RESEARCH ARTICLE

Mutational Analysis of Key EGFR Pathway Genes in Chinese Breast Cancer Patients

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Abstract

Background: The epidermal growth factor receptor (EGFR) is a potential therapeutic target for breast cancer treatment; however, its use does not lead to a marked clinical response. Studies of non-small cell lung cancer and colorectal cancer showed that mutations of genes in the PIK3CA/AKT and RAS/RAF/MEK pathways, two major signalling cascades downstream of EGFR, might predict resistance to EGFR-targeted agents. Therefore, we examined the frequencies of mutations in these key EGFR pathway genes in Chinese breast cancer patients. Methods: We used a high-throughput mass-spectrometric based cancer gene mutation profiling platform to detect 22 mutations of the PIK3CA, AKT1, BRAF, EGFR, HRAS, and KRAS genes in 120 Chinese women with breast cancer. Results: Thirteen mutations were detected in 12 (10%) of the samples, all of which were invasive ductal carcinomas (two stage I, six stage II, three stage III, and one stage IV). These included one mutation (0.83%) in the EGFR gene (rs121913445-rs121913432), three (2.50%) in the KRAS gene (rs121913530, rs112445441), and nine (7.50%) in the PIK3CA gene (rs121913273, rs104886003, and rs121913279). No mutations were found in the AKT1, BRAF, and HRAS genes. Six (27.27%) of the 22 genotyping assays called mutations in at least one sample and three (50%) of the six assays queried were found to be mutated more than once. Conclusions: Mutations in the EGFR pathway occurred in a small fraction of Chinese breast cancers. However, therapeutics targeting these potential predictive markers should be investigated in depth, especially in Oriental populations.

Keywords: Breast cancer - PIK3CA - EGFR - KRAS - mutation

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Introduction

Breast cancer is one of the most common cancers of women worldwide; the cumulative life-time incidence is ~11% (Kadota et al., 2009). The development of breast cancer is related to diverse genetic and environmental factors, thus it is a heterogeneous disease. Compared with Western countries, the incidence of breast cancer in China is lower. The age of peak breast cancer incidence is much earlier and the mortality rate is increasing in Asian populations (Leong et al., 2010). Therefore, effective measures to control breast cancer in China are becoming increasingly important.

In the last 20 years, the treatment of breast cancer has evolved very rapidly and become increasingly complex. Systematic treatment consists of surgery, chemotherapy, hormone therapy, radiotherapy, and molecular-targeted therapy and requires a comprehensive assessment and review of multiple issues. Some novel biological targeted agents have been developed in recent years, resulting in great advances in breast cancer treatment. The epidermal growth factor receptor (EGFR) is one potential therapeutic target. It encodes a transmembrane glycoprotein, a member of the protein kinase superfamily, which is a receptor for members of the epidermal growth factor family and is involved in the survival and proliferation of cancer cells. EGFR tyrosine kinase inhibitors are thought to inhibit activated EGFR, blocking one of the key drivers of the disease and consequently improving patient outcomes. EGFR inhibitors include gefitinib, erlotinib, and lapatinib, which have been investigated in non-small cell lung cancer (NSCLC) and colorectal cancer.

Mutations that lead to EGFR overexpression or overactivity have been associated with lung breast, colon, and pancreatic cancers. Compared with NSCLC and colorectal cancer, however, anti-EGFR targeted therapy does not produce a dramatic clinical response in breast cancer (Modi et al., 2006; Dickler et al., 2008; Dickler et al., 2009; Green et al., 2009; Gutteridge et al., 2010). Furthermore, few studies (Walker et al., 1999; Tsutsui et al., 2002; Tsuda et al., 2005) have reported on EGFR expression in breast cancer. Studies of the response of NSCLC to anti-EGFR therapy indicate that the presence of EGFR mutations is a better indicator of a response to specific EGFR inhibitors than is EGFR expression (Sholl et al., 2010). Therefore, evaluation of the presence of

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EGFR mutations in breast cancer is critical.

