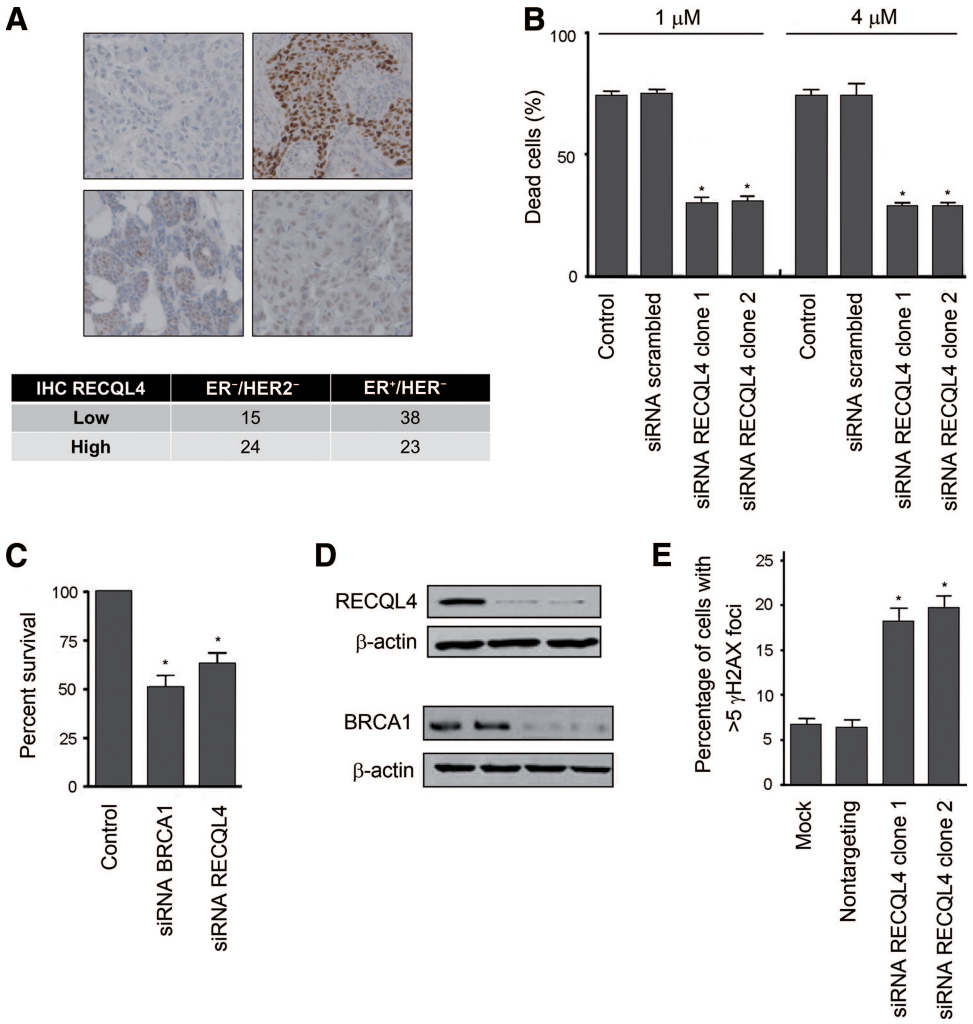
Discussion

Different clinical subtypes of BC are characterized by different gene expression profiles, DNA copy number alterations, and mutation distributions [1–5, 23, 24]. In this study, we examined the expression of 145 DNA repair-related genes independently in each of the main molecular subtypes of BC. We focused our comparisons on ER-negative and ER-positive cancers after adjusting for HER2 status because these groups showed large-scale differences regardless of HER2 status [20]. Moreover, more complex molecular classification methods have not yet been standardized [1].

We found that 5 DNA repair genes were significantly overexpressed in ER-positive tumors and that 22 DNA repair genes were significantly overexpressed in ER-negative tumors. All of the overexpressed DNA repair gene candidates were confirmed ($p < .05$) using three independent validation datasets. The ER-negative overexpressed genes are involved in several DNA repair pathways including MMR (*MSH2* and *MSH6*), HR (*RAD51*, *RAD54L*, *RAD54B*, and *SHFM1*), and Fanconi anemia (*FANCA* and *FANCI*) pathways as well as in other cellular processes that cooperate with DNA repair pathways such as chromatin remodeling (*H2AFX* and *CHAF1A*) and DNA polymerization, synthesis, editing, and processing (*PCNA*, *POLD1*, *TOP2A*, *BLM, FEN1,* and *EXO1*). These genes could play important biological functions in the ER-negative group contributing to the ER-negative-associated clinical characteristics of higher rate of early recurrence, younger age at diagnosis, higher histological grade, and higher chemotherapy benefit [25, 26]. Notably, the same genes were not similarly differentially expressed in a series of 51 BC cell lines, underscoring that the expression of some genes in cancer cell lines does not mimic the corresponding human BC.

