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ORIGINAL ARTICLE

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Somatic alterations of *TP53*, *ERBB2*, *PIK3CA* and *CCND1* are associated with chemosensitivity for breast cancers

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The correlation of genetic alterations with response to neoadjuvant chemotherapy (NAC) has not been fully revealed. In this study, we enrolled 247 breast cancer patients receiving anthracycline-taxane-based NAC treatment. A next generation sequencing (NGS) panel containing 36 hotspot breast cancer-related genes was used in this study. Two different standards for the extent of pathologic complete response (pCR), ypT0/isypN0 and ypT0/is, were used as indicators for NAC treatment. *TP53* mutation (n = 149, 60.3%), *PIK3CA* mutation (n = 109, 44.1%) and *MYC* amplification (n = 95, 38.5%) were frequently detected in enrolled cases. *TP53* mutation ($P = 0.019$ for ypT0/isypN0 and $P = 0.003$ for ypT0/is) and *ERBB2* amplification ($P < 0.001$ for both ypT0/isypN0 and ypT0/is) were related to higher pCR rates. *PIK3CA* mutation ($P = 0.040$ for ypT0/isypN0) and *CCND2* amplification ($P = 0.042$ for ypT0/is) showed reduced sensitivity to NAC. Patients with MAPK pathway alteration had low pCR rates ($P = 0.043$ for ypT0/is). Patients with *TP53* mutation (–) *PIK3CA* mutation (–) *ERBB2* amplification (+) *CCND1* amplification (–), *TP53* mutation (+) *PIK3CA* mutation (–) *ERBB2* amplification (+) *CCND1* amplification (–) or *TP53* mutation (+) *PIK3CA* mutation (+) *ERBB2* amplification (+) *CCND1* amplification (–)had significantly higher pCR rates ($P < 0.05$ for ypT0/isypN0 and ypT0/is) than wild type genotype tumors. Some

cancer genetic alterations as well as pathway alterations were associated with chemosensitivity to NAC treatment. Our study may shed light on the molecular characteristics of breast cancer for prediction of NAC expectations when breast cancer is first diagnosed by biopsy.

KEYWORDS
breast neoplasm, genetic variation, high-throughput nucleotide sequencing, neoadjuvant therapy, pathologic complete response

1 | INTRODUCTION

Neoadjuvant chemotherapy (NAC) is being increasingly used in locally advanced breast cancers to limit the extent of surgery in the breast and axilla. Some patients are sensitive to chemotherapy and reach pathologic complete response (pCR) after NAC. Those patients who reach pCR after NAC have more favorable prognosis than those who do not.[1] Many biomarkers are reported to be associated with pCR, including negative ER expression,[2] positive HER2 expression[3] and high Ki67 expression.[4] Some studies have developed prediction models of pCR based on gene expression analysis with cDNA microarrays, such as Genomic Grade Index[5] and DLDA-30 Pharmacogenomic Predictor.[6] These models had a predictive value in breast cancer patients receiving NAC treatment. Apart from those immunochemistry (IHC) biomarkers and prediction models, genetic polymorphism of a few genes, such as *TP53*,[7] *BRCA1/2*[8] and *FGFR4*,[9] was also reported to predict pCR. However, these studies based on conventional DNA sequencing only focused on limited DNA sites.

Next-generation sequencing (NGS) is a high-throughput, time-effective and cost-effective method for parallel analyses. Through NGS, whole-exome sequencing was conducted for individual patients and portrayed mutation maps for breast cancers.[10] For clinical application, many NGS gene panels containing mutational hotspots varied for different sequencing platforms. It is common to use NGS panels containing *BRCA1/2* or other related genes for auxiliary diagnosis of hereditary breast cancers or to assess breast cancer risk.[11-14] Moreover, NGS also can be used for archived, formalin-fixed and paraffin-embedded tissue (FFPE) samples to analyze genetic mutation over time. Some designed NGS panels have also been used to depict gene mutation profiling for different purposes. For example, Park et al[15], using FFPE samples and NGS panels, found that several actionable mutations were different in NAC patients who reached pCR compared with non-pCR patients. However, the panel they used did not contain high frequency altered genes which were specific for breast cancers.

In the present study, we aimed to analyze the relationship between cancer alterations with chemosensitivity in stage II-III breast cancer patients who received anthracycline-taxane-based NAC treatment. A commercial breast cancer-specific gene panel, which contains hotspot genes for breast cancer, was used for deep sequencing on FFPE samples.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

A total of 247 breast cancer patients who received anthracycline-taxane-based NAC from January 2015 to March 2017 were retrospectively enrolled in this study. No patients received neoadjuvant anti–HER2 targeted therapy or endocrine therapy before surgery. All patients were in clinical stages II or III and underwent a tumor core needle biopsy (CNB) under ultrasonographic guidance before NAC. Approval for this study was granted by the Ethics Committee of West China Hospital (IRS no.: 2017-476) and the clinical trial was registered with the Chinese Clinical Trial Registry (http://www.chictr.org.cn/; registration no.: ChiCTR1800016763). The flowchart is shown in Figure S1.

In the present study, we used 2 different definitions of pCR: ypT0/isypN0 (no residual invasive disease in the breast and node) and ypT0/is (no residual invasive disease in the breast but possibly with nodal involvement). For each patient, estrogen receptor (ER) and progesterone receptor (PR) were evaluated using pre–NAC FFPE tissue blocks by IHC. For ER-positive and PR-positive disease, there was ≥1% positively stained nuclei in tumor tissues. ER and/or PR positive disease was regarded as hormone receptor (HR) positive disease. ER and PR negative disease was regarded as HR negative disease. Human epidermal growth factor receptor 2 (HER2) status was determined by IHC and FISH. Only HER2 IHC (3+) and/or FISH amplified disease was considered as HER2 positive disease. Ki67 expression was divided into a high expression group (>20%) and a low expression group (≤20%). Apart from those IHC biomarkers, age of patient at diagnosis, tumor size and lymph node status before NAC were collected from the patient medical histories.

