

Clinical Impact of Tumor DNA Repair Expression and T-cell Infiltration in Breast Cancers

Andrew R. Green¹, Mohammed A. Aleskandarany¹, Reem Ali², Eleanor Grace Hodgson¹, Suha Atabani¹, Karen De Souza³, Emad A. Rakha¹, Ian O. Ellis¹, and Srinivasan Madhusudan^{2,3}



Abstract

Impaired DNA repair drives mutagenicity, which increases neoantigen load and immunogenicity. We investigated the expression of proteins involved in the DNA damage response (ATM, Chk2), double-strand break repair (BRCA1, BLM, WRN, RECQL4, RECQL5, TOPO2A, DNA-PKcs, Ku70/Ku80), nucleotide excision repair (ERCC1), base excision repair (XRCC1, pol β , FEN1, PARP1), and immune responses (CD8, PD-1, PD-L1, FOXP3) in 1,269 breast cancers and validated our findings in an independent estrogen receptor–negative (ER[−]) cohort ($n = 279$). Patients with tumors that expressed low XRCC1, low ATM, and low BRCA1 were not only associated with high numbers of CD8⁺ tumor-infiltrating lymphocytes, but were also linked to higher grades, high proliferation indexes, pres-

ence of dedifferentiated cells, ER[−] cells, and poor survival (all $P \leq 0.01$). PD-1⁺ or PD-L1⁺ breast cancers with low XRCC1 were also linked to an aggressive phenotype that was high grade, had high proliferation indexes, contained dedifferentiated cells and ER[−] (all with P values ≤ 0.01), and poor survival ($P = 0.00021$ and $P = 0.00022$, for PD-1⁺ and PD-L1⁺ cancers, respectively) including in an independent ER[−] validation cohort ($P = 0.007$ and $P = 0.047$, respectively). We conclude that the interplay between DNA repair, CD8, PD-L1, and PD-1 can promote aggressive tumor phenotypes. XRCC1-directed personalization of immune checkpoint inhibitor therapy may be feasible and warrants further investigation in breast cancer. *Cancer Immunol Res*; 5(4); 292–9. ©2017 AACR.

Introduction

The breast cancer tumor microenvironment includes infiltrating inflammatory cells, such as lymphocytes and macrophages. CD8⁺ T lymphocytes are critical for tumor-specific adaptive immunity (1). We have previously investigated the clinicopathologic and prognostic significance of tumor-infiltrating CD8⁺ T lymphocytes (TIL) in a large cohort of breast cancers (2). CD8⁺ TILs were correlated to high tumor grade, hormone receptor negative, and basal-like phenotype. High total CD8⁺ counts were independently associated with favorable clinical outcome (2). A recent large study in 12,439 breast cancers has provided confirmatory evidence that CD8⁺ TILs are associated with significant reduction in the relative risk of death in estrogen receptor (ER)–negative as well as in ER⁺/HER-2⁺ breast cancers (3).

Breast cancers with enhanced immunogenicity will be prone to attack by CD8⁺ T lymphocytes. Impaired DNA repair and the associated genomic instability not only leads to increased mutagenicity/carcinogenicity but can also increase neoantigen load on tumor cell surface, resulting in increased immunogenicity. This concept of DNA repair deficiency and enhanced immunogenicity was shown in mismatch repair (MMR)-deficient colorectal cancers that had a good response to PD-1 blockade (pembrolizumab) therapy compared with tumors that are MMR proficient (4, 5). Whether a similar mechanism can also operate in breast cancers is currently unknown. In the current study, we profiled proteins involved in the DNA damage response (ATM, Chk2), double-strand break repair (BRCA1, BLM, WRN, RECQL4, RECQL5, TOPO2A, DNA-PKcs, Ku70/Ku80), nucleotide excision repair (ERCC1), base excision repair (XRCC1, pol β , FEN1, PARP1) and immune response [CD8, programmed death-1 (PD-1), programmed death ligand-1 (PD-L1), FOXP3] in 1,269 breast cancers and validated in an independent ER[−] cohort ($n = 279$).

Patients and Methods

The study was performed in a consecutive series of 1,650 patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1998 and enrolled into the Nottingham Tenovus Primary Breast Carcinoma series. Patient demographics are summarized in Supplementary Table S1. This is a well-characterized series of patients with long-term follow-up that have been investigated in a wide range of biomarker studies (6–14). All patients were treated in a uniform way in a single institution with standard surgery (mastectomy or wide local excision), followed by radiotherapy. Prior to 1989, patients did not receive systemic adjuvant treatment. After 1989, adjuvant treatment was scheduled

¹Department of Pathology, Division of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham, United Kingdom. ²Academic Unit of Oncology, Division of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham, United Kingdom. ³Department of Oncology, Nottingham University Hospitals, Nottingham, United Kingdom.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

A.R. Green and M.A. Aleskandarany are co-first authors of this article.

Corresponding Author: Srinivasan Madhusudan, School of Medicine, University of Nottingham, Nottingham University Hospitals, Nottingham NG51PB, United Kingdom. Phone: 4411-5823-1850; Fax: 4411-5823-1849; E-mail: srinivasan.madhusudan@nottingham.ac.uk

doi: 10.1158/2326-6066.CIR-16-0195

©2017 American Association for Cancer Research.

on the basis of prognostic and predictive factor status, including Nottingham prognostic index (NPI), estrogen receptor- α (ER- α) status, and menopausal status. Patients with NPI scores of <3.4 (low risk) did not receive adjuvant treatment. In premenopausal patients with NPI scores of ≥ 3.4 (high risk), classical cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy was given; patients with ER- α^+ tumors were also offered endocrine therapy. Postmenopausal patients with NPI scores of ≥ 3.4 and ER positivity were offered endocrine therapy, whereas ER $^-$ patients received classical CMF chemotherapy. Median follow-up time was 111 months (range, 1–233 months). Breast cancer-specific survival (BCSS) data were maintained on a prospective basis and were defined as the number of months from diagnosis to the occurrence of breast cancer-related death. Survival was censored if the patient was still alive at the time of analysis, lost to follow-up, or died from other causes.

We also evaluated an independent series of 279 ER- α^- invasive breast cancers diagnosed and managed at the Nottingham University Hospitals (Nottingham, United Kingdom) between 1999 and 2007. All patients were primarily treated with surgery, followed by radiotherapy and anthracycline/CMF chemotherapy. The characteristics of this cohort are summarized in Supplementary Table S2.

