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Immunogenomic profiling and pathological response results from a clinical trial of docetaxel and carboplatin in triple negative breast cancer

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Authors' contributions

FOA, IC, MJE, WG were involved in the conception and design of the study. FOA, MFR, CXM, KW, RS, LLP, RB, NB, CER, AF, TPR, LFH, AR,KC, MO participated in the acquisition of data (acquired and managed patients, provided facilities, etc.). FOA, IC, JL, MFR, ISH, BF, GJ, ZLS, AB, MR, CXM, KW, JD, LLP, RB, MO, OA, BHL, SF, SC, MA, FG, WG, OLG, MG provided analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis). All authors were involved in writing, reviewing, and/or revision of the manuscript. FOA, BF, GJ, ZLS, AB, MR, SC, MA, OLG, MG provided administrative, technical, or material support (i.e., reporting or organizing data, constructing databases). The authors read and approved the final manuscript.

Ethical Approval and Consent to participate

The protocol and informed consent documents were approved by WUSM and BCM. Upon approval, all participating institutions agreed to follow the Declaration of Helsinki, good clinical practice guidelines, and the applicable parts of the U.S. Code of Federal Regulations. Written informed consent was required for enrollment.

Consent for publication

Not applicable.

Availability of supporting data

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests

FOA reports consulting for Eisai, Immunomedics, Astra Zeneca, Athenex, Cardinal Health, Pfizer, Abbvie, Best Doctors, and Advance Medical. FOA reports contracted research for Immunomedics, Pfizer, Seattle Genetics, NeoImmuneTech, RNA Diagnostics, and Astellas. MFR reports consulting for Genentech, MacroGenics, Daiichi, Seattle Genetics, and Novartis. MFR reports contracted research for Pfizer. RB consulting for Genentech. RB reports contracted research for Puma Biotechnology, Inc.

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Abstract

Purpose: Patients with triple negative breast cancer (TNBC) who do not achieve pathological complete response (pCR) following neoadjuvant chemotherapy have a high risk of recurrence and death. Molecular characterization may identify patients unlikely to achieve pCR. This neoadjuvant trial was conducted to determine the pCR rate with docetaxel and carboplatin, and to identify molecular alterations and/or immune gene signatures predicting pCR.

Experimental Design: Patients with clinical stages II/III TNBC received 6 cycles of docetaxel and carboplatin. The primary objective was to determine if neoadjuvant docetaxel and carboplatin would increase the pCR rate in TNBC compared to historical expectations. We performed whole exome sequencing (WES) and immune profiling on pre-treatment tumor samples to identify alterations that may predict pCR. Thirteen matching on-treatment samples were also analyzed to assess changes in molecular profiles.

Results: Fifty-eight of 127 (45.7%) patients achieved pCR. There was a non-significant trend towards higher mutation burden for patients with residual cancer burden (RCB) 0/I versus RCB II/III (median 80, versus 68 variants, p 0.88). *TP53* was the most frequently mutated gene, observed in 85.7% of tumors. *EGFR*, *RB1*, *RAD51AP2*, *SDK2*, *L1CAM*, *KPRP*, *PCDHA1*, *CACNA1S*, *CFAP58*, *COL22A1*, and *COL4A5* mutations were observed almost exclusively in pre-treatment samples from patients who achieved pCR. Seven mutations in *PCDHA1* were observed in pre-treatment samples from patients who did not achieve pCR. Several immune gene signatures including IDO1, PD-L1, interferon gamma signaling, CTLA4, cytotoxicity, tumor inflammation signature, inflammatory chemokines, cytotoxic cells, lymphoid, PD-L2, exhausted CD8, Tregs, and immunoproteosome were upregulated in pre-treatment samples from patients who achieved pCR.

Conclusions: Neoadjuvant docetaxel and carboplatin resulted in a pCR of 45.7%. WES and immune profiling differentiated patients with and without pCR.

Trial registration: Clinical trial information: NCT02124902, Registered 24 April 2014, & NCT02547987, Registered 10 September 2015.

Keywords

Breast cancer; clinical trials; combination chemotherapy; immune biomarkers; genomic biomarkers

Introduction

Triple negative breast cancer (TNBC) is a heterogeneous clinical breast cancer subtype characterized by the absence of expression of receptors for estrogen (ER), progesterone

(PR), and the lack of overexpression of the tyrosine kinase cell surface receptor HER2/*Neu* (HER2). This disease subset accounts for approximately 15 - 20% of all patients with primary breast cancer.[1] TNBC is characterized by a poorer prognosis compared to other clinical subtypes, and because of the absence of targetable receptors, chemotherapy remains the principal systemic therapy.

Neoadjuvant chemotherapy is widely used in early stage patients with TNBC who are being treated with curative intent. The preoperative approach provides an opportunity to assess *in vivo* responses, enables rapid identification of effective drugs, and allows tailoring of adjuvant systemic therapy.[2] Due to improvements in mortality, anthracycline and taxane-based regimens are widely used in the neoadjuvant treatment of patients with TNBC.[3] Unfortunately, anthracyclines are associated with increased cardiac mortality, myelodysplastic syndromes, and treatment-related leukemia.[4,3] Thus, there is interest in evaluating other effective regimens for patients with TNBC. Platinum salts induce double-strand DNA damage and are active in *BRCA*-associated breast cancers.[5,6] Sporadic TNBC and *BRCA*-associated breast cancers share similar molecular features,[7] suggesting possible benefits of platinum salts in patients with TNBC. Recent studies have shown that platinum plus taxane-based non-anthracycline regimens may be an alternative in TNBC patients.[8–11]

The failure of chemotherapy to eradicate disease is believed to be due to selection of cells intrinsically resistant to chemotherapy.[12] Only 30-50% of patients with TNBC who receive neoadjuvant chemotherapy achieve a pathological complete response (pCR).[13–16] Patients who do not achieve a pCR tend to have a higher rate of recurrence and poorer overall survival than patients who do achieve pCR.[17–20] The 3-year risk of distant recurrence for non-pCR patients is 27% versus 9% for those achieving pCR.[21] Three year survival probability is only 68% in non-pCR versus 94% in TNBC patients who achieve pCR.[17] The median survival once TNBC has recurred is only 13-25 months.[22–24]

There is no reliable method for predicting which individual patient will achieve pCR, consequently, many early stage patients with TNBC may be exposed to several months of ineffective chemotherapy prior to definitive breast surgery. The ability to individualize chemotherapy is yet to be achieved because robust predictive markers for chemotherapy response have not been identified. We therefore sought to determine the pCR rate with a non-anthracycline regimen of docetaxel and carboplatin in TNBC. Using this clinical trial as a platform for biomarker discovery, we also sought to understand the role of the host and tumor immune profile and tumor genomics in pathological responses.

Materials and Methods

Patient population

Eligible patients included pre- or post-menopausal women at least 18 years old, with clinical stages II or III ER negative (Allred score < 3 or less than 1% positive staining cells in the invasive component of the tumor) and HER2 negative (0 or 1+ by IHC or FISH negative) invasive breast cancer. Additional eligibility criteria include: Eastern Cooperative Oncology Group Performance Status of 0 to 2, adequate organ and marrow

function, tumor size ≥ 2cm in one dimension by clinical or radiographic exam (World Health Organization criteria), and patients with palpable lymph nodes regardless of tumor size. Exclusion criteria included prior treatment of the current cancer, uncontrolled intercurrent illness, bilateral or inflammatory cancer, pregnant/nursing, and prior sentinel lymph node biopsy. The study was approved by the Institutional Review Boards at the participating sites (Washington University School of Medicine [WUSM] and Baylor College of Medicine [BCM]), and followed the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was required. Both institutions ran parallel protocols. Clinical trial information: NCT02124902 & NCT02547987.