2.2 | DNA and library preparation

DNA was extracted from FFPE samples of CNB using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden). The concentration of extracted DNA was detected by Qubit 3.0 using a Qubit dsDNA HS Kit (Life Technologies). The quality of DNA was assessed using gel electrophoresis. Only samples with a total yield of more than 50 ng of DNA and no laddering effects (the most concentrated band above 1000 bp) were used for further analysis.

For library preparation, there were 2 main steps for quality control required for successful sequencing. After DNA extraction, DNA

TABLE 1 Patients' characteristics

Characteristics	Number (%)
Age, y	
<50	136 (52.6)
≥50	111 (47.4)
Tumor size	
T1	30 (12.1)
T2	137 (55.5)
T3	32 (13.0)
T4	47 (19.0)
Unknown	1 (.4)
Node status	
Negative	58 (23.5)
Positive	189 (76.5)
Hormone receptor status	
Negative	55 (22.3)
Positive	190 (76.9)
Unknown	2 (.8)
Human epidermal growth factor receptor 2 status	
Negative	177 (71.7)
Positive	68 (27.5)
Unknown	2 (.8)
Ki67 status	
≤20%	58 (23.5)
>20%	187 (75.7)
Unknown	2 (.8)
ypT0/isypN0	
No	217 (87.9)
Yes	30 (12.1)
ypT0/is	
No	205 (83.0)
Yes	42 (17.0)

was purified using magnetic beads and then amplified using a PCR Cycler (Bio-Rad Laboratories, Inc., CA, USA) with AmpliSeq HiFi Master Mix. The total yield of this pre–library of more than 500 ng was required for the next step. Then, this pre–library was processed for hybridization, capture and amplification using a PCR Cycler with HotStart Taq MasterMix. The final library should have concentration of no less than 4.5 ng/μL and fragment size between 280 and 500 bp, which was detected by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., CA, USA). Only qualified library was accepted for NGS sequencing.

2.3 | Next generation sequencing gene panel and gene alteration analysis

The panel (Beikang, Burning Rock Dx) consists of 36 breast cancer-related genes, spanning 140 kb of the human genome. Details regarding detected regions of those selected genes are shown in Table S1. NGS sequencing was performed by MiSeq (Illumina Technologies) using Miseq Reagent Kit V3. Sequence data were mapped to the human genome (hg19) using BWA aligner 0.7.10. Local alignment optimization, variant calling and annotation were performed using GATK 3.2, MuTect and VarScan. Variants were filtered using the VarScan filter pipeline, with loci with depth less than 100 filtered out. For hotspot insertions and deletions (INDEL), a minimum of 5 supporting reads are needed at an allelic fraction (AF) >.5%; while 10 supporting reads are required for non-hotspot INDEL at an AF >2%. A minimum of 8 supporting reads are needed for hotspot single nucleotide variants (SNV) to be called at an AF >1%, while 16 supporting reads are needed for non-hotspot SNV at an AF >2%. According to the ExAC, 1000 Genomes, dbSNP, ESP6500SI-V2 database, variants with population frequency over .1% were grouped as SNP and excluded from further analysis. Remaining variants were annotated with ANNOVAR and SnpEff v3.6. DNA translocation analysis was performed using Factera 1.4.3 as previously described. The limit of detection for SNV is 2% for hotspots and 5% for non-hotspots. Copy number variation was detected by in-house analysis scripts based on depth of coverage data of capture intervals. Coverage data were corrected against sequencing bias resulting from GC content and probe design. The average coverage of all captured regions was used to normalize the coverage of different samples to comparable scales. The copy number was calculated based on the ratio between the depth of coverage in tumor samples and the average coverage of an adequate number (n > 50) of samples without copy number variation as references for each capture interval. Copy number variation was confirmed if the coverage data of the gene region was quantitatively and statistically significantly different from its reference control. The limit of detection for CNVs is 1.5 for deletion and 2.64 for amplification.

2.4 | Statistical analysis

Pearson's χ^2 test and Fisher's exact test were used to set features, including clinical features and genetic alterations, associated with pCR. A multivariate analysis was performed using binary logistic regression analysis for genetic alterations to identify which were related to pCR. Nonsynonymous genetic alterations in at least 1 sample were mainly classified into 3 tumor-associated pathways, including: (i) the retinoblastoma (RB) pathway (*RB1*, *CCND1*, *CDK4*); (ii) the mitogen-activated protein kinase (MAPK) pathway (*MAP2K4*, *MAP3K1*, *BRAF*, *KRAS*, *EGFR*, *FGFR1*, *FGFR2*); (iii) and the phosphatidylinositol-3-kinase (PI3K) pathway (*AKT1*, *AKT3*, *PTEN*, *PIK3CA*, *PIK3R1*). Alterations of these 3 pathways were also included in the analysis. To further analyze the influence of genetic alterations on pCR, samples with complete records of HR and HER2 were classified into 4 subtypes: (i) HR−HER2− (n = 30); (ii) HR−HER2+ (n = 25); (iii) HR+HER2+ (n = 43); and (iv) HR+HER2− (n = 147). The associations between genetic alterations and pCR were analyzed in different subtypes, respectively. Statistical

analysis was performed with SPSS 16.0 (SPSS, Chicago, IL, USA). The 2-sided significance level was set at $P < .05$.

3 | RESULTS

3.1 | Patients' characteristics

Patients' characteristics are shown in Table 1. The median age of enrolled patients was 63 years (range from 26-71 years). A total of 167 patients (67.6%) had tumor sizes of T1 or T2. A total of 189 patients (76.5%) had positive node status before treatment; 76.9% of all tumors were HR positive (ER and/or PR positive) and 27.5% of all tumors were HER2 positive. High Ki67 expression was detected in 187 patients (75.7%). After receiving anthracycline-taxane-based NAC, 30 patients (12.1%) reached ypT0/isypN0 and 42 patients (17.0%) had no residual invasive tumors in the breast (ypT0/is).

