Modern Pathology (2014) 27, 1212–1222

High *EGFR* gene copy number predicts poor outcome in triple-negative breast cancer

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Epidermal growth factor receptor (EGFR) is frequently overexpressed in triple-negative breast cancer and is emerging as a therapeutic target. *EGFR* gene copy number alteration and mutation are highly variable and scientists have been challenged to define their prognostic significance in triple-negative breast cancer. We examined EGFR protein expression, *EGFR* gene copy number alteration and mutation of exon 18 to 21 in 151 cases of triple-negative breast cancer and correlated these findings with clinical outcomes. In addition, intratumoral agreement of EGFR protein overexpression and gene copy number alteration was evaluated. EGFR overexpression was found in 97 of 151 cases (64%) and high *EGFR* gene copy number was detected in 50 cases (33%), including 3 gene amplification (2%) and 47 high polysomy (31%). Five *EGFR* mutations were detected in 4 of 151 cases (3%) and included G719A in exon 18 ($n=1$), V786M in exon 20 ($n=1$), and L858R in exon 21 ($n=3$). One case had two mutations (G719A and L858R). High *EGFR* copy number, but not *EGFR* mutation, correlated with EGFR protein overexpression. Intratumoral heterogeneity of EGFR protein overexpression and *EGFR* copy number alteration was not significant. In survival analyses, high *EGFR* copy number was found to be an independent prognostic factor for poor disease-free survival in patients with triple-negative breast cancer. Our findings showed that *EGFR* mutation was a rare event, but high *EGFR* copy number was relatively frequent and correlated with EGFR overexpression in triple-negative breast cancer. Moreover, high *EGFR* copy number was associated with poor clinical outcome in triple-negative breast cancer, suggesting that evaluation of *EGFR* copy number may be useful for predicting outcomes in patients with triple-negative breast cancer and for selecting patients for anti-EGFR-targeted therapy.
Modern Pathology (2014) **27,** 1212–1222; doi:10.1038/modpathol.2013.251; published online 10 January 2014

Keywords: breast carcinoma; copy number gain; EGFR; fluorescence *in situ* hybridization; mutation

Introduction

Triple-negative breast cancer is a subtype of breast cancer characterized by the absence of expression of estrogen and progesterone receptors and human epidermal growth factor receptor 2 (HER2), accounting for 10–20% of all breast cancers.[1–3] Histopathologically, the majority of triple-negative breast cancers are high-grade invasive carcinomas of no special type, metaplastic carcinomas, and medullary carcinomas.[3] Triple-negative breast cancer carries a poorer prognosis than other subtypes of invasive breast cancer.[1] It is relatively sensitive to chemotherapy, but early relapse and visceral metastases are common and overall survival remains poor.[4,5] In addition, current systemic therapeutic options for triple-negative breast cancer are limited to conventional cytotoxic chemotherapy, whereas non-triple-negative breast cancer, that is, hormone receptor-positive, or HER2-positive breast cancer may have benefit from anti-hormonal or HER2-targeted therapy. The search for specific molecular targets in triple-negative breast cancer and the development of therapeutics for these targets are ongoing.

Epidermal growth factor receptor (EGFR) alterations have been implicated in the pathogenesis and

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Received 15 August 2013; revised 15 October 2013; accepted 16 October 2014; published online 10 January 2014

progression of many malignancies including non-small cell lung cancer[6,7] and glioblastoma.[8] EGFR is a well-established treatment target for colorectal cancer, non-small cell lung cancer, and squamous cell carcinoma of the head and neck. In breast cancer, EGFR overexpression has been reported in up to 78% of triple-negative breast cancers,[9–20] more than in non-triple-negative breast cancers,[12,15] suggesting that EGFR is a potential therapeutic target for triple-negative breast cancer. EGFR tyrosine kinase inhibitors have yielded insignificant response rates in breast cancer,[21–23] possibly due to the lack of patient selection in these studies; they were not restricted to breast cancers with EGFR overexpression or triple-negative breast cancers. Recently, however, EGFR-targeting monoclonal antibody, cetuximab, has improved outcomes in triple-negative breast cancers.[24]

EGFR gene amplification, one of the mechanisms of EGFR overexpression, is highly variable and found in up to 24% of triple-negative breast cancer.[10,11,13,14,19,25] *EGFR* gene mutation, another mechanism of EGFR overexpression, has been reported to be rare,[11,17,19,25,26] although a recent study reported that it was present in 11% of triple-negative breast cancers.[16] EGFR immunoreactivity has been presented as an independent indicator of poor prognosis in patients with triple-negative breast cancer.[18,20] However, there have been no reports on the prognostic impact of *EGFR* copy number alteration or mutation in triple-negative breast cancer. Intratumoral heterogeneity of EGFR protein overexpression and copy number alteration has also not been studied in triple-negative breast cancer, although it may be associated with responsiveness to EGFR-targeted therapy.

The primary goals of this study were to (1) evaluate the rates and prognostic significance of *EGFR* copy number alteration and mutation in triple-negative breast cancers and (2) assess intratumoral heterogeneity of EGFR protein overexpression and copy number alteration.