Study procedures

All patients were treated with neoadjuvant intravenous docetaxel 75mg/m^2 and carboplatin AUC 6 cycled every 21 days for 6 cycles, with granulocyte colony-stimulating factor support. Dose adjustments for toxicity were at the discretion of the treating physician. Definitive surgery was performed 3-5 weeks after completion of chemotherapy. Patients received adjuvant radiation when indicated, and adjuvant chemotherapy for patients without pCR was left to the discretion of the treating physician. NCI CTCAE 4.0 was used to record severity and attribution of toxicities. Research tumor biopsies for correlative studies were obtained at baseline prior to chemotherapy, on cycle 1 day 3 (C1D3), and at time of definitive surgery following neoadjuvant chemotherapy in those patients with residual disease. On-treatment biopsy on C1D3 and biopsy at time of relapse were optional. Figure 1 shows the trial schema.

Residual cancer burden (RCB) scoring

Histologic slides from surgical cases post neoadjuvant therapy were reviewed by a breast pathologist (IH), to determine tumor bed size, percent neoplastic cellularity within the tumor bed, percent of residual tumor that was in situ, number of positive nodes, and largest lymph node deposit. RCB score and category were assigned by the MD Anderson method using a publicly available web calculator (http://www3.mdanderson.org/app/medcalc/index.cfm?pagename=jsconvert3, accessed 9/9/20).

Tumor infiltrating lymphocytes (TILs) assessment

TILs were quantitated according to the method recommended by the International Immuno-Oncology Biomarker Working Group on Breast Cancer.[25] A breast pathologist (IH) independently reviewed whole slide scans (blinded to treatment status) and documented the percentage of TIL infiltration in increments of 5% in tumor-adjacent stroma.

Whole exome sequencing (WES)

Tumor DNA was extracted from fresh-frozen biopsies and matched leukocyte germline DNA from blood samples. WES libraries were enriched using the NimbleGen Roche VCRome v2.1 hybrid capture reagent supplemented with a “panel killer” spike-in designed by BCM. Paired-end (2×150 base pair) next generation sequencing was performed using the Illumina platform to a target depth of coverage of 100x (mean target coverage achieved was 70 - 130x across the cohort). Sequence data processing pipelines utilized tools

implemented in Docker containers (published to DockerHub), input parameters and data specified in YAML configuration files, pipeline steps and dependencies expressed using the Common Workflow Language (CWL), and compute tasks submitted to a compute cluster using Cromwell and platform LSF.[26] All associated CWL files, example YAML files, and docker files are version tracked using Git and GitHub (https://github.com/genome/analysis-workflows). Metadata on analysis runs was tracked using an Analysis Information Management System developed at the Washington University McDonnell Genome Institute.[27] Briefly, WES analysis was performed by aligning sequence reads to the human reference genome build GRCh38 using the BWA-MEM aligner.[28] Alignments were subjected to base quality score recalibration,[29] sorted by chromosome position, duplicates marked with Picard (http://broadinstitute.github.io/picard/), and converted to a lossless CRAM format (https://github.com/samtools/hts-specs) to reduce disk usage. All samples in each cohort were tested for sample swaps and contamination using Somalier (https://github.com/brentp/somalier) and subjected to a QC analysis consisting of Picard, samtools flagstat,[30] and VerifyBamID (https://github.com/statgen/verifyBamID). Somatic single nucleotide variants (SNVs) and small insertions and deletions were called using an ensemble approach involving MuTect2, Strelka2, Varscan2 and Pindel.[31–33] Somatic variants from each of these callers were left aligned and trimmed using GATK LeftAlignAndTrim,[29] merged into a single variant call format file using GATK CombineVariants and multiallelic sites separated using vt decompose.[34] Somatic variants were subjected to a false positive filter to flag variants of high frequency in the population according to GNOMAD,[35] lacking minimum sequence read support from both sequence strands, corresponding to regions of the genome with ambiguous read mapping, and those that fail a log likelihood test that models the observed read support relative to known error rates of the sequencing platform and conservative sample specific assumptions of tumor purity. The resulting candidate somatic variants were subjected to a formal manual review standard operating procedure.[36] The resulting high confidence somatic variants were annotated for transcript variant effect using VEP and Ensembl transcripts.[37,38] Variant allele frequencies were computed using bam-readcount (https://github.com/genome/bam-readcount). Pathway analyses for genes harboring sub-clonal variants selected under treatment pressure were performed using WEB-based GEne SeT AnaLysis Toolkit [39] using an Over-Representation Analysis (ORA) approach and “Wikipathway cancer” as the source of pathways.

NanoString gene expression analysis

RNA extracted from formalin-fixed paraffin embedded (FFPE) tissue samples were analyzed on the nCounter® analysis system using the PanCancer IO 360™ panel (for research use only). Raw data counts were normalized using the geomean of 20 housekeeping genes in the IO360 panel and each gene was adjusted based on IO360 panel standards to adjust for batch-to-batch variation. The housekeeper-normalized and panel standard-normalized data is Log(2) transformed. IO360 gene analysis for 48 signatures measuring immune cell abundance, immune signaling, tumor and stromal biology were calculated as previously described.[40,41] A constant of 8 is added to the tumor inflammation signature (TIS) so that it is on the same scale as investigational use only (IUO) TIS, making scores comparable

across research use only (RUO) and IUO assays. Other non-TIS signatures are also adjusted with constants to express values in a similar range.

Statistical analyses

The primary endpoint of the clinical trial is pCR rate calculated as the percentage of patients who achieve pCR among all evaluable patients. pCR is defined as absence of residual invasive disease in the breast and lymph nodes following neoadjuvant chemotherapy. Exploratory aims were to investigate immune and genomic changes with the intent to identify predictors of response. A sample size of 100 patients provides 82.1% power to test a pCR rate of 40% against the null rate of 28% with standard chemotherapy, based on 1-sided binomial exact test at a target 0.05 alpha level. If 36 or more patients achieve a pCR, we conclude that the investigational regimen yields better efficacy than standard chemotherapy.

Patient characteristics were summarized by descriptive statistics, counts and percentages for categorical characteristics, and median with inter-quartile ranges (IQR) for quantitative characteristics. Tumor burden was compared between pCR (RCB 0) versus non-pCR patients, and between RCB 0/I versus RCB II/III patients, by Wilcoxon rank sum test, and compared between paired pre-treatment and C1D3 samples by Wilcoxon signed rank test. Mutation landscape waterfall plot was generated using the R package “GenVisR” (Version 1.16.1). Gene sets defined in the Molecular Signatures Database v7.1 (MSigDB v7.1 released March 2020) were downloaded and extracted using R package “msigdbr”. The overall effect of gene mutations of a gene set on pathological outcome was evaluated using the sequence kernel based association (SKAT) test method in the logistic regression framework. SKAT p value was reported for each gene set.

NanoString IO360 signatures were compared based on pathological response and time point. Differential expression based on response was fit on a per gene or per signature basis using a linear model for analyses without a blocking factor. The statistical model uses the expression value or signature score as the dependent variable and fits a grouping variable as a fixed effect to test for differences in the levels of that grouping variable.

$$\text{Expression}_{(\text{gene or signature})} = \mu + \text{Response} + \varepsilon$$

For differential expression for time series analysis the duplicateCorrelation function within the limma R package is used to assess the correlation between subsequent time points. This correlation estimate is fit into the linear mixed effect model with subject as the random effect and the correlation between the repeated temporal measurements.

$$\text{Expression}\_{(\text{gene or signature})} = \mu + \text{SubjectID} + \text{Group} + \varepsilon$$

For all differential expression analysis. All models are fit using the limma package in R.

