Published in final edited form as:

Clin Cancer Res. 2010 December 1; 16(23): 5796–5804. doi:10.1158/1078-0432.CCR-10-0292.

DNA Repair Protein Biomarkers Associated with Time to Recurrence in Triple Negative Breast Cancer

Brian M. Alexander^{1,3}, Kam Sprott⁴, D. Allan Farrow⁴, XiaoZhe Wang⁴, Alan D. D'Andrea¹, Stuart J. Schnitt⁵, Laura C. Collins⁵, David T. Weaver⁴, and Judy E. Garber²

¹Dana Farber/Brigham and Women's Cancer Center Departments of Radiation Oncology, Harvard Medical School, Boston, MA

²Dana Farber/Brigham and Women's Cancer Center Department of Medical Oncology, Harvard Medical School, Boston, MA

³Harvard Radiation Oncology Program, Boston, MA

⁴On-Q-ity, Inc., Waltham, MA

⁵Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

Abstract

Purpose—To evaluate the prognostic utility of immunohistochemical assessment of key proteins in multiple DNA repair pathways in triple negative breast cancer (TNBC; estrogen receptor negative, progesterone receptor negative, and HER2/neu negative by immunohistochemistry).

Experimental design—Archived clinically-annotated tumor specimens from 112 women with TNBC were immunostained with antibodies against DNA repair proteins and scored using digital image analysis. The cohort was divided into training and test sets for development of a multi-antibody model. Scores were combined with clinical data to assess association with outcome.

Results—Low XPF ($p=0.005$), pMK2 ($p=0.01$), MLH ($p=0.002$) and FANCD2 ($p=0.001$) were each associated with shorter time to recurrence (TTR) in univariate analysis. A four-antibody model was able to segregate high risk and low risk groups based on TTR in both the training (RR 3.52, $p=9.05E-07$) and test (RR 2.67, $p=0.019$) cohorts.

Conclusions—DNA repair proteins may be useful as prognostic markers in triple negative breast cancer. Further study in larger, uniformly treated cohorts with additional clinical parameters is warranted.

Keywords

Triple negative; Biomarker; DNA repair; Breast cancer

Introduction

'Triple negative' breast cancers (TNBCs), those that are estrogen receptor (ER) negative, progesterone receptor (PR) negative, and HER-2 low by immunohistochemistry or fluorescent in-situ hybridization, comprise approximately 15% of all breast cancers and have an aggressive clinical course with high rates of local and systemic relapse(1). The clinical course appears to reflect the intrinsic biology of this group of tumors as well as the absence of specific targeted treatments to supplement conventional cytotoxic chemotherapy, such as hormonal therapies used for ER or PR positive patients and trastuzumab for HER-2 over-expressing tumors. In addition, these cancers may have different sensitivity to common chemotherapeutic agents(2). Thus, there is a great deal of interest in determining novel therapeutic regimens for this aggressive breast cancer subtype. Although triple negative breast cancers comprise an established subtype of breast cancer, they are not uniform and relatively little biomarker information is available with which to stratify patients and to direct treatment decisions.

Deficits in DNA repair are a characteristic of at least a subset of triple negative breast cancers(3, 4). These tumors exhibit more DNA copy alterations(5) and loss of heterozygosity(6) than other breast cancers- features suggestive of genomic instability. Furthermore, sporadic triple negative tumors share phenotypic and cytogenetic features with familial *BRCA1*-associated breast cancers(1) the majority of which are also basal-like using microarray RNA expression data(7). *BRCA1* mutant tumors have been shown to be deficient in DNA repair(3, 4), particularly homologous recombination (HR). Taken together, these similarities suggest that a DNA repair deficiency may underlie the development of at least a subset of triple negative tumors. Deficits in DNA repair may have implications for prognosis and could influence response to both current DNA damage-inducing therapies as well as novel targeted agents such as poly-ADP ribose polymerase (PARP) inhibitors (8–12).

Given the possible association of triple negative breast cancers with DNA repair deficits and the relationship of DNA repair to phenotypic aggressiveness and therapy, we sought to evaluate the prognostic value of key proteins from multiple DNA repair pathways in TNBC. The goal of the study was to evaluate whether a pattern of immunohistochemistry-assessed DNA repair proteins from multiple repair pathways in pre-treatment breast cancer specimens was correlated with clinical outcome.

Materials and Methods

Patients

We identified 143 women diagnosed between 1991 and 2006 whose primary breast cancers had been reported to be negative for estrogen and progesterone receptors and HER2/neu by clinical immunohistochemistry at the time of their initial visit to the Breast Oncology Center at the Dana Farber Cancer Institute, Boston MA. Women were eligible if they had signed the informed consent document, provided a blood specimen to the Dana Farber/Harvard Cancer Center SPORE in Breast Cancer specimen bank and given permission to use clinical data, a blood specimen and their unused stored tumor specimens for research. Pathology was

reviewed for histology and ER/PR/Her2 status by a breast pathologist at the Brigham and Women's Hospital or Beth Israel Deaconess Medical Center and all grades were confirmed. Clinical data including date of diagnosis, age at diagnosis, clinical TNM stage, *BRCA1* mutation status, adjuvant chemotherapy regimen and dates of administration and pathologic data including histologic type, histologic grade, lymphovascular invasion and pathologic nodal status were collected and transferred with identifiers removed in accordance with procedures specified in a protocol approved by the Dana Farber/Harvard Cancer Center Institutional Review Board. *BRCA1* mutation carriers had been identified previously by Exon Grouping Analysis (EGAN), a method based on Conformation Specific Gel Electrophoresis (CSGE)(13, 14). Of the 143 eligible women identified with triple negative tumors in the Breast Cancer specimen bank, 112 had adequate tissue and clinical follow up data available for analysis.

