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ORIGINAL ARTICLE

The Gene Mutation Spectrum of Breast Cancer Analyzed by Semiconductor Sequencing Platform

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Abstract

To use the semiconductor sequencing platform (SSP) to analyze the gene mutate spectrum of breast cancer patients. We recruited 46 breast cancer patients, and detected the ER/PR/HER2 expression level of the tumor tissue by immunohistochemistry. In addition, combined with SSP technology, we detected 207 hot mutation regions of 50 breast cancer related genes with multiple polymerase chain reaction (PCR) technology. There were 8 cases of grade I, 18 cases of grade II, 20 cases of grade III in 46 breast cancer patients according to histological grade and 12 cases of ER/PR $^{+}$ HER2 $^{+}$, 18 cases of ER/PR $^{+}$ HER2^{-}, 13 cases of ER/PR $^{-}$ HER2 $^{+}$, 20 cases of ER/PR $^{-}$ HER2^{-} according to ER/PR/HER2 status classification. Moreover, we found that there were 33 gene locus mutations of 8 genes including *AKT1*, *APC*, *BRAF*, *CDKN2A*, *KRAS*, *PTEN*, *PIK3CA* and *TP53*, but difference was not statistically significant ($P > 0.05$) when compared these gene mutations (except for *PIK3CA*) in each groups according to the histological classification of breast cancer and the ER/PR/HER2 classification. *PIK3CA* mutation rate of grade I was obviously higher than that of grade II ~ III histological grading in breast cancer patients ($P < 0.05$). Based on our results, we drew a conclusion that the occurrence and development of breast cancer was a process involved multiple genes. Here, we found that *PIK3CA* played a role in the development of the early stage of breast cancer, which could provide clinical basis for treatment of breast cancer. Moreover, SSP technology could be an effective and sensitive method for detection of gene mutation spectrum in breast cancer.

Keywords Semiconductor sequencing platform (SSP) · Breast cancer · Mutate spectrum · Genetic screening

Introduction

Breast cancer is the most common cancer in women currently, with more than 1.3 million new cases occurring every year around the worldwide, and meanwhile about 450,000 persons died because of it [1]. Breast cancer is one of the major malignant tumors that threaten women's life and health. According to clinical data, breast cancer had become the fourth cause of death for women who due to malignant tumors in China by 2013 [2]. Its occurrence and development are related to many factors, including genetic factors, living habits and environmental factors, among which genetic factors are particularly important [3]. The treatment of breast cancer at present is mainly according to the disease grade, tumor stage, histological subtypes, the expression of hormone such as classifying patients [4, 5].

Clinically, according to estrogen receptor (ER) and/or progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression level, patients with breast cancer can be divided into four groups [6, 7]: (1) the ER/PR $^{+}$ HER2 $^{+}$ type, the hormone receptors and expression of HER2 receptor is high; (2) ER/PR^{+}HER2 $^{-}$ type, high expression of

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hormone receptor but no expression of HER2 receptor; (3) ER/PR $^{-}$ HER2^{+} type, hormone receptor is not expressed and HER2 is highly expressed; (4) triple negative breast cancer (TNBCs) is defined as lack of expression of ER, PR and HER2 (ER/PR $^{-}$ HER2 $^{-}$). At present, it is believed that the change of expression level of breast cancer related genes is far earlier than morphological changes [8]. A characteristic gene expression profile can help pathologists and oncologists diagnose of breast cancer before they can observe morphological changes in the tumor. In addition, studies have shown that different gene expression profiles may reflect different tumor subtypes, involving different phenotypes and clinical features [8, 9].

With the development of technology, next generation sequencing (NGS) technology play an important role in detecting the expression of thousands of genes at the same time. Meantime, the molecular mechanism of breast cancer has a deeper understanding [10]. New molecular tumor markers may be used to more accurately diagnose and could help develop more effective individualized targeted therapy drugs.