Results

Patient characteristics and clinical efficacy

Between 8/2014 and 1/2020, 168 patients were screened and 132 ultimately received protocol therapy. Thirty-five did not meet inclusion criteria/were not registered due to reasons such as being found to have estrogen receptor positive breast cancer, metastatic disease, or abnormal laboratory values. Five patients were not evaluable for pCR due to a variety of reasons, including patient or physicians choice to withdraw protocol therapy. One hundred and twenty seven patients were evaluable. Median follow up is 27 months (IQR 14.4 - 39.5 months). Additional file 1 shows the patient disposition. Median age at diagnosis was 52.5 years (IQR 42 - 61). African Americans comprised 23.5% of the study population, 93.2% had high grade disease, and 43.9% had clinical nodal involvement prior to chemotherapy. Patient characteristics are shown in Table 1.

Fifty-eight of 127 (45.7%) evaluable patients achieved pCR (95% CI 36.9% - 54.7%), and pCR was similar by accrual site (WUSM: 45.6%, 95% CI 35.9% - 55.7% and BCM: 45.8%, 95% CI 26.2% - 66.8%). There were no differences in age, race, clinical stage, grade, or clinical node status according to pathological response (Table 1).

Toxicity

All patients who received at least 1 cycle of combination chemotherapy are evaluable for toxicity. Due to toxicities, 13 patients did not complete all 6 cycles of protocol specified therapy (five had 4 cycles, and eight patients had 5 cycles of combination therapy). Treatment-emergent adverse events of any grade occurred in 123 patients, with 1,947 events reported. Table 2 shows the incidence of grade 3 and 4 adverse events occurring in at least two patients. The incidence of grade 3 to 4 anemia was 18.2%, thrombocytopenia was 13.6%, diarrhea 9.1%, and febrile neutropenia 7.6%. Adverse events leading to discontinuation of the regimen were reported in 15 patients (11.4%). Nineteen patients (14.4%) had at least one SAE. (Table 2). No treatment-related deaths occurred.

Immune profiling and pathological response

We evaluated the association of immune parameters with pathological response on 82 patients. Stromal TILs, CD4, CD8, CD79a, and PD-L1 levels from tumor samples were assessed by hematoxylin and eosin (H&E)-stained tumor sections and immunohistochemistry. Higher baseline stromal TILs (OR 1.31; 95% CI: 1.00 - 1.70, p=0.05) and CD8 (OR 1.14; 95% CI: 1.03 −1.26, p=0.02) values were associated with pCR.

Patients with higher post-treatment absolute lymphocyte counts (ALCs) from peripheral blood were more likely to achieve pCR than those with lower ALCs (OR 5.5; 95% CI: 1.5-20.7, p=0.011). Post-treatment median ALCs was 1.55 cells/mm3 (range 0.8 - 3.6 cells/mm3) in pCR patients; and 1.4 cells/mm3 (range 0.4 - 3.9 cells/mm3) in those with residual disease. Similarly, patients with higher minimum ALCs were also more likely to achieve pCR than those with a lower ALC nadir (OR 9.1, 95% CI 1.5-54.9, p=0.016). The minimum ALC is the nadir patients experienced during the period of neoadjuvant chemotherapy. The

associations of post-treatment and minimum ALCs with pCR remained significant after adjusting for age and clinical stage at diagnosis (post-treatment ALC OR 7.6; 95% CI: 1.7 - 34.8, p=0.009; minimum ALC OR 9.0; 95% CI: 1.5 - 55.2, p=0.018). Baseline ALC values were not associated with pCR (OR 1.0; 95% CI: 0.6 - 1.9, p=0.863).

Analysis of gene expression in samples collected at baseline using the NanoString's nCounter® PanCancer IO 360 Gene Expression Panel, demonstrated that several immune signatures, including IDO1 (p=0.004), PD-L1 (p=0.001), interferon gamma signaling (p=0.016), CTLA4 (p=0.038), cytotoxicity (p=0.019), TIS (p=0.026), inflammatory chemokines (p=0.013), cytotoxic cells (p=0.028), lymphoid (p=0.040), PD-L2 (p=0.018), exhausted CD8 (p=0.042), Tregs (p=0.035), and immunoproteosome (p=0.028) were upregulated in pCR versus non-pCR samples (Figure 2). Key immune signatures of borderline significance included T cells, CD8 T cells, IL7R, and TIGIT. A heatmap displayed as Additional file 2 uses unsupervised hierarchical clustering to show relatedness among signature scores for baseline samples according to pathological response.

In a longitudinal analyses, differences between pre- and on-treatment (C1D3) paired samples were analyzed. Many immune-related signatures were significantly higher in baseline samples compared to on-treatment (Figure 3 and Additional file 3). The signatures with the greatest significance covered four categories of immune biology, including interferon-related (TIS: $p<0.001$, Interferon gamma signaling: $p<0.001$, Interferon signaling: $p<0.005$), T cell abundance (Exhausted CD8: $p<0.001$, T cells: $p<0.0005$, CD8 T cells: $p<0.005$, Th1 T cells: $p<0.005$), cytotoxicity (Cytotoxicity: $p<0.001$, Cytotoxic Cells: $p<0.001$) and checkpoint molecules (PD-L2: $p<0.001$, TIGIT: $p<0.001$, PD-L1: $p<0.001$, CTLA4:$p<0.001$, PD-1: $p<0.005$). Interestingly, baseline pCR samples had slightly higher expression of IDO1, inflammatory chemokines, and CD56dim cell abundance signatures at compared to non-pCR samples, and showed a larger change in signatures scores from baseline to on-treatment time points.

Tumor mutation profiling and pathological response

WES was performed on baseline pre-treatment samples collected from a subset (N=56 patients) of the clinical trial population. Due to funding limitations, only samples with the highest tumor cellularity for sequencing were selected for WES. Thirteen patients had baseline-matched samples from C1D3 that were analyzed. 9063 variants were detected in 5386 unique genes. The mutation landscape waterfall plot of genes with a mutation frequency of >5% is shown in Figure 4. The overall mutation burden for patients who achieved pCR was not significantly different from non-pCR patients (median of 78.5 variants, IQR 43 - 134 in pCR, vs median 72, IQR 47.8 - 103.8 in non-pCR, Wilcoxon rank sum test p=0.98). Similarly, there was no difference in the overall mutation burden for patients with RCB 0/I versus those with RCB II/III (median of 80 variants, IQR 40 - 134 in RCB 0/I, vs median 68, IQR 53.5 - 87.8 in RCB II/III, Wilcoxon rank sum test p=0.88) (Figure 5).

Table 3 shows the genes with variants occurring in at least 10% of the biomarker population, according to pathological response. *TP53* was the most frequently mutated gene observed in 48 of 56 patients sequenced (85.7%) As expected for *TP53*, we observed mutations

primarily within the DNA-binding domain, and many of the mutations are likely loss-of-function variants caused by frameshift insertion/deletions, frameshift splice variants, and nonsense mutations (Additional file 4). There was a non-significant trend with fewer *TP53* mutations occurring in 80% of patients with pCR, versus 88.9% of non-pCR patients (OR 0.51, 95% CI 0.08 - 3.09; p value 0.44). There were no differences in *TP53* mutation frequency in patients with RCB 0/I (85%), versus RCB II/III (87.5%) (OR 1.23, 95% CI 0.19 - 13.92; p value 1.00). *EGFR*, *RB1*, *RAD51AP2*, *SDK2*, *L1CAM*, *KPRP*, *PCDHA1*, *CACNA1S*, *CFAP58*, *COL22A1*, and *COL4A5* were differentially mutated, and almost exclusively found in pre-treatment pCR samples (except *PCDHA1*). The seven mutations in *PCDHA1* were observed in non-pCR samples only (19.4%). We observed four variants within *EGFR* in three patients. Three of these variants occurred within the tyrosine kinase catalytic domain region, and all three have documented clinical significance with respect to targeted therapy response in CIViC (Additional file 5). One of the patients with an *EGFR* mutation has recurred, despite pCR.

