

Clinical Utility of Targeted Next-Generation Sequencing for Determining Human Epidermal Growth Factor Receptor 2 Status and Optimizing Targeted Therapy in Breast Cancer

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Abstract

Background: The development of targeted next-generation sequencing (NGS) technologies has contributed to precision medicine, as evidenced by the growing interest in evaluating human epidermal growth factor receptor 2 (HER2) expression status to treat unresectable/metastatic HER2-low breast cancer (BC). However, the concordance between erb-b2 receptor tyrosine kinase 2 (*ERBB2*) copy number alteration (CNA) and HER2 immunohistochemistry (IHC) has never been determined. The aim of this study was to evaluate the utility of targeted NGS for determining HER2 status and optimizing targeted therapies for BC.

Methods: *ERBB2* CNAs were examined by targeted NGS in 41 formalin-fixed paraffin-embedded (FFPE) BC tissues. *ERBB2* CNA was compared with HER2 status evaluated by IHC in tissue sections,

which were identical to those subjected to targeted NGS, using the Ventana 4B5 antibody.

Results: The median fold changes (FCs) for *ERBB2* CNAs in tumors with an IHC score of 3+, 2+, 1+, and 0 were 4.81, 1.49, 1.00, and 1.00, respectively. The difference in the FC for *ERBB2* CNA according to HER2 status was statistically significant ($P < 0.001$). An FC greater than 1.0 for *ERBB2* CNA was established as the cutoff value to differentiate between tumors with an IHC score of 3+, 2+, or 1+ and tumors with an IHC score of 0, on the basis of receiver operating characteristic curve analysis. The overall percent agreement, positive percent agreement, negative percent agreement, and Cohen's kappa between *ERBB2* CNA and HER2 status were 68.3%, 57.7%, 86.7%, and 0.39, respectively. The numbers of patients with mutations in *ERBB2*, estrogen receptor 1 (*ESR1*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), serine/threonine kinase 1 (*AKT1*), and phosphatase and tensin homolog (*PTEN*) were 7, 3, 6, 1, and 5, respectively. Targeted NGS detected additional gene mutations and presented treatment options for seven of 22 patients (31.8%) with an FC of *ERBB2* CNA = 1.00.

Conclusions: Targeted NGS has the potential in distinguishing HER2 IHC 3+, 2+, and 1+ tumors from IHC 0 in patients with BC; however, differentiating between HER2 IHC 1+ and 0 remains challenging. Additionally, targeted NGS may aid in the identification of actionable mutations, thereby contributing to the selection of optimal treatment strategies in BC management.

Keywords: Breast cancer; Copy number alteration; Human epidermal growth factor receptor 2; Next-generation sequencing; Precision medicine

Introduction

Breast cancer (BC) is the leading cause of cancer-related death among women worldwide, and the estimated number of deaths in the USA was 42,170 in 2025 [1]. Strategies for BC

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treatment based on biomarkers, including hormone receptors (HRs), human epidermal growth factor receptor 2 (HER2), and programmed cell death ligand 1 (PD-L1), have improved in terms of progression-free survival (PFS) and/or overall survival (OS) in the last decade [2-4]. However, treatment strategies consisting of conventional cytotoxic agents, molecularly targeted therapies, and immune checkpoint inhibitors have become more complex than ever before, especially for metastatic BC (mBC). Multiple biomarkers, including those tested in companion diagnostics, must be evaluated to select the optimal treatment from among multiple complex treatment strategies. More accurate and simplified biomarker assessments could improve the quality of BC care.

HER2-positive BC [5], defined as BC with erb-b2 receptor tyrosine kinase 2 (*ERBB2*) amplification and/or protein overexpression, is identified in 15-25% of all invasive BCs [5-7] and is diagnosed by immunohistochemistry (IHC) and/or fluorescence *in situ* hybridization (FISH). The evaluation of HER2 status is necessary for selecting anti-HER2 treatments, including trastuzumab and pertuzumab [8-11]. Recently, the DESTINY-Breast04 phase III trial demonstrated the remarkable activity of trastuzumab deruxtecan (T-DXd), an antibody-drug conjugate (ADC) that targets HER2, to substantially improve PFS and OS compared with traditional chemotherapy among patients with HER2-low expression, which is defined as IHC 2+/FISH negativity or an IHC score of 1+ [12]. The indication for T-DXd was expanded for not only HER2-positive BC but also triple-negative and HR-positive BCs with HER2-low expression, as determined by companion diagnostic tests using IHC with a proprietary anti-HER2 antibody named as the VENTANA ultraView pathway HER2 (4B5).

In addition to HER2 status, several important actionable mutations in the additional genes including *ERBB2*, estrogen receptor 1 (*ESR1*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), serine/threonine kinase 1 (*AKT1*), and phosphatase and tensin homolog (*PTEN*) have become biomarkers for selecting candidates with mBC for novel targeted therapies. Recently, *ERBB2*-mutant BC was suggested to be sensitive to neratinib [13], a potent irreversible pan-*ERBB2* inhibitor. Although neratinib is not currently indicated for BC treatment in Japan, some studies, including the phase II SUMMIT basket trial, are ongoing [13]. In 2023, elacestrant was approved for the treatment of patients with *ESR1*-mutant HR-positive mBC [14]. Capivasertib (an *AKT* inhibitor) was also approved for patients who present with mBC with *PIK3CA*, *AKT1*, or *PTEN* mutations [15]. Alpelisib (an PI3K α -specific inhibitor) was approved for patients with *PIK3CA*-mutated, HR-positive, HER2-negative advanced BC who had received endocrine therapy previously [16]. Thus, drug therapies targeting these oncogenic pathways have become treatment options for early mBC [17]. In addition to HER2 and HR status, the evaluation of oncogenic mutations in these genes is essential for developing treatment strategies for early mBC.