In the present study, the main purpose was to investigate the semiconductor sequencing platform (SSP) to detect breast cancer gene mutation spectrum, and at the same time analyzed the relationship between breast cancer mutation, the pathological classification and the histological grading, which could provide clinical basis for further treatment.

Materials and Methods

Patients

This study was approved by the Ethics Committee of Dongguan Maternal and Child Health Care Hospital and the informed consent signed by the patients. 46 cases without chemotherapy of breast cancer patients was recruited in both Maternal and child health care hospital, Dongguan, Guangdong province and Houjie hospital, Dongguan, Guangdong province from February 2016 to August 2017. The cohort aged from 31 to 86 years old, with an average of 47.22 years old. Among the 46 cases, there were 45 females and 1 male.

Histological Specimens and Immunohistochemistry

The breast tissues removed from surgical and the corresponding normal breast tissue were both fixed with 4% formalin, paraffin embedding, and cut into 4 μM size. Tissue sections were dewaxed by conventional xylene, and dehydrated by gradient ethanol, followed by 3% hydrogen peroxide was used to block the endogenous peroxidase. Closed by goat antigens with 0.01 mol/L citrate buffer (pH = 6.0) under high pressure, then incubation with primary antibody at 4 °C over night; the second antibody labeled horseradish enzyme was then incubated and also streptomyces affinity peroxidase (SP) was added. Cyanobacterium essence was redyeing after DAB staining. Finally, a neutral gum seal was applied. Phosphate buffer solution (PBS) was used as the negative control instead of monoclonal antibody, and the breast tissue samples with normal positive staining were known as the positive control. Specimens were graded by two pathologists independently, and the evaluation of immunohistochemitry results was according to the proportion of positive cells, the cell nucleus of samples stained yellow were determine as positive. The samples with positive cells was less than 5% in 5 X view of microscope were considered as negative, and 5%~40% was for ++, 40%~60% was for +++, more than 60% for ++++.

Semiconductor Sequencing and Genetic Mutation Analysis

The peripheral blood from the patients were collected, and followed by genomic DNA extracted, ultrasonic interrupt, enzyme supplement, polyA tail was added and joint, build sequencing library by multiple polymerase chain reaction (PCR) amplification method. Built DNA libraries were quantitative determined by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA), Qubit and 1% agarose gel electrophoresis, respectively. In the present study, we amplified 207 focus mutation areas in 50 tumor related genes, including *BRAF*, *EGFR*, *HER2*, *KIT*, *KRAS*, *NRAS* and *PDGFRA* that seven genes recommend in National Comprehensive Cancer Network (NCCN) guide. Finally, the samples tested by BioelectronSeq 4000 high throughput sequencing machines (CapitalBio Genomics Co., Ltd). When for data analysis, the gene mutation sites from our samples would be matched with database such as Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/) and the COSMIC database (https://cancer.sanger.ac.uk/cosmic) to obtain their mutation significance.

Statistical Analysis

Based on our obtained results, we used SPSS 19.0 software for statistical data analysis, Pearson chi-square and fisher exact probability methods to compare the difference between groups, when P value was ≤0.05 meant statistically significant, but P value was >0.05 meant no difference in statistical significant.

Results

In the present study, we collected 46 breast cancer patients, including 45 females and one male, which range from 31 to 86 years old, with the average age of 47.22 years old (Table 1). These 46 breast cancer patients were classified into three

Table 1 Clinicopathologic features of patients with breast carcinoma($n = 46$)

Characteristic	Value
Age (years)	
Median	47.22
Range	31–86
Sex	
Male	1
Female	45
Stage at diagnosis	
I	8
II	18
III	20
ER/PR/HER2 status	
ER/PR + HER2+	23
ER/PR + HER2–	10
ER/PR–HER2+	8
ER/PR–HER2–	5
Number of mutation sites	
0	8
1	28
2	8
3	2

