Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Genetic Alterations in HER2-Positive and Equivocal Breast Cancer by Immunohistochemistry

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Purpose: We aimed to identify genetic alterations in groups with different HER2 immunohistochemical (IHC) scores.

Patients and Methods: A total of 120 patients with HER2-positive breast cancers, including 89 cases with IHC 3+ tumors and 31 cases with IHC 2+ and positive for in situ hybridization (ISH) were enrolled. Molecular profiles were determined using Thermo Fisher TMO comprehensive assay on surgically removed tissues. All called variants were compared between IHC3+ and IHC2+/ISH+ groups by Fisher exact test.

Results: There was a significantly higher sample frequency 94.4% (84/89) of *ERBB2* amplification in IHC3+ group than that in IHC2+/ISH+ group 45.2% (14/31). By contrast, there was a significantly lower sample frequency of *MYC*_AMP_CNA 10.1% (9/89) and *CCND3*_AMP_CNA 0% (0/89) in IHC3+ group than those in IHC2+/ISH+ group with sample frequency 25.8% (8/31), and 9.7% (3/31), respectively.

Conclusion: We conclude that HER2 IHC3+ tumors have higher frequency of *ERBB2*_ AMP_CNA and lower frequency of *CCND3*_ AMP_CNA and *MYC*_AMP_CNA than IHC2+/ISH+ tumors. These results provide therapeutic strategies in treatment of HER2-positive breast cancer.

Keywords: genetic alteration, CNA, erbb2, breast cancer, immunohistochemistry

Introduction

Breast cancer is the most common invasive female cancer worldwide.[1,2] Rapid advances made using genomic approaches, molecular analysis using immunohistochemistry, measurement of proliferative capacity, and gene expression profiles have allowed the further categorization of breast cancers into four types; these are the luminal A, luminal B, HER2 and basal-like subtypes.[3–6] Biological subtyping not only helps to provides feasible treatment strategies that correlate well with clinical outcome, but also helps to predict whether the primary tumor has a preferential distant metastasis site.[7]

Precision medicine is a concept to take individual variability into account in disease prevention and treatment. Thanks to the rapid advance of human genome sequencing and the application of large-scale biologic databases to characterize patients and guide clinical practice, an effective therapy accurately determined for each cancer patient, increasing their survival rates, has become possible. Among many genetic alterations, some of them defined as actionable mutations have been identified in patients with melanoma[8] and non-small cell lung cancer[9] responsive to a specific target therapy with demonstrable clinical benefit.[10]

According to the HER2 testing guideline from the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP), HER2-positive breast cancer is defined when there is HER2 overexpression on an

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immunohistochemistry (IHC) assay (score 3+) or gene amplification on an in situ hybridization (ISH) assay. In the case of a 2+ score on IHC, reflex ISH testing is required to define the HER2 status.[11] Patients with IHC 3+ or IHC 2+/ISH+ tumors both are allowed for anti-HER2 therapy, but the efficacy of treatment may differ according the HER2 IHC scores.

Tumor Mutational Burden (TMB) is defined as total number of mutations in a genomic target region. Recent study has demonstrated that further analysis on tumor mutation burden in different subtypes in breast cancer is necessary to provide therapeutic prediction.[12] We previously demonstrated that IHC 3+ tumors presented a trend of higher pathological complete response (pCR) rate and better outcome in HER2-positive breast cancer patients who receive neoadjuvant systemic therapy (NST).[13] However, information concerning genetic alterations between different HER2 IHC scores remains limited. This study was aimed to identify genetic variants in groups with different HER2 IHC scores.

Subjects and Methods

Study Population

Under approval of the Institutional Review Board of Taipei Veterans General Hospital (2021–07-003CC), a prospective collected database was established and comprehensive genetic variants in Taiwan breast cancers by target sequencing was profiled.[14] In brief, all female (≥ 20 y/o) patients with pathology-proved breast cancer in Taipei Veterans General Hospital were included. Subjects who had primary cancer other than breast cancer within 5 years prior to screening were excluded. The interpretation of tumor biopsy, immunohistochemistry (IHC), in situ hybridization (ISH) testing was performed by the experienced pathologist (Hsu, CY). The positivity of IHC stains for oestrogen receptor (ER) (clone 6F11; Leica Biosystems, Newcastle, UK), progesterone receptor (PR) (clone 16; Leica Biosystems, Newcastle, UK) and HER2 (A0485; Dako, Glostrup, Denmark), were defined as ≥1% of tumor nuclear staining. HER2 IHC (score 3+) was defined by intense membrane staining in >10% of tumor cells, while fluorescence ISH testing (PathVysion *HER2* DNA Probe Kit; Abbott Laboratories, Des Plaines, IL, USA) was performed for patients with equivocal HER2 IHC (score 2+) tumors. Cases with *HER2* copy numbers of ≥6 signals per cell or a *HER2* ISH ratio (*HER2* gene signals to chromosome 17 centromere signals) of ≥2 were defined as ISH-positive by 2013 ASCO/CAP guidelines.[15] For subjects who received neoadjuvant therapy, core needle biopsy was feasible for pathological diagnosis. The clinical and pathological information including age, tumor size, node status, staging, grade, estrogen receptor status, progesterone receptors status and the percentage of Ki-67 were reviewed from the above-mentioned database.