Specimen characteristics

A tissue microarray (TMA) was constructed from stored formalin-fixed paraffin-embedded breast tumor specimens obtained from either biopsy tissue taken prior to neoadjuvant chemotherapy (n=6) or primary surgical excisions performed before the administration of adjuvant radiation or chemotherapy (n=105). The one patient whose specimen had been obtained after neoadjuvant chemotherapy was ultimately excluded from immunohistochemical analysis. The TMA was created in order to efficiently evaluate the markers and minimize the effects of artifact-based staining variation between slides. For TMA construction, three 0.6mm cores were obtained from a paraffin block of each breast cancer and were inserted into the recipient TMA block.

Assay methods

Immunohistochemistry was conducted on 5 micron sections cut from the TMA block using primary antibodies against proteins in multiple DNA repair pathways, including XPF [nucleotide excision repair (NER); AbCam, Ab17798], FANCD2 [Fanconi Anemia/homologous recombination (FA/HR); Santa Cruz, sc-20022], MLH1 [mismatch repair (MMR); BioCare Medical, CM220], PARP1 [base excision repair (BER); AbD Serotec, MCA1522G], PAR [base excision repair (BER); Millipore, MAB3192], pMK2 [MAPKAPKinase2, DNA damage response (DDR); Cell Signaling Technology, 3007], p53 and Ki67. These downstream DNA repair pathway proteins were chosen as surrogates for the integrity of the entire pathway. For example, in the FA pathway, a critical step is the monoubiquitination of FANCD2 and the formation of nuclear foci(15–17). Therefore antibodies against FANCD2, assessing for nuclear staining, were used to represent all of the upstream events that occur in the FA pathway. XPF is a protein that forms a heterodimer with ERCC1 to create an endonuclease that is critical in the removal of helix-distorting DNA lesions through the NER pathway(18). Cells with absent XPF are hypersensitive to UV irradiation and chemicals that cause interstrand crosslinks(19–22). Likewise, PAR, MLH1 and pMK2 are proteins that are critical actors in the BER and MMR pathways(23, 24) and DNA damage response(25), respectively. Ki-67 was added to investigate the possible dependence of DNA repair protein expression on proliferation rate.

Positive assay controls, consisting of human breast cancer sections previously determined to express each antigen of interest, were run for each antibody as was a negative control lacking primary antibody. Tissue sections were deparaffinized and rehydrated using standard techniques. Antigen retrieval was performed in a high pressure cooker using Citrate Buffer (pH 6.5) for XPF, FANCD2, PAR, PARP1, pMK2, MLH1, and Ki67. For p53, antigen retrieval was performed in a high pressure cooker using Tris-EDTA (pH 7.8). Antibody incubations were overnight for XPF (1:1200), p53 (1:400), and FANCD2 (1:100). Two hour antibody incubations were performed for PAR (1:6400), PARP1 (1:10000), pMK2 (1:100), MLH1 (1:100), and Ki67 (1:100). Renaissance TSA™ (Tyramide Signal Amplification) Biotin System (Perkin Elmer) was used for detection of XPF and FANCD2. Super Sensitive™ IHC Detection System (BioGenex) was used for detection of MLH1, PARP1, PAR, pMK2, and Ki67. Envision + System-HRP (Dako) was used for detection of p53. Antibody dilution was optimized such that the antibody level was appropriate to explore a dynamic range.

Scoring

Microscope slides were scanned into a digital pathology platform (Aperio). A nuclear score was reported for FANCD2, XPF, PAR, MLH1, PARP1, p53, and Ki67 each. Image analysis macros were developed by modifying the Aperio IHC nuclear macro and were used to identify tumor nuclei for the nuclear staining markers. Additionally, the percentage of nuclei staining at 4 different intensities (high, moderate, low, and negative) was outputted and used to create an H-score (0–300 scale) for XPF, PAR, PARP1, FANCD2, p53, and MLH1(26). For Ki67, the score was based on the standard method of percentage of positively staining nuclei. Nuclear and cytoplasmic scores were separately compiled for pMK2 using a modified Aperio colocalization macro. Cytoplasmic staining was additionally scored for pMK2 based on the prior observation that kinase activation is coupled with nuclear export(27). The tumor area was manually annotated. The presence of hematoxylin was used to define the nuclear area while the lack of hematoxylin is used to define the cytoplasmic area. Not all patients had IHC scoring data generated for each of the antibodies because of technical reasons or lack of tumor tissue on specific TMA slides.

