Serial Tumor Molecular Profiling of Newly Diagnosed HER2-Negative Breast Cancers During Chemotherapy in Combination with Angiogenesis Inhibitors

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Abstract

Background  Breast cancers are heterogeneous with variable clinical courses and treatment responses.

Objective  We sought to evaluate dynamic changes in the molecular landscape of HER2-negative tumors treated with chemotherapy and anti-angiogenic agents.

Patients and Methods  Newly diagnosed HER2-negative breast cancer patients received low-dose sunitinib or bevacizumab prior to four 2-weekly cycles of dose-dense doxorubicin and cyclophosphamide. Tumor biopsies were obtained at baseline, after 2 weeks and after 8 weeks of chemotherapy. Next-generation sequencing was performed to assess for single nucleotide variants (SNVs) and copy number alterations (CNAs) of 440 cancer-related genes (ACTOnco®). Observed genomic changes were correlated with the Miller-Payne histological response to treatment.

Results  Thirty-four patients received sunitinib and 18 received bevacizumab. In total, 77% were hormone receptor positive (HER2−/HR+) and 23% were triple negative breast cancers (TNBC). New therapy-induced mutations were infrequent, occurring only in 13%, and appeared early after a single cycle of treatment. Seventy-two percent developed changes in the variant allele frequency (VAF) of pathogenic SNVs; the majority (51%) of these changes occurred early at 2 weeks and were sustained for 8 weeks. Changes in VAF of SNVs were most commonly seen in the PI3K/mTOR/AKT pathway; 13% developed changes in pathogenic mutations, which potentially confer sensitivity to PIK3CA inhibitors. Tumors with poor Miller-Payne response to treatment were less likely to experience changes in VAF of SNVs compared with those with good response (50% [7/14] vs 15% [4/24] had no changes observed at any timepoint, \( p = 0.029 \)).

Conclusions  Serial molecular profiling identifies early therapy-induced genomic alterations, which may guide future selection of targeted therapies in breast cancer patients who progress after standard chemotherapy.

Clinical trial registration  ClinicalTrials.gov: NCT02790580 (first posted June 6, 2016).

Key Points

The molecular landscape of HER2− breast cancer is heterogenous.

Allele frequencies of many targetable alterations change early during treatment; PI3K pathway and RTK genes were often altered in those tumors with poor histological response.

Early intensification with addition of targeted agents could benefit these patients.
1 Introduction

Breast cancer is one of the most common cancers with more than 2 million new cases and 650,000 deaths worldwide in 2020 [1]. Breast cancer is a heterogenous group of diseases with variable clinical courses and treatment responses. Treatment decisions are typically guided by the clinical subtypes: the hormone receptor positive (HR+) group, HER2 amplified (HER2+) group, and triple-negative breast cancers (TNBC). However, it is recognized that heterogeneity also exists within these broad classifications. Analysis of tumor genomic alterations, gene expression profiles, and immune markers have been shown to provide additional prognostic and predictive value [2–4].

In the era of precision medicine, comprehensive understanding of the genomic landscape of breast cancer and incorporation of genomic findings into patient care is crucial. The success of poly (ADP-ribose) polymerase (PARP) inhibitors [5, 6] in germline BRCA-mutated breast cancer and phosphoinositide 3-kinase (PI3K) inhibitors [3] in PIK3CA-mutated HR+ breast cancer together highlight the importance of genomic testing to guide therapy. Genomic analysis can also identify inducible oncogenic drivers such as ESR1 that confer resistance to certain therapeutic strategies [7, 8].

Multiple large studies including The Cancer Genome Atlas (TCGA) Network [9] and Lang et al [10] have extensively reported tumor mutational profiles demonstrating significant mutational heterogeneity within individual subtypes of breast cancer. Hormone receptor positive breast cancers were found to be the most heterogenous in terms of gene expression, mutational spectrum, copy number changes and patient outcomes [10]. Of note, most molecular studies of breast cancer have focused on single timepoint analysis of DNA sequencing of tumors [9–11]: however, tumors are dynamic and undergo evolution driven by selective treatment pressures [12], but there are limited studies on serial molecular changes that occur in tumors during and after anti-cancer treatment in the clinic.

Our group has previously studied the strategy of pretreatment with low-dose anti-angiogenic agents to normalize tumor vasculature to improve intra-tumoral delivery of standard neoadjuvant chemotherapy in breast cancer in two clinical trials [13, 14]. The first trial tested low-dose short-course sunitinib and confirmed vascular normalization on functional imaging and immunohistochemistry. The second trial tested low-dose short-course sunitinib in one cohort and low-dose bevacizumab in a second cohort, and again confirmed tumor vascularization effect by both agents. Serial tumor biopsies were collected in the trials, and these were used to carry out tumor mutational profiling to determine dynamic changes induced by systemic therapy and to compare changes between good versus poor responders. We hope to gain deeper insights into biological changes in breast cancer when exposed to treatment pressure in the clinic and to uncover potential therapeutic targets or pathways in resistant patients.

