Clinical Cancer Research

A Randomized Phase II Neoadjuvant Study of Cisplatin, Paclitaxel With or Without Everolimus in Patients with Stage II/III Triple-Negative Breast Cancer (TNBC): Responses and Long-term Outcome Correlated with Increased Frequency of DNA Damage Response Gene Mutations, TNBC Subtype, AR Status, and Ki67

Bojana Jovanović[1], Ingrid A. Mayer[2], Erica L. Mayer[1], Vandana G. Abramson[2], Aditya Bardia[3], Melinda E. Sanders[2], M. Gabriela Kuba[2], Monica V. Estrada[2], J. Scott Beeler[2], Timothy M. Shaver[2], Kimberly C. Johnson[2], Violeta Sanchez[2], Jennifer M. Rosenbluth[1], Patrick M. Dillon[4], Andres Forero-Torres[5], Jenny C. Chang[6], Ingrid M. Meszoely[2], Ana M. Grau[2], Brian D. Lehmann[2], Yu Shyr[2], Quanhu Sheng[2], Sheau-Chiann Chen[2], Carlos L. Arteaga[2], and Jennifer A. Pietenpol[2]

Abstract

Purpose: Because of inherent disease heterogeneity, targeted therapies have eluded triple-negative breast cancer (TNBC), and biomarkers predictive of treatment response have not yet been identified. This study was designed to determine whether the mTOR inhibitor everolimus with cisplatin and paclitaxel would provide synergistic antitumor effects in TNBC.

Methods: Patients with stage II/III TNBC were enrolled in a randomized phase II trial of preoperative weekly cisplatin, paclitaxel and daily everolimus or placebo for 12 weeks, until definitive surgery. Tumor specimens were obtained at baseline, cycle 1, and surgery. Primary endpoint was pathologic complete response (pCR); secondary endpoints included clinical responses, breast conservation rate, safety, and discovery of molecular features associated with outcome.

Results: Between 2009 and 2013, 145 patients were accrued; 36% of patients in the everolimus arm and 49% of patients in the placebo arm achieved pCR; in each arm, 50% of patients achieved complete responses by imaging. Higher rates of neutropenia, mucositis, and transaminase elevation were seen with everolimus. Clinical response to therapy and long-term outcome correlated with increased frequency of DNA damage response (DDR) gene mutations, Basal-like1 and Mesenchymal TNBC-subtypes, AR-negative status, and high Ki67, but not with tumor-infiltrating lymphocytes.

Conclusions: The paclitaxel/cisplatin combination was well tolerated and active, but addition of everolimus was associated with more adverse events without improvement in pCR or clinical response. However, discoveries made from correlative studies could lead to predictive TNBC biomarkers that may impact clinical decision-making and provide new avenues for mechanistic exploration that could lead to clinical utility. *Clin Cancer Res; 23(15); 4035–45. ©2017 AACR.*

Introduction

Triple-negative breast cancer (TNBC) defines approximately 15% of breast cancers; TNBC lacks expression of estrogen, progesterone, and HER2 receptors (1). Patients with TNBC have an increased likelihood of distant recurrence and death compared with other types of breast cancer (2). Using gene expression (GE) analyses, six distinct TNBC subtypes (3) have been identified (TNBCtype). These include two basal-like (BL1 and BL2), an

[1]Dana-Farber Cancer Institute, Boston, Massachusetts. [2]Vanderbilt-Ingram Cancer Center, Nashville, Tennessee. [3]Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, Massachusetts. [4]University of Virginia Health Sciences Center, Charlottesville, Virginia. [5]University of Alabama, Birmingham, Alabama. [6]Methodist Hospital Research Institute, Houston, Texas.

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Corresponding Authors: Ingrid A. Mayer, Vanderbilt-Ingram Cancer Center, 2220 Pierce Avenue, 777 PRB, Nashville, TN 37232-6307. Phone: 615-322-4967; Fax: 615-343-7602; E-mail: ingrid.mayer@vanderbilt.edu; and Jennifer A. Pietenpol, jennifer.pietenpol@vanderbilt.edu

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Translational Relevance

To our knowledge, this is the largest randomized neoadjuvant study in TNBC with a PI3K/mTOR pathway inhibitor. Clinically, the combination of paclitaxel and cisplatin is well tolerated and active in TNBC, with pathologic responses comparable with traditional anthracycline/taxane combinations, and could be considered as an option for patients that cannot tolerate a more dose-intense chemotherapy regimen. The addition of everolimus did not improve response rates and was associated with more adverse events. This is the first study to prospectively demonstrate that patients whose tumors had high Ki67, no expression of androgen receptor (AR), and contained higher frequencies of DDR gene alterations (in particular Basal-like 1 and mesenchymal TNBC subtypes) had significant benefit from a platinum-containing chemotherapy regimen. The discoveries made from the correlative studies in this trial could lead to predictive TNBC biomarkers that may impact clinical decision-making.

immunomodulatory (IM), two mesenchymal (M and MSL), and luminal androgen receptor (LAR) subtypes; the last being characterized by AR signaling (3). The benefits of targeted therapies have largely eluded TNBC because of disease heterogeneity. Distinguishing one subtype of TNBC from another is histologically challenging, and prospective validation of which subset of TNBC benefits from a given chemotherapy, with or without molecularly targeted agents, is greatly needed.

On the basis of preclinical data supporting the roles of mTOR inhibitors, paclitaxel and cisplatin in activating proapoptotic signaling in tumor cells (4–9), we postulated that in TNBCs that express both p63 and p73, p63 promotes tumor cell survival through repression of p73. Furthermore, the combined use of drugs that impinge on the p63/p73 signaling axis (such as cisplatin and mTOR inhibitors; ref. 10) would have synergistic activity. To test preclinical observations *in vivo* and identify new therapeutic options for patients with TNBC, we conducted a randomized phase II study of neoadjuvant cisplatin and paclitaxel with or without everolimus (a TORC1 inhibitor) for 12 weeks in patients with stage II/III TNBC (NCT00930930). The goals of the study were to determine: (i) the rates of complete pathologic response (pCR) and clinical response, and (ii) if genomic and molecular analyses, including TNBCtype, p53, p63, and p73 status or other molecular features of the tumors, predict sensitivity and response to neoadjuvant therapy.

Materials and Methods

Study design

This was a multicenter, randomized, double-blinded, placebo-controlled, phase II clinical trial evaluating the combination of preoperative cisplatin, paclitaxel, with or without everolimus for a total of 12 weeks in patients with TNBC. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was obtained from all patients before enrollment, in agreement with approved protocols from respective ethics committees at every site.

Participants

Eligible patients were $\geq$18 years old, with clinical stage II or III triple-negative [defined as ER and PR none or weak staining in <10% cells by IHC and HER2-negative by Herceptest (0, 1+) or FISH (not amplified); by local assessment] invasive mammary carcinoma. Other key inclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and adequate hematologic and end-organ function. Key exclusion criteria included prior or concurrent treatment for the newly diagnosed breast cancer, and clinically significant cardiac, pulmonary, or liver dysfunction, malabsorption symptoms, active autoimmune disease, and immunocompromised status.