Tissue microarrays and IHC

Tumors were arrayed in tissue microarrays (TMA) constructed with 0.6-mm cores. The TMAs were immunohistochemically profiled for ATM, Chk2, BRCA1, BLM, WRN, RECQL4, RECQL5, TOPO2A, DNA-PKcs, Ku70/Ku80, ERCC XRCC1, pol β , FEN1, PARP1, CD8, and FOXP3 expression as described previously (2, 6–15). Supplementary Table S3 summarizes antigens, primary antibodies, clone, source, optimal dilution, scoring system, and cutoffs used for each DNA repair marker, ER, progesterone receptor (PR), and HER-2 expression. The specificity of the antibodies used is described in our recent publications (2, 6–15).

IHC protocol

Detailed IHC protocol and evaluation of immune staining is summarized in Supplementary Table S3. Immunohistochemical staining was performed using the Thermo Scientific Shandon Sequenza chamber system (REF: 72110017), in combination with the Novolink Max Polymer Detection System (RE7280-K: 1250 tests), and the Leica Bond Primary Antibody Diluent (AR9352), each used according to the manufacturer's instructions (Leica Microsystems). Leica Autostainer XL machine was used to dewax and rehydrate the slides. Pretreatment antigen retrieval was performed on the TMA sections using sodium citrate buffer (pH 6.0) and heated for 20 minutes at 95°C in a microwave (Whirlpool JT359 Jet Chef 1000W). Negative and positive (by omission of the primary antibody and IgG-matched serum) controls were included for each marker in each run. The negative control ensured that all the staining was produced from the specific interaction between antibody and antigen. HER2 expression was assessed according to the new ASCO/CAP guidelines using IHC and FISH (16).

Evaluation of immune staining

Whole-field inspection of the core was scored, and intensities of nuclear staining for DNA repair markers were grouped as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The percentage of each category was estimated

(0%–100%). H-score (range, 0–300) was calculated by multiplying intensity of staining and percentage staining. The number of CD8 $^+$ T lymphocytes was counted in each tumor core by using a Nikon Eclipse 80i microscope (Nikon) and an eyepiece graticule. CD8 $^+$ and FOXP3 $^+$ T cells were counted in three locations in each tumor: intratumoral compartment (within the tumor cell nests), within the distant stroma (defined as more than one tumor cell diameter away from the tumor), and within the adjacent stroma (defined as CD8 $^+$ cells within one tumor cell diameter of the tumor). The total number of CD8 $^+$ T cells was determined by combining the counts for these three compartments.

Not all cores within the TMA were suitable for IHC assessments, as some cores were missing or containing inadequate invasive tumor ($<15\%$ of whole core surface area). Tumor marker prognostic studies (REMARK) criteria, recommended by McShane and colleagues (17), were followed throughout this study. Ethical approval was obtained from the Nottingham Research Ethics Committee (C202313).

Statistical analysis

Data analysis was performed using SPSS (SPSS, version 21). Where appropriate, Pearson χ^2 , Fisher exact, the Student t , and one-way ANOVA tests were used. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazards model. The proportional hazards assumption was tested using standard log–log plots. HRs and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI and $P < 0.05$ considered significant. For multiple comparisons, P values were adjusted according to the Benjamini–Hochberg method (18).

Results

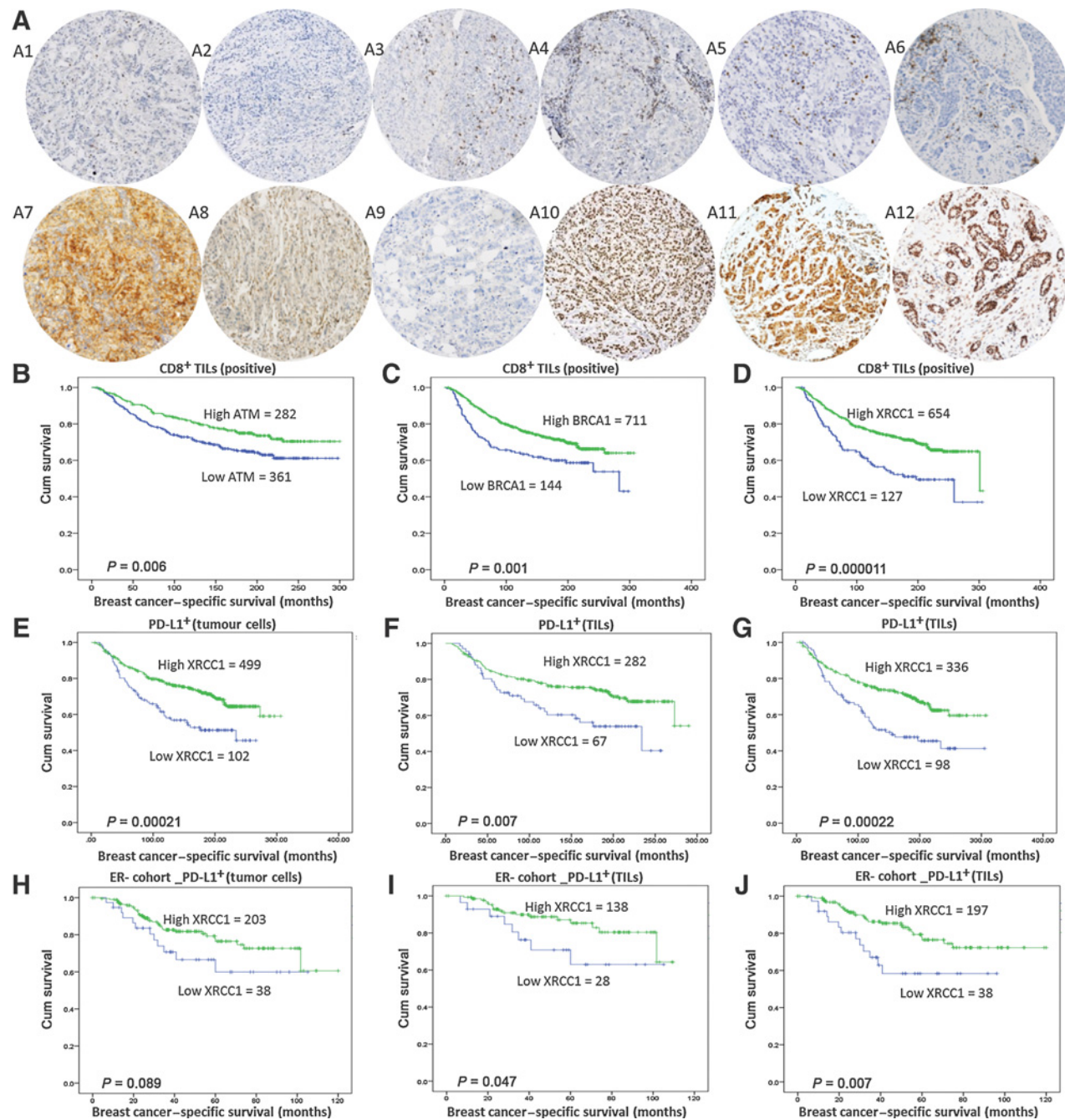
Significance of ATM, BRCA1, and XRCC1 in CD8 $^+$ TIL-positive breast cancers