Materials and methods

Patients and Tissue Samples

We retrospectively examined the records of the Department of Pathology, Seoul National University Bundang Hospital from 2003 to 2011 and searched for cases of invasive triple-negative breast cancer using immunohistochemical data for standard biomarkers. Estrogen and progesterone receptors were regarded as negative if there were $<1\%$ positive tumor nuclei.[27] Expression of HER2 was scored according to 2007 American Society of Clinical Oncology/College of American Pathologist guidelines[28] and immunohistochemical scores of 0 or 1+ were regarded as negative. For the equivocal (2+) cases, HER2 negative status was confirmed by fluorescence *in situ* hybridization (FISH). After excluding cases with initial metastases, we selected 151 invasive triple-negative breast cancers from cases of surgically resected primary breast cancer. Baseline patient characteristics are summarized in Table 1. Hematoxylin- and eosin-stained slides were reviewed for each case, and the following histopathologic variables were determined: histologic subtype, T stage, nodal status, Nottingham combined histologic grade, venous invasion, lymphatic invasion, tumor border, and presence or absence of ductal carcinoma *in situ* component. All cases were independently reviewed by two breast pathologists (SYP and HJL). The study was approved by the institutional review board of Seoul

Table 1 Baseline characteristics of 151 patients with triple-negative breast cancer

Characteristics	*Number (%)*
Age, years	
Median	54
Range	25–87
Sex	
Female	151 (100)
Male	0
Stage	
I	47 (31)
II	88 (58)
III	16 (11)
T stage	
T1	58 (38)
T2	85 (56)
T3	6 (4)
T4	2 (1)
N stage	
N0	111 (74)
N1	25 (17)
N2	8 (5)
N3	7 (5)
Histologic subtype	
Invasive carcinoma of no special type	137 (91)
Metaplastic carcinoma	12 (8)
Medullary carcinoma	2 (1)
Histologic grade	
I	0
II	10 (7)
III	141 (93)
Ki-67 proliferation index	
$<50\%$	74 (49)
$\geq 50\%$	77 (51)
P53 overexpression	
Absent	67 (44)
Present	84 (56)
Basal-like phenotype[a]	
Absent	19 (13)
Present	132 (87)

[a]As documented by immunoreactivity of CK5/6 and/or EGFR.

National University Bundang Hospital (IRB No. B-1005/100-303), which waived the requirement for informed consent.

Tissue Microarray Construction

We used tissue microarrays to evaluate EGFR protein expression and *EGFR* copy number alteration. All slides were reviewed and the most representative tumor section was selected for each case. Tissue microarrays were conducted in two different ways. At first, we constructed large core (4-mm diameter) tissue microarrays (Superbiochips Laboratories, Seoul, Korea) using 42 cases of triple-negative breast cancer to test EGFR protein overexpression and gene copy number. We then constructed tissue microarrays from three different representative tissue cores (2-mm diameter) of 109 triple-negative breast cancers to evaluate the heterogeneity of EGFR protein expression and copy number alteration.

Immunohistochemical Analyses and Scoring

Expression of standard biomarkers including estrogen receptor, progesterone receptor, HER2, p53, and Ki-67 was evaluated in full sections at the time of diagnosis or in tissue microarray sections for missing data during the study. EGFR and cytokeratin 5/6 were evaluated using tissue microarrays. Tissue sections (4 μm) were cut, dried, deparaffinized, and rehydrated following standard procedures. EGFR expression was detected by using EGFR pharmDx^{TM} (Dako). Immunohistochemical staining for the other biomarkers was performed in a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ) using an i-View detection kit (Ventana Medical Systems) for estrogen receptor (1:100; clone SP1; Labvision), progesterone receptor (1:70; PgR 636; Dako), HER2 (1:700; polyclonal; Dako), p53 (1:600; D07; Dako), Ki-67 (1:250; MIB-1; Dako), and cytokeratin 5/6 (1:50; clone D5/16 B4; Dako). EGFR expression was scored as follows: 0, no staining or weak membranous staining in $<10\%$ of the tumor cells; $1+$, weak membranous staining in $\geq 10\%$ of the tumor cells; $2+$, moderate, membranous staining in $\geq 10\%$ of the tumor cells; $3+$, strong membranous staining in $\geq 10\%$ of the tumor cells. Both complete and incomplete membranous staining was accepted, and $2+$ or more staining was considered to represent EGFR overexpression. If the tissue microarray cores yielded a different score, the highest score for the case was used. For cytokeratin 5/6, cases with any positive membranous staining were defined as positive. For p53, cases with 10% or more positive staining were grouped as positive. For the Ki-67 proliferation index, cases with 50% or more positive tumor cells were regarded as having high indices.

FISH Assays for *EGFR*

To evaluate *EGFR* copy number alteration, we performed FISH on tissue microarray samples with commercially available locus-specific and chromosome enumeration probes (CEPs) (LSI EGFR SpectrumOrange probe (7p12) and CEP 7 SpectrumGreen probe (7p11.1-q11.1)) (Abbott Molecular, Des Plaines, IL).

FISH was performed as reported for *HER2* amplification.[29] Briefly, 4-μm deparaffinized tissue microarray sections were incubated in pretreatment solution (Abbott Molecular) at 80 °C for 30 min, then in protease solution (Abbott Molecular) for 25 min at 37 °C. Probes were diluted in tDen-Hyb-2 hybridization buffer (InSitus Biotechnologies, Albuquerque, NM). Co-denaturation of the probes and DNA was achieved by incubating at 75 °C for 5 min in a HYBrite^{TM} (Abbott Molecular) followed by 16-h hybridization at 37 °C. Post-hybridization washes were performed according to supplier protocols. Slides were mounted in 4′,6-diamidino-2-phenylindole/anti-fade and viewed with a fluorescence microscope.