Statistical Analysis

IHC scoring to measure expression levels of DNA repair proteins was analyzed with clinical data to assess for correlation with clinical outcome. Time to recurrence (TTR) was defined as the time from diagnosis until the time of first recurrence. Patients without evidence of recurrence were censored at last follow up. The 112 patients available for analysis were randomly assigned to training (n=68) and test (n=44) sets; the test set was withheld for final verification at the end of the modeling process. The training and test sets were generated to ensure an equal fraction of recurrences in each group with a primary consideration to provide adequate numbers in the training set to facilitate discovery. We estimated that 6–8 samples would be required per model parameter; this allows 8 independent parameters to be explored (at the high end) with 64 patients in the training set. Ensuring equal outcome fractions in the training and test set led to the actual numbers of 68 and 44. Each antibody IHC score was examined by univariate analysis and its ability to discriminate patient

outcomes assessed. Antibodies that showed significant discriminatory ability were moved forward to further multivariate analysis.

To assess the ability of the antibody scores to segregate patients into high and low recurrence risk groups, thresholds for each were determined which separated patients into two groups with maximally different survival curves. These optimal divisions were selected by choosing the threshold that generated minimum recurrence-free survival curve p-value and had at least five samples per group.

A multi-antibody model was developed to determine whether combinations of antibodies from different repair pathways were more informative than was any individual antibody from a single pathway. To determine which combinations of antibody scores enhanced performance, all pairs were analyzed using a single-node multivariate decision tree with the impurity of the partition given by the survival curve p-value(28). The optimal scoring thresholds for any two antibodies divide the samples into four groups; the group with the largest proportion of recurrent samples was labeled as “high-risk” and the remaining three groups together form the “low-risk” group. Combinations that significantly segregated low versus high-risk groups were used for development of a four-antibody model. The optimal scoring thresholds for the four antibodies were determined simultaneously by dividing the range of each antibody score into 20 equal intervals and establishing a grid of thresholds to be analyzed. The set of thresholds that generated the minimum survival curve p-value were determined to be the optimal set. The number of patients available for each marker combination varied- not all patients had data available for every marker.

The antibodies and optimal thresholds determined from the training dataset were then applied to the test dataset. Survival curves for the low and high-risk groups were compared using Kaplan-Meier models and the p-value reported. Additionally, the apparent error rate (AER), ROC curve and AUC (for univariate analyses), sensitivity, specificity, positive predictive value, negative predictive value, and relative risk (RR), were computed and reported.

The correlation of additional clinical and pathologic data with outcome was examined with Cox models using all data from test and training sets to provide maximal statistical power. Clinical data in addition to the single multi-antibody model was examined to determine if there was any correlation with outcome using univariate and multivariate analysis to control for known prognostic factors.

Results

Patient cohort

Primary treatment and follow-up data was available for 112 patients with a median follow up of 61.5 months (Table 1). Median age for the cohort was 49.2 years. Sixty-four patients were treated with breast conserving therapy and 47 with mastectomy, 17 of which received post mastectomy radiation. Treatment details were unavailable for one patient. There were 110 grade 3 tumors, 1 grade 2 tumor and one grade unknown. LVI was present in 48 (43%) of tumors and there was nodal involvement in 50 (47%) cases. One hundred eight patients

received chemotherapy as part of their treatment: 41 with anthracycline/cyclophosphamide, 49 with anthracycline/cyclophosphamide/taxane, 15 with cyclophosphamide/methotrexate/5-FU based regimens and 3 with other regimens. Eighteen patients had *BRCA1* mutations and 5 had unknown variants, 11 patients *BRCA1* status was unknown. Testing for *BRCA2* mutations was not performed. There were 37 recurrences classified by site of first recurrence: 19 were distant, 12 local, and 7 were local and distant simultaneously. The median time to recurrence was 16.9 months (range 2.3–109.2 months).

IHC scoring

Antibody staining from a single patient is shown in Figure 1. To demonstrate that the variability of scoring among patients was greater than the scoring within the triplicate cores of a single patient, the triplicate variation was determined and scored against the patient rank value for each antibody. For the eight DNA repair and proliferation antibodies tested, the average rank error was a low percentage of the total (8.8 – 11.1% DNA Repair, 11.1% Ki67). The observed variations between triplicate specimen cores per patient did not significantly change the patient rank order for any of the antibodies tested so the antibody score assigned by averaging cores was therefore a robust measure of expression. The distribution of each antibody score with intra-patient variation among cores is shown graphically in Figure 2.

Association of DNA repair IHC scoring with recurrence of cancer

Eight antibodies were analyzed for association with recurrence. P53 protein expression ($p=0.02$), low XPF ($p=0.005$), pMK2.C ($p=0.01$), MLH ($p=0.007$) and FANCD2 ($p=0.001$) were each associated with shorter TTR on univariate analysis in the training cohort (Table 2). Scoring for PAR, PARP1 and Ki67 were not significantly correlated with TTR.

Development of a multiple DNA repair antibody panel that distinguishes recurrence groups

Pair-wise combinations of various markers to maximally segregate low versus high-risk groups in the training set yielded four proteins- XPF, FANCD2, pMK2.C, and PAR- that were more discriminatory when used in combination than individually. Not all patients had data available for every antibody; only patients with all four antibody values were included in the analysis. The training set had 50 patients with scoring available for XPF, FANCD2, PAR and pMK2.C, inclusive, (down from 68 patients in the randomized set) while the test set had 35 patients (down from 44). The thresholds that best segregated the samples into outcome groups were: XPF=229, FANCD2=42, PAR=56, pMK2.C=0.36 corresponding to the 0.39, 0.61, 0.71, and 0.62 quantiles of the scoring data, respectively.

