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ORIGINAL ARTICLE

PIK3CA Mutations and Neoadjuvant Therapy Outcome in Patients with Human Epidermal Growth Factor Receptor 2-Positive Breast Cancer: A Sequential Analysis

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Purpose: *PIK3CA* mutation is considered to be a possible cause for resistance to neoadjuvant chemotherapy (NAC) in human epidermal growth factor receptor 2 (HER2)-positive breast cancer. We investigated the association between *PIK3CA* mutations and the outcome of NAC in HER2-positive breast cancers. **Methods:** A total of 100 HER2-positive breast cancer patients who had undergone NAC and surgery between 2004 and 2016 were examined. Mutation status was sequentially assessed in pre-NAC, post-NAC, and recurrent specimens taken from these patients. **Results:** *PIK3CA* mutations were identified in the sequential specimens of 17 patients (17.0%). These 17 patients experienced shorter disease-free survival (DFS) than the rest of the patients (58.3 months vs. 119.3 months, $p=0.020$); however, there was no significant difference in pathologic complete response (pCR) and overall survival (OS) (pCR, 17.6% vs. 33.7%, $p=0.191$; OS, 84.5 months vs. 118.0 months, $p=0.984$). While there was no difference in pCR between the wild-type and mutant *PIK3CA* groups in pre-NAC specimens (25.0% vs. 31.8%, $p=0.199$), *PIK3CA* mutations correlated with lower pCR in post-NAC specimens (0.0% vs. 24.3%, $p<0.001$). Multivariate analysis revealed significantly worse DFS in the mutant *PIK3CA* group than in the wild-type group (hazard ratio, 3.540; 95% confidence interval, 1.001–12.589; $p=0.050$). Moreover, the DFS curves of the change of *PIK3CA* mutation status in sequential specimens were significantly different ($p=0.016$). **Conclusion:** *PIK3CA* mutation in HER2-positive breast cancer was correlated with a lower pCR rate and shorter DFS. These results suggest that *PIK3CA* mutation is a prognostic marker for NAC in HER2-positive breast cancer, especially in post-NAC specimens.

Key Words: *Breast neoplasms, ErbB-2 receptor, Mutation, Neoadjuvant therapy, Phosphatidylinositol 3-kinases*

INTRODUCTION

The *PIK3CA* gene encodes the phosphoinositide 3-kinase (PI3K) catalytic subunit and is involved in the PI3K/protein kinase B (AKT) pathway, affecting cell growth, division, and differentiation. Mutations in this gene can activate the PI3K/AKT pathway and affect tumorigenesis [1,2]. *PIK3CA* mutation is one of the most common mutations in breast cancer [3]. Although the frequency of the mutation varies according to molecular subtype, this mutation has been described in approximately 12% to 39% of human epidermal growth factor receptor 2 (HER2)-positive breast cancers [3-8]. The *PIK3CA* mutation is being examined as a causative candidate for trastuzumab resistance in HER2-positive breast cancer due to the relevance of the PI3K/AKT pathway and HER2 [1,9,10]; however, previous studies have been inconclusive [7,8,11-21].

In previous studies, *PIK3CA* was analyzed using the first biopsy specimen, especially in patients who had received neoadjuvant chemotherapy (NAC) [11,13,15,16,19]. There was one study in which sequential *PIK3CA* mutations were analyzed and compared; however, it was conducted in a population with general breast cancer not in a population with HER2-positive breast cancer [11].

The aim of this study was to determine whether the *PIK3CA* mutation has clinical significance in assessing pathologic complete response (pCR), overall survival (OS), and disease-free survival (DFS) in HER2-positive breast cancer patients

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undergoing NAC. More specifically, we attempted to identify the effect of the mutation according to site or time at which it occurred, as well as the change of the mutation status, through sequential analysis using pre-NAC, post-NAC, or recurrent specimens from each patient.

METHODS

Study population and clinicopathologic data

We retrospectively selected patients with invasive breast cancer who underwent surgery after NAC at the general surgery unit of Samsung Medical Center between December 2004 and June 2016. There were 287 patients with HER2-positive breast cancer among 967 selected patients. All patients underwent at least three cycles of NAC before surgery. The NAC regimens without trastuzumab included anthracycline-based (doxorubicin with cyclophosphamide) or anthracycline and taxane-based (doxorubicin and docetaxel with or without cyclophosphamide) regimens. The NAC regimens including trastuzumab were anthracycline-based, taxane-based (docetaxel), or anthracycline and taxane-based (doxorubicin with docetaxel and cyclophosphamide) regimen. We collected the sequential specimen blocks including pre-NAC, post-NAC, and recurrent specimens of each patient. Patients were excluded if they did not have either pre-NAC or post-NAC specimen blocks. Finally, a total of 100 patients were included in this study. All clinical parameters, including patient age, menopause status, clinical tumor-node-metastasis (TNM) stage, NAC regimen, surgery type, follow-up period after diagnosis, survival, and tumor recurrence were reviewed based on electronic medical records from initial diagnosis to last recorded follow-up until July 2016. All hematoxylin and eosin (H&E)-stained slides and immunohistochemistry slides were reviewed by two pathologists (Y.S. and E.Y.C.). Tumor recurrence was defined as involving both local recurrence and distant metastasis, while pCR was defined as no invasive tumor in the breast or lymph nodes, and no lymphovascular invasion. This study was approved by the Institutional Review Board (IRB) of Samsung Medical Center (SMC 2016-08-005). The requirement for formal written informed consent was waived by the IRB.