ER, estrogen receptor; *HER2*, human epidermal growth factor receptor 2; *PR*, progesterone receptor; +, positive; −, negative

grades including grade I, II and III according to postoperative pathological and immunohistochemistry results of the patients and WHO histological classification for breast cancer (2012 version). There were 8 cases for grade I, 18 cases for grade II, and 20 cases for grade III. In the aspect of histological type, there were 46 cases non-specific invasive ductal carcinoma and 1 case of invasive ductal carcinoma characterized by myeloid carcinoma. As for the state classification of ER/PR/HER2 (Fig. 1), there were 23 cases of ER/PR$^+$HER2$^+$, among which 20 had gene mutation; 10 cases of ER/PR$^+$HER2$^-$, and 8 patients had gene mutation; 8 cases of ER/PR$^-$HER2$^+$, including 7 patients with gene mutation; 5 cases with ER/PR$^-$HER2$^-$, including 3 patients with gene mutation (Fig. 1).

Among the 46 patients, we found that there were 33 mutation sites of 8 genes that belonged to 50 common breast cancer related genes in 39 patients (84.78%). The 8 mutated genes including *AKT1*, *APC*, *BRAF*, *CDKN2A*, *KRAS*, *PTEN*, *PIK3CA* and *TP53*. Among of them, the mutation of *PIK3CA* gene took the most of 26 cases, followed by *TP53* (19 cases), *CDKN2A* (2 cases), and one case in the remaining five genes, respectively. For every patient, there were 2 cases of three site mutations, 8 cases of two site mutations, 28 cases of single site mutation, and 7 cases without gene mutation (Table 2).

We compared the gene mutations in each groups according to the histological classification of breast cancer and the ER/PR/HER2 classification, but found that the difference was not statistically significant ($\boldsymbol{P} > 0.05$) (Table 3). Then we analyzed the mutation situation of *PIK3CA* gene and learnt its relationship with the histological classification of breast cancer and ER, PR and HER2. There were 7 cases of *PIK3CA* gene mutation in grade I, non-mutation of 1 case; 9 cases of mutation in grade II, 9 cases of non-mutation; 6 cases of mutation in grade III, 14 cases of non-mutation. Based on our results, we had knowledge that the lower the histological classification level, the higher *PIK3CA* gene mutation frequency ($\boldsymbol{P} < 0.05$) (Table 4). Moreover, there was no significant correlation between *PIK3CA* gene and other clinical pathological features, and the difference was not statistically significant (Table 4).