The PIK3CA/AKT and RAS/RAF/MEK pathways are two major signalling cascades downstream of EGFR that participate in many pathological and physiological processes, including cell proliferation, migration, and resistance to apoptosis, angiogenesis, and tumour cell invasion (Lowenstein et al., 1992; Batzer et al., 1994; Chan et al., 1999). The clinical responses of patients differ according to the genetic variant of the drug target. Downstream drug-resistant genes and PIK3CA, KRAS, and BRAF mutations are now part of the Quest Diagnostics Colorectal Cancer Mutation Panel. Quest reports that testing for mutations in both BRAF and KRAS, two key genes in EGFR downstream signalling pathways, increases the ability to predict sensitivity or resistance to colon cancer drugs. However, current guidelines for the treatment of breast cancer do not include testing for these mutations. Therefore, evaluation of not only EGFR mutations, but also mutations of the downstream signalling pathway genes, is necessary to determine the mechanism of drug action.

Therefore, we determined the frequency of AKT1, BRAF, EGFR, HRAS, KRAS, and PIK3CA mutations in 120 breast cancers using a high-throughput mass-spectrometry-based cancer gene mutation-profiling platform (Macconaill et al., 2009) to detect these mutations with high specificity and sensitivity.

Materials and Methods

Patients and samples

Fresh frozen samples from patients whose tumours were diagnosed as invasive breast cancer by haematoxylin and eosin (H&E) staining were retrieved from Nanfang Hospital, Southern Medical University (Guangzhou, Guangdong Province, China) from January 2010 to July 2011, and reviewed by at least two pathologists. The selected patients had primary unilateral breast cancer, with full clinical and histological data. The clinical information included age, tumour type, disease stage, mass size, and axillary lymph node metastasis status. The histological information included ER, PR, and HER-2 status determined immunohistochemically. This study included 120 cases of breast cancer.

The study was approved by the Nanfang Hospital Ethics Committee and written informed consent was obtained from all participants.

Candidate mutations

The genes investigated (AKT1, BRAF, EGFR, HRAS, KRAS, and PIK3CA) play important roles in the PIK3CA/AKT and RAS/RAF/MEK pathways, two major signalling cascades downstream of EGFR. Based on their relevance, 22 candidate mutations were selected (Table 1). These do not usually occur randomly, but are more frequent in certain genomic regions and affect gene function, which is important in the natural selection process that takes place during tumorigenesis or once an individual undergoes treatment.

Mutation detection

The Sequenom platform was used for mutation detection, following the manufacturer's protocol (Sequenom; San Diego, CA, USA). DNA was extracted from each breast cancer sample included in this study using an E.Z.N.A.™ Tissue DNA kit (Omega Bio-Tek, USA) according to the manufacturer's instructions, and then stored at –70°C. The tumour DNA was examined for the candidate mutations.

Genomic DNA was amplified by PCR in 5-μl volumes containing 0.2 μl of Taq polymerase, 5 ng of genomic DNA, 2.5 pmol of each PCR primer, and 2.5 mmol of dNTP. Thermocycling was performed at 94°C for 2 min followed by 45 cycles of 94°C for 30 s, 56°C for 30 s,