CD8 $^+$ T lymphocytes are critical for tumor-specific adaptive immunity (1). A total of 1,269 invasive breast carcinomas [ER $^+$ ($n = 928$), ER $^-$ ($n = 341$), triple negative ($n = 219$), HER2 $^+$ /ER $^-$ ($n = 92$), and HER2 $^+$ /ER $^+$ ($n = 89$)] were suitable for CD8 $^+$ TIL assessments; 1,032 were positive for CD8 $^+$ TILs, and 237 cases were negative for CD8 $^+$ TILs (Fig. 1A1–A4).

Low ATM, BRCA1, and XRCC1 expression was associated with poor BCSS in tumors with CD8 $^+$ TILs ($P = 0.006$, 0.001, and 0.000011, respectively; Fig. 1A9–A12, B, C, and D), but not in tumors negative for CD8 $^+$ TILs ($P = 0.217$, 0.723, and 0.249, respectively; Supplementary Fig. S1A–S1C). Expression of pol β , ERCC1, RECQL4, RECQL5, BLM, PARP1, FEN1, TOPO2A, Ku70/Ku80, and Chk2 was not significantly associated with survival in CD8 $^+$ TIL-positive or negative breast cancers (Supplementary Fig. S2A–S2T). In tumors positive for CD8 $^+$ TILs, WRN did not influence survival ($P = 0.332$, Supplementary Fig. S2U), but in tumors negative for CD8 $^+$ TILs, low WRN influenced survival ($P = 0.026$, Supplementary Fig. S2V). Similarly, in tumors positive for CD8 $^+$ TILs, DNA-PKcs did not influence survival ($P = 0.996$, Supplementary Fig. S2W), but in tumors negative for CD8 $^+$ TILs, low DNA-PKcs influenced poor survival ($P = 0.044$, Supplementary Fig. S2X).

To investigate whether low tumor ATM, BRCA1, or XRCC1 expression increased CD8 $^+$ TILs counts and resulted in an aggressive phenotype, we proceeded to investigate clinicopathologic

Green et al.

**Figure 1.**

A, Immunohistochemical expression of CD8, FOXP3, PD-1, PD-L1, ATM, BRCA1, and XRCC1 in breast cancers (all images are at $\times 20$ magnification). **A1**, Invasive carcinoma, infiltrate with minimal lymphocytic infiltrate. **A2**, Invasive carcinoma with extensive CD8⁺ lymphocytic infiltrate. **A3**, Invasive carcinoma showing CD8⁺ intratumoral lymphocytic infiltrate. **A4**, Invasive carcinoma with extensive CD8⁺ peritumoral lymphocytic infiltrate. **A5**, FOXP3⁺ (TIL staining) invasive carcinoma. **A6**, PD-1⁺ (TIL staining) invasive carcinoma. **A7**, PD-L1⁺ (tumour cell staining) invasive carcinoma. **A8**, PD-L1⁺ (TIL staining) invasive carcinoma. **A9**, ATM⁻ invasive carcinoma. **A10**, ATM⁺ invasive carcinoma. **A11**, BRCA1⁺ invasive carcinoma. **A12**, XRCC1⁺ invasive carcinoma. **B**, Prognostic significance of ATM expression in CD8⁺ TIL-positive breast cancers (Kaplan–Meier survival curves is shown here). Cum, cumulative. **C**, Prognostic significance of BRCA1 expression in CD8⁺ TIL-positive breast cancers. **D**, Prognostic significance of XRCC1 expression in CD8⁺ TIL-positive breast cancers. **E**, Prognostic significance of XRCC1 expression in PD-L1⁺ (tumour cells) breast cancers. **F**, Prognostic significance of XRCC1 expression in PD-L1⁺ (TILs) breast cancers. **G**, Prognostic significance of XRCC1 expression in PD-L1⁺ (TILs) breast cancers. **H**, Prognostic significance of XRCC1 expression in PD-L1⁺ (tumour cells) ER⁻ breast cancers. **I**, Prognostic significance of XRCC1 expression in PD-L1⁺ (TILs) ER⁻ breast cancers. **J**, Prognostic significance of XRCC1 expression in PD-L1⁺ (TILs) ER⁻ breast cancers.

associations. The mean CD8⁺TIL counts were significantly higher in tumors with low ATM ($P = 0.004$), low BRCA1 ($P = 2.4 \times 10^{-9}$), and low XRCC1 ($P = 0.007$; Supplementary Fig. S3). Tumors with low ATM, low BRCA1, or low XRCC1 and that contained CD8⁺TILs were significantly more likely to manifest aggressive features, including high grade, high mitotic index, dedifferentiation, ER negativity, and PR negativity (all adjusted P values ≤ 0.05 ; Supplementary Tables S4–S6).