From Nov. 2018 to Nov. 2021, a total of 120 consecutive patients with HER2 (+) breast cancers, including 89 cases (74.2%) with IHC 3+ tumors and 31 cases (25.8%) with IHC 2+/ISH+ tumors. Molecular profiles were determined using Oncomine™ comprehensive assay (Thermo Fisher TMO comprehensive assay Scientific, Waltham, MA) feasible to detect thousands of variants across 161 cancer-related genes.[16] In summary, the extraction of DNA from tumor frozen tissues or formalin-fixed paraffin-embedded (FFEP) tissues for next generation target sequencing were prepared as described previously.[17] The DNA quality check followed the manual of the TMO assay requirement. Approximately seven unstained sections of tumors FPE tissue per subject were retrieved with one unstained section for H&E staining, and six unstained sections for the TMO comprehensive assay. This was followed by data processing, alignment and variant calling using Torrent Suite™ Software and the Torrent Variant Caller plug-in.[14] The workflow "Oncomine Comprehensive v3 - w3.2 - DNA and Fusions-Single Sample" version 5.10 used hg19 as reference genome. The gene list used in this platform was provided as Supplementary Table 1. Called variants and types of mutation included single nucleotide alteration (SNA) such as synonymous, missense, insertion/deletion (Indel), or frameshift, structure alteration (SA) such as fusion, truncation, and copy number alteration (CNA) in each enrolled subject. All called variants were annotated by 1000 genome projects, COSMIC, CLINVA, SHIF, POLYPHEN2 using an integrated Vari-ED database.[18] Those variants with poor quality, minor allele frequency (MAF) > 0.05 and variants of unknown significance (VUS) such as synonymous mutations or benign/likely benign pathogenicity were filtered out in this study (Supplementary Figure 1).

Actionable Gene-Based Classification

In the current study, the ESMO Scale of clinical actionability of molecular targets (ESCAT) was used to define the level of actionability of called variants.[19] Briefly, actionable gene-based classification is defined as Tier I, an alteration-drug match associated with improved outcome in clinical trials; Tier II, an antitumor activity associated with the matched

alteration-drug but lacks prospective outcome data; and Tier III, the matched drug-alteration leads to clinical benefit in another tumor type other than the tumor of interest.

Statistics

Continuous variables were presented as the number of observations, mean, standard deviation, minimum, maximum, and 95% confidence interval, while categorical variables were summarized as counts and percentages. All called variants were compared between IHC3+ and IHC2+/ISH+ groups by Fisher exact test. Odds ratio between IHC3+ and IHC2+/ISH+ groups was analyzed by logistic regression model. A two-sided p value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the Python 3.11.2 software.

Results

Clinical and Pathological Features by HER2 IHC Scores

A total of 120 consecutive patients with HER2 (+), stage I–IV breast cancer were enrolled. The tumor specimens obtained from these 120 patients were sent for targets NGS analysis. The clinical presentation and pathological features of this cohort study stratified by HER2 IHC scores were demonstrated previously.[13] The forest plot of the clinical and pathological parameters by logistic regression analysis (Odds ratio) disclosed that there were significant N1 lymph node involvement favoring IHC 2+/ISH+ group with OR=0.333 and higher Ki67 level (>30%) favoring IHC 3+ tumors with OR=2.64 (Figure 1).

Mutation Types Across the Level of HER2 Expression

The results of 120 TMO assay revealed that the average number of called variants in HER2 2+/ISH+ group was 10.9 (SD: 14.3, range: 2 ~ 53) while those in HER2 3+ group was 6.6 (SD: 8.4, range: 1 ~ 53). The mutation types of called variants were SNA such as synonymous, missense, insertion/deletion (Indel), or frameshift, SA such as fusion, truncation, and CNA. The average number of each mutation type among HER2 (+), including IHC 2+/ISH+ or IHC 3+breast cancers with at least one variant was demonstrated in Table 1.

