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ORIGINAL RESEARCH ARTICLE

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.

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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 pre-treatment 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/m^2) 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 800×, 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 (C_n), tumor copy number (C_t), 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 $AF_g = (pM + (1-p))/(pC_t + C_n(1-p))$; for somatic mutations, the expected $AF_s = pM/(pC_t + C_n(1-p))$. 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.

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 two weeks). As decrease in tumor purity was an expected consequence in the setting of tumor response to chemotherapy, and samples with <30% tumor purity could not be accurately assessed for CNA changes, a smaller number of patients had serial samples suitable for CNAs analysis: 11 patients had 3 serial samples while 19 patients had 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%), *SYNE1* (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

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

[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

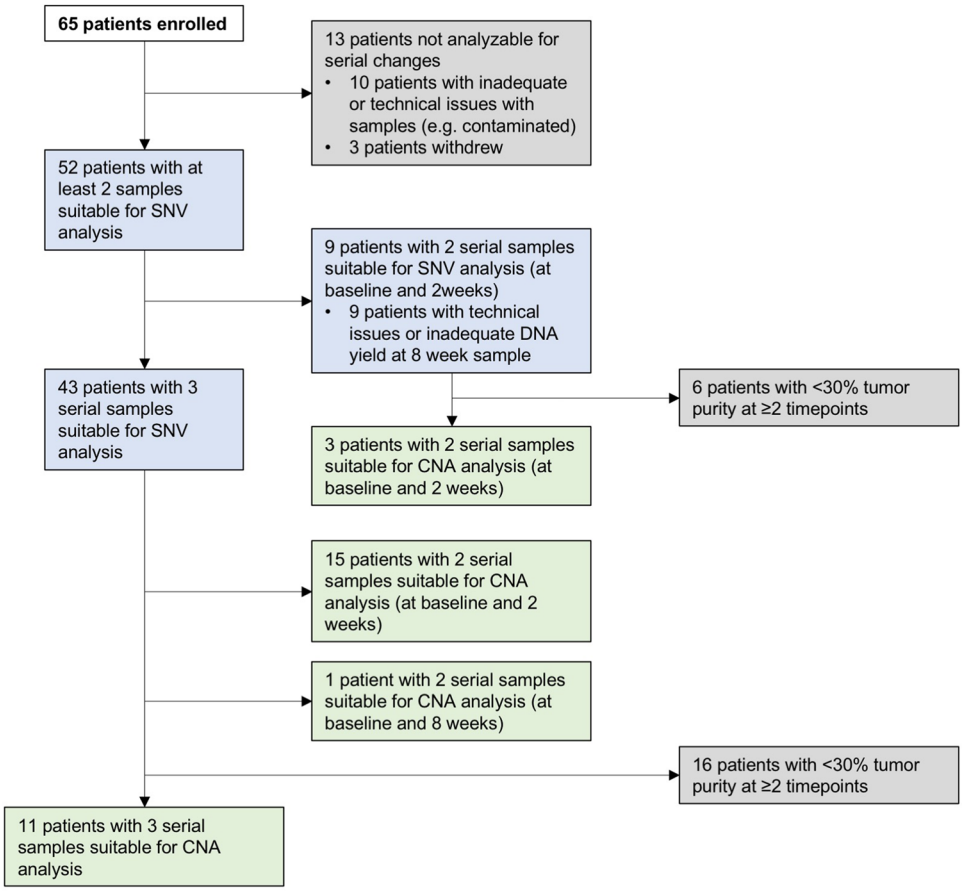

Fig. 1 Consort diagram of sample disposition

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 *PIK3CA* alteration at baseline; five tumors had ≥2 pathogenic *PIK3CA* mutations. There was no significant difference in frequency of *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 *ARID1A*, *ATM, ATR, BAP1, BARD1, BLM, BRCA1/2, BRIP1, CHEK1/2, FANCA/C/D2/E/F/G/L, MRE11, NBN, PALB2, RAD50, RAD51, 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 *MLH1, MSH2, MSH6, 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/AKT pathway where seven patients developed significant increase in VAF of pathogenic *PIK3CA* alterations, which confer sensitivity to *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 (≥2- or ≤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 ≥2-fold gain or ≤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 ≥ 2-fold gain were *TSC2, AKT1, AKT3, FLCN, CDK5, FH* and *IKBKE*; many of these are part of the PIK3CA/AKT/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+