3.2 | Breast cancer genetic alterations and pathway involved

For sequencing depth, 234 cases (94.7%) had high median coverage depth (>500×) and 233 (94.3%) cases had high unique coverage depth (>100×). A total of 245 patients (99.2%) harbored at least 1 somatic alteration. The most frequently nonsynonymous alterations were detected on *TP53* (n = 152, 61.5%), *PIK3CA* (n = 109, 44.1%), *MYC* (n = 99, 40.1%) and *ERBB2* (n = 76, 30.8%). Gene amplifications were frequently detected on *MYC* (n = 95, 38.5%), *ERBB2* (n = 67, 27.1%) and *CCND1* (n = 37, 15.0%). All genetic alterations in this study are shown in Figure 1. The schematic structure of *TP53* and *PIK3CA* is illustrated in Figure S2.

Based on the known function of genes, genetic alterations were mainly classified into 3 pathways (Figure 2). Sixty-two cases (25.1%) had at least 1 related genetic alteration in the RB pathway. In this pathway, 49 of 62 cases (79.0%) harbored amplified genes (*CCND1* and *CDK4*). *RB1* was the most frequently (11 of 62 cases, 17.7%) mutated gene in this pathway. The second gene set was MAPK pathway related genes. Eighty cases (32.4%) had alterations in at least 1 gene related to MAPK pathway. *FGFR1* was the most altered gene, and had 29 amplified cases and 3 mutated cases. The third gene set was associated with the PI3K pathway. Of 247 cases, 139 (56.3%) had a least 1 genetic alteration related to the PI3K pathway. In this pathway, *PIK3CA* was the most frequently (109 of 139 cases, 78.4%) altered gene, including 103 mutated cases and 6 amplified cases. Twenty-six cases which harbored mutated *PIK3CA* also had other altered genes related to the PI3K pathway.

In addition, we compared the frequency of genetic alterations among different subtypes of breast cancer (Table S2). The distribution of altered *TP53* was statistically different among subtypes ($P < .001$). *TP53* alterations were lower in HR+HER2− cancers (47.6%) than in the other 3 subtypes (76.7%-90.0%). *GATA3* alterations were only found in HR+ tumors ($P = .013$). *TOP2A* alterations were not detected in HR−HER2− tumors ($P < .001$). Altered MAPK pathway was detected in only 1 case (4.0%) in HR−HER2+ cancers; however, alterations of the MAPK pathway were found in 40.0% of HR−HER2− cancers. Moreover, alterations of the PI3K pathway were more frequent (63.9%) in HR+HER2− breast cancers than other subtypes ($P = .048$).

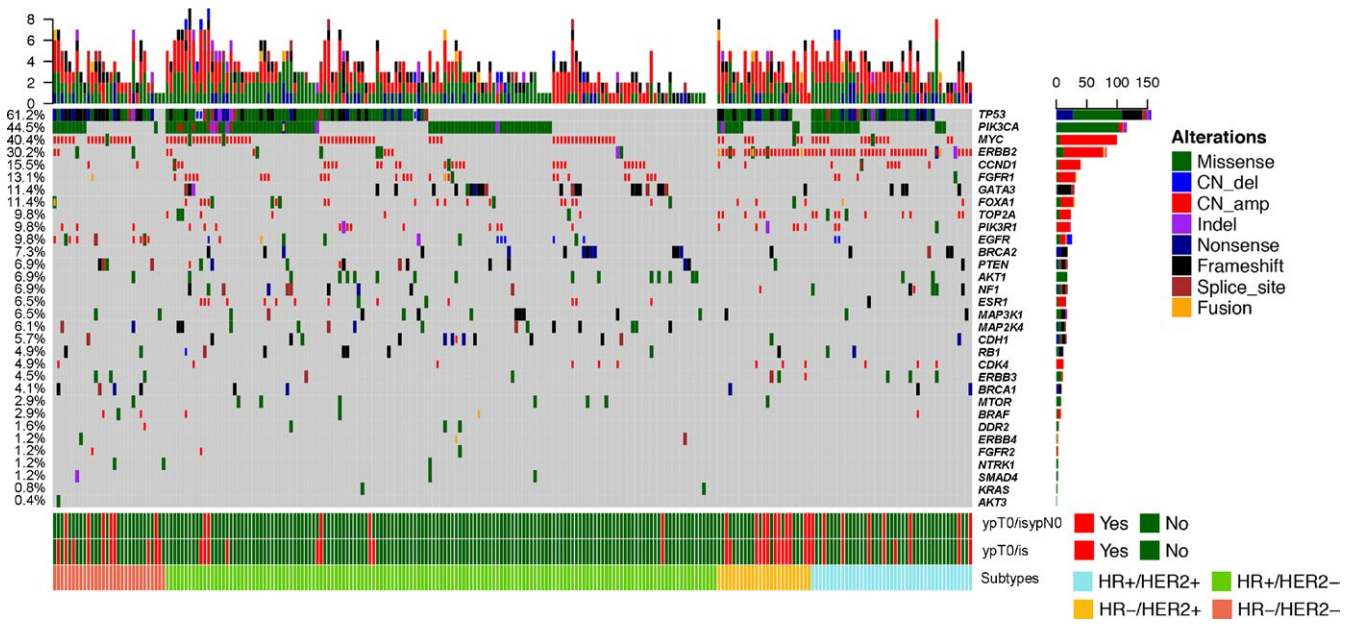

FIGURE 1 Heat map of gene alterations. Percentage of gene alterations is shown in the left row. Two cases with no records of hormone receptor (HR) and HER2 status were not included in this figure. The absolute count of altered genes in each case are shown in the upper bars. The absolute counts of cases which harbor genetic alterations of each detected gene are shown in the right bars