DNA Repair Genes as Prognostic Factors in Breast Cancer Subtypes

The prognostic value of DNA repair genes has been assessed in untreated, node-negative BCs. We found that nine genes (*BUB1*, *FANCI*, *MNAT1*, *PARP2*, *PCNA*, *POLQ*, *RPA3*, *TOP2A*, and *UBE2V2*) were significantly associated with poor prognosis in ER-positive/HER2-negative cancers and that one gene (*ATM*) was significantly associated with good prognosis in ER-positive/HER2-negative cancers. In ER-positive/HER2-negative cancers, several markers of proliferation have already been shown to provide clinically relevant prognostic information (e.g., Oncotype DX, Genomic Health, Redwood City, CA, http://www.genomichealth.com/; MammaPrint, Agendia, Amsterdam, the Netherlands, http://www.agendia.com/; MapQuant Dx Genomic Grade, Qiagen Marseille, Marseille, France, http://www.qiagenmarseille.com/) [1, 27]. We assessed whether these markers were associated with proliferation. Only *ATM* and *MNAT1* did not correlate or only weakly correlated with proliferation markers. We also assessed



IHC RECQL4	ER⁻/HER2⁻	ER⁺/HER⁻
Low	15	38
High	24	23

Figure 2. Loss of RECQL4 is associated with resistance to taxanes and spontaneous DNA damage response activation. (A): IHC performed with anti-RECQL4 antibody in ER-positive/HER2-negative tumors (61 cases) and ER-negative/HER2-negative tumors (39 cases). RECQL4 was expressed to higher levels in ER-negative/HER2-negative tumors ($p = .0264$). (B): Death of *RECQL4*-silenced cells compared with scrambled or untransfected control cells after taxane treatment (1 μM or 4 μM) (*$p < .001$). Error bars indicate the standard deviation (SD; $n = 3$). (C): Survival of *RECQL4*-silenced cells compared with untransfected or *BRCA1* siRNA control cells after PARP inhibition (*$p <$ .001). (D): Immunoblot analysis of protein knockdown 96 hours after transfection of control, *RECQL4,* and *BRCA1* siRNA constructs. (E): Nontargeting and *RECQL4* siRNA were transfected into MDA-MB-231 cells, and γH2AX foci were counted 96 hours after transfection (mock, transfection with no siRNA) (*$p < .001$). Error bars represent the SD ($n = 3$).

Abbreviations: IHC, immunohistochemistry; siRNA, small interfering RNA.

whether these prognostic genes provide independent information in multivariate models including proliferation marker and PGR expression, which are other established prognostic factors in this group of cancers [28]. Interestingly, *MNAT1* retained significant prognostic value ($p = .0006$) in the multivariate model, suggesting potential value in assessing prognosis over conventional proliferation markers; however, the value of *MNAT1* expression requires independent confirmation. Expression levels of *TOP2A* and *BUB1* closely reflect the proliferative activity of cells [18, 29] and have been previously associated with recurrence in ER-positive/HER2-negative BC [30, 31]. PCNA and POLQ are involved in genomic replication and DNA repair [32, 33], and their overexpression has been correlated with poor clinical outcome in BC patients [34, 35]. Another study has shown that the DNA repair gene *UBE2V2* contributes to the overall resistance of cancer cells to genotoxic agents [36].

Even if *ATM* expression does not provide independent prognostic information in multivariate analyses, *ATM* expression is not associated with proliferation, which could indicate that *ATM* expression has a relevant biological function in cell dedifferentiation. In fact, low *ATM* expression in BC tissue has been correlated with a higher rate of DNA mutations, a progressive cancer phenotype, and increased angiogenesis [37]. The expression of *ATM* has been associated with favorable patient outcomes [37], which is in agreement with our results. None of the DNA repair genes considered in our study was consistently associated with prognosis in both ER-negative/HER2-negative and HER2-positive tumors. These findings suggest that by including the expression of the *MNAT1* gene in the clinical assessment, it could be possible to refine the risk of relapse prediction performed using established molecular markers of proliferation and PGR expression.