Immunohistochemistry and molecular tests

Immunohistochemical tests for estrogen receptor (ER), progesterone receptor (PR), and HER2 were performed on 10% formalin-fixed and paraffin-embedded (FFPE) blocks of pre-NAC and post-NAC biopsy specimens. After rehydration and antigen retrieval in citrate buffer, the tissue sections were stained for ER (clone 6F11; Novocastra, Newcastle upon Tyne, UK), and PR (clone 16; Novocastra). Immunohistochemical positivity for ER and PR was determined according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines using a cutoff of >1% stained tumor nuclei and evaluated by Allred score [22]. HER2 positivity was defined as HER2 3+ expression by immunohistochemistry (clone 4B5; Ventana, Tucson, USA) or 2+ expression by immunohistochemistry with amplification by silver *in situ* hybridization (SISH) [23]. HER2 SISH analysis was performed using INFORM DDISH HER-2 DNA SISH probe kits (BenchMark XT; Ventana) on 10% FFPE blocks. HER2 positivity was determined according to ASCO/CAP guidelines using a cutoff HER2 amplification ratio of ≥2.0 and/or an average HER2 copy number of ≥6.0 signals per cell.

PIK3CA mutation analysis was performed on corresponding FFPE blocks. All H&E slides were reviewed, and the target area containing invasive carcinoma and ductal carcinoma *in situ* (DCIS) was marked on the slides. Genomic DNA was extracted from the 5-μm-thick unstained slides of the FFPE block using manual microdissection. Mutational analysis of *PIK3CA* exon 9 and 20, which were reported as hotspots, was performed by directional sequencing of polymerase chain reaction (PCR) fragments amplified from genomic DNA using sequencing probes, similar to that reported in a previous study [24]. The forward primer for exon 9 was 5′-TGTGAATCCAGAGGGGAAAA-3′, and the reverse primer was 5′-TGCTGAGATCAGCCAAATTCA-3′; the forward primer for exon 20 was 5′-TTGCATACATTCGAAAGACC-3′, and the reverse primer was 5′-CCTATGCAATCGGTCTTGC-3′. The PCR products were subjected to Sanger sequencing to detect the mutation.

Statistical analysis

The associations between *PIK3CA* mutations and clinicopathological parameters were statistically evaluated using several tests, including chi-square test, Fisher exact test, Student t-test, Mann-Whitney U-test, and Kruskal-Wallis test. For the survival analyses, OS was defined as the time between the date of diagnosis and the date of death or last follow-up. DFS was defined as the time between the date of diagnosis and the date of tumor recurrence or last follow-up. Survival analyses were assessed using a Kaplan-Meier curve and the log-rank test. Multivariate analysis was used to evaluate the prognostic value of OS and DFS using a Cox proportional hazards model. Binary logistic analysis was performed to evaluate the predictive significance of pCR. A two-sided *p*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, USA).

RESULTS

Patient clinicopathologic characteristics according to *PIK3CA* mutation status

Average patient age was 47.5 ± 10.0 years. *PIK3CA* mutations were observed in the sequential specimens of 17 patients. Among these, five patients had mutations in exon 9, and the rest had mutations in exon 20. The frequency of *PIK3CA* mutation in the pre-NAC, post-NAC, and recurrent specimens was 12.0% (12/100), 13.6% (11/81), and 20.0% (3/15), respectively. *PIK3CA* mutation analysis could not be performed on the post-NAC specimens of 19 patients due to insufficient amount of residual tumor. Among these 19 uninvestigated patients, 14 showed pCR but low DCIS volume, and five had invasive carcinoma smaller than 3 mm in post-NAC specimens.

In 23 cases, post-NAC specimens did not provide enough residual invasive carcinoma in the resected breast for analysis. In these cases, post-NAC *PIK3CA* mutation analysis was conducted in residual DCIS. Not all of the analyzed residual DCIS specimens revealed a *PIK3CA* mutation.

Patients in the mutant *PIK3CA* group had a higher pathologic N stage (ypN stage, $p = 0.008$) and underwent total mastectomy more frequently than those in the wild-type group ($p = 0.034$). Patients with *PIK3CA* mutations tended to have larger tumor size after NAC ($p = 0.075$). There was no significant difference between the two groups when comparing other parameters. The baseline characteristics of the study population are summarized in Table 1.

Pathologic response with neoadjuvant chemotherapy and *PIK3CA* mutation status in sequential specimens

In total, 31 patients achieved pCR after NAC. Table 2 summarizes the pCR rate under varying conditions. There was no difference between the pCR rate of patients who had at least one *PIK3CA* mutation in sequential specimens and that of patients with no *PIK3CA* mutation in all sequential specimens (17.6% vs. 33.7%, $p = 0.191$). *PIK3CA* mutation in post-NAC specimens correlated with a lower pCR rate than that observed in the post-NAC wild-type specimens (0.0% vs. 24.3%, $p < 0.001$). However, there was no statistical difference in pCR rate between the pre-NAC mutant group and the pre-NAC wild-type group (25.0% vs. 31.8%, $p = 0.750$).