In the training dataset, the high-risk group defined by the four-antibody model contained 12 patients with 10 recurrences while the low-risk group contained 38 patients with 9 recurrences. The difference in mean TTR (28 months versus 103 months) was highly significant ($p=9.05E-07$)(Figure 3). Using thresholds obtained from the training dataset to assign patients in the test set to recurrence risk groups yielded a high-risk group with 5 patients and 4 recurrences and a low-risk group containing 30 patients with 9 recurrences. The difference in mean TTR (31 months versus 129 months) in the test set was also

statistically significant ($p = 0.0186$) (Figure 4). To verify that the high and low risk groups in both datasets are similar, the TTR curves were compared. There was no difference between the high-risk groups from test and training datasets ($p = 0.625$) or between the two low-risk groups ($p = 0.606$). There was also no statistically significant difference in the distribution of antibody scores between the training and test sets.

We also determined whether the four-antibody model improved the assignment of patients into the expected recurrence groups based on several statistical criteria. The area under Receiver Operator Characteristic (ROC) curves (AUC values) for the four individual antibody scores were FANCD2- 0.71, pMK2- 0.65, XPF- 0.67, and PAR- 0.54 (Table 2). However, using a probability analysis for the four-antibody model, a significantly higher AUC value of 0.77 was calculated. Positive predictive value (PPV; 0.45–0.57) and negative predictive value (NPV; 0.7–0.85) of the individual antibodies were inferior to the PPV (0.83) and NPV (0.76) of the same four-antibody model. The apparent error rate (AER), the fraction of samples incorrectly classified, for the four-antibody model was lower (0.22) than the individual antibodies (0.30–0.42) or other markers such as p53 (0.35) and Ki67 (0.39). The four-antibody model yielded sensitivities of 53% and 31% and specificities of 94% and 95% in the training and test sets, respectively.

DNA repair IHC scoring in addition to clinical variables

To compare the discriminatory ability of the four-antibody model to available clinical data, univariate and multivariate Cox proportional hazard models were constructed. Univariate results presented in Table 2 indicate that of the clinical parameters available, higher T stage, N stage and positive LVI were each associated with increased risk of recurrence. The four-antibody model is more highly associated with increased risk of recurrence than any single clinical variable ($p = 1.5 \times 10^{-6}$).

All clinical variables and the four-antibody model score (0=low recurrence risk or 1=high recurrence risk) were combined into a single multivariate Cox model to isolate the effect of the DNA repair markers from other clinical data. The four-antibody model was highly significant ($p = 0.007$) while only T stage (0.038) retained a statistically significant association with recurrence (Table 2).

Discussion

Both normal cellular processes and exogenous agents contribute to the accumulation of DNA damage for which eukaryotic cells have evolved complex and redundant repair mechanisms to ensure stability and high fidelity replication of the genetic material (23, 24, 29, 30). There are six identified major DNA repair pathways: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR)/Fanconi Anemia (FA), non-homologous end joining (NHEJ) and translesion synthesis (TLS) (23, 24, 31). The six DNA repair pathways may also operate in cell survival and chemotherapy responses in coordinated and concerted ways (23, 24, 31, 32). Cancer cells frequently exhibit dysfunctional DNA repair, with genome instability a hallmark of many cancers (31). Dysfunctional DNA repair can lead to a 'mutator' phenotype where cells accumulate damage at an accelerated rate, leading to oncogenesis, tumor aggressiveness and

an enhanced ability to become resistant to therapy(23, 33, 34). Assessment of the cellular DNA repair machinery may therefore have prognostic implications. While these repair deficits may contribute to genomic instability and aggressiveness, they may also sensitize tumor cells to damage by exogenous DNA damaging agents such as chemotherapy and ionizing radiation(35), suggesting a possible predictive utility to the analysis of tumor DNA repair capacity (36). Finally, DNA repair pathway assessment may also suggest new targets for therapy or identify subsets of patients that may respond to novel therapeutics that are targeted to DNA repair, such as PARP inhibitors.

Triple negative and basal-like breast cancers have been shown to harbor deficits in DNA repair. When loss of heterozygosity (LOH) is used as a marker for genomic instability, basal-like breast cancers have been shown to have the highest rate of LOH of all breast cancer subtypes(6). There is also a high degree of DNA copy number gains and losses associated with the basal-like subtype when analyzed by genome-wide array-based comparative genomic hybridization(5, 37–39). Furthermore, basal-like cancers have been shown to have comparatively more genomic losses on chromosome 5q, a site near a number of DNA repair and checkpoint genes (6, 37–39). Finally, familial *BRCA1* related cancers share many clinical and phenotypic features with triple negative cancers including high-grade, EGFR expression, *p53* mutations, ER/PR and Her2 negativity, cytogenetic abnormalities and cluster with basal-like tumors by DNA microarray analysis(1). The *BRCA1* protein is a key constituent of the HR/FA pathway and integral in the repair of DNA double strand breaks (DSBs)(4).

