Molecular Profiling of the Residual Disease of Triple-Negative Breast Cancers after Neoadjuvant Chemotherapy Identifies Actionable Therapeutic Targets

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Neoadjuvant chemotherapy (NAC) induces a pathologic complete response (pCR) in approximately 30% of patients with triple-negative breast cancers (TNBC). In patients lacking a pCR, NAC selects a subpopulation of chemotherapy-resistant tumor cells. To understand the molecular underpinnings driving treatment-resistant TNBCs, we performed comprehensive molecular analyses on the residual disease of 74 clinically defined TNBCs after NAC, including next-generation sequencing (NGS) on 20 matched pretreatment biopsies. Combined NGS and digital RNA expression analysis identified diverse molecular lesions and pathway activation in drug-resistant tumor cells. Ninety percent of the tumors contained a genetic alteration potentially treatable with a currently available targeted therapy. Thus, profiling residual TNBCs after NAC identifies targetable molecular lesions in the chemotherapy-resistant component of the tumor, which may mirror micrometastases destined to recur clinically. These data can guide biomarker-driven adjuvant studies targeting these micrometastases to improve the outcome of patients with TNBC who do not respond completely to NAC.

SIGNIFICANCE: This study demonstrates the spectrum of genomic alterations present in residual TNBC after NAC. Because TNBCs that do not achieve a CR after NAC are likely to recur as metastatic disease at variable times after surgery, these alterations may guide the selection of targeted therapies immediately after mastectomy before these metastases become evident. Cancer Discov; 4(2); 232-45. ©2013 AACR.
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centroids (7) revealed a predominance of tumors with basal-like gene expression (Supplementary Fig. S1A). After adjusting for seven tumors that exhibited HER2 amplification (see below), 70% of TNBCs were basal-like, which is similar to previously published rates in larger datasets (12). Basal-like status was associated with a trend toward worse RFS and OS (log-rank test, \( P = 0.12 \) and 0.58, respectively; Supplementary Fig. S1B). As we have previously demonstrated (10), the Ki67 score in the residual disease varied significantly among molecular subtypes within this TNBC cohort, but was not prognostic (Supplementary Fig. S1C and S1D). Ki67 staining decreased significantly in response to chemotheraphy (\( P < 0.0001 \), paired \( t \) test; Supplementary Fig. S1E), but this change was not different among the molecular subtypes (Supplementary Fig. S1F). Tumor cellularity was significantly decreased between the pre- and post-NAC samples (paired \( t \) test, \( P < 0.0001 \); Supplementary Fig. S1G). Node status at surgery (an established prognostic marker), but not a change in Ki67, was predictive of both RFS and OS, although this effect seemed to be confined only to postmenopausal women (Supplementary Fig. S2). These data suggest that the underlying molecular subtype may confound the prognostic ability of Ki67 score in the residual disease after NAC.

Genomic Alterations in Drug-Resistant Residual Cancers after NAC

To identify targetable molecular lesions present in breast cancers after NAC, we performed targeted NGS of 3,320 exons in 85 formalin-fixed, paraffin-embedded (FFPE) cancers frequently rearranged in cancer (Supplementary Table S4). Compared with basal-like primary tumors in the TCGA, we detected a higher frequency of amplifications (54% in post-NAC TNBC vs. 10% in TCGA basal-like tumors). This difference suggests that these alterations are present at higher frequency in chemotherapy-treated TNBCs, and may play a role in de novo or acquired therapeutic resistance. However, it is important to note that these comparisons of CNAs with the TCGA data are made between platforms [NGS vs. Affymetrix single-nucleotide polymorphism (SNP) arrays], and thus some variation in calling rates and detection of alterations may be platform-specific.

Identified alterations were categorized into several key pathway or functional groups: cell-cycle alterations (amplifications in CDK4, CDK6, CCND1, CCND2, CCND3, CCNE1, or AURKA and loss of CDKN2A, CDKN2B, or RB1); phosphoinositide 3-kinase (PI3K)/mTOR alterations (amplifications of AKT1, AKT2, AKT3, PIK3CA, RAPIR, or RICTOR; loss or mutation of PTEN; truncations or nonsense mutations in TSC1; amplifications or mutations in PIK3CA or PIK3R1); growth factor receptor (GFR) amplifications (EGFR, MET, KIT, FGFRI, 2, and 4, or IGFIR); RAS/mitogen-activated protein kinase (MAPK) alterations (amplifications/gains of KRAS, BRAF, or RAF1, or truncations of NF1); or DNA repair alterations (truncations, loss or mutations of BRCA1 or BRCA2, or mutations in ATM; Fig. 1B). Importantly, more than 90% of the patients had alterations in at least one of these clinically targetable pathways.