		Subject no.	Odds ratio (95% CI)	p-value
Age (years)	<55	55	Reference	
	≥55	65	0.72 (0.3, 1.73)	0.463
Tumor size	T1	35	Reference	
	T2	65	1.231 (0.447, 3.393)	0.688
	T3	10	1.077 (0.185, 6.259)	0.934
	T4	10	0.308 (0.071, 1.34)	0.116
Lymph node	N0	68	Reference	
	N1	25	0.333 (0.116, 0.959)	0.042*
	N2–N3	27	1.1 (0.35, 3.459)	0.87
Stage	1	28	Reference	
	2	54	1.051 (0.365, 3.031)	0.926
	3–4	31	1.143 (0.344, 3.795)	0.827
Neoadjuvant therapy	No	75	Reference	
	Yes	38	1.272 (0.498, 3.249)	0.615
Histologic grade	1–2	69	Reference	
	3	51	1.098 (0.456, 2.644)	0.834
Estrogen receptor	ER−	52	Reference	
	ER+	68	0.462 (0.183, 1.169)	0.103
Progesterone receptor	PR−	72	Reference	
	PR+	48	0.451 (0.188, 1.084)	0.075
Ki67	≤30%	34	Reference	
	>30%	86	2.64 (1.064, 6.553)	0.036*

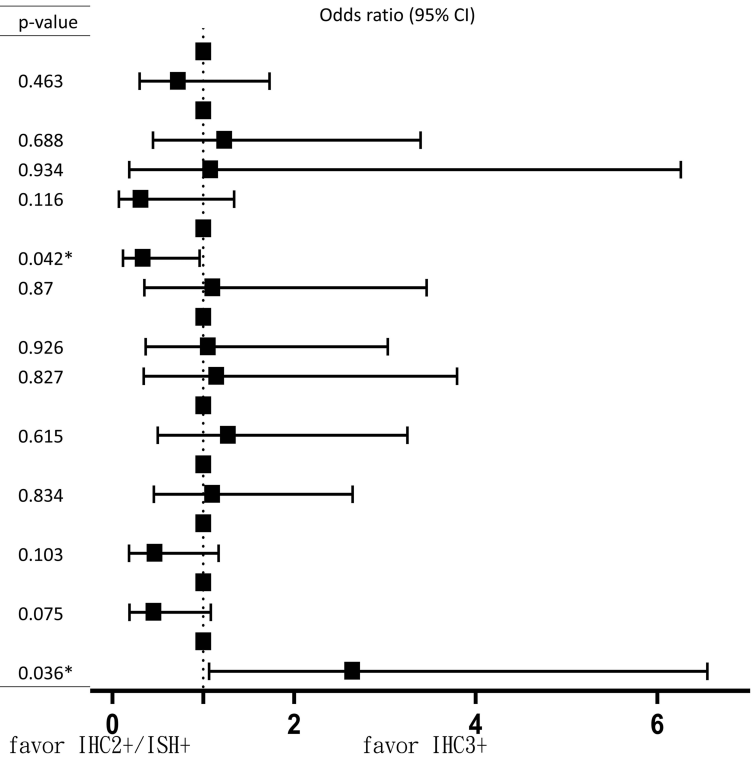

Figure 1 The forest plot of the clinical and pathological parameters between patients with IHC 2+/ISH+ or IHC 3+ tumors. Odds ratio and 95% confidence interval were analyzed by logistic regression model. Odds ratio > 1 favours IHC 3+ while Odds ratio < 1 favours IHC 2+/ISH+. Asterisk indicates a p value less than 0.05.

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Table 1 Mutation Types Across the Level of HER2 Expression

Subtype	Mutation Type	Mean	SD	Min	Max
IHC2+/ISH+ (31/338)*	CNA	2.55	2.35	0	9
(Mean: 10.9	Frameshift insertion	0.06	0.25	0	1
SD: 14.3	Fusion	0.39	0.80	0	2
Min: 2	Inframe	0.03	0.18	0	1
Max: 53)	Missense	6.71	12.49	0	44
	Nonframeshift deletion	0.03	0.18	0	1
	Truncation	0.71	1.01	0	3
IHC3+ (89/584)*	CNA	2.22	1.99	0	11
(Mean: 6.6	Frameshift insertion	0.02	0.15	0	1
SD: 8.4	Fusion	0.22	0.77	0	4
Min: 1	Inframe	0.02	0.21	0	2
Max: 53)	Missense	3.42	6.98	0	43
	Nonframeshift deletion	0	0	0	0
	Truncation	0.58	0.86	0	4

Note: *Subtype (Subject number/Called variant number).