In the longitudinal analyses, there was evidence of tumor heterogeneity and shifts in clonal architecture under treatment pressure from pre-treatment to C1D3, due to both selection and depletion of subclones. However, more variants were enriched suggesting possible subclone emergence under treatment pressure (Figure 6). The overall variant counts in the matched samples at C1D3 trended higher (median of 82, IQR 49 - 157) than corresponding pre-treatment samples (median of 72, IQR 42 - 92), p=0.29. As expected, all tumor pairs had a substantial set of shared clonal variants with *TP53* variants exhibiting high variant allele frequency (VAF) reflecting its driver status. Across all patients, 289 genes harbored such emerging variants including a second *TP53* Y220C variant in patient NTN022 (0% VAF at baseline and 13.0% VAF at C1D3), and an *EGFR* S768I variant in NTN046 (0.07% VAF at baseline and 29.5% VAF at C1D3). Pathway analysis of the entire set of mutated genes emerging at C1D3 (green points in Figure 6) showed significant enrichment for DNA damage and nucleotide synthesis pathways suggesting possible selection for cells with molecular mechanisms of resistance to the DNA damaging agent carboplatin.

Last, we explored whether mutations in different gene families would cluster in recognized pathways. Using the Molecular Signatures Database (MsigDB) v7.1, several gene families involved in immune-signature related gene sets (MsigDB C7 set) showed differences between RCB 0/I and RCB II/III samples (Additional file 6). Additionally, borderline differences in PI3K AKT MTOR signaling pathway among MsigDB 50 hallmark gene sets were identified between RCB 0/I and RCB II/III samples. There were no differences in inflammatory response, angiogenesis, apoptosis, NOTCH signaling, TNF alpha, or androgen response pathways.

Discussion

In this multicenter single-arm phase II trial of neoadjuvant docetaxel and carboplatin in patients with newly diagnosed clinical stage II to III TNBC, we observed a pCR rate of 45.7%. These data are consistent with previous observations that non-anthracycline based regimens in TNBC patients achieve similar pCR rates as anthracycline plus taxane based regimens.[10,11,9,8] Recent randomized trials demonstrate that the addition of carboplatin

to anthracycline taxane based regimens increase pCR rates, but also increase toxicity.[13,14] More recently, Sharma et al. compared neoadjuvant carboplatin with docetaxel to carboplatin with paclitaxel followed by anthracyclines in patients with early stage TNBC.[42] This study demonstrated similar pCR rates in both study groups: carboplatin with docetaxel group pCR 52%, versus anthracycline group pCR 55%, p=0.84. Patients who did not receive anthracyclines had a more favorable toxicity profile and higher treatment completion rate compared with patients who received anthracyclines. The effects of the addition of carboplatin to anthracycline based regimens on longer-term clinical outcomes such as event-free and overall survival are conflicting.[43] GeparSixto, a randomized phase 3 trial that evaluated the addition of carboplatin to anthracycline and taxanes in TNBC, showed a higher 3-year disease-free survival rate in the carboplatin group vs the non-carboplatin group (85% vs 76%, p = 0.03).[14,44] Conversely, Cancer and Leukemia Group B (CALGB) 40603 randomized phase II trial demonstrated no difference in 3-year event-free survival with the addition of carboplatin to anthracycline taxane based neoadjuvant therapy (71% versus 76%, p = 0.36).[45] As a result, carboplatin has not been incorporated into the routine clinical management of early stage TNBC. Ongoing clinical trials may address long-term outcomes in the future.[46–48] The similarity in antitumor activity with docetaxel and carboplatin compared with standard anthracycline taxane based regimens, and the increased toxicities with adding carboplatin to anthracycline taxane based regimens provide rationale for considering docetaxel and carboplatin chemotherapy for patients with early stage TNBC. Moreover, the long-term risk of cardiotoxicity, myelodysplasia, and therapy-associated leukemia is minimized with non-anthracycline regimens.

pCR is a surrogate of long-term outcomes in patients with TNBC, and the extent of residual disease is linked to the risk of recurrence.[17,49,50,16,51] Individuals who do not achieve pCR or RCB I have a high risk of recurrent disease and subsequent death, with a hazard ratio for an overall survival event reported to be as high as 12.4 (95% CI 5.8 - 26.5, p= 0.001).[17,16] More recently, results from the ECOG-ACRIN EA 1131 showed that patients who did not achieve pCR had an extremely poor 3-year invasive disease free survival of less than 50%.[52] Other than *BRCA* germline status,[53,44,54] there are no predictive factors for pCR. There are promising data using early imaging changes in positron emission or computed tomography, tumor morphological changes, and ctDNA to predict pathological response to neoadjuvant chemotherapy.[55–58] From a clinical perspective, understanding variables that predict response is an urgent unmet need. If TNBC patients who will not achieve pCR can be identified earlier, they may be triaged to innovative trials or definitive surgery, with a view to changing the natural history of resistant TNBC whilst sparing them the toxicity of ineffective chemotherapy. Therefore, we examined tumor immune and genomic profiles to identify molecular factors that may predict response. Our study did not identify mutation burden as a predictor for pathological response to docetaxel and carboplatin chemotherapy. An interesting observation was the identification of *EGFR* mutations in pCR patients. Although *EGFR* mutations have previously been described in TNBC,[59] the clinical relevance has not been described. These results need to be confirmed in a larger study, but may suggest a potential rationale for evaluating *EGFR* tyrosine kinase inhibitor therapy in TNBC patients harboring *EGFR* mutations.

Due to the absence of recurrently mutated genes other than *TP53*,[60] the aggregation of individual genes at the pathway level may be a more practical way to evaluate predictors of response in TNBC. On-treatment samples had a suggestion of clone emergence, with increased variants compered to baseline samples. Pathway analysis of the emerging genes showed enrichment for DNA damage pathway, suggesting selection for resistance to the DNA damaging agents. These hypothesis-generating results suggest that early on-treatment tumor assessment may be used in the future to identify patients who may be more likely to respond to chemotherapy. We also found that several immune-related pathway gene signatures showed differences between pathological outcomes, suggesting the possible utility of immune perturbation in early TNBC. Patients with higher baseline TILs, baseline upregulation of immune-related (IDO, PD-L1, lymphoid, Tregs), inflammation-related (inflammatory cytokines, immunoproteasome) and cytotoxicity-related (interferon gamma signaling, cytotoxic cells, exhausted CD8) gene signatures were more likely to achieve pCR. In addition, patients that were able to maintain higher post-treatment absolute lymphocyte counts despite the lymphodepleting effects of chemotherapy, were also more likely to achieve pCR. Taken together, these results suggest that patients with an inflamed tumor microenvironment (TME) that experience less treatment-related lymphopenia may be more responsive to neoadjuvant chemotherapy with docetaxel and carboplatin. Thus, immunomodulatory interventions such as IL-7 treatment which is aimed at increasing the persistence, survival and trafficking of lymphocytes may provide a significant clinical benefit when combined with neoadjuvant strategies. Long-acting recombinant human IL-7 (rhIL-7-hyFc) has shown to be safe and well tolerated in human, while significantly increasing the frequency of lymphocytes.[61] rhIL-7-hyFc has also proven to increase T cell infiltration and inflammation within the TME in cancer-bearing mouse models.[62] Using rhIL-7-hyFc treatment to improve the rates of pCR in TNBC patients undergoing adjuvant chemotherapy is an appealing strategy that needs further investigation.