Imaging and tissue collection

Participants underwent breast imaging (diagnostic ultrasound) prior to treatment initiation and prior to definitive surgery, for clinical response assessment. Imaging response assessments were based on unidimensional ultrasound measurements and were defined as follows: complete response, no radiologic evidence of residual tumor; partial response, reduction in size of the tumor more than 30%; stable disease, reduction in size of the tumor inferior than 30%; and progressive disease, increase in size of the tumor or appearance of new lesions.

Paired snap-frozen and formalin-fixed paraffin-embedded (FFPE) biopsies were collected at baseline (baseline tumor collection) and 3–5 days after day 1/cycle 1 of treatment (cycle 1 tumor collection) for correlative analysis. At the time of definitive surgery, additional snap-frozen and formalin-fixed paraffin-embedded (FFPE) tissues were collected in patients that did not achieve a pCR (surgery tissue collection).

Randomization and blinding

Eligible patients were randomized according to a stratified permuted block scheme with 2:1 ratio (two-thirds in the everolimus arm and one-third in the placebo arm). Stratification was based on initial lymph node status assessment (positive or negative involvement) and grade (low/intermediate versus high).

Unblinding was allowed if the treating investigator deemed identification of the study drug necessary for the purpose of providing urgent patient care. Once unblinded, patient could continue study drugs until completion of 12 weeks of therapy if medically safe.

Treatment

We had previously completed a phase I/II study of paclitaxel, cisplatin, and weekly everolimus in patients with HER2-negative metastatic breast cancer at Vanderbilt University Medical Center (Nashville, TN; refs. 11, 12). The final recommended doses for the everolimus/cisplatin/paclitaxel combination in a phase II trial were everolimus 30 mg weekly or the equivalent 5 mg daily, paclitaxel 80 mg/m^2 weekly, and cisplatin 25 mg/m^2 weekly. To be able to differentiate the individual activity of cisplatin and everolimus in the p63/p73 apoptotic axis, we chose to delay initiation of paclitaxel for one week, to obtain the second tumor biopsy while patients were exposed to cisplatin and everolimus/placebo without paclitaxel interference.

Participants were randomized to receive:

- Arm A: cisplatin 25 mg/m^2 IV weekly for 12 weeks, everolimus 5 mg orally daily for 12 weeks, and paclitaxel 80 mg/m^2 IV

weekly for 11 weeks (starting 1 week after cisplatin initiation); or

- Arm B: cisplatin 25 mg/m^2 IV weekly for 12 weeks, placebo orally daily for 12 weeks, and paclitaxel 80 mg/m^2 IV weekly for 11 weeks (starting 1 week after cisplatin initiation)

Definitive surgery was scheduled 3–6 weeks after treatment completion. Postoperative adjuvant treatment was offered at the discretion of the treating team (not part of protocol procedures), but it is of note that over 90% of all patients in the trial (in both arms), regardless of pathologic response, received an anthracycline-containing regimen postoperatively.

Efficacy endpoints

The primary endpoint was to determine pCR (defined as absence of invasive carcinoma in the breast and axillary lymph nodes) rate for each individual treatment arm on an intention-to-treat basis. Secondary endpoints included rate of near pCR (residual invasive carcinoma in the breast of less than 0.5 cm in diameter with no lymph node involvement), rate of breast conservation surgery, clinical response [by breast imaging; RECIST (13)] immediately prior to surgery, safety profile of the treatment combinations, and therapy-mediated changes in the tumor. *A posteriori*, we also evaluated the Residual Cancer Burden (RCB) Index (14, 15) for each individual treatment arm.

Statistical analysis

Approximately 30% of early TNBCs treated with standard neoadjuvant anthracycline and taxane-based chemotherapy regimens without platinum agents achieve a pCR after treatment (16). The sample size estimation of this randomized phase II trial was completed using the Fisher exact test. With the proposed sample size of 145 (96 for everolimus and 49 for placebo), it provides at least 80% power to detect 20% difference of the pCR rates between two arms with one-sided type I error = 10%.

For lifetime data analyses, for example, disease-free survival (DFS; defined as the interval between initial diagnosis and evidence of locoregional or distant disease recurrence), possible risk factors for survival were compared using Kaplan–Meier estimates and log-rank tests. The correlative studies, including the IHC, TILs, and sequencing data, were analyzed using the following methods: (i) the Fisher exact test was used for between-group comparisons among the categorical variables of interest; (ii) the Wilcoxon signed rank test and Friedman test were applied to the paired and correlated (more than two groups) continuous outcomes; and (iii) the Kruskal–Wallis test was employed for testing the continuous outcomes between the independent study groups.

All tests were two-tailed and statistically significant level was set at 0.05. All analyses were performed with R statistical package (http:// www.r-project.org/).

Correlative studies

IHC. IHC for AR (Dako, catalog no. M3562 mouse), Ki67 (Dako, catalog no. M7240 mouse), p63 (Sigma, catalog no. P3737 mouse), p73 (Epitomics, catalog no. 1636-1 rabbit), and pS6 (Cell Signaling Technology, catalog no. 4857 rabbit) was performed on FFPE tissues obtained at baseline, completion of cycle 1, and definitive surgery. Antigen retrieval for AR was performed using citrate buffer (pH 6.0) in a decloaking chamber (Biocare Medical). The anti-AR antibody (Dako, catalog no. M3562) was used at 1:200 dilution overnight at 4°C. Visualization was achieved using the 4plus Detection System (BioCare) and 3,3′-diaminobenzidine (DAB; DAKO) as the chromogen. Antigen retrieval for Ki67 was performed using high pH buffer (pH 8.0) in a decloaking chamber (Biocare). The anti-Ki67 antibody (Dako, catalog no. M7240) was used at a 1:75 dilution overnight at 4°C. Visualization was achieved using the 4plus Detection System (BioCare) and DAB (DAKO). Antigen retrieval for p63 was performed using buffer (pH 6.0) in a decloaking chamber (Biocare). The anti-p63 antibody (Sigma, catalog no. P3737) was used at a 1:500 dilution overnight at 4°C. Visualization was achieved using the Envision detection system (DAKO) and DAB (DAKO). Antigen retrieval for p73 was performed using citrate buffer (pH 6.0) in a decloaking chamber (Biocare). The anti-p73 antibody (Sigma, catalog no. P3737) was used at a 1:200 dilution at room temperature for 1 hour. Visualization was achieved using the Envision detection system (DAKO) and DAB (DAKO). Antigen retrieval for pS6 (Cell Signaling Technology, catalog no. 4857) was performed using citrate buffer (pH 6.0) in a decloaking chamber (Biocare). The anti-pS6 antibody (Cell Signaling Technology, catalog no. 4857) was used at a dilution of 1:80 overnight at 4°C. Visualization was achieved using the Envision detection system (DAKO) and DAB (DAKO). AR-, Ki67-, p63-, and p73-stained sections were evaluated for nuclear staining. For Ki67, the percentage of tumor cells demonstrating nuclear staining of any intensity in hotspot regions was scored. For AR, p63, and p73, the percentage of total tumor cells stained and the average intensity (0, 1+, 2+, 3+) among all staining cells was recorded. For pS6, tumor cells were scored for cytoplasmic expression. The percentage of total tumor cell staining and the average intensity (0, 1+, 2+, 3+) among all stained cells was recorded. All IHC slides were evaluated and scored for by dedicated breast pathologists (M.E. Sanders, M.G. Kuba, and M.V. Estrada).