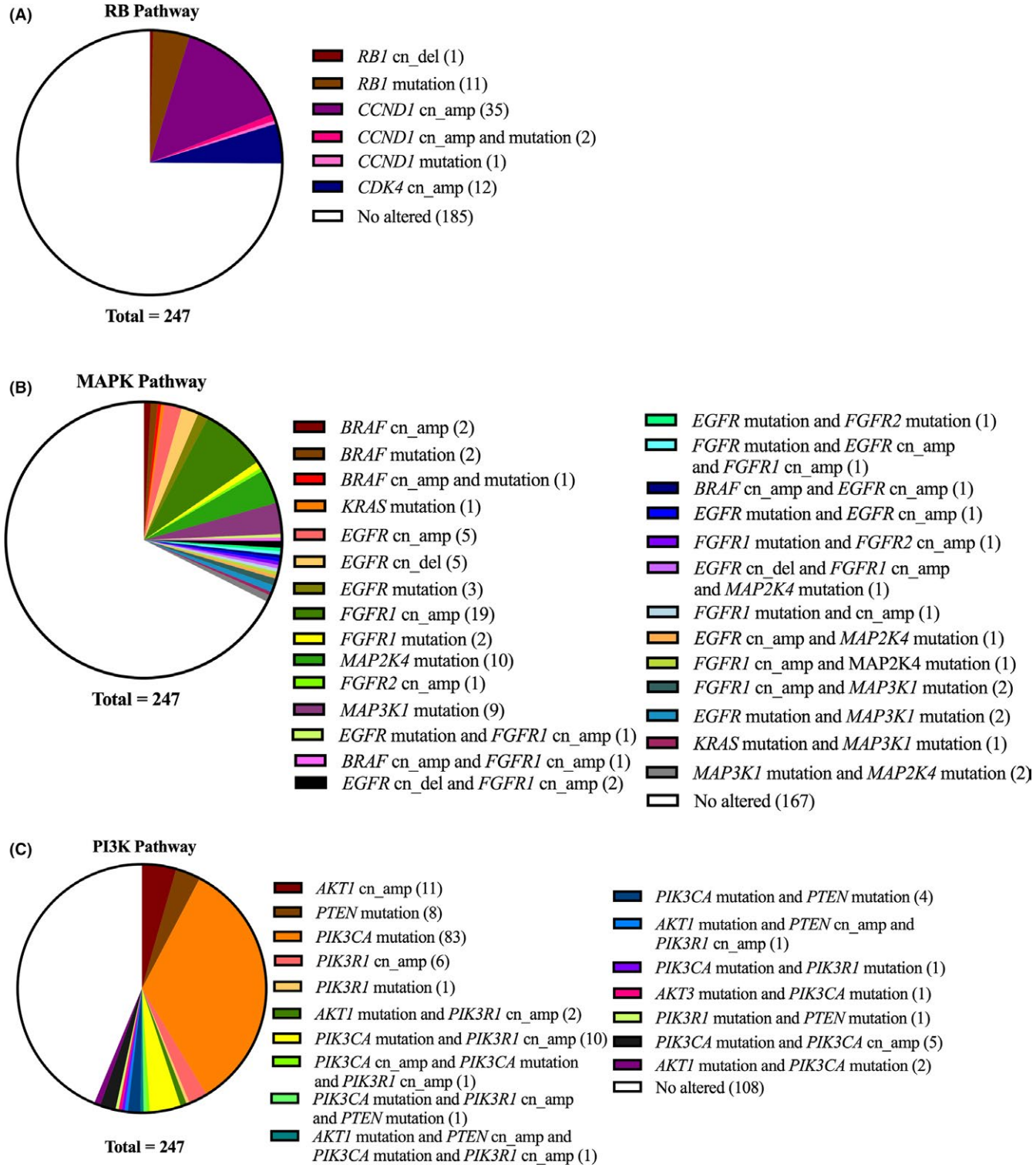

FIGURE 2 Distribution of gene alterations in 3 functional pathways. A, retinoblastoma (RB) pathway. B, mitogen activated protein kinase (MAPK) pathway. C, phosphatidylinositol-3-kinase (PI3K) pathway

3.3 | Clinical and genetic features associated with pathologic complete response

Clinical features and high-frequency genetic alterations were included to analyze the relationship with ypT0/isypN0 or ypT0/is, respectively (Table 2). In this analysis, clinical features, such as positive node status ($P = .005$ for ypT0/isypN0; $P < .001$ for ypT0/is), negative HR expression ($P < .001$ for ypT0/isypN0 or ypT0/is) and positive HER2 status ($P < .001$ for ypT0/isypN0 or ypT0/is), were associated with higher possibility of pCR.