The significance of pCR rates associated with the site of *PIK3CA* mutation was assessed in the group with at least one mutation in sequential specimens and in the group with mutant *PIK3CA* in the pre-NAC specimens. No significant difference was observed in the pCR rates of these two groups (at least one mutation in sequential specimen: wild-type vs. exon 9 vs. exon 20, 33.7% vs. 20.0% vs. 16.7%, $p = 0.199$; pre-NAC

Table 1. Clinicopathologic parameters according to *PIK3CA* mutation status

Characteristic	Wild type (n = 83) No. (%)	Mutant type (n = 17) No. (%)	*p*-value
Age (yr)*	47.4 ± 10.2	48.1 ± 9.2	0.762
Menopause			0.812
Premenopause	47 (56.1)	9 (52.9)	
Postmenopause	36 (43.9)	8 (47.1)	
Surgery type			0.034
Partial mastectomy	44 (53.0)	4 (23.5)	
Total mastectomy	39 (47.0)	13 (76.5)	
Usage of trastuzumab			0.594
Yes	58 (69.9)	10 (58.8)	
No	25 (30.1)	7 (41.2)	
ER			0.876
Positive	26 (31.3)	5 (29.4)	
Negative	57 (68.7)	12 (70.6)	
PR			0.734
Positive	15 (18.1)	4 (23.5)	
Negative	68 (81.9)	13 (76.5)	
Subgroup			0.802
Luminal B†	27 (32.5)	5 (29.4)	
HER2 enriched‡	56 (67.5)	12 (70.6)	
Pre-NAC size (mm)	51.18	51.24	0.992
Post-NAC size (mm)	14.15	25.56	0.075
Clinical N stage (cN)			0.847
cN0	3 (3.6)	0	
cN1	20 (24.1)	3 (17.6)	
cN2	42 (50.6)	9 (52.9)	
cN3	18 (21.7)	5 (29.4)	
Pathologic N stage (ypN)			0.008
ypN0	53 (63.9)	6 (35.3)	
ypN0(itc)	8 (9.6)	1 (5.9)	
ypN1(mi)	5 (6.0)	2 (11.8)	
ypN1	8 (9.6)	2 (11.8)	
ypN2	4 (4.8)	5 (29.4)	
ypN3	5 (6.0)	0	
ypNx§	0	1 (5.9)	
Pre-NAC cellularity (%)	53.7	53.5	0.976
Post-NAC cellularity (%)	18.7	37.8	0.037
Multifocality			0.424
Yes	10 (10.8)	3 (17.6)	
No	73 (89.2)	14 (82.4)	
LVI			0.236
Yes	21 (25.3)	7 (41.2)	
No	62 (74.7)	10 (58.8)	
Follow-up period (mo)*	29.1 ± 30.6	34.3 ± 22.2	0.422

ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; NAC = neoadjuvant chemotherapy; itc = isolated tumor cells; mi = microinvasion; LVI = lymphovascular invasion.
*Mean ± SD; †Luminal B: immunonistochemical stain for estrogen receptor or progesterone receptor (+); ‡HER2 enriched: immunonistochemical stain for estrogen receptor and progesterone receptor (–); §The one of the ypNx case did not undergo node dissection.

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Table 2. Pathologic complete response rates by *PIK3CA* mutation status and site

Characteristic	pCR No. (%)	Non-pCR No. (%)	*p*-value
Sequential specimen			
Mutation status			0.191
Wild type (n=83)	28 (33.7)	55 (66.3)	
Mutant type (n=17)	3 (17.6)	14 (82.4)	
Mutation site			0.199
Wild type (n=83)	28 (33.7)	55 (66.3)	
Exon 9 (n=5)	1 (20.0)	4 (80.0)	
Exon 20 (n=12)	2 (16.7)	10 (83.3)	
Pre-NAC specimen			
Mutation status			0.750
Wild type (n=88)	28 (31.8)	60 (68.2)	
Mutant type (n=12)	3 (25.0)	9 (75.0)	
Mutation site			>0.999
Wild type (n=88)	28 (31.8)	60 (68.2)	
Exon 9 (n=4)	1 (25.0)	3 (75.0)	
Exon 20 (n=8)	2 (25.0)	6 (75.0)	
Post-NAC specimen			
Mutation status			<0.001
Wild type (n=70)	17 (24.3)	53 (75.7)	
Mutant type (n=11)	0	11 (100)	
Mutation site			<0.001
Wild type (n=70)	17 (24.3)	53 (75.7)	
Exon 9 (n=3)	0	3 (100)	
Exon 20 (n=8)	0	8 (100)	

pCR=pathologic complete response; NAC=neoadjuvant chemotherapy.

specimen: wild-type vs. exon 9 vs. exon 20, 31.8% vs. 25.0% vs. 25.0%, $p>0.999$).

Survival analysis

The survival curves for each condition are depicted in Figure 1. Patients with at least one *PIK3CA* mutation had a significantly shorter median DFS than those in the wild-type group (58.3 months vs.119.3 months, $p=0.020$) (Figure 1A). Survival analysis of the post-NAC specimens revealed a trend for shorter DFS in the mutant *PIK3CA* group (60.2 months vs. 113.5 months, $p=0.148$) (Figure 1B). There was no significant difference between the DFS of the mutant group and that of the wild-type group in pre-NAC specimens (51.8 months vs. 103.9 months, $p=0.477$) (Figure 1C).