In this study, we hypothesized that immunohistochemical scoring of critical proteins in various DNA repair pathways could be used in triple negative breast cancer to identify subsets of patients with worse clinical outcome. We showed that basal expression of DNA repair proteins was variable in triple negative breast cancer, that the pattern of variability was antibody dependent and that DNA repair protein expression from different pathways was related in some cases. For example, no tumors with low XPF staining had high FANCD2 staining (data not shown). This finding is consistent with recent cell line data showing that XPF-ERCC1 deficient cells show dramatically reduced levels of chromatin bound FANCD2 and interstrand cross-link induced FANCD2 foci(40).

Low expression of four proteins- XPF, FANCD2, MLH1 and pMK2 was associated with shorter recurrence free survival on univariate analysis. These findings are consistent with the idea that tumors with reduced DNA repair capacity will have a higher degree of genomic instability and therefore behave more aggressively(23, 31, 33–35). The association of low expression of DNA repair proteins with more aggressive behavior is similar to the results of another study that used immunohistochemistry to assess DNA repair. In a scientific correlate to the International Adjuvant Lung Trial (IALT Bio), low ERCC1 expression, while predictive of response to cisplatin, was also associated with worse overall outcomes(36). Potentially, tumors that have reduced DNA repair capacity may be more susceptible to DNA damaging agents (leading to a predictive effect), but also may be able to develop more mutations that lead to aggressiveness and eventual resistance to therapy. We were unable to test this hypothesis in this study, which was not derived from a randomized trial in which specific treatment regimens were compared. The IALT Bio study was able to show this

relationship because the parent trial was a randomized study with no therapy in one arm and a uniform DNA damaging agent (cisplatin) in the experimental arm. Patients with low levels of ERCC1, a critical protein involved in the repair of cisplatin induced intra-strand cross-links through the NER pathway(41), comprised the subset that benefited from adjuvant cisplatin(36). Antibody panels such as ours should be further examined in similar in similar large datasets.

In the present study, we found that the assessing multiple DNA repair proteins from distinct pathways provided additional prognostic information than any one protein alone. Analysis of proteins in multiple pathways may provide independent information given the complexity of DNA repair processes. For example, repair of cisplatin-induced intrastand cross-links involves not only NER, but also coordination with other pathways such as the HR/FA pathway(15, 41–44). In addition to NER related sensitivity, defective HR/FA has been shown to be associated with platinum sensitivity(45, 46) and restoration of HR/FA is one mechanism of acquired platinum resistance(47–49). Preclinical models have further demonstrated that deficiencies in a number of DNA repair genes are associated with sensitivity to platinum(50–54). Therefore, deficits in either pathway might be associated with platinum sensitivity, necessitating analysis of both.

A potential utility of IHC assessment of DNA repair pathways is as a biomarker of response to novel therapeutics. Targeting DNA repair pathways with such therapeutics offers the potential for synthetic lethality. One example is the use of poly(ADP-ribose) polymerase (PARP) inhibitors in recurrent *BRCA*-deficient breast and ovarian cancers, and in unselected metastatic triple-negative breast cancers(9, 10, 12, 55). This strategy relies on the fact that base damage unrepaired by BER is converted to the more lethal DNA DSB, which is repaired by HR/FA. The synthetic lethality with PARP inhibitors is not limited to *BRCA1/2* deficient cells, but also extends to cells with deficits in a variety of proteins involved in HR(11). PARP inhibition is currently being evaluated in clinical trials of patients with *BRCA* mutations(12, 56, 57), but profiling of the HR/FA pathway, as we have done in this study using immunohistochemistry, might identify other subsets of patients who might benefit from PARP inhibitors.

Limitations of this study are several. The study is retrospective and the relatively small sample size permits only exploratory assessments. Additionally, the cohort is a convenience sample and follow up was therefore not uniform. The breast tumor tissue in this cohort was sampled prior to treatment with DNA damaging agents so analysis was an assessment of basal levels of protein expression rather than quantification of response to DNA damage. For some proteins (such as FANCD2) assessment under normal conditions can illustrate pathway integrity(4). But for others, the inducible level of protein in response to DNA damage measured in post-treatment tissue might provide additional information, albeit not as useful clinically. Finally, target sensitivity and specificity is a common concern with IHC. By using antibodies that were well characterized in other published work and/or by testing antibodies in FFPE gene deficient and complemented cell lines (pMK2, XPF, MLH1 and FANCD2, data not shown), we sought to mitigate this limitation. Further work in this area is ongoing.

In summary, we hypothesized that triple negative breast cancers have a high degree of associated DNA repair defects, that we could use antibodies against critical proteins involved in DNA repair pathways to characterize these defects, and that the IHC scoring of those antibodies could be used as prognostic markers. We were able to show that IHC scoring of DNA repair protein showed variability among a cohort of triple negative breast cancers and that this variability correlated with clinical outcome. Expressions of DNA repair proteins were not independent of each other, and combinations of antibodies showed more explanatory power than individual antibodies. The results of this study may reflect an underlying biology of these tumors and suggest a potential utility in identifying subsets of patients that might be sensitive to DNA damaging agents or novel therapeutics targeting DNA repair. The findings require further confirmation in larger datasets.