2 Materials and Methods

2.1 Patients and Tumor Specimens

Patients with newly diagnosed HER2− breast cancer were enrolled into this prospective, Phase II open-label study of chemotherapy in combination with low-dose antiangiogenic agents to normalize tumor vasculature, conducted at the National University Cancer Institute, Singapore (NCIS) [14]. The study was approved by the institution’s ethics review board and all patients signed written informed consent (ClinicalTrials.gov identifier: NCT02790580). HER2 negativity was defined as HER2 score 0 or 1+ on immunohistochemistry (IHC), or HER2 IHC 2+ but HER2 fluorescence in situ hybridization (FISH) negative (HER2/CEP17 ratio <2.0 with gene copy number <4.0 signals/cell) [15].

There were two sequential cohorts of patients – cohort 1 received dose-dense doxorubicin/cyclophosphamide (ddAC) in combination with sunitinib, cohort 2 received ddAC with bevacizumab. Low-dose sunitinib 12.5 mg daily orally was administered for 7 days prior to cycle 1 ddAC, and for 5 days prior to each subsequent cycle of ddAC. Bevacizumab 5 mg/kg was administered intravenously, 1 week before each cycle of ddAC (±1 day), every 2 weeks. Patients received 4 cycles of ddAC at standard doses (60/600 mg/m²) every 2 weeks, supported by subcutaneous pegfilgrastim 6 mg after each dose of chemotherapy.

Tumor samples from three timepoints were obtained from each subject. Ultrasound-guided core biopsies of breast tumors were obtained at baseline prior to chemotherapy and after two weeks of ddAC chemotherapy in combination with the anti-angiogenesis agent. Tumor samples from the third timepoint were obtained intra-operatively from a wide-local excision or mastectomy performed after eight weeks of the combination treatment.

Scoring of the histological response to treatment was done using Miller–Payne grading (MPG) classification, which compared the tumor cellularity between baseline and post-treatment samples. Good histological response was defined as a score of ≥3, which corresponds to more than 30% reduction in tumor cellularity [16].
2.2 Next-generation Sequencing and Sequence Data Analysis

Targeted exon sequencing was used to determine the mutational landscape of each specimen including single nucleotide variants, small insertions and deletions, and copy number variations of 440 cancer-related genes using the ACTOnco® comprehensive cancer gene panel. The 440-gene panel comprises biomarkers that are known to be associated with the pathogenesis, progression, and response to targeted, hormonal, or immuno-therapies across different solid tumors.

Forty nanograms of extracted genomic DNA from formalin-fixed paraffin-embedded (FFPE) tumor samples were amplified with four pools of primer pairs. The library was prepared using the Ion AmpliSeq Library Kit (Thermo Fisher Scientific). Amplicons were ligated with barcoded adaptors using the Ion Amplicon Library Kit (Life Technologies). Barcoded libraries were subsequently conjugated with sequencing beads by emulsion PCR and enriched using IonChef (Life Technologies) according to the Ion Torrent protocol (Life Technologies). The quality and quantity of amplified libraries were determined using the fragment analyzer (AATI) and Qubit (Invitrogen). Sequencing was performed on the Ion Proton sequencer using the Ion PI chip (Life Technologies) according to the manufacturer’s protocol. The mean sequencing depth was more than 800x, and the mean uniformity was more than 80%.

Sequencing raw reads were mapped to the hg19 human reference genome using Torrent Suite Server version 5.10, base calling and variant calling were performed with the Torrent Suite Variant Caller plug-in version 5.10. Variant Effect Predictor (VEP, version 88) was used to annotate every variant with databases from COSMIC v.86 (RRID:SCR_002260) and Genome Aggregation database r2.0.2 (RRID:SCR_014964). Variants with an allele frequency of at least 1% in Genome Aggregation database r2.0.2 and those detected in 25 peripheral blood mononuclear cell (PBMC) ACT Genomics in-house samples from healthy volunteers were disregarded as polymorphisms and excluded from further analysis. Criteria for variant analysis were coverage ≥25 and an allele frequency of ≥2% for actionable variants and ≥5% for other variants.

To quantify the serial changes in variant allele frequency of somatic mutations while accounting for differences in tumor purity, we calculated the cancer cell fraction (CCF) of each somatic mutation using methods as described by Loh et al [17]. A CCF value of 1 implies that mutation was present in 100% of cancer cells within that biopsy sample. Mutations with CCF<0.8 were categorized as subclonal and CCF≥0.8 were clonal as previously described [18]. By calculating the CCF slope (post-treatment CCF/baseline CCF) of each somatic mutation, we systematically quantified CCF changes in response to treatment. We used a slope cut-off of ≤0.8 to indicate a decrease in CCF and ≥1.25 to indicate an increase in CCF.

Copy number alterations (CNAs) were processed by removing amplicons with a coefficient of variation ≥0.3 and read counts in the lowest 5th percentile. Each pool was normalized to correct for samples from different amplicon pool designs. ONCOCNV (Boeva et al. 2014) was applied to normalize total amplicon number, guanine-cytosine content of each amplicon region, amplicon length, technology-related biases, and to segment the sample with a gene-aware model. The diploid reference baseline was established according to ACT Genomics’ in-house PBMC samples from healthy volunteers. Aberration Detection in Tumor Exome (ADTEx, RRID:SCR_012059) was applied for estimating tumor purity and correcting baseline shifts based on SNP information. Copy number gain was defined as copy number ≥4, whereas copy number loss was defined as an observed copy number ≤1. Copy number loss estimation was not provided for the samples with tumor purity less than 30% as previous studies have shown that interpretation of copy number loss is inaccurate when tumor purity is <30% [19].