The development of targeted next-generation sequencing (NGS) technologies could provide an opportunity to implement genomics in clinical practice and to reveal an overall picture of genetic abnormalities such as single nucleotide variations, copy number alterations (CNAs), chromosomal

abnormalities, and fusion genes in a wide range of cancers [18]. *ERBB2* is the key gene in BC, and thus confirming the agreement between *ERBB2* CNA evaluated by targeted NGS and HER2 status determined by IHC is crucial in the use of targeted NGS to suggest the standard of care in mBC. Targeted NGS can also analyze multiple genes, which could reveal simultaneous treatment strategies for targeted therapy [18]. Due to complex treatment strategies dictated by an increasing number of biomarkers, targeted NGS has the potential to play a central role in selecting drug therapies for patients with mBC.

In this study, we investigated the concordance between *ERBB2* CNA and HER2 status, including HER2-low expression, in BC via genomic profiles constructed using targeted NGS. In addition, we delineated the landscape of additional gene mutations in *ERBB2*, *ESR1*, *PIK3CA*, *AKT1*, and *PTEN* in relation to HER2 status to select optimal treatment strategies consisting of T-DXd and novel targeted therapies, including neratinib, elacestrant, capivasertib, and alpelisib, for mBC.

Materials and Methods

Patient tissue samples

We conducted a single-center retrospective study. This study was approved by the Institutional Review Board of Niigata University (G2020-0038, 2018-0137).

Among the 589 patients with BC who underwent breast surgery at Niigata University Medical and Dental Hospital between 2016 and 2020, we enrolled 41 patients who had not received any other treatment, including chemotherapy or hormone therapy. Informed consent was obtained from all participants. All samples were derived from surgical specimens from patients. Among the cohort, 11 patients were included in our previous targeted NGS study of BC [19]. One patient diagnosed with stage IV BC underwent surgical resection for local control. Tumor stage was evaluated according to the American Joint Committee on Cancer (eighth edition) [20] and the general rules for clinical and pathological recording of BC (18th edition) [21].

IHC

Archival tissue from formalin-fixed paraffin-embedded (FFPE) tumors obtained from surgical specimens was used for analysis. Four-micrometer-thick slides were prepared, and the tumor tissues were stained with hematoxylin and eosin. To ensure the presence of > 50% tumor content in each section, the tumor content on hematoxylin and eosin-stained slides for each study sample was evaluated by an independent pathologist. The levels of estrogen receptor (ER) (1D5, Nichirei, Tokyo, Japan), progesterone receptor (PgR) (A9621A, Nichirei, Tokyo, Japan), and HER2 (clone 4B5, Ventana Roche Diagnostics, Tokyo, Japan) were determined by IHC. IHC staining was assessed according to the American Society of Clinical Oncology (ASCO) and College of American Pathologists

(CAP) guidelines [22]. HER2 scores were given according to the following criteria: 3+ for moderate to strong complete or basolateral membrane staining in 10% or more invasive tumor cells; 2+ for weak to moderate complete or basolateral membrane staining in 10% or more invasive tumor cells; 1+ for weak membrane staining in 10% or more invasive tumor cells; and 0 for no staining overall or membrane staining in fewer than 10% invasive tumor cells. *ERBB2* amplification was evaluated by FISH in patients with an IHC score of 2+. FISH was performed using a PathVysion HER2 DNA probe kit (Abbott Molecular, Inc., Illinois, USA), and gene amplification was evaluated according to the fluorescence signal ratio of *ERBB2* to chromosome enumeration probe 17 (CEP17). FISH positivity with gene amplification was defined as an *ERBB2*-to-CEP17 ratio of at least 2.0. HER2 positivity was defined as an IHC score of 3+ or an IHC score of 2+ in addition to FISH positivity, while HER2-low was defined as IHC 2+/FISH negativity or an IHC score of 1+, and HER2 negativity was defined as an IHC score of 0 [23].

NGS

Genomic profiles of BC tissues were generated by targeted NGS (CANCERPLEX, KEW Inc., Cambridge, MA, USA) using 435 cancer-associated genes, as previously described [24]. First, genomic DNA was extracted from FFPE BC tissues. Next, DNA fragment libraries were enriched for the coding regions and selected introns of the 435 genes and were then subjected to the Illumina MiSeq and NextSeq platforms (Illumina, San Diego, CA, USA) for genomic sequencing with an average depth of $\times 500$. We extracted information on *ERBB2* CNAs and additional gene mutations defined as single nucleotide variants (SNVs) and short insertions and deletions (indels) in genes related to BC targeted therapies including *ERBB2*, *ESR1*, *PIK3CA*, *AKT1*, and *PTEN*. The pathogenicity of the extracted mutations was identified via the ClinVar [25] and OncoKB [26] databases. Targeted therapies were recommended for cases harboring additional gene mutations classified as OncoKB therapeutic evidence level 1 (FDA-approved drug) [27].

