SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 14: 6562-6570, 2017

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

PENG GUO[1], TIANJIE PU[1,2], SHINAN CHEN[1], YAN QIU[1,2], XIAORONG ZHONG[3,4], HONG ZHENG[3,4], LINA CHEN[5], HONG BU[1,2] and FENG YE[1]

[1]Laboratory of Pathology; [2]Department of Pathology; [3]Cancer Center; [4]Laboratory of Molecular Diagnosis of Cancer, State Key Laboratory of Biotherapy, National Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University; [5]Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China

Received August 14, 2015; Accepted February 28, 2017

DOI: 10.3892/ol.2017.7051

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.

Correspondence to: Professor Feng Ye, Laboratory of Pathology, West China Hospital, Sichuan University, Guo Xue Xiang 37 Hao, Chengdu, Sichuan 610041, P.R. China
E-mail: fengye@scu.edu.cn

Key words: breast neoplasms, *EGFR*, *HER2*, gene amplification, metastasis, prognosis

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.

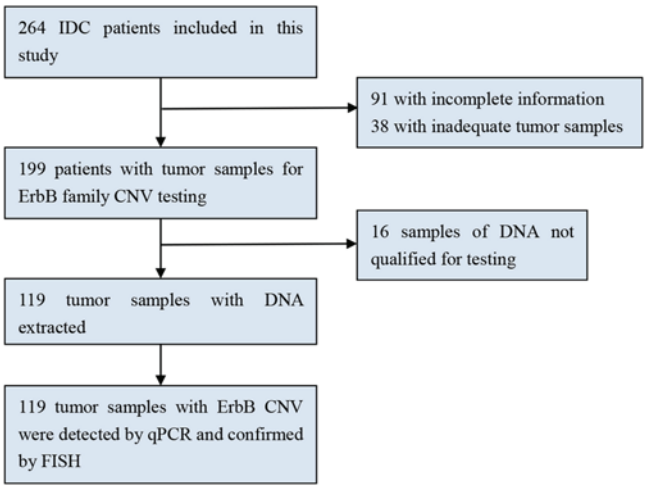

Figure 1. Flowchart showing the inclusion criteria and results of the present study. IDC, invasive ductal carcinoma; qPCR, quantitative polymerase chain reaction; FISH, fluorescence *in situ* hybridization; CNV, copy-number variations.

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.

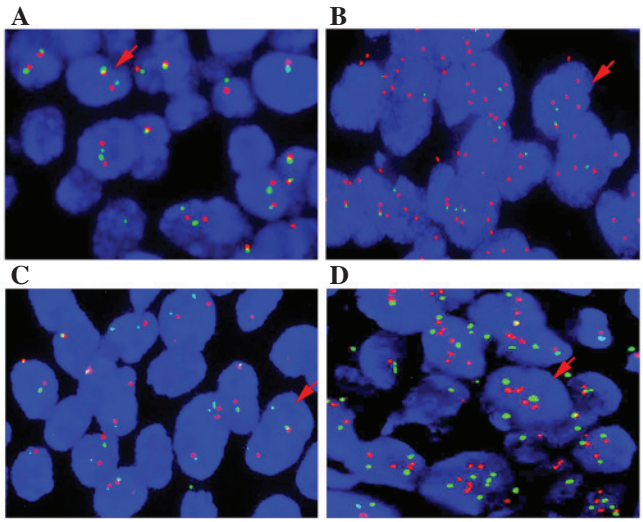

Figure 2. *EGFR* and *HER2* amplification by FISH. FISH shows *EGFR* and *HER2* gene amplification in invasive ductal carcinoma patients. Signals of the *EGFR* probe are illustrated in red, while signals of the centromere 7 probe are shown in green. The arrow focuses on a representative cell, with a 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.