Significance of ATM, BRCA1, and XRCC1 in FOXP3⁺ breast cancers

T regulatory cells (Treg) can inhibit antitumor responses. FOXP3, a member of the forkhead family of transcription factors, is restricted to specific population of Tregs (19). In FOXP3⁺ breast cancers (Fig. 1A5), low BRCA1 ($P = 0.016$) and low XRCC1 ($P = 0.00002$) expression influenced survival but ATM did not ($P = 0.536$; Supplementary Fig. S4A–S4C). On the other hand, in FOXP3[−] breast cancers, low ATM influenced poor BCSS ($P = 0.001$; Supplementary Fig. S4D), but BRCA1 and XRCC1 amounts did not ($P = 0.556$ and 0.084 , respectively; Supplementary Fig. S4E and S4F). Low ATM, low BRCA1, or low XRCC1 and FOXP3⁺ breast cancers were highly significantly associated with high grade, high-risk NPI, high mitotic index, pleomorphism, HER-2⁺, and ER[−] and PR[−] phenotypes (all adjusted P values < 0.0001 ; Supplementary Tables S7–S9). The data provide compelling evidence that Treg infiltration along with tumor DNA repair expression can influence breast cancer pathology and outcomes.

Significance of ATM, BRCA1, and XRCC1 in PD-L1⁺/PD-1⁺ breast cancers

PD-L1 and PD-1 are key members of the programmed death pathway involved in immune regulation. The interaction of PD-L1 with PD-1 induces T-cell suppression. Accordingly, the PD-L1/PD-1 pathway has emerged as a key target for immune checkpoint inhibitor therapy (20). We investigated ATM, BRCA1, and XRCC1 expression in PD-L1⁺/PD-1⁺ or PD-L1[−]/PD-1[−] breast cancers (Fig. 1A6–A8).

Low XRCC1 expression was associated with poor BCSS in PD-L1⁺ (tumor cells), PD-L1⁺ (TILs), and PD-1⁺ (TILs) breast cancers ($P = 0.00021$, 0.007 , and 0.00022 , respectively; Fig. 1E–G). ATM and BRCA1 amounts did not influence survival in PD-L1⁺ or PD-1⁺ breast cancers (Supplementary Fig. S5A–S5F). In PD-L1[−] breast cancers, ATM, BRCA1, and XRCC1 did not influence survival (Supplementary Fig. S6A–S6F). In PD-1[−] breast cancers, low ATM, low BRCA1, and low XRCC1 were associated with poor BCSS (Supplementary Fig. S6G–S6I).

In PD-L1⁺ (tumor cells; Table 1), PD-L1⁺ (TILs; Table 2), or PD-1⁺ (TILs; Table 3) breast cancers, low XRCC1 amounts were significantly associated with aggressive features, including high grade, high-risk NPI, high mitotic index, pleomorphism, ER negativity, and PR negativity (all adjusted P values < 0.001). Taken together, the data provide evidence that low XRCC1 expression was associated with aggressive phenotype and poor outcomes in PD-L1⁺ and PD-1⁺ breast cancers. We then proceeded to investigate in breast cancer subgroups.

Prognostic significance of ATM, BRCA1, and XRCC1 in ER⁺ or HER-2⁺ breast cancers

In ER⁺ tumors, although CD8⁺TILs alone did not influence survival (Supplementary Fig. S7A–S7C), low BRCA1 ($P = 0.002$) and low XRCC1 ($P < 0.0001$) were linked to poor BCSS (Sup-

plementary Fig. S8A and S8B). Low ATM expression was not significant ($P = 0.080$; Supplementary Fig. S8C).

In PD-1⁺/PD-L1⁺ (TILs) or PD-L1⁺ (tumor cells) ER⁺ breast cancers that received endocrine therapy, ATM, BRCA1, or XRCC1 did not influence survival (unpublished observations). However, XRCC1 expression influenced survival in CD8⁺, PD-1⁺, or FOXP3⁺ ER⁺ breast cancers that received no endocrine therapy ($P = 0.02$, 0.038 , and 0.026 respectively; Supplementary Fig. S9A–S9C) but was not significant to PD-L1⁺ (tumor cells) breast cancers (Supplementary Fig. S9D, $P = 0.078$).

XRCC1 amounts influenced survival in PD-1⁺ HER-2⁺ breast cancers ($P = 0.011$; Supplementary Fig. S10A). ATM and BRCA1 did not associate with survival in CD8⁺, FOXP3⁺, or PD-1⁺ HER-2⁺ breast cancers (unpublished observations). BRCA1 expression was borderline associated with survival in FOXP3⁺ HER-2⁺ breast cancers ($P = 0.05$; Supplementary Fig. S10B). ATM and XRCC1 did not associate with survival in FOXP3⁺ HER-2⁺ breast cancers (unpublished observations). ATM, BRCA1, or XRCC1 did not influence survival in PD-L1⁺ (TILs) or PD-L1⁺ (tumor cells) HER2⁺ breast cancers (unpublished observations).

Prognostic significance of ATM, BRCA1, and XRCC1 in ER[−] breast cancers

As expected, CD8⁺TIL alone was associated with longer survival in ER[−] tumors ($P = 0.013$; Supplementary Fig. S11A), including in patients who received no chemotherapy ($P = 0.029$; Supplementary Fig. S11B), but was not significant to patients who received CMF-based chemotherapy ($P = 0.081$; Supplementary Fig. S11C). In ER[−] tumors with CD8⁺TILs that received CMF chemotherapy, low ATM, low BRCA1, or low XRCC1 did not influence survival (unpublished observations).

In PD-L1⁺ (TILs) ER[−] breast cancers that received no chemotherapy, BRCA1 amounts influenced survival (Supplementary Fig. S12), but ATM and XRCC1 did not (unpublished observations). ATM, BRCA1, or XRCC1 did not influence survival in FOXP3⁺, PD-1⁺, or PD-L1⁺ (tumor cells) ER[−] breast cancers that received no chemotherapy (unpublished observations). Similarly, ATM, BRCA1, or XRCC1 did not influence survival in FOXP3⁺, PD-1⁺, or PD-L1⁺ (tumor cells) ER[−] breast cancers that received CMF chemotherapy (unpublished observations).

We then proceeded to investigate an independent ER[−] cohort that received modern anthracycline-based chemotherapy. Low XRCC1 expression was associated with poor survival in PD-L1⁺ (TILs) and PD-1⁺ (TILs) ER[−] breast cancers ($P = 0.047$ and $P = 0.007$ respectively; Fig. 1I and J). Low XRCC1 expression was not significant ($P = 0.089$) to PD-L1⁺ (tumor cells) ER[−] breast cancers (Fig. 1H). ATM or BRCA1 did not influence survival in this cohort (unpublished observations).