Statistical analysis

The Kruskal-Wallis test was performed to assess the significance of differences in continuous data among the multiple groups. The Games-Howell test was applied to the *post hoc* tests for multiple comparisons. The cutoff value of *ERBB2* CNA was determined via receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) indicated accuracy according to the following criteria: AUC > 0.9, high accuracy; $0.7 < \text{AUC} \leq 0.9$, moderate accuracy; and $0.5 < \text{AUC} \leq 0.7$, low accuracy [28]. The concordance between *ERBB2* CNA and HER2 IHC status was evaluated using overall percent agreement (OPA), positive percent agreement (PPA), negative percent agreement (NPA), and Cohen's kappa with 95% confidence intervals (CIs). The relative strength of

Table 1. Patient Background

Variables	Number of patients (%)
Age	
Median (range)	57 (28 - 84)
T classification*	
T1	15 (37)
T2	21 (51)
T3	4 (10)
T4	1 (2)
N classification*	
N0	22 (54)
N1	14 (34)
N2	3 (7)
N3	2 (5)
M classification*	
M0	40 (98)
M1	1 (2)
Estrogen receptor status	
Negative	22 (54)
Positive	19 (46)
Progesterone receptor	
Negative	26 (63)
Positive	15 (37)
Ki67 labeling index	
< 25%	19 (46)
$\geq 25\%$	22 (53)

*Union for International Cancer Control (UICC) TNM classification, eighth edition.

agreement was assessed according to the following criteria: kappa > 0.8, almost perfect; $0.6 < \text{kappa} \leq 0.8$, substantial; $0.4 < \text{kappa} \leq 0.6$, moderate; $0.2 < \text{kappa} \leq 0.4$, fair; $0 \leq \text{kappa} \leq 0.2$, slight; and kappa < 0, poor [29]. Statistical evaluations were performed using the IBM SPSS version 28.0 (SPSS Japan, Tokyo, Japan) and the R programming language and environment (version 4.3.1) [30]. All tests were two-sided, and a P value less than 0.05 indicated statistical significance.

Results

Histologic and molecular characteristics of the 41 patients

In all, 41 patients were enrolled in this study. The patient backgrounds at diagnosis are summarized in Table 1, and the histologic and molecular characteristics of the tumors in each patient are detailed in Supplementary Material 1 (wjon.elmerpub.com). Only 15 (37%) patients had T1 classification and only 22 (54%) patients did not present with lymph node me-

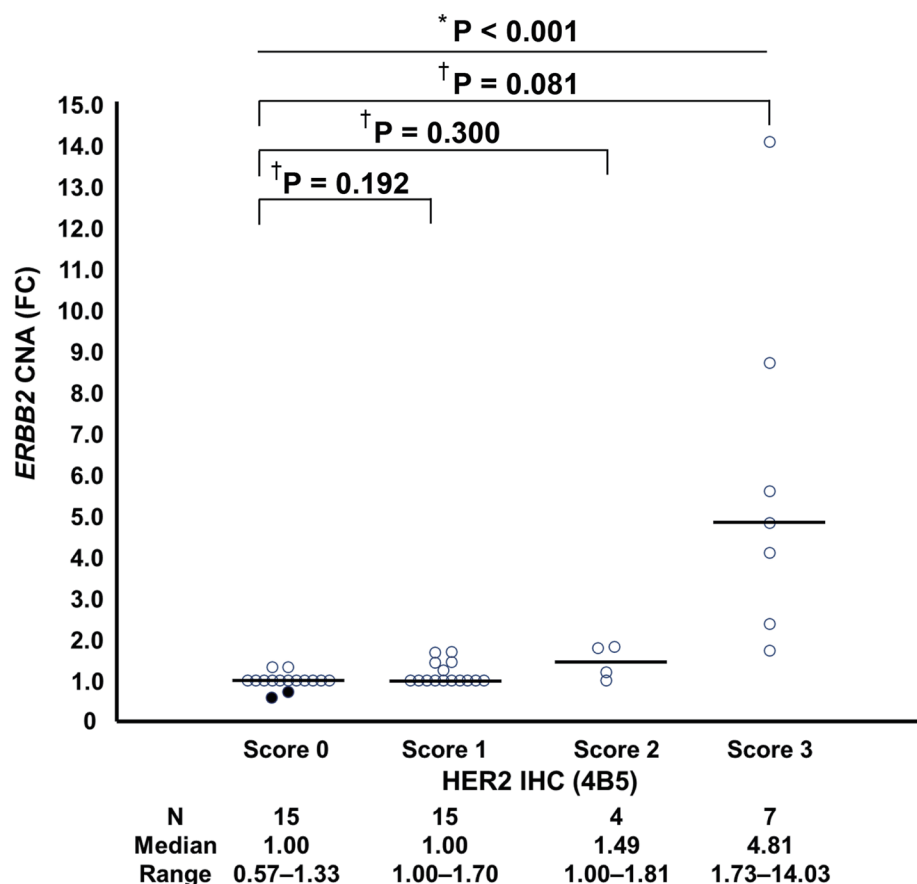


Figure 1. Distribution of *ERBB2* CNAs according to HER2 IHC (4B5) expression status. Comparison of *ERBB2* CNAs (FC) among patients with HER2 IHC (4B5) scores of 3+, 2+, 1+, and 0. The Kruskal-Wallis test was performed to assess the significance of differences in continuous data among the multiple groups (*). The Games-Howell test was applied to the *post hoc* tests for multiple comparisons (†). All tests were two-tailed, and $P < 0.05$ was considered significant. CNA: copy number alteration; *ERBB2*: erb-b2 receptor tyrosine kinase 2; FC: fold change; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry.

