Cancer Science
Japanese Cancer Association JCA
Open Access

Epidermal growth factor receptor and AKT1 gene copy numbers by multi-gene fluorescence *in situ* hybridization impact on prognosis in breast cancer

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Key words

AKT1, breast cancer, epidermal growth factor receptor, gene copy number, survival analysis

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Funding information

National Science and Technology Support Program (2013BAI09B08). Tianjin municipal Major Scientific and Technological Special Project for Significant Anticancer Development (12ZCDZSY15700). Chinese National Natural Sciences Foundation (81402480).

Received October 14, 2014; Revised February 10, 2015; Accepted February 12, 2015

Cancer Sci **106** (2015) 642–649

doi: 10.1111/cas.12637

The epidermal growth factor receptor (EGFR)/PI3K/AKT signaling pathway aberrations play significant roles in breast cancer occurrence and development. However, the status of EGFR and AKT1 gene copy numbers remains unclear. In this study, we showed that the rates of EGFR and AKT1 gene copy number alterations were associated with the prognosis of breast cancer. Among 205 patients, high EGFR and AKT1 gene copy numbers were observed in 34.6% and 27.8% of cases by multi-gene fluorescence *in situ* hybridization, respectively. Co-heightened EGFR/AKT1 gene copy numbers were identified in 11.7% cases. No changes were found in 49.3% of patients. Although changes in EGFR and AKT1 gene copy numbers had no correlation with patients' age, tumor stage, histological grade and the expression status of other molecular makers, high EGFR ($P = 0.0002$) but not AKT1 ($P = 0.1177$) gene copy numbers correlated with poor 5-year overall survival. The patients with co-heightened EGFR/AKT1 gene copy numbers displayed a poorer prognosis than those with tumors with only high EGFR gene copy numbers ($P = 0.0383$). Both Univariate (U) and COX multivariate (C) analyses revealed that high EGFR and AKT1 gene copy numbers ($P = 0.000$ [U], $P = 0.0001$ [C]), similar to histological grade ($P = 0.001$ [U], $P = 0.012$ [C]) and lymph node metastasis ($P = 0.046$ [U], $P = 0.158$ [C]), were independent prognostic indicators of 5-year overall survival. These results indicate that high EGFR and AKT1 gene copy numbers were relatively frequent in breast cancer. Co-heightened EGFR/AKT1 gene copy numbers had a worse outcome than those with only high EGFR gene copy numbers, suggesting that evaluation of these two genes together may be useful for selecting patients for anti-EGFR-targeted therapy or anti-EGFR/AKT1-targeted therapy and for predicting outcomes.

Much progress has been made to determine gene copy number alterations and to assess their biological consequences. It is known that gene copy number alterations are frequently observed in solid tumors. Changes in the gene copy numbers can have a remarkable impact on tumor development through gene copy number-induced alterations of gene expression. Over the past 20 years, study of the molecular characteristics and genomic structure of breast cancer has attracted a great deal of interest.[1–4] Increasing evidence indicates that a number of gene copy number aberrations correlate with poor survival in breast cancer patients. Thus, determination of specific gene copy number changes and identification of specific aneuploidy status are considerably significant for the diagnosis and treatment of breast cancer.

The epidermal growth factor receptor (EGFR) gene, which is located at chromosome 7p12, was the first tyrosine kinase transmembrane receptor to be directly linked with human cancers.[5] The EGFR signaling plays important roles in the regulation of multiple cellular processes, including cell proliferation, apoptosis, angiogenesis and metastasis.[6] The effects on cell proliferation and survival are known to be mediated by phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) pathways.[7,8] Studies have demonstrated that overexpression of EGFR is frequent in triple-negative breast cancer and is associated with a poor prognosis.[9,10] However, the EGFR gene amplification is rare and cannot account for the EGFR overexpression that is commonly found in breast cancer.[11,12] Recent studies indicate that an increase in EGFR

gene copy number caused by aneuploidy is common in metastatic breast cancer, suggesting that heightened EGFR gene copy numbers may attribute to its overexpression in breast cancer.[13] Currently, EGFR has been selected as a molecular therapeutic target for several cancers, such as colorectal cancer, non-small cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC). Patients with EGFR gene mutations or increased gene copy numbers are more sensitive to anti-EGFR therapies, including tyrosine kinase inhibitors and monoclonal antibodies.[14,15] With the development of various types of EGFR tyrosine kinase inhibitors, EGFR is becoming a potential target for the treatment of breast carcinomas.[16] The capability of selecting drug-sensitive breast cancer types, such as in patients with increased EGFR gene copy numbers, would be essential for achieving effective personalized cancer therapy.