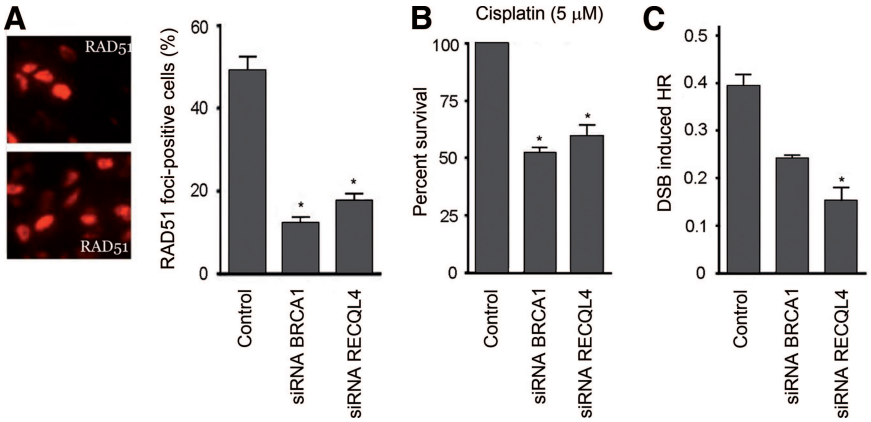


Figure 3. *RECQL4* silencing impairs HR repair at DSBs. (A): RAD51 foci were scored by immunofluorescent imaging 8 hours after treatment with IR. Quantification of nuclei containing RAD51 foci was determined based on 200 cells. Error bars indicate the standard deviation (SD; $n = 3$; $*p < .001$). (B): Control, *BRCA1*- and *RECQL4*-silenced MDA-MB-231 cells were treated with cisplatin (5 μM) for 96 hours and then were measured for viability ($*p < .05$). Error bars indicate the SD ($n = 5$). (C): Control, *BRCA1*- and *RECQL4*-silenced MDA-MB-231 cells containing an integrated HR repair substrate were transfected with constructs expressing I-SceI to induce a DSB in the *GFP* reporter gene. The percentage of GFP-positive cells was scored by flow cytometry. Error bars indicate the SD ($n = 3$; $*p < .05$).

Abbreviations: DSB, double-strand break; HR, homologous recombination; IR, ionizing radiation; siRNA, small interfering RNA.

DNA Repair Genes as Predictors of Likelihood of Response to Chemotherapy

We also examined the association between expression of DNA repair genes and the likelihood of achieving a pCR in patients treated with preoperative taxane/anthracycline- or anthracycline-based chemotherapy regimens. In BC patients treated with anthracycline-based chemotherapy, we found that only higher expression of *TOP2A* was consistently associated with pCR, and this association was found only in the HER2-positive tumor group. This finding confirms previous data regarding the association between *TOP2A* expression and response to anthracycline-based regimens in HER2-positive tumors [38]. This consistency indirectly confirmed the validity of the discovery approach adopted in our study. The chromosomal region that incorporates *HER2* is a gene-rich region of genetic instability that is pivotal to tumor progression and poor prognosis in BCs [39]. These data reinforce the clinical need to explore the inclusion of the assessment of *TOP2A* gene expression for tailoring the group of patients in the HER2-positive molecular subtype at higher likelihood to benefit from anthracycline administration [38, 40]. The amplification of *HER2* is associated with important amplifications and/or deletions of neighboring *TOP2A* DNA repair genes and require further investigation for the identification of additional therapeutic targets. Notably, *TOP2A* expression was not associated with pCR when other drugs were administered concurrently with taxanes. This may suggest that the group of patients who show lower expression of *TOP2A* and less benefit from anthracycline-based therapy could derive substantial benefits from other drugs. This difference, correlated with *TOP2A* expression and chemotherapy regimen, also underscores the inherent limitation of assessing predictive markers for polychemotherapy regimens because of the potential different predictive value of each marker according to the drugs administered. Overall, these findings provide a supportive explanation for the additional clinical benefit of taxanes to anthracyclines in HER2-positive BCs [41]. In fact, our biomarker analysis in HER2-positive tumors indirectly suggests that different molecular subgroups of tumors, such as high and low *TOP2A* expression, may derive diverse benefits from anthracyclines or taxanes, respectively. Consequently, our data contribute to the current active debate about whether anthracycline should be abandoned as adjuvant treatment for HER2-positive disease [42, 43]. Because it seems that not all patients will benefit from the association of both drugs, our data also support the need for use of specific biomarkers to tailor selection of the appropriate regimen, which will account for greater advantages during adjuvant treatment.

The novel gene *PMS2* is consistently associated with RD in ER-negative/HER2-negative tumors treated with anthracycline-based regimens. PMS2 is a major component of the DNA MMR system that heterodimerizes with MLH1 to form MutLα [44]. Sporadic BCs demonstrate microsatellite instability, reflecting the presence of MMR-deficient cells in approximately one-fourth of cases at the time of diagnosis [45]. MMR-deficient cells show resistance to doxorubicin, suggesting a role for MMR in topoisomerase II poison-mediated cell killing [45]. Although much of the current data have supported the involvement of MLH1 in topoisomerase II poison-mediated cytotoxicity, our results suggest that the MLH1 binding partner PMS2 warrants further investigation and should be considered as a potential biomarker for anthracycline resistance.

