Breast cancers with *EGFR* and *HER2* co-amplification favor distant metastasis and poor clinical outcome

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Abstract. ErbB signaling serves essential roles in invasive ductal carcinoma (IDC). The aim of the present study was to assess gene amplification in ErbB family members in IDC with clinical implications. Quantitative polymerase chain reaction and fluorescence *in situ* hybridization were performed on formalin-fixed paraffin-embedded tumor samples for gene amplification detection. The clinical and histopathological characteristics, as well as the prognostic significance, were analyzed. Among the 119 IDC patients evaluated, epidermal growth factor receptor [*EGFR*; also known as human epidermal growth factor receptor (HER)1], *HER2*, *HER3* and *HER4* gene amplification was observed in 30 (25.2%), 44 (36.9%), 0 (0.0%) and 1 (0.8%) patients, respectively. *EGFR* amplification was associated with estrogen receptor status (P=0.028) and higher possibilities of recurrence (P=0.015) and distant metastasis (following initial surgery) (P=0.011). In survival analysis, *EGFR* amplification was also associated with disease-free survival (DFS) (P=0.001) and overall survival (OS) (P=0.003). *HER2* amplification was associated with larger tumor size (P=0.006), later clinical stage (P=0.003) and distant metastasis (following initial surgery) (P=0.006). In survival analysis, *HER2* amplification was also associated with DFS (P=0.011). Notably, the present study identified a group of patients in whom *EGFR* and *HER2* were co-amplified. This group of patients appeared to have a higher possibility of metastasis (when diagnosed) (P=0.014) and distant metastasis (following initial surgery) (P<0.001). In survival analysis, these patients were noticed to be associated with DFS (P<0.001) and OS (P=0.002). With respect to treatment regimen, this was also true for the DFS association with chemotherapy (P<0.001), radiotherapy (P<0.001) and hormonal therapy (P=0.001). The present results suggest that *EGFR* and *HER2* amplification favor distant metastasis following initial surgery and are significantly associated with poor clinical outcome in breast cancer patients.

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Introduction

Based on the 2014 World Health Organization report, breast cancer is the second most life-threatening tumor (following lung cancer) for women in China (1). Numerous genes were identified to be abnormal in breast cancer, with different biological significance (2). It is well known that the Erb-B2 receptor tyrosine kinase 2 gene [also known as human epidermal growth factor receptor (HER)2], which encodes a member of the ErbB family, serves essential roles in breast cancer carcinogenesis, invasion and metastasis (3,4). Additionally, *HER2* amplification is a well-established biomarker for the treatment of breast and gastric carcinomas with trastuzumab (5,6).

Epidermal growth factor receptor (*EGFR*), which also encodes a family member of the ErbB family, serves essential roles in breast cancer. *EGFR* is a well-established treatment target for colorectal cancer, non-small cell lung cancer, and squamous cell carcinoma of the head and neck (7). Furthermore, a high *EGFR* gene copy number was significantly associated with poor clinical outcome (8-10). *EGFR* overexpression was reported to be significantly correlated with poor clinical outcome in breast cancer (11). *EGFR* is also a target for EGFR-tyrosine kinase inhibitor therapy for *EGFR* mutation and *EGFR* amplification of cancer patients (5,6).

Since both *EGFR* and *HER2* belong to the same family and share a high degree of structural and functional homology (12), the present study evaluated the gene amplification status and clinical significance in breast cancer of other members, including *HER3* and *HER4*. It has been reported that *EGFR*, *HER2*, *HER3* and *HER4* constitute a complex network, coupling various extracellular ligands to intracellular signal transduction pathways, resulting in receptor interaction and cross-activation (12). Members of the ErbB family

are critically involved in the development and progression of breast cancer. Amplification of the four members of the ErbB family has been detected by droplet digital polymerase chain reaction (ddPCR) (13), fluorescence *in situ* hybridization (FISH) (12) and next-generation sequencing (NGS) (2,6) at different rates, with no clinical outcome implications. Since these molecules belong to the same family and share certain homologous domains, the present study aimed to assess whether there are invasive ductal carcinoma (IDC) patients with amplification of ≥2 ErbB family members. Additionally, the current study sought to determine the clinical significance of the amplification of multiple gene (such as, tumor genesis, invasion and metastasis), as well as their prognostic values and therapeutic responses.

Thus, the quantification of all four ErbB family member receptors as a whole panel in IDC may shed light on their amplification status. Therefore, the amplification status of the four ErbB family members and their clinical implications was detected in 119 breast carcinoma patients with an average follow-up of 27.0 months in the present study.