Copy number alterations index was calculated by the percentage of the regions of genes altered in a tumor and the total regions of the genes that were covered in the test on the chromosome to measure degree of genomic instability across the entire genome of a tumor. Statistical analysis of CNA index distribution with differences between each group was assessed with unpaired t-test using SPSS Statistics (RRID:SCR_002865).

To distinguish between somatic and germline mutations, a prediction algorithm integrating rule-based and analytical methods was performed. The rule-based method assigned somatic or germline mutational status to a mutation by matching it to records in population databases. The analytical method follows approaches described in primary literature [20, 21], which utilize tumor purity (p), normal copy number (Cn), tumor copy number (Ct), and all possible mutated allele counts (M) based on zygosity to calculate all possible expected allele frequencies (AFs) if the mutation is somatic or germline. For germline mutations, the expected AFg = (pM + (1 − p))/((pCt + Cn(1 − p))) for somatic mutations, the expected AFs = pM/(pCt + Cn(1 − p)). By comparing the expected AFs to the observed AF, it assigns the status of the most likely expected AF to the mutation. Subsequently, by using a set of training data with known somatic and germline mutations, the algorithm integrates the two methods by optimizing the relative weights and ordering through iterative tuning.

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2.3 Statistical Analysis

Statistical analyses were performed using SPSS Statistics v23. All calculations were two-sided tests, with a $p$ value <0.05 considered as statistically significant.

3 Results

3.1 Baseline Characteristics of the Patients

Sixty-five patients with newly diagnosed HER2− stage I–IV breast cancer were enrolled into this study. We report the analysis of genomic changes in the 52 patients who had at least 2 pre- and post-treatment tumor biopsies performed. Table 1 summarizes the baseline characteristics and clinical outcomes of these patients. Thirty-four patients were treated with sunitinib (Cohort 1) and 18 patients with bevacizumab (Cohort 2). Of these, 77% had hormone receptor positive disease (HER2−/HR+) and 23% had triple negative breast cancer (TNBC). The majority of the patients (90%) were non-metastatic at diagnosis and received the study treatment as neoadjuvant therapy before curative surgery. Median duration of follow-up was 39.5 months (range 12–54). At the time of analysis, 4/47 (9%) patients who were non-metastatic at diagnosis had relapsed, at a median of 22 months (range 7–36) from the time of curative breast cancer surgery. Patients with metastatic disease at diagnosis were all HER2−/HR+ and underwent primary chemotherapy followed by palliative mastectomy as well as hormonal therapy. Metastatic disease progressed in 3/5 (60%) of patients 17, 18, and 38 months after mastectomy, while the remaining two patients with metastatic disease remained progression-free at 36 and 38 months of follow-up, respectively, after initial mastectomy and radiotherapy to oligometastatic disease (bone and contralateral axilla) and hormonal therapy.

3.2 Sample Disposition (Fig. 1)

In total, we sequenced 174 biopsy samples. Fifty-two patients had pre- and post-treatment tumor biopsy samples suitable for analysis of genomic changes. For analysis of SNV changes, there were 43 patients with 3 serial samples and 9 patients with 2 serial samples (baseline and after 2 weeks $n=18$; baseline and after 8 weeks $n=1$). These samples form the basis of analysis for the following results.

3.3 Baseline Genomic Landscape (Fig. 2)

Median number of SNVs per tumor sample was 18 (range 7–29); the percentage and median number of pathogenic SNVs was 37.5% (IQR 29–46%) and 7 (range 1–19), respectively.

Copy number alterations had a higher prevalence than mutations, with a median number of 23 (range 0–101) amplifications and/or deletions per tumor sample.

3.3.1 Comparing the SNVs between TNBC and HER2−/HR+ Tumors Present at Baseline

There was no significant difference in the number of total SNVs (median 14 [range 11–25] vs 18 [range 7–29], $p = 0.060$) or pathogenic SNVs (median 5.5 [range 0–10] vs 7 [range 1–19], $p = 0.376$) for TNBC versus HER2−/HR+ tumors. The most frequent mutations occurring in ≥25% of samples in TNBC tumors were in $TP53$ (100%), $KMT2C$ (50%), $BRCA2$ (33.3%), $SYNE1$ (33.3%), $PIK3CA$ (25.0%), $MUC16$ (25.0%) and SETD2 (25.0%); on the other hand, HER2−/HR+ tumors had mutations in $MUC16$ (45.0%), $TP53$ (42.5%), $TP53$ (42.5%), $PIK3CA$ (37.5%) and ATM (30.0%). Pathogenic $TP53$ mutations were significantly more common in TNBC tumors compared to HER2−/HR+ tumors, occurring in 12/12 (100%) versus 17/40 (42.5%) ($p = 0.014$).