TABLE 2 Clinical features and genetic alteration related to chemosensitivity

Characteristics	N (%)	ypT0/isypN0				ypT0/is			
		No	Yes	χ^2	*P*	No	Yes	χ^2	*P*
Age, y									
<50	136 (52.6)	119 (87.5)	17 (12.5)	.036	.850	112 (82.4)	24 (17.6)	.089	.766
≥50	111 (47.4)	98 (88.3)	13 (11.7)			93 (83.8)	18 (16.2)		
Tumor stage									
T1-T2	167 (67.6)	145 (86.8)	22 (13.2)	.465	.495	136 (81.4)	31 (18.6)	.815	.367
T3-T4	79 (32.0)	71 (89.9)	8 (10.1)			68 (86.1)	11 (13.9)		
Unknown	1 (.4)	–	–			–	–		
Node status									
Negative	58 (23.5)	57 (98.3)	1 (1.7)	7.715	.005	57 (98.3)	1 (1.7)	12.540	<.001
Positive	189 (76.5)	160 (84.7)	29 (15.3)			148 (78.3)	41 (21.7)		
Hormone receptor status									
Negative	55 (22.3)	39 (70.9)	16 (29.1)	20.232	<.001	32 (58.2)	23 (41.8)	32.024	<.001
Positive	190 (76.9)	177 (93.2)	13 (6.8)			172 (90.5)	18 (9.5)		
Unknown	2 (.8)	–	–			–	–		
Human epidermal growth factor receptor 2 status									
Negative	177 (71.7)	166 (93.8)	11 (6.2)	19.315	<.001	158 (89.3)	19 (10.7)	16.477	<.001
Positive	68 (27.5)	50 (73.5)	18 (26.5)			46 (67.6)	22 (32.4)		
Unknown	2 (.8)	–	–			–	–		
Ki67 status									
≤20%	58 (23.5)	54 (93.1)	4 (6.9)	1.777	.183	52 (89.7)	6 (10.3)	2.227	.136
>20%	187 (75.7)	162 (86.6)	25 (13.4)			152 (81.3)	35 (18.7)		
Unknown	2 (.8)	–	–			–	–		
TP53 mutation									
No	98 (39.7)	92 (93.9)	6 (6.1)	5.524	.019	90 (91.8)	8 (8.2)	8.997	.003
Yes	149 (60.3)	128 (83.9)	24 (16.1)			115 (77.2)	34 (22.8)		
PIK3CA mutation									
No	138 (55.9)	116 (84.1)	22 (15.9)	4.224	.040	110 (79.7)	28 (20.3)	2.392	.122
Yes	109 (44.1)	101 (92.7)	8 (7.3)			95 (87.2)	14 (12.8)		
ERBB2 amplification									
No	180 (72.9)	169 (93.9)	11 (6.1)	22.647	<.001	162 (90.0)	18 (10.0)	23.067	<.001
Yes	67 (27.1)	48 (71.6)	19 (28.4)			43 (64.2)	24 (35.8)		
MYC amplification									
No	152 (61.5)	135 (88.8)	17 (11.2)	.342	.558	128 (84.2)	24 (15.8)	.413	.520
Yes	95 (38.5)	82 (86.3)	13 (13.7)			77 (81.1)	18 (18.9)		
CCND1 amplification									
No	210 (85.0)	181 (86.2)	29 (13.8)	3.637	.057	170 (81.0)	40 (19.0)	4.148	.042
Yes	37 (15.0)	36 (97.3)	1 (2.7)			35 (94.6)	2 (5.4)		
Retinoblastoma pathway alteration									
No	185 (74.9)	161 (87.0)	24 (13.0)	.473	.654	152 (82.2)	33 (17.8)	.363	.696
Yes	62 (25.1)	56 (90.3)	6 (9.7)			53 (85.5)	9 (14.5)		
Mitogen-activated protein kinase pathway alteration									
No	167 (67.6)	142 (85.0)	25 (15.0)	3.854	.050	133 (79.6)	34 (20.4)	4.113	.043
Yes	80 (32.4)	75 (93.8)	5 (6.2)			72 (90.0)	8 (10.0)		
Phosphatidylinositol-3-kinase pathway alteration									
No	108 (43.7)	91 (84.3)	17 (15.7)	2.324	.127	85 (78.7)	23 (21.3)	2.505	.113
Yes	139 (56.3)	126 (90.6)	13 (9.4)			120 (86.3)	19 (13.7)		

For genetic alterations, patients with mutated *TP53* status tended to have higher possibility of pCR ($P = .019$ for ypT0/isypN0; $P = .003$ for ypT0/is). *ERBB2* amplification tested by NGS ($P < .001$ for both ypT0/isypN0 and ypT0/is) was also related to higher pCR rates. Mutated *PIK3CA* or amplified *CCND1* was related to lower pCR rates with statistically significance (*PIK3CA* mutation, $P = .040$ for ypT0/isypN0; *CCND1* amplification, $P = .042$ for ypT0/is). However, in analyzing the correlation between pathway alterations with pCR (Table 2), alterations of PI3K and RB pathways showed trends of lower pCR rates with no statistical significance. Altered MAPK pathway was significantly associated with pCR ($P = .043$ for ypT0/is). In multivariate analyses for genetic alterations (Table S3), *ERBB2* amplification and *CCND1* amplification demonstrated a relationship with pCR (*ERBB2* amplification, $P = .002$ for ypT0/isypN0 and $P = .003$ for ypT0/is; *CCND1* amplification, $P = .032$ for ypT0/is).

3.4 | Different somatic mutation subtypes leading to diverse neoadjuvant chemotherapy effect

To further analyze the association between high frequency alterations and the NAC effect, we classified the patients with different status of 4 altered genes, which showed a relationship with pCR. We further analyzed these different somatic mutation subtypes with the NAC effect (pCR or non-pCR). Sixteen different combinations