Discussion

The presence of TILs is a marker for good prognosis (1–3, 21, 22) and predicts a favorable response to neoadjuvant chemotherapy in breast cancer (23). Although the biological mechanisms are poorly understood, immune effector cells, their cytokine secretions, or cancer cell immunogenicity may influence biology and antitumor response in breast cancer. In addition, chemotherapy-induced Treg depletion could also allow preexisting immune effector cells to operate effectively and induce antitumor responses (1). CD8⁺TILs can be good markers of response to neoadjuvant chemotherapy, providing evidence

Green et al.

Table 1. Clinicopathologic significance of XRCC1 expression in PD-L1⁺ (tumor cells) and PD-L1⁻ breast cancers

	PD-L1 (tumor cells) and XRCC1 expression				P	P _{adj}
	XRCC1 ⁺ /PD-L1 ⁺	XRCC1 ⁺ /PD-L1 ⁻	XRCC1 ⁻ /PD-L1 ⁺	XRCC1 ⁻ /PD-L1 ⁻		
(A) Pathologic parameters						
Tumor size						
<1 cm	39 (69.6%)	7 (12.5%)	9 (16.1%)	1 (1.8%)	0.018	0.022
>1-2 cm	258 (66.7%)	76 (19.6%)	37 (9.6%)	16 (4.1%)		
>2-5 cm	208 (67.1%)	35 (11.3%)	57 (18.4%)	10 (3.2%)		
>5 cm	8 (72.7%)	1 (9.1%)	2 (18.2%)	0 (0.0%)		
Tumor stage						
1	297 (65.4%)	87 (19.2%)	50 (11.0%)	20 (4.4%)	0.000966	0.00015
2	167 (69.0%)	23 (9.5%)	47 (19.4%)	5 (2.1%)		
3	52 (74.3%)	9 (12.9%)	8 (11.4%)	1 (1.4%)		
Tumor grade						
G1	80 (71.4%)	21 (18.8%)	9 (8.0%)	2 (1.8%)	7.45 × 10 ⁻⁷	< 0.00001
G2	183 (71.5%)	50 (19.5%)	14 (5.5%)	9 (3.5%)		
G3	251 (63.2%)	48 (12.1%)	82 (20.7%)	16 (4.0%)		
NPI						
≤3.4	145 (70.0%)	46 (22.2%)	10 (4.8%)	6 (2.9%)	0.000017	< 0.00001
>3.4	338 (65.3%)	70 (13.5%)	90 (17.4%)	20 (3.9%)		
Mitotic index						
M1 (low; mitoses < 10)	172 (72.6%)	45 (19.0%)	16 (6.8%)	4 (1.7%)	0.000029	0.0001
M2 (medium; mitoses 10-18)	102 (67.5%)	23 (15.2%)	17 (11.3%)	9 (6.0%)		
M3 (high; mitosis >18)	231 (64.9%)	40 (11.2%)	71 (19.9%)	14 (3.9%)		
Tubule formation						
1 (>75% definite tubule)	31 (79.5%)	4 (10.3%)	4 (10.3%)	0 (0.0%)	0.175	1.925
2 (10%-75% definite tubule)	175 (72.9%)	31 (12.9%)	26 (10.8%)	8 (3.3%)		
3 (<10% definite tubule)	299 (64.3%)	73 (15.7%)	74 (15.9%)	19 (4.1%)		
Pleomorphism						
1 (Small-regular uniform)	7 (63.6%)	3 (27.3%)	1 (9.1%)	0 (0.0%)	0.000108	0.0002
2 (Moderate variation)	203 (70.7%)	54 (18.8%)	19 (6.6%)	11 (3.8%)		
3 (Marked variation)	294 (66.1%)	51 (11.5%)	84 (18.9%)	16 (3.6%)		
Tumor type						
IDC-NST	313 (66.5%)	65 (13.8%)	76 (16.1%)	17 (3.6%)	0.002	0.0028
Tubular	103 (74.1%)	18 (12.9%)	14 (10.0%)	4 (2.9%)		
Medullary	9 (45.0%)	2 (10.0%)	7 (35.0%)	2 (10.0%)		
ILC	48 (67.6%)	19 (26.8%)	3 (4.2%)	1 (1.4%)		
Others	4 (44.4%)	3 (33.3%)	2 (22.2%)	0 (0.0%)		
Mixed NST & lobular/special type	26 (61.9%)	10 (23.8%)	3 (7.1%)	3 (7.1%)		
Her2 overexpression						
No	449 (68.1%)	100 (15.2%)	86 (13.1%)	24 (3.6%)	0.022	0.0242
Yes	64 (64.0%)	17 (17.0%)	16 (16.0%)	3 (3.0%)		
ER status						
Negative	117 (58.8%)	23 (11.6%)	46 (23.1%)	13 (6.5%)	6.9 × 10 ⁻⁷	< 0.00001
Positive	391 (71.2%)	90 (16.4%)	55 (10.0%)	13 (2.4%)		
PR						
Negative	185 (60.3%)	41 (13.4%)	64 (20.8%)	17 (5.5%)	0.000001	< 0.00001
Positive	316 (73.1%)	69 (16.0%)	39 (9.0%)	8 (1.9%)		

NOTE: Values in bold are statistically significant.

Abbreviations: IDC-NST, invasive ductal carcinoma-non-specific type; ILC, invasive lobular carcinoma.

that specific immune effector cells could be essential (23). Another possibility is that the immunogenicity of tumor cells themselves could influence immune effector cell anticancer activity. Tumor cells with abundant surface neoantigens will be prone to immune attack compared with tumor cells with low neoantigen load. Emerging data provide evidence that tumors with many somatic mutations accumulate neoantigens and are highly immunogenic (5). A key determinant of mutation load is the DNA repair capacity in cancer cells. DNA repair-deficient cancers have increased genomic instability, leading to a "mutator phenotype" characterized by the accumulation of mutations. For example, MMR-deficient colorectal cancers not only have 10 to 100 times more somatic mutations compared with MMR-proficient colorectal tumors, but also have prominent lymphocytic infiltration (5). A pivotal phase II study of PD-1 blockade by pembrolizumab provided the first compelling evidence that MMR-deficient colorectal cancers are more

responsive to immune checkpoint inhibitor therapy compared with MMR-proficient tumors (5). In breast cancers, however, MMR deficiency is rare (24), suggesting that impairment of other DNA repair factors may influence prognosis in tumors with immune cell infiltration.