Actionable Genes Across the Level of HER2 Expression

Based on ESCAT criteria, the average number of actionable gene variants in HER2 2+/ISH+ group was 1.97 (SD: 7.8, range: 2 ~ 9) while those in HER2 3+ group was 1.92 (SD: 1.19, range: 0 ~ 8). The actionable gene variants were AKT2, BRCA1, BRCA2, ERBB2, ERBB3, PI3KCA, MDM2. The average actionable gene variants among IHC 2+/ISH+ or IHC 3+breast cancers were shown in (Supplementary Table 2). The forest plot of the actionable gene by logistic regression analysis (Odds ratio) between patients with IHC 2+/ISH+ or IHC 3+ tumors was shown in Figure 2.

Comparison of Mutation Alterations Across the Level of HER2 Expression

When called gene alterations in IHC2+/ISH+ group were compared with those identified in IHC3+ group, there was a significantly higher sample frequency 94.4% (84/89) of *ERBB2 AMPLIFICATION* in IHC3+ group than IHC2+/ISH+ group [45.2% (14/31)] (p<0.001, Fisher exact test). By contrast, there was a significantly lower sample frequency of *MYC*_AMP_CNA [10.1% (9/89)] and *CCND3*_AMP_CNA [0% (0/89)] amplification in IHC3+ group than IHC2+/ISH+

	IHC3+		IHC2+/ISH+			
Actionable gene	Variant (+) no.	Subject total	Variant (+) no.	Subject total	Odds ratio (95% CI)	p-value
AKT2	1	89	1	31	0.3409 (0.01778, 6.679)	0.4515
BRCA1	3	89	3	31	0.3256 (0.07357, 1.473)	0.1778
BRCA2	10	89	6	31	0.5274 (0.1806, 1.664)	0.3559
ERBB2	87	89	17	31	35.82 (7.703, 163)	<0.0001*
ERBB3	2	89	1	31	0.6897 (0.07831, 10.3)	>0.9999
PIK3CA	36	89	16	31	0.6368 (0.2823, 1.416)	0.2999
MDM2	2	89	2	31	0.3333 (0.05103, 2.225)	0.2742

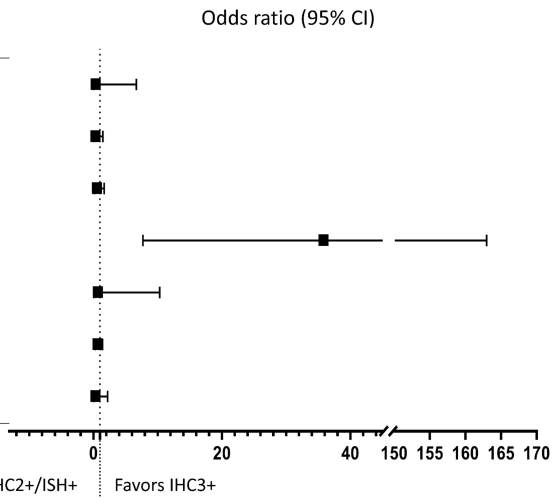

Figure 2 The forest plot of the actionable gene mutation between patients with IHC 2+/ISH+ or IHC 3+ tumors. Odds ratio and 95% confidence interval were analyzed by logistic regression model. Odds ratio > 1 favours IHC 3+ while Odds ratio < 1 favours IHC 2+/ISH+. Asterisk indicates a *p* value less than 0.05.

Table 2 Comparison of Mutation Alterations Across the Level of HER2 Expression

#Chrom.	Type	ALT	Gene Symbol	IHC2+/ISH+			IHC3+			p-value
				#variant (+)	#total subjects	SF(%)	#variant (+)	#total subjects	SF(%)	
17	CNA	AMP	ERBB2	14	31	45.2	84	89	94.4	<0.0001
8	CNA	AMP	MYC	8	31	25.8	9	89	10.1	0.04
6	CNA	AMP	CCND3	3	31	9.7	0	89	0	0.016

Note: #number.

Abbreviations: ALT, alteration; AMP, amplification; Chrom, chromosome; CNA, copy number amplification; IHC, immunohistochemistry score; SF, sample frequency.

group [25.8% (8/31)] (p=0.04, Fisher exact test), and [9.7% (3/31)] (p=0.016, Fisher exact test), respectively (Table 2). The oncoplot result was shown in Figure 3, while the top 20 commonly mutated alterations between IHC3+ group and IHC2+/ISH+ groups are shown in Supplementary Figure 2.

Mutation Types Across the HER2(+) Subtypes

There is consensus that patients with HER2 subtypes breast cancer, namely, estrogen receptor (+)/HER2(+) and estrogen receptor (-)/HER2(+), have different clinical outcomes. The average number of called variants in estrogen receptor (+)/HER2(+) group was 7.88 (SD: 10.98, range: 1 ~ 53) while those in estrogen receptor (-)/HER2(+) group was 7.41 (SD: 9.61, range: 1 ~ 48). The average number of each mutation type among HER2 (+) subtype, including estrogen receptor (+)/HER2(+) and estrogen receptor (-)/HER2(+) breast cancers with at least one variant was demonstrated in Table 3.