Tumor purity(%)
Mutation count
Subtype
8
90
7
29
HER2-/HR+
TNBC
Alterations
Missense (driver)
Missense (VUS)
In-frame (driver)
In-frame (VUS)
Truncating (driver)
Truncating (VUS)
Splice region
Amplification
Gain
No alterations
Deep loss
Shallow deletion
TP53 56% 42.5% 100%
SYNE1 40% 42.5% 33.3%
MUC16 40% 45.0% 25.0%
PIK3CA 35% 37.5% 25.0%
USH2A 29% 35.0% 8.3%
KMT2C 27% 20.0% 50.0%
ATM 25% 30.0% 8.3%
BRCA2 19% 15.0% 33.3%
NOTCH4 17% 20.0% 8.3%
KMT2D 15% 17.5% 8.3%
POLE 15% 15.0% 16.7%
SETD2 15% 12.5% 25.0%
ADGRA2 15% 17.5% 8.3%
RECQL4 40% 40.0% 41.7%
RUNX1T1 29% 32.5% 16.7%
MYC 27% 30.0% 16.7%
NBN 27% 30.0% 16.7%
CCNE2 27% 30.0% 16.7%
USH2A 27% 32.5% 8.3%
MCL1 25% 22.5% 33.3%
BCL9 25% 25.0% 25.0%
UBR5 25% 27.5% 16.7%
TSC2 25% 22.5% 33.3%
IKBKE 25% 30.0% 8.3%
NTRK1 23% 20.0% 33.3%
ADGRA2 23% 27.5% 8.3%
IKBKB 21% 22.5% 16.7%
POLB 21% 22.5% 16.7%
CCND1 21% 22.5% 16.7%
PARP1 21% 25.0% 8.3%
AKT3 21% 22.5% 16.7%
CDK5 21% 17.5% 33.3%
FCGR2B 19% 17.5% 25.0%
DDR2 19% 17.5% 25.0%
PRKDC 19% 22.5% 8.3%
FGF19 19% 20.0% 16.7%
FGF3 19% 20.0% 16.7%
BTG2 19% 22.5% 8.3%
PIK3C2B 19% 22.5% 8.3%
MDM4 19% 22.5% 8.3%
FH 19% 22.5% 8.3%
LYN 17% 20.0% 8.3%
NOTCH1 17% 15.0% 25.0%
ABL2 17% 20.0% 8.3%
PTGS2 17% 20.0% 8.3%
FGFR1 17% 20.0% 8.3%
KAT6A 17% 20.0% 8.3%
SDHC 15% 15.0% 16.7%
CDC73 15% 17.5% 8.3%
RXRA 15% 15.0% 16.7%
TNFRSF14 15% 15.0% 16.7%

◂**Fig. 2** Tumor SNV and CNA landscape at baseline. Commonly detected SNVs and CNAs by NGS are shown in decreasing order of prevalence. Pathogenic SNVs are labeled as driver mutations while SNVs of unknown significance are labeled as VUS. Copy number gain was defined as copy number between 4–7 while copy number amplification was defined as copy number ≥8. Copy number loss was defined as an observed copy number ≤1 where heterozygous loss (shallow deletion) was defined as observed copy number 1 while homozygous loss (deep deletion) was defined as observed copy number 0s. *CNAs* copy number alterations; *NGS* next-generation sequencing; *SNV* single nucleotide variants; *VUS* variants of uncertain significance