3.3.2 Comparing the CNAs between TNBC and HER2−/HR+ Tumors Present at Baseline

There was no significant difference in the median number of CNAs in TNBC and HER2−/HR+ tumors at baseline (26 [range 2–63] vs 22 [range 0–101] for TNBC vs HER2−/HR+, $p = 0.857$). The most frequent CNAs in TNBCs were in $RECQL4$ (41.7%), $MCL1$ (33.3%), $TSC2$ (33.3%), $NTRK1$ (33.3%) and $CDK5$ (33.3%); HER2−/HR+ tumors had frequent CNAs in $RECQL4$ (40.0%), $RUNX1T1$ (32.5%), $USH2A$ (32.5%), $MYC$ (30.0%), $NBN$ (30.0%), $CCNE2$ (30.0%). However, there was no significant difference in the frequency of commonly occurring CNAs between TNBC and HER2−/HR+ tumors at baseline including in $RUNX1T1$ ($p = 0.288$), $MYC/NBN/CCNE2$ ($p = 0.361$) and $USH2A$ ($p = 0.098$).
Table 1  Clinicopathological characteristics and treatment outcomes

| Clinicopathological characteristics | No. of patients (%) |
|-------------------------------------|---------------------|
|                                    | All patients; \(N=52\) | Sunitinib; \(N=34\) | Bevacizumab; \(N=18\) |
| Median age                          | 52; range 29–70      | 52; range 30–69      | 52; range 29–70      |
| Post-menopausal                     | 30 (58%)             | 20 (59%)             | 10 (55%)             |
| Pre-menopausal                      | 22 (42%)             | 14 (41%)             | 8 (45%)              |
| Chinese                             | 34 (65%)             | 23 (68%)             | 11 (61%)             |
| Malay                               | 10 (19%)             | 5 (15%)              | 5 (28%)              |
| Indian                              | 3 (6%)               | 3 (9%)               | 0 (0%)               |
| Others                              | 5 (10%)              | 3 (9%)               | 2 (11%)              |
| TNBC                                | 12 (23%)             | 8 (24%)              | 4 (22%)              |
| Non TNBC (HER2−/HR+)                | 40 (77%)             | 26 (76%)             | 14 (78%)             |
| Grade 1                             | 2 (4%)               | 2 (6%)               | 0 (0%)               |
| Grade 2                             | 19 (36%)             | 9 (26%)              | 10 (55%)             |
| Grade 3                             | 31 (60%)             | 23 (68%)             | 8 (45%)              |
| Clinical stage                      |                      |                     |                     |
| Stage I                             | 1 (2%)               | 0 (0%)               | 1 (6%)               |
| Stage II                            | 29 (55%)             | 19 (56%)             | 10 (55%)             |
| Stage III                           | 17 (33%)             | 10 (29%)             | 7 (39%)              |
| Stage IV                            | 5 (10%)              | 5 (15%)              | 0 (0%)               |
| Non metastatic                     | 47 (90%)             | 29 (85%)             | 18 (100%)            |
| Metastatic                          | 5 (10%)              | 5 (15%)              | 0 (0%)               |
| Pathological response               |                      |                     |                     |
| Pathological complete response      | 2 (4%)               | 1 (3%)               | 1 (6%)               |
| Residual tumor present             | 50 (96%)             | 33 (97%)             | 17 (94%)             |
| Miller Payne histological response |                      |                     |                     |
| Good                                | 29 (56%)             | 18 (53%)             | 11 (61%)             |
| Poor                                | 15 (29%)             | 11 (32%)             | 4 (22%)              |
| Unable to assess\(^a\)             | 8 (15%)              | 5 (15%)              | 3 (17%)              |
| Clinical objective response on CT imaging by RECIST | | | |
| Clinical CR/PR                      | 38 (73%)             | 23 (67%)             | 15 (83%)             |
| Clinical stable disease             | 12 (23%)             | 9 (27%)              | 3 (17%)              |
| Primary progression of disease      | 0 (0%)               | 0 (0%)               | 0 (0%)               |
| No imaging for RECIST assessment done after treatment | 2 (4%) | 2 (6%) | 0 (0%) |
| Dead                                | 2 (4%)               | 1 (3%)               | 1 (6%)               |
| Alive                               | 50 (96%)             | 33 (97%)             | 17 (94%)             |
| Stage I–III patients who developed relapse | 4 (9%) | 3 (10%) | 1 (6%) |
| Stage I–III patients who remained disease free | 43 (91%) | 26 (90%) | 17 (94%) |
| Stage IV patients who developed progression of disease | 3 (60%) | 3 (60%) | 0 |
| Stage IV patients who remained progression free after mastectomy and radiotherapy | 2 (40%) | 2 (40%) | 0 |

\(^a\)Miller Payne score could not be assessed in patients if there was insufficient tissue after prior sectioning for IHC analysis or if the baseline tumor content was <5% in the baseline samples

CR/PR complete response/partial response; CT computed tomography; HER2– human epidermal growth factor receptor 2 negative; HR+ hormone receptor positive; IHC immunohistochemistry; RECIST response evaluation criteria in solid tumors
3.3.3 Comparing Mutations in Key Pathways between TNBC and HER2−/HR+ Tumors Present at Baseline

Baseline tumor SNVs and CNAs were further categorized by key pathways including phosphoinositide 3-kinase (PI3K)/mTOR alterations, DNA repair alterations, cell-cycle alterations, RTK/RAS/mitogen-activated protein kinase (MAPK) alterations and growth factor receptor (GFR) pathway (Supplemental Fig. 1).