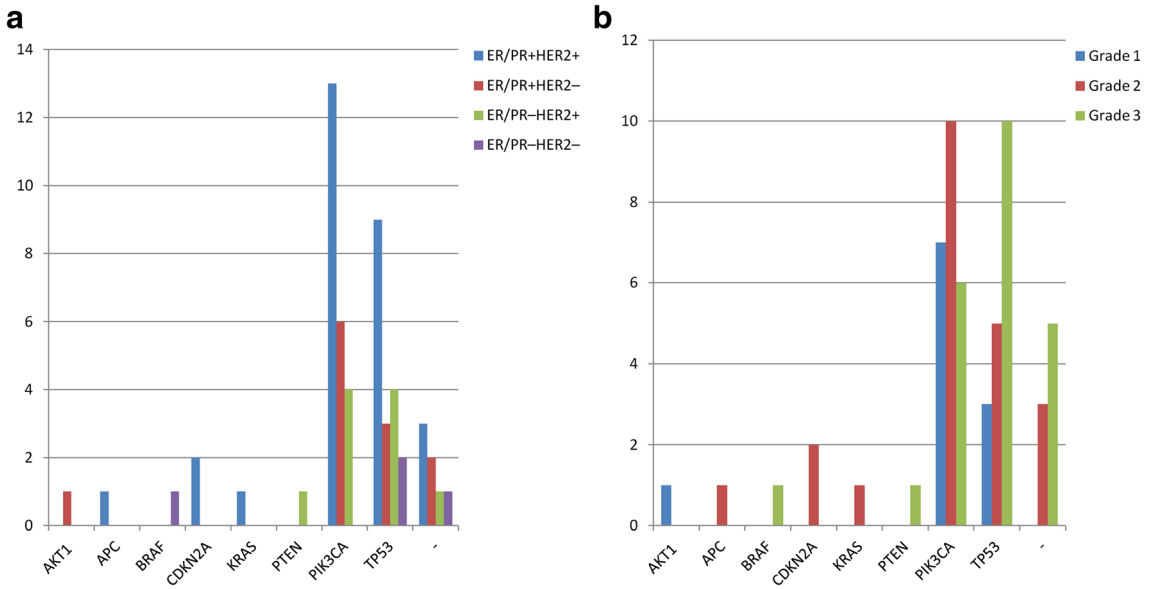

Fig. 1 Statistics about the relationship between gene mutations and clinical characteristics in 46 breast cancer patients. **a** Gene mutations and ER/PR/HER2 classification; **b** Gene mutations and histological grade

Table 2 Mutational spectrum detected by next generation sequencing among 46 breast carcinoma patients

Gene	Nucleotide change	Consequence of amino acid change	Mutation type	Category	Number of patients (n)	Mutation frequency (100%)
AKT1	c.49G > A	p.E17K	Missense	Pathogenic	1	29.46
APC	c.3313C > T	p.R1105W	Missense	Unknown significance	1	40.56
BRAF	c.1796C > T	p.T599I	Missense	Pathogenic	1	3.15
CDKN2A	c.385C > T	p.R129C	Missense	Unknown significance	1	50.07
	c.296G > A	p.R99Q	Missense	Unknown significance	1	50.56
KRAS	c.40G > A	p.V14I	Missense	Pathogenic	1	4.05
PTEN	c.511C > T	p.Q171X	Stop gained	Unknown significance	1	16.52
PIK3CA	c.3140A > T	p.H1047L	Missense	Pathogenic	3	24.71~47.49
	c.3140A > G	p.H1047R	Missense	Pathogenic	9	6.71~81.96
	c.241G > A	p.E81K	Missense	Likely pathogenic	1	14.89
	c.1636C > A	p.Q546K	Missense	Pathogenic	1	33.11
	c.1633G > A	p.E545K	Missense	Pathogenic	3	18.27~32.29
	c.1624G > A	p.E542K	Missense	Pathogenic	3	8.79~45.08
	c.1616C > G	p.P539R	Missense	Likely pathogenic	1	30.89
	c.1258 T > C	p.C420R	Missense	Pathogenic	1	62.82
	c.1214C > T	p.S405F	Missense	Pathogenic	1	4.11
	c.1035 T > A	p.N345 K	Missense	Pathogenic	2	8.07~25.57
TP53	c.856G > A	p.E286K	Missense	Pathogenic	1	49.77
	c.823delT	p.C275fs	Deletion	Unknown significance	1	19.74
	c.817C > T	p.R273C	Missense	Pathogenic	2	25.05~65.83
	c.768_769del	p.T256 fs	Deletion	Unknown significance	1	13.43
	c.743G > A	p.R248Q	Missense	Pathogenic	2	8.67~46.92
	c.711G > A	p.M237I	Missense	Likely pathogenic	1	40.27
	c.659A > G	p.Y220C	Missense	Pathogenic	1	29.38
	c.524G > A	p.R175H	Missense	Pathogenic/Likely pathogenic	1	68.22
	c.517G > A	p.V173 M	Missense	Pathogenic/Likely pathogenic	1	3.35
	c.488A > G	p.Y163C	Missense	Likely pathogenic	1	17.05
	c.404G > A	p.C135Y	Missense	Likely pathogenic	1	8.76
	c.329G > C	p.R110P	Missense	Likely pathogenic	1	12.45
	c.229C > T	p.P77S	Missense	Unknown significance	1	33
	c.206C > T	p.A69V	Missense	Unknown significance	1	3.03
	c.1025G > C	p.R342P	Missense	Pathogenic/Likely pathogenic	2	34.53~59.15
	c.1009C > G	p.R337G	Missense	Unknown significance	1	37.02