AKT1 (commonly called AKT) is an important downstream effector that mediates EGFR/PI3K signals to modulate diverse cellular events. Activation of the AKT1 signaling pathway is a significant contributor to the pathogenesis of cancer.[17] Upregulation of AKT1 has been found in a number of cancers, including gastric, prostatic, ovarian and breast carcinomas, and this upregulation is often associated with poor prognosis.[18–20] AKT1 gene amplification is one of the underlying mechanisms that cause AKT1 overexpression in sporadic cases of lung, gastric, breast and prostate carcinomas. However, AKT1 gene amplification only accounts for 1% of estrogen receptor (ER)-positive breast carcinomas.[21] Whether or not gene copy number alterations induce AKT1 overexpression in breast cancer is currently unknown.

Due to a low resolution of common methods, such as comparative genomic hybridization, the ability to precisely determine multiple gene copy numbers is limited. The latest molecular techniques, such as multi-gene FISH (M-FISH), have made a high-resolution analysis of DNA copy numbers possible. The M-FISH technique can simultaneously identify the changes in DNA copy numbers of multiple genes in a single cell, which provides a new and effective platform for genetic studies of breast cancer.[22] The primary goals of the present study were to evaluate the rates of EGFR and AKT1-gene copy number alterations by M-FISH and their association with the well-established prognostic parameters in breast cancer.

Materials and Methods

Clinical samples. A total of 223 randomly selected surgical pathology mammary specimens were collected from female patients with pathologically diagnosed primary breast invasive carcinoma who were admitted to Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) during August 2007 to August 2008. Eight cases were excluded because they had biopsies performed in other hosptials or received new adjuvant chemotherapy. Ten cases failed to follow up (the deaths unrelated to breast cancer within 5 years were regarded as failed to follow up). As such, there were 205 cases in this study. All specimens were fixed with 10% formaldehyde, dehydrated, and then embedded in paraffin. Paraffin sections were sliced at 4-μm thickness.

All patients were followed up by telephone calls, letter interviews or clinic visitations every quarter during the first 2 years of the study and semi-annually thereafter. Breast cancer death was regarded as the follow-up end, and the cut-off time was 5 years from the first diagnosis. The overall survival (OS) rate was defined as from the first operation to death caused by breast cancer or 5 years until the end of follow up. The pathological characteristics as listed in Table 1 of these breast cancer patients enrolled in this study were obtained from Tianjin Medical University Cancer Institute and Hospital. This study involving human subjects was approved by an institutional review board of Tianjin Medical University Cancer Institute and Hospital.

Preparation of probes. The UCSC (University of California, Santa Cruz) genome browser database (http://genome.ucsc.edu/) was used to design probes for all genes. All probes are bacterial artificial chromosome (BAC) plasmid DNA. Two to three different but continuous binding sites were chosen for every gene to boost the hybridization signal and enhance resolution. BAC clones RP11-339F13 and RP11-815K24, and CTD-3022N7, RP11-150I16 and RP11-817G24, which encompass EGFR (7p11.2) and AKT1 (14q32.33) genes, respectively, were used. The reference probes that were adjacent to the centromeres included RP11-203M5 (14q11.2) for AKT1, and RP11-251I15 and RP11-97P11 (7p11.1-11.2) for EGFR. A plasmid purification kit (Qiagen, Hilden, Germany) was used to extract these BAC plasmid DNA according to the instructions suggested by the manufacturer. The plasmid DNA were digested with EcoRI (Sigma, St. Louis, MO, USA). The purified DNA fragments to be used as probes were labeled by nick translation with the following four different color fluorophores: PromoFluor-590-aadUTP (red) for EGFR probes, PromoFluor-415-aadUTP (light blue) for AKT1 probes, PromoFluor-555-aadUTP (orange) for chromosome 14 centromere probe (PromoKine, Heidelberg, Germany) and Green dUTP (green) for chromosome 7 centromere probe (Abbott Molecular, Abbott Park, IL, USA). The specificity and sensitivity of these probes were verified using human dermal fibroblast (HDF) cell line prior to hybridization with breast cancer samples.