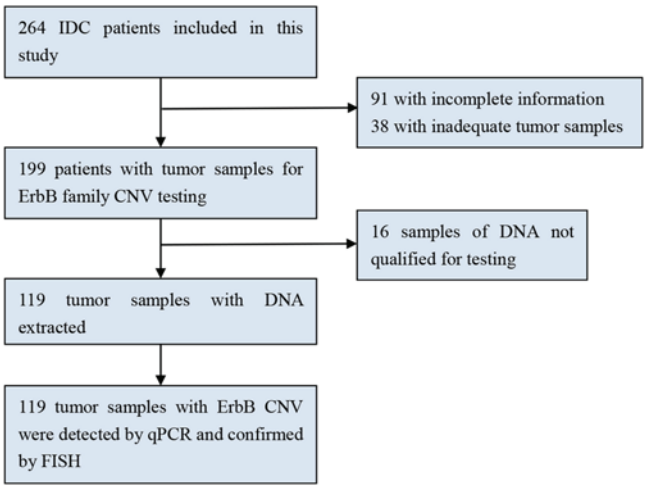

Figure 1. Flowchart showing the inclusion criteria and results of the present study. IDC, invasive ductal carcinoma; qPCR, quantitative polymerase chain reaction; FISH, fluorescence *in situ* hybridization; CNV, copy-number variations.

Materials and methods

Patients and sample preparation. The samples were human breast neoplasm tissue specimens removed during surgery. Patients anonymity was preserved in all cases. Approval for the study was granted by the Ethics Committee of West China Hospital (Chengdu, China; approval no. 2013-191), who also waived the requirement for patient consent. Formalin-fixed paraffin-embedded (FFPE) samples from 119 patients with breast cancer who underwent breast mastectomy between January 2010 and December 2012 at West China Hospital were analyzed in the present study (Fig. 1). Surgical specimens were obtained prior to systemic treatment, and paraffin embedding was performed within the framework of diagnostic procedures. Disease-free survival (DFS) and overall survival (OS) were defined as the time between the initial surgery and local or distant metastatic relapse, and the time between surgery and mortality, respectively.

DNA isolation and quantitative PCR (*qPCR*). Tumor areas (≥1 cm^2) from 4.0 μm-thick unstained FFPE sections were macrodissected. DNA was isolated from two 4 μm-thick tissue sections using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA quantitation was performed using a NanoDrop 2000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Finally, DNA purity was confirmed by measuring the absorbance (A)260/A280 ratio. Good-quality DNA was indicated by a ratio of A260/A280 nm = 1.70-1.95. Reactions were carried out using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions for qPCR were as follows: 98˚C for 2 min, followed by 39 amplification cycles at 98˚C for 15 sec and 60˚C for 15 sec. Each gene was measured in triplicate and normalized relative to a set of two reference genes [GAPDH and transferrin receptor (TFRC)] (Table I). Relative quantitation of ErbB gene amplification in IDC was calculated by the $2^{-\Delta\Delta Cq}$ method (14) using the mean copy number in 50 normal control samples and reference genes (GAPDH and TFRC). A sample was considered positive for *EGFR*, *HER2*, *HER3* and *HER4* gene amplification if the above ratio was >2, whereas a ratio of <2 indicated that the sample was negative for *EGFR*, *HER2*, *HER3* and *HER4* gene amplification (15,16) (Table I).

FISH. To confirm the *EGFR* and *HER2* copy number, FISH was conducted using EGFR and HER-2 DNA Probe kits (LBP China, Inc., Guangzhou, China). FFPE tissues were prepared in serial 4-μm sections on microscope slides. A set of tissue was used for two-color FISH. SpectrumOrange-labeled gene-specific probes were used together with SpectrumGreen-labeled probes (LBP China, Inc.) for the respective centromere region as references. The probe combinations were as follows: *HER2*, LBP EGFR SpectrumOrange/centromere (CEP) 17 SpectrumGreen; and *EGFR*, LBP EGFR SpectrumOrange/CEP7 SpectrumGreen. Prior to hybridization, the tissues were deparaffinized, air dried, and dehydrated in 70, 85 and 100% ethanol, followed by denaturation for 5 min at 85˚C. Upon overnight hybridization at 37˚C in a humidified chamber, the slides were washed and counterstained with 0.1% NP-40 in an antifade solution (LBP China, Inc.), and viewed under a fluorescence microscope. For each tumor, the predominant gene and centromere copy numbers were estimated. Under a fluorescence microscope, signals of the *EGFR* probe appear red, while signals of the centromere 7 probe appear green. Red and green signals were counted in 40 tumor cells, and the ratio of red:green signals was calculated. *EGFR* and *HER2* were considered amplified if the oncogene/centromere ratio was >2 (17,18).