Pathologic assessment of tumor-infiltrating lymphocytes. Evaluation of tumor-infiltrating lymphocytes (TIL) was performed independently by two breast pathologists (M.V. Estrada and M.E. Sanders), who were blinded to clinical response information. Analysis of both stromal and intratumoral TILs was performed on the basis of the criteria recommended by the International TILs Working Group (17). This consisted of evaluating the percentages of TILs on a single hematoxylin and eosin (H&E)-stained baseline tumor breast biopsy section. All H&E sections were jointly evaluated, first for stromal TILs (percent of tumor stroma containing lymphocytes) then for intratumoral TILs (percent of lymphocytes in direct contact with tumor cells). The percentage of TILs was broken down into three categories: mild (0%–10%), moderate (20%–40%), and intense (>50%), and results were reported in increments of 10.

Sample preparation for next-generation sequencing. DNA was extracted from 98 FFPE breast core biopsies (sections ranged between 10 μm and 60 μm, depending on tumor cellularity) via QIAamp DNA FFPE Tissue Kit (Qiagen, catalog no. 56404). Extracted DNA was quantified with the Qubit 2.0 Fluorometer using the Qubit dsDNA HS Assay Kit (Life Technologies, catalog no. Q32854). DNA from genes in the OncoGxOne Breast Cancer Discovery panel ($n = 278$) was captured for each sample using [Agilent SureSelect Enrichment] 200 ng of captured DNA to generate sequencing libraries using the [OncoGxOne, GeneWiz]. Sequencing was performed on the Illumina HiSeq 2500 in high output mode with a 2 × 100 bp paired-end (PE) configuration.

RNA RNA was extracted from tissue sections of 50 fresh frozen breast core biopsies (a total number of sections ranged between 15 μm and 30 μm, depending on tumor cellularity) using the RNAqueous-Micro Total RNA Isolation Kit (Life Technologies| Ambion, catalog no. AM1931). Extracted RNA was quantified with the Qubit 2.0 Fluorometer using the Qubit RNA HS Assay Kit (Life Technologies, catalog no. Q32852) and assessed with the Agilent 2100 Bioanalyzer. Five-hundred nanograms of RNA were used for Directional rRNA Reduction RNA-seq library reactions on all samples (Illumina, catalog no. MRZE724 and NEB catalog no. E7550L). All samples were sequenced to a depth of 50e6 reads using the Illumina HiSeq 2500 on high output mode with a 2 × 100 bp PE configuration.

DNA sequencing analysis. DNA sequencing (DNA-seq) analysis was performed following GATK Best Practices workflow (18, 19). DNA-seq reads were aligned to hg19 (20) using BWA-MEM and duplicates were marked with PICARD.v.1.9 (http://sourceforge.net/p/picard) GATK v.3.3 was used to perform local realignment around indels, recalibrate base quality scores, and call variants. Variant calling was first performed on individual samples using HaplotypeCaller in gVCF mode and then samples were jointly genotyped. Variant filtering was performed with VQSR (21). Variant annotation was conducted with VEP and GEMINI (22, 23). Variants with low predicted severity impact and/or raw allele frequency ≥1% in ExAC [Exome Aggregation Consortium: http://exac.broadinstitute.org (accessed on May, 2015)] data were filtered out to enrich for somatic mutations with a role in tumorigenesis.

RNA sequencing analysis. Reads were aligned to the hg19 using the STAR (24) using the 2-pass method (25) and gene-level reads counts were quantified using HTSeq-Count (26). The FPKM values of each gene were calculated by in-house software then used for determining TNBC subtypes (27) as well as gene expression analysis. Variant calling was performed following the GATK Best Practices recommendations (18, 19) for RNA sequencing (RNA-seq). GATK (21) was used to perform base quality score recalibration, indel realignment, duplicate removal, and variant/indel calling. Variant/indel calling was performed across all samples simultaneously using variant quality score recalibration.

Results

A total of 145 women were randomized to everolimus ($n = 96$) or placebo ($n = 49$) between June 2009 and May 2013. Data cutoff was May 2015. Baseline characteristics were well balanced between arms (Table 1). A total of 115 patients completed 12 weeks of therapy as planned; 30 patients discontinued systemic treatment due to adverse events, withdrawal of consent, noncompliance or disease progression (Fig. 1A). Patients with adequate tissue availability at baseline, cycle 1, and surgery (when applicable) were included in the correlative studies: 87 patients for DNA/NGS analysis, 48 for RNA sequencing, and 115 for IHC and TIL analysis.

Pathologic and clinical outcome

Thirty-five of 96 (36%) patients in the everolimus arm and 24 of 49 (48%) patients in the placebo arm achieved a pCR, and 15 of 96 (16%) patients in the everolimus arm and 5 of 49 (10%) patients in the placebo arm achieved a near pCR ($P = 0.4084$).

Table 1. Baseline patient/disease characteristics

Treatment arm	Everolimus ($N = 96$)	Placebo ($N = 49$)	*P*
Median age	52 (43–57.25)	52 (43–58)	ns
Race/Ethnicity			ns
White	71 (75%) (1 Hispanic)	39 (78%) (2 Hispanics)	
Black	19 (20%)	8 (16%)	
Asian	1 (1%)	0	
BRCA1 or *BRCA2* Mutation			
Present	2 (2%)	4 (8%)	0.049
Absent	17 (18%)	3 (6%)	ns
Unknown	77 (80%)	42 (86%)	ns
Tumor grade			ns
II	18 (19%)	7 (14%)	
III	66 (70%)	38 (76%)	
Not reported	11 (11%)	5 (10%)	
AJCC Clinical stage			ns
II	33 (34%)	18 (37%)	
IIIA	56 (58%)	29 (59%)	
IIIB	6 (6%)	1 (2%)	
IIIC	1 (1%)	1 (2%)	
Median primary tumor size (cm)	2 (0.3–7.6)	1.4 (0.1–6.5)	ns

Fifty-two of 96 (54%) patients in the everolimus arm and 30 of 49 (61%) patients in the placebo arm had an RCB of 0–I ($P = 0.3905$; Table 2).