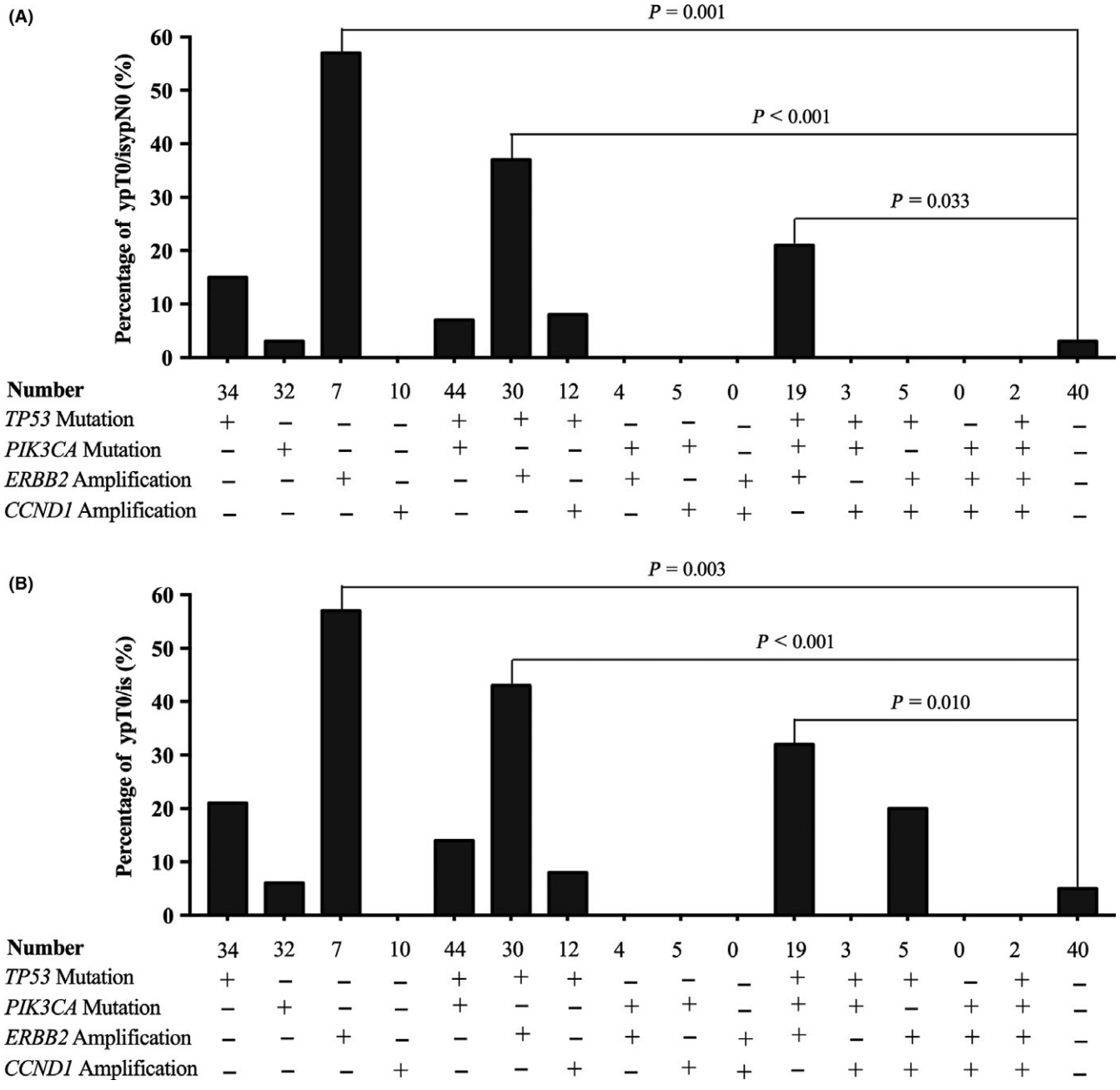

FIGURE 3 Different rates of pathologic complete response (pCR) (ypT0/isypN0 [A] or ypT0/is [B]) between patients with any combination of altered genes

TABLE 3 Association between genetic alterations and ypT0/isypN0 in different breast cancer subtypes

		HR−HER2−				HR−HER2+				HR+HER2+				HR+HER2−			
Characteristics	N (%)[a]	No	Yes	χ^2	*P*	No	Yes	χ^2	*P*	No	Yes	χ^2	*P*	No	Yes	χ^2	*P*
TP53 mutation																	
No	98 (40.0)	2 (66.7)	1 (33.3)	.370	.501	3 (60.0)	2 (40.0)	<.001	1.000	8 (80.0)	2 (20.0)	.017	.897	79 (98.8)	1 (1.2)	2.472	.178
Yes	147 (60.0)	22 (81.5)	5 (18.5)			12 (60.0)	8 (40.0)			27 (81.8)	6 (18.2)			63 (94.0)	4 (6.0)		
PIK3CA mutation																	
No	136 (55.5)	16 (80.0)	4 (20.0)	<.001	1.000	7 (43.8)	9 (56.2)	4.890	.040	22 (81.5)	5 (18.5)	<.001	1.000	70 (95.9)	3 (4.1)	.221	.681
Yes	109 (44.5)	8 (80.0)	2 (20.0)			8 (88.9)	1 (11.1)			13 (81.2)	3 (18.8)			72 (97.3)	2 (2.7)		
ERBB2 amplification																	
No	180 (73.5)	22 (78.6)	6 (21.4)	.536	1.000	2 (66.7)	1 (33.3)	.063	1.000	9 (100.0)	0 (0)	2.602	.171	136 (97.1)	4 (2.9)	2.650	.219
Yes	65 (26.5)	2 (100.0)	0 (0)			13 (59.1)	9 (40.9)			26 (76.5)	8 (23.5)			6 (85.7)	1 (14.3)		
MYC amplification																	
No	150 (61.2)	12 (92.3)	1 (7.7)	2.172	.196	8 (44.4)	10 (55.6)	6.481	.020	25 (86.2)	4 (13.8)	1.362	.404	89 (98.9)	1 (1.1)	3.705	.074
Yes	95 (38.8)	12 (70.6)	5 (29.4)			7 (100.0)	0 (0)			10 (71.4)	4 (28.6)			53 (53.0)	4 (7.0)		
CCND1 amplification																	
No	208 (84.9)	23 (82.1)	5 (17.9)	1.205	.366	13 (56.5)	10 (43.5)	1.449	.500	28 (77.8)	8 (22.2)	1.911	.315	116 (95.9)	5 (4.1)	1.112	.586
Yes	37 (15.1)	1 (50.0)	1 (50.0)			2 (100.0)	0 (0)			7 (100.0)	0 (0)			26 (100.0)	0 (0)		
Retinoblastoma pathway alteration																	
No	183 (74.7)	21 (84.0)	4 (16.0)	1.500	.254	12 (60.0)	8 (40.0)	<.001	1.000	23 (74.2)	8 (25.8)	3.805	.082	104 (97.2)	3 (2.8)	.427	.613
Yes	62 (23.7)	3 (60.0)	2 (40.0)			3 (60.0)	2 (40.0)			12 (100.0)	0 (0)			38 (95.0)	2 (5.0)		
Mitogen-activated protein kinase pathway alteration																	
No	165 (67.3)	12 (75.0)	4 (25.0)	.536	.657	15 (62.5)	9 (37.5)	1.563	.400	28 (77.8)	8 (22.2)	1.911	.315	86 (96.6)	3 (3.4)	.001	1.000
Yes	80 (32.7)	12 (85.7)	2 (14.3)			0 (0)	1 (100.0)			7 (100.0)	0 (0)			56 (96.6)	2 (3.4)		
Phosphatidylinositol-3-kinase pathway alteration																	
No	106 (43.3)	13 (81.2)	3 (18.8)	.033	1.000	7 (53.8)	6 (46.2)	.427	.688	19 (79.2)	5 (20.8)	.178	1.000	51 (96.2)	2 (3.8)	.035	1.000
Yes	139 (56.7)	11 (78.6)	3 (21.4)			8 (66.7)	4 (33.3)			16 (84.2)	3 (15.8)			91 (96.8)	3 (2.2)		