Statistical analysis. Statistical analyses were conducted using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA), and two-tailed P<0.05 was considered to indicate a statistically significant difference. Associations between the prevalence of *EGFR* and *HER2* amplification and clinical parameters were evaluated using the χ^2 test. Univariate survival analysis was conducted using the Kaplan-Meier method, and multivariate survival analysis was carried out using the Cox proportional hazards model.

Table I. Quantitative polymerase chain reaction primers of the ErbB family.

Gene	GenBank no.	Oligo name	Oligo sequence	Target size (bp)
TFRC	NC_000003.12	TFRC-F	5'-ACTTCCTCTCTCCCTACGTATC-3'	105
		TFRC-R	5'-GCAGTTTCAAGTTCTCCAGTAAAG-3'	
GAPDH	NG_007073.2	GAPDH-F	5'-CCTCAAGATCATCAGCAATGCCTC-3'	100
		GAPDH-R	5'-GTGGTCATGAGTCCTTCCACGATA-3'	
EGFR	NG_007726.3	EGFR-F	5'-CGGGACGTTTCGTTCTTCGG-3'	130
		EGFR-R	5'-GAAAGTTGGGAGCGGTTCGG-3'	
HER2	NG_007503.1	HER2-F	5'-ATGAGCTACCTGGAGGATGT-3'	103
		HER2-R	5'-CCAGCCCGAAGTCTGTAATTT-3'	
HER3	NG_011529.1	HER3-F	5'-CCTCAACCTGCTCCTCTTTATT-3'	168
		HER3-R	5'-GGCTACAACAGTGAGACCATAG-3'	
HER4	NG_011805.1	HER4-F	5'-TTGCACGACTTTCTCACGGC-3'	130
		HER4-R	5'-GCTGCTGACCTGAAGGCACT-3'	

-F, forward; -R, reverse; *TFRC*, transferrin receptor; *EGFR*, epidermal growth factor receptor; *HER*, human epidermal growth factor receptor.

Table II. Baseline clinical characteristics of the study subjects (n=119).

		Disease-free survival		Overall survival	
Characteristic	No. (%)	Log-rank	P-value	Log-rank	P-value
Age, years	49.3 (29-74)[b]	0.658	0.417	0.756	0.385
≤50	70 (58.8)				
>50	49 (41.2)				
Grade		2.245	0.134	2.633	0.105
G1-G2	40 (33.6)				
G3	79 (66.4)				
Tumor size[a]		4.696	0.032[c]	2.491	0.114
T0-2	111 (93.7)				
T3-4	7 (5.9)				
Nodal status[a]		5.065	0.024[c]	1.567	0.211
N0	54 (45.8)				
N1-N3	64 (54.2)				
Metastasis		118.000	<0.001[c]	0.026	0.871
M0	118 (98.3)				
M1	1 (0.8)				
Clinical stage[a]		5.020	0.025[c]	0.725	0.394
I-II	90 (76.3)				
III-IV	28 (23.7)				
ER status		0.156	0.692	1.619	0.203
ER^+	40 (33.6)				
ER^-	79 (66.4)				
PR status[a]		1.685	0.194	0.290	0.590
PR^+	43 (36.8)				
PR^-	74 (63.2)				
HER2[a]		1.975	0.372	0.046	0.977
0-1+	65 (54.6)				
2+	25 (21.0)				
3+	28 (23.5)				

[a]Differences in total patient numbers reflect missing data. [b]Data are presented as median (range). [c]Statistically significant. ER, estrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor.

Table III. *EGFR* and *HER2* gene amplification in the present cohort.

	HER2, no. (%)		
EGFR	Amp.	No amp.	Total
Amp.	17	13	30 (25.2)
No amp.	27	62	89 (74.8)
Total	44 (36.9)	75 (63.1)	119 (100.0)

EGFR, epidermal growth factor receptor; *HER*, human epidermal growth factor receptor; amp., amplification.