Actionable Genes Across the HER2 (+) Subtypes

The average number of actionable gene variants in estrogen receptor (+)/HER2(+) group was 1.84 (SD: 1.37, range: 0 ~ 8) while those in estrogen receptor (-)/HER2(+) group was 2.06 (SD: 1.36, range: 1 ~ 9). The average actionable gene variants among estrogen receptor (+)/HER2(+) and estrogen receptor (-)/HER2(+) breast cancers is shown in Table 4.

Comparison of Mutation Alterations Across the HER2 (+) Subtypes

When called gene alterations in estrogen receptor (+)/HER2(+) group were compared with those identified in estrogen receptor (-)/HER2(+) group, there was a significantly higher sample frequency, 17.4% (12/69) of *FGF*3_AMP_CNA in receptor (+)/HER2(+) group than estrogen receptor (-)/HER2(+) group with sample frequency 3.9% (2/51) (p=0.041, Fisher exact test) (Table 5). The oncoplot result is shown in Figure 4.

Discussion

There is consensus that next generation sequencing (NGS) is a strong tool not only to detect familial disease mutation carriers and to identify novel germline or somatic mutations in cancer, but also provide important information for personalized treatment clinically.[20,21] While whole exosome sequencing (WES) has advantages in providing unprecedented coverage of the coding region of the whole genome, target sequencing is designed to identify mutation hotspots and related specific proteins or pathways that can be counteracted by targeted agents.[22,23] Nevertheless, such technologies have limitations due to inherent sequencing biases, technical artefacts, and inability to detect rare variants.[24,25] Recently, by using several NGS-based platforms on a wide spectrum of clinical scenarios such as breast cancer subtypes and different clinical settings such as neoadjuvant, adjuvant and metastatic etc. have been established to identify biomarkers

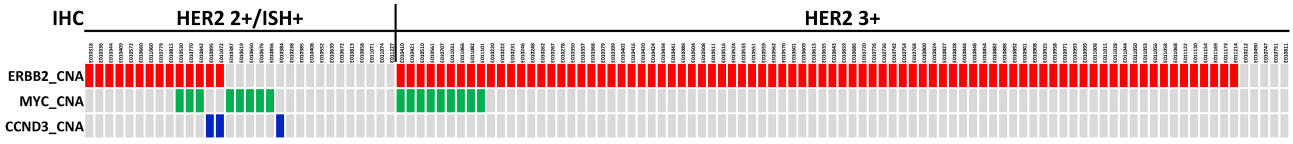

Figure 3 The oncoplot results of mutation alterations across the level of HER2 expression. ERBB2, *ERBB2*_CNA; MYC, *MYC*_CNA; CCND3, *CCND3*_CNA.

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Table 3 Mutation Types Across Estrogen Receptor(+)/HER2(+) and Estrogen Receptor(-)/HER2(+) Subtype

Subtype	Mutation Type	Mean	SD	Min	Max
ER(+)/HER2(+) (69/544)*	CNA	2.62	2.31	0	11
(Mean: 7.88	Frameshift insertion	0.04	0.21	0	1
SD: 10.98	Fusion	0.32	0.88	0	4
Min: 1	Inframe	0.04	0.27	0	2
Max: 53)	Missense	4.09	8.89	0	43
	Nonframeshift deletion	0	0	0	0
	Truncation	0.59	0.86	0	4
ER(-)/HER2(+) (51/378)*	CNA	1.88	1.66	0	8
(Mean: 7.41	Frameshift insertion	0.02	0.14	0	1
SD: 9.61	Fusion	0.20	0.60	0	2
Min:1	Inframe	0	0	0	0
Max: 48)	Missense	4.51	8.77	0	44
	Nonframeshift deletion	0.02	0.14	0	1
	Truncation	0.65	0.96	0	3

Note: *Subtype (Subject number/Called variant number).
Abbreviations: ER(+)/HER2(+), Estrogen Receptor(+)/HER2(+); ER(-)/HER2(+), Estrogen Receptor (-)/HER2(+).