Gene Expression Analysis

NanoString gene expression analysis was performed in 104 of 111 samples; 89 samples (86%) passed quality control metrics (Supplementary Table S5). Sixty-five samples were analyzed by both NGS and NanoString. Overall, 450 transcripts were quantified. These 450 transcripts were selected on the basis of their inclusion in published gene expression signatures or based on their association with the post-NAC Ki67 score we reported recently (10). Specifically, we included the PAM50 genes (7), a signature of MAP-ERK kinase (MEK) activation (15), a signature of TGF-β activation (16), and genes we have previously shown to correlate with the post-NAC Ki67 score (10). These signatures were selected on the basis of our previous studies demonstrating association of DUSP4 loss with the MEK activation signature, and with the enrichment of TGF-β–inducible genes after NAC (10, 17). There was excellent concordance between gene expression and CNAs or mutations in cases where both were assessed (Supplementary Fig. S3). Gene expression data were used to predict the molecular subtype using the PAM50 centroids. Of the 89 samples, 10 were predicted to be of the luminal subtype. These samples were confirmed ER- and PR-negative by IHC. Gene expression analysis confirmed low ESRI and PGR expression for all samples, with the exception of two outliers for ESRI mRNA expression (basal-like and luminal B, respectively, both ER-negative by IHC; data not shown). Neither of these samples was included in the NGS analysis. This phenomenon has been noted and discussed elsewhere (18), and one possible explanation to the presence of ER/PR-negative samples with luminal-like gene expression patterns as the “Luminal Androgen Receptor” subtype that expresses the androgen receptor (AR) hormone receptor (11).
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**Figure 1.** Targetable alterations and pathways in TNBCs after NAC. **A,** the most common recurrently altered genes detected by NGS, representing amplifications, deletions, rearrangements, and known somatic mutations. **B,** organization and representation of altered genes (n = 81) into five functional and targetable pathways. A total of 118 genomic alterations were identified across 81 tumors (1.5 alterations/tumor). **C,** integrated molecular analysis of residual tumors, using unsupervised clustering based on gene expression patterns (NanoString). **D,** scatterplots depicting the differences among the clusters identified in **C** for cellularity in the entire FFPE block cross-section; cellularity in the sampled (macrodissected) hotspot; Ki67 score; TGF-β response signature; MEK signature; and DUSP4 gene expression. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Visualization of expression patterns identified distinct gene signatures that did not correlate with the breast cancer molecular subtype or pathway alterations identified by NGS, but seemed to correlate with the MEK signature score (Fig. 1C). To explore these patterns, we identified the three most prominent gene expression clusters (clusters I–III; Fig. 1C) by hierarchical clustering. These clusters contained core gene sets of 84, 6, and 30 genes, respectively, expressed within each cluster (Supplementary Table S6). Tumor cellularity in the gross specimen seemed to be a defining factor of these clusters, where cluster II represented the most paucicellular tumors with the lowest Ki67 staining (Fig. 1D). Importantly, the cellularity was less of a defining feature after considering the cellularity of the “hotspot” regions that were macrodissected for gene expression analysis. Thus, the gene expression patterns may be influenced by tumor sampling but could also be reflective of the underlying microenvironment resulting from a strong antitumor effect from neoadjuvant therapy. Clusters I and III seemed more similar in terms of cellularity and Ki67 staining (Fig. 1D). However, cluster I had a distinct lack of expression of TGF-β-responsive genes. Cluster III had a high MEK signature score. This cluster contained a group of tumors with low expression of DUSP4, a negative-regulator of the MAPK pathway, offering at least one possible mechanism of MEK activation (Fig. 1D). Importantly, survival of the patients comprising these clusters was not significantly different (data not shown).

Bioinformatic exploration of these gene sets with the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb) suggested that cluster I was driven primarily by luminal-like breast expression patterns (Supplementary Table S7), despite a lack of ER, PR, or AR IHC staining in these tumors. Furthermore, several overlapping gene signatures suggested that trimethylated H3K27 (H3K27me3) genes were highly expressed in this cluster. H3K27me3 maintains epigenetic silencing of developmental genes in stem cells, leaving them poised for expression upon differentiation (19). Thus, we speculate that cluster I is composed of more differentiated tumors with high Ki67 and low TGF-β and MEK activity. In contrast, cluster III–expressed genes overlapped with invasive signatures across many types of cancer, including signatures of poorly differentiated cancers, suggesting that this cluster reflects tumors maintained in a less differentiated state and toward a higher stem cell–like hierarchy. The expression patterns in cluster III, reflective of high MEK and TGF-β activation (including tumors with DUSP4 loss), are consistent with stem-like phenotypes induced by these pathways, as we have previously demonstrated (10, 17, 20).

Selection of Oncogenic Alterations by Chemotherapy

To quantify enrichment of alterations during NAC, we analyzed 20 matched pretreatment biopsies by NGS. We detected gain (not detected in pretreatment sample but detected in posttreatment sample) and enrichment (detected in pretreatment sample but increased in posttreatment sample) in mutational allele frequencies and copy-number estimations in 41 patient-specific alterations (Supplementary Fig. S4). Many of these enrichments and gains occurred in genes comprising cell-cycle regulators and PI3K/mTOR pathway genes. Although the number of alterations in GFRs and DNA repair genes were low in this subset, significant gains and enrichments were noted in these pathways as well.