[a]Two cases with no records of hormone receptor (HR) and human epidermal growth factor receptor 2 (HER2) status were not included in this table.

of gene alterations among *TP53* mutation, *PIK3CA* mutation, *ERBB2* amplification and *CCND1* amplification are listed in Figure 3, but only 6 combinations had more than 15 patients in each category. When compared with wild type genotype (*TP53* mutation [–] *PIK3CA* mutation [–] *ERBB2* amplification [–] *CCND1* amplification [–]) tumors, patients with *TP53* mutation (–) *PIK3CA* mutation (–) *ERBB2* amplification (+) *CCND1* amplification (–), *TP53* mutation (+) *PIK3CA* mutation (–) *ERBB2* amplification (+) *CCND1* amplification (–) or *TP53* mutation (+) *PIK3CA* mutation (+) *ERBB2* amplification (+) *CCND1* amplification (–) had significantly higher pCR rates ($P < .05$ for ypT0/isypN0 and ypT0/is, Figure 3A and B).

3.5 | Genetic alteration and related pathway that predispose to pathologic complete response in different subtypes

In addition, we analyzed the rates of ypT0/isypN0 (Table 3) and ypT0/is (Table S4) in genetic alterations within different subtypes to reveal relationships between genetic alterations and chemosensitivity. In HR−HER2+ tumors, *PIK3CA* mutation ($P = .040$ for ypT0/isypN0; $P = .041$ for ypT0/is) and *MYC* amplification ($P = .020$ for ypT0/isypN0; $P = .002$ for ypT0/is) were associated with lower pCR rates. However, in HR+HER2− tumors, *MYC* amplification was related to higher pCR rates ($P = .074$ for ypT0/isypN0; $P = .028$ for ypT0/is). For HR−HER2− and HR+HER2+ tumors, no genetic alterations had statistically significant association with pCR.

4 | DISCUSSION

In this study, we detected cancer-related genes using CNB tissues of breast cancer patients and the NGS method. To focus on the hotspot genes with high frequency in breast cancer samples and to obtain high quality data with deep sequence results, we used commercially available breast cancer-specific NGS panel sequencing (Beikang, Burning Rock Biotech).

For 36 candidate genes, 32 genes had altered in at least 1 patient. *TP53*, *PIK3CA* and *MYC* had high frequency (>40%) of alterations, which was in accordance with previous studies.[10,16,17] Most genes (24/32, 75.0%) had low-frequency (<10%) of alterations.[10] The alteration frequency of some genes was different among clinical subtypes. For example, *TOP2A* was closed to HER2 on chromosome 17 and was reported to be frequently co-amplified in HER2 positive breast cancers.[18,19] We also found that *TOP2A* had higher frequency of alterations in HER2-positive subtypes (Table S2). As for *GATA3* and *FOXA1*, which have been proven to have functional effects on the ESR1 pathway[20] and to have high expression levels in ER positive breast cancers,[21,22] they were also found to be altered frequently in HR-positive tumors (Table S2).

There were 3 main definitions for pCR, which were ypT0/isypN0 (no residual invasive disease in the breast and node), ypT0ypN0 (no residual disease in the breast and node) and ypT0/is (no residual invasive disease in the breast but possibly with nodal involvement). For breast cancer patients who received NAC, pCR (no matter which definition was used) could be a favorable prognosis indicator.[1,23,24] In particular, there was no statistically significant difference in the prognosis between ypT0/isypN0 and ypT0ypN0.[23,24] Although ypT0/is was not a better indicator for prognosis when compared with ypT0/isypN0 and ypT0ypN0,[23] it could be used as a chemosensitivity indicator for breast cancer patients with NAC.[1] Therefore, we used ypT0/isypN0 and ypT0/is simultaneously as definitions for pCR in this study.

Then we calculated associations between gene mutations or amplifications with pCR in all cases and different subtypes. Only genes with frequency of alteration over 15% were enrolled in this analysis. Therefore, high frequency gene mutation, such as for *TP53* and *PIK3CA*, and high frequency gene amplification, such as for *ERBB2*, *MYC* and *CCND1*, were further analyzed.

It is known that *TP53* is frequently altered in the majority of human cancers.[25] Along with *TP53* alterations, breast cancers were more sensitive to DNA-damaging therapy because of loss of p53-dependent transcriptional control.[26] We found that *TP53* was altered in 61.2% of cases and it had higher alteration rates in HR−HER2− tumors (Table S2). For breast cancers that harbored *TP53* mutation, it was easier to reach pCR (Table 2). However, when analyzing the relationship between *TP53* mutation and pCR in different subtypes, *TP53* mutation had higher pCR rates in HR+HER2− tumors with no statistical significance (Table 3 and Table S4).