Seven patients did not undergo imaging assessments prior to surgery due to withdrawal of consent ($n = 2$) and disease progression ($n = 5$). Prior to definitive surgery, approximately 50% of patients in both arms achieved a complete imaging response, and about 30% of patients had a partial reduction of tumor volume ($P = 0.43$). Three patients had disease progression in the placebo arm, and 2 patients had disease progression in the everolimus arm (one of them developed meningeal carcinomatosis, which resulted in death). Forty percent (40%) and 32% of patients underwent breast conservation surgery in the everolimus and placebo treatment arms, respectively ($P = 0.99$). The remainder of patients underwent mastectomy for a variety of reasons, including multicentric disease, presence of calcifications, patient preference, or unfavorable tumor/breast volume ratio (Table 2).

As of May 2015, after a median of 42 months of follow-up, 84 (87%) of patients in the everolimus arm and 38 (78%) of patients in the placebo arm were alive, without evidence of locoregional or distant disease recurrence [$P = 0.28$; HR = 0.6450; 95% confidence interval (CI), 0.2863–1.4530; Fig. 1B]. One patient in the placebo arm developed pancreatic cancer and died in 2014, two years after her breast cancer diagnosis. In both arms, 95% of patients with pCR and near pCR are alive, without evidence of locoregional or distant disease recurrence, in contrast to 68% of patients with no pCR ($P < 0.0001$; HR = 0.6209; 95% CI, 0.0645–5.9750 for near pCR vs. pCR and HR = 7.2646; 95% CI, 2.14570–24.5950 for no pCR vs. pCR; Fig. 1C).

Safety

The addition of everolimus to cisplatin and paclitaxel was responsible for higher rates of oral mucositis (39 vs. 20%, $P = 0.03$), transaminase elevation (63 vs. 18%, $P = 0.0001$), rash (49 vs. 29%, $P = 0.0214$), nonfebrile neutropenia (52 vs. 38%, $P = 0.16$), and grade 3 nonfebrile neutropenia (26 vs. 11%). Everolimus use was associated with a higher rate of treatment discontinuation (21 vs. 6%). Only one patient developed grade 2

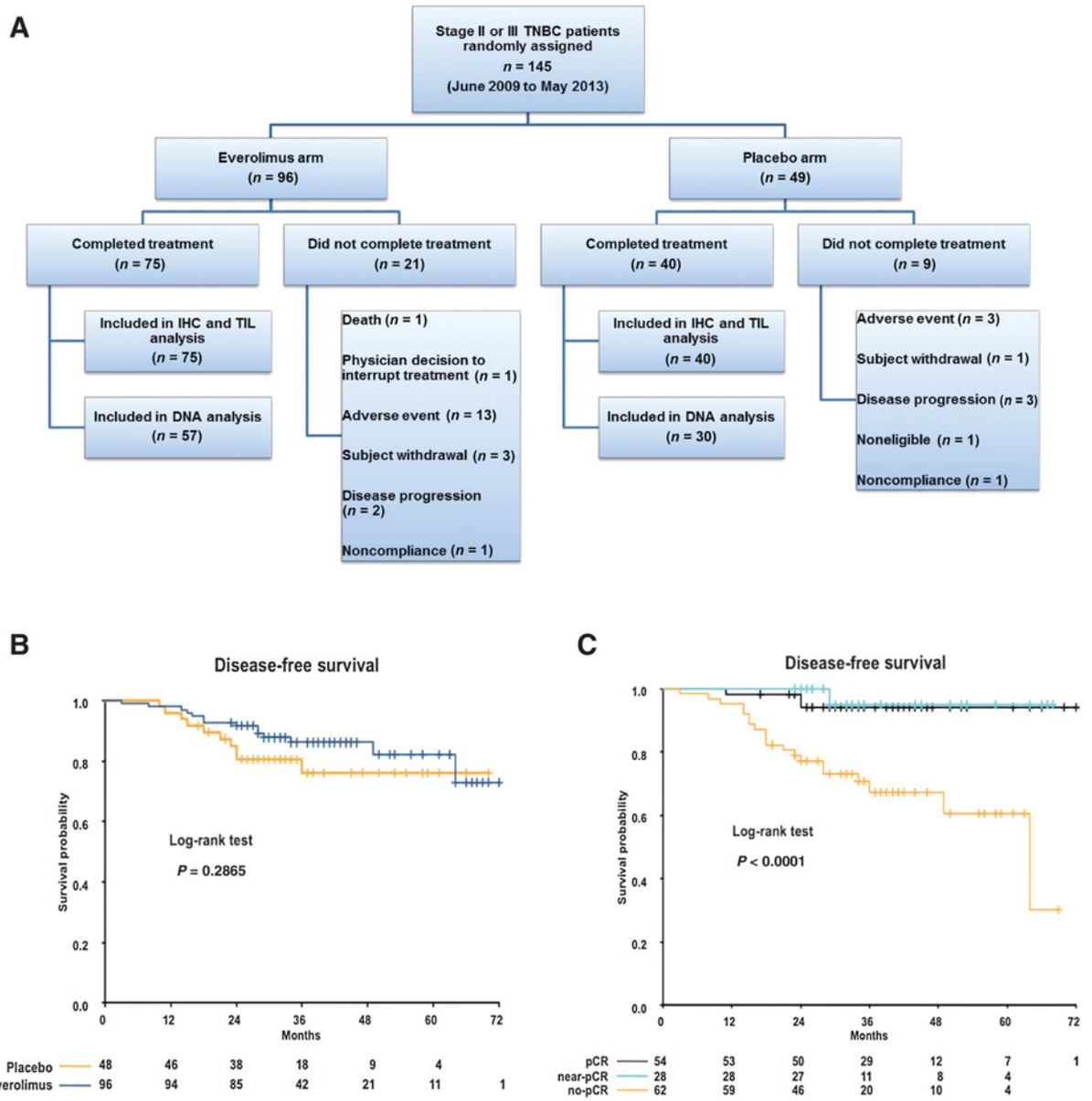


Figure 1.

Consort diagram of patient flow and analysis of disease-free survival by treatment arm and pathologic response. **A,** Consort Diagram of Patient Flow. **B,** Kaplan–Meier plot represents DFS of patients with TNBC in the placebo arm (orange) and everolimus arm (blue). Numbers of patients at risk over time are depicted under graph. No disease-free survival difference was detected between the treatment and placebo arms [log-rank test, $P = 0.2865$; Cox regression, HR = 0.6450, 95% CI = (0.2863–1.4530)]. **C,** Kaplan–Meier plot represents DFS of patients with TNBC achieving pCR (dark blue) near-pCR (light-blue) and no-pCR (orange). Numbers of patients at risk over time are depicted under graph. A disease-free survival difference was detected between the treatment and placebo arms [log-rank test, $P < 0.0001$; Cox regression, HR = 0.6209, 95% CI = (0.0645–5.9750) for near-pCR vs. pCR; HR = 7.2646, 95% CI = (2.14570–24.5950) for no-pCR vs. pCR].

everolimus-related pneumonitis, which resolved with everolimus interruption (Table 3).