References

1. Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. *Lancet Oncol.* 2007; 8:235–244. [PubMed: 17329194]
2. Rouzier R, Perou CM, Symmans WF, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res.* 2005; 11:5678–5685. [PubMed: 16115903]
3. Schneider BP, Winer EP, Foulkes WD, et al. Triple-negative breast cancer: risk factors to potential targets. *Clin Cancer Res.* 2008; 14:8010–8018. [PubMed: 19088017]
4. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nature reviews.* 2004; 4:814–819.
5. Bergamaschi A, Kim YH, Wang P, et al. Distinct patterns of DNA copy number alteration are associated with different clinicopathological features and gene-expression subtypes of breast cancer. *Genes Chromosomes Cancer.* 2006; 45:1033–1040. [PubMed: 16897746]
6. Wang ZC, Lin M, Wei LJ, et al. Loss of heterozygosity and its correlation with expression profiles in subclasses of invasive breast cancers. *Cancer Res.* 2004; 64:64–71. [PubMed: 14729609]
7. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A.* 2003; 100:8418–8423. [PubMed: 12829800]
8. Bryant HE, Helleday T. Inhibition of poly (ADP-ribose) polymerase activates ATM which is required for subsequent homologous recombination repair. *Nucleic acids research.* 2006; 34:1685–1691. [PubMed: 16556909]
9. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature.* 2005; 434:913–917. [PubMed: 15829966]
10. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature.* 2005; 434:917–921. [PubMed: 15829967]
11. McCabe N, Turner NC, Lord CJ, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res.* 2006; 66:8109–8115. [PubMed: 16912188]
12. Yap T. First in human phase I pharmacokinetic (PK) and pharmacodynamic (PD) study of KU-0059436 (Ku), a small molecule inhibitor of poly ADP-ribose polymerase (PARP) in cancer patients (p), including BRCA1/2 mutation carriers. *Journal of Clinical Oncology, 2007 ASCO Annual Meeting Proceedings Part I.* 2007; 25
13. Ganguly A, Rock MJ, Prockop DJ. Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci U S A.* 1993; 90:10325–10329. [PubMed: 8234293]
14. Korkko J, Milunsky J, Prockop DJ, Ala-Kokko L. Use of conformation sensitive gel electrophoresis to detect single-base changes in the gene for COL10A1. *Hum Mutat.* 1998; (Suppl 1):S201–S203. [PubMed: 9452086]
15. D'Andrea AD, Grompe M. The Fanconi anaemia/BRCA pathway. *Nature reviews.* 2003; 3:23–34.

16. Moldovan GL, D'Andrea AD. How the Fanconi Anemia Pathway Guards the Genome. *Annu Rev Genet.* 2009
17. Shimamura A, Montes de Oca R, Svenson JL, et al. A novel diagnostic screen for defects in the Fanconi anemia pathway. *Blood.* 2002; 100:4649–4654. [PubMed: 12393398]
18. Sijbers AM, de Laat WL, Ariza RR, et al. Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. *Cell.* 1996; 86:811–822. [PubMed: 8797827]
19. Bhagwat N, Olsen A, Wang A, et al. XPF-ERCC1 participates in the Fanconi anemia pathway of crosslink repair. *Mol Cell Biol.* 2009
20. De Silva IU, McHugh PJ, Clingen PH, Hartley JA. Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol Cell Biol.* 2000; 20:7980–7990. [PubMed: 11027268]
21. Hoy CA, Thompson LH, Mooney CL, Salazar EP. Defective DNA cross-link removal in Chinese hamster cell mutants hypersensitive to bifunctional alkylating agents. *Cancer Res.* 1985; 45:1737–1743. [PubMed: 3919945]
22. Niedernhofer LJ, Garinis GA, Raams A, et al. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. *Nature.* 2006; 444:1038–1043. [PubMed: 17183314]
23. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature.* 2001; 411:366–374. [PubMed: 11357144]
24. Kennedy RD, D'Andrea AD. DNA repair pathways in clinical practice: lessons from pediatric cancer susceptibility syndromes. *J Clin Oncol.* 2006; 24:3799–3808. [PubMed: 16896009]
25. Manke IA, Nguyen A, Lim D, Stewart MQ, Elia AEH, Yaffe MB. MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. *Mol Cell.* 2005; 17:37–48. [PubMed: 15629715]
26. Kerfoot C, Huang W, Rotenberg SA. Immunohistochemical analysis of advanced human breast carcinomas reveals downregulation of protein kinase C alpha. *J Histochem Cytochem.* 2004; 52:419–422. [PubMed: 14966210]
27. Meng W, Swenson LL, Fitzgibbon MJ, et al. Structure of mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 suggests a bifunctional switch that couples kinase activation with nuclear export. *J Biol Chem.* 2002; 277:37401–37405. [PubMed: 12171911]
28. Li Y, Dong M, Kothari R. Classifiability-based omnivariate decision trees. *IEEE Trans Neural Netw.* 2005; 16:1547–1560. [PubMed: 16342495]
29. Eisen JA, Hanawalt PC. A phylogenomic study of DNA repair genes, proteins, and processes. *Mutat Res.* 1999; 435:171–213. [PubMed: 10606811]
30. Friedberg EC. DNA damage and repair. *Nature.* 2003; 421:436–440. [PubMed: 12540918]
31. Hoeijmakers JH. DNA damage, aging, and cancer. *N Engl J Med.* 2009; 361:1475–1485. [PubMed: 19812404]
32. Cohn MA, D'Andrea AD. Chromatin recruitment of DNA repair proteins: lessons from the fanconi anemia and double-strand break repair pathways. *Mol Cell.* 2008; 32:306–312. [PubMed: 18995829]
33. Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. *Proc Natl Acad Sci U S A.* 2003; 100:776–781. [PubMed: 12552134]
34. Loeb LA, Bielas JH, Beckman RA. Cancers exhibit a mutator phenotype: clinical implications. *Cancer Res.* 2008; 68:3551–3557. discussion 7. [PubMed: 18483233]
35. Madhusudan S, Middleton MR. The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer. *Cancer Treat Rev.* 2005; 31:603–617. [PubMed: 16298073]
36. Olausson KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med.* 2006; 355:983–991. [PubMed: 16957145]
37. Adélaïde J, Finetti P, Bekhouche I, et al. Integrated profiling of basal and luminal breast cancers. *Cancer Res.* 2007; 67:11565–11575. [PubMed: 18089785]
38. Andre F, Job B, Dessen P, et al. Molecular characterization of breast cancer with high-resolution oligonucleotide comparative genomic hybridization array. *Clin Cancer Res.* 2009; 15:441–451. [PubMed: 19147748]