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
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)	

SPANDIDOS PUBLICATIONS
ONCOLOGY LETTERS 14: 6562-6570, 2017

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

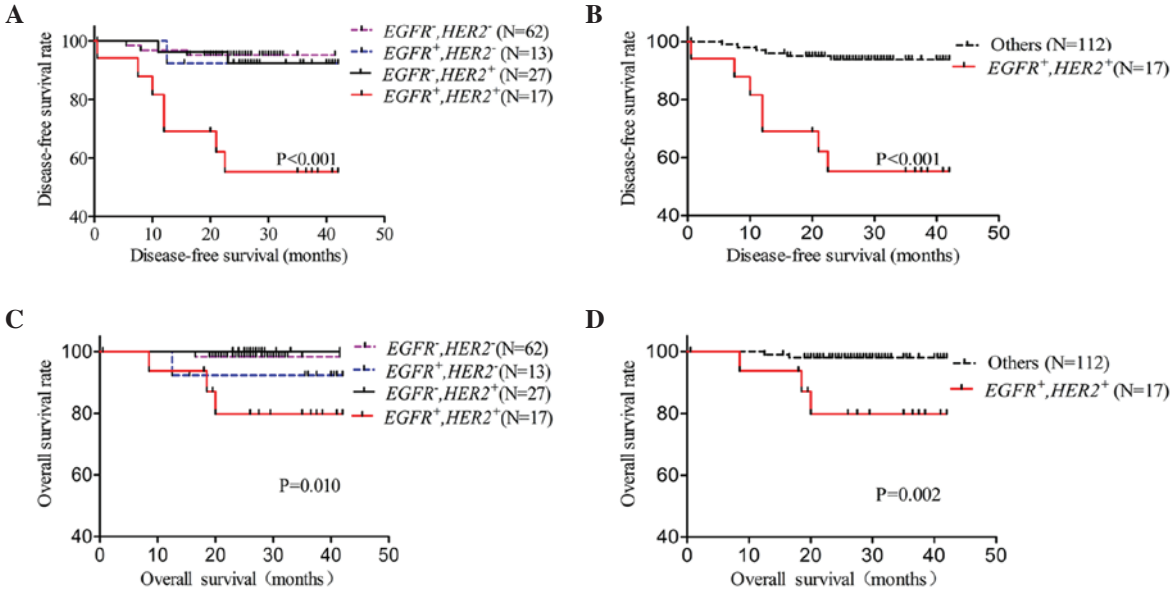

Figure 3. DFS and OS according to *EGFR* and *HER2* gene amplification. Association of *EGFR* and *HER2* gene amplification with prognosis in invasive ductal carcinoma calculated by the log-rank test and shown by Kaplan-Meier curves. The 119 breast cancer patients were divided into four groups: Nor *EGFR* 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.

Acknowledgements

The present study was funded by grants from the National Natural Science Foundation of China (grant nos. 31000601 and 81200461) and the Young Investigator Scholarship in Sichuan University (grant no. 2012SCU04A14).