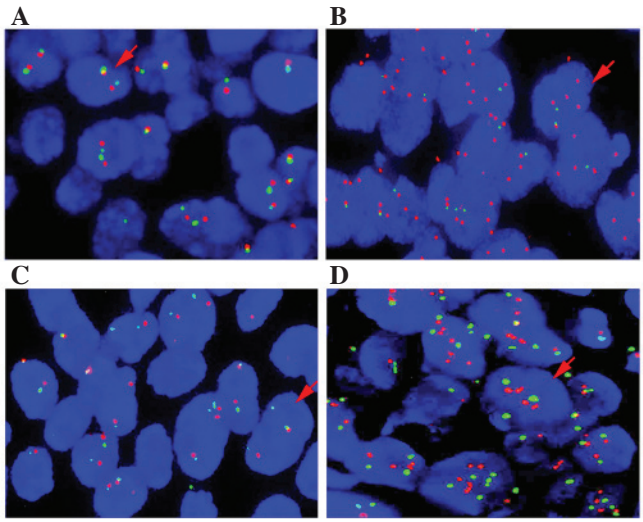

Figure 2. *EGFR* and *HER2* amplification by FISH. FISH shows *EGFR* and *HER2* gene amplification in invasive ductal carcinoma patients. Signals of the *EGFR* probe are illustrated in red, while signals of the centromere 7 probe are shown in green. The arrow focuses on a representative cell, with a representative example of (A) *EGFR* non-amplification and (B) *EGFR* amplification. Signals of the *HER2* probe are illustrated in red, while signals of the centromere 17 probe are shown in green. The arrow focuses on a representative cell, with a representative example of (C) *HER2* non-amplified and (D) *HER2*-amplified breast tumors. Original magnification, x100. *EGFR*, epidermal growth factor receptor; *HER*, human epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization.

Results

Baseline clinical characteristics. All the patients included in the present study were females, ranging in age from 29 to 74 years (mean, 49.3 years). The mean DFS was 25.6 months, and the mean OS was 27.0 months. The DFS and OS of the 119 patients are listed in Table II with respect to histopathological characteristics and prognostic factors, including age, histological grading, tumor size, nodal status, metastasis, clinical stage, and estrogen receptor (ER), progesterone receptor (PR) and HER2/neu status. As expected, nodal metastasis status, clinical state (P=0.025) and distant metastasis status (when diagnosed) (P<0.001) were observed to be significantly correlated with DFS. Larger tumor size, positive-node status, higher clinical state and metastasis (when diagnosed) were associated with DFS. However, none of the histopathological characteristics was significantly associated with OS (Table II).

Gene amplification of ErbB family members by qPCR and FISH. The gene amplification of 119 patients was detected using qPCR (Table III) and was confirmed by FISH (Fig. 2). The relative quantitation of ErbB amplification in IDC was calculated by the $2^{-\Delta\Delta Cq}$ method using the mean copy number in 50 normal control samples and reference genes (GAPDH and TFRC). A sample was considered positive for *EGFR*, *HER2*, *HER3* and *HER4* gene amplification if the ratio was >2, whereas a ratio <2 indicated that the sample was negative for *EGFR*, *HER2*, *HER3* and *HER4* gene amplification. *EGFR* amplification was detected in 30 patients (25.2%), while *HER2* amplification was detected in 44 (36.9%) patients. However, in the present study, only one patient was detected to have *HER4* amplification but no amplification of *HER3*. Furthermore, a group of 17 patients (14.2%) with both *EGFR* and *HER2* gene amplification was identified. A total of 62 patients (52.1%) were identified to have neither *EGFR* nor *HER2* genes amplified. In one patient with *HER4* gene amplification, the *EGFR*, *HER2* and *HER3* genes were not observed to be amplified.

Clinicohistopathological features of EGFR and HER2 amplification in breast cancer. To identify any correlation between the gene amplification status of the ErbB family and clinical characteristics (Table IV), the correlation between *EGFR* and *HER2* amplification and clinical features was analyzed. Patients with *EGFR* and *HER2* amplification, as well as those with *EGFR* and *HER2* co-amplification, were analyzed regarding age, histological grading, tumor size, nodal status, metastasis, clinical stage, ER, PR and HER2/*neu* status, local recurrence, and distant metastasis. In the present study, *EGFR* amplification was significantly associated with ER expression (P=0.028), local recurrence (P=0.015) and distant metastasis (following initial surgery) (P=0.011). Additionally, *EGFR* amplification primarily occurred in tumors with a high histological grade (Table IV). *HER2* amplification was associated with larger tumor size (P=0.006), later clinical stage (P=0.003) and distant metastasis (following initial surgery) (P=0.006). *HER2* amplification, as expected, was also significantly associated with HER2 expression (P<0.001) and distant metastasis (following initial surgery) (P=0.006) (Table IV).