Sequential mutation analysis indicated no significant difference in median DFS between the group exhibiting exon 9 mutation and the group exhibiting exon 20 mutation (119.3 months vs. 47.2 months vs. 59.8 months, $p=0.063$) (Figure 1D). Survival analysis of post-NAC specimens showed significant median DFS differences among the three mutation site groups (exon 9 vs. exon 20 vs. post-NAC wild-type: 41.8 months vs. 74.0 months vs. 113.5 months, $p=0.039$) (Figure 1E). Among the mutation sites, a *PIK3CA* mutation in exon 9 was found to yield a significantly shorter median DFS than other groups (exon 9 vs. exon 20, $p=0.048$; exon 9 vs. post-NAC wild-type, $p=0.017$). There was no significant difference in median OS according to *PIK3CA* mutation status (84.5 months vs. 118.0 months, $p=0.984$) (Figure 1F).

Prognostic or predictive effectiveness of *PIK3CA* mutation

Univariate analysis revealed that *PIK3CA* mutation and post-NAC tumor size were significantly associated with a worse DFS in sequential specimens (*PIK3CA* mutation: hazard ratio [HR], 3.143, 95% confidence interval [CI], 1.137–8.689, $p=0.027$; post-NAC tumor size: HR, 1.022, 95% CI, 1.009–1.036, $p=0.001$). These results are presented in Supplementary Table 1 (available online). Multivariate analysis also determined that *PIK3CA* mutation was a significant contributing factor for a shorter DFS (HR, 3.550; 95% CI, 1.001–12.589; $p=0.050$). Post-NAC tumor size and the presence of pre-NAC clinical node metastasis were also determined to be significant contributors by multivariate analysis (post-NAC tumor size: HR, 1.029, 95% CI, 1.007–1.052, $p=0.010$; pre-NAC clinical node metastasis: HR, 0.003, 95% CI, 0.000–0.047, $p<0.001$). These results are presented in Table 3. All the patients in this study underwent NAC, and a total of 97 patients had lymph node metastasis at the time of diagnosis due to characteristics of NAC. One out of three patients (33.3%) who were not clinically diagnosed with lymph node metastasis (cN0) had tumor recurrence at a higher rate than that observed at other stages. This could explain why a shorter DFS was observed in the cN0 stages.

Univariate analysis for OS in the sequential specimens revealed the significance of post-NAC tumor size (HR, 1.031; 95% CI, 1.012–1.051; $p=0.001$) and the presence of lymphovascular invasion (HR, 4.140; 95% CI, 1.035–16.562; $p=0.045$). Supplementary Table 2 (available online) summarizes these results. However, these factors were not found to be significant in multivariate analysis. As shown in Table 4, older age and the presence of pre-NAC clinical lymph node metastasis were additional significant factors for shorter OS (age: HR, 1.109, 95% CI, 1.004–1.225, $p=0.042$; pre-NAC clinical lymph node metastasis: HR, 0.027, 95% CI, 0.001–0.633, $p=0.025$).

Due to the small sample size, it was difficult to identify all variables for pCR analysis. Supplementary Table 3 (available online) summarizes the univariate analysis for pCR. According to univariate evaluation, trastuzumab treatment as part of NAC was a significant factor for pCR (HR, 4.610; 95% CI, 1.453–14.628; $p=0.009$). Similar significance was obtained using multivariate analysis for pCR (HR, 4.766; 95% CI,