FISH. The dewaxed sections were heated at 65°C for 1 h, treated with dimethyl benzene three times for 15 min each and then washed with 100% ethanol twice. Subsequently, the sections were rehydrated in a decreasing ethanol gradient and distilled H_2O, incubated in 0.2 M HCl at room temperature (RT) for 10 min, washed with PBS for 5 min and incubated in 0.01 M citrate buffer at 80°C for 1 h, followed by a rinse with 2× saline–sodium citrate buffer (Invitrogen, Carlsbad, CA, USA) and distilled H_2O. The processed sections were treated with 300 μL pre-warmed pepsin solution (0.25 mg/mL pepsin and 0.01 M HCl) at 37°C for 10 min, washed with 2× saline-sodium citrate buffer (SSC) twice for 5 min each, fixed with

Table 1. Epidermal growth factor receptor (EGFR) and AKT1 gene copy number alterations detected by M-FISH

Gene	Low gene copy number (%)				High gene copy number (%)	
	Disomy	Low trisomy	High trisomy	Low polysomy	High polysomy	Gene amplification
EGFR	78 (38.0)	7 (3.4)	5 (2.4)	44 (21.5)	65 (31.7)	6 (2.9)
AKT1	86 (41.9)	5 (2.4)	11 (5.4)	46 (22.4)	53 (25.9)	4 (1.9)

0.4% methanol for 10 min followed by a wash with PBS and dehydrated through an increasing ethanol gradient. The sections were then air-dried at RT. Each prepared section was incubated with 10 μL of the probes, covered with a cover slip and placed in the StatSpin Thermo Brite hybridization system (Abbott Laboratories, Abbott Park, IL, USA) under the following conditions: denatured at 90°C for 10 min and hybridized at 47°C for 48 h. After hybridization, the samples were washed with saline sodium phosphate-EDTA (Invitrogen, Grand Island, NY, USA) for 5 min at 47°C and again at 55°C for 10 min. The dehydrated and air-dried sections were counterstained with DAPI, and coverslips were mounted with an aqueous mounting medium and stored in a dark box at 4°C.

Image acquisition and analysis. The hybridized sections were examined and imaged under a Zeiss Axioplan2 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany). The microscope was equipped with a high-resolution charge-coupled device camera and a computer loaded with Axio Vision software for image analysis. Fields were chosen within the DAPI stained nuclear outline (dark blue), and the position was recorded for image acquisition. Images of DAPI, Green, Cy3v1, Texas Red and Zeiss PF415 were taken with a ×630 objective lens. Each field was documented for 30 slices and then merged using the background extension. At least 100 non-overlapping tumor cells were scored per sample, and the signals of EGFR (Red), AKT1 (light Blue), chromosome 14 centromere (Orange) and chromosome 7 centromere (Green) were recorded, respectively.

The following criteria were used to assess the alterations of EGFR and AKT1 gene copy numbers, as described previously:[23,24] disomy (≤2 copies in >90% of cells); low trisomy (≤2 copies in ≥40% of cells, three copies in 10–40% of cells and ≥4 copies in <10% of cells); high trisomy (≤2 copies in ≥40% of cells, three copies in ≥40% of cells and ≥4 copies in <10% of cells); low polysomy (≥4copies in 10–40% of cells); high polysomy (≥4 copies in ≥40% of cells) and gene amplification (presence of tight gene clusters and a ratio of the gene copies to chromosome 7 centromere [EGFR] or chromosome 14 centromere [AKT1] was ≥2, or ≥15 copies of the gene per cell in ≥10% of cells). The results were classified into two major groups, high and low for analyses of their associations with clinicopathological characteristics of these breast cancer patients. A high EGFR or high AKT1 gene copy number group includes high polysomy and gene amplification, and a low EGFR or low AKT1 gene copy number group consists of disomy, low trisomy, high trisomy and low polysomy.