At least 50 non-overlapping tumor cells were evaluated for each tissue microarray core. *EGFR* copy number was classified into six categories, as described previously[30,31]: disomy (≤ 2 copies in $>90\%$ of cells); low trisomy (≤ 2 copies in $\geq 40\%$ of cells, three copies in 10–40% of cells, and ≥ 4 copies in $<10\%$ of cells); high trisomy (≤ 2 copies in $\geq 40\%$ of cells, three copies in $\geq 40\%$ of cells, and ≥ 4 copies in $<10\%$ of cells); low polysomy (≥ 4 copies in 10–40% of cells); high polysomy (≥ 4 copies in $\geq 40\%$ of cells); and gene amplification (presence of tight *EGFR* gene clusters and a ratio of the *EGFR* gene to chromosome 7 of ≥ 2, or ≥ 15 copies of *EGFR* per cell in $\geq 10\%$ of cells). For further analysis, the patients were divided into groups according to *EGFR* copy number as follows: low *EGFR* gene copy number (disomy, low trisomy, high trisomy, and low polysomy) and high *EGFR* gene copy number (high polysomy and gene amplification).

Analysis of *EGFR* Mutation

DNA was extracted from five formalin-fixed, paraffin-embedded tissue sections (10 μm) containing a representative portion of tumor tissue using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA (50 ng) was amplified in a 20-μl reaction containing 10 μl of $2\times$ HotStarTaq Master Mix (Qiagen), including PCR Buffer with 3 mM $MgCl_2$, 400 μM each dNTP, and 0.3 μM of each primer (Exon 18F: 5′-CCA TGT CTG GCA CTG CTT T-3′, 18R: 5′-CAG CTT GCA AGG ACT CTG G-3′; Exon 19F: 5′-TGT GGC ACC ATC TCA CAA TTG-3′, 19R: 5′-GGA CCC CCA CAC AGC AA-3′; Exon 20F: 5′-GGT CCA TGT GCC CCT CCT-3′, 20R: 5′-TGG CTC CTT ATC TCC CCT CC-3′; Exon 21F: 5′-CCA TGA TGA TCT

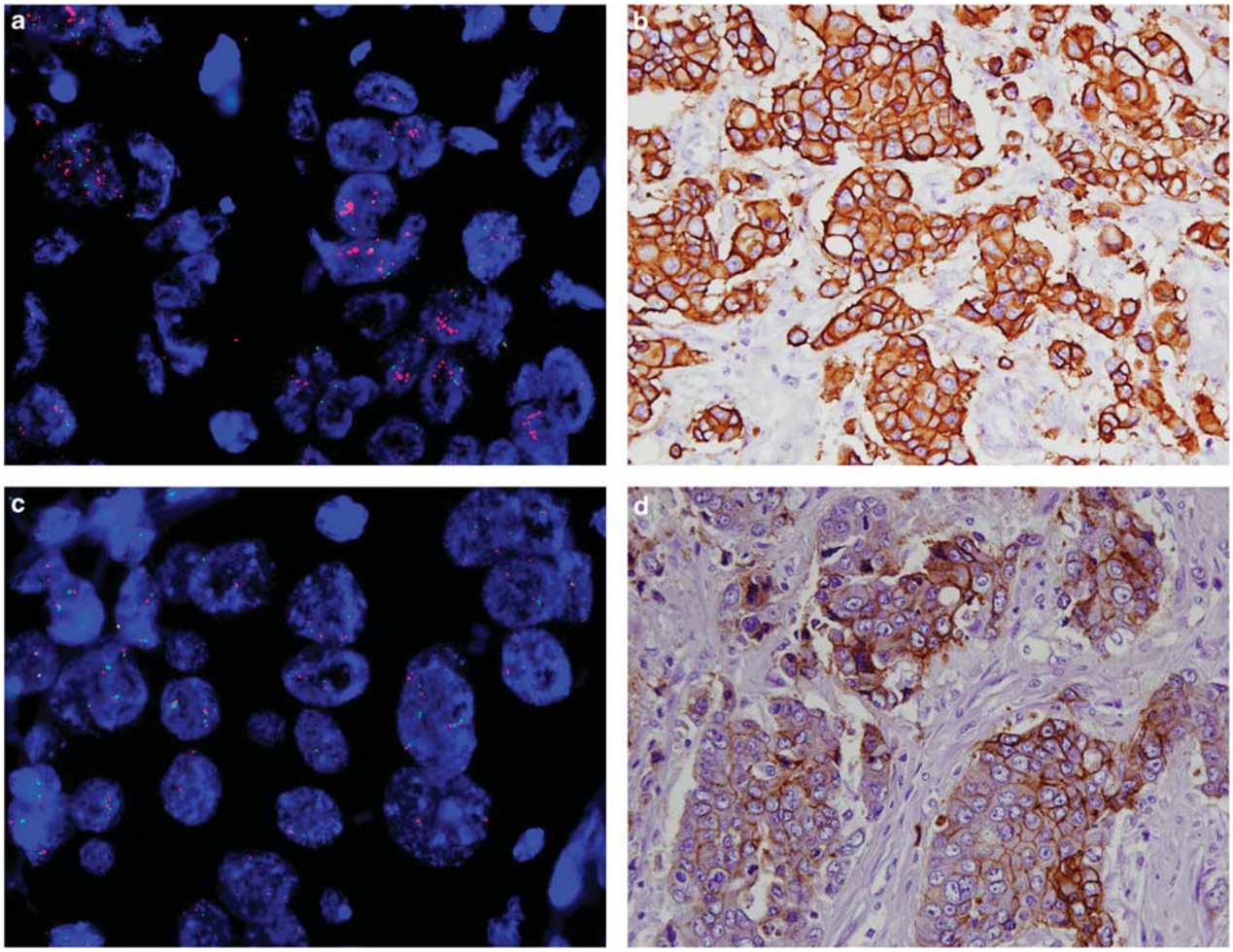

Figure 1 Two representative examples of high *EGFR* copy number. A case of triple-negative breast cancer with *EGFR* amplification (**a**) and strong (3+) EGFR overexpression (**b**). Another case of triple-negative breast cancer with *EGFR* high polysomy (**c**) and strong (3+) EGFR overexpression (**d**).