PI3K/mTOR/ AKT pathway was most commonly affected with 20/52 (38%) of tumors harboring a pathogenic or likely pathogenic \textit{PIK3CA} alteration at baseline; five tumors had ≥2 pathogenic \textit{PIK3CA} mutations. There was no significant difference in frequency of \textit{PIK3CA} mutations in TNBC versus HER2−/HR+ tumors (3/12 [25.0%] vs 15/40 [37.5%], \(p = 0.274\)).

Pathogenic mutations in homologous recombination DNA damage repair (HR-DDR) genes (including \textit{ARID1A}, \textit{ATM}, \textit{ATR}, \textit{BAP1}, \textit{BARD1}, \textit{BLM}, \textit{BRCA1/2}, \textit{BRIP1}, \textit{CHEK1/2}, \textit{FANCA/C/D2/E/F/G/L}, \textit{MRE11}, \textit{NBN}, \textit{PALB2}, \textit{RAD50}, \textit{RAD51}, \textit{RAD51B}) were detected in 56% of tumors. The prevalence of HR-DDR mutations at baseline was not significantly different between TNBCs and HER2−/HR+ breast cancers (\(p = 0.386\)).

Pathogenic DNA mismatch repair gene mutations (in \textit{MLH1}, \textit{MSH2}, \textit{MSH6}, \textit{PMS2}) were present at baseline in 23% (12/52) of tumors. Again, the prevalence of mismatch repair mutations was not significantly different between TNBCs and HER2−/HR+ tumors (\(p = 0.336\)).

We investigated whether the presence of HR-DDR mutations or mismatch repair mutations contributed to higher somatic mutation burden in tumors. Indeed, tumors harboring HR-DDR mutations had more somatic mutations compared to those without HR-DDR mutations (median number of pathogenic SNVs 7.5 [range 4–19] vs 6.0 [range 0–1], \(p = 0.036\)). On the other hand, presence of mismatch repair mutations was not associated with increase in number of pathogenic SNVs (median 6.5 [range 3–10] vs 6.5 [range 0–19], \(p = 0.962\)).
3.3.4 Alterations in Genes of Signaling Molecules that Interact with the VEGF Angiogenesis Pathway at Baseline

Vascular endothelial growth factor (VEGF)-A and VEGF-B amplification was present in 5/30 (16.7%) and 7/30 (23.3%) of tumors, respectively, at baseline. A higher proportion of TNBC compared to HER2−/HR+ patients had amplification of VEGF-A (33.3% vs 2.5%, Fisher’s Exact \( p = 0.008 \)) and VEGF-B (16.7% vs 10.0%, \( p = 0.612 \)) in the baseline tumor (Fig. 3). Vascular endothelial growth factor-receptor activation is regulated by and interacts with a complex network of signaling cascades to promote cell growth, migration and survival [22]. Figure 3 is a module diagram that shows the percentage of TNBC versus HER2−/HR+ samples which have alterations of relevant oncogene molecules in signaling cascades that are documented to interact with angiogenesis pathway molecules. Apart from differences in amplification of VEGF-A, there were no other significant differences in incidence of genomic alterations in individual genes at baseline between HER2−/HR+ and TNBC tumors. However, HER2−/HR+ tumors have a higher proportion of samples with activating mutations in receptor tyrosine kinase genes compared to TNBC tumors (\( p = 0.049 \)).

3.4 Changes in Molecular Landscape that Occur after Chemotherapy and Anti-angiogenesis Therapy

3.4.1 Chemotherapy and Anti-angiogenesis Therapy Led to a Handful of New Mutations and Changes in VAF of Multiple Pathogenic SNVs

To evaluate how the genomic landscape changed in response to systemic therapy, we searched for new treatment-induced mutations. Seven patients (13%) developed a single new treatment-induced mutation and one patient (2%) developed two mutations, which all appeared early, after a single cycle of treatment. These mutations occurred in ARID2, FRG1, FAT1, MAP3K1, PIK3R2, KIT, HER2, RB1, CTNNB1 and SPEN; majority were likely benign, but FRG1 E63K and KIT C673R which are likely pathogenic, occurred in patients treated with chemotherapy in combination with sunitinib.

Although few new mutations were found, 31 patients (72%) developed significant changes in the VAF of pathogenic SNVs measured by cancer cell fraction (CCF) slope after exposure to chemotherapy and anti-VEGF treatment; 37% (accounting for the majority of patients with change) had early and sustained changes, while 21% had transient changes and 14% had late changes (Fig. 4). There was no significant difference in pattern of timing of changes in TNBC vs HER2−/HR+ patients (\( p = 0.869 \)) or in sunitinib versus bevacizumab cohorts (\( p = 0.276 \)).