PIK3CA is the second most commonly mutated gene next to TP53 in many human cancers.[25] In breast cancers, *PIK3CA* mutated frequently[10] and was enriched in luminal tumors, especially in luminal A subtype.[10,25,27] The same phenomenon was also found in our study (Table S2). We found that tumors with mutated *PIK3CA* were less sensitive to NAC (Table 2), as previously reported.[28] Majewski et al[29] reported that the pCR rate of HER2-targeted therapy for *PIK3CA* wild-type tumors was higher in ER−HER2+ subtypes than that in ER+HER2+ tumors. In our study, *PIK3CA* wild-type tumors had higher pCR rates in HR-HER2+ subtype than the other 3 subtypes and the association between *PIK3CA* mutation and pCR had statistical significance only in HR−HER2+ tumors (Table 3 and Table S4). Although patients with mutated *PIK3CA* were not sensitive to traditional chemotherapy, they may be benefit from dual inhibition of B cell lymphoma-extra large (BCL-X_L) and mTOR/4E-BP axes, which could sensitize these patients to traditional chemotherapy.[30]

In the present study, we investigated *ERBB2* gene status using the NGS method. Among 65 cases (26.5%) with *ERBB2* amplification, 56 (86.0%) were detected in HER2-positive diseases. In this study, both HER2-positive status (IHC-3+ and/or amplified FISH results) and *ERBB2* amplification tested by NGS were significantly related to higher pCR rates (Table 2). We also found 9 (3.6%) cases harboring mutated *ERBB2* without *ERBB2* amplification. Only 1 case (1/9, 11.1%) reached pCR after NAC treatment.

The *MYC* gene is overexpressed in many breast cancers. In our study, we found that its amplification rate was 38.5%. As for *MYC* amplification, it showed significant correlation with pCR in both HR−HER2+ and HR+HER2− tumors (Table 3 and Table S4). However,

cases with *MYC* amplification had a higher pCR rate in HR+HER2− subtype and had a lower pCR rate in HR−HER2+ subtype. Yasojima et al[31] also reported that c-myc amplification was related to a higher pCR rate in ER-positive but not ER-negative breast cancers. Apart from sample size of HR+HER2− and HR−HER2+ breast cancers in our study, there may be different mechanisms of amplified *MYC* in responding to chemotherapy.

Overexpression of *CCND1* may be related to gene amplification[32] and result in tumor formation.[33] *CCND1* is a member of the cyclin D family and promotes G1-S transition in cell proliferation.[34] In the TransATAC substudy, breast cancers with *CCND1* amplification were found to have a poor prognosis.[32] In addition, overexpression of *CCND1* demonstrates resistance to chemotherapy in vitro.[34-36] In this study, *CCND1* was amplified in 37 (15.0%) cases, with reported amplification rates from 8.7% to 30%.[32,37,38] Cases with amplified *CCND1* were negatively related to pCR (Table 2).

Moreover, we also classified different altered genes into 3 tumor-related pathways. In our study, the RB pathway contained 3 major altered genes, which were *RB1*, *CCND1* and *CDK4*. The distribution of RB pathway alteration was slightly more in HR-positive disease. However, the RB pathway alteration showed lower sensitivity to chemotherapy with no statistical significance (Table 2). As for the MAPK pathway, which was altered significantly more in triple-negative breast cancers in this study (Table S2), its aberrant activity is important for the progression of tumors. *KRAS* belonging to Ras families, which are small GTPases activated indirectly by external stimuli,[39] have low frequency of mutation in our study (.8%) and in other published studies.[10,40] *BRAF* are also an important component in the MAPK pathway. It mutates frequently in melanoma and papillary thyroid cancer.[39] Targeted $BRAF^{V600E}$ treatment, such vemurafenib[41,42] and dacarbazine[43,44], is approved to be effective in melanoma. In breast cancers, *BRAF* is rarely reported to be mutated.[45] The mutation rate was also under 3.0% in our study. In addition, MAPK pathway alteration was significantly related to a lower pCR rate (Table 2). Aberrant MAPK pathway is also reported to be involved in tamoxifen resistance.[46] The third pathway analyzed in our study was the PI3K pathway, which was found altered in 139 (56.3%) cases. It is reported that alteration of the PI3K pathway is mainly observed in HR-positive diseases.[47] The highest alteration rate of the PI3K pathway was found in HR+HER2− breast cancers (Table S2). In addition, aberrant activation of the PI3K pathway is related to resistance to endocrine therapy and chemotherapy.[48,49] The resistance to endocrine therapy may be on account of inducing estrogen-independent transcriptional activity.[50] Activation of the PI3K pathway may overcome proapoptotic signals of anti−cancer drugs and leads to chemoresistance.[51] Therefore, inhibition of the PI3K pathway is proved to promote cytotoxic effects in chemotherapy.[52] In this study, cases with PI3K pathway alterations had lower pCR rates than non-alteration cases with no statistical significance (Table 2).

With the development of targeted sequencing leading to lower cost and higher depth of coverage, more NGS panels are being applied in clinical practice. Multiple gene alterations can be detected at one time. Some gene alterations have been proven to be related to clinical outcomes, as discussed above. Moreover, patients with specific gene alterations may have the opportunity to receive targeted therapy, which may be approved or investigational. The targeted therapies of detected genes in this study are summarized in Table S5. It is a challenge in clinical practice to choose a suitable therapy for patients who harbor gene alterations.

In conclusion, we used NGS panels to detect breast cancer-related gene alterations in FFPE CNB samples. Gene alterations, such as *TP53* mutation, *PIK3CA* mutation, *ERBB2* amplification and *CCND1* amplification, and MAPK pathway alteration were associated with pCR in our study. Our study may shed light on the molecular characteristics of breast cancer for prediction of NAC expectations when breast cancer is first diagnosed by biopsy.

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SUPPORTING INFORMATION

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