tastasis (Table 1). We evaluated HER2 status by IHC and FISH in tumor tissue sections derived from FFPE blocks that were identical to those subjected to targeted NGS. HER2 positivity (IHC 3+ or IHC 2+/FISH positivity) was detected in the tumors of nine patients (22.0%), including seven (17.1%) with IHC 3+ and two (4.9%) with IHC 2+/FISH-positive tumors. HER2-low expression (IHC 2+/FISH negativity, IHC 1+) was detected in the tumors of 17 patients (41.5%), including two (4.9%) IHC 2+/FISH-negative and 15 (36.6%) IHC 1+ tumors. HER2 negativity (IHC 0) was detected in the tumors of the remaining 15 patients (36.6%) (Supplementary Material 1, wjon.elmerpub.com). All patients who were HR-positive were ER-positive. We compared the *ERBB2* CNA according to HER2 IHC (Fig. 1). The median fold changes (FCs) (range) for *ERBB2* CNAs in HER2 IHC 3+, 2+, 1+, and IHC 0 were 4.81 (range: 1.73 - 14.03), 1.49 (range: 1.00 - 1.81), 1.00 (range: 1.00 - 1.70), and 1.00 (range: 0.57 - 1.33), respectively (Fig. 1). The difference in the FC for *ERBB2* CNA according to HER2 status was statistically significant ($P < 0.001$). However, there were no significant differences between the groups of HER2 3+ and HER2 0 ($P = 0.081$), HER2 IHC 2+ and HER2

IHC 0 ($P = 0.300$), and HER2 IHC 1+ and HER2 IHC 0 ($P = 0.192$) (Fig. 1).

Concordance between *ERBB2* CNA evaluated by targeted NGS and HER2 status determined by IHC

To clarify the concordance between *ERBB2* CNA evaluated by targeted NGS and HER2 IHC, we determined the cutoff value of *ERBB2* CNA using receiver operating characteristic (ROC) curve analysis (Table 2, Supplementary Materials 2 and 3, wjon.elmerpub.com). ROC curve analysis revealed that FC of *ERBB2* CNA over 1.00 (AUC = 0.78) was a fair discrimination point for distinguishing IHC scores of 3+, 2+, and 1+ from IHC 0. Fifteen of the 26 patients with an IHC score of 3+, 2+, or 1+ presented with an FC of *ERBB2* CNA > 1.00, and the PPA was calculated to be 57.7% (Table 2). Among the 15 patients with an IHC score of 0, 13 patients presented an FC of *ERBB2* CNA ≤ 1.00, and the NPA was calculated to be 86.7% (Table 2). The OPA was 68.3%, and fair agreement was determined with a Cohen's kappa of 0.39 (95% CI:

Table 2. Concordance Between *ERBB2* CNA Evaluated by Targeted Next-Generation Sequencing and HER2 IHC (4B5)

		Number of patients		
		HER2 IHC (4B5)		Total
		Score 1 - 3	Score 0	
<i>ERBB2</i> CNA evaluated by targeted next-generation sequencing	> 1.00-fold change	15	2	17
	≤ 1.00-fold change	11	13	24
	Total	26	15	41
Positive percent agreement		57.7%		
Negative percent agreement		86.7%		
Overall percent agreement		68.3%		
Cohen's kappa (95% CI)		0.39 (0.14 - 0.64)		

CI: confidence interval; CNA: copy number alteration; *ERBB2*: erb-b2 receptor tyrosine kinase 2; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry.

0.14 - 0.64) (Table 2). We show representative examples of IHC based on *ERBB2* CNA (Supplementary Material 4, wjon.elmerpub.com). No patients had an FC of *ERBB2* CNA ≤ 1.00 for IHC 1+, an FC of CNA ≤ 1.00 for IHC 2+, an FC of CNA ≤ 1.00 for IHC 3+, or an FC of *ERBB2* CNA = 1.00 for IHC 3+. As representative examples of concordance between the *ERBB2* CNA, as evaluated by targeted NGS, and IHC, we identified the following patients: #1 (FC of *ERBB2* CNA < 1.00, IHC 0), #4 (FC of *ERBB2* CNA = 1.00, IHC 0), #26 (FC of *ERBB2* CNA > 1.00, IHC 1+), #25 (FC of *ERBB2* CNA > 1.00, IHC 2+), and #41 (FC of *ERBB2* CNA > 1.00, IHC 2+). On the contrary, we show patient #14 (FC of *ERBB2* CNA = 1.00, IHC 1+), patient #24 (FC of *ERBB2* CNA = 1.00, IHC 2+), and patient #27 (FC of *ERBB2* CNA > 1.00, IHC 0) as representative examples of discordance between *ERBB2* CNA and HER2 IHC.

Landscape of additional gene mutations and proposed targeted therapies in relation to *ERBB2* CNA

T-DXd and novel targeted therapies proposed for the enrolled patients according to *ERBB2* CNA and additional gene mutations with ER status are shown in Figure 2. Lollipop plots depicting amino acid changes are presented in Figure 3, and a comprehensive summary of additional gene mutations along with corresponding treatment recommendations based on OncoKB therapeutic evidence levels is provided in Supplementary Material 5 (wjon.elmerpub.com). Additional gene mutations were detected in three of 17 patients (17.6%) with an FC of *ERBB2* CNA > 1.00, in 15 of 22 patients (68.2%) with an FC of *ERBB2* CNA = 1.00, and in neither of the two patients (0%) with an FC of *ERBB2* CNA < 1.00.