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

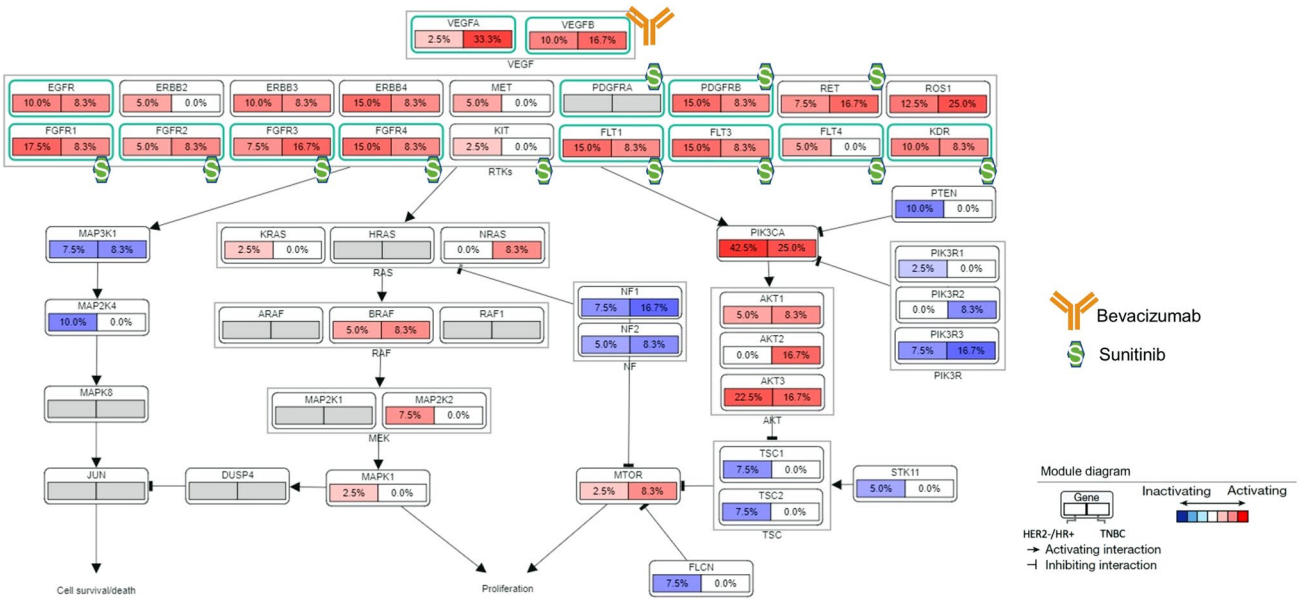

Fig. 3 Genomic alterations in signaling molecules that interact with the VEGF angiogenesis pathway at baseline. Percentages of HER2−/HR+ and TNBC tumors (represented on the left and right of each module diagram, respectively) with alterations in signaling molecules that interact with the angiogenesis pathway are shown. Blue highlights the presence of inactivating mutations while red highlights the presence of activating mutations within that gene. The arrows describe whether the interactions are activating or inhibitory upon downstream signaling molecules. Sunitinib and bevacizumab have direct inhibitory effects on certain targets within the signaling cascades which are marked by the symbols. *HER2−* human epidermal growth factor receptor 2 negative; *HR+* hormone receptor positive; *TNBC* triple-negative breast cancer; *VEGF* vascular endothelial growth factor

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 *PDGFRB* 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

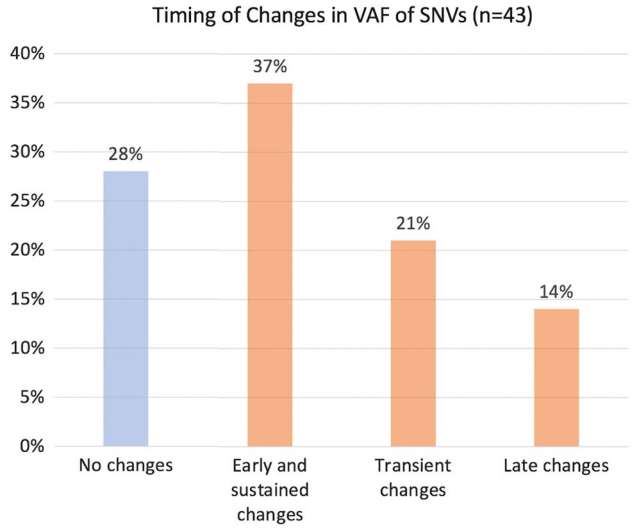

Fig. 4 Timing of changes in CCF of SNVs. Patients with 3 serial tumor biopsies were analysed for timing of significant changes in VAF of SNVs [measured by the CCF slope (post-treatment CCF/baseline CCF)]. Patients were classified into 4 groups: no changes, early and sustained changes (those who experienced 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 highlight 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* variant allele frequency