Correlative studies

Gene alterations and TNBC subtypes. A total of 84 baseline TNBC tumor biopsies were evaluable for targeted DNA sequencing on 278 known breast cancer-associated genes (ref. 28; Supplementary Table S1). As previously reported (29, 30), we identified a high frequency of *TP53* mutations (54% of all sequenced tumors), regardless of clinical response (Fig. 2A). However, we observed that the proportion of mutations in genes with known functional roles in DNA damage repair (DDR) signaling and/or genome maintenance was higher in patients with pCR/near pCR than in patients with no pCR [71% vs. 31%; $P < 0.01$; OR = 0.2035; 95% CI = (0.0698–0.5584)], where definition of mutation was at least one mutation among *BRCA1* and BRCA2, *PALB2*, *ATM*, *NBN*,

Table 2. Pathologic and clinical responses by treatment arm

Treatment arm	Everolimus	Placebo	Total	*P*
Enrolled patients (N)	96	49	145	
Pathologic responses				0.4084
Complete (pCR)	35 (36%)	24 (48%)	59 (40%)	
Near	15 (16%)	5 (10%)	20 (14%)	
Residual disease	38 (39%)	19 (38%)	57 (39%)	
Tissue not available for analysis	9 (9%)	2 (4%)	11 (7%)	
Residual cancer burden				0.3905
0 (pCR)	35 (36%)	24 (48%)	59 (40%)	
I	17 (18%)	6 (12%)	23 (16%)	
II	29 (30%)	12 (24%)	41 (28%)	
III	7 (7%)	6 (12%)	13 (9%)	
Tissue not available for analysis	9 (9%)	2 (4%)	11 (7%)	
Clinical responses (breast imaging; RECIST)				0.43
Complete	48 (50%)	23 (48%)	71 (49%)	
Partial	27 (28%)	19 (40%)	46 (32%)	
No change	18 (19%)	5 (10%)	23 (16%)	
Disease progression	2 (2%)	0 (0%)	2 (1%)	
Nonevaluable	1 (1%)	1 (2%)	2 (1%)	
Definitive surgery				0.9999
Breast conservation	38 (40%)	19 (32%)	57 (39%)	
Mastectomy	57 (59%)	30 (61%)	87 (60%)	
Metastatic disease progression (No surgery)	1 (1%)	-	1 (1%)	

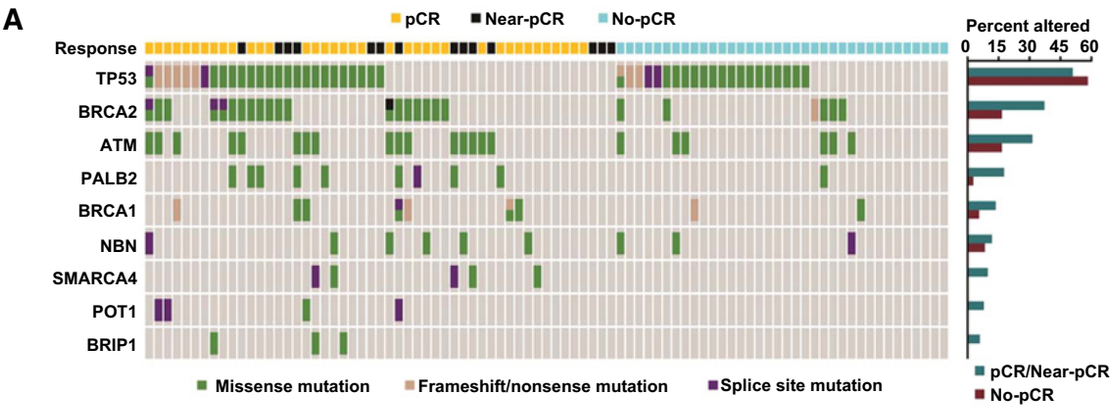
NOTE: Complete pathologic response = absence of invasive carcinoma in the breast and axilla; near pathologic response = $\leq$ 0.5 cm residual invasive carcinoma in the breast, negative lymph nodes; residual disease = > 0.5 cm of residual invasive carcinoma in the breast or lymph node involvement; RCB scores determined by the methods of Symmans (15) and Bossuyt (49). Test used: Fisher exact test.

SMARCA4, *POT1*, and *BRIP1* (Fig. 2A). No difference in the proportion of mutations in PI3K/AKT/mTOR signaling genes (*TSC1*, *TSC2*, *PIK3CA*, *PIK3CB*, *MTOR*, *AKT1* or *AKT2*) was observed between the pCR/near pCR and no pCR groups [35% vs. 33%; $P > 0.05$; OR = 0.9176; 95% CI = (0.3350–2.4617); Supplementary Fig. S1].

When we first subtyped TNBC (3), we reported that the immunomodulatory (IM) subtype is enriched for gene ontologies linked to immune cell processes. These processes include immune cell signaling, cytokine signaling, antigen processing and presentation, and signaling through core immune signal transduction pathways. Thus, TNBC tumors with IM subtype most likely represent a compilation of gene expression from tumor cells and TILs rather than tumor cell intrinsic, as described previously (3). Given several reports showing that TILs correlate with increased expression of genes involved in immune response (31–34), we hypothesized that the IM TNBC subtype defines tumors that have significant immune infiltrates, and that the immune response and resulting gene expression variably impact gene expression profiles of the tumor biopsy, and thus ability to subtype the actual tumor cells. To test this hypothesis, we correlated the initial TNBC subtype of 48 cases (Fig. 2B) with the pathologists' scoring of

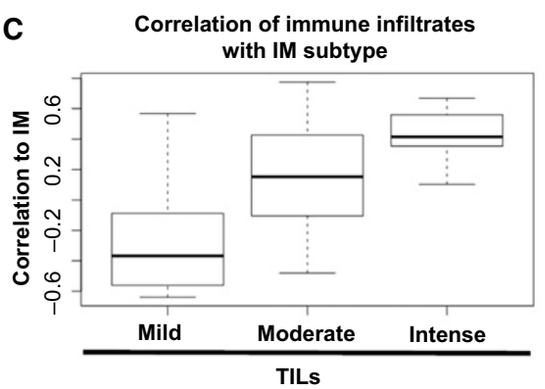
Table 3. Total adverse events by treatment arm

	Everolimus ($n = 96$)		Placebo ($n = 49$)		
	N (%) of subjects				*P* (Fisher
Toxicity category, Toxicity (CTCAE v.4)	Total	Grade 3,4	Total	Grade 3,4	exact test)
Gastrointestinal disorders					
Nausea	58 (60%)	—	32 (66%)	1 (2%)	0.5924
Diarrhea	31 (32%)	2 (2%)	14 (29%)	—	0.7071
Mucositis (oral)	37 (39%)	—	10 (20%)	—	0.0384
Dyspepsia	29 (30%)	—	17 (35%)	—	0.5783
General disorders					
Fatigue	61 (64%)	3 (3%)	37 (75%)	—	0.1894
Investigations					
Transaminase elevation	60 (63%)	3 (3%)	9 (18%)	—	0.0001
Creatinine elevation	6 (6%)	3 (3%)	5 (10%)	—	0.5089
Metabolism and nutrition disorders					
Hyperglycemia	49 (51%)	—	21 (42%)	1 (2%)	0.3834
Hypercholesterolemia	9 (9%)	—	3 (7%)	—	0.7513
Skin and subcutaneous tissue disorders					
Rash	47 (49%)	—	14 (29%)	1 (2%)	0.0214
Blood and lymphatic system disorders					
Anemia	62 (65%)	6 (6%)	37 (77%)	1 (2%)	0.1935
Neutropenia; no fever	50 (52%)	25 (26%)	19 (38%)	5 (11%)	0.1602
Thrombocytopenia	38 (40%)	—	4 (9%)	—	0.0001
Special interest					
Pneumonitis	1 (1%)	—	—	—	—
Nervous system disorders					
Neuropathy	47 (49%)	—	29 (59%)	—	0.2926