Statistical analyses. IBM SPSS Statistics Version 21 (IBM Corp, Armonk, NY, USA) and Graphpad 5.0 (Graphpad Software, San Diego, CA, USA) were used to analyze the data. Pearson's χ^2-test with cross tables or Fisher's exact test was applied for evaluation of comparisons between high

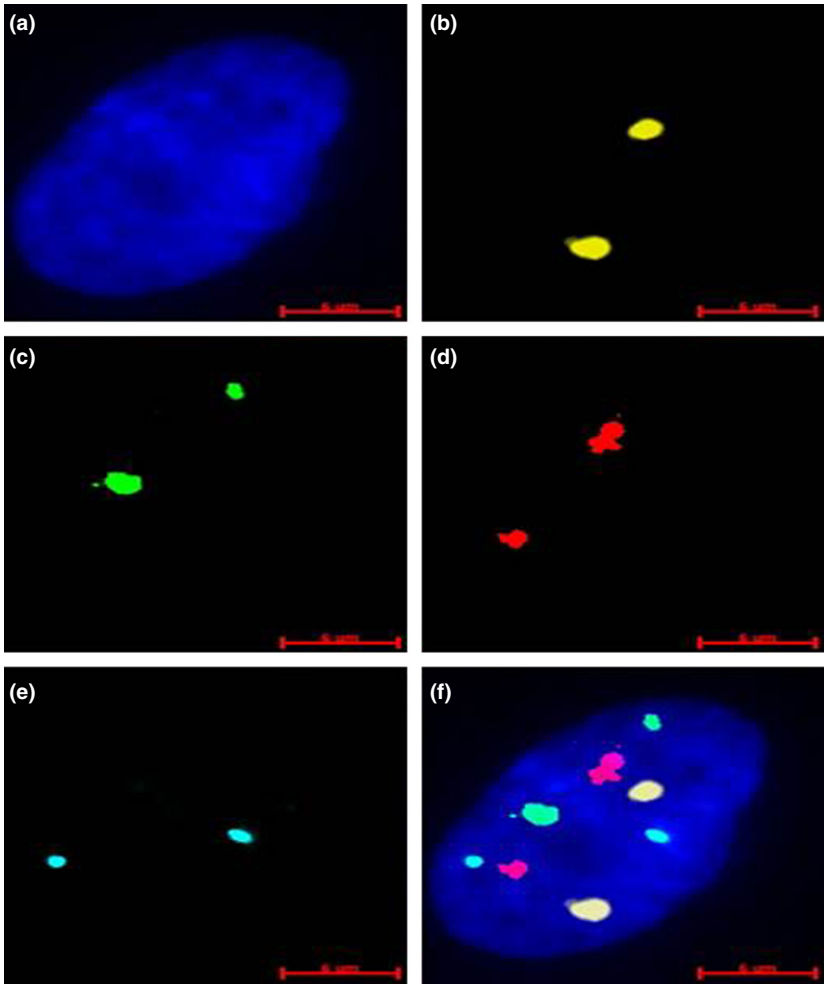

Fig. 1. Verification of the specificity and sensitivity of these FISH probes in human dermal fibroblast (HDF) at division phase. (a) DAPI stained HDF nucleus; (b) chromosome 14 centromere hybridized by RP11-203M5 (orange color); (c) chromosome 7 centromere hybridized by RP11-251I15 and RP11-97P11 (green color); (d) the epidermal growth factor receptor gene hybridized by RP11-339F13 and RP11-815K24 (red color); (e) the AKT1 gene hybridized by CTD-3022N7, RP11-150I16 and RP11-817G24 (light blue color); and (f) composed picture of a, b, c, d and e.

EGFR or AKT1 copy number and clinicopathological features. Kaplan–Meier life tables with log-rank testing were plotted to assess OS at the optimal cut point and prognostic multivariate analysis was performed using the Cox regression model. A P-value < 0.05 was considered statistically significant.

Results

Clinicopathological analysis of epidermal growth factor receptor and AKT1 gene copy numbers in breast carcinomas. As shown in Figure 1, discrete and robust FISH signals were detected using the probes prepared from BAC clones RP11-339F13 and RP11-815K24 (EGFR), CTD-3022N7, RP11-150I16 and RP11-817G24 (AKT1), RP11-203M5 (chromosome 14 centromere) and RP11-251I15 and RP11-97P11 (chromosome 7 centromere) in dividing nuclei of HDF cells. Hence, these four different color fluorophore-labeled probes were chosen for M-FISH in breast cancer tissue sections.