39. Chin K, DeVries S, Fridlyand J, et al. Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. *Cancer Cell*. 2006; 10:529–541. [PubMed: 17157792]
40. Bhagwat N, Olsen AL, Wang AT, et al. XPF-ERCC1 participates in the Fanconi anemia pathway of crosslink repair. *Mol Cell Biol*. 2009
41. McHugh PJ, Spanswick VJ, Hartley JA. Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol*. 2001; 2:483–490. [PubMed: 11905724]
42. Kennedy RD, D'Andrea AD. The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes Dev*. 2005; 19:2925–2940. [PubMed: 16357213]
43. Timmers C, Taniguchi T, Hejna J, et al. Positional cloning of a novel Fanconi anemia gene, FANCD2. *Mol Cell*. 2001; 7:241–248. [PubMed: 11239453]
44. Garcia-Higuera I, Taniguchi T, Ganesan S, et al. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell*. 2001; 7:249–262. [PubMed: 11239454]
45. Byrski T, Huzarski T, Dent R, et al. Response to neoadjuvant therapy with cisplatin in BRCA1-positive breast cancer patients. *Breast Cancer Res Treat*. 2008
46. Fong PC, Yap TA, Boss DS, et al. Poly(ADP)-ribose polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. *J Clin Oncol*. 2010; 28:2512–2519. [PubMed: 20406929]
47. Edwards S, Brough R, Lord C, et al. Resistance to therapy caused by intragenic deletion in BRCA2. *Nature*. 2008
48. Sakai W, Swisher EM, Jacquemont C, et al. Functional restoration of BRCA2 protein by secondary BRCA2 mutations in BRCA2-mutated ovarian carcinoma. *Cancer Res*. 2009; 69:6381–6386. [PubMed: 19654294]
49. Sakai W, Swisher EM, Karlan BY, et al. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature*. 2008; 451:1116–1120. [PubMed: 18264087]
50. Albertella MR, Green CM, Lehmann AR, O'Connor MJ. A role for polymerase eta in the cellular tolerance to cisplatin-induced damage. *Cancer Res*. 2005; 65:9799–9806. [PubMed: 16267001]
51. Tassone P, Di Martino MT, Ventura M, et al. Loss of BRCA1 function increases the antitumor activity of cisplatin against human breast cancer xenografts in vivo. *Cancer Biol Ther*. 2009; 8
52. Sgagias MK, Wagner KU, Hamik B, et al. Brca1-deficient murine mammary epithelial cells have increased sensitivity to CDDP and MMS. *Cell Cycle*. 2004; 3:1451–1456. [PubMed: 15492509]
53. Clingen PH, Arlett CF, Hartley JA, Parris CN. Chemosensitivity of primary human fibroblasts with defective unhooking of DNA interstrand cross-links. *Exp Cell Res*. 2007; 313:753–760. [PubMed: 17188678]
54. Bulmer JT, Zagal NJ, Rainbow AJ. Human cells deficient in transcription-coupled repair show prolonged activation of the Jun N-terminal kinase and increased sensitivity following cisplatin treatment. *Cancer Chemother Pharmacol*. 2005; 56:189–198. [PubMed: 15841377]
55. O'Shaughnessy J, Osborne C, Pippen J, et al. Efficacy of BSI-201, a poly (ADP-ribose) polymerase-1 (PARP1) inhibitor, in combination with gemcitabine/carboplatin (G/C) in patients with metastatic triple-negative breast cancer (TNBC): Results of a randomized phase II trial. *J Clin Oncol*. 2009; 27
56. Audeh MW, Penson RT, Friedlander M, et al. Phase II trial of the oral PARP inhibitor olaparib (AZD2281) in BRCA-deficient advanced ovarian cancer. *J Clin Oncol*. 2009; 27
57. Tutt A, Robson M, Garber JE, et al. Phase II trial of the oral PARP inhibitor olaparib in BRCA-deficient advanced breast cancer. *J Clin Oncol*. 2009; 27

Statement of translational relevance

This study investigates the use of antibodies against critical proteins in DNA repair pathways as molecular biomarkers. We show that in triple negative breast cancers, low expression of several DNA repair proteins (as assessed in FFPE tumor specimens by semi-quantitative immunohistochemistry using these antibodies), is associated with worse progression-free survival. We also show the development of a multi-antibody model that is further able to stratify patients into recurrence risk categories.