Table 4 Actionable Genes Alterations Across Estrogen Receptor (+)/HER2(+) and Estrogen Receptor(-)/HER2(+) Subtype

Subtype	Actionable Gene	Mean	SD	Min	Max
ER(+)/HER2(+) (69)*	AKT2	0.01	0.12	0	1
(Mean: 1.84	BRCA1	0.14	0.77	0	5
SD: 1.37	BRCA2	0.16	0.41	0	2
Min: 0	ERBB2	0.99	0.56	0	2
Max: 8)	ERBB3	0.03	0.17	0	1
	PIK3CA	0.45	0.58	0	2
	MDM2	0.06	0.24	0	1
ER(-)/HER2(+) (51)*	AKT2	0.02	0.14	0	1
(Mean: 2.06	BRCA1	0.18	0.79	0	4
SD: 1.36	BRCA2	0.18	0.52	0	2
Min: 1	ERBB2	1.18	0.59	0	2
Max: 9)	ERBB3	0.02	0.14	0	1
	PIK3CA	0.49	0.54	0	2
	MDM2	0	0	0	0

Note: *Subtype (Subject number).
Abbreviations: ER(+)/HER2(+), Estrogen Receptor(+)/HER2(+); ER(-)/HER2(+), Estrogen Receptor(-)/HER2(+).

Table 5 Comparisons of Mutation Alterations Across Estrogen Receptor(+)/HER2(+) and Estrogen Receptor(-)/HER2(+) Subtype

#Chrom.	Type	ALT	Gene Symbol	ER(+)/HER2(+)			ER(-)/HER2 (+)			p-value
				#variant (+)	#total subjects	SF(%)	#variant (+)	#total subjects	SF(%)	
11	CNA	AMP	FGF3	12	69	17.4	2	51	3.9	0.041

Note: #number.
Abbreviations: ALT, alteration; AMP, amplification; Chrom, chromosome; CNA, copy number amplification; FGF3, fibroblast growth factor 3; ER(+)/HER2(+), Estrogen Receptor(+)/HER2(+); ER(-)/HER2(+), Estrogen Receptor(-)/HER2(+); SF, sample frequency.

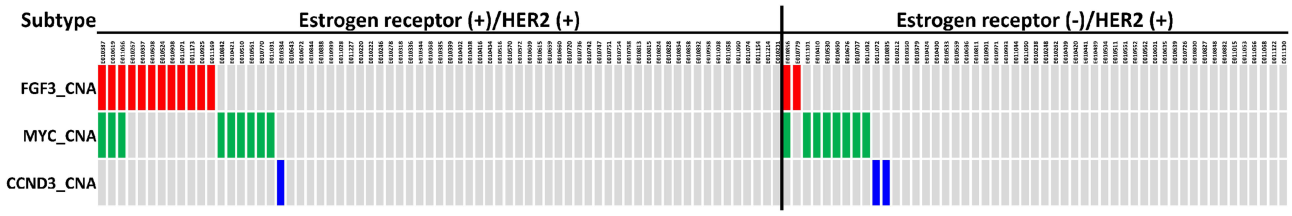

Figure 4 The oncoplot results of mutation alterations across the HER2 (+) subtypes. HER2 (+) subtypes include estrogen receptor(+)/HER2(+) and estrogen receptor (-)/HER2(+). FGF3, *FGF3*_CNA; CCND3, *CCND3*_CNA; MYC, *MYC*_CNA.

for Taiwanese patients with highly heterogeneous breast cancers.[14,26] The results showed that the prevalence of BRCA1/2 mutations was far lower than that observed in the Western population[27] and the T-cell receptor repertoire and potential effects of immunotherapy in breast cancer provide important information for personalized treatment regimens and outcome prediction.[28] In this study, we found that HER2 IHC3+ tumors have higher frequency of *ERBB2*_AMP_CNA and lower frequency of *CCND3*_ AMP_CNA and *MYC*_AMP_CNA than IHC2+/ISH+ tumors. These results provide clues in individualized treatment strategies for HER2-positive breast cancer.

The range of TMB is defined as low, ≤5 mut/Mb, intermediate, 5–20 mut/Mb, high, 20–50 mut/Mb and very high >50 mut/Mb. TMB is calculated by counting the number of somatic mutations in a database obtained from WGS, WES or even target sequencing.[29,30] However, many factors such as the amount of genome interrogated, the read depth, intratumor heterogeneity, the tissue-fixation methodologies and the lack of standardization of the genes of interest, can impact TMB reproducibility [8]. Considering target-based NGS as an optimal and labor-efficient workflow that combines DNA/RNA gene variant and fusion detection is particularly important when small biopsy samples are submitted for comprehensive genomic profiling in clinical settings.