Among the 17 patients with an FC of *ERBB2* CNA > 1.00, one patient (#39) with a *PTEN* mutation and one patient (#35) with an *ERBB2* mutation were not eligible for targeted therapy recommendations (Supplementary Material 5, wjon.elmerpub.com). Additionally, one patient (#28) with a *PIK3CA* E542K mutation was not considered for targeted therapy due to ER negativity. Overall, 15 patients (88.2%) with an FC of *ERBB2*

CNA > 1.00 were eligible for T-DXd treatment.

Among the 22 patients with an FC of *ERBB2* CNA = 1.00, 15 patients (68.2%) harbored additional gene mutations and seven patients (31.8%) were recommended targeted therapies. Four patients (#17, #18, #19, and #20) with *PIK3CA* mutations were considered potential candidates for capivasertib and alpelisib. Although one patient (#18) also harbored an *ESR1* mutation, elacestrant was not recommended; instead, capivasertib or alpelisib was proposed as a more suitable option. Two patients (#7 and #8) presented with *ESR1* mutations along with either *AKT1* or *PTEN* mutations. Of these, patient #7 was considered a candidate for capivasertib rather than elacestrant, whereas patient #8 was deemed a candidate for elacestrant over capivasertib according to OncoKB therapeutic evidence levels (Supplementary Material 5, wjon.elmerpub.com). One patient (#6) with a *PTEN* mutation was also considered a candidate for capivasertib. Conversely, no targeted therapies were recommended for one patient (#15) with *ERBB2* and *PIK3CA* mutations and HER2-low, five patients (#9, #10, #11, #12, and #13) with *ERBB2* mutations, and two patients (#5 and #23) with *PTEN* mutations (Supplementary Material 5, wjon.elmerpub.com). Additionally, two patients with an FC of *ERBB2* CNA < 1.00 were not recommended any targeted therapy.

Discussion

Targeted NGS technologies, which can identify additional therapeutic targets in patients with recurrence or metastasis, have exploded on a worldwide scale. NGS libraries are prepared from only 100 ng of extracted tumor DNA and cover molecular target genes of BC, including *ERBB2*, *ESR1*, *PIK3CA*, *AKT1*, and *PTEN* [31]; however, HER2-low status is defined not by targeted NGS but by HER2 IHC. Although several reports have shown the genomic characterization of HER2-low BC via NGS [17, 32], the degree of concordance between *ERBB2* CNA and HER2 status determined by IHC has never been examined. This is the first study to evaluate the degree of concordance between *ERBB2* CNA determined by targeted NGS and HER2 status determined by IHC.