Strengths of this translational study include the high proportion of African Americans accrued, which is likely reflective of the demographics of both accrual sites, and the commitment of the investigators to accrue minorities to clinical trials. Furthermore, this was a multicenter collaborative study, which simultaneously served as a platform for biomarker analyses. Additionally, this was a homogenously treated, clinically well-annotated TNBC cohort. All samples were processed similarly at the same institution, the whole exome sequencing approach utilized was comprehensive, and we had normal tissue on all patients for accurate variant calling. However, several limitations deserve comment. First, this is a phase 2 single-arm non-randomized study. Therefore, a larger randomized trial is necessary for further evaluation of non-athracycline based regimens in TNBC. Second, the relatively small sample size of the correlative study limits the power of the analyses. Follow up is short but ongoing, and long-term clinical outcomes will be reported as results become available. Despite these limitations, this is the first hybrid study with comprehensive tumor profiling comparing a homogeneously treated cohort of TNBC patients to identify biomarkers predicting pCR.

Conclusions

Our results demonstrate a robust pCR rate of 45.7% with this alternative non-anthracycline chemotherapy regimen administered to patients with TNBC. Tumor mutation assessment for *EGFR*, *RB1*, *RAD51AP2*, *SDK2*, *L1CAM*, *KPRP*, *PCDHA1*, *CACNA1S*, *CFAP58*, *COL22A1*, *COL4A5* mutations, and immune-related gene signatures may discriminate patients who will achieve pCR following platinum-based neoadjuvant chemotherapy. While larger confirmatory studies are needed, these may be potential biomarkers for predicting pathological response and therapeutic efficacy in patients with TNBC, and emphasize the need for molecular analyses in therapeutic clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TNBC	Triple negative breast cancer
ER	Estrogen receptor
PR	Progesterone receptor
HER2	HER2/*Neu* gene
DNA	Deoxyribonucleic acid
pCR	Pathological complete response
IHC	Immunohistochemistry

FISH	Fluorescence in situ hybridization
WUSM	Washington University School of Medicine
BCM	Baylor College of Medicine
AUC	Area under the curve
NCI CTCAE	National Cancer Institute Common Terminology Criteria for Adverse Events
C1D3	Cycle 1 day 3
RCB	Residual cancer burden
TIL	Tumor infiltrating lymphocytes
WES	Whole exome sequencing
SNV	Single nucleotide variant
RNA	Ribonucleic acid
FFPE	Formalin fixed paraffin embedded
TIS	Tumor inflammation signature
IUO	Investigational use only
RUO	Research use only
SKAT	Sequence kernel based association
SAE	Serious adverse events
ALC	Absolute lymphocyte count
OR	Odds ratio
VAF	Variant allele frequency
TME	Tumor microenvironment

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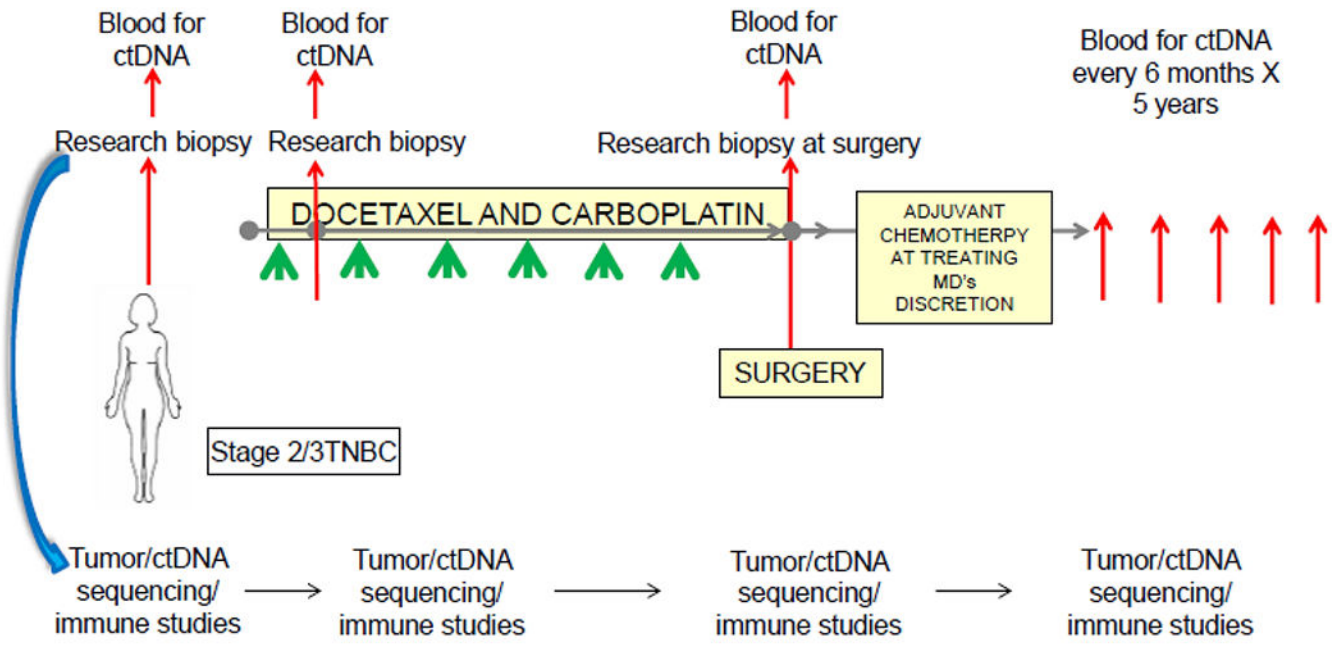

Figure 1.

Clinical trial schema. Diagram of clinical trial showing chemotherapy regimen and biospecimen collection time points.