In the current study, a key initial observation was that low amounts of ATM, BRCA1, and XRCC1 increased CD8⁺ TIL infiltration and were associated with aggressive pathology, leading to poor patient survival. Germline mutations in ATM or BRCA1 predispose to hereditary breast cancers. In sporadic breast cancers, epigenetic silencing of the BRCA1 promoter has been reported in up to 11% to 14% of tumors. About 25% of breast cancers may have a dysfunctional BRCA pathway in which they do not harbor germline BRCA mutations, but display similar phenotypes, including homologous recombination (HR) deficiency. XRCC1 deficiency delays SSB rejoining, induces mutations, and results in elevated numbers of sister chromatid exchanges, a hallmark of

Table 2. Clinicopathologic significance of XRCC1 expression in PD-L1⁺ (TILs) and PD-L1⁻ breast cancers

	PD-L1 (TILs) and XRCC1 expression				P	P _{adj}
	XRCC1 ⁻ /PD-L1 ⁻	XRCC1 ⁺ /PD-L1 ⁻	XRCC1 ⁻ /PD-L1 ⁺	XRCC1 ⁺ /PD-L1 ⁺		
(A) Pathologic parameters						
Tumor size						
<1 cm	4 (7.4%)	21 (38.9%)	6 (11.1%)	23 (42.6%)	0.044	0.0484
>1-2 cm	25 (7.3%)	160 (46.5%)	23 (6.7%)	136 (39.5%)		
>2-5 cm	23 (8.4%)	89 (32.5%)	37 (13.5%)	125 (45.6%)		
>5 cm	1 (9.1%)	3 (27.3%)	1 (9.1%)	6 (54.5%)		
Tumor stage						
1	31 (7.8%)	172 (43.1%)	32 (8.0%)	164 (41.1%)	0.075	0.825
2	20 (9.1%)	75 (34.1%)	29 (13.2%)	96 (43.6%)		
3	1 (1.5%)	27 (40.9%)	6 (9.1%)	32 (48.5%)		
Tumor grade						
G1	7 (7.0%)	51 (51%)	4 (4%)	38 (38%)	1.57 × 10 ⁻¹²	<0.00001
G2	12 (5.3%)	125 (55.6%)	6 (2.7%)	82 (36.4%)		
G3	34 (9.5%)	98 (27.3%)	57 (15.9%)	170 (47.4%)		
NPI						
≤3.4	10 (5.4%)	106 (57.6%)	4 (2.2%)	64 (34.8%)	3.8 × 10 ⁻⁸	<0.00001
>3.4	41 (8.8%)	159 (34.3%)	59 (12.7%)	205 (44.2%)		
Mitotic index						
M1 (low; mitoses < 10)	12 (5.5%)	112 (51.6%)	8 (3.7%)	85 (39.2%)	4.1 × 10 ⁻⁸	<0.00001
M2 (medium; mitoses 10-18)	13 (10.1%)	57 (44.2%)	7 (5.4%)	52 (40.3%)		
M3 (high; mitosis >18)	28 (8.7%)	94 (29.1%)	52 (16.1%)	149 (46.1%)		
Tubule formation						
1 (>75% definite tubule)	3 (8.6%)	17 (48.6%)	1 (2.9%)	14 (40%)	0.021	0.0257
2 (10%-75% definite tubule)	13 (6.1%)	102 (47.9%)	16 (7.5%)	82 (38.5%)		
3 (<10% definite tubule)	37 (8.8%)	144 (34.2%)	50 (11.9%)	190 (45.1%)		
Pleomorphism						
1 (Small-regular uniform)	1 (10.0%)	4 (40.0%)	0 (0%)	5 (50.0%)	8.5 × 10 ⁻⁹	<0.00001
2 (Moderate variation)	18 (7.2%)	136 (54.2%)	8 (3.2%)	89 (35.5%)		
3 (Marked variation)	34 (8.4%)	123 (30.2%)	59 (14.5%)	191 (46.9%)		
Tumor type						
IDC-NST	33 (7.8%)	132 (31.4%)	51 (12.1%)	205 (48.7%)	1.9 × 10 ⁻⁸	<0.00001
Tubular	9 (7.4%)	69 (56.6%)	7 (5.7%)	37 (30.3%)		
Medullary	3 (15.8%)	2 (10.5%)	6 (31.6%)	8 (42.1%)		
ILC	3 (4.7%)	39 (60.9%)	1 (1.6%)	21 (32.8%)		
Others	1 (20.0%)	2 (40.0%)	0 (0%)	2 (40.0%)		
Mixed NST & lobular/special type	4 (10.0%)	24 (60.0%)	2 (5.0%)	10 (25.0%)		
Her2 overexpression						
No	44 (7.5%)	248 (42.2%)	55 (9.4%)	241 (41.0%)	0.01	0.0138
Yes	10 (11.0%)	22 (24.2%)	9 (9.9%)	50 (54.9%)		
ER status						
ER ⁻	19 (10.5%)	34 (18.8%)	35 (19.3%)	93 (51.4%)	2.4 × 10 ⁻¹²	<0.00001
ER ⁺	31 (6.4%)	232 (47.5%)	31 (6.4%)	194 (39.8%)		
PR						
Negative	29 (10.7%)	78 (28.7%)	43 (15.8%)	122 (44.9%)	4.8 × 10 ⁻⁸	<0.00001
Positive	22 (5.7%)	185 (47.7%)	22 (5.7%)	159 (41.0%)		

genomic instability. Polymorphism in XRCC1 gene may increase the risk of cancer. We have previously shown that having little ATM or XRCC1 protein in somatic tumors is associated with aggressive breast cancers and poor survival (6, 14). We therefore speculate that reduced protein expression of ATM, BRCA1, or XRCC1 could lead to a "mutator phenotype," increase immunogenicity, promote CD8⁺ TILs, and influence tumor biology and outcome. However, a limitation to the current study is that we have not directly shown an increased mutational load in ATM/BRCA1/XRCC1-deficient tumors compared with ATM/BRCA1/XRCC1-proficient tumors. This will be an important area for future investigation. Given the essential role of Tregs in attenuating immune response in the tumor microenvironment (19), we also investigated the expression of ATM, BRCA1, and XRCC1 in FOXP3⁺ breast cancers. We observed highly significant associations with aggressive phenotypes and outcome, implying that ATM-, BRCA1-, and XRCC1-deficient breast

cancers elicit complex immune responses, including cytotoxic T-cell, as well as Treg, infiltration.