References

1. World Health Organization: Cancer Country Profiles, 2014. http://www.who.int/cancer/country-profiles/chn_en.pdf.
2. Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. Nature 490: 61-70, 2012.
3. Callahan R: Genetic alterations in primary breast cancer. Breast Cancer Res Treat 13: 191-203, 1989.
4. Eccles SA: The role of c-erbB-2/HER2/neu in breast cancer progression and metastasis. J Mammary Gland Biol Neoplasia 6: 393-406, 2001.
5. Lemmon MA: The EGF receptor family as therapeutic targets in breast cancer. Breast Dis 18: 33-43, 2003.
6. Vasan N, Yelensky R, Wang K, Moulder S, Dzimitrowicz H, Avritscher R, Wang B, Wu Y, Cronin MT, Palmer G, *et al*: A targeted next-generation sequencing assay detects a high frequency of therapeutically targetable alterations in primary and metastatic breast cancers: Implications for clinical practice. Oncologist 19: 453-458, 2014.
7. Chong CR and Jänne PA: The quest to overcome resistance to EGFR-targeted therapies in cancer. Nat Med 19: 1389-1400, 2013.
8. Park HS, Jang MH, Kim EJ, Kim HJ, Lee HJ, Kim YJ, Kim JH, Kang E, Kim SW, Kim IA and Park SY: High EGFR gene copy number predicts poor outcome in triple-negative breast cancer. Mod Pathol 27: 1212-1222, 2014.
9. Cho EY, Choi YL, Han JJ, Kim KM and Oh YL: Expression and amplification of Her2, EGFR and cyclin D1 in breast cancer: Immunohistochemistry and chromogenic in situ hybridization. Pathol Int 58: 17-25, 2008.
10. Lv N, Xie X, Ge Q, Lin S, Wang X, Kong Y, Shi H, Xie X and Wei W: Epidermal growth factor receptor in breast carcinoma: Association between gene copy number and mutations. Diagn Pathol 6: 118, 2011.
11. Lee HJ, Seo AN, Kim EJ, Jang MH, Kim YJ, Kim JH, Kim SW, Ryu HS, Park IA, Im SA, *et al*: Prognostic and predictive values of EGFR overexpression and EGFR copy number alteration in HER2-positive breast cancer. Br J Cancer 112: 103-111, 2015.
12. Sassen A, Rochon J, Wild P, Hartmann A, Hofstaedter F, Schwarz S and Brockhoff G: Cytogenetic analysis of HER1/EGFR, HER2, HER3 and HER4 in 278 breast cancer patients. Breast Cancer Res 10: R2, 2008.
13. Zaczek A, Wełnicka-Jaśkiewicz M, Bielawski KP, Jaśkiewicz J, Badzio A, Olszewski W, Rhone P and Jassem J: Gene copy numbers of HER family in breast cancer. J Cancer Res Clin Oncol 134: 271-279, 2008.
14. Livak and Schmittgen: Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 25: 402-408, 2001.
15. Gjerdrum LM, Sorensen BS, Kjeldsen E, Sorensen FB, Nexo E and Hamilton-Dutoit S: Real-time quantitative PCR of micro-dissected paraffin-embedded breast carcinoma: An alternative method for HER-2/neu analysis. J Mol Diagn 6: 42-51, 2004.
16. Bernardi CC, Ribeiro Ede S, Cavalli IJ, Chautard-Freire-Maia EA and Souza RL: Amplification and deletion of the ACHE and BCHE cholinesterase genes in sporadic breast cancer. Cancer Genet Cytogenet 197: 158-165, 2010.
17. Park K, Han S, Shin E, Kim HJ and Kim JY: EGFR gene and protein expression in breast cancers. Eur J Surg Oncol 33: 956-960, 2007.
18. Burkhardt L, Grob TJ, Hermann I, Burandt E, Choschzick M, Jänicke F, Müller V, Bokemeyer C, Simon R, Sauter G, *et al*: Gene amplification in ductal carcinoma in situ of the breast. Breast Cancer Res Treat 123: 757-765, 2010.
19. Bauer KR, Brown M, Cress RD, Parise CA and Caggiano V: Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: A population-based study from the California cancer Registry. Cancer 109: 1721-1728, 2007.
20. Brandt B, Vogt U, Schlotter CM, Jackisch C, Werkmeister R, Thomas M, von Eiff M, Bosse U, Assmann G and Zänker KS: Prognostic relevance of aberrations in the erbB oncogenes from breast, ovarian, oral and lung cancers: Double-differential polymerase chain reaction (ddPCR) for clinical diagnosis. Gene 159: 35-42, 1995.
21. Nanda R: Targeting the human epidermal growth factor receptor 2 (HER2) in the treatment of breast cancer: Recent advances and future directions. Rev Recent Clin Trials 2: 111-116, 2007.
22. Belgrader P, Tanner SC, Regan JF, Koehler R, Hindson BJ and Brown AS: Droplet digital PCR measurement of HER2 copy number alteration in formalin-fixed paraffin-embedded breast carcinoma tissue. Clin Chem 59: 991-994, 2013.
23. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182, 1987.