Some paired samples demonstrated gains or enrichments across several lesions, suggesting a difference in regional sampling or tumor purity between the pre- and posttherapy specimens. To accommodate these variations, we normalized each sample to its estimated tumor purity (see Methods) to calculate a fold change in allele or copy-number frequency across each tumor pair. This produced an expected pattern of normal distribution around zero of changes in alterations as a result of NAC, assuming that most alterations should not be selected for or against by chemotherapy (Fig. 2A and B). When analyzed by this method, several alterations were highly enriched relative to other within-sample alterations. These included two mutations in ATM: R337H and R2443Q, TP53 T253fs*11, a CDH1 splice site deletion, KDM6A L214fs*, AR A401V, and DPYD S175W. When examining CNAs in tumor pairs, we found that copy numbers of AKT and CCND family members were increased in three of four tumors each. Although copy number of MYC and MCL1 was enriched in several cases following NAC, this effect was not consistent in all tumor pairs. Furthermore, there was no clear concordance of case-specific enrichment with the therapeutic agents used for NAC. However, because the frequency of MCL1 amplifications was higher in this post-NAC cohort relative to primary tumors in the TCGA, this discordance suggests that MCL1 amplification may be associated with de novo resistance to chemotherapy, but is not enriched further upon treatment.

Amplification of MYC and MCL1 in the Residual Disease of TNBC

The antiapoptosis MCL1 protein is dynamically regulated during cell-cycle progression and shows rapid turnover rates in cancer cells (21). To determine whether MCL1 CNAs contribute to higher protein levels in breast cancer, we performed IHC for MCL1 on tissue microarrays (TMA) of this cohort. MCL1 amplification was significantly associated with increased protein expression (P = 0.01; Fig. 3A and B). However, MCL1 amplification does not seem to be the sole factor in modulating protein expression in breast cancer, as several samples showed high MCL1 protein levels by IHC in the absence of CNAs. We also detected three frameshift or nonsense mutations in FBXW7, encoding the E3 ubiquitin ligase responsible for targeting MCL-1 (and MYC) for proteasome-mediated degradation (22). However, presence of these mutations was not associated with higher protein levels of MCL-1 (Fig. 3A).

We detected a high degree of concordance between CNAs in both MYC and MCL1. MCL1 expression has been shown to facilitate MYC-induced lung cancers and leukemogenesis (23–25), although this interaction has not been shown in breast cancer. Indeed, 83% of MYC-amplified tumors also showed CNAs at MCL1 (P = 0.001; Fig. 3C). Cooccurrence of MYC and MCL1 amplification was not associated with altered prognosis (DFS or OS) as compared with the patients with amplification of either gene alone in this dataset (data not shown). This co-occurrence was also present in the basal-like breast cancers in the TCGA.
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**Figure 2.** Quantitative changes in gene alterations in TNBC tumor pairs before and after NAC. **A**, change in allele frequency of known and likely somatic alterations and gene expression signatures identified by NGS and NanoString analysis, respectively (15, 17). Gene-specific alterations occurring in at least eight (>10%) analyzed tumors were tested for prognostic impact (RFS and OS) by the setting. However, knockdown of these oncogenes increased the fractional killing at lower doses of doxorubicin relative to nontargeting siRNA (siCONTROL)–treated cells (Fig. 3I). Furthermore, lentiviral-mediated overexpression of MCL1 increased resistance to doxorubicin and docetaxel (Fig. 3J and Supplementary Fig. S5A and S5B). Resistance to doxorubicin was mediated in part by decreased baseline and doxorubicin-mediated apoptosis (Fig. 3K). Thus, MYC and MCL1 enhance cell fitness, and MCL1 additionally protects TNBCs from chemotherapy-induced apoptosis.

**Molecular Alterations in the Residual Disease after NAC Correlate with Patient Outcome**

Next, we explored the prognostic impact of genomic alterations and gene expression signatures identified by NGS and NanoString analysis, respectively (15, 17). Gene-specific alterations occurring in at least eight (>10%) analyzed tumors were tested for prognostic impact (RFS and OS) by the...
Importantly, the RAS–MAPK pathway has been shown to function as a risk factor for reduced RFS and OS. An association with poor OS, whereas high MEK transcriptional output was a risk factor for reduced RFS and OS. Prognostic Interaction of MEK

**Figure 3.** Coamplification and interaction of MYC and MCL1 in TNBCs. A, MCL1 IHC score as quantified on TMAs of TNBCs after NAC. Signal intensity (a.u., arbitrary units) was normalized for tumor area and number of nuclei. FBXW7-mutant tumors are shown as green triangles. B, example images of high and low MCL1-expressing tumors by IHC. C, coamplification of MCL1 and MYC in residual breast tumors assessed in this study. Absolute numbers of tumors are shown in bars (P = 0.001, Fisher exact test). D, coamplification of MCL1 and MYC in primary basal-like breast tumors in the TCGA (13, 37). E, Western blot analysis of MCF10A cells expressing pLX302–GFP (control) or pLX302–MCL1 (V5-tagged) and pINDUCER22–MYC (HA tagged) in soft agar colony formation assay of MCF10A cells in E doxycycline (DOX) where indicated. F, quantification of colonies from each bar represents the mean colony number of triplicate wells ± SD. H, siRNA knockdown of MYC and MCL1 in HCC1143 (MYC-amplified and MCL1 gain), HCC1395 (MYC-amplified), and MDA436 (MYC- and MCL1-amplified) cells (13). (continued on following page)

likely ratio test (Table 1). Alterations in pathways, gene expression signature scores, and clinical variables were also tested. Of note, JAK2 amplification was strongly associated with poor RFS (P = 0.006; HR, 3.36), whereas BRCA1 truncations/mutations and JAK2 amplification predicted poor OS (P = 0.041; HR, 2.5 and P = 0.002; HR, 4.16, respectively). JAK2 amplification was strongly associated with poor OS (P = 0.03; HR, 0.14). MYC amplifications also demonstrated a trend toward worse OS (P = 0.084; HR, 1.78). When alterations were categorized into functional pathways, DNA repair alterations were weakly associated with poor OS (P = 0.09; HR, 1.89). Interestingly, a high MEK activation score (15) predicted poor RFS and OS (P = 0.059; HR, 1.758 and P = 0.013; HR, 2.264, respectively). These data offer insights into molecular alterations that may predict the natural history of TNBC after NAC.