Table 2 Summary of triple-negative breast cancer patients harboring *EGFR* mutations

				EGFR IHC score			*EGFR FISH*		
Serial no.	*Age*	*pTNM*	*EGFR mutation*	*A core*	*B core*	*C core*	*A core*	*B core*	*C core*
22	27	pT1N3	Exon 21, L858R	0	0	0	LP	LT	D
23	38	pT1N0	Exon 21, L858R	2	2	2	HT	NA	LP
29	87	pT2N0	Exon 20, V786M	3	3	3	HP	HP	HP
37	32	pT3N0	Exon 18, G719A						
			Exon 21, L858R	0	0	0	LP	NA	LT

Abbreviations: IHC, immunohistochemistry; LP, low polysomy; LT, low trisomy; D, disomy; HT, high trisomy; HP, high polysomy; NA, not available due to the failure of FISH.

GTC CCT CA-3′, 21R: 5′-AAT GCT GGC TGA CCT AAA GC-3′). Amplifications of *EGFR* exons 18–21 were performed using a 15-min initial denaturation at 95 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C, and a 10-min final extension at 72 °C. PCR products were purified with a HiYield^{TM}Gel/PCR DNA Extraction Kit (Real Biotech Corporation, Taiwan).

DNA templates were processed for sequencing with ABI-PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster, CA) with both forward and reverse sequence-specific primers. Purified PCR products (20 ng) were used in a 10-μl sequencing reaction containing 1 μl BigDye Terminator v3.1 and 0.1 μM PCR primer. Sequencing reactions were performed using 25 cycles of 10 s at 96 °C, 5 s at

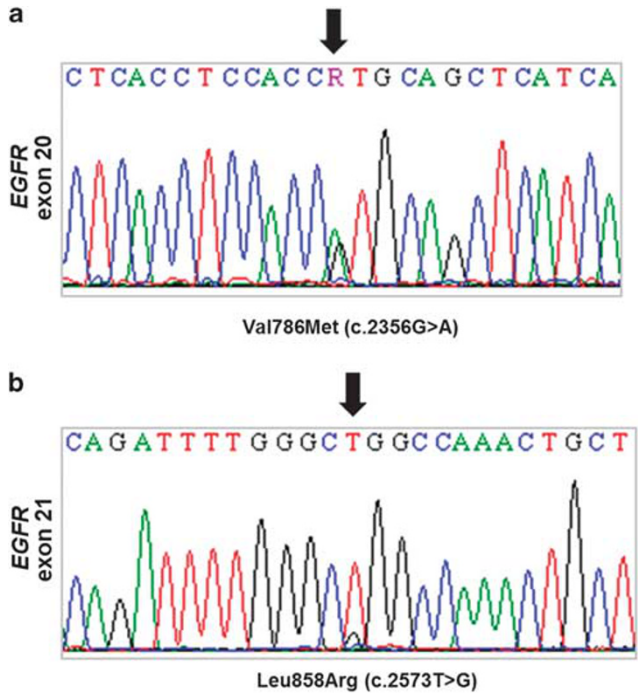

Figure 2 Missense mutations of *EGFR* exon 20 and exon 21. (**a**) Substitution of G to A at mRNA coding nucleotide sequence 2356, resulting in valine to methionine substitution at amino acid codon position 786 (V786M). (**b**) Substitution of T to G at mRNA coding nucleotide sequence 2573, resulting in leucine to arginine substitution at amino acid 858 (L858R).

Table 3 Correlation of EGFR protein expression and copy number alterations in each tissue microarray core

	EGFR FISH		
	Low gene copy number[a] *(%)*	*High polysomy (%)*	*Gene amplification (%)*
EGFR IHC			
0	73 (30)	11 (13)	0
1+	43 (18)	5 (6)	0
2+	90 (37)	32 (38)	0
3+	36 (15)	36 (43)	8 (100)

Abbreviations: IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.
[a]Include disomy, trisomy, and low polysomy; Of the 369 TMA cores (one core for 42 cases and three cores for 109 cases), data of 334 cores were available due to tissue loss, inadequate hybridization or failure of FISH.

50 °C, and 4 min at 60 °C. Sequence data were generated with the ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Sequences were analyzed with Sequencing analysis 5.4. software (Applied Biosystems).

Statistical Analysis

Statistical significance was assessed using Statistical Package, SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL). Concordance of EGFR protein overexpression or *EGFR* copy number alteration in different tissue microarray cores of a tumor were analyzed by the kappa test. The associations of EGFR protein expression or copy number alteration with clinicopathologic tumor characteristics were analyzed by Fisher's exact test or the Chi-square test, depending on test conditions. Survival curves were estimated using the Kaplan–Meier product-limit method, and the significance of differences between survival curves was determined using the log-rank test. Covariates that were statistically significant in the univariate analysis were then included in the multivariate analysis using the Cox proportional hazards regression model, and the hazard ratio and its 95% confidence interval were assessed for each factor. *P*-values <0.05 were considered statistically significant. All reported *P*-values are two-sided.

