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

INTRODUCTION

Neoadjuvant chemotherapy (NAC) is used increasingly in patients with triple-negative breast cancer (TNBC), a subtype lacking expression of estrogen receptor (ER), progesterone receptor (PR), or HER2 amplification. The goals of NAC are to increase the likelihood of breast-conserving surgery and to eliminate clinically silent micrometastases. Approximately 30% of TNBC patients who receive NAC achieve a pathologic complete response (pCR). These patients have a favorable recurrence-free survival (RFS) and overall survival (OS; refs. 1-3). The remaining patients with residual viable cancer in the breast or lymph nodes exhibit high rates of metastatic recurrence and an overall poor long-term outcome (1-3).

Immunohistochemistry (IHC) of the proliferation marker Ki67 in post-NAC residual disease has been shown to correlate with patient outcome (4-6). Previous studies showing the prognostic ability of Ki67 after NAC included all subtypes of breast cancer (i.e., HER2-enriched, luminal A, luminal B, and basal-like), which also offer prognostic information (7-9). We have recently shown that these subtypes differ vastly in their post-NAC Ki67 scores, confounding the prognostic utility of Ki67 in this setting (10), and this has been confirmed by other investigators (9). Furthermore, Ki67 scoring is difficult to standardize among clinical laboratories and many studies have defined different “cutoffs” for patient stratification, ranging from 14% to 50% (4-6). Finally, the Ki67 scoring of the post-NAC residual tumor is not actionable as it does not identify a pathogenic driver of the tumor and, as such, a drug target and rational treatment decision.

Intuition suggests that tumor cells remaining after NAC contain the cancer cell population intrinsically resistant to chemotherapy. These tumor cells likely mirror the micrometastatic component of the disease that is ultimately responsible for distant metastases, and is unlikely to be highly sensitive to further chemotherapy once clinical metastases become evident. The standard of care for patients with TNBC who have residual disease after NAC is observation, as therapies that would be effective in reducing recurrences are unknown. Thus, we molecularly profiled the residual disease remaining after NAC in a cohort of 111 TNBCs [including gene expression analysis of 89 tumors and next-generation sequencing (NGS) of 80 tumors, 74 of which were TNBC] to identify lesions that could be therapeutically targeted in adjuvant trials.

RESULTS

Ki67 Does Not Predict Clinical Outcome in TNBCs

Because TNBC is a heterogeneous subtype of breast cancer (11), we determined whether Ki67 could predict patient outcome within this clinical subtype by scoring Ki67 in the residual disease of a cohort of 111 TNBCs after NAC. Patient demographics are listed in Supplementary Table S1. Molecular subtyping based on gene expression using the PAM50...
cancers after NAC, we performed targeted NGS of 3,320 exons from seven tumors that exhibited residual disease significantly among molecular subtypes within this TNBC cohort, but was not prognostic (Supplementary Fig. S1C and S1D). Ki67 staining decreased significantly in response to chemotherapy (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 of 182 oncogenes and tumor suppressors plus 37 introns of 14 genes frequently rearranged in cancer (Supplementary Table S2), including intergenic targets throughout the genome for copy-number alteration (CNA) analysis. NGS analysis was attempted in 85 formalin-fixed, paraffin-embedded (FFPE) cancers with sufficiently high tumor cellularity (>20%); 81 (95%) were successfully analyzed. Paucicellular samples were enriched by macrodissociation such that the sampled region was as close to 20% tumor nuclei as possible, or greater. Mean depth of coverage across all samples was 609× (range, 131–1,215). Six samples lacked sufficient depth of coverage (<200×) to make calls in CNAs with confidence. Seven tumors harbored HER2 amplification (confirmed by FISH) and were excluded from further analysis. All remaining post-NAC tumors were ER- and PR-negative by IHC. Thus, 74 tumors had evaluable NGS data, 68 of which also had CNA data. No obvious differences between the NGS-evaluable population and the entire cohort were observed in terms of outcome or clinical characteristics. NGS analysis revealed a diversity of lesions, many of which were present in less than 5% of samples (Fig. 1A and Supplementary Table S3).