It is well known that HER2 status is an important factor to define the molecular subtypes of breast cancer. Our previous studies have demonstrated that the outcomes of IHC 3+ patients are significantly better than those of IHC 2+/ISH+ patients[31] and HER2 IHC score correlates the treatment outcome in HER2 (+) breast cancer patients receiving neoadjuvant therapy.[13] HER2 IHC is a semi-quantitative measure of protein levels co-existing with interlaboratory variation in interpretation.[32] The ASCO/CAP guidelines had optimized thresholds for several times to ensure the targeted populations who can truly benefit from anti-HER2 agents.[15,33] In the past two decades, IHC 3+ and IHC 2+/ISH + both were allocated as a disease in the same spectrum no matter how the guidelines updated in the definition of ISH positivity. However, it seems somehow different in the treatment efficacy or outcome for the two categories based on the findings of our previous research. Other literature also proposed the similar viewpoint that the pCR rate was significantly higher in the patients with IHC 3+ tumors than that with IHC 2+/ISH + tumors ((46.0% vs 25%, p=0.016).[34] In addition, previous study had found that the significant association between HER2 heterogeneity, IHC scores and pCR rate. IHC 3+ tumors accounted for the majority (81%) of the non-heterogeneous cases and a significantly higher pCR rate (55%, 77/141 cases) compared to the heterogeneous subgroup.[35]

It is noteworthy that the IHC2+/ISH+ group carried more average alterations, but the number of actionable alterations is very similar between both groups. We attribute this result to the fact that many gene alterations such as *MYC*_CNA etc. play an important role in combined gene alterations, but it does not belong to actionable gene mutation defined by the 4-Tier classification. Although there is a lack of general consensus, the 4-Tier classification system[36] is feasible for most clinical samples with tumor-only sequencing data.[37] ERBB2 amplification is a Tier I actionable gene alteration, defined as variants of strong clinical significance with being therapeutically relevant.[36] Among 120 patients with IHC HER2 (+), 98 (81.7%) were with ERBB2 amplification. In those 22 patients with IHC HER2 (+)/ERBB2 amplification (-), 17 (77.3%) were HER2 2+/ISH+ and 5 (22.7%) were HER2 3+ tumors. In contrast, our previous cohort study has demonstrated that among patients with ERBB2 amplification, 18.8% were clinically IHC HER2 (-), suggesting that targeted NGS may identify more HER2 (+) breast cancers than the traditional IHC assay.[14] Knowing the fact that there was a significantly higher sample frequency 94.4% (84/89) of *ERBB2 AMPLIFICATION* in HER2 3+ group than IHC 2+/ISH+ group with sample frequency 45.2% (14/31), it is speculated that the former group benefits more from the merit

of targeted therapy than the latter one. Our previous findings that the outcomes, in terms of relapse-free survival and overall survival, of IHC 3+ patients are significantly better than those of IHC 2+/ISH+ patients with 5-year survival (confident interval) being 93.7% (91.2–95.6) and 90.2% (84.1–94.1), respectively, supports our speculation.[31]

Women with BRCA1/2 germline mutation (Tier I gene alteration) have a risk of breast cancer[38] and are candidates for poly (ADP-ribose) polymerases (PARP) inhibitors (talazoparib) therapy.[39] Among 120 hER2(+) breast cancers, there is no significant difference in BRCA1/2 mutation between HER2 2+/ISH+ and HER2 3+ groups. The prevalence of BRCA1 (4.2%) and BRCA2 (11.7%) mutations in our series was far lower than that observed in the Western population,[27,38] which is consistent with those observations that social, economic and genetic factors play important roles in tumor behavior and cancer subtype among different ethnic groups.[40,41] Interestingly, all five patients with BRCA1 mutation have also co-concurred with BRCA2 mutation ($p<0.001$).

CCND3 gene encodes the cyclin D family proteins that controls the G1-S phase of cell cycle and generally is recognized as a target of genomic gain/amplification. CCND3 amplification, presenting in 0.59% of AACR GENIE cases, is commonly found in breast cancer, lung cancer, and osteosarcoma, etc.[42] Recently, gene alterations including CDK4/6, CCNE1, CCND3and HRAS and the presence of co-occurrence CDK4/6 amplification correlate with poor patient's outcome and can serve as a predictive biomarker for de novo EGFR TKI resistance in sensitizing EGFR mutation non-small cell lung cancer.[43] In the present study, there was a significantly higher sample frequency (3/31) of CCND3 amplification in HER2 2+/ISH+ group than HER2 3+ group (0/89) ($p=0.016$), indicating the presence of CCND3 amplification suppresses the HER2 gene expression, which is consistent with the phenomenon described previously.[44] Despite the lack of clinical trial support, our results suggest that target NGS sequencing is advisable for those HER2-positive patients who are resistant to HER2-targeted therapy and they are potential candidates for CDK4/6 inhibitor or neratinib.[45]

The prevalence of PI3KCA gene alterations is 37.1% ~ 44.0% of breast cancers reported in Chinese population.[14,46] These gene alterations occurred in hotspots E545K and E542K (helical region) or hotspot H1047R (kinase domain) of the PIK3CA-encoded p110α2 were 38.7% (n=12) in HER2 2+/ISH+ and 34.8% (n=31) in HER2 3+ groups, respectively. For subtype analysis, there is also no significant difference between ER(+)/HER2(+) and ER(-)/HER2(+) groups with sample frequency 36.2% (n=25) and 35.2% (n=18), respectively.