**Prognostic Interaction of MEK Activation and MYC Amplification**

MYC amplifications in the residual disease trended toward an association with poor OS, whereas high MEK transcriptional output was a risk factor for reduced RFS and OS. Importantly, the RAS–MAPK pathway has been shown to cooperate with the MYC oncogene (26, 27). Thus, we tested the possibility that these perturbations may interact with one another. When the interaction term was tested by Cox proportional hazards analysis, a significant interaction was noted for RFS but not for OS (P = 0.03 and 0.83, respectively). Kaplan–Meier analysis confirmed this association (Fig. 4A–C).

The effect of the interaction between MYC and MEK activation on patient outcome suggested a mechanistic interaction linking these pathways to tumor progression. This cooperation has been demonstrated in experimental models, where MEK stabilizes MYC expression (27–34). For example, c-MYC overexpression in transgenic mice results in spontaneous breast tumors that activate MEK through the generation of KRAS mutations (30). To test this interaction on a molecular level, we used MCF10A cells stably transduced with MYC (5XMYC; ref. 35). Stable expression of MYC induced the formation of anchorage-independent MCF10A colonies. However, treatment with a single dose of a MEK1/2 inhibitor (GSK1120212/trametinib or AZD6244/selumetinib) completely abolished the ability of MYC to induce MCF10A colonies (Fig. 4D and Supplementary Fig. S6A and S6B). This effect was MEK-specific, as treatment with the
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The unmet need suggests that the identification of actionable molecular targets in the residual disease could, in turn, be explored in adjuvant trials after NAC and mastectomy.

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**DISCUSSION**

Herein, we have described the genomic landscape of drug-resistant tumor cells remaining in the breasts of patients with TNBC after anticancer chemotherapy. We also performed serial analysis to detect changes in CNAs and mutations before and after NAC. These data provide insights into genomic alterations that may predict de novo or acquired resistance to standard anticancer therapies in TNBC and could inform on the effective use of rational molecularly targeted agents in adjuvant trials. In an effort not to confound...
Table 1. Prognostic ability of clinical factors and molecular alterations

<table>
<thead>
<tr>
<th></th>
<th>RFS</th>
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<tr>
<td></td>
<td>n</td>
<td>Events</td>
<td>HR</td>
<td>P</td>
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<tr>
<td>Clinical (all patients)</td>
<td></td>
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<tr>
<td>Ki67 score &gt; 15</td>
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<td>60</td>
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<td>0.772</td>
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<tr>
<td>Genes (TNBC only)</td>
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<tr>
<td>MCL1 = amp</td>
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<td>1.700</td>
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<tr>
<td>Cell-cycle altered</td>
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<tr>
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</table>

NOTE: P ≤ 0.05 (significant) or P ≤ 0.1 (statistical trend) defined in bold italics.

*Clinical data analysis of clinically defined TNBC includes 7 samples later identified as HER2 amplified by NGS.

Our results with tumors with variable residual cancer burden, we focused on a cohort of cancers with significant macroscopic residual disease after NAC. Indeed, this represents a cohort with a particularly poor prognosis (median survival ~18 months).

Several molecular insights were gained through this analysis. We showed that the Ki67 score after NAC does not provide prognostic information in patients with TNBC. Furthermore, we confirmed our previous report demonstrating that Ki67 in the residual disease is intimately related to the underlying molecular subtype (10). We also found frequent coamplification of MCL1 and MYC that conferred an advantage in anchorage-independent growth. Importantly, these coamplifications were more frequent in this study as compared with those previously reported in primary basal-like breast tumors. Amplification of the MYC oncogene coinciding with gene expression signatures of MEK activity identified a group of patients with very poor prognosis. Furthermore, MEK inhibitors potently inhibited three-dimensional growth of MYC-overexpressing cells, suggesting a role for MEK inhibitors in MYC-amplified breast cancers.

We also detected a higher frequency of several potentially targetable alterations in this cohort of posttreatment TNBCs compared with basal-like primary breast cancers in the TCGA. These included PTEN alterations (PI3K and AKT inhibitors), and amplifications of JAK2 (ruxolitinib or tofacitinib), CDK6, CCND1, CCND2, CCND3 (CDK4/6 inhibitors), and IGF1R (dalotuzumab). Importantly, several patients’ tumors showed an enrichment of AKT family CNAs and CCND family CNAs after NAC, suggesting an association of these alterations with resistance to chemotherapy. TSC1 truncations and mutations were also identified. These alterations have been associated with high sensitivity to the TORC1 inhibitor everolimus in other tumor types (36), suggesting they generate tumor dependence on the mTOR pathway.