Table 1. Character of Mutations Detected by Present Study

Gene	Mutation	Allele	Reference SNP ID
AKT1	rs11555435	A	rs11555435
	rs34409589	DEL	rs34409589
BRAF	F595L	C	rs121913341
	V600L	A(BRAF_15) & G(BRAF_16)	rs121913378
	D594V|G	C	rs121913338
HRAS	G12D	T	rs104894230
	G13S	T	rs104894228
KRAS	G12C	C(KRAS_1) & A(KRAS_2)	rs121913530
	G12V	A(KRAS_1) & C(KRAS_2)	rs121913529
	G13D	T	rs112445441
PIK3CA	H1047Y	T	rs121913281
	N345K	A	rs121913284
	E542K	A	rs121913273
	E545K	A	rs104886003
	H1047L	T	rs121913279
	H1047R	G	rs121913279
EGFR	T790M	T	rs121434569
	A289V	T	rs149840192
	D770_N771insG	GGT	rs147149347-rs121913445
	N771_P772>SVDNR	GCGT	rs121913445-rs121913432
	H773_V774insNPH	AA..AC	rs121913432-rs142999400
	E746_T751del, I ins	DEL(EGFR_M01F) & T(EGFR_M06R)	rs121913426-rs121913463

Table 2. Summary of the Mutations Detected in 120 Invasive Breast Cancer Samples

Sample ID	Gene	SNP ID	Mutation	Allele	Age	Stage	Pathological diagnosis	ER	PR	Her-2	Ki-67(%)
2	EGFR	rs121913445-rs121913432	N771_P772>SVDNR	GCGT	53	I	invasive dutal breast carcinomas	negative	negative	positive	>16
30	KRAS	rs121913530	G12C	A	47	I	invasive dutal breast carcinomas	positive	negative	negative	>16
22	KRAS	rs112445441	G13D	T	57	IIA	invasive dutal breast carcinomas	positive	positive	negative	<14
99	KRAS	rs112445441	G13D	T	31	IIIC	invasive dutal breast carcinomas	positive	positive	negative	>16
3	PIK3CA	rs121913273	E542K	A	44	IIB	invasive dutal breast carcinomas	positive	positive	positive	<14
42	PIK3CA	rs121913273	E542K	A	57	IIIA	invasive dutal breast carcinomas	positive	negative	positive	>16
47	PIK3CA	rs121913273	E542K	A	53	IV	invasive dutal breast carcinomas	positive	negative	positive	>16
99	PIK3CA	rs121913273	E542K	A	31	IIIC	invasive dutal breast carcinomas	positive	positive	negative	>16
52	PIK3CA	rs104886003	E545K	A	56	IIA	invasive dutal breast carcinomas	negative	negative	positive	>16
57	PIK3CA	rs104886003	E545K	A	40	IIA	invasive dutal breast carcinomas	positive	positive	positive	>16
101	PIK3CA	rs104886003	E545K	A	44	IIB	invasive dutal breast carcinomas	positive	positive	positive	<14
20	PIK3CA	rs121913279	H1047R	G	50	IIIA	invasive dutal breast carcinomas	positive	positive	positive	>16
68	PIK3CA	rs121913279	H1047R	G	36	IIB	invasive dutal breast carcinomas	negative	negative	positive	>16

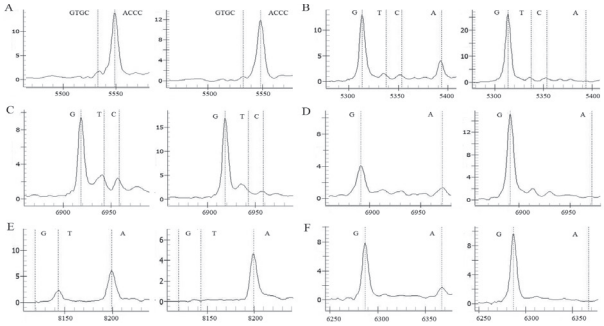

Figure 1. The Left Spectrum of Each Gene Shows A Mutation Allele, the Right One Shows a Wild Type Allele. A) EGFR N771_P772>SVDNR B) KRAS G12D C) KRAS G13D D) PIK3CA E545K E) PIK3CA H1047R F) PIK3CA E542K