24. Menard S, Fortis S, Castiglioni F, Agresti R and Balsari A: HER2 as a prognostic factor in breast cancer. Oncology 61 (Suppl 2): S67-S72, 2001.
25. Hoang MP, Sahin AA, Ordòñez NG and Sneige N: HER-2/neu gene amplification compared with HER-2/neu protein over-expression and interobserver reproducibility in invasive breast carcinoma. Am J Clin Pathol 113: 852-859, 2000.
26. Bhatti AB, Khan AI, Siddiqui N, Muzaffar N, Syed AA, Shah MA and Jamshed A: Outcomes of triple-negative versus non-triple-negative breast cancers managed with breast-conserving therapy. Asian Pac J Cancer Prev 15: 2577-2581, 2014.
27. Stern DF: Tyrosine kinase signalling in breast cancer: ErbB family receptor tyrosine kinases. Breast Cancer Res 2: 176-183, 2000.
28. Ge H, Gong X and Tang CK: Evidence of high incidence of EGFRvIII expression and coexpression with EGFR in human invasive breast cancer by laser capture microdissection and immunohistochemical analysis. Int J Cancer 98: 357-361, 2002.
29. Olayioye MA: Update on HER-2 as a target for cancer therapy: Intracellular signaling pathways of ErbB2/HER-2 and family members. Breast Cancer Res 3: 385-389, 2001.
30. Ellis MJ: Neoadjuvant endocrine therapy for breast cancer: Medical perspectives. Clin Cancer Res 7: s4388-s4391, 2001.
31. Lemoine NR, Barnes DM, Hollywood DP, Hughes CM, Smith P, Dublin E, Prigent SA, Gullick WJ and Hurst HC: Expression of the ERBB3 gene product in breast cancer. Br J Cancer 66: 1116-1121, 1992.
32. Kew TY, Bell JA, Pinder SE, Denley H, Srinivasan R, Gullick WJ, Nicholson RI, Blamey RW and Ellis IO: c-erbB-4 protein expression in human breast cancer. Br J Cancer 82: 1163-1170, 2000.
33. Nguyen PL, Taghian AG, Katz MS, Niemierko A, Abi Raad RF, Boon WL, Bellon JR, Wong JS, Smith BL and Harris JR: Breast cancer subtype approximated by estrogen receptor, progesterone receptor and HER-2 is associated with local and distant recurrence after breast-conserving therapy. J Clin Oncol 26: 2373-2378, 2008.
34. Li CY, Zhang S, Zhang XB, Wang P, Hou GF and Zhang J: Clinicopathological and prognostic characteristics of triple-negative breast cancer (TNBC) in Chinese patients: A retrospective study. Asian Pac J Cancer Prev 14: 3779-3784, 2013.
35. Diver EJ, Foster R, Rueda BR and Growdon WB: The therapeutic challenge of targeting HER2 in endometrial cancer. Oncologist 20: 1058-1068, 2015.
36. Feldinger K and Kong A: Profile of neratinib and its potential in the treatment of breast cancer. Breast Cancer (Dove Med Press) 7: 147-162, 2015.
37. Black JC, Atabakhsh E, Kim J, Biette KM, Van Rechem C, Ladd B, Burrowes PD, Donado C, Mattoo H, Kleinstiver BP, *et al*: Hypoxia drives transient site-specific copy gain and drug-resistant gene expression. Genes Dev 29: 1018-1031, 2015.
38. Wang S, Mou Z, Ma Y, Li J, Li J, Ji X, Wu K, Li L, Lu W and Zhou T: Dopamine enhances the response of sunitinib in the treatment of drug-resistant breast cancer: Involvement of eradicating cancer stem-like cells. Biochem Pharmacol 95: 98-109, 2015.
39. Chiotaki R, Polioudaki H and Theodoropoulos PA: Cancer stem cells in solid and liquid tissues of breast cancer patients: Characterization and therapeutic perspectives. Curr Cancer Drug Targets 15: 256-269, 2015.
40. Zhang P, Sun Y and Ma L: ZEB1: At the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. Cell Cycle 14: 481-487, 2015.
41. Jaspers JE, Sol W, Kersbergen A, Schlicker A, Guyader C, Xu G, Wessels L, Borst P, Jonkers J and Rottenberg S: BRCA2-deficient sarcomatoid mammary tumors exhibit multidrug resistance. Cancer Res 75: 732-741, 2015.
42. Montemurro F and Scaltriti M: Biomarkers of drugs targeting HER-family signalling in cancer. J Pathol 232: 219-229, 2014.