Overall, this analysis provides new information on the molecular alterations present in chemotherapy-resistant tumor cells within TNBCs. As supported by the poor outcome of patients with TNBC that recurs with metastatic disease after an incomplete response to NAC, we surmise that these persistent tumor cells are resistant to conventional cytotoxic chemotherapies without the addition of novel agents targeting these oncogenic pathways. Furthermore, these data suggest that molecular analysis of TNBCs not achieving a pCR to NAC should be performed routinely to stratify patients according to this information to rational adjuvant trials with molecularly targeted agents.
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**METHODS**

**Patients and Tumor Specimens**

Surgically resected tumor samples (N = 111) were from patients with TNBC diagnosed and treated with NAC at the Instituto Nacional de Enfermedades Neoplásicas (Lima, Perú). Clinical and pathologic data were retrieved from medical records under an institutionally approved protocol (INEN 10-018). Tumors were determined to be triple-negative if they were negative for ER, PR, and HER2 overexpression measured by IHC. A subset of cases was subjected to HER2 FISH to resolve discrepant findings between the HER2 IHC results and the PAM50 subtype assignment. The results were further verified by comparison with the NGS results. The diagnostic biopsy (pre-NAC) was obtained for NGS analysis in a subset (n = 20) of these patients.

**Immunohistochemistry**

Antigen retrieval for Ki67 was performed using HpH Buffer (pH 9.0) in a decloaking chamber (Biocare Medical). The Ki67 antibody (m7240; DAKO) was used at a 1:75 dilution overnight. Visualization was performed using the 4 Plus Detection System (Biocare) and 3,3’-diaminobenzidine (DAB; DAKO) as the chromogen. The section was scanned at x100 magnification and the area containing the highest number of positive cells was selected. Positive and negative tumor cells were manually counted at x400; the percentage of positive cells was calculated with at least 700 viable cells.

Antigen retrieval for ER and PR was performed using citrate buffer (pH 6) in a decloaking chamber (Biocare Medical). The ER (6F11; Vector Laboratories) and PR (PgR636; DAKO) antibodies were used at 1:200 and 1:50 dilutions, respectively, for a 1-hour incubation. Visualization for both antibodies was performed using the Envision Detection System (DAKO) and DAB (DAKO) as the chromogen. The percentage of invasive tumor cells with nuclear staining and the average intensity of all positively staining tumor cells in the section were manually counted as per the CAP/ASCO (College of American Pathologists/American Society of Clinical Oncology) guidelines (38).

**Figure 4.** Interaction of MYC amplification with MEK pathway activity correlates with poor prognosis in TNBCs. A, Kaplan–Meier analysis of RFS in patients with a high MEK transcriptional signature (ref. 15; highest 66%) versus all others (lowest 33%). B, Kaplan–Meier analysis of RFS in MYC-amplified tumors versus those with normal MYC copy number. C, combined Kaplan–Meier analysis of patients with a high MYC transcriptional signature and MYC amplification versus those with either or neither alteration. D, quantification of 3-week soft-agar colony formation assays using MCF10A cells stably transduced with MYC (5x MYC) versus vector control, plated in the presence or absence of a single dose of AZD6244/selumetinib, GSK1120212/trametinib, or the pan-PI3K inhibitor BKM120 at the indicated concentrations. Bars represent the mean colony number ± SD of three replicates. E, immunofluorescence of E-cadherin, vimentin, and DAPI in cells from D grown on chamber slides and treated with 100 nmol/L GSK1120212/trametinib. Scale bars represent 50 μm.
Antigen retrieval for HER2 was performed using pH Buffer (pH 9.0) in a decloaking chamber (Biocare Medical). The HER2 antibody (#2242; Cell Signaling Technology) was used at a 1:200 dilution overnight. Visualization was performed using the Envision Detection System (DAKO) and DAB (DAKO) as the chromogen. The percentage of invasive tumor cells with membranous staining at the highest intensity level was manually assessed and recorded as per the CAP/ASCO guidelines (39).

Antigen retrieval for MCL1 was performed in citrate buffer (pH 6.0) under pressure for 15 minutes; endogenous peroxidase activity was blocked by incubating with 3% H2O2 for 10 minutes. The sections were incubated with MCL1 antibody (Santa Cruz Biotechnology; sc-819) at 1:800 dilution overnight at 4°C and developed using DAB substrate (Vector Laboratories). Automated slide scanning and scoring were performed at the Vanderbilt Epithelial Biology Center Imaging Resource (Nashville, TN). Images were captured and quantitated using the Ariol SL-50 automated microscope system (Leica Microsystems) at ×20. Selected areas at original resolution are displayed. Immunoreactivity intensity scores were determined in areas of residual tumor cells selected by expert breast pathologists (J.M. Giltnane and M.G. Kuba) and averaged for redundant tissue cores.

HER2 FISH

FISH for detection of amplification of HER2 was performed using the PathVysion HER-2 DNA Probe Kit (PathVysion Kit; Abbott Molecular) using the Vyssis LSI HER-2/neu 17q11.2-12 SpectrumOrange and Vyssis CEP 17 17p11.1-q11.1 SpectrumGreen Alpha Satellite DNA probes. Images were visualized on a Fluorescence Olympus BX60 Microscope and analyzed using the Genus for Genetic Image Analysis software, version 3.6. The ratio of HER2 to CEP 17 signals was recorded and reported as an average ratio as per the CAP/ASCO guidelines (39).