B

		pCR N = 20		Near-pCR N = 9		No-pCR N = 19		Total N = 48	
		Primary n (%)	After removal of IM n (%)	Primary n (%)	After removal of IM n (%)	Primary n (%)	After removal of IM n (%)	Primary n (%)	After removal of IM n (%)
TNBC-Subtypes	BL1	2 (33%)	8 (53%)	2 (33%)	3 (20%)	2 (33%)	4 (27%)	6 (13%)	15 (31%)
	BL2	1 (100%)	1 (33%)	0	1 (33%)	0	1 (33%)	1 (2%)	3 (6%)
	IM	4 (40%)	----------	1 (10%)	----------	5 (50%)	----------	10 (21%)	----------
	M	7 (50%)	7 (47%)	2 (14%)	2 (13%)	5 (36%)	6 (40%)	14 (29%)	15 (31%)
	MSL	2 (33%)	3 (33%)	3 (50%)	3 (33%)	1 (17%)	3 (33%)	6 (13%)	9 (19%)
	LAR	1 (33%)	1 (25%)	0	0	2 (67%)	3 (75%)	3 (6%)	4 (8%)
	UNS	3 (38%)	0	1 (13%)	0	4 (50%)	2 (100%)	8 (17%)	2 (4%)



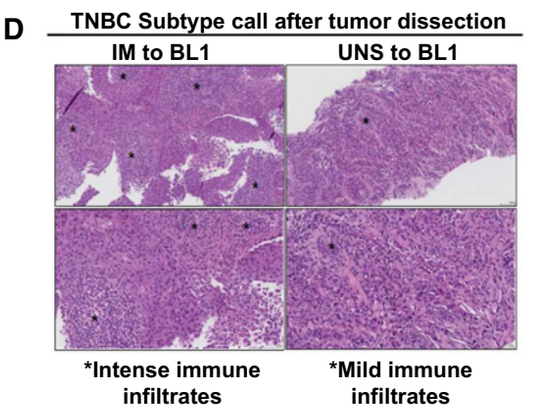


Figure 2.

Alterations in DNA damage response (DDR) genes and BL1 and M subtypes are enriched in clinical responders. **A,** Histogram of patient-specific mutations colored according to the mutation status (green, missense; brown, frameshift/nonsense; purple, splice site). Patients ($n = 87$) are annotated in the map according to their clinical response category: pCR ($N = 36$, gold), near pCR ($N = 15$, black), and no pCR ($N = 36$, teal). The graph on the right represents alteration percentage of pCR/near pCR versus no pCR. *TP53* mutation was seen in 54% of all sequenced tumors, regardless of clinical response. However, we found a higher proportion of mutations in genes with known functional roles in DNA damage repair signaling and maintenance of genomic instability in patients with a pCR/near pCR compared with patients with no pCR [71% vs. 31%; Fisher exact test, $P < 0.01$; OR = 0.2035, 95% CI = (0.0698–0.5584)]. **B,** The TNBC subtypes across 48 patients grouped into clinical response categories presented as initial output or "primary" and second output or "after removal of IM" from the TNBC subtype. The number of patients within in each subtype is presented along with the percent within its response category. **C,** Box and whisker plot for correlation of immune infiltrates with IM subtype across mild, moderate, and intense stain of TILs. *P* values are as follows: 0.0007 (mild vs. moderate), 0.0005 (mild vs. intense), 0.1432 (moderate vs. intense), 0.0001 (mild vs. moderate/intense) for unpaired *t* test. **D,** H&E representative images for TNBC tumor tissues with intense immune infiltrates versus mild immune infiltrates. The areas with immune infiltrates are marked with (*).

immune infiltrates (intense, moderate, mild), and found a statistically significant correlation between the IM TNBC-subtype and the level of TILs (Fig. 2C). To determine the extent to which surrounding nontumor cells contributed to gene expression profiles of the TNBC subtypes in the study, we performed laser capture microdissection (LCM) on cases that were subtyped as IM or unclassified (UNS) and for which sufficient tissue was available, followed by RNA isolation, gene expression analysis, and TNBC subtyping. Representative cases are shown in Fig. 2D, in which the subtype switched from IM or UNS to BL1 after dissection of as much stroma as possible from the tumor sections. Given these results, we reanalyzed the same 48 cases, and for those with an initial IM or UNS classification, we reassigned the second most statistically significant subtype generated by the TNBCtype algorithm (Fig. 2B). With removal of the IM features, we observed that there was an enrichment of patients with BL1 subtype tumors in the pCR group, whereas the majority of patients with LAR subtype were in the no pCR group.

All patients with BL1 TNBC subtype were alive, without evidence of locoregional or distant disease recurrence after a median of 42 months follow-up (May 2015 data cutoff), in contrast to 75% of patients with MSL and 65% of patients with BL2 TNBC subtypes ($P < 0.0001$; HR = 22.2367; 95% CI, 1.1663–3270.962 for BL2 vs. BL1; HR = 35.4715;95% CI, 3.3619–4805.502 for LAR vs. BL1; HR = 3.2085; 95% CI, 0.1711–468.249 for M vs. BL1; HR = 11.2859; 95% CI, 1.0763–1523.4870 for MSL vs. BL1; Supplementary Fig. S2). This pattern is consistent with favorable outcome to DNA-damaging therapies such as cisplatin, which target cell replication processes.

Ki67, AR protein expression, and TILs in tumor biopsies. In accordance with previous studies (35, 36), we observed a significantly higher pretreatment/baseline Ki67 expression level in tumors from patients with pCR/near pCR, compared with those from patients with no pCR (Fig. 3A). Ki67 expression levels remained unchanged throughout the course of treatment in the pCR patients, while a decrease was observed after treatment in the near pCR (significant) and no pCR (nonsignificant) groups. We also found a trend toward higher median expression levels of Ki67 in BL1 and M compared with the rest of the TNBCtypes (Fig. 3B).