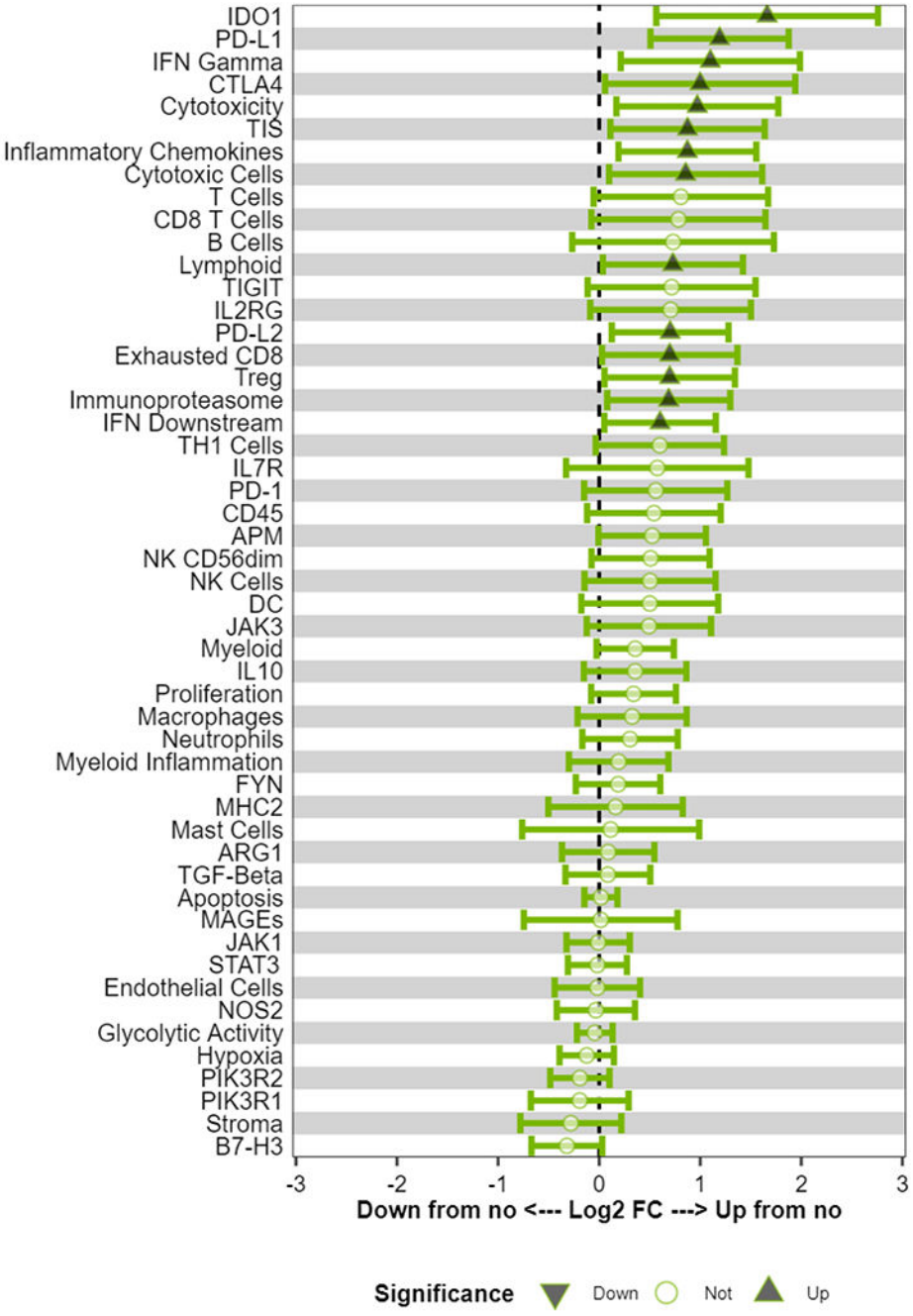

Figure 2.

Differential expression of gene signatures' forest plot based on pCR in baseline samples only (N = 66 patients). This shows the differential expression means and 95% confidence intervals between response variables, for each signature on an unadjusted scale. The vertical axis is shown at fold change equal to zero, indicating equivalent expression between pCR and no pCR (no). As the marker shifts from the center line there is an increase (shift to the right), or decrease (shift to the left), in the differential expression of that signature when compared to the no pCR group (represented as the vertical line at zero). The shape of

the marker in each box indicates whether there is a significant difference in the signature as assessed by univariate analysis (note that this significance is not adjusted for multiple comparisons). A signature is considered significant if the 95% confidence interval (the horizontal line of the signature) does not cross the vertical axis representing the no response group.

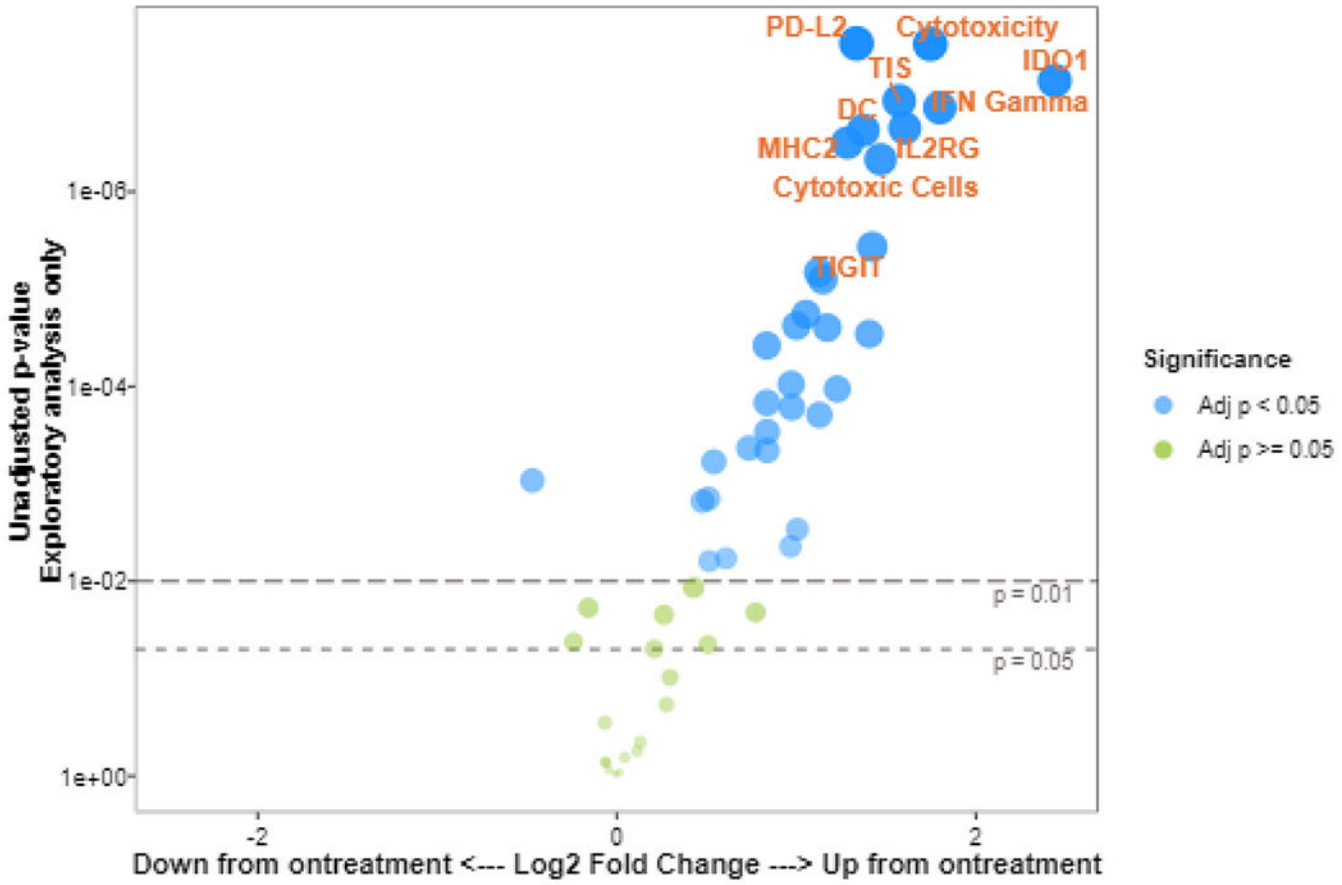

Figure 3.

Differential expression of IO360 gene signatures before and on-treatment (N = 66 patients). A) Volcano plot showing differential expression of IO360 immune signatures from baseline or on treatment samples. Signatures in blue were significantly different using an adjusted p-value ($p<0.05$). Dotted lines indicate an unadjusted p-value of $p<0.05$ and $p<0.01$. Larger circles indicate greater significance.