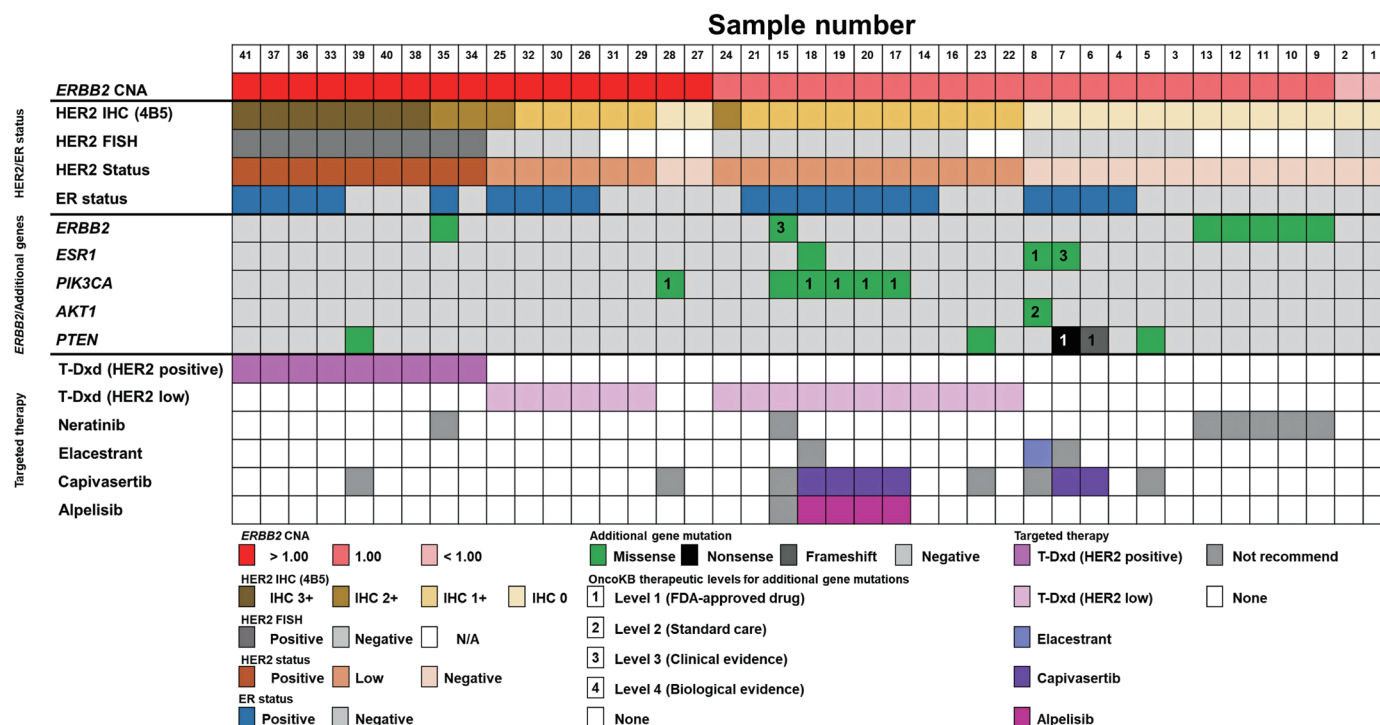


Figure 2. Landscape of additional gene mutations and proposed targeted therapies by targeted NGS, HER2, and ER status. *ERBB2* CNA, HER2 status determined by HER2 IHC (4B5) and FISH, ER status, and additional gene mutations in *ERBB2*, *ESR1*, *PIK3CA*, *AKT1*, and *PTEN* along with the recommended targeted therapies based on OncoKB therapeutic evidence levels are depicted in color tiles. *AKT1*: serine/threonine kinase 1; CNA: copy number alteration; ER: estrogen receptor; *ERBB2*: erb-b2 receptor tyrosine kinase 2; FISH: fluorescence *in situ* hybridization; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry; NGS: next-generation sequencing; *PTEN*: phosphatase and tensin homolog.

We demonstrated the distribution of *ERBB2* CNAs determined by targeted NGS for different HER2 IHC scores (Fig. 1). Similar to some previous reports [32, 33], the median FC of *ERBB2* CNAs tends to be higher in patients with higher HER2 IHC scores. In this study, an FC cutoff value of *ERBB2* CNA > 1.00 demonstrated potential to distinguish HER2 IHC 3+, 2+, and 1+ tumors from IHC 0 in patients with BC; however, differentiation between HER2 IHC 1+ and HER2 0 remains challenging. As shown in Figure 1, two patients (#1 and #2) whose *ERBB2* gene was deleted according to targeted NGS were determined to have an IHC score of 0. Considering that these two patients presented an FC of *ERBB2* CNAs greater than 0.50 (Supplementary Material 1, wjon.elmerpub.com), the type of gene deletion might not be a homozygous deletion. Given that the frequency of *ERBB2* single-copy deletions among HER2-negative BC patients was greater than that among HER2-low BC patients [31], our results also support this finding.

In this study, we showed that an FC of *ERBB2* CNA ≤ 1.00 indicated an IHC score of 0, with a NPA of 86.7% (Table 2). This result indicated that patients with an FC of *ERBB2* CNA > 1.00 might have an IHC score of 3+, 2+, or 1+, which would increase their chances of receiving T-DXd treatment. In contrast to the NPA, an FC of *ERBB2* CNA > 1.00 indicated HER2 IHC scores of 3+, 2+, and 1+ with a PPA of 57.7%, which was lower than expected. Several HER2 IHC 1+ cases, potential

candidates for T-DXd treatment, exhibited FC values of ≤ 1.00, potentially missing the opportunity for treatment. This raises the question of whether an FC cutoff value of *ERBB2* CNA > 1.00 is truly optimal for distinguishing HER2 IHC 0 from higher expression levels (IHC 1+ to 3+). In contrast, we were able to effectively differentiate HER2 IHC 3+ or HER2 IHC 2+/FISH-positive tumors from others (Supplementary Material 2, wjon.elmerpub.com), and to distinguish the group of HER2 IHC 3+ or IHC 2+ from the group of HER2 IHC 1+ or IHC 0 using an FC cutoff of > 1.70, which demonstrated near-perfect concordance (Supplementary Material 3, wjon.elmerpub.com). Taken together, *ERBB2* CNAs examined by targeted NGS may have limited utility in precisely identifying HER2-low status. Furthermore, confirmation of HER2 IHC expression may be necessary for tumors with *ERBB2* CNA ≤ 1.00. However, IHC assessment is subject to variability due to staining conditions and inter-observer differences among pathologists, and thus cannot be considered an absolute standard. The optimal FC cutoff for *ERBB2* CNA should be determined through further studies incorporating therapeutic outcomes, particularly the efficacy of T-DXd.

This inconsistent result may be explained by HER2 heterogeneity, which is defined as the presence of 5-50% cells with amplified *ERBB2* or the presence of an area of the tumor that is HER2-negative [34], and increases the difficulty in evaluating HER2 expression status. Wu et al reported that