and 72°C for 60 s, then a final 5 min at 72°C. Excess nucleotides in sample wells were deactivated using 2 μl of a shrimp alkaline phosphatase cocktail containing 1.53 μl of water, 0.17 μl of reaction buffer (Sequenom), and 0.3 μl of shrimp alkaline phosphatase (Sequenom) at 37°C for 40 min and 85°C for 5 min. Primer extension was performed using 0.755 μl of water, 0.2 μl of TypePLEX 10× buffer (Sequenom), 0.2 μl of TypePLEX terminator mix (Sequenom), 0.804 μl of primer extension mixture, and 0.041 μl of TypePLEX enzyme (Sequenom). The reactions were heated at 94°C for 30 s, followed by 40 cycles of 94°C for 5 s, five cycles of 52°C for 5 s and 80°C for 3 s, and then 3 min at 72°C. Salts were removed by adding a cation exchange resin, and the analytes were spotted onto a SpectroCHIP (Sequenom) and analysed using a MassARRAY matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry platform (Sequenom).

Analytical and statistical methods

Mutation calls for each sample were analysed by the MassARRAY Typer Analyser software version 4.0.4.20 (Sequenom). Mutations were identified in two ways. Automated mutation calls identified by the typer were generated using computational algorithms by quantifying the height ratio of raw spectral peaks corresponding to the mutant and wild-type signals, noise-to-peak-height ratios, and areas under the curve. In addition, all mutations from the report were reviewed manually by three investigators (Lin Tong, Xue-Xi Yang, and Guang-Yu Yao). Manual review of the individual calls was necessary to distinguish real mutant peaks from salt adduct or other background peaks.

Results

All patients were females ranging in age from 20–70 (mean 48.06) years. The TNM Cancer Staging Manual, 7th edition (Sinn et al., 2010) was used to classify the cancer staging, and there were 26, 61, 30, and 3 cases of stages I to IV, respectively. The majority (n=112) of the cases were invasive ductal carcinomas. In addition, we analysed three invasive lobular carcinomas and one case each of adenocarcinoma, tubular carcinoma, secretory carcinoma, invasive papillary carcinoma, and clear cell carcinoma.

Thirteen mutations were identified in 12 (10%) of the samples, all of which were invasive ductal carcinomas and consisted of 2, 6, 3, and 1 at stages I to IV, respectively. Of the 22 genotyping assays used here, six (27.27%) called a mutation in at least one sample (Figure 1), and three (50%) of the six queried were mutated more than once. The identified mutations are outlined in Table 2. Sample #99 had one KRAS and one PIK3CA mutation.

Discussion

The EGFR is a member of the family of cell-membrane receptors. When EGFR is increased significantly in a cancer, it indicates a more aggressive tumour and a poorer patient prognosis. Drugs that target specific receptors have been developed. Patients are classified as EGFR-positive or -negative, based upon whether a tissue test shows a mutation. EGFR-positive patients have an impressive 60% response rate to treatment with EGFR inhibitors. However, many patients develop resistance, especially those with breast cancer. This might be due to multiple mutations in the important PI3K/AKT and RAS/RAF/MEK signalling pathways downstream of EGFR. The importance of these signalling pathways for cellular processes is evidenced by their frequent mutational activation in malignant tumours; mutations of important genes in these pathways explain various biological processes and might be predictive of sensitivity or resistance to anti-EGFR targeted therapies. However, few studies have investigated mutational activation of both the PIK3CA/AKT and RAS/RAF/MEK pathways in breast cancer. Therefore, we determined the

frequencies of common mutations of the AKT1, BRAF, EGFR, HRAS, KRAS, and PIK3CA genes in EGFR downstream pathways in Chinese women with breast cancer. Mutations in only EGFR (0.83%), KRAS (2.50%), and PIK3CA (7.50%) were identified.

To date, few studies have focused on EGFR gene mutations in Chinese women with breast cancer. Lv (Lv et al., 2011) identified EGFR gene mutations in exons 19 and 21 in 2 of 139 (1.4%) Chinese breast cancer cases. Uramoto (Uramoto et al., 2010) reported no EGFR-activating mutations in Japanese breast cancers. We found a single EGFR gene mutation in 120 cases (0.83%). Therefore, EGFR gene mutations appear to be rare in Asian patients. Further research is necessary to validate these results.