Results

EGFR Protein Expression, Copy Number Alteration, and mutation

Of the 151 triple-negative breast cancers, 41 cases (27%) were scored as 3+, 56 (37%) were scored as 2+, 24 (16%) were scored as 1+, and the remaining 30 (20%) were scored as 0 by EGFR immunohistochemistry (IHC) (Figure 1). *EGFR* FISH revealed gene amplification in 3 (2%) cases, high polysomy in 47 (31%) cases, low polysomy in 69 (46%) cases, high trisomy in 4 (3%) cases, low trisomy in 25 (17%) cases, and disomy in 3 (2%) cases (Figure 1). *EGFR* mutation was observed in 4 (3%) out of 151 triple-negative breast cancers (Table 2; Figure 2). Three cases had L858R mutation in *EGFR* exon 21, of which one harbored another mutation, G719A in *EGFR* exon 18. The remaining case had a V786M mutation in *EGFR* exon 20, along with *EGFR* high polysomy and strong (3+) EGFR overexpression.

The results of EGFR IHC and *EGFR* FISH were compared in each tissue microarray core from 151 cases. EGFR protein overexpression correlated with *EGFR* gene amplification and high polysomy ($P<0.001$; Table 3). The sensitivity and specificity of EGFR overexpression (immunoreactivity of more than 2+) for high *EGFR* gene copy number were 83% and 48%, respectively. The sensitivity and specificity of EGFR IHC 3+ for *EGFR* gene amplification were 100% and 78%, respectively.

Intratumoral Comparison of EGFR Protein Expression and *EGFR* Copy Number

Of the 109 triple-negative breast cancers with three tissue microarray cores, 86 cases with three different cores and 19 cases with two different cores were available for comparison of EGFR protein expression within a tumor; the remainder was excluded owing

Table 4 Analysis of intratumoral concordance of EGFR protein expression in triple-negative breast cancers

	EGFR IHC, B core			
	0, 1+	*2+, 3+*	*Kappa value*	P-*value*
EGFR IHC, A core				
0, 1+	33	4	0.812	<0.001
2+, 3+	4	46		
	EGFR IHC, C core			
	0, 1+	*2+, 3+*	*Kappa value*	P-*value*
EGFR IHC, B core				
0, 1+	34	5	0.825	<0.001
2+, 3+	3	53		
	EGFR IHC, C core			
	0, 1+	*2+, 3+*	*Kappa value*	P-*value*
EGFR IHC, A core				
0, 1+	36	4	0.870	<0.001
2+, 3+	2	53		

Abbreviation: IHC, immunohistochemistry.

Table 5 Analysis of intratumoral concordance of *EGFR* copy number alteration in triple-negative breast cancers

	EGFR FISH, B core			
	Low gene copy number	*High gene copy number*	*Kappa value*	P-*value*
EGFR FISH, A core				
Low gene copy number	63	5	0.734	<0.001
High gene copy number	5	21		
	EGFR FISH, C core			
	Low gene copy number	*High gene copy number*	*Kappa value*	P-*value*
EGFR FISH, B core				
Low gene copy number	69	1	0.859	<0.001
High gene copy number	4	21		
	EGFR FISH, C core			
	Low gene copy number	*High gene copy number*	*Kappa value*	P-*value*
EGFR FISH, A core				
Low gene copy number	70	2	0.786	<0.001
High gene copy number	6	21		

Abbreviation: FISH, fluorescence *in situ* hybridization.

to the loss of tissue microarray cores. For *EGFR* FISH, 94 cases with three tissue microarray cores and 9 cases with two tissue microarray cores were available; the remainder was excluded due to FISH failure or core detachment. Intratumoral agreement of EGFR protein overexpression or copy number alteration was analyzed in these cases. Overall, the concordance rate of intratumoral EGFR expression (0, 1+ *vs* 2+, 3+) was 90% (94/105) with a mean kappa value of 0.836 ($P<0.001$) (Table 4). The concordance rate of intratumoral *EGFR* copy number alteration (low gene copy number *vs* high gene copy number) was 86% (89/103) with a mean kappa value of 0.793 ($P<0.001$) (Table 5). Specifically, the

Table 6 Clinicopathological correlation according to EGFR alteration in 151 triple-negative breast cancer patients