Furthermore, a subgroup of patients who harbored *EGFR* and *HER2* gene co-amplification was identified. This group of patients was significantly correlated with metastasis (at diagnosis) (P=0.014) and distant metastasis (subsequent to initial surgery) (P<0.001). They were almost significantly correlated with clinical stage (P=0.062), HER2 overexpression (P=0.062) and local recurrence (P=0.053) (Table IV).

EGFR and HER2 amplification for IDC prognosis. To further reveal the prognostic value of gene amplification for *EGFR* or/and *HER2* in IDC patients, the *EGFR* and/or *HER2* amplification status were evaluated in association with DFS and OS by Kaplan-Meier analysis (Fig. 3). The 119 patients were divided into four groups: Nor *EGFR* or *HER2* amplification; *EGFR* amplification but no *HER2* amplification; no *EGFR* amplification but *HER2* amplification; and *EGFR* and *HER2* co-amplification. The present study revealed that patients with *EGFR* and *HER2* co-amplification had a significantly shorter DFS (P<0.001) and OS (P=0.010) than any other group (Fig. 3A and C). Next, differences in the *EGFR* and *HER2*

Table IV. Prevalence of *EGFR* and *HER2* amplification in breast tumors stratified according to clinical characteristics.

	EGFR amplification			*HER2* amplification			*EGFR* and *HER2* co-amplification		
Characteristic	P (n=30) No. (%)	N (n=89) No. (%)	P-value	P (n=44) No. (%)	N (n=75) No. (%)	P-value	P (n=17) No. (%)	N (n=102) No. (%)	P-value
Age, years			0.781			0.468			0.595
≤50	17 (56.7)	53 (59.6)		24 (54.5)	46 (61.3)		11 (64.7)	59 (57.8)	
>50	13 (43.3)	36 (40.4)		20 (45.5)	29 (38.7)		6 (35.3)	43 (42.2)	
Grading			0.628			0.128			0.416
G1-G2	9 (30.0)	31 (34.8)		11 (25.0)	29 (38.7)		4 (23.5)	36 (35.3)	
G3	21 (70.0)	58 (65.2)		33 (75.0)	46 (61.3)		13 (76.5)	66 (64.7)	
Tumor size[a]			0.844			0.006[b]			0.271
T0-2	28 (93.3)	83 (94.3)		38 (86.4)	73 (98.6)		15 (88.2)	96 (95.0)	
T3-4	2 (6.7)	5 (5.7)		6 (24.4)	1 (1.4)		2 (11.8)	5 (5.0)	
Nodal status[a]			0.590			0.414			0.349
N0	15 (50.0)	39 (44.3)		18 (40.9)	36 (48.6)		6 (35.3)	48 (47.5)	
N1-N3	15 (50.0)	49 (55.7)		26 (59.1)	38 (51.4)		11 (64.7)	53 (52.5)	
Metastasis			0.084			0.190			0.014[b]
M0	29 (96.7)	89 (100.0)		43 (97.7)	75 (100.0)		16 (94.1)	102 (100.0)	
M1	1 (3.3)	0 (0.0)		1 (2.3)	0 (0.0)		1 (5.9)	0 (0.0)	
Clinical stage[a]			0.661			0.003[b]			0.068
I-II	22 (73.3)	68 (77.3)		27 (61.4)	63 (85.1)		10 (58.8)	80 (79.2)	
III-IV	8 (26.7)	20 (22.7)		17 (38.6)	11 (14.9)		7 (41.2)	21 (20.8)	
ER status			0.028[b]			0.197			0.476
ER^+	15 (50.0)	25 (28.1)		18 (40.9)	22 (29.3)		10 (58.8)	69 (67.6)	
ER^-	15 (50.0)	64 (71.9)		26 (59.1)	53 (70.7)		7 (41.2)	33 (32.4)	
PR status[a]			0.298			0.689			0.532
PR^+	13 (44.8)	30 (34.1)		19 (45.2)	24 (32.0)		9 (56.2)	65 (64.4)	
PR^-	16 (55.2)	58 (65.9)		23 (54.8)	51 (68.0)		7 (43.8)	36 (35.6)	
HER2[a]			0.753			<0.001[b]			
0-1+	18 (60.0)	47 (53.4)		9 (20.5)	56 (75.7)		5 (29.4)	60 (59.4)	0.062
2+	5 (16.7)	20 (22.7)		8 (18.2)	17 (23.0)		5 (29.4)	20 (19.8)	
3+	7 (25.4)	21 (23.9)		27 (61.4)	1 (1.3)		7 (41.2)	21 (20.8)	
Recurrence			0.015[b]			0.554			0.053
Yes	3 (10.0)	0 (0.0)		2 (4.5)	1 (1.3)		2 (11.8)	1 (1.0)	
No	27 (90.0)	89 (100.0)		42 (95.5)	74 (98.7)		15 (88.2)	101 (99.0)	

Table IV. Continued.

Characteristic	*EGFR* amplification			*HER2* amplification			*EGFR* and *HER2* co-amplification		
	P (n=30) No. (%)	N (n=89) No. (%)	P-value	P (n=44) No. (%)	N (n=75) No. (%)	P-value	P (n=17) No. (%)	N (n=102) No. (%)	P-value
Distant metastasis			0.011[b]			0.006[b]			<0.001[b]
Yes	7 (23.3)	5 (5.6)		9 (20.5)	3 (4.0)		7 (41.2)	5 (4.9)	
No	23 (76.7)	85 (94.4)		35 (79.5)	72 (96.0)		10 (58.8)	97 (95.1)	

[a]Number differences reflect missing data. [b]Statistically significant. *EGFR*, epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone receptor; *HER*, human epidermal growth factor receptor. P, amplification patients; N, non-amplification patients.

Table V. Prevalence of epidermal growth factor receptor and human epidermal growth factor receptor 2 co-amplification and treatment response.

Treatment	No. (%)	Disease-free survival	
		Log-rank	P-value
Chemotherapy	117		
Co-amplification	17 (14.5)	22.219	<0.001[a]
No co-amplification	100 (85.5)		
Radiotherapy	40	15.694	<0.001[a]
Co-amplification	6 (15.0)		
No co-amplification	34 (85.0)		
Hormonal therapy	74	13.330	0.001[a]
Co-amplification	9 (12.2)		
No co-amplification	65 (87.8)		

[a]Statistically significant.

co-amplification group vs. the no co-amplification group were analyzed for DFS and OS. In the present study, *EGFR* and *HER2* co-amplification was observed to be correlated with both DFS (P<0.001) and OS (P=0.002) (Fig. 3B and D). DFS and OS were also calculated by Kaplan-Meier analysis for triple-negative breast cancer (TNBC) (ER^-, PR^- and $HER2^-$) (19) in the present study as the control. The TNBC group did not exhibit any significant difference with the non-TNBC group for DFS (P=0.538) or OS (P=0.633) (data not show).

Furthermore, multivariate analysis indicated that *EGFR* and *HER2* co-amplification was associated with both DFS (co-amplification vs. no co-amplification; hazard ratio, 10.145; 95% confidence interval, 2.820-36.499; P<0.001) and OS (co-amplification vs. no co-amplification; hazard ratio, 51.564; 95% confidence interval, 1.467-1,890.000; P=0.032) (data not shown). Concerning the treatment regimen, *EGFR* and *HER2* co-amplification patients were significantly correlated with poor DFS regarding chemotherapy (P<0.001), radiotherapy (P<0.001) and hormonal therapy (P=0.001) (Table V).

Discussion

The gene copy number of ErbB family members has been determined in a group of 119 IDC patients with an average follow-up of 27.0 months, and has been compared with clinicopathological features. The reliability of all of the amplification-positive tumors for *EGFR* and *HER2* was confirmed by FISH. Of the four detected ErbB family members of IDC in the present study, 14.2% (17/119) represented an *EGFR* and *HER2* co-amplification subgroup. This subgroup was significantly correlated with a higher possibility of metastasis (when diagnosed) (P=0.014) and distant metastasis (following initial surgery) (P<0.001), while they were almost significantly associated with local recurrence (P=0.053). *EGFR* and *HER2* co-amplification was noticed to be significantly associated with DFS (P<0.001) and OS (P=0.002). Concerning the treatment regimen, *EGFR* and *HER2* co-amplification patients were significantly correlated with poor DFS regarding chemotherapy (P<0.001), radiotherapy

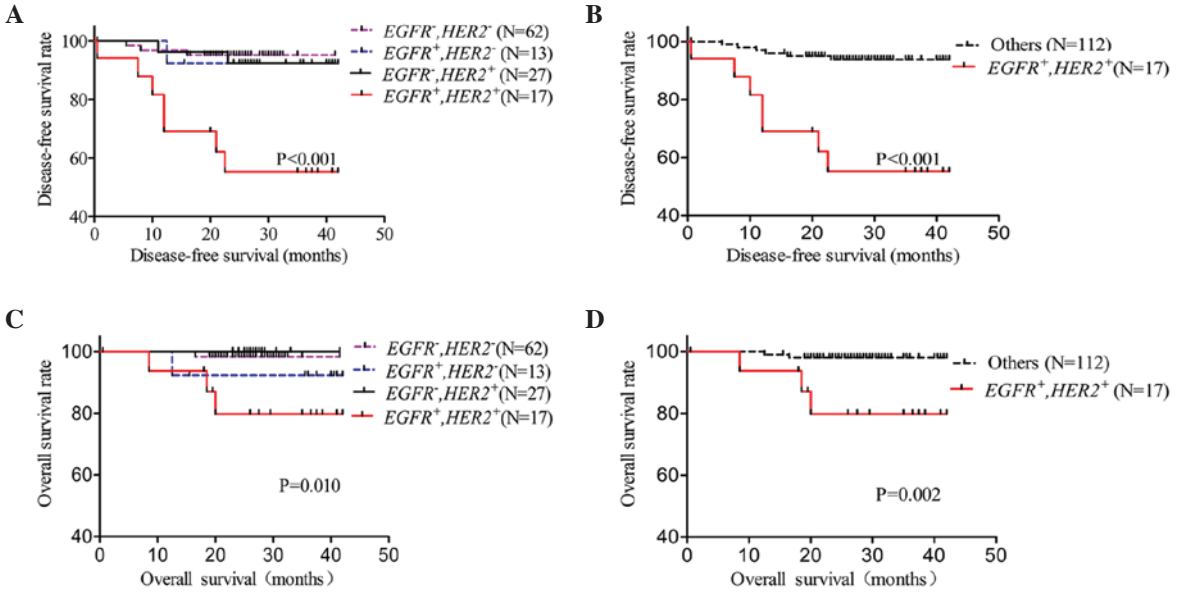

Figure 3. DFS and OS according to *EGFR* and *HER2* gene amplification. Association of *EGFR* and *HER2* gene amplification with prognosis in invasive ductal carcinoma calculated by the log-rank test and shown by Kaplan-Meier curves. The 119 breast cancer patients were divided into four groups: Nor *EGFR* or *HER2* amplification (*EGFR*$^-$ and *HER2*$^-$); *EGFR* amplification but no *HER2* amplification (*EGFR*$^+$ and *HER2*$^-$); no *EGFR* amplification but *HER2* amplification (*EGFR*$^-$ and *HER2*$^+$); and *EGFR* and *HER2* co-amplification (*EGFR*$^+$ and *HER2*$^+$). (A) Univariate survival analysis of DFS was performed in patients with *EGFR* and *HER2* gene amplification. (B) Differences in DFS between the *EGFR* and *HER2* co-amplification group vs. the no co-amplification group (others) were analyzed. (C) Univariate survival analysis of OS was performed in patients with *EGFR* and *HER2* gene amplification. (D) Differences in OS between the *EGFR* and *HER2* co-amplification group vs. the no co-amplification group (others) were analyzed for DFS. *EGFR*, epidermal growth factor receptor; *HER*, human epidermal growth factor receptor; DFS, disease-free survival; OS, overall survival.

(P<0.001) and hormonal therapy (P=0.001). Thus, *EGFR* and *HER2* co-amplification may be an independent prognostic indicator of poor DFS and OS.

In the present study, the *EGFR* amplification rate was 25.2%, a value similar to that reported in previous studies (7.9-33.1%) (10,17,20). The rate of *HER2* amplification (36.9%) in the present study was higher than that reported in previous studies (21-26), suggesting that the frequency of *HER2* may vary according to the different detection methods (*e.g.* qPCR-based methods vs. FISH-based assays). According to the FISH assay, the *HER2* status can be classified as non-amplified (HER2/CEP17 ratio <1.8), amplified (HER2/CEP17 ratio >2.2) or equivocal (1.8 <HER2/CEP17 <2.2). However, qPCR-based assays can only identify certain patients as equivocally amplified cases (22). Thus, by combining qPCR-based and FISH assays, more *HER2*-amplified cases were identified, which revealed that certain FISH equivocal patients were actually amplified cases (22). A rate of *EGFR* and *HER2* co-amplification of 14.2% (17/119) was detected in the present study, but no *HER3* amplification was detected. Only one patient had *HER4* amplification in the present study. Previous reports had mentioned the frequency of *HER3* and *HER4* in breast cancer. However, this frequency varies depending on the cut-off value for FISH, ddPCR and NGS-based assays. The cut-off value is difficult to determine. For *HER2* FISH assay, the cut-off value was not well defined, and the cut-off values for *HER3* and *HER4* amplification were not defined either. There were almost no patients in whom the *HER3* and *HER4* FISH ratio was >2.0 (12).

It was previously shown that *EGFR* or *HER2* amplification was an independent poor clinical prognostic indicator in breast cancer (10,23,24). However, to date, no study has been reported concerning *EGFR* and *HER2* co-amplification in breast cancer. The present study further investigated the association between *EGFR* and *HER2* co-amplification with the clinical prognosis of breast cancer. The present study confirmed the association of *HER2* amplification with HER2 overexpression (Table IV) (25). In the current study, *HER2* amplification was also significantly associated with DFS and OS, as has been reported previously (3,24). It was also confirmed that a variable *EGFR* copy number can be useful for predicting outcomes in patients (8,10). Certain clinicopathological analyses of ErbB family receptors in breast cancer were limited to single ErbB family members (8,27-32). The present study detected gene amplification of four members of the ErbB family, and observed that *EGFR* and *HER2* co-amplification in the present study was significantly associated with short DFS and OS (Fig. 3). When analyzing the co-amplification subgroup with chemotherapy, radiotherapy and hormonal therapy, it was observed that the co-amplification subgroup was significantly correlated with DFS. However, due to the relatively short follow-up, the association between the co-amplification subgroup and OS regarding the treatment regimens could not be determined.

To assess the prognostic value of *EGFR* and *HER2* co-amplification in breast cancer patients, the present study analyzed DFS and OS for this subgroup of patients. Another classification by expression profile, *e.g.* TNBC patients, were also analyzed as a control (19). In the present study, *EGFR* and *HER2* co-amplification exhibited a significant difference compared with the non-co-amplified group for both DFS and OS, but the TNBC group did not show any significant difference compared with the non-TNBC group for DFS in such a relatively short follow-up period (26,33,34). This result suggests that *EGFR* and *HER2* co-amplification can be considered to indicate a poor prognosis.

Resistance to treatment regiments, including chemotherapy, radiotherapy, hormonal therapy and target therapy, is a nearly universal and ultimately lethal consequence for breast cancer patients (35-37). Numerous theories have attempted to explain drug resistance during treatment, including the cancer stem cell theory, the epithelial-mesenchymal transition theory and certain somatic tumor cell mutations (38-41). Since *EGFR* and *HER2* co-amplified tumor cells were abnormal in the corresponding signaling pathway, the patients may respond differently to treatment regimens (5,42). In the present study, patients with *EGFR* and *HER2* co-amplification exhibited poor clinical outcome for both DFS and OS. Notably, this is also true for DFS with respect to treatment regimen for chemotherapy (P<0.001), radiotherapy (P<0.001) and hormonal therapy (P=0.001). However, the response to current treatment of this group of patients requires further detailed studies. In addition, the present study explored the response to target therapy of this subgroup of patients, including Herceptin treatment for *HER2*-amplified patients. Only 8 patients in the present study received Herceptin treatment. Of these, 3 patients were *EGFR* and *HER2* co-amplified. Although all 3 patients exhibited distant metastasis following initial surgery, there are not statistically significant data showing resistance to Herceptin treatment in *EGFR* and *HER2* co-amplified patients due to the limited number of patients included in the present study. The other 5 patients who received target therapy were not co-amplified patients, who did not show any recurrence or distant metastasis in a mean of 26.2 months of follow-up. All the 8 patients exhibited *HER2* amplification and HER2 over-expression (3+), but there were no other statistically significant deferences between the two groups. Further studies on the response to different treatment of this particular subgroup of patients should be carried out, although the present data strongly suggest that *EGFR* and *HER2* co-amplified cancer cells may be the cell source responsible for drug resistance.

In summary, the present study detected ErbB family gene amplification using qPCR and FISH, and the results suggested that *EGFR* and *HER2* co-amplification has a considerable prognostic relevance regarding clinical outcomes in breast cancer. *EGFR* and *HER2* co-amplification may be a novel particular subgroup in IDC that can be considered predictive of poor clinical outcomes. Regarding treatment regimen analysis, the results of the present study indicate that patients with *EGFR* and *HER2* co-amplification exhibit resistance to chemotherapy, radiotherapy and hormonal therapy. Specific treatment regimens may be required for this particular subgroup of patients.

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