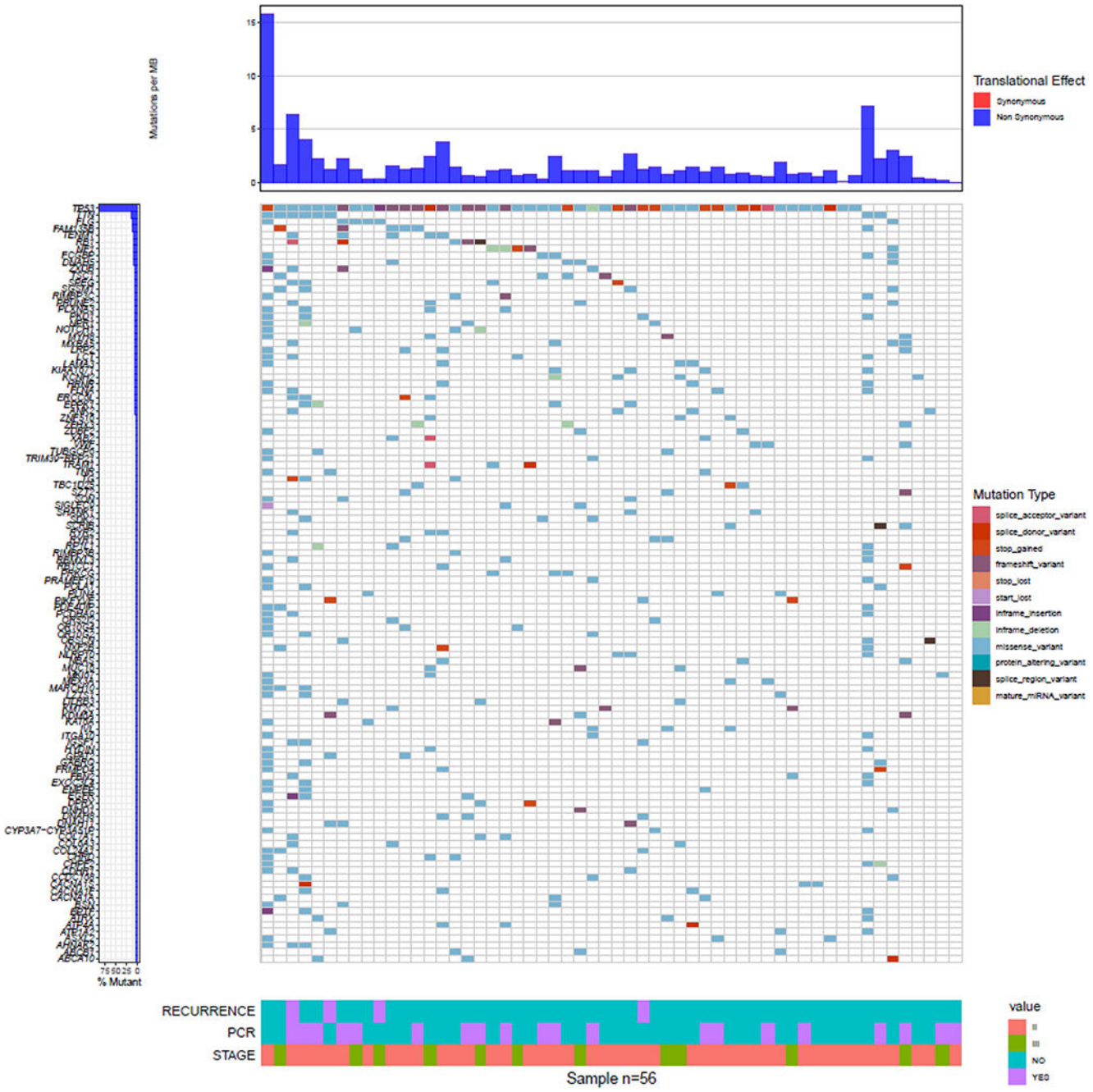

Figure 4.

The mutation landscape waterfall plot of genes with a non-synonymous (frameshift, in-frame deletion, in-frame insertion, mature miRNA, missense, protein altering, splice acceptor, splice donor, splice region, start lost, stop gained, and stop lost) mutation frequency >5%. Top bar plot indicates mutation burden, left bar plot indicates mutation frequency, and lower panel provides clinical annotation.

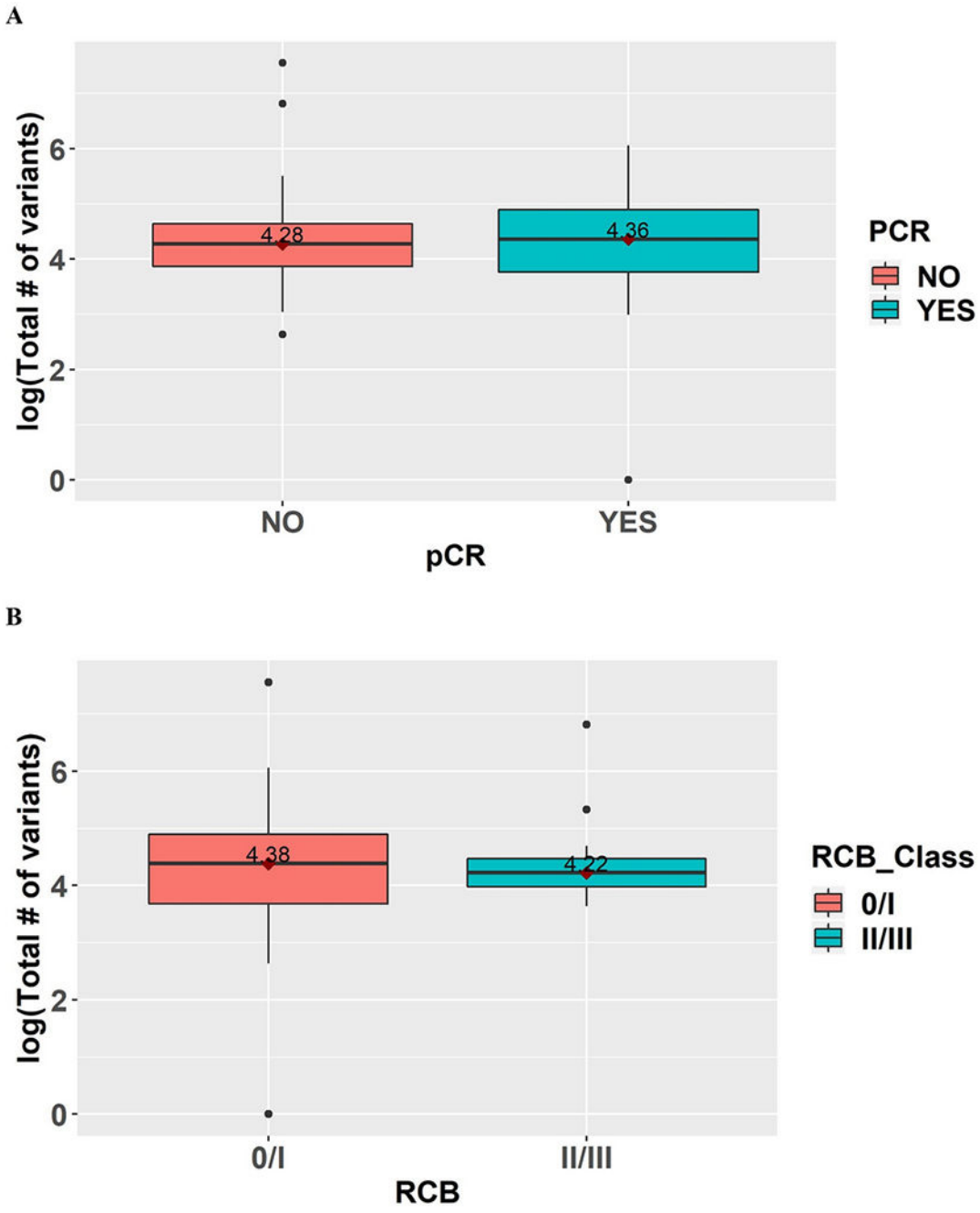

Figure 5.

Overall mutation burden according to pathological response (N = 56 patients). Log scale of somatic mutation burden according to pathological response following neoadjuvant chemotherapy. Red bar indicates patients without pCR. Green bar indicates patients with pCR. pCR, pathological complete response.