The PD-1 pathway is critical for immune regulation. PD-L1/PD-1 interaction induces T-cell repression. PD-L1/PD-1-targeted immune checkpoint inhibitor therapy is an exciting approach in cancers (20). Although durable responses have been seen in PD-L1⁺ non-small cell lung cancer (25), not all patients respond to pembrolizumab (a humanized mAb to PD-1). Therefore, evaluation of potential biomarkers that could allow personalization of PD-L1⁺ solid tumors is a high priority. We provide clinical evidence that XRCC1 expression can stratify patients into distinct prognostic groups in PD-L1⁺ and PD-L1⁻ breast cancers, including in ER⁻ breast cancers. In addition, low XRCC1 expression also promoted aggressive PD-L1⁺ and PD-L1⁻ breast cancer phenotypes, implying potential roles in breast cancer biology. Although none of the patients investigated in the current study received

Green et al.

Table 3. Clinicopathologic significance of XRCC1 expression in PD-1⁺ (TILs) and PD-1⁻ breast cancers

	PD-1 and XRCC1 expression				P	P _{adj}
	XRCC1 ⁻ /PD-1 ⁻	XRCC1 ⁺ /PD-1 ⁻	XRCC1 ⁻ /PD-1 ⁺	XRCC1 ⁺ /PD-1 ⁺		
(A) Pathologic parameters						
Tumor size						
<1 cm	4 (6.2%)	32 (49.2%)	7 (10.8%)	22 (33.8%)	0.111	0.137
>1-2 cm	19 (4.3%)	210 (47.9%)	41 (9.4%)	168 (38.4%)		
>2-5 cm	25 (7.1%)	129 (36.9%)	48 (13.7%)	148 (42.3%)		
>5 cm	1 (7.7%)	4 (30.8%)	2 (15.4%)	6 (46.2%)		
Tumor stage						
1	26 (5.2%)	232 (46.2%)	49 (9.8%)	195 (38.8%)	0.239	2.64
2	19 (6.6%)	118 (40.8%)	40 (13.8%)	112 (38.8%)		
3	4 (5.2%)	27 (35.1%)	8 (10.4%)	38 (49.4%)		
Tumor grade						
G1	5 (4.0%)	83 (66.9%)	6 (4.8%)	30 (24.2%)	1 × 10 ⁻¹³	<0.00001
G2	11 (4.1%)	152 (56.1%)	13 (4.8%)	95 (35.1%)		
G3	33 (7.0%)	141 (29.9%)	79 (16.7%)	219 (46.4%)		
NPI						
≤3.4	8 (3.6%)	139 (62.6%)	9 (4.1%)	66 (29.7%)	2.63 × 10 ⁻¹⁰	<0.00001
>3.4	39 (6.4%)	224 (37.0%)	85 (14%)	257 (42.5%)		
Mitotic index						
M1 (low; mitoses < 10)	7 (2.7%)	155 (59.6%)	14 (5.4%)	84 (32.3%)	3.7 × 10 ⁻¹²	<0.00001
M2 (medium; mitoses 10-18)	15 (9.1%)	76 (46.1%)	13 (7.9%)	61 (37.0%)		
M3 (high; mitosis >18)	27 (6.4%)	132 (31.4%)	70 (16.7%)	191 (45.5%)		
Tubule formation						
1 (>75% definite tubule)	2 (4.7%)	30 (69.8%)	3 (7.0%)	8 (18.6%)	0.000001	<0.00001
2 (10%-75% definite tubule)	20 (7.4%)	139 (51.5%)	17 (6.3%)	94 (34.8%)		
3 (<10% definite tubule)	27 (5.1%)	194 (36.5%)	77 (14.5%)	234 (44%)		
Pleomorphism						
1 (Small-regular uniform)	1 (8.3%)	7 (58.3%)	0 (0%)	4 (33.3%)	1 × 10 ⁻¹³	<0.00001
2 (Moderate variation)	15 (4.9%)	195 (63.5%)	15 (4.9%)	82 (26.7%)		
3 (Marked variation)	33 (6.3%)	160 (30.5%)	82 (15.6%)	250 (47.6%)		
Tumor type						
IDC-NST	35 (6.4%)	200 (36.5%)	71 (13.0%)	242 (44.2%)	3.3 × 10 ⁻⁸	<0.00001
Tubular	8 (5.2%)	93 (60.8%)	9 (5.9%)	43 (28.1%)		
Medullary	1 (4.3%)	2 (8.7%)	8 (34.8%)	12 (52.2%)		
ILC	2 (2.6%)	49 (62.8%)	3 (3.8%)	24 (30.8%)		
Others	0 (0%)	6 (66.7%)	1 (11.1%)	2 (22.2%)		
Mixed NST & lobular/special type	3 (6.5%)	19 (41.3%)	6 (13.0%)	18 (39.1%)		
Her2 overexpression						
No	40 (5.4%)	328 (44.5%)	80 (10.9%)	289 (39.2%)	0.123	0.1353
Yes	9 (7.1%)	42 (33.3%)	18 (14.3%)	57 (45.2%)		
ER status						
Negative	18 (7.6%)	60 (25.4%)	49 (20.8%)	109 (46.2%)	2.87 × 10 ⁻¹²	<0.00001
Positive	28 (4.6%)	308 (50.1%)	46 (7.5%)	233 (37.9%)		
PR						
Negative	26 (7.1%)	124 (33.8%)	67 (18.3%)	150 (40.9%)	8.1 × 10 ⁻⁹	<0.00001
Positive	19 (4%)	236 (50.2%)	31 (6.6%)	184 (39.1%)		

anti-PD-1 therapy, our data, taken together, would suggest that XRCC1 could aid in the personalization of anti-PD-1 therapies that are currently under investigation in PD-L1⁺ breast cancers. Prospective evaluation of this possibility is warranted in the context of clinical trials.