	EGFR overexpression			*EGFR FISH*			*EGFR mutation*		
Clinicopathologic parameters	*Absent* (n = *54*)	*Present* (n = *97*)	P-*value*	*Low gene copy* (n = *101*)	*High gene copy* (n = *50*)	*P-value*	*Wild type* (n = *147*)	*Mutant* (n = *4*)	P-*value*
pT			0.388			0.297			0.197
pT1, T2	50 (93)	93 (96)		97 (96)	46 (92)		140 (95)	3 (75)	
pT3, T4	4 (7)	4 (4)		4 (4)	4 (8)		7 (5)	1 (25)	
pN			0.300			0.141			1.000
pN0	37 (69)	74 (76)		78 (77)	33 (66)		108 (73)	3 (75)	
pN1, N2, N3	17 (31)	23 (24)		23 (23)	17 (34)		39 (27)	1 (25)	
Stage			0.018			0.693			0.364
I, II	44 (81)	91 (94)		91 (90)	44 (88)		132 (90)	3 (75)	
III	10 (19)	6 (6)		10 (10)	6 (12)		15 (10)	1 (25)	
Histologic grade			0.331			0.632			1.000
II	5 (9)	5 (5)		6 (6)	4 (8)		10 (7)	0 (0)	
III	49 (91)	92 (95)		95 (94)	46 (92)		137 (93)	4 (100)	
Venous invasion			0.177			0.669			0.263
Absent	48 (89)	92 (95)		93 (92)	47 (94)		137 (93)	3 (75)	
Present	6 (11)	5 (5)		8 (8)	3 (6)		10 (7)	1 (25)	
Lymphatic invasion			0.124			0.363			1.000
Absent	33 (61)	71 (73)		72 (71)	32 (64)		101 (69)	3 (75)	
Present	21 (39)	26 (27)		29 (29)	18 (36)		46 (31)	1 (25)	
Tumor border			0.660			0.088			0.638
Pushing	32 (59)	61 (63)		67 (66)	26 (52)		91 (62)	2 (50)	
Infiltrative	22 (41)	36 (37)		34 (34)	24 (48)		56 (38)	2 (50)	
Ki-67 proliferation index			0.403			0.387			0.620
$<50\%$	24 (44)	50 (52)		52 (51)	22 (44)		73 (50)	1 (25)	
$\geq 50\%$	30 (56)	47 (48)		49 (49)	28 (56)		74 (50)	3 (75)	
P53 overexpression			1.000			0.528			0.630
Absent	24 (44)	43 (44)		43 (43)	24 (48)		66 (45)	1 (25)	
Present	30 (56)	54 (56)		58 (57)	26 (52)		81 (55)	3 (75)	
Basal-like phenotype[a]			0.000			0.501			1.000
Absent	16 (30)	3 (3)		14 (14)	5 (10)		19 (13)	0	
Present	38 (70)	94 (97)		87 (86)	45 (90)		128 (87)	4 (100)	

Numbers in parentheses indicate column percentages.
[a]As documented by immunoreactivity for CK5/6 and/or EGFR.

three cases with *EGFR* gene amplification showed homogenous *EGFR* gene amplification and strong (3+) EGFR protein expression in all tissue microarray cores. There was no clinicopathologic difference between cases with intratumoral heterogeneity and those with homogeneity for EGFR protein expression or *EGFR* copy number alteration.

Clinicopathologic Features According to EGFR Protein Expression, Copy Number, and Mutation

We explored the relationship of EGFR protein expression, copy number gain, and mutation with the clinicopathologic variables of triple-negative breast cancer (Table 6). EGFR overexpression (2+ or 3+) was significantly associated with lower stage ($P = 0.018$). However, *EGFR* copy number and mutation did not correlate with any clinicopathologic variable. We also investigated the prognostic utility of EGFR alteration. At the time of analysis, the median follow-up was 4.9 years (range, 0.1–9.0 years). There were 7 (5%) loco-regional recurrences, 12 (8%) distant metastases, and 1 (1%) cancer-related death as the first event. In Kaplan–Meier survival analyses, the patients with high *EGFR* copy number had shorter disease-free survival than those without it ($P = 0.027$; Figure 3; Table 7). However, EGFR overexpression and *EGFR* mutation were not associated with disease-free survival (Table 7). In multivariate analysis including stage and *EGFR* copy number alteration, high stage (stage I and II *vs* stage III; hazard ratio, 2.815; 95% confidence interval, 1.022–7.751; $P = 0.045$) and high *EGFR*

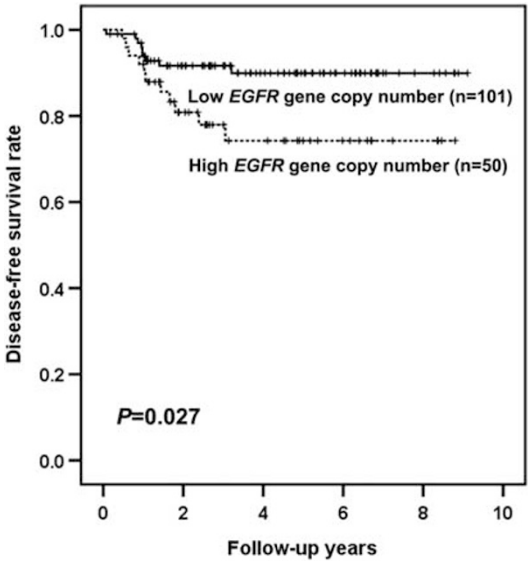

Figure 3 Disease-free survival and *EGFR* copy number in triple-negative breast cancer. Cases with high *EGRF* copy number show significantly poorer disease-free survival in comparison to subjects with low *EGFR* copy number.

Table 7 Univariate analyses for disease-free survival

Variables	P-*value*
pT T1, T2 *vs* T3, T4	0.033
pN N0 *vs* N1, N2, N3	0.058
Stage I, II *vs* III	0.033
Histologic grade II *vs* III	0.663
Ki-67 index <50 *vs* $\geq 50\%$	0.924
P53 overexpression absent *vs* present	0.334
Basal-like phenotype absent *vs* present	0.354
EGFR overexpression absent *vs* present	0.743
EGFR FISH low gene copy number *vs* high gene copy number	0.027
EGFR mutation wild type *vs* mutant type	0.438

Abbreviations: IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

gene copy number (low gene copy number *vs* high gene copy number; hazard ratio, 2.569; 95% confidence interval, 1.063–6.208; $P=0.036$) remained as independent prognostic indicators of poor disease-free survival.

Discussion

EGFR is frequently overexpressed in triple-negative breast cancer and clinical trials of EGFR-targeting agents are underway in patients with triple-negative breast cancer. However, the rate of *EGFR* copy number alteration and mutation, and the underlying mechanisms of EGFR overexpression are unclear, and their prognostic significance is poorly defined in triple-negative breast cancer. We evaluated the rates of *EGFR* gene alteration, their clinical implications in prognosis, and the intratumoral agreement of EGFR protein overexpression and gene copy number in triple-negative breast cancer.