Changes in VAF of SNVs after chemotherapy and bevacizumab/sunitinib were most commonly seen in the PI3K/mTOR/PIK3CA pathway where seven patients developed significant increase in VAF of pathogenic PIK3CA alterations, which confer sensitivity in PIK3CA inhibitors such as alpelisib or taselisib [3]. Two patients had changes in more than one PI3K pathway alteration. Figure 5 shows the change in VAF of PIK3CA mutations by cohort. Most of the tumors with significant rise in VAF of PIK3CA mutations were HER2−/HR+. Majority (60%) of these had a poor Miller–Payne response to treatment.

Majority of patients also experienced decrease in VAF of pathogenic TP53 mutations in response to treatment; the overall response rate (ORR) was 80% in these patients and a decrease in VAF of TP53 mutations was also associated with a good Miller-Payne response to treatment in 75% of patients (9/12).

3.4.2 Combination Chemotherapy with Angiogenesis Inhibitors Resulted in Copy Numbers Changes in Multiple Genes

Fifty-seven percent (17/30) experienced fold change in copy numbers (\( \geq 2 \)- or \( \leq 0.5 \)-fold compared to baseline) of clinically significant genes that were classified as actionable based on the OncoKB database (Supplementary Table 1). The median number of genes with significant \( \geq 2 \)-fold gain or \( \leq 0.5 \)-fold decrease compared to baseline was 9 (range 0–98), respectively. Majority (57%, 17/30) experienced fold changes in <10 genes; however, 8 patients experienced numerous changes with gains in 12–95 genes while 5 patients experienced numerous losses in 15–29 genes (Supplementary Table 1). There was no significant difference in copy number change between the sunitinib versus bevacizumab cohorts (median number of genes with significant fold change 8 [range 0–98] vs 12 [range 0–43], \( p = 0.972 \)), HER2−/HR+ versus TNBC tumors (median number of genes with significant fold change 12 [range 0–98] vs 7 [range 1–29], \( p = 0.707 \)) and Miller-Payne good vs poor response to treatment (median number of genes with significant fold change 12 [range 0–99] vs 8 [range 0–18], \( p = 0.285 \)).

The most common genes with significant \( \geq 2 \)-fold gain were TSC2, AKT1, AKT3, FLCN, CDK5, FH and IKBKE; many of these are part of the PIK3CA/PI3K/mTOR pathway (Supplementary Fig. 2). These PI3K pathway copy number gains occurred in both sunitinib and bevacizumab treatment cohorts in 9/21 and 4/9 patients, respectively. Most of these PI3K pathway copy number gains occurred in HER2−/HR+...
patients (84%, 11/13 patients); 61% (8/13) had a poor Miller–Payne response to treatment.

3.5 Correlative Analysis of Genomic Alterations with Treatment Outcomes

As few relapse events (9%, 4/47 stage I–III patients) had occurred at the time of analysis, we did not correlate survival outcomes with observed genomic changes. The pathological complete response (pCR) rate was low (4%, 2/52); a substantial proportion had locally advanced cancer (44.6% cT3–4, 70.7% cN+), the majority of patients had HER2−/HR+ disease, which tends to have low pCR from neoadjuvant chemotherapy, and all patients underwent surgery after just 4 cycles of doxorubicin-cyclophosphamide chemotherapy without neoadjuvant taxanes. Hence, correlative analysis was performed with histological response based on the Miller–Payne grading classification.

3.5.1 Correlative Analysis of Baseline SNVs with Miller–Payne Grading of Histological Response

Patients with good Miller–Payne histological response to treatment had a higher median number of SNVs (19 [range 10–29] vs 13 [range 7–29], \( p = 0.032 \)) and pathological SNVs (8 [range 1–11] vs 4.5 [range 0–19], \( p = 0.014 \)) present at baseline. However, there was no significant difference in median number of genes with CNAs at baseline between patients with good vs poor histological response to treatment (22 [range 0–101] vs 24 [range 2–63], \( p = 0.797 \)).

3.5.2 Correlative Analysis of Changes in VAF of SNVs and CNAs with Miller–Payne Grading of Histological Response

Patients with good Miller–Payne histological response were more likely to experience changes in VAF of SNVs after treatment compared with those with poor response (85% [4/24] vs 50% [7/14] had changes observed at any timepoint, \( p = 0.029 \)).

There was no significant difference in the median number of genes with CNAs after 2 weeks of treatment between those with good versus poor Miller-Payne response (22 [range 0–100] vs 35 [range 0–106], \( p = 0.605 \)). However, after 8 weeks, those with a good Miller-Payne response had fewer genes with CNAs (lower CNA burden) compared to those with a poor response (12 [range 3–26] vs 32 [range 15–73], \( p = 0.042 \)); of note, patients with good response had fewer genes with copy number gain compared to those with poor response (11 [range 3–11] vs 30 [range 14–65], \( p = 0.012 \)).

4 Discussion

In this study, a total of 174 tumor biopsy samples from 52 patients with HER2− breast cancer were sequenced for genomic alterations – 147 samples (84%) were analyzable for SNV changes and 82 samples (47%) for CNA changes. We describe the genomic landscape of breast tumor cells at 3 timepoints – prior to starting systemic chemotherapy (in combination with an anti-angiogenesis inhibitor), after 2 weeks of treatment, and after 8 weeks of treatment. To our knowledge, this is the first analysis that assesses dynamic changes in SNVs/CNAs before, during, and after neoadjuvant or primary chemotherapy. These data provide insights on genomic alternations that occur in response to chemotherapy, which may confer acquired resistance and can uncover potential therapeutic targets in patients who progress after chemotherapy.