Epidermal growth factor receptor, AKT1, chromosome 7 centromere and chromosome 14 centromere DNA copies were clearly and specifically detected by the corresponding probes in the nuclei of breast cancer cells using M-FISH (Fig. 2). The six karyotypes and numbers of patients in each type of EGFR and AKT1 gene copy numbers are summarized in Table 1. Among 205 female patients with breast cancer analyzed, high EGFR and AKT1 gene copy numbers were observed in 71 (34.6%) and 57 (27.8%) cases, respectively. Twenty-four (11.7%) cases were identified with co-heightened EGFR/AKT1 gene copy numbers. No changes in either gene were found in 101 (49.3%) patients. Clinicopathological analysis showed that the EGFR and AKT1 copy numbers were not significantly associated with the age at diagnosis, pathological T (pT) stage, lymph node metastasis, histologic grade, estrogen receptor and progesterone receptor (PR) status, HER2 status, Ki-67 proliferation index, p53 overexpression and the subtypes (Table 2).

Relationship between epidermal growth factor receptor and AKT1 gene copy numbers and the prognosis of breast cancers. Univariate survival analysis revealed that co-existing high gene copy numbers of EGFR and AKT1 (P < 0.0001), lymph node metastasis (P = 0.046) and histologic grade (P = 0.001) were prognostic factors that affected patients' 5-year OS. Although high EGFR (P = 0.0002) but not high AKT1 (P = 0.1177) gene copy numbers significantly affected patients' 5-year OS, co-heightened EGFR and AKT1 gene copy numbers displayed a worse prognosis than tumors with high EGFR gene copy number alone (P = 0.0383) (Fig. 3). COX multivariate analysis of EGFR and AKT1 gene copy numbers (P = 0.0001) and histologic grade (III versus II; hazard ratio, 1.918; 95% confidence interval, 1.154–3.189; P = 0.012) showed that high EGFR/high AKT1 gene copy numbers and high histologic grade were significantly associated with a poor prognosis for breast cancers and remained as independent prognostic indicators of poor 5-year OS for the patients (Table 3).

Discussion

Breast cancer has become the world's highest incidence of female cancer.[25] Breast cancer is a complex, multi-step process and is associated with multiple genes. A better understanding of these genetic alterations can provide a more accurate diagnosis and appropriate treatment options for patients.

Dysregulation of the EGFR/PI3K/AKT1 signaling pathway promotes tumor angiogenesis, progress, invasion and metastasis. Growing evidence indicates that gene copy number alterations are closely related to inappropriate activation of the EGFR/PI3K/AKT1 cascade in various tumors. EGFR gene amplification and high EGFR expression have been recognized as biological predictors of poor prognosis for breast carcinoma, HNSCC and NSCLC.[26–28] However, the significance of EGFR and AKT1 gene copy numbers in breast cancer has not been well characterized. In two cohort studies, Gilbert *et al.*