To date, several biomarkers have been evaluated for their potential value in the prediction of BC patient responses to taxanes such as p53, the microtubule-associated protein tau, and others, although different studies have yielded inconsistent results [46]. In our analysis, we found that higher expression of the MMR genes *MSH2* and *MSH6* is significantly associated with pCR in ER-negative/HER2-negative BC patients receiving neoadjuvant taxane/anthracycline-containing regimens. MSH2 and MSH6 could reside in a protein complex with BRCA1 and other DNA repair proteins as well as checkpoint signaling proteins (BASC) [45]. The members of this complex may regulate DNA repair and p53-mediated apoptosis following DNA damage. Although *BRCA1* is a DNA damage response gene, BRCA1 also appears to regulate mito-

sis by interacting with the spindle microtubule protein γ-tubulin and thus playing a role in modulating the response to spindle poisons such as taxanes. Cells lacking a functional MMR system are unable to undergo G2/M arrest in response to several genotoxic agents, suggesting a link between MMR and G2/M arrest. The association between MMR proteins and BASC could contribute to the BRCA1-mediated modulation of apoptosis in response to spindle damage and could participate in BRCA1-dependent taxane sensitivity [45]. The expression of these biomarkers could help rationalize therapy decisions, although further prospective and independent evaluations are needed.

We hypothesized that DNA repair gene expression with bimodal distribution can indicate biologically relevant pathways that can be targeted. Within bimodal genes, only *RECQL4* showed a significant association with clinical outcome. High *RECQL4* expression is associated with poor prognosis and higher pCR after taxane/anthracycline regimens in ER-positive/HER2-negative tumors, and a similar trend was observed in ER-negative/HER2-negative tumors. Mutations in the *RECQL4* gene are associated with the Rothmund-Thomson syndrome, a genetic disorder characterized by genomic instability, including trisomy, aneuploidy, and chromosomal rearrangements [47], suggesting a role for *RECQL4* in preventing tumorigenesis and maintaining genome integrity in humans. RECQL4 has important biological functions in several DNA repair pathways and has been shown to form complexes with RAD51, which is a crucial factor for DSB repair through the HR pathway [47]. We confirmed that RECQL4 is involved in the HR pathway at DNA DSBs (Fig. 2D and 3A). ER-negative/HER2-negative tumors tend to be BRCA1 defective [1, 4]. Accordingly, loss of BRCA1 function leads to a tumor-specific dysfunction in DSB repair by HR, and agents that cause an increase in DSBs should selectively affect HR-deficient cells. We demonstrated that *RECQL4* knockdown sensitizes BC cell lines to DNA damaging agents such as platinum and PARP inhibitors. These results demonstrate a novel mechanism of sensitivity to DNA damaging agents because of defects of RECQL4-mediated HR in BC cells, suggesting that RECQL4-deficient tumors may be hypersensitive to platinum-based or PARP inhibitor-based chemotherapy. PARP inhibitors target HR-based DNA repair defects similarly to cisplatin chemotherapy [48], and studies support the efficacy for the PARP inhibitor olaparib in *BRCA1*- and *BRCA2*-deficient BCs that have been previously treated with chemotherapy [49]. Despite their promise to make chemotherapy treatments more effective for patients with chemoresistant cancers, PARP inhibitors currently available through clinical trials are based on a single gene or biomarker that targets only those patients with *BRCA1/2*-mutated and basal-like TNBCs, which accounts for roughly 5% of all BC cases. RECQL4/HR-deficient tumors may benefit from PARP inhibitors that specifically target this DNA repair defect. Consequently, the new identified biomarker may broaden the scope of eligible patients.

We performed biomarker assessments based on molecular tumor subgroups and chemotherapy regimens to reduce the heterogeneity and increase the reliability of our results; however, the small sample size and small number of events in each tumor subset increased the risk of both false-negative and false-positive findings. Furthermore, multidrug regimens reduced the possibility of linking single markers to specific drug activity. Despite these limitations, our observations are consistent with the hypothesis that prognosis and chemotherapy response are at least partially associated with differential expression of some DNA repair-related genes. We confirmed the association of *TOP2A* gene expression with pCR to anthracyclines in HER2-positive tumors, but we also identified new genes associated with the likelihood of response to anthracycline- or taxane/anthracycline-based regimens. These prognostic and predictive markers warrant further investigation and independent validation in prospective studies. Because some DNA repair pathways can enable tumor cells to survive DNA damage induced by chemotherapy [50], inhibitors of specific DNA repair pathways could prove efficacious when used in combination with DNA-damaging chemotherapeutic drugs [50].

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Disclosures

W. Fraser Symmans: Nuvera Biosciences, Inc. (IP); Nuvera Biosciences, Inc. (OI). **Gabriel N. Hortobágyi:** AstraZeneca, Allergan, Novartis, Genentech, SanofiAventis (C/A); Novartis (RF). The other authors indicated no financial relationships.

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