TP53 and PIK3CA were the most commonly mutated genes that were different between TNBC versus HER2−/HR+ breast cancer subtypes. All TNBC tumor samples (100%) harbored pathogenic TP53 alterations at baseline while less than half (43%) of HER2−/HR+ had such mutations; this is consistent with other studies that reported TP53 mutations to occur in at least 80–90% of TNBC tumors [23, 24] and in only about 30–40% of HER2−/HR+ breast cancers [9, 11]. The presence of mutations in TP53 is prognostic and has previously been correlated with responses to chemotherapy [25]. Interestingly, we found a decrease in VAF of TP53 mutations to be associated with a high ORR (80%) and a good Miller–Payne response (75%) to treatment.

We found a higher prevalence of CNAs compared to SNVs present in the tumor samples prior to treatment, consistent with other studies that have shown breast cancer to be driven largely by CNAs [26]. Stephens et al [27] previously showed that breast tumors were caused by combinations of at least 40 different driver mutations or CNAs of uncommon genes. Similarly, we observed that the tumors in our study were genomically heterogeneous—with a
median of 7 likely pathogenic driver SNVs and 28 CNAs present at baseline. However, as Shah and colleagues described [23], although breast tumors are genetically heterogeneous, there are phenotypic similarities due to mutations in multiple similar pathways. Mutations can be grouped into the dysregulation of pathways involving TP53, PI3K, DNA repair and chromatin remodeling [23].

A higher proportion of TNBC compared to HER2−/HR+ patients had amplification of VEGF-A in the baseline tumor. Other studies have correspondingly shown that TNBC tumors have higher levels of intratumoral VEGF levels compared to non-TNBC tumors [28]. Accordingly, clinical trials in the neoadjuvant setting have incorporated anti-angiogenesis agents in the treatment of TNBC with improvements seen in pathological complete response although overall survival benefit is still uncertain [29]. In this trial, although no TNBC patients achieved pCR with anti-angiogenesis treatment in combination with 4 cycles of chemotherapy, the majority (60%) had a good Miller-Payne histological response.

A higher proportion of HER2−/HR+ tumors compared to TNBC tumors, had activating mutations in receptor tyrosine kinase (RTK) genes at baseline. There was no significant difference in RTK gene CNAs at baseline between the tumor subtypes; however a higher proportion of HER2−/HR+ tumors had copy number gain in RTK genes after treatment compared to TNBC tumors. These observations suggest tumor dependence on RTK pathways in HER2−/HR+ tumors. About 20% of HER2−/HR+ tumors acquire resistance to hormonal therapy via activation of escape signaling pathways [30]. For instance, amplification and activating mutations of FGFR1 are associated with increased resistance to hormonal therapy [31, 32], while aberrant PDGFR expression has been associated with poorer survival in HER2−/HR+ breast cancer [33]. Several trials are currently ongoing exploring the strategy of inhibiting RTK targets like PDGFR and FGFR in breast cancer with several early phase trials showing meaningful efficacy in terms of response rate [34, 35] with the addition of anti-FGFR therapy. Tumor somatic genomic analysis can thus identify potential alternate therapeutic targets in the setting of resistance to systemic therapy.

We showed that the genomic mutational status is largely stable in terms of the SNVs that are present throughout treatment, with only a small proportion of patients (15%) developing 1–2 new mutations following treatment. However, we found that the VAF of clinically relevant targetable alterations changed in response to chemotherapy. These changes are seen as early as 2 weeks for the majority of tumors that experience a significant fold change in the VAF.
ant allele frequency

Treatment changes in mutation VAF and/or copy numbers

individual tumors [40]. Thus, we believe that the post-
are subclonal [39] and unevenly distributed spatially among
majority of mutations detected in primary breast cancers
tumor subpopulations [37, 38], with others showing that a
strate intratumoral spatial heterogeneity and contain clonal
[36]. Several studies have shown that breast tumors demon-
strate intratumoral spatial heterogeneity and contain clonal
tumor subpopulations [37, 38], with others showing that a
majority of mutations detected in primary breast cancers
are subclonal [39] and unevenly distributed spatially among
individual tumors [40]. Thus, we believe that the post-
treatment change in mutation VAF and/or copy numbers

observed in our study is predominantly due to subclonal
proliferation from selective pressure, although it is difficult
to ascertain this with confidence. Nonetheless, regardless
of the underlying mechanism, the emergence of a dominant
clonal subpopulation of cells with PIK3CA mutations fol-
lowing treatment would suggest that a PIK3CA-inhibitor
might be a treatment option against residual cancer cells in
patients with a poor response to chemotherapy, and this has
relevant clinical implications.