Changes in PIK3CA are common in a number of cancers, including colorectal, breast, and liver cancers. Notably, its mutations are some of the most common genetic changes found in breast cancer. We found an unusually low PIK3CA mutation rate of 7.50% compared to the 26% reported by COSMIC (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bycancer&ln=PIK3CA&sn=breast). This might be because first, we assessed only six hotspot mutations in PIK3CA. Second, PIK3CA mutations are correlated with old age, and the mean age of our patients was 48.06 years, nearly 10 years younger than their Caucasian counterparts (Kalinsky et al., 2009). The third, and most importantly, the COSMIC data originates mostly from studies of Caucasians. Compared with Caucasians, breast cancer in Oriental populations is a heterogeneous disease with divergent molecular mechanisms of pathogenesis. One study (Wang et al., 2011) also found a lower PIK3CA mutation rate (12.3%) in Chinese breast cancers. In addition, of the PIK3CA mutations detected in our study, 44.44% were E542K mutations, 33.33% were E545K, and 22.22% were H1047R; no mutations of other sites were found. However, according to a meta-analysis, the majority of PIK3CA mutations are located at H1047R/L (54%) and E542/5K (39%) (Lee et al., 2005; Saal et al., 2005; Li et al., 2006; Liang et al., 2006; Maruyama et al., 2007; Perez-Tenorio et al., 2007; Stemke-Hale et al., 2008; Dunlap et al., 2010). This also suggests differences in the breast cancer that develops in Chinese and Western women.

The KRAS gene is important for cell proliferation and survival. When KRAS is activated by mutation, the resulting uncontrolled cell growth and division can result in cancer. KRAS mutations are identified in ~30% of colorectal carcinomas. Mutant KRAS is an absolute predictor of resistance to EGFR-targeted agents in ~30% of colon cancer patients (Benvenuti et al., 2007). We found that 2.5% of the samples had KRAS mutations, which is much lower than in colorectal carcinomas. Therefore, KRAS mutations may not be the main reason for the lack of therapeutic benefit of anti-EGFR monoclonal antibodies in breast cancer.

In this study, we also investigated the BRAF, AKT1, and HRAS genes but found no mutations. In other studies (Kononen et al., 1998; Davies et al., 2002; Cheang et al., 2008; Gollamudi et al., 2010; Kan et al., 2010), BRAF mutations were identified only in breast cancer cell lines and other primary tumours, but not in breast cancer patients. AKT1 mutations have been identified in 1–8% of breast carcinomas, in which they occur early (Dunlap et al., 2010); however, mucinous breast carcinomas have been reported to lack AKT1 mutations (Kehr et al., 2012). In a triple-negative breast cancer study, no HRAS mutation was found (Martin et al., 2012); however, 1 of 45 papillary breast neoplasm cases exhibited an HRAS mutation (Troxell et al., 2010). Since little data regarding these markers has been published, we strongly recommend a systematic review of the frequency of the various mutations and their association with distinct populations.

In conclusion, we investigated the prevalence of AKT1, BRAF, EGFR, HRAS, KRAS, and PIK3CA mutations in breast cancer samples, and found few mutations in the PI3K/AKT and RAS/RAF/MEK pathways in breast cancers in Chinese patients. This study provides useful information regarding Chinese breast cancer patients. A number of mutations in the EGFR pathways were identified; these might play a role in driving the proliferation of breast cancer cells. Therefore, these potential predictive markers may facilitate treatment of individual Chinese patients with breast cancer. However, the limitations of this study include the small number of samples and the fact that only function-affecting mutations of key genes were investigated. Therefore, further studies with a larger number of samples and that screen for mutations in full-length sequences should be conducted.

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