Table 2. Actionability of lesions identified in at least three post-NAC specimens

<table>
<thead>
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<th>Gene symbol</th>
<th># Altered</th>
<th>Category</th>
<th>Potential therapy</th>
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<td>TP53</td>
<td>73</td>
<td>D</td>
<td>Prognostic (poor, potentially sensitive to WEE1 inhibitors, e.g., MK1775)</td>
</tr>
<tr>
<td>MCL1</td>
<td>40</td>
<td>C</td>
<td>Resistance to anti-tubulins, e.g., paclitaxel, MCL1 inhibitor in development</td>
</tr>
<tr>
<td>MYC</td>
<td>24</td>
<td>C</td>
<td>Aurora kinase inhibitors, e.g., MLN8237, AMG 900, possible sensitivity to CDK inhibitors</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>13</td>
<td>B</td>
<td>PI3K/mTOR inhibitors, e.g., everolimus, temsirolimus, and others</td>
</tr>
<tr>
<td>PTEN</td>
<td>12</td>
<td>B</td>
<td>PI3K/mTOR inhibitors, e.g., GSK2636771, everolimus, temsirolimus, and others</td>
</tr>
<tr>
<td>BRCA1</td>
<td>9</td>
<td>B</td>
<td>PARP inhibitors, e.g., olaparib, CEP-9722, rucaparib, and others</td>
</tr>
<tr>
<td>RB1</td>
<td>9</td>
<td>D</td>
<td>Prognostic</td>
</tr>
<tr>
<td>JAK2</td>
<td>8</td>
<td>D</td>
<td>JAK2 inhibitors, e.g., ruxolitinib, and others</td>
</tr>
<tr>
<td>ERBB2</td>
<td>7</td>
<td>A</td>
<td>Herceptin, lapatinib, and others</td>
</tr>
<tr>
<td>CDKN2A/B</td>
<td>7</td>
<td>E</td>
<td>CDK4/6 inhibitors, e.g., PD0332991, LEE011, P276-00</td>
</tr>
<tr>
<td>NF1</td>
<td>5</td>
<td>C</td>
<td>MAPK/PI3K/mTOR inhibitors, e.g., MSC1936369B, everolimus, temsirolimus, and others</td>
</tr>
<tr>
<td>AKT3</td>
<td>5</td>
<td>C</td>
<td>AKT inhibitors, e.g., MK2206, PI3K/mTOR inhibitors, e.g., everolimus, temsirolimus</td>
</tr>
<tr>
<td>KRAS</td>
<td>5</td>
<td>A</td>
<td>Resistance to cetuximab, MEK inhibitors, e.g., MEK162</td>
</tr>
<tr>
<td>CCND1</td>
<td>5</td>
<td>C</td>
<td>CDK4/6 inhibitors, e.g., PD0332991, LEE011, P276-00</td>
</tr>
<tr>
<td>CCND3</td>
<td>4</td>
<td>C</td>
<td>CDK inhibitors, kinetin riboside</td>
</tr>
<tr>
<td>CCNE1</td>
<td>4</td>
<td>C</td>
<td>CDK2/4/6 inhibitors, e.g., ABT-888, PD0332991, LEE011, P276-00</td>
</tr>
<tr>
<td>CCND2</td>
<td>4</td>
<td>C</td>
<td>CDK inhibitors, kinetin riboside</td>
</tr>
<tr>
<td>CDK6</td>
<td>4</td>
<td>C</td>
<td>CDK4/6 inhibitors, e.g., PD0332991, LEE011, P276-00</td>
</tr>
<tr>
<td>IGF1R</td>
<td>4</td>
<td>C</td>
<td>IGF-IR inhibitors, e.g., AMG-479, BMS-754808, MK-0646, IMC A12, and others</td>
</tr>
<tr>
<td>LRP1B</td>
<td>3</td>
<td>E</td>
<td>Biologically relevant, presently no known targeted therapies</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>3</td>
<td>C</td>
<td>PI3K pathway inhibitors</td>
</tr>
<tr>
<td>ATM</td>
<td>3</td>
<td>C</td>
<td>PARP inhibitors, e.g., olaparib, CEP-9722, rucaparib</td>
</tr>
<tr>
<td>BRCA2</td>
<td>3</td>
<td>B</td>
<td>PARP inhibitors, e.g., olaparib, CEP-9722, rucaparib, and others</td>
</tr>
<tr>
<td>EGFR</td>
<td>3</td>
<td>A</td>
<td>Cetuximab, panitumumab, and others</td>
</tr>
<tr>
<td>FBXW7</td>
<td>3</td>
<td>C</td>
<td>Resistance to anti-tubulins, potential sensitivity to PI3K/mTOR inhibitors</td>
</tr>
<tr>
<td>CDK4</td>
<td>3</td>
<td>C</td>
<td>CDK4/6 inhibitors, e.g., PD0332991, LEE011, P276-00</td>
</tr>
<tr>
<td>RPTOR</td>
<td>3</td>
<td>E</td>
<td>Biologically relevant, possible sensitivity to mTORC1 and mTORC2 inhibitors</td>
</tr>
</tbody>
</table>