Correlative analysis of genomic alterations that were pre-
sent at baseline demonstrated that patients with good his-
tological response to treatment were those who had a high
number of SNVs present at baseline. In addition, patients
with good histological response had more changes in VAF
of SNVs and also a lower CNA burden with fewer genes
exhibiting CNAs after exposure to treatment compared to
those with poor response. These observations are concord-
ant with studies of the TCGA and METABRIC dataset that
have demonstrated that lower CNA burden in breast cancer
patients predicts for improved survival outcomes [41].

One of the limitations of this study was that as most
patients had HER2−/HR+ tumors with a median follow-up
of about 3 years, few relapse events had occurred at the time
of analysis. Hence, our study is not yet mature to correlate
tumor genomic changes with survival outcomes. Also, the
pathological complete response rate was low in this study,
likely contributed by the fact that patients underwent surgery
after just 4 cycles of ddAC without neoadjuvant taxanes and
the large proportion of HER2−/HR+ and locally advanced
breast cancers included in the cohort. Further directions
would be to obtain continual follow-up of these patients
to assess for biomarkers predictive of relapse and overall
survival.

Another limitation was that genomic changes observed
in serial biopsies from the primary breast tumors may have
been contributed by intra-tumoral spatial heterogeneity. Shah
and colleagues showed that intra-tumor genetic heterogene-
ity appeared to sometimes affect even known driver genetic
aberrations, such as TP53 and PIK3CA mutations [23].
However, other studies have demonstrated a high degree
of reproducibility (high intraclass correlation coefficients
ranging 0.90–0.98) in genome-wide expression profiling of
single gene predictors of estrogen receptor and progester-
one receptor expression [42]. Also, tumor biopsies from the
primary breast tumor alone may be less representative of
the systemic tumor genomic landscape. For instance, non-
concordant results between circulating tumor and primary
tumor samples for HER2 status have been described [43].
Perhaps future directions could be to perform correlative
analysis on corresponding serial liquid biopsies to evaluate
the genomic landscape of circulating tumor cells as well.

Fig. 4 Timing of changes in CCF of SNVs. Patients with 3 serial
tumor biopsies were analysed for timing of significant changes in
VAF of SNVs [measured by the CCF slope (post-treatment CCF/
baseline CCF)]. Patients were classified into 4 groups: no changes,
early and sustained changes (those who experienced changes at 2
weeks which were persist at 8 weeks), transient changes (those who
experienced changes at 2 weeks which subsequently normalized at
8 weeks) and those who experienced late changes alone. The blue
bar highlights the patients with no changes, while orange bars high-
light those with changes in VAF of SNVs. Of those who experienced
changes in VAF of SNVs, majority had early and sustained changes.
CCF cancer cell fraction; SNVs single nucleotide variants; VAF vari-
ant allele frequency;
5 Conclusion

This is the first study that characterizes dynamic changes in SNVs/CNAs before, during, and after neoadjuvant or primary chemotherapy. As anticipated, there were few new mutations occurring after just eight weeks of systemic therapy, but intriguingly, the VAF of numerous targetable alterations changed significantly with treatment and most changes were observed as early as after just two weeks of treatment. In patients with poor histological response to treatment, potentially targetable alterations were identified in the PI3K pathway and in RTK genes. Early identification of targetable alterations during the course of treatment may select a specific group of patients with primary resistance to standard chemotherapy who could benefit from intensification of therapy with the addition of targeted agents. In the era of precision medicine, we hope these tailored treatment strategies will be increasingly adopted in the clinic.

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Declarations

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Conflict of interest/competing interests Soo Chin Lee has received Honoraria and consulting fees from Astra Zeneca, Pfizer, Novartis, Eli Lilly, Roche, ACT Genomics, Eisai; research funding from Taiho, Eisai, Pfizer and ACT Genomics. Samuel GW Ow has received honoraria from AstraZeneca, Pfizer, Lilly, Roche and Novartis. Andrea Wong has received research funding from Otsuka Pharmaceuticals, and has advisory roles with Pfizer, Novartis and Eisai. Natalie Ngoi has received honoraria from AstraZeneca. Joline SJ Lim has consultancy with Pfizer, Novartis; research funding from Synthon. Goh Boon Cher has received honoraria from Novartis, Merck Serono, MSD; has consultancy with Adagene, research support and funding from Bayer, MSD, BMS, Adagene, and Taiho; stock interests in Gilead Sciences and Avantor. Yi-Hua Jan, Shu-Jen Chen and Kien Thiam Tan are under employment by ACT Genomics. Joan RE Choo, Matilda Xinwei Lee, Kritika Yadav, Siew Eng Lim, Ching Wan Chan, Mikael Hartman, Siau Wei Tang, Hon Lyn Tan, Wan Qin Chong, Ang Li En Yvonne, Gloria HJ Chan declared no conflicts of interest.
Ethics approval  This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the National Healthcare Group Domain Specific Ethics Review Board in 2016 (DSRB 2016/00327).

Consent to participate and consent for publication  Signed written informed consent was obtained from all individual participants included in the study.

Data availability  All data generated or analyzed during this current study are included in this published article.

Code availability  Not applicable.

Author contributions  All authors contributed to at least one of the following: study conception, design, data acquisition, analysis, and/or interpretation. The first draft of the manuscript was written by JC and supervision/critical revision of the work was done by LSC. All authors have read and approved the final manuscript.

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