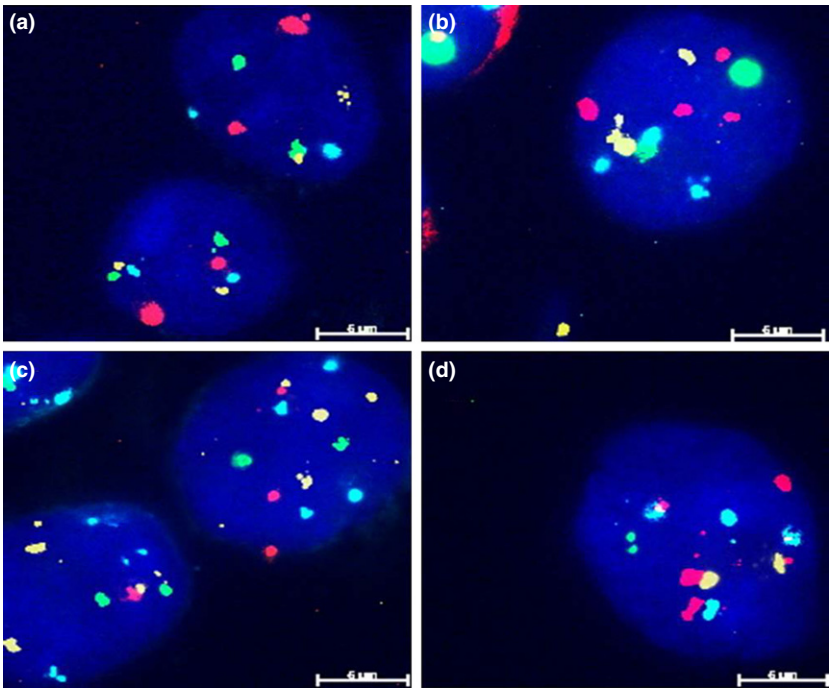

Fig. 2. Alterations of epidermal growth factor receptor (EGFR) and AKT1 gene copy numbers in breast cancer cells detected by M-FISH. The images show that the four-color probes detect EGFR gene (red), chromosome 7 centromere (green), AKT1 gene (light blue) and chromosome14 centromere (orange) in the nuclei (dark blue) of breast cancer cells. Representative FISH pictures (a–d) for different types of alternations are shown: (a) low EGFR/low AKT1 gene copy numbers; (b) high EGFR/low AKT1 gene copy numbers; (c) low EGFR/high AKT1 gene copy numbers; and (d) high EGFR/high AKT1 gene copy numbers.

Table 2. Summary of the relationship between epidermal growth factor receptor (EGFR) and AKT1 gene copy number alterations and the patients' clinical-pathological characteristics

Clinical-pathological characteristics	Copy number				*P*
	Low EGFR/Low AKT1 (%)	High EGFR/Low AKT1 (%)	Low EGFR/High AKT1 (%)	High EGFR/High AKT1 (%)	
Age (years)					
<60	45 (48.4)	25 (26.9)	17 (18.3)	6 (6.5)	0.127
≥60	56 (50.0)	22 (19.6)	16 (14.3)	18 (16.1)	
pT stage					
pT1, pT2	93 (51.7)	40 (22.2)	26 (14.4)	21 (11.7)	0.208
pT3, pT4	8 (32.0)	7 (28.0)	7 (28.0)	3 (12.0)	
Lymph node metastasis					
Positive	27 (39.7)	19 (27.9)	15 (22.1)	7 (10.3)	0.143
Negative	74 (54.0)	28 (20.4)	18 (13.1)	17 (12.4)	
Histologic grade					
II	74 (53.2)	28 (20.1)	21 (15.1)	16 (11.5)	0.374
III	27 (40.9)	19 (28.8)	12 (18.2)	8 (12.1)	
ER					
Positive	72 (48.3)	37 (24.8)	25 (16.8)	15 (10.1)	0.499
Negative	29 (51.8)	10 (17.9)	8 (14.3)	9 (16.1)	
PR					
Positive	66 (48.9)	33 (24.4)	19 (14.1)	17 (12.6)	0.642
Negative	35 (50.0)	14 (20.0)	14 (20.0)	7 (10.0)	
HER2 status					
Positive	24 (40.0)	12 (20.0)	13 (21.7)	11 (18.3)	0.085
Negative	77 (53.1)	35 (24.1)	20 (13.8)	13 (9.0)	
Ki-67 proliferation index					
<14%	68 (56.7)	23 (19.2)	18 (15.0)	11 (9.2)	0.078
≥14%	33 (38.8)	24 (28.2)	15 (17.6)	13 (15.3)	
P53 overexpression					
Positive	70 (47.0)	31 (20.8)	3 (5.4)	6 (10.7)	0.065
Negative	31 (55.4)	16 (28.6)	33 (20.1)	18 (12.1)	
Subtypes					
Luminal A	61 (52.6)	30 (25.9)	15 (12.9)	10 (8.6)	0.112
Luminal B	23 (42.6)	11 (20.4)	12 (22.2)	8 (14.8)	
HER2	1 (16.7)	1 (16.7)	1 (16.7)	3 (50.0)	
Triple-negative	16 (55.2)	5 (17.2)	5 (17.2)	3 (10.3)	

ER,estrogen receptor; PR,progesterone receptor; pT,pathological T.

and Reis-Filho *et al.*[13,29] report that high EGFR gene copy numbers in metastatic breast carcinomas were 26% and 28%, respectively. The current study evaluated the frequency of EGFR and AKT1 gene copy number alterations in 205 female patients with breast cancer and their clinical implications for prognosis. Our data showed that an increase in EGFR gene copy numbers in breast cancer is frequent (34.6%), but the frequency of gene amplification is uncommon (2.9%), which is consistent with the findings reported by Lv *et al.*[30] Our findings also demonstrated that patients with high EGFR gene copy numbers were associated with reduced OS compared to those patients whose tumors showed low EGFR and AKT1 gene copy number ($P = 0.0002$).