Alterations in TP53 were identified in 72 of 81 samples (89%), which is similar to other studies of basal-like breast cancer or TNBC, and The Cancer Genome Atlas (TCGA) dataset (~85%; refs. 13, 14). The next most common alterations included MCL1 (54%) and MYC (35%) gene amplifications. MYC amplifications were detected primarily in basal-like tumors (42% basal vs. 10% all others; Fisher exact test, P = 0.018) and with a similar frequency as in the basal-like cohort in the TCGA (Supplementary Table S4). Compared with basal-like primary tumors in the TCGA, we detected a higher frequency of MCL1 amplifications (54% in post-NAC TNBC vs. 19% in TCGA basal-like tumors, P = 0.0006), PTEN deletions or mutations (trend, P = 0.0697), and JAK2 amplifications (trend, P = 0.08) in the residual disease. Amplifications in CDK6 and CCND1, CCND2, and CCND3 were collectively enriched as well (24% 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, RAPTOR, or RICTOR; loss or mutation of PTEN; truncations or nonsense mutations in TSCI; amplifications or mutations in PIK3CA or PIK3R1); growth factor receptor (GFR) amplifications (EGFR, MET, KIT, FGFR1, 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 PAMS05 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 ESR1 and PGR expression for all samples, with the exception of two outliers for ESR1 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 is the “Luminal Androgen Receptor” subtype that expresses the androgen receptor (AR) hormone receptor (11).
Figure 1. Targetable alterations and pathways in TNBCs after NAC. A, 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 tumors) 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 macroadissected 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 DPD4 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.

**Coamplification 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 MCL1 (and MYC) for proteasome-mediated degradation (22). However, presence of these mutations was not associated with higher protein levels of MCL1 (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, 88% 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 (RFS 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.
Furthermore, in TNBC cell lines demonstrating gains or edly increased MCF10A colony formation (Fig. 3F and G). growth assays, concurrent overexpression of MCL1 marked the cells with a doxycycline-inducible MYC vector (Fig. 3E). Although MYC induction induced sporadic transformation of MCF10A ascertained by anchorage-independent (Fig. 3E). Although MYC induction induced sporadic transformation of MCF10A ascertained by anchorage-independent growth assays, concurrent overexpression of MCL1 markedly increased MCF10A colony formation (Fig. 3F and G). Furthermore, in TNBC cell lines demonstrating gains or amplifications in MYC and/or MCL1 including HCC1143 (MYC-amplified and MCL1 gains), HCC1395 (MYC-amplified), and MDA-436 (MYC-amplified and MCL1-amplified; ref. 13), siRNA knockdown of MYC and MCL1 reduced cell viability (Fig. 3H and I). siRNA-targeting of MYC and MCL1 did not alter the relative sensitivity to doxorubicin, a commonly used chemotherapeutic agent in the neoadjuvant 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. SS and 5B). 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 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 (SXMYC; 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
pan-PI3K inhibitor BKM120 had no effect on MCF10A anchorage-independent growth. Treatment of 5XMYC cells with a MEK inhibitor resulted in the formation of polarized normal acini, as observed by immunofluorescence for basal (CK5, vimentin), luminal (CK8, e-cadherin), and a tight-junction marker (ZO-1; Fig. 4E and Supplementary Fig. S6C and S6D). These data suggest that MYC cooperates with RAS/MAPK to drive anchorage-independent growth in breast cancer.

**Molecular Profiling for Rational Selection of Adjuvant Targeted Therapies**

Despite the high likelihood of recurrence, the current standard of care for patients with TNBC who do not achieve a pCR after NAC is a watchful waiting. Patients who recur with metastatic cancer are less likely to exhibit prolonged responses to conventional anticancer therapy, as the micrometastases that generated these clinical recurrences have already been exposed to chemotherapy in the neoadjuvant setting. This 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. Because the spectrum of alterations present in such tumors is highly diverse, we integrated existing preclinical and clinical data into an “actionability” table of rational therapies for the identified alterations (Table 2 and Supplementary Table S6).

Of note, we detected two tumors (2.5%) with presumed loss-of-function alterations in TSC1: a truncation of an intergenic region between intron 8 and the 3′-untranslated region (3′-UTR) contained in the 23rd intron (Supplementary Fig. S7), which was detected in matched pre- and post-NAC samples in 1 patient, and a nonsense mutation (TSC1_Q516*, 20% allele frequency) in another patient (Supplementary Table S2). Loss of TSC1 function has been proposed as a basis for significant clinical responses in metastatic bladder cancer to the TORC1 inhibitor everolimus (36), and may also represent a therapeutically actionable target in patients with breast cancer. Importantly, loss-of-function TSC1 deletions, truncations, or nonsense mutations were not identified in the TCGA breast cancer study (13, 37).