Category A: approved/standard alterations that predict sensitivity or resistance to approved/standard therapies
Category B: alterations that are inclusion or exclusion criteria for specific experimental therapies
Category C: alterations with limited evidence that predict sensitivity or resistance to standard or experimental therapies
Category D: alterations with prognostic or diagnostic utility
Category E: alterations with clear biologic significance in cancer (i.e., driver mutations) without clear clinical implications to date

Abbreviation: IGF-IR, insulin-like growth factor-I receptor.
Sequencing of Triple-Negative Breast Tumors after Chemotherapy

**Immunofluorescence**

Immunofluorescence staining was performed as described previously (40). All primary and secondary antibodies were diluted in 12% Fraction V BSA (RPI-Cat#A30075). The following antibodies and dilutions were used: ZO-1 (Life Technologies; Cat#61673001) 1:200; E-cadherin (BD-Cat#610182) 1:200, vimentin (Covance; Cat#PCK-594P) 1:500; CK5 (Covance; Cat#PRB-160P-100) 1:500, and CK8 (RD1 Fitzgerald; Cat#20R-CP004) 1:500. Secondary goat antibodies were highly cross-absorbed and used at 1:200 (Molecular Probes/Life Technologies). Chamber slides were briefly rinsed in PBS and fixed in 4% paraformaldehyde with 0.1% Triton X-100 for 20 minutes. Cells were rinsed three times with PBS and primary antibodies were applied overnight. Secondary antibodies were incubated for 20 minutes and rinsed four times in PBS then mounted in SlowFade plus DAPI (4',6-diamidino-2-phenylindole; Molecular Probes/Life Technologies).

**Tissue Microarrays**

Tissue block arrays were prepared using post-NAC FFPE tumor tissue from patients with a known triple-negative breast cancer primary tumor. Tissue microarray blocks were created at the Beecher Manual Tissue Arrayer MTA-1 (Beecher Scientific). Three foci were analyzed from each sample to generate tissue microarrays from three to six 10-mm cores. Six replicate tissue microarrays per tumor were created. Immunofluorescence staining was performed as described previously (45). Cell cycle alterations were identified and filtered using the Single Nucleotide Polymorphism Database (dbSNP; version 135; http://www.ncbi.nlm.nih.gov/projects/SNP), 1000 Genomes (http://www.1000genomes.org), and, subsequently annotated for known and likely somatic mutations using the COSMIC database (version 62; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Detection of CNAs was performed by obtaining a log-ratio profile of the sample by normalizing the sequence coverage obtained at all exons against a process-matched normal control. The profile was segmented and interpreted using allele frequencies of approximately 1,800 additional genome-wide SNPs to estimate tumor purity and copy number based on estimated methods (46–48) by fitting parameters of the equation

\[
\log_{\text{base}} \left( \frac{C_{\text{exp}} + (1-p) \times 2}{p \times \text{mutant reads} + (1-p) \times 2} \right)
\]

where \(b_{\text{log}} \), \(C_{\text{exp}} \), and \(p \) are the log-ratios and copy numbers at each segment and sample purity, respectively. Focal amplifications are called at segments with ≥6 copies and homozygous deletions at 0 copies, in samples with tumor cell purity ≥20%.

To normalize for tumor content between pre- and post-NAC-matched samples, allele frequencies or copy-number estimations were divided by fractional tumor purity to calculate normalized allele frequency or copy number for the individual sample. The pre-NAC sample-normalized frequency/copy number was subtracted from the post-NAC sample-normalized frequency or copy number to calculate the absolute change in allele frequency or copy number.

**TSC1 Deletion Verification**

PCR primers amplifying across the predicted breakpoint in intron 23 (F: ACCCAATCTCAGAAAGCTC; R: CAGTCTTCTCGCGTA) were used for PCR detection of the truncated allele.

**Cell Culture**

MCF10A cells were cultured in DMEM/F12 nutrient mix with 5% horse serum (GIBCO), 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin (all from Sigma). HCC1143 and HCC1395 were cultured in RPMI + 10% FBS (GIBCO); MDA-436 and MDA-468 were cultured in Dulbecco’s Modified Eagle Medium (DMEM) + 10% FBS. SUM159P cells were cultured in DMEM + 5% FBS and 0.5 μg/mL hydrocortisone. All cells were cultured at 37°C in 5% CO₂. MCF10A cells were purchased from the American Type Culture Collection (ATCC). All TNBC cell lines (HCC1143, HCC1395, MDA-436, MDA-468, and SUM159P) were obtained from the sources described in ref. 11 and were confirmed.

**Genomic Alteration Detection**

Base substitution detection was performed using a Bayesian methodology, which allows detection of somatic mutations at a low mutation allele frequency and increased sensitivity for mutations at hotspot sites (44) through the incorporation of tissue-specific prior expectations.