AKT1 is a serine/threonine kinase. It can be activated by EGFR/PI3K signaling cascade through site-specific phosphorylation. Activated AKT1 plays important roles in regulation of several cellular functions. It has been well established that aberrant overactivation of AKT1 signaling promotes tumor growth.[31] In a previous study, researchers found that 6% of lung carcinomas exhibited AKT1gene amplification and 7% exhibited high-level polysomy of chromosome 14.[32] Other studies have shown AKT1 amplification in 20% of gastric cancers.[33] The impact of changes in AKT1 gene copy number on OS of these cancers is not yet fully elucidated. Our current data revealed that high AKT1 gene copy numbers in breast cancer are relatively common (27.8%), but the frequency of AKT1 gene amplification is very low (1.9%). However, univariate analyses did not find significant association of high AKT1 gene copy number and reduced OS compared to those patients with any status of EGFR gene copy numbers ($P = 0.1177$). Interestingly, the present study did find that patients with co-heightened EGFR/AKT1 gene copy numbers had a poorer prognosis than those only showing a high EGFR copy number ($P = 0.0383$). Spears *et al.*[34] recently reported that excessive AKT1 activation in breast cancer was associated with decreased OS compared to other patients. Because AKT1 can be activated by the EGFR signaling to enhance cancer cell survival, it is possible that the AKT1 activity may be further exaggerated due to the augment of both EGFR and AKT1 gene copy numbers and result in a worse 5-year OS than patients with an increase in EGFR gene copy numbers alone. These findings suggest that analysis of the alterations of both EGFR and AKT1 gene copy numbers together may provide better molecular classifications of breast cancers and facilitate the decision-making for individual therapeutic plans.

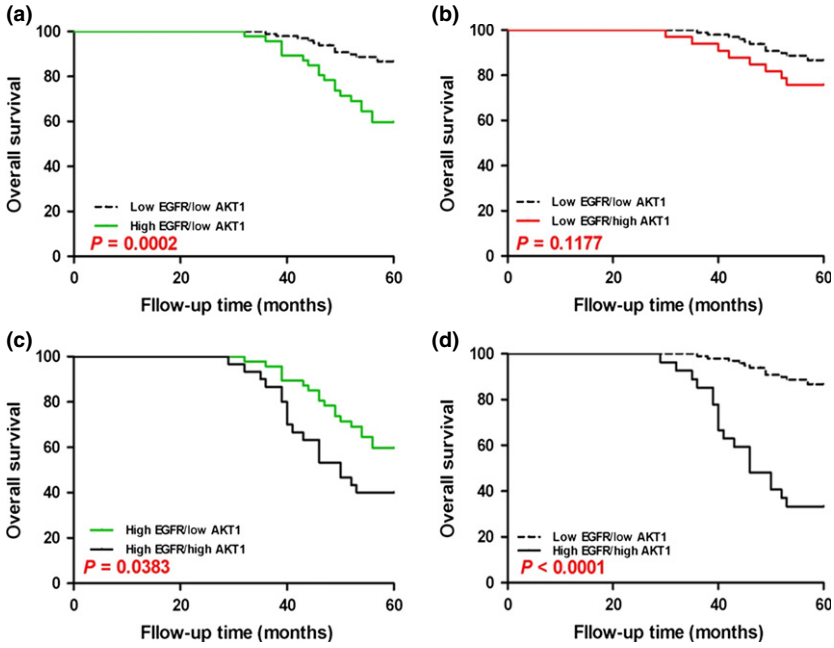

Fig. 3. Kaplan–Meier analyses of the association of patient overall survival rate (OS) and epidermal growth factor receptor (EGFR) and AKT1gene copy numbers in breast cancers. (a) Cases with high EGFR gene copy number show significantly poorer 5-year OS compared to subjects with low EGFR copy number ($P = 0.0002$). (b) Alterations of AKT1 gene copy number do not significantly affect patient 5-year OS ($P = 0.1177$). (c) Breast cancers with co-heightened EGFR and AKT1 gene copy numbers exhibit a worse prognosis than those with only heightened EGFR gene copy number ($P = 0.0383$). (d) Breast cancers with co-heightened EGFR and AKT1 gene copy numbers display a worse prognosis than those with low copy numbers of both EGFR and AKT1genes ($P < 0.0001$).