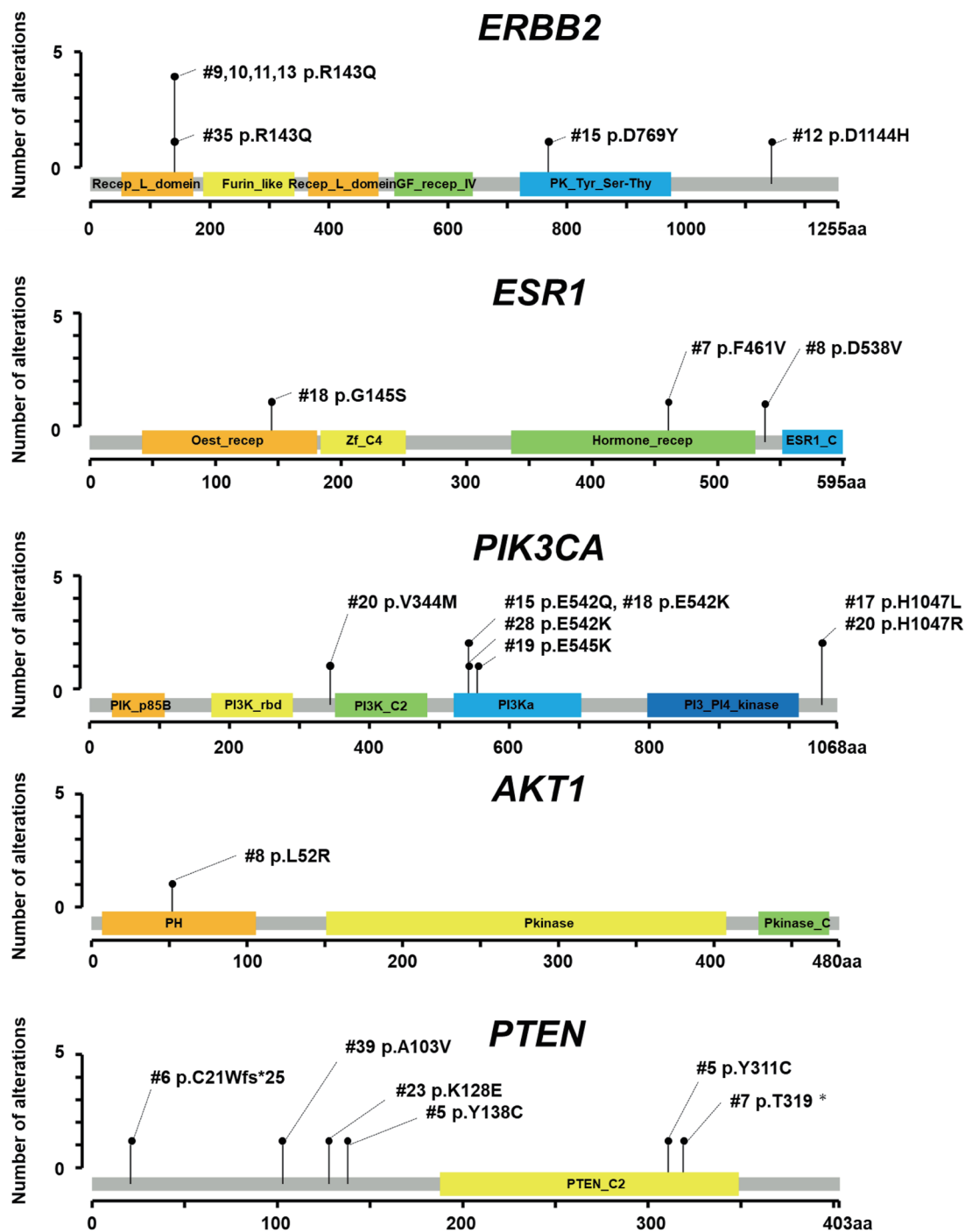


Figure 3. Mutations in *ERBB2*, *ESR1*, *PIK3CA*, *AKT1*, and *PTEN* detected in 41 patients. Lollipop plots, which show amino acid changes, are illustrated for *ERBB2*, *ESR1*, *PIK3CA*, *AKT1*, and *PTEN*. *AKT1*: serine/threonine kinase 1; *ERBB2*: erb-b2 receptor tyrosine kinase 2; *ESR1*: estrogen receptor 1; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry; *PIK3CA*: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *PTEN*: phosphatase and tensin homolog.

HER2 IHC heterogeneity was more frequent in patients with HER2-low BC [35]. Moreover, since targeted NGS was performed on nucleic acids extracted from tumor tissues, it might be inappropriate to use targeted NGS to assess the heterogeneity of HER2 expression rather than HER2 IHC. The heterogeneity

of HER2 expression might lead to discordance between *ERBB2* CNA status and HER2 IHC scores. Taken together, although HER2 heterogeneity remains a concern, targeted NGS could indicate T-DXd treatment for patients with an FC of *ERBB2* CNA > 1.00.

Although targeted NGS has raised concerns in the field of precision medicine, it has also presented opportunities to select optimal treatments for BC patients (Fig. 2). Interestingly, despite having *ERBB2* CNA > 1.00, some patients received no recommendation for targeted therapy, even though T-DXd typically plays a central role in the treatment of mBC. In contrast, among patients with *ERBB2* CNA = 1.00, seven of 22 (31.8%) were recommended targeted therapies. Notably, one patient (#8) harboring the *ESR1* D538V mutation was identified as potentially responsive to elacestrant. *ESR1* is one of the major drivers of endocrine resistance [36]. Elacestrant, the first oral selective estrogen receptor degrader (SERD), was approved for *ESR1*-mutant mBC in the USA. The phase III EMERALD study demonstrated that elacestrant was associated with longer median PFS than estrogen treatments in all patients (2.8 vs. 1.9 months for elacestrant and estrogen treatments, respectively), with a 45% relative risk reduction in disease progression or death in patients with the *ESR1* mutation detected by Guardant 360 companion diagnostics (3.8 vs. 1.9 months, respectively) [14]. We utilized tumor biopsy rather than liquid biopsy for the detection of *ESR1* mutation, in contrast to the approach used in the EMERALD study. Since prevalence of *ESR1* mutation of liquid biopsy was higher than that of tumor biopsy [37], it is possible that the current study includes more cases with *ESR1* mutations detected by liquid biopsy. Elacestrant therapy is a new effective option and has led to advancements in precision medicine for ER-positive and HER2-negative mBC, including HER2-low mBC.