EGFR gene amplification and high polysomy were reported in up to 24% and 27% of triple-negative breast cancer, respectively (Table 8).[10,11,13,14,19,25] However, the frequency of *EGFR* gene amplification is quite variable, even though most studies used the same, University of Colorado Cancer Center criteria.[30,31] We also assessed *EGFR* amplification by the University of Colorado Cancer Center criteria and found that *EGFR* amplification was quite rare, being present in only 2% of cases, which is consistent with some previous studies.[10,19,25] However, *EGFR* high polysomy was found in 31% of cases, which is relatively higher than in previous studies. This result may be related to the heterogeneity of high polysomy in some cases, which will be discussed later. The frequency of *EGFR* gene copy number gain in the previous studies appears to be greater in a subset of triple-negative breast cancers, ie, metaplastic carcinoma and triple-negative breast cancer with basal-like feature. In this study, the rate of high *EGFR* gene copy tended to be higher in metaplastic carcinoma than the other tumors (58% *vs* 30%, $P=0.063$, data not shown); however, there were no significant differences in *EGFR* copy number gain between basal-like and non-basal-like, triple-negative breast cancers.

The correlation between EGFR protein expression by IHC and *EGFR* gene copy number gain is controversial. Several groups that used *in situ* hybridization technique reported significant correlations between EGFR protein overexpression and high gene copy in triple-negative breast cancer.[10,13,14,19] However, Martin *et al*[11] and Toyama *et al*[17] showed no correlation between EGFR immunoexpression and increased *EGFR* gene copy number. In this study, EGFR overexpression was generally correlated with high *EGFR* copy number. However, about half of the cases with low *EGFR* gene copy number showed EGFR overexpression. Of the 202 tumor cores showing EGFR overexpression, only 76 (38%) revealed high *EGFR* copy number. Even if EGFR overexpression was defined as IHC 3+, only 44 (55%) of 80 cores with EGFR IHC 3+ showed *EGFR* high polysomy or gene amplification. That is, the specificity and positive predictive value of EGFR overexpression for high *EGFR* gene copy number were relatively low. These findings are contrary to the close correlation between HER2 overexpression and *HER2* amplification; HER2 overexpression is mostly attributable to *HER2* gene amplification.[28,32] Moreover, eight cases (11 tumor cores) with no EGFR immunoreactivity had high *EGFR* polysomy. Therefore, EGFR IHC alone has limited value in defining the group of triple-negative breast cancer patients with increased *EGFR* gene copy number.

Most studies encompassing Caucasian, European, and Japanese patients report a lack of *EGFR* mutation

Table 8 Review of previous studies on EGFR alterations in triple-negative breast cancer

Authors	*Study population*	*Study*	*Method (antibody/probes/interpretation)*	*Results (% of positive cases)*
Choi *et al*[9]	122 TNBCs	IHC	Novocastra: 2+ to 3+ membranous staining in ≥10% tumor cells	13%
Rakha *et al*[12]	282 TNBCs	IHC	Novocastra: any membranous staining in ≥10% tumor cells	37%
Tan *et al*[15]	31 TNBCs	IHC	Zymed: 2+ to 3+ membranous staining in ≥10% tumor cells	52%
Viale *et al*[18]	284 TNBCs	IHC	Zymed: membranous and cytoplasmic staining in ≥50% tumor cells	13%; EGFR expression correlates with worse prognosis
Liu *et al*[20]	287 TNBCs	IHC	Dako: any staining in ≥10% tumor cells	36%; EGFR expression correlates with worse prognosis
Gumuskaya *et al*[10]	62 TNBCs	IHC	Zymed and Novocastra: any cytoplasmic and/or membranous staining	61% (Zymed), 78% (Novocastra). Concordant result in two different EGFR clones
		FISH	Abbot Molecular: UCCC criteria	Amplification in 2%, high polysomy in 15%. Correlation between membranous staining of EGFR and FISH
Martin *et al*[11]	38TN-BCBLs, European	IHC	EGFR PharmDx™, Dako: any membranous staining	76%; no correlation between IHC and FISH
		FISH	Abbot molecular: UCCC criteria	Amplification in 24%, high polysomy in 27%
		Mutation	Direct sequencing	No activating mutations
Reis-Filho *et al*[13]	47 metaplastic carcinomas, Caucasian	IHC	Zymed: 2+ to 3+ membranous staining in >10% tumor cells	68%
		CISH	Zymed: >5 signals / nuclei or large gene copy clusters in >50% tumor cells	Amplification in 23%. Correlation between IHC and CISH
		Mutation	Direct sequencing	No activating mutations
Toyama *et al*[17]	110 TNBCs, Japanese	IHC	EGFR pharmDx™, Dako: 3+ membranous staining in ≥10% tumor cells	31%
		RT-PCR	Copy number changes relative to LINE 1 gene>3	21%; no correlation between IHC and RT-PCR
		Mutation	TaqMan genotype assays	None of 58 cases showed 14 known EGFR mutation
Nakajima *et al*[19]	84 TNBCs, Japanese	IHC	Ventana: any membranous staining in ≥10% tumor cells	33%; correlation between IHC and DISH
		DISH	Ventana: UCCC criteria	No amplification, high polysomy in 7%
		Mutation	EGFR mutation detection kit, DNAFORM	No activating mutations in 55 samples
Shao *et al*[14]	59TN-BCBLs	FISH	IHC—Dako: membranous staining, H-score FISH—GP medical Technologies, UCCC criteria	Amplification in 12%. Correlation between IHC score and EGFR/chromosome 7 ratio
Grob *et al*[25]	65 TNBCs	FISH	Abbott Molecular: UCCC criteria	Amplification in 2%, high polysomy in 8%
		Mutation	Direct sequencing	No activating mutations
Teng *et al*[16]	70 TNBCs, Chinese	IHC	Dako: any membranous staining	Mutation in 11%.
		Mutation	Direct sequencing	Incongruity between immunostaining and gene mutation
Jacot *et al*[26]	229 TNBCs, European	Mutation	Direct sequencing	No activating mutations