We performed AR IHC analysis in 115 evaluable tumor specimens from the trial. As anticipated, about 10% of specimens had high expression of AR (ranging from 30% to 99% positive nuclei). We noted low AR expression (median $\leq$ 10% positive nuclei) in patients with pCR/near pCR on both arms of the trial; however, higher AR expression (median 63%; range 4–99% positive nuclei) was observed in the tumors of no pCR patients ($P = 0.09$; Fig. 3C). AR expression did not change in subsequent tissue sampling (biopsy at cycle 1) in either arm (data not shown). After a 42-month follow-up, 15% of patients with AR-negative TNBC had a locoregional or distant disease recurrence, in contrast to 43% of patients with AR-positive tumors ($P = 0.05$; HR = 2.78; 95% CI, 0.9394–8.2260; Fig. 3D), further suggesting lack of response to chemotherapy $\pm$ everolimus in luminal TNBC.

After evaluation of both stromal and intratumoral TILs at baseline biopsy specimens, we found that neither correlated with pathologic responses observed in this trial (Supplementary Fig. S4A and S4B).

Everolimus and cisplatin modulation of p73 and p63 expression in vivo. Higher expression levels of p73 were seen in cycle 1 biopsy specimens of pCR patients treated with everolimus ($P = 0.03$) and in no pCR patients treated with placebo ($P = 0.004$; Supplementary Fig. S4A). Baseline specimens of patients that achieved a pCR tended to have higher levels of p63 expression. There was a significant decrease in p63 expression levels in specimens from pCR patients after treatment with everolimus and cisplatin ($P < 0.01$; Supplementary Fig. S4B). Of note, the average percentage of p63-positive cells was almost six times lower than percent of p73-positive cells. No positive correlation was observed between p73 and p63, nor between clinical response and levels of expression of either p73 or p63 (Supplementary Fig. S4A and S4B).

mTOR activity assessment by pS6 expression levels. To evaluate whether everolimus decreased mTOR activity *in vivo* (37, 38), we performed IHC analysis of phospho-S6 (pS6) on baseline and cycle 1 biopsy specimens. We detected robust baseline pS6 expression across all patients and observed a statistically significant decrease at cycle 1 biopsy (Supplementary Fig. S5A and S5B), suggesting drug-induced inhibition of TORC1. However, no significant correlation was observed between clinical response and decrease in pS6 expression within the evaluable patient cohort (data not shown).

Discussion

Patients with breast cancer who achieve a pCR after neoadjuvant chemotherapy have been demonstrated to exhibit favorable long-term outcomes (39). In our study, a relatively short course of neoadjuvant chemotherapy with cisplatin and paclitaxel in patients with stage II/III TNBC was safe and well tolerated and yielded pCR rates comparable with traditional anthracycline/taxane combinations (16). Therefore, a cisplatin/paclitaxel combination could be considered an option for TNBC patients that cannot tolerate a more dose-intense anthracycline/taxane-containing chemotherapy regimen. The addition of everolimus at 5 mg daily, despite an observed decrease in pS6, added toxicity and did not improve overall pathologic and clinical outcomes. A potential limitation of our study was the inability to combine all three drugs at a dose that enabled the targeted agent (everolimus) to more effectively "hit" target and be evaluated in TNBC. As administered, the addition of everolimus did not seem to be associated with a negative chemotherapy benefit and did not have a negative effect on long-term outcome.

The intrinsic genomic instability of certain TNBCs (most notably basal-like and *BRCA1/2*-mutant breast cancers; ref. 40) results from deficient DNA repair mechanisms (41) and may be responsible for their sensitivity to platinum agents (42). This is the first study to prospectively demonstrate that patients whose tumors contain higher frequencies of DDR gene alterations (in particular BL1 and M TNBC subtypes) are more likely to benefit from a platinum-containing chemotherapy regimen. Interestingly, genomic alterations in the PI3K/mTOR pathway were unable to predict benefit from everolimus or chemotherapy.

Previously, we showed that the LAR subtype tumors comprise about 10% of TNBC, exhibit transcriptional features of AR receptor signaling, and are sensitive to the AR antagonist bicalutamide, but not to cisplatin (3). In a previous study (43), investigators reported that the LAR TNBC-subtype had a good long-term outcome despite lack of response to chemotherapy, most likely due to slow growth as demonstrated by low Ki67 expression levels typically seen in these tumors. In our current study, several

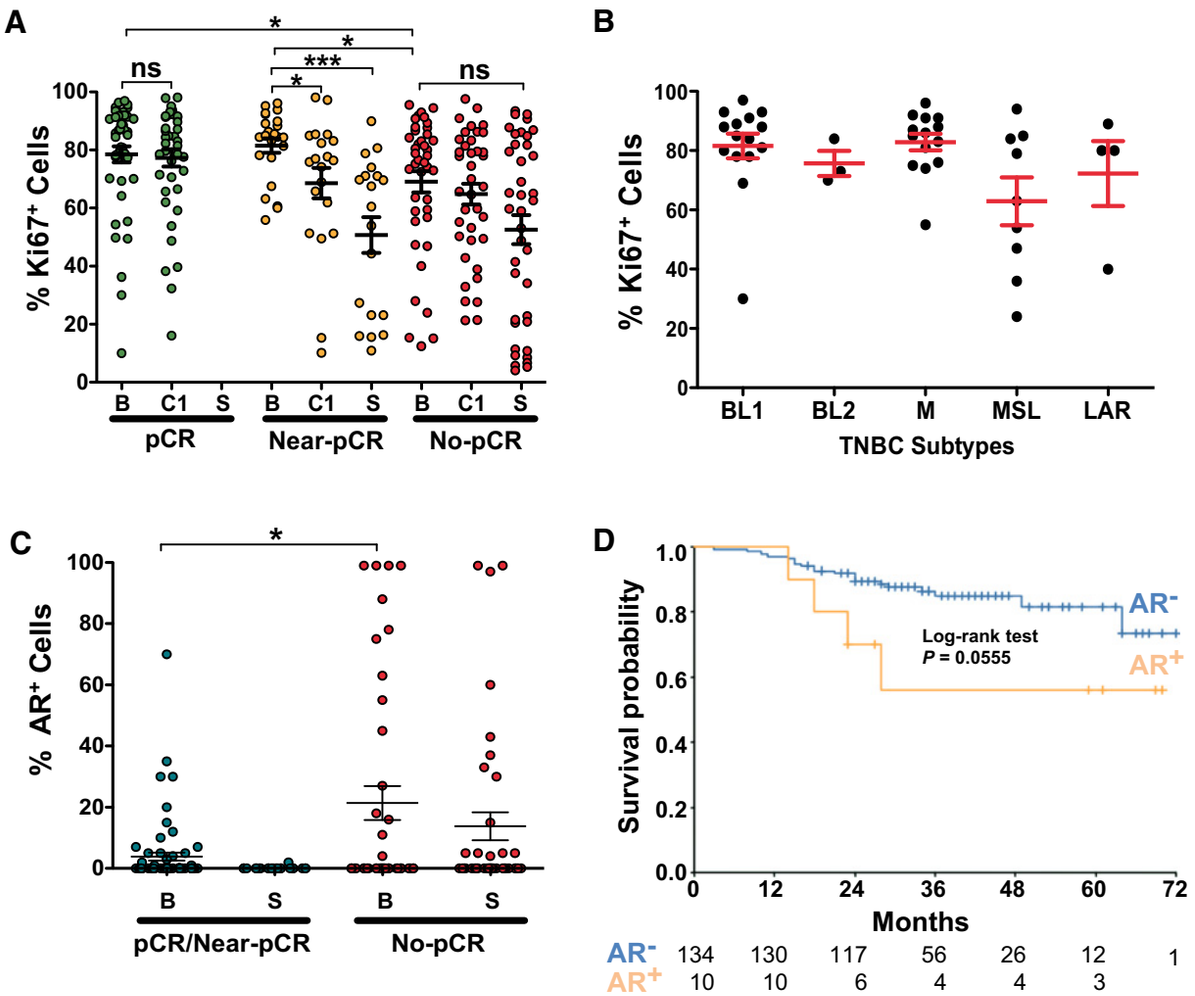


Figure 3.