Six patients (#6, #7, #17, #18, #19, and #20) harboring *PIK3CA/AKT1/PTEN* mutations were identified as potential candidates for capivasertib (Fig. 2). Overactivation of the *PI3K-AKT-PTEN* signaling pathway occurs in approximately half of HR-positive and HER2-negative BCs, including HER2-low BCs, and leads to worse outcomes [38, 39]. The CAPItello-291 study demonstrated that capivasertib plus fulvestrant was associated with a longer median PFS than fulvestrant alone (7.3 vs. 3.1 months) in mBC patients who were HR-positive, which is defined as ER positivity with or without PgR and HER2 negativity. The *PIK3CA/AKT1/PTEN* mutation group was defined as a molecular subgroup of tumors harboring at least one *PIK3CA/AKT1/PTEN* qualifying mutation [15]. In the CAPItello-291 study, at least one *PIK3CA/AKT1/PTEN* mutation was detected in 289/708 patients (40.8%). Mutations in *PIK3CA* only, *AKT1* only, *PTEN* only, *PIK3CA* and *AKT1*, and *PIK3CA* and *PTEN* were detected in 202/708 (28.5%), 33/708 (4.7%), 37/708 (5.2%), 4/708 (0.6%), and 13/708 (1.8%) patients, respectively [15]. In this study, at least one *PIK3CA/AKT1/PTEN* mutation was detected in 8/15 patients (52.3%), while double gene mutations were not detected. Given that *PIK3CA/AKT1/PTEN* mutations were detected in 1,038/1,967 (52.8%) Japanese patients by FoundationOne [40], our results are expected. Four patients (#17, #18, #19, and #20) harboring *PIK3CA* mutations were identified as potential candidates for alpelisib therapy. The SOLAR-1 study demonstrated that alpelisib in combination with fulvestrant significantly prolonged median PFS than compared to fulvestrant alone (11.0 vs. 5.7 months) [16].

Thus, targeted NGS could be used to identify multiple tar-

gets and to develop strategies for cancer treatment. Moreover, since both targeted NGS and IHC are needed to select a treatment strategy for HR-positive mBC at present, further studies are needed to detect HER2-low BC by targeted NGS to avoid additional work needed to confirm each biomarker for drug therapy.

We acknowledge that the current study has limitations. First, we recognized cohort bias. Since we collected tissue samples without adversely affecting the diagnosis, we did not collect tissue samples from tumors < 10 mm in diameter or from cases of ductal carcinoma in situ. We also recognized the small cohort limited to Japanese patients. Second, although HER2 IHC heterogeneity was more common in HER2-low BC patients, we did not evaluate heterogeneity in this study or in the real world. Third, the targeted NGS utilized in this study lacks approval for clinical utilization worldwide. Nonetheless, we believe that our findings regarding the use of targeted NGS for BC are informative for cancer treatment.

In conclusion, targeted NGS has the potential in distinguishing HER2 IHC 3+, 2+, and 1+ tumors from IHC 0 in patients with BC; however, differentiating between HER2 IHC 1+ and 0 remains challenging. Additionally, targeted NGS may aid in the identification of actionable mutations, thereby contributing to the selection of optimal treatment strategies in BC management.

Supplementary Material

Suppl 1. Histologic and molecular characteristics of the tumors.

Suppl 2. Concordance between *ERBB2* CNA evaluated by targeted next-generation sequencing and HER2 IHC (4B5).

Suppl 3. Concordance between *ERBB2* CNA evaluated by targeted next-generation sequencing and HER2 IHC (4B5).

Suppl 4. Immunohistochemistry for HER2 (4B5). Representative images of HER2 IHC (4B5, × 400) in patients with *ERBB2* CNAs with < 1, 1, and > 1 FC are shown. *ERBB2*, erb-b2 receptor tyrosine kinase 2; CNA, copy number alteration; FC, fold change; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

Suppl 5. Additional gene mutations and treatment recommendation referred to OncoKB therapeutic evidence.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper and that they approved the final version of the manuscript being submitted.

Informed Consent

Written informed consent for publication of individual patient data was obtained from the subjects for case presentation.

Author contributions

Y. Hara, K. Moro, H. Ichikawa, J. Tsuchida, and T. Wakai contributed to the conception and design of the study. K. Moro, H. Uchida, K. Naruse, H. Otake, Y. Obata, J. Tsuchida, and H. Ichikawa contributed to the collection and assembly of the data. M. Sugai and H. Umezumi supervised the histopathological investigation. K. Moro, J. Tsuchida, H. Ichikawa, and S. Okuda performed the statistical and bioinformatic analyses. Y. Hara, K. Moro, J. Tsuchida, H. Ichikawa, Y. Shimada, J. Sakata, Y. Koyama, K. Takabe, and T. Wakai contributed to the drafting of the article. T. Wakai gave final approval for submission of the article.

Data Availability

The authors declare that the data supporting the findings of this study are available within the article. The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Abbreviations

AKT1: serine/threonine kinase 1; AUC: area under the ROC curve; BC: breast cancer; CDx: companion diagnostics; CNA: copy number alteration; EGFR: epidermal growth factor receptor; ER: estrogen receptor; *ERBB2*: erb-b2 receptor tyrosine kinase 2; *ESR1*: estrogen receptor 1; FC: fold change; FISH: fluorescence *in situ* hybridization; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry; NGS: next-generation sequencing; NPA: negative percent agreement; OPA: overall percent agreement; PD-L1: programmed cell death ligand 1; *PIK3CA*: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PPA: positive percent agreement; *PTEN*: phosphatase and tensin homolog; ROC: receiver operating characteristic; SERD: selective estrogen receptor degrader

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