Table 3. Univariate and multivariate analyses for overall survival

Variables	Univariate analysis *P*-value	Multivariate analysis		
		P-value	HR	95% CI
EGFR and AKT1 copy number	0.0000	0.0001		
High/high versus low/low	0.0000	0.0001	4.319	2.101–8.880
High/low versus low/low	0.0002	0.0010	2.808	1.495–5.274
Low/high versus low/low	0.1177	0.5150	1.321	0.572–3.050
High/low versus high/high	0.0383	0.2120	0.650	0.331–1.278
pT stage (T3,T4 versus T1,T2)	0.451	—	—	—
Histologic grade (III versus II)	0.001	0.012	1.918	1.154–3.189
Lymph node metastasis (pos versus neg)	0.046	0.158	1.442	0.867–2.397
ER status (pos versus neg)	0.090	—	—	—
PR status (pos versus neg)	0.408	—	—	—
Her-2 status (pos versus neg)	0.145	—	—	—
Ki-67 index (≥14% versus < 14%)	0.291	—	—	—
P53 overexpression (pos versus neg)	0.804	—	—	—
Subtypes	0.167	—	—	—

CI, confidence interval; HR, hazard ratio; neg, negative; pos, positive; ER,estrogen receptor; PR,progesterone receptor; pT,pathological T.

A number of EGFR tyrosine kinase inhibitors such as gefitinib, erlotinib and other targeted drugs have been produced during the past decades. Several selective AKT1 inhibitors are also under investigation.[35,36] Determining the best way to select patients who are sensitive to these targeted drugs in order to improve the prognosis of patients has become a research focus. Previous studies have found that EGFR protein expression measured by immunohistochemistry does not correlate with EGFR gene copy number status determined by FISH assay in metaplastic breast carcinomas.[13] A number of clinical studies demonstrate that the gene copy number status rather than the protein expression of EGFR is a critical parameter for selection of cancer patients to receive anti-EGFR therapy. Promising results obtained from studies in solid tumors, both squamous and non-squamous, reveal that whether an increase in the EGFR gene copy numbers is present or not may forecast the outcome of anti-EGFR therapies.[37,38] Cappuzzo *et al.*[39] report a decrease in mortality of NSCLC patients with high EGFR gene copy numbers who received gefitinib treatment. A recent study reported that an increase in EGFR gene copy numbers is likely to be a suitable marker for identification of a subset of breast cancer patients who are particularly responsive to the dual tyrosine kinase inhibitor lapatinib.[40] In the present study, the breast cancer patients with high EGFR gene copy numbers had a poor prognosis. It stands to reason that the 5-year OS can be improved if these patients are placed under anti-EGFR therapy. More importantly, our study further demonstrated that there was an even worse prognosis if both high EGFR and high AKT1 gene copy numbers are present, which is tempting us to speculate that a combination of anti-EGFR and anti-AKT1 treatment of these breast cancer patients may meliorate the 5-year OS. In line with this speculation, an additive suppression of cell growth with combined administration of EGFR and AKT1 inhibitors compared to individual treatments has been observed in breast cancer MCF-7 cells

that are forced to overexpress both EGFR and AKT1 (our unpublished data).

The present study suggests that the use of M-FISH to simultaneously detect both EGFR and AKT1 gene copy numbers is an effective approach to identify patients who may benefit from anti-EGFR or both anti-EGFR and anti-AKT1 therapy. We are currently carrying out laboratory and clinical studies to validate this conjecture. PI3KCA mutations are common in breast cancer and these mutations might confer the sensitivity to inhibitors as anti-EGFR or anti-AKT1, although the relationship of PI3KCA mutations and prognosis of breast cancer patients has not been unequivocally reported.[41–45] Due to the very small number of breast cancer patients with co-heightened EGFR/AKT1 gene copy numbers found in the current study, whether there is a significant co-existence of PI3KCA mutations and EGFR/AKT gene number alterations in breast cancer patients is not analyzed. This is warranted to be investigated in the future.

Acknowledgments

This study was supported by the National Science and Technology Support Program (2013BAI09B08), the Tianjin municipal Major Scientific and Technological Special Project for Significant Anticancer Development (12ZCDZSY15700) and the Chinese National Natural Sciences Foundation (81402480).

Disclosure Statement

The authors have no conflict of interest to declare.

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