In conclusion, we provide clinical evidence that the interplay between DNA repair, CD8, PD-L1, and PD-1 can promote aggressive tumor phenotypes. XRCC1-directed personalization of immune checkpoint inhibitor therapy may be feasible in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Ali, I.O. Ellis, S. Madhusudan
Development of methodology: E.A. Rakha, S. Madhusudan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.R. Green, M.A. Aleskandarany, R. Ali, E.G. Hodgson, E.A. Rakha, I.O. Ellis, S. Madhusudan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.R. Green, M.A. Aleskandarany, S. Atabani, I.O. Ellis, S. Madhusudan

Writing, review, and/or revision of the manuscript: A.R. Green, M.A. Aleskandarany, S. Atabani, K. De Souza, E.A. Rakha, I.O. Ellis, S. Madhusudan

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.G. Hodgson, E.A. Rakha

Study supervision: E.A. Rakha, I.O. Ellis, S. Madhusudan

Acknowledgments

The authors are grateful to "Breast Cancer Now Tissue Bank" for tissue provision.

Received August 4, 2016; revised September 22, 2016; accepted February 20, 2017; published OnlineFirst March 2, 2017.

References

- Dushyanthen S, Beavis PA, Savas P, Teo ZL, Zhou C, Mansour M, et al. Relevance of tumor-infiltrating lymphocytes in breast cancer. *BMC Med* 2015;13:202.
- Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol* 2011;29:1949–55.
- Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, et al. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. *Ann Oncol* 2014;25:1536–43.
- Dudley JC, Lin MT, Le DT, Eshleman JR. Microsatellite Instability as a Biomarker for PD-1 Blockade. *Clin Cancer Res* 2016;22:813–20.
- Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 2015;372:2509–20.
- Abdel-Fatah TM, Arora A, Alsubhi N, Agarwal D, Moseley PM, Perry C, et al. Clinicopathological significance of ATM-Chk2 expression in sporadic breast cancers: a comprehensive analysis in large cohorts. *Neoplasia* 2014;16:982–91.
- Abdel-Fatah TM, Arora A, Moseley PM, Perry C, Rakha EA, Green AR, et al. DNA repair prognostic index modelling reveals an essential role for base excision repair in influencing clinical outcomes in ER negative and triple negative breast cancers. *Oncotarget* 2015;6:21964–78.
- Abdel-Fatah TM, Perry C, Arora A, Thompson N, Doherty R, Moseley PM, et al. Is there a role for base excision repair in estrogen/estrogen receptor-driven breast cancers? *Antioxid Redox Signal* 2014;21:2262–8.
- Albarakati N, Abdel-Fatah TM, Doherty R, Russell R, Agarwal D, Moseley P, et al. Targeting BRCA1-BER deficient breast cancer by ATM or DNA-PKcs blockade either alone or in combination with cisplatin for personalized therapy. *Mol Oncol* 2015;9:204–17.
- Arora A, Abdel-Fatah TM, Agarwal D, Doherty R, Croteau DL, Moseley PM, et al. Clinicopathological and prognostic significance of RECQL5 helicase expression in breast cancers. *Carcinogenesis* 2016;37:63–71.
- Arora A, Abdel-Fatah TM, Agarwal D, Doherty R, Moseley PM, Aleskandarany MA, et al. Transcriptomic and protein expression analysis reveals clinicopathological significance of bloom syndrome helicase (BLM) in breast cancer. *Mol Cancer Ther* 2015;14:1057–65.
- Arora A, Agarwal D, Abdel-Fatah TM, Lu H, Croteau DL, Moseley P, et al. RECQL4 helicase has oncogenic potential in sporadic breast cancers. *J Pathol* 2016;238:495–501.
- Shamanna RA, Lu H, Croteau DL, Arora A, Agarwal D, Ball G, et al. Camptothecin targets WRN protein: mechanism and relevance in clinical breast cancer. *Oncotarget* 2016;7:13269–84.
- Sultana R, Abdel-Fatah T, Abbotts R, Hawkes C, Albarakati N, Seedhouse C, et al. Targeting XRCC1 deficiency in breast cancer for personalized therapy. *Cancer Res* 2013;73:1621–34.
- Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Lee AH, Ellis IO, et al. An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. *Breast Cancer Res Treat* 2011;127:99–108.
- Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007;25:118–45.
- McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst* 2005;97:1180–4.
- Holm S. A simple sequentially rejective multiple test procedure. *Scand J Stat* 1979;6:65–70.
- Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008;9:239–44.
- Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Invest* 2015;125:3384–91.
- Denkert C, Wienert S, Poterie A, Loibl S, Budczies J, Badve S, et al. Standardized evaluation of tumor-infiltrating lymphocytes in breast cancer: results of the ring studies of the international immuno-oncology biomarker working group. *Mod Pathol* 2016;29:1155–64.
- Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol* 2015;26:259–71.
- Melichar B, Studentova H, Kalabova H, Vitaskova D, Cermakova P, Hornychova H, et al. Predictive and prognostic significance of tumor-infiltrating lymphocytes in patients with breast cancer treated with neoadjuvant systemic therapy. *Anticancer Res* 2014;34:1115–25.
- Caldes T, Perez-Segura P, Tosar A, De La Hoya M, Diaz-Rubio E. Low frequency of microsatellite instability in sporadic breast cancer. *Int J Oncol* 2000;16:1235–42.
- Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Czoszi T, Fulop A, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med* 2016;375:1823–33.

Cancer Immunology Research

Clinical Impact of Tumor DNA Repair Expression and T-cell Infiltration in Breast Cancers

Andrew R. Green, Mohammed A. Aleskandarany, Reem Ali, et al.

Cancer Immunol Res 2017;5:292-299. Published OnlineFirst March 2, 2017.

Updated version Access the most recent version of this article at:
doi:[10.1158/2326-6066.CIR-16-0195](https://doi.org/10.1158/2326-6066.CIR-16-0195)

Supplementary Material Access the most recent supplemental material at:
<http://cancerimmunolres.aacrjournals.org/content/suppl/2017/03/02/2326-6066.CIR-16-0195.DC1>

Cited articles This article cites 25 articles, 6 of which you can access for free at:
<http://cancerimmunolres.aacrjournals.org/content/5/4/292.full#ref-list-1>

Citing articles This article has been cited by 2 HighWire-hosted articles. Access the articles at:
<http://cancerimmunolres.aacrjournals.org/content/5/4/292.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerimmunolres.aacrjournals.org/content/5/4/292>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.