Unknown significance: conflicting interpretations of pathogenicity

Discussion

The incidence of breast cancer ranks the first among female malignant tumors in China, which seriously endangers women's life and health. Traditional treatment planning and the prognosis of patients with breast cancer is mostly based on anatomical features such as tumor size, local tumor infiltration, lymph node pathological condition, and some biological indexes such as histologic grade, ER, PR, and HER2 [11]. Because of the heterogeneity of breast cancer, the patient's

Table 3 Mutations detected by next generation sequencing in different histologic tumor grades and different ER/PR/HER2 status of breast carcinoma

Characteristic		No. (%) of patients		*P* value*
		+	–	
Histologic Tumor Grades	Grade 1 and 2	23	3	>0.05
	Grade 3	15	5	
ER/PR/HER2	ER/PR + HER2+	20	3	>0.05
	ER/PR + HER2-	8	2	
	ER/PR-HER2+	7	1	
	ER/PR-HER2–	3	2	
ER	+	27	5	>0.05
	–	11	3	
PR	+	26	5	>0.05
	–	12	3	
HER2	+	30	4	>0.05
	–	8	4	

ER, estrogen receptor; *HER2*, human epidermal growth factor receptor 2; *PR*, progesterone receptor; +, positive; –, negative; * Fisher exact probability method

Table 4 *PIK3CA* mutation detected by next generation sequencing in different histologic tumor grades and different ER/PR/HER2 status of breast carcinoma

Characteristic		No. (%) of Patients		$\chi 2$	*P* Value
		PIK3CA+	*PIK3CA-*		
Histologic Tumor Grades	Grade 1	7	1	–	<0.05[a]
	Grade 2	9	9		
	Grade 3	6	14		
ER	+	19	13	–	0.054[a]
	–	4	10		
PR	+	18	13	2.473	>0.05[b]
	–	5	10		
HER	+	18	16	0.451	>0.05[b]
	–	5	7		

ER, estrogen receptor; *HER2*, human epidermal growth factor receptor 2; *PR*, progesterone receptor; +, positive; –, negative; [a], Fisher exact probability method; [b] chi-square test

response to treatment exist certain differences between different types of breast cancer patients and even the different stages of the same patient, even if installment and molecular classification is the same. So some patients was applied the best treatment might have progress in disease.

In the present study, we divided the 46 breast patients into three groups according to inmunohistochemistry results, with 8 cases of grade I, 18 cases of grade II and 20 cases of grade III (Table 1 and Fig. 1). Breast histological classification was often used as a single prognostic indicator, so histological classification combined with gene mutation could be used as a prognostic indicator for breast cancer [7, 11]. Studies had confirmed that the histological classification of breast cancer was associated with cancer gene expression [12]. Grade III breast cancer patients often accompanied by the expression of epidermal growth factor, prompt with poor prognosis [13].

In this study, we found that the gene mutations of breast cancer were not related to the histological classification and had no statistical significance ($\boldsymbol{P} > 0.05$) (Table 3). We also classified the breast cancer patients according to the states of $ER^{-/+}$, $PR^{-/+}$ and $HER2^{-/+}$ (Table 1, Fig. 1). The result showed that $ER/PR^{+}HER2^{+}$ had the largest number of patients that of 24 cases and $ER/PR^{+}HER2^{-}$ had 10 cases; there were 8 cases of ER/PR $^{-}$ $HER2^{+}$ type and 4 cases of triple negative breast cancer (ER/PR^{-} $HER2^{-}$). Statistical analysis was conducted on these 4 groups patients, and there was no difference in them ($\boldsymbol{P} > 0.05$) (Table 3). Van't Veer, L. J. et al. [8] found one gene subset with 70 genes when analyzing 69 ER negative and 226 positive breast cancer patients, and this gene subset was significantly associated with disease free survival and overall survival of breast cancer patients. Paik, S. et al. [14] got 21 breast cancer related genes with real time quantitative reverse transcription polymerase (Q-RT-PCR