after exposure to systemic treatment. Mutations of note were in the PI3K pathway. All the patients with sustained rise in VAF of pathogenic PI3K pathway mutations were HER2−/HR+ and the majority (60%) had a poor Miller-Payne response to treatment. This may suggest that some HER2−/HR+ patients who are resistant to chemotherapy may become sensitized to *PIK3CA* inhibitors. Also, significant gains in copy numbers after exposure to systemic therapy commonly occurred in genes within PI3K pathway. These changes occurred in about 40% of patients in both sunitinib and bevacizumab treatment cohorts. These changes may have been induced by treatment or may reflect selection pressure of existing subclones following treatment. It is recognized that acquired treatment resistance is linked to intratumoral heterogeneity and clonal evolution [36]. Several studies have shown that breast tumors demonstrate 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 following 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 present at baseline demonstrated that patients with good histological 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 concordant 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 heterogeneity 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 progesterone 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.

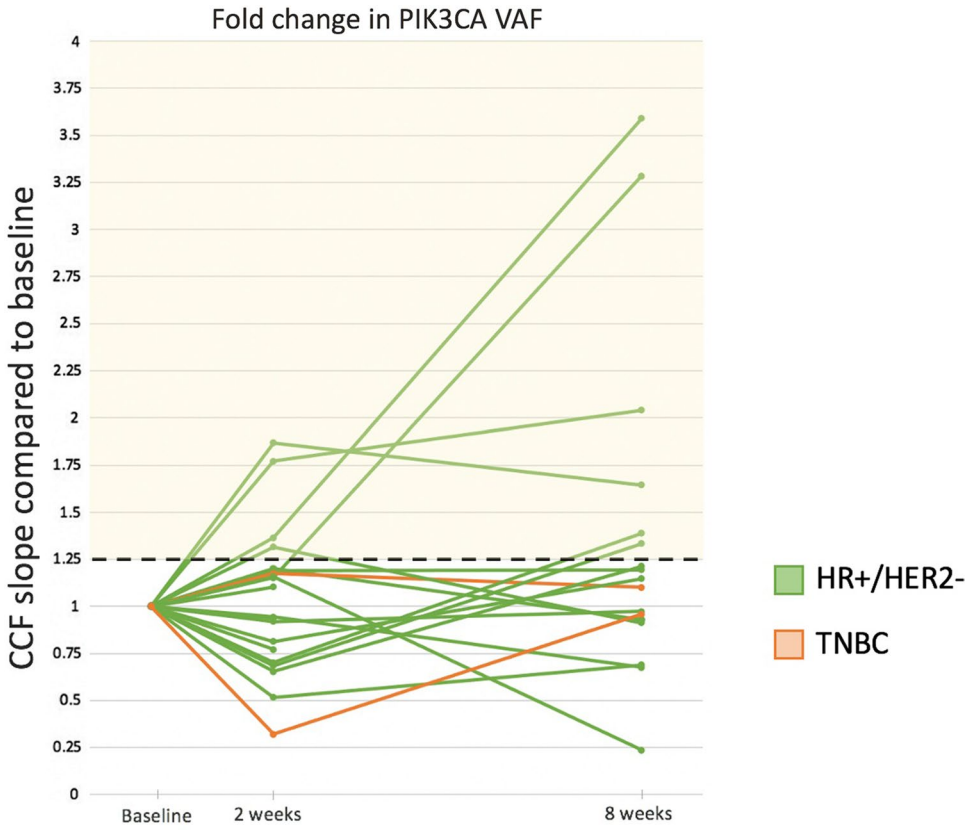

Fig. 5 Change in VAF measured by CCF slope (post-treatment CCF/baseline CCF) of pathogenic PIK3CA mutations by treatment cohort. The change in VAF measured by the CCF slope (post-treatment CCF/baseline CCF) of each patient with pathogenic PIK3CA mutations are represented here. A slope of ≤0.8 indicates a decrease in VAF and ≥1.25 indicates an increase in VAF. Of the 20 patients with pathogenic PIK3CA mutations at baseline, 35% had sustained increase in VAF of pathogenic PIK3CA alterations. Significant rise in VAF of pathogenic PI3K mutations only occurred in HER2−/HR+ tumors. *CCF* cancer cell fraction; *VAF* variant 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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