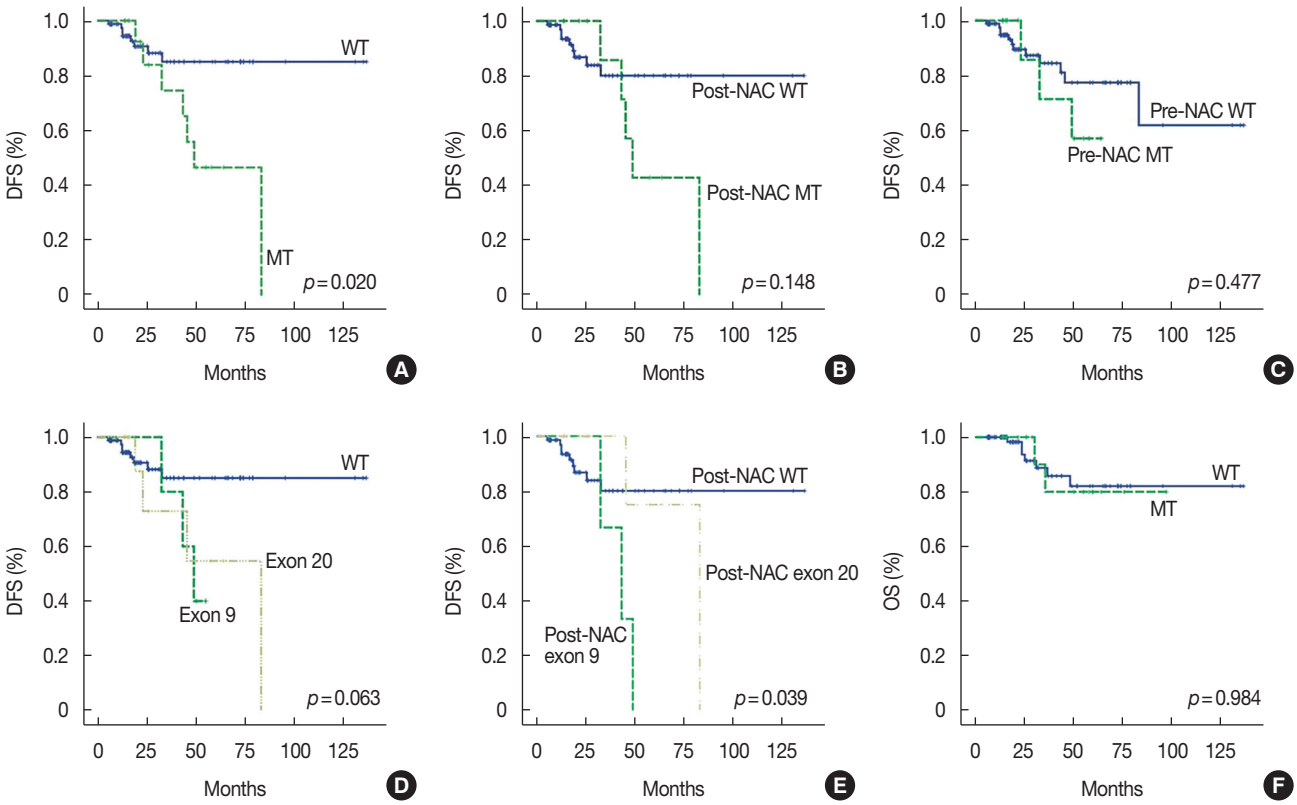

Figure 1. Survival curves for disease-free survival (DFS) and overall survival (OS). (A) *PIK3CA* mutant group (MT) in any sequential specimen had a shorter median DFS than the purely wild-type group (WT) in sequential specimens (58.3 months vs. 119.3 months, $p=0.020$). (B) In post-neoadjuvant chemotherapy (NAC) specimens, MT showed a shorter median DFS tendency than WT (60.2 months vs. 113.5 months, $p=0.148$). (C) Median DFS was not significantly different in pre-NAC WT and pre-NAC MT (51.8 months vs. 103.9 months, $p=0.477$). (D) There was no difference in median DFS depending on mutation site in sequential analysis (119.3 months vs. 47.2 months vs. 59.8 months, $p=0.063$). (E) Mutation site was associated with different median DFS in post-NAC specimens (post-NAC WT: 113.5 months vs. post-NAC exon 9: 41.8 months vs. post-NAC exon 20: 74.0 months, $p=0.039$). In subgroup analysis, post-NAC exon 9 mutation was found to have significantly shorter DFS than other groups (vs. post-NAC WT: $p=0.017$ vs. post-NAC exon 20: $p=0.048$). (F) OS was not correlated with *PIK3CA* mutation status (84.5 months vs. 118.0 months, $p=0.984$).

Table 3. Multivariate analysis for disease-free survival

Variable	*p*-value	HR	95% CI
Age	0.958	0.958	0.899–1.020
Usage of trastuzumab	0.665	0.766	0.228–2.567
HER2 enriched subgroup*	0.742	0.795	0.203–3.107
Pre-NAC tumor size	0.699	0.993	0.958–1.029
Post-NAC tumor size	0.010	1.029	1.007–1.052
Presence of cN metastasis	<0.001	0.003	0.000–0.047
Presence of ypN metastasis	0.593	0.658	0.141–3.060
Presence of LVI	0.312	2.075	0.503–8.549
PIK3CA mutation	0.050	3.550	1.001–12.589

HR=hazard ratio; CI=confidence interval; HER2=human epidermal growth factor receptor 2; NAC=neoadjuvant chemotherapy; cN=clinical node; ypN=post-NAC pathologic node; LVI=lymphovascular invasion.
*HER2 enriched subgroup versus luminal B subgroup.

Table 4. Multivariate analysis for overall survival

Variable	*p*-value	HR	95% CI
Age	0.042	1.109	1.004–1.225
Usage of trastuzumab	0.242	0.348	0.059–2.041
HER2 enriched subgroup*	0.179	5.306	0.466–60.469
Pre-NAC tumor size	0.879	0.996	0.948–1.047
Post-NAC tumor size	0.142	1.024	0.992–1.058
Presence of cN metastasis	0.025	0.027	0.001–0.633
Presence of ypN metastasis	0.852	1.207	0.168–8.665
Presence of LVI	0.137	4.527	0.619–33.091
PIK3CA mutation	0.336	0.387	0.056–2.676

HR=hazard ratio; CI=confidence interval; HER2=human epidermal growth factor receptor 2; NAC=neoadjuvant chemotherapy; cN=clinical node; ypN=post-NAC pathologic node; LVI=lymphovascular invasion.
*HER2 enriched subgroup versus luminal B subgroup.

1.464–15.519; $p=0.010$). In both univariate and multivariate analyses, *PIK3CA* mutation did not correlate with pCR (univariate: HR, 0.714, 95% CI, 0.179–2.843, $p=0.633$; multivariate: HR, 0.988, 95% CI, 0.228–4.279, $p=0.987$). These results can be viewed in Table 5.