Besides, fibroblast growth factor 3 (FGF3), a gene located at the 11q13.3 locus, regulates important developmental processes. When amplification of the 11q13.3 locus occurs, FGF3 is frequently coamplified with CCND1 and decreases antitumor activity during the carcinogenesis of breast cancer, particularly in luminal B subtype.[47] Our results demonstrate a higher frequency of *FGF3*_AMP_CNV in ER(+)/HER2(+) than in ER (-)/HER2(+) tumors with sample frequency being 17.4% (12/69) and 3.9% (2/51), respectively, suggesting FGF3 can be considered as a potential target for ER (+)/HER2(+) or target therapy-resistant HER2(+) breast cancer.[48] Further analysis on tumor mutation burden in different subtypes in breast cancer is mandatory to provide therapeutic responses in the near future.[12] In our series, the prevalence of *ERBB2*_CNA, *CCND3*_CNA, *MYC*-CNA, and *FGF3*_CNA breast cancer patients are 17.4%, 1.4%, 11.7%, 11.3%, respectively, which is consistent with data obtained from publicly available datasets that the prevalence of *ERBB2*_CNA, *CCND3*_CNA, *MYC*_CNA, and *FGF3*_CNA breast cancer patients are 14%, 3%, 19%, 15% in TCGA dataset and 18%, 2%, 25%, 16% in METABRIC dataset, respectively (Supplementary Figure 3). In addition, TP53, KRAS, and PIK3CA mutations are well known to be present in HER2-positive tumors. The prevalence of TP53 and KRAS mutations in HER2 2+/ISH+ and HER2 3+ tumors are 38.7%, 25.8% and 58.4%, 12.4%, respectively. The PIK3CA gene alterations are 38.7% in HER2 2+/ISH+ and 34.8% in HER2 3+ groups. These results also provide the validity of our dataset.

There is consensus that both patients with IHC 3+ and IHC 2+/ISH+ tumors are suitable for anti-HER2 target therapy, but with different treatment efficacy.[13] For example, we have demonstrated that IHC 3+ tumors presented a trend of higher pathological complete response (pCR) rate and better outcome in HER2-positive breast cancer patients who receive neoadjuvant systemic therapy (NST).[13] We hypothesize that different gene alterations between IHC 3+ and HIHC 2+/ISH+ tumors are expected. The mutation types and actionable genes across non_pCR and pCR in HER2 (+) breast cancer are shown in Supplementary Tables 3 and 4, respectively. When called gene alterations in non_pCR tumors were compared with those identified in pCR ones, there was a trend of higher sample frequency 94.7% (18/19) of *ERBB2 AMPLIFICATION* in pCR group than non_pCR group [71.4% (15/21)] ($p=0.053$, Fisher exact test). By contrast, there

was a significantly lower sample frequency of *FGFR1*_AMP_CNA [0% (09/19)] and *PI3KCA*_MISSENSE_H1047R [10.5% (2/19)] in pCR group than non_pCR group [19.0% (4/21)] (p=0.045, Fisher exact test), and [38.1% (4/21)] (p=0.044, Fisher exact test), respectively (Supplementary Table 5). The oncoplot result was shown in Supplementary Figure 4.

In conclusion, in addition to the fact that the HER2 IHC score is an independent predictor of survival outcomes, HER2 IHC3+ tumors have higher frequency of *ERBB2*_ AMP_CNA and lower frequency of *CCND3*_ AMP_CNA and *MYC*_AMP_CNA than IHC2+/ISH+ tumors. These results provide therapeutic strategies in treatment of HER2-positive breast cancer.

Abbreviations

CNA, copy number alteration; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; Indel, insertion/deletion; ISH, in situ hybridization; NGS, next generation sequencing; NST, neoadjuvant systemic therapy; pCR, pathological complete response; PR, progesterone receptor; SA, structure alteration; SNA, single nucleotide alteration; VUS, variants of unknown significance; WES, whole exosome sequencing; WGS, whole genome sequencing.

Data Sharing Statement

All data generated or analysed during this study are included in this published article (and its Supplementary Information files). The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Patient Consent for Publication

Since the clinical records and the tumor tissue utilization was released from the bio-bank in the same hospital, the need for consent to participate was waived with the approval of the Institutional Review Board of Taipei Veterans General Hospital. This study has been approved by the Institutional Review Board of this hospital (#2021-07-003CC, 2022-01-016CC).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare they have no relevant financial or non-financial interests to disclose.

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