\[
P(\text{Mutation present} | \text{Read data} = \text{R}) = P(\text{Frequency of mutation} = F > 0 | F) \propto 1 - P(R | F = 0) P(F = 0)
\]

where \(P(R|F)\) is evaluated with a multinomial distribution of the observed allele counts using empirically observed error rates and \(P(F=0)\) is the prior expectation of mutation in the tumor type. To detect indels, de novo local assembly in each targeted exon was performed using the de-Brujin approach (45). Candidate calls were filtered using a series of quality metrics, including strand bias, read location bias, and a custom database of sequencing artifacts derived from normal controls. Germ-line alterations were identified and filtered using the Single Nucleotide Polymorphism Database (dbSNP; version 135; http://www.ncbi.nlm.nih.gov/projects/SNP), 1000 Genomes (http://www.1000genomes.org), and, subsequently annotated for known and likely somatic mutations using the COSMIC database (version 62; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). Detection of CNAs was performed by obtaining a log-ratio profile of the sample by normalizing the sequence coverage obtained at all exons against a process-matched normal control. The profile was segmented and interpreted using allele frequencies of approximately 1,800 additional genome-wide SNPs to estimate tumor purity and copy number based on established methods (46–48) by fitting parameters of the equation

\[
\log_{\text{base}} \left( \frac{C_{\text{exp}} + (1-p) \times 2}{p \times \text{mutant reads} + (1-p) \times 2} \right)
\]

where \(b_{\text{log}} \), \(C_{\text{exp}} \), and \(p \) are the log-ratios and copy numbers at each segment and sample purity, respectively. Focal amplifications are called at segments with ≥6 copies and homozygous deletions at 0 copies, in samples with tumor cell purity ≥20%.

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by DNA fingerprinting (Cell Line Genetics) on March 24, 2011, to consist of a single cell line and to match the DNA fingerprint on file at ATCC. Frozen stocks were archived shortly after fingerprinting from the confirmed cell lines were used in these studies.

**Chemicals**

GSK1120212 (trameretinib), AZD6244 (selumetinib), and BKM-120 were purchased from Selleckchem, dissolved in dimethyl sulfoxide (DMSO) and used at dilutions resulting in a final concentration of <0.1% DMSO in all studies in vitro. Doxorubicin was purchased from Sigma and was solubilized in DMSO at a concentration of 100 mM/mL.

**siRNA Knockdown**

siRNA knockdown was performed as previously described (20). Cells were transfected with 20 nmol/L siCONTROL (nontargeting siRNA), 10 nmol/L siMYC + 10 nmol/L siCTRL, 10 nmol/L siMCL1 + 10 nmol/L siCTRL, or 10 nmol/L siMYC + 10 nmol/L siMCLI. Constructs for siMYC and siMCLI were purchased from Ambion (s9129 and s8583, respectively).

**MCL-1 Overexpression**

Cells were transduced with lentiviral particles derived from 293FT cells transfected with pLX302-MCL1 or GFP, GFP, and MCL1 vectors were purchased from Thermo Scientific (Open Biosystems).

**Soft Agar Colony Formation Assays**

These assays were carried out in 6- or 12-well dishes using 5 × 10⁴ or 1 × 10⁴ cells, respectively. A single-cell suspension in 0.4% agarose in 1× media was layered on the top of a bottom layer of 0.8% agarose in 1× media in the presence of inhibitors or DMSO (control). Fresh 1× media (no drug) was applied to cells every 3 to 4 days to protect against dehydration. Colonies measuring >80 μm were counted after 2 to 3 weeks on a Gelcount Scanner (Oxford Optronix).

**Immunoblotting**

Immunoblotting was carried out as described previously (43). Antibodies used for immunoblotting were: p-ERK1/2 (p-T202/Y204; #9101), ERK1/2 (#9102), p-AKT (p-S473; #9271), AKT (# 9272), HA-tag (#2367), calnexin (#2433; all from Cell Signaling Technology), and V5-tag (Invitrogen R960-25).

**Statistical Analysis**

Statistics were performed where indicated using R (49) or Graph-Pad Prism (GraphPad Software). P < 0.05 was considered statistically significant and P > 0.1 was considered a statistical trend.

**Disclosure of Potential Conflicts of Interest**

J.S. Ross is employed as Medical Director at Foundation Medicine, Inc., has received a research grant from Foundation Medicine, Inc., and has ownership interest (including patents) in the same. G.A. Palmer, R. Yelensky, and M. Cronin have ownership interest (including patents) in the same. G.A. Palmer, R. Yelensky, and M. Cronin have ownership interest (including patents) in Foundation Medicine, Inc., and has received a commercial research grant from Foundation Medicine, Inc., and has ownership interest (including patents) in the same. G.A. Palmer, R. Yelensky, and M. Cronin have ownership interest (including patents) in Foundation Medicine, Inc., and has ownership interest (including patents) in the same. G.A. Palmer, R. Yelensky, and M. Cronin have ownership interest (including patents) in Foundation Medicine, Inc., and has ownership interest (including patents) in the same. G.A. Palmer, R. Yelensky, and M. Cronin have ownership interest (including patents) in Foundation Medicine, Inc., and has ownership interest (including patents) in the same.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Balko, J.M. Giltnane, K. Wang, M.E. Sanders, P.D. Moore, J.A. Pinto, H. Gómez, J.A. Bauer, J.A. Pietenpol, J.S. Ross, R. Yelensky, V.A. Miller, C.L. Arteaga

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Balko, L.J. Schwarz, R.S. Cook, P. Owens, V. Sánchez, P.D. Moore, D. Hornuchi, A. Goga, C.L. Arteaga

Study supervision: J.M. Balko, M. Cronin, C.L. Arteaga

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**REFERENCES**


Sequencing of Triple-Negative Breast Tumors after Chemotherapy


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