Abbreviations: TNBC, triple-negative breast cancer; TN-BCBL, triple-negative breast cancer with basal-like feature; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; DISH, dual-color in situ hybridization; CISH, chromogenic in situ hybridization; UCCC, University of Colorado Cancer Center.

in triple-negative breast cancer (Table 8).[11,17,19,25,26] However, Teng *et al* reported the presence of *EGFR* mutation, specifically exon 19 deletions and exon 21 missense (L858R) mutations, in 11% (8/70) of triple-negative breast cancer samples from predominantly Chinese patients.[16] In this study, *EGFR* mutation was found in 3% (4/151) of triple-negative breast cancer samples from Korean patients. As suggested by Lamy and Jacot,[33] wide variations in the rate of *EGFR* mutation in different populations may reflect geographic or ethnic differences in the presence of *EGFR* mutation. However, most studies encompass small series; well-organized, large-scale studies are needed to determine the origin of *EGFR* mutation variation in triple-negative breast cancer. Of the four cases with *EGFR* mutation, three carried a missense mutation of exon 21 (L858R), one of which with a coexisting

missense mutation of exon 18 (G719A), and remaining case carried a missense mutation of exon 20 (V786M). Missense mutations such as G719A/S and L858R and exon 19 deletions are well-known predictors of sensitivity to tyrosine kinase inhibitors in non-small cell lung cancer,[34] so the presence of L858R and G719A mutations in this study population suggests gefitinib or erotinib therapy may be beneficial in these selected triple-negative breast cancer patients. In our study, there was disagreement between EGFR immunostaining and the presence of *EGFR* mutation, similar to the report of Teng and colleagues,[16] suggesting that positivity in EGFR IHC cannot predict *EGFR* mutation in triple-negative breast cancer.

In this study, high *EGFR* copy number was significantly associated with poor disease-free survival and acted as an independent poor prognostic factor. Although EGFR overexpression has been presented as a poor prognostic indicator in triple-negative breast cancer,[18,20] high *EGFR* copy number has no reported prognostic impact in patients with triple-negative breast cancer. The mechanism by which high *EGFR* copy number contributes to the progression of triple-negative breast cancer is unclear. However, *EGFR* copy number gain might be one of the accumulating genetic alterations during tumor progression and EGFR activation induced by *EGFR* copy number gain may contribute to tumor aggressiveness in triple-negative breast cancer. It was suggested that EGFR activation drives migration and invasion of tumor cells through epithelial–mesenchymal transition and alters chemosensitivity by rewiring the apoptotic signaling network.[35] However, the utility of high *EGFR* copy number as a predictive biomarker for EGFR-targeted therapy and as a prognostic factor for triple-negative breast cancer should be validated in large studies.

EGFR protein expression in triple-negative breast cancer is quite variable, ranging from 13 to 78% (Table 8).[9–20] In this study, we used EGFR pharmDx™ (Dako), which is an approved anti-EGFR antibody for identification of colorectal cancer patients eligible for treatment with cetuximab or panitumumab. Moderate to strong membranous staining in >10% of tumor cells was regarded as EGFR overexpression and was found in 97 (64%) of 151 triple-negative breast cancer cases, comparable to previous studies. However, in contrast to previous studies suggesting prognostic implications of EGFR overexpression in triple-negative breast cancer,[18,20] we did not observe a survival difference associated with EGFR protein expression. This conflicting result may be attributable to differences in the antibodies used and different definitions of EGFR overexpression.

We evaluated intratumoral concordance of EGFR protein expression and gene copy number alteration in triple-negative breast cancer using different tissue microarray cores within a tumor. EGFR protein overexpression and *EGFR* copy number alteration were highly concordant in different tumor areas within each triple-negative breast cancer. All tumors with *EGFR* gene amplification demonstrated homogeneous EGFR protein overexpression within a tumor, implying triple-negative breast cancer patients with *EGFR* gene amplification would be excellent candidates for EGFR-targeted therapy. On the other hand, 12 of 30 cases with high polysomy showed discordant copy number results in at least one core of three tumor cores. It appears that *EGFR* high polysomy is relatively heterogeneous within a tumor, possibly due to the chromosomal instability of triple-negative breast cancer.

In summary, *EGFR* mutation was a rare event in triple-negative breast cancers, but high *EGFR* copy number including *EGFR* amplification and high polysomy was relatively frequent and correlated with EGFR overexpression. Intratumoral heterogeneity of EGFR protein overexpression and *EGFR* copy number alteration was not significant. More importantly, high *EGFR* copy number was associated with poor clinical outcome of the patients with triple-negative breast cancer. Our results suggest that evaluation of *EGFR* copy number can be useful for predicting outcomes in patients with triple-negative breast cancer and selecting patients for anti-EGFR-targeted therapy.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (Grant No. 2012R1A1B4000557) and by a grant from Seoul National University Bundang Hospital, Republic of Korea (03-2012-007).

Disclosure/conflict of interest

The authors declare no conflict of interest.

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