For evaluation of the predictive impact of *PIK3CA* muta-

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tion, the difference in the rate of pCR after NAC was statistically analyzed. In the trastuzumab-treated group, patients who had at least one *PIK3CA* gene mutation in sequential specimens experienced a 20.0% pCR rate, whereas patients who had purely wild-type *PIK3CA* experienced a 43.1% pCR rate; this difference, however, was not significant ($p=0.294$). Pre-NAC subgroup analysis yielded corresponding results with no significant predictive effect associated with the *PIK3CA* mutation (pre-NAC mutant group: 33.3% vs. pre-NAC wild-type: 40.3%, $p>0.999$). Supplementary Table 4 (available online) summarizes these results.

Change of *PIK3CA* mutation status in sequential specimens

In nine out of 100 cases, *PIK3CA* mutation status changed in sequential specimens. In four cases, the pre-NAC *PIK3CA* mutation was lost after NAC (MT-WT group). New *PIK3CA* mutations developed in five cases (WT-MT group), and of these, tumor recurrence was observed in four. In 83 cases, *PIK3CA* remained purely wild-type in sequential specimens (WT-WT group), while purely *PIK3CA* mutants were found in the sequential specimens (MT-MT group) of eight cases. The clinicopathological details of patients who experienced changes in their mutation status are described in Table 6.

The DFS curves of the mutation change groups are plotted in Figure 2. Because there was no tumor recurrence in the MT-WT group, the Kaplan-Meier method could not be em-

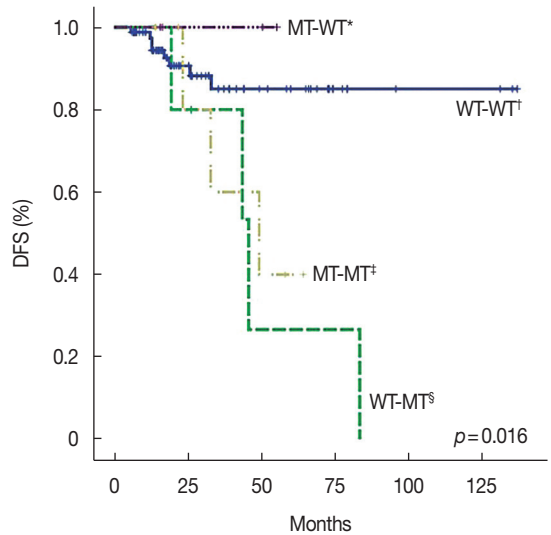

Figure 2. Survival curve for disease-free survival (DFS) according to mutation change status. There are four groups of mutation change status in sequential analysis. Due to lack of recurrence in the mutant-wild (MT-WT) group, Kaplan-Meier methods could not be utilized. The log-rank test was used to reveal significantly different survival curves for DFS ($p=0.016$). Subgroup analysis determined a significant DFS difference between the WT-WT group and WT-MT groups ($p=0.005$).
*MT-WT: *PIK3CA* mutation lost after neoadjuvant chemotherapy; †WT-WT: purely *PIK3CA* wild-type in all of the sequential specimens; ‡MT-MT: purely *PIK3CA* mutant type in all of the sequential specimens; §WT-MT: gained *PIK3CA* mutation after neoadjuvant chemotherapy.

Table 5. Multivariate analysis for pathologic complete response

Variable	*p*-value	HR	95% CI
Age	0.864	0.996	0.952–1.042
Usage of trastuzumab	0.010	4.766	1.464–15.519
Pre-NAC tumor size	0.165	0.986	0.967–1.006
HER2 enriched subgroup*	0.970	1.019	0.384–2.701
Pre-NAC *PIK3CA* mutation	0.987	0.988	0.228–4.279

HR=hazard ratio; CI=confidence interval; NAC=neoadjuvant chemotherapy; HER2=human epidermal growth factor receptor 2.
*HER2 enriched subgroup versus luminal B subgroup.

Table 6. Clinicopathologic characteristics of cases with mutation change in sequential analysis

No.	Age (yr)	Pre-NAC mutation	Post-NAC mutation	Recurrent specimen mutation	pCR	Recur	Subgroup	Usage of trastuzumab	Status	OS	DFS	cTN	ypTN
1	48	Wild	Wild	H1047R	-	+	HER2*	-	Death	27.5	16.6	T3N3	T4N2
2	53	Wild	E542K	Wild	-	+	HER2	+	F/U loss	53.2	36.7	T3N2	T2N0
3	55	Wild	H1047R	H1047R	-	+	HER2	+	Alive	57.6	43.6	T2N3	T2Nx
4	28	Wild	H1047R	NA†	-	+	Luminal B‡	+	Alive	54.2	37.0	T2N3	T1N1
5	46	Wild	H1047R	-	-	-	HER2	+	Alive	20.2	20.2	T2N2	T2N2
6	55	E542A	Wild	-	+	-	HER2	-	Alive	49.3	49.3	T3N2	TisN0
7	46	H1047R	Wild	-	+	-	Luminal B	+	Alive	9.0	9.0	T2N2	TisN0
8	53	E542K	Wild	-	-	-	Luminal B	-	Alive	43.8	43.8	T4N3	T1N0(i)
9	58	H1047R	Wild	-	-	-	HER2	+	Alive	8.9	8.9	T3N2	T3N1(mi)

NAC=neoadjuvant chemotherapy; pCR=pathologic complete response; OS=overall survival; DFS=disease-free survival; cTN=clinical T&N stage; ypTN=post-NAC pathological T&N stage; HER2=human epidermal growth factor receptor 2; F/U=follow-up; i=isolated tumor cell; mi=microinvasion.
*HER2 enriched: immunonistochemical stain for estrogen receptor and progesterone receptor (–); †NA: multiple distant metastases were found in the brain in this case, but further tests could not be performed because no samples were obtained; ‡Luminal B: immunonistochemical stain for estrogen receptor or progesterone receptor (+).

ployed for survival analysis. The log-rank test was used, revealing a significant difference in the survival curves (p = 0.016). More specifically, there was a statistically significant difference between the purely wild-type group and developed *PIK3CA* mutation group (WT-WT vs. WT-MT, p = 0.005). While the survival curve for the MT-MT group showed a worse trend than that for the MT-WT group (MT-MT vs. MT-WT, p = 0.079), further analysis between the subgroups showed no significant differences.