Lack of clinical response was associated with low Ki67 and high AR protein expression frequency. **A,** Scatter plot for percent of Ki67-positive cells in baseline (B), cycle 1 (C1) and surgical (S) biopsies in pCR, near pCR, and no pCR patients (trial arms combined). Horizontal lines indicate the mean of the percent of Ki67-positive cells; error bars, SEM (ns = not significant, *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$ for a two-sided Wilcoxon signed rank or rank sum test with Bonferroni adjustment). **B,** Scatter plot for percent of Ki67-positive cells across baseline tissues TNBC subtypes abbreviated as follows: basal-like (BL1), immunomodulatory (IM), mesenchymal-like (M), and luminal androgen receptor (LAR); error bar represents SEM (ns = not significant for Kruskal–Wallis test). **C,** Scatter plot for percent of AR-positive cells in baseline and surgical biopsies in pCR/near pCR versus no pCR patients (trial arms combined). Horizontal lines represent the mean percent of AR-positive cells; error bars, SEM (*, $P = 0.0998$ for Wilcoxon rank sum test). **D,** Kaplan–Meier plot represents DFS for patients with AR expression-negative (blue) and AR expression-positive (orange) TNBC. Numbers of patients at risk over time are depicted under graph (log-rank test, $P > 0.05$; Cox regression, HR = 2.78, 95% CI = 0.9394–8.2260).

patients with strongly AR-expressing tumors developed disease recurrence, consistent with their lack of pCR. These data suggest that AR positivity is not an adequate surrogate marker for LAR subtype, consistent with results of a trial for AR-positive TNBC patients treated with the AR-blocker enzalutamide (44). Nevertheless, AR positivity appears to be a reliable marker of lack of response to cisplatin/paclitaxel chemotherapy.

On the basis of our preclinical studies (8, 9), we hypothesized that both everolimus and cisplatin would stimulate proapoptotic signaling via p73 and p63; however, similar to a single-agent platinum trial in metastatic TNBC (45), our results did not show an association between p63 and p73 expression status and response to cisplatin or everolimus.

Although Ki67 staining is not currently standardized as a prognostic or predictive biomarker for routine clinical use in TNBC, partially due to mixed results for its ability to predict patient outcome (46), certain studies have shown that higher levels of Ki67 expression levels prior to neoadjuvant chemotherapy are significantly correlated with higher pCR (35, 36). We found that a high Ki67 expression level both at baseline and subsequent biopsy was predictive of a pCR, and not surprisingly, correlated with BL1 and M subtypes (3). We postulate that the subsequent decrease seen in Ki67 expression level in near pCR tumors, despite good clinical response, reflects a higher degree of tumor heterogeneity, where the prevailing cancer cell population posttherapy is less proliferative and therefore less chemosensitive.

The predictive value of pretreatment Ki67 seen in our study, concordant with prior reports (35, 36), lays the groundwork for incorporating Ki67 as an important measure in future neoadjuvant clinical trials.

Unlike previous neoadjuvant trials demonstrating that TILs correlate with response to chemotherapy (31–34, 47, 48), no correlation was seen between intratumoral or stromal TILs and pCR/near pCR. As all patients received cisplatin, it is possible that the high frequency of DDR gene alterations conferred good clinical outcome regardless of quantification of TILs. We were unable to measure the impact of everolimus on T-cell activity, which could have affected the contribution of an immune response. However, the presence of higher scores of immune infiltrates correlated with the IM TNBC subtype. Considering that the IM TNBC subtype is associated with high levels of PD-1, PD-L1, and CTLA4 (48), alignment to this subtype should be further explored for patient selection in trials investigating immune-checkpoint inhibitors.

Irrespective of the clinical endpoint outcomes, the discoveries made from the correlative studies in this trial provide new avenues for mechanistic exploration in ongoing clinical trials, such as the utility of DDR gene variants in selecting patients more likely to respond to PARP inhibitors or other DNA-damaging agents, alone or in combination with checkpoint inhibitors. AR positivity or low Ki67 expression, once validated, may significantly impact clinical decision-making by predicting resistance to taxane/platinum-based chemotherapy.

In summary, this is a large neoadjuvant trial with significant number of patients for which a substantial fraction had comprehensive genomic analyses, as well as correlative histologic and immunohistologic protein analyses. These data are of significance to the field, and sharing the results of the trial (safety, toxicity, and outcome as well as molecular and genomic data) will allow comparison with other studies, and importantly, expand the genomic datasets for TNBC.

Disclosure of Potential Conflicts of Interest

V.G. Abramson is a consultant/advisory board member for Novartis. A. Bardia is a consultant/advisory board member for Genentech and Novartis. B.D. Lehmann is listed as a coinventor of intellectual property related to TNBCtype that is licensed to Insight Genetics, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: B. Jovanović, I.A. Mayer, J.M. Rosenbluth, J.C. Chang, Y. Shyr, J.A. Pietenpol

Development of methodology: B. Jovanović, I.A. Mayer, K.C. Johnson, V. Sanchez, J.M. Rosenbluth, J.C. Chang, J.A. Pietenpol

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Jovanović, I.A. Mayer, E.L. Mayer, V.G. Abramson, A. Bardia, M.E. Sanders, M.V. Estrada, K.C. Johnson, V. Sanchez, P.M. Dillon, A. Forero-Torres, J.C. Chang, I.M. Meszoely, A.M. Grau, J.A. Pietenpol

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Jovanović, I.A. Mayer, A. Bardia, M.E. Sanders, M.G. Kuba, M.V. Estrada, J.S. Beeler, T.M. Shaver, J.M. Rosenbluth, A. Forero-Torres, J.C. Chang, B.D. Lehmann, Y. Shyr, Q. Sheng, S.-C. Chen, J.A. Pietenpol

Writing, review, and/or revision of the manuscript: B. Jovanović, I.A. Mayer, E.L. Mayer, V.G. Abramson, A. Bardia, M.E. Sanders, J.S. Beeler, T.M. Shaver, K.C. Johnson, P.M. Dillon, A. Forero-Torres, A.M. Grau, S.-C. Chen, C.L. Arteaga, J.A. Pietenpol

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Jovanović, J.A. Pietenpol

Study supervision: I.A. Mayer, J.A. Pietenpol

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