**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

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

aClinical 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.
Sequencing of Triple-Negative Breast Tumors after Chemotherapy

RESEARCH ARTICLE

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 DAB (DAKO) as the chromogen. The section was scanned at ×100 magnification and the area containing the highest number of positive cells was selected. Positive and negative tumor cells were manually counted at ×400; 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 MEK 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.

A

B

C

D

E

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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 by 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 Vysis LSI HER-2/neu 17q11.2-12 SpectrumOrange and Vysis 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>
<thead>
<tr>
<th>Gene symbol</th>
<th># Altered</th>
<th>Category</th>
<th>Potential therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>73</td>
<td>D</td>
<td>Prognostic (poor, potentially sensitive to WEE1 inhibitors, e.g., MK1775)</td>
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<tr>
<td>MCL1</td>
<td>40</td>
<td>C</td>
<td>Resistance to anti-tubulins, e.g., paclitaxel, MCL1 inhibitor in development</td>
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<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>
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<tr>
<td>PIK3CA</td>
<td>13</td>
<td>B</td>
<td>PI3K/mTOR inhibitors, e.g., everolimus, temsirolimus, and others</td>
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<tr>
<td>PTEN</td>
<td>12</td>
<td>B</td>
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<tr>
<td>BRCA1</td>
<td>9</td>
<td>B</td>
<td>PARP inhibitors, e.g., olaparib, CEP-9722, rucaparib, and others</td>
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<tr>
<td>RB1</td>
<td>9</td>
<td>D</td>
<td>Prognostic</td>
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<tr>
<td>JAK2</td>
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<td>D</td>
<td>JAK2 inhibitors, e.g., ruxolitinib, and others</td>
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<tr>
<td>ERBB2</td>
<td>7</td>
<td>A</td>
<td>Herceptin, lapatinib, and others</td>
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<td>CDK4/6 inhibitors, e.g., PD0332991, LEE011, P276-00</td>
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<td>C</td>
<td>MAPK/PI3K/mTOR inhibitors, e.g., MSC193639B, everolimus, temsirolimus, and others</td>
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<tr>
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<td>AKT inhibitors, e.g., MK2206, PI3K/mTOR inhibitors, e.g., everolimus, temsirolimus</td>
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<td>KRAS</td>
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<td>A</td>
<td>Resistance to cetuximab, MEK inhibitors, e.g., MEK162</td>
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<tr>
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<td>C</td>
<td>CDK4/6 inhibitors, e.g., PD0332991, LEE011, P276-00</td>
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<td>C</td>
<td>CDK inhibitors, kinetin riboside</td>
</tr>
<tr>
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<td>C</td>
<td>CDK2/4/6 inhibitors, e.g., ABT-888, PD0332991, LEE011, P276-00</td>
</tr>
<tr>
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<td>C</td>
<td>CDK inhibitors, kinetin riboside</td>
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<td>E</td>
<td>Biologically relevant, presently no known targeted therapies</td>
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<td>PI3K pathway inhibitors</td>
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<tr>
<td>ATM</td>
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<td>B</td>
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<tr>
<td>EGFR</td>
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<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>
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<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 (RFP-Chat®A30075). The following antibodies and dilutions were used: ZO-1 (Life Technologies; Cat#617300) 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 (RDI 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 and Prolong Gold (Invitrogen; 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 + DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes/Life Technologies).

Tissue Microarrays

Triple 0.6-mm cores were punched from post-NAC FFPE tumor blocks from the patient’s surgical resection specimen using the Beecher Manual Tissue Arrayer MTA-1 (Beecher Scientific). Three arrays containing approximately 100 cores per array were generated.

Gene Expression Analyses

RNA was isolated from FFPE tumor blocks by macrodissecting tumor-rich regions from three to six 10-μm sections. RNA was purified using the RNeasy FFPE Kits (Qiagen). Gene expression analysis was performed by NanoString as previously described (10). Raw transcript counts were subtracted from background (negative input control). Normalization of raw transcript counts was performed by dividing the geometric mean of seven housekeeper-control genes: NPS1, NAGA, POLR1B, CD40, WAS, B2M, and TUBB. Housekeeper-normalized transcript counts were log transformed and data were row z-score standardized before further analysis. PAM50 analysis was performed using the PAM50 quantitative real-time PCR (qRT-PCR) centroids (7) on the normalized log, gene expression data before z-standardization.