DISCUSSION

In our study, the frequency of *PIK3CA* mutation in the pre-NAC, post-NAC, and recurrent specimens was 12.0%, 13.6%, and 20.0%, respectively. Previous studies have chronicled a 12%–39% *PIK3CA* mutation rate in HER2-positive breast cancer. In particular, the mutation rate of initial biopsy specimens was reported as approximately 15%–20%, that of surgically resected specimens as 12%–29%, and that of metastatic breast cancer as 19%–33% [3-8]. The ratio of patients with *PIK3CA* mutation was slightly lower in our study than in other studies.

The association between *PIK3CA* mutations and HER2-positive breast cancer is still unclear, as previous studies have related *PIK3CA* mutations with unfavorable clinical outcomes [7,8,11,16,17,19], favorable outcomes [18], or found no significant impact [12-15,20,21]. Many studies that concluded that *PIK3CA* mutations corresponded with poor clinical outcomes found lower pCR rates in the mutant group than in the wild-type group [7,8,11,17,19], and in some cases, the difference was more pronounced in the ER-positive group [7,19].

In this study, a lower pCR rate was observed in the post-NAC mutant *PIK3CA* group than in the post-NAC wild-type group (p < 0.001). However, this correlation did not occur in pre-NAC specimen groups. ER-positivity and of the mutation site also did not correlate with changes in the pCR rate. The median DFS was significantly shorter in the mutant group than in the wild-type group (p = 0.020), as well as in post-NAC *PIK3CA* exon 9 mutation than in other mutation or wild-type groups (exon 9 vs. exon 20 vs. post-NAC wild-type: 41.8 months vs. 74.0 months vs. 113.5 months, p = 0.039). On the other hand, there was no significant difference in median OS in any subgroup analysis.

The presence of a *PIK3CA* mutation was statistically associated with a short DFS in both univariate and multivariate analyses (univariate: p = 0.027; multivariate: p = 0.050). Nevertheless, the mutation did not significantly affect pCR and OS and provided no significant predictive effect after NAC, regardless of trastuzumab usage. A previous meta-analysis also failed to demonstrate any predictive effect associated with *PIK3CA* mutation [8].

Post-NAC *PIK3CA* mutation analysis could not be performed in 19 cases due to insufficient data. Specifically, pCR was achieved in 14 cases, while a minimal volume of invasive carcinoma up to 3 mm was found in the remaining five cases. All of these cases had an initially good clinical response to NAC and showed good prognosis. However, because true mutation status or change could not be analyzed in post-NAC specimens, the uninvestigated group was excluded from statistical evaluation. One patient who had pre-NAC *PIK3CA* mutation achieved pCR after NAC but experienced tumor recurrence 23 months later. The recurrent specimen had the same mutation (H1047R) as observed in the patient's pre-NAC specimen.

Sequential analysis revealed nine cases involving change in *PIK3CA* mutation status. There are two hypotheses for this occurrence. The first is tumor heterogeneity, which posits that several clusters with different mutational statuses were present in the tumor, and that the initial biopsy could not identify all these clusters. The second hypothesis is that NAC could affect the gain or loss of mutations in the tumor clusters. Further study is required to clarify the mechanisms underlying mutation change.

In most previous studies, pre-NAC specimens were used to analyze *PIK3CA* mutations, but in this study, mutation status in pre-NAC specimens was determined not to be clinically significant. Instead, clinical significance, including pCR rate (post-NAC mutant-type vs. post-NAC wild-type: 0.0% vs. 24.3%, p < 0.001) and median DFS (post-NAC mutant-type vs. post-NAC wild-type: 60.2 months vs. 113.5 months, p = 0.108), was more correlated with post-NAC mutation. In particular, the DFS curve developed from the group in which *PIK3CA* mutations were gained was significantly worse than that developed from the group with purely wild-type *PIK3CA* (WT-MT vs. WT-WT, p = 0.005). This may also support the clinical significance of the mutations found in post-NAC specimens.

According to Yuan et al. [11], breast cancer patients in whom the pre-NAC mutation was maintained had a worse pCR rate and borderline worse survival than patients who lost the mutation. In our study, the worst survival curve was obtained in the group that gained the mutation after NAC, while the group in which the *PIK3CA* mutation was preserved had a worse survival curve trend than the group in which the mutation was lost (MT-MT vs. MT-WT, p = 0.079). Although previous studies examined all molecular subtypes of breast cancer, it was considered that post-NAC mutations would hold more clinical significance.