The results of this study may be applied to the future practice of cancer medicine in a number of ways. First, our data suggest that these biomarkers have prognostic relevance. Second, many therapeutic regimens in oncology involve DNA damaging agents, so these DNA repair antibody biomarkers could have predictive capacity as has been suggested by other studies. Finally, these antibodies may prove to be useful as biomarkers in clinical studies of novel therapeutics that target DNA repair, such as poly(ADP-ribose) polymerase (PARP) inhibitors.

Figure 1. Immunohistochemistry patterns for the several DNA repair antibodies in one triple negative breast tumor

Figure 2. Inter and intra-patient immunohistochemical scoring variation

For each antibody, the X axis represents the sorted patient ranks according to mean score among the triplicate core specimens while the Y axis represents the immunohistochemical score. Error bars display the intra patient variability between each of the three cores for each individual sample.

Figure 3. Kaplan-Meier plot for Time to Recurrence According to High Versus Low Recurrence Risk Group as Defined by the Four-Antibody Model in the Training Set

Figure 4. Kaplan-Meier plot for Time to Recurrence According to High Versus Low Recurrence Risk Group as Defined by the Four-Antibody Model in the Test Set

Table 1

Patient distribution within the cohort

	Total	Training	Test		Total	Training	Test
Age				Treatment			
< 40	25	12	13	Unknown	1	1	0
<=50	34	26	8	BCT	64	42	22
>50	53	30	23	PMRT	17	9	8
				Mastectomy	30	16	14
T stage							
0	1	0	1	Chemotherapy			
1	60	38	22	None	4	4	0
2	38	22	16	Anthracycline	41	25	16
3	8	5	3	Anthracycline+taxane	49	28	21
4	5	3	2	CMF	15	9	6
				Other	3	2	1
Nodes positive							
0	62	38	24	BRCA1 mutation status			
1-3	32	21	11	Negative	78	45	33
>=4	18	9	9	Positive	18	14	4
				Unknown variant	5	3	2
M stage				Unknown	11	6	5
0	110	66	44				
1	2	2	0	Neoadjuvant chemo			
				Yes	7	4	3
Grade				No	105	64	41
unknown	1	1	0	LVI			
1	0	0	0	-	57	37	20
2	1	1	0	+	48	27	21
3	110	66	44	unknown	7	4	3

Table 2

A. Univariate and partition analysis biomarker output data from training cohort							
Biomarker	p-value ^a	AER ^b	Relative Risk	Odds Ratio	Positive Power	Negative Power	AUC ^c
FANCD2	0.001	0.3	3.83	7.52	0.57	0.85	0.71
XPF	0.005	0.3	2.66	4.77	0.56	0.79	0.67
PAR	0.293	0.35	1.64	2.29	0.5	0.7	0.54
pMK2	0.012	0.42	3.02	4.68	0.45	0.85	0.65
PARP1	0.259	0.41	1.5	1.88	0.43	0.71	0.53
MLH1	0.017	0.37	2.34	3.61	0.48	0.79	0.61
P53	0.024	0.35	2.06	3.11	0.5	0.76	0.6
Ki67	0.070	0.39	2.36	3.5	0.45	0.81	0.59
4-marker	9.05E-07	0.22	3.52	16.11	0.83	0.76	na

B. Univariate and multivariate analysis of clinical factors and biomarker panel in all patients for recurrence							
	Univariate analysis			Multivariate analysis			
	Value	95% CI		p-value	Value	95% CI	p-value
4-marker	5.8	2.84	11.9	1.50E-06	4.74	1.5	0.0017
Age	1	0.97	1.03	0.77	1.01	0.97	0.43
T stage	1.69	1.17	2.43	4.10E-03	1.54	0.83	0.038
N stage	2.15	1.44	3.22	1.90E-05	1.12	0.55	0.28
Tx	0.98	0.68	1.41	0.91	1.32	0.78	0.21
Chemo	1.23	0.87	1.74	0.24	1.1	0.65	0.8
BRCA	1.34	0.74	2.41	0.33	1.26	0.61	0.4
LVI	4.24	1.96	9.17	2.40E-04	1.57	0.55	0.36
Grade	55.6	4.50E-09	6.90E+11	0.73	167.4	3.00E-15	0.75

^a. p-value for separation of Early Recurrence from Late Recurrence groups;

^b. AER, Apparent Error Rate

^c. AUC, Area Under Curve value from Receiver Operator Characteristics; &. Ki67 quantity score, weighting used is 0111 for 0, 1+2+, 3+ bins #, 4-marker, multi-marker model containing FANCD2, XPF, PAR, pMK2