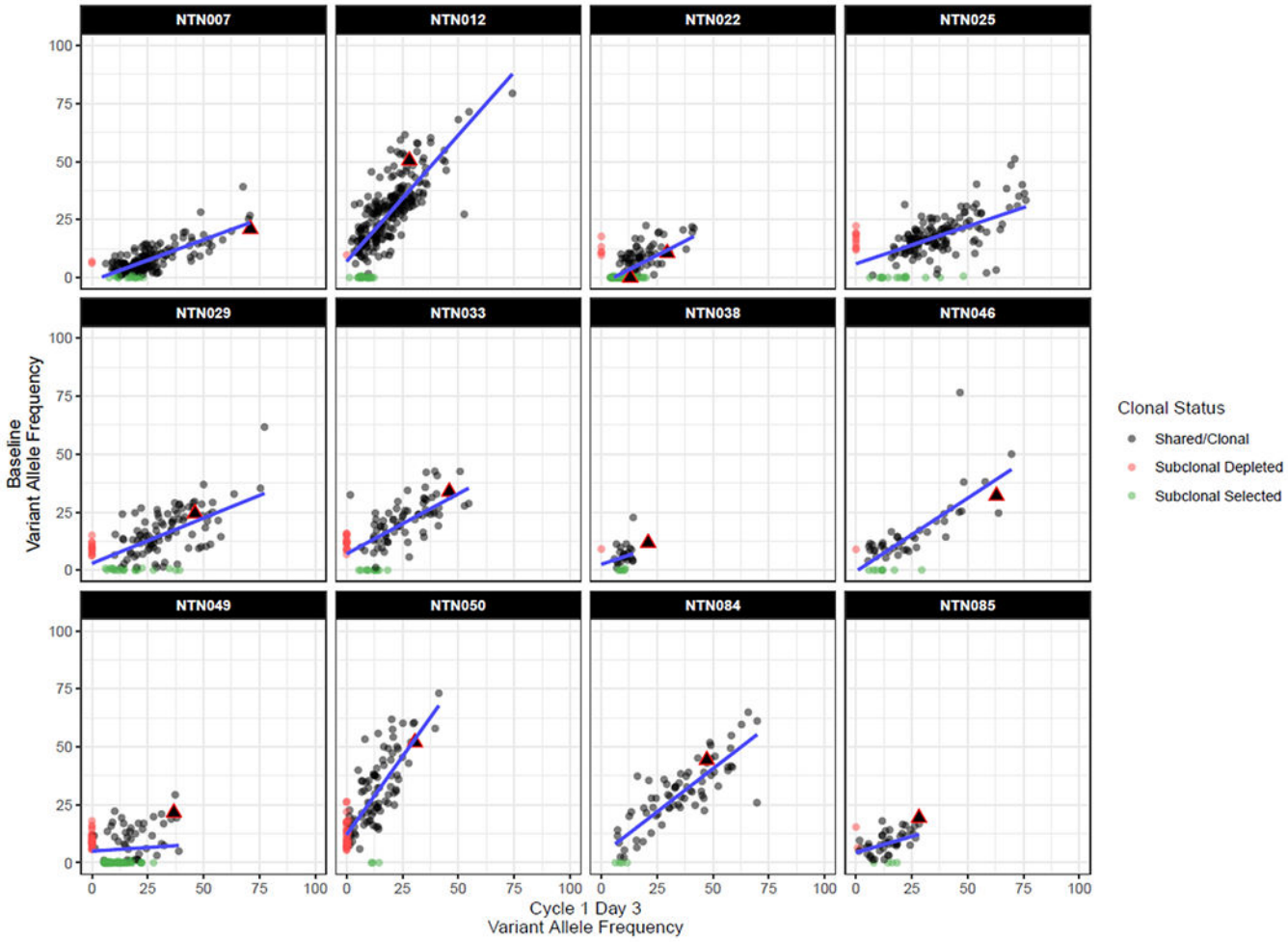

Figure 6.

Comparison of variant detection in pre-treatment and on-treatment paired tumor samples. Of the 13 participants for which WES was performed at both baseline and C1D3, 12 matched samples are plotted. One paired sample set is not shown due to low variant counts. For the variants plotted, the minimum coverage was set to 30 reads. The clonal status of variants in each sample is indicated by color. Red represents subclonal depleted mutations, green represents subclonal selected mutations, and grey represents shared clonal mutations. *TP53* variants were called in 11 of the participants' samples and are highlighted in the plots by the triangle.

Table 1.

Characteristics of study population

	Total study population (%)	Non-pCR N=69 (%)	pCR N=58 (%)	*p* value
Age at diagnosis, median (IQR)	52.5 (42-61)	53.0 (44.0-62.0)	51.5 (40.2-58.8)	0.301
Race Caucasian Black Other	 98 (74.2) 31 (23.5) 3 (2.3)	 49 (71.0) 18 (26.1) 2 (2.9)	 44 (75.9) 13 (22.4) 1 (1.7)	 0.913
Clinical stage II III	 107 (81.1) 25 (18.9)	 54 (78.3) 15 (21.7)	 48 (82.8) 10 (17.2)	 0.648
Tumor grade II III	 9 (6.8) 123 (93.2)	 7 (10.1) 62 (89.9)	 2 (3.5) 56 (96.5)	 0.179
Clinical node status Positive Negative	 58 (43.9) 74 (56.1)	 34 (49.3) 35 (50.7)	 22 (37.9) 36 (62.1)	 0.301

Table 2.

Treatment-emergent adverse events of ≥ grade 3 in study population

	All patients, N=132 N (%)
Total number of grade ≥3 TEAEs occurring in ≥ 2 patients	**56 (42.4)**
Anemia	24 (18.2)
Thrombocytopenia	18 (13.6)
Lymphopenia	14 (10.6)
Diarrhea	12 (9.1)
Febrile neutropenia	10 (7.6)
Neutropenia	10 (7.6)
Hyponatremia	7 (5.3)
Leucopenia	6 (4.5)
Hypokalemia	5 (3.8)
Fatigue	2 (1.5)
Oral mucositis	2 (1.5)
Anorexia	2 (1.5)
Syncope	2 (1.5)
Renal disorders	2 (1.5)
Patients with any SAEs	**19 (14.4)**
SAEs occurring in ≥ 2 patients	
Syncope	2 (1.5)
Febrile neutropenia	7 (5.3)
Colitis	4 (3.0)
Nausea	2 (1.5)
Neutropenia	3 (2.3)
Urinary tract infections	3 (2.3)
Diarrhea	3 (2.3)
Acute kidney injury	2 (1.5)

Table 3.

Genes with variants occurring in at least 10% of the biomarker study population.

Gene symbol	Variant/N (%)	Non-pCR N=36 (%)	pCR N=20 (%)	*p* value
TP53	48 (85.7)	32 (88.7)	16 (80.0)	0.44
TTN	13 (23.2)	9 (25.0)	4 (20.0)	0.75
FLG	8 (14.3)	6 (16.7)	2 (10.0)	0.69
PCDHA1	7 (12.5)	7 (19.4)	0 (0.0)	0.04
TRMT9B	6 (10.7)	5 (13.9)	1 (5.0)	0.40
PCDHGA2	6 (10.7)	3 (8.3)	3 (15.0)	0.66
FAM135B	6 (10.7)	4 (11.1)	2 (10.0)	1.00
KIAA1671	6 (10.7)	4 (11.1)	2 (10.0)	1.00
SPEG	6 (10.7)	4 (11.1)	2 (10.0)	1.00
TENM1	6 (10.7)	4 (11.1)	2 (10.0)	1.00