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Several studies have focused on a *PIK3CA* inhibitor in HER2-positive breast cancer [15,25,26]. One NAC clinical trial tested the mutation in pre-NAC specimens [15]. However, in our study, pre-NAC mutation did not correlate with poor NAC outcome and did not function as a predictive marker for the efficacy of NAC. In metastatic trastuzumab-refractory HER2-positive breast cancer, a combination of pilaralisib, trastuzumab, and paclitaxel yielded a partial response of 20% [26]. In our study, the mutation rate of recurrent specimens was 20.0%, which was higher than the rates observed for pre-NAC and post-NAC specimens. With the exception of two cases, the mutation status of the recurrent specimens was the same in post-NAC specimens. One of the two exceptions achieved pCR; therefore, post-NAC mutation analysis was not performed. The other case gained the *PIK3CA* mutation in recurrence. Post-NAC mutations resulted in a worse effect for DFS in multivariate analysis ($p = 0.050$). Post-NAC, rather than pre-NAC, mutational analysis may be more helpful for clinical trials using *PIK3CA* inhibitor.

There were some statistical limitations regarding multivariate and survival analyses due to the small sample size and short follow-up period. Nevertheless, to the best of our knowledge, this study analyzes the largest number of sequential *PIK3CA* mutations in HER2-positive breast cancer.

In conclusion, *PIK3CA* mutation in HER2-positive breast cancer was correlated with a lower pCR rate and shorter DFS in our study. These results suggest that *PIK3CA* mutation could be a prognostic marker for NAC in HER2-positive breast cancer, especially in post-NAC specimens. Further studies should be conducted with a larger number of post-NAC cases and clinical outcomes of NAC and should assess the effects of *PIK3CA* inhibitors in adjuvant chemotherapy.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Supplementary Table 1. Univariate analysis for disease-free survival

Variable	*p*-value	HR	95% CI
Age	0.211	0.967	0.918–1.019
Usage of trastuzumab	0.240	0.535	0.188–1.520
HER2 enriched subgroup*	0.715	1.222	0.416–3.588
Pre-NAC tumor size	0.850	0.998	0.976–1.020
Post-NAC tumor size	0.001	1.022	1.009–1.036
Presence of cN metastasis	<0.001	0.018	0.002–0.135
Presence of ypN metastasis	0.047	3.019	1.017–8.964
Presence of LVI	0.082	2.481	0.890–6.912
PIK3CA mutation	0.027	3.143	1.137–8.689

HR = hazard ratio; CI = confidence interval; HER2 = human epidermal growth factor receptor 2; NAC = neoadjuvant chemotherapy; cN = clinical node; ypN = post-NAC pathologic node; LVI = lymphovascular invasion.
*HER2 enriched subgroup versus luminal B subgroup.

Supplementary Table 2. Univariate analysis for overall survival

Variable	*p*-value	HR	95% CI
Age	0.252	1.042	0.971–1.118
Usage of trastuzumab	0.133	0.299	0.062–1.446
HER2 enriched subgroup*	0.122	5.152	0.644–41.224
Pre-NAC tumor size	0.539	1.009	0.982–1.036
Post-NAC tumor size	0.001	1.031	1.012–1.051
Presence of cN metastasis	0.074	0.140	0.016–1.212
Presence of ypN metastasis	0.140	2.840	0.709–11.377
Presence of LVI	0.045	4.140	1.035–16.562
PIK3CA mutation	0.984	1.017	0.211–4.901

HR = hazard ratio; CI = confidence interval; HER2 = human epidermal growth factor receptor 2; NAC = neoadjuvant chemotherapy; cN = clinical node; ypN = post-NAC pathologic node; LVI = lymphovascular invasion.
*HER2 enriched subgroup versus luminal B subgroup.

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Supplementary Table 3. Univariate analysis for pathologic complete response

Variable	*p*-value	HR	95% CI
Age	0.667	0.991	0.949–1.034
Usage of trastuzumab	0.009	4.610	1.453–14.628
Pre-NAC tumor size	0.199	0.987	0.969–1.007
HER2 enriched subgroup*	0.983	0.970	0.397–2.436
Pre-NAC *PIK3CA* mutation	0.633	0.714	0.179–2.843

HR = hazard ratio; CI = confidence interval; NAC = neoadjuvant chemotherapy; HER2 = human epidermal growth factor receptor 2.
*HER2 enriched subgroup versus luminal B subgroup.

Supplementary Table 4. Predictive effect of *PIK3CA* mutation on pCR

Usage of trastuzumab	Mutation status	pCR No. (%)	Non-pCR No. (%)	*p*-value
At least one *PIK3CA* mutation				
Yes (n=68)	Mutant type (n=10)	2 (20.0)	8 (80.0)	0.294
	Wild type (n=58)	25 (43.1)	33 (56.9)	
No (n=32)	Mutant type (n=7)	1 (14.3)	6 (85.7)	>0.999
	Wild type (n=25)	3 (12.0)	22 (78.0)	
Pre-NAC *PIK3CA* mutation				
Yes (n=68)	Mutant type (n=6)	2 (33.3)	4 (66.7)	>0.999
	Wild type (n=62)	25 (40.3)	37 (59.7)	
No (n=32)	Mutant type (n=6)	1 (18.8)	5 (81.2)	0.750
	Wild type (n=26)	3 (11.5)	23 (88.5)	

pCR=pathologic complete response; NAC=neoadjuvant chemotherapy.

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