Sequencing and Primary Sequence Data Analysis

Eighty-five post-NAC tumors and 26 pre-NAC biopsies were submitted for NGS at Foundation Medicine Inc. A 4-μm of hematoxylin and eosin–stained slide was reviewed by an expert pathologist to ensure (i) a sample volume of ≥1 mm³, (ii) cellularularity ≥80% or ≥50,000 cells, and (iii) that ≥20% of the nucleated cells in the sample are derived from the tumor. DNA was extracted from 40 μm of unstained FFPE sections, typically 4 μm × 10 μm sections by digestion in a Proteinase K buffer for 12 to 24 hours and then purified with the Promega Maxwell 16 Tissue LEV DNA Kit; 50 to 200 ng of double-stranded DNA (dsDNA) in 50 to 100 μL of water in microTUBEs were fragmented to approximately 200 bp by sonication (3 minutes, 10% duty, intensity = 5, 200 cycles/burst; Covaris E210) before purification with a 1.8x volume of AMPure XP Beads (Agencourt). Solid Phase Reversible Immobilisation (SPRI) purification and subsequent library construction with the NEBNext Kits (E6040S, NEB), containing mixes for end repair, dA addition, and ligation, were performed in 96-well plates (Eppendorf) on a Bravo Benchbot (Agilent) using the “with-bead” protocol 43 to maximize reproducibility and library yield. Indexed (6 bp barcodes) sequencing libraries were PCR-amplified with HiFi (Kapa) for 10 cycles, 1.8x SPRI-purified and quantified by quantitative PCR (qPCR; Kapa SYBR Fast) and sized on a LabChip GX (Caliper); size selection was not performed. Paired-end sequencing (49 × 49 cycles) was performed using the HiSeq2000 (Illumina). Sequence data from genomic DNA were mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner (BWA) (41). PCR duplicate read removal and sequence metric collection was performed using Picard (http://picard.sourceforge.net) and SAMtools (42). Local alignment optimization was performed using the Genome Analysis toolkit (GATK; ref. 43).

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.

\[ P(Mutation\ present \mid \text{Read~data \text{"}R\text{"}}) = P(Frequency\ of\ mutation\ > 0 \mid F) × \frac{1 - P(R \mid F = 0)}{P(F = 0)} \]

where \( P(R\mid F = 0) \) 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-Bruijn 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

\[ \log \left( \frac{C_{\text{tumor}}}{{C_{\text{normal}}} + 1} \right) = \log \left( \frac{p \times \text{normal~tumor~ploidy} + (1 - p) \times 2}{p \times \text{tumor~ploidy} + (1 - p) \times 2} \right) \]

where \( p \) is the log-ratio and \( C_{\text{tumor}} \) and \( C_{\text{normal}} \) are the copy numbers at each segment and sample purity, respectively. Focal amplifications are called at segments with ≥26 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: ACCCAAATCTCACAAAGCTCC; R: CACCTTTTCTGGTGAAA GCA, product: 100 bp) and spanning the truncation from intron 23-intron 8 (F: AACAATCTTCAAGAGTATCCGCAG R: TGTTGC CGAAAAGACGTCTGCTT, product: 170 bp) were used for PCR detection of the truncated allele.

Cell Culture

MC101A 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. SUM159PT cells were cultured in DMEM + 5% FBS and 0.5 μg/mL hydrocortisone. All cells were cultured at 37°C in at 5% CO₂. MC101A cells were purchased from the American Type Culture Collection (ATCC). All TNBC cell lines (HCC1143, HCC1395, MDA-436, MDA-468, and SUM159PT) were obtained from the sources described in ref. 11 and were confirmed.
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 archived shortly after fingerprinting from the confirmed cell lines were used in these studies.

**Chemicals**

GSK1120212 (trameztinib), 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 mmol/L.

**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 siCONTROL, 10 nmol/L siMCL1 + 10 nmol/L siCONTROL, or 10 nmol/L siMYC + 10 nmol/L siMCL1. Constructs for siMYC and siMCL1 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 × 10^4 or 1 × 10^5 cells, respectively. A single-cell suspension in 0.4% agarose confirmed cell lines were used in these studies. A single-cell suspension in 0.4% agarose was purchased from Sigma and was solubilized in DMSO at a concentration of 100 mmol/L.

**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 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. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Balko, J.M. Gilnane, 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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Study supervision: J.M. Balko, M. Cronin, C.L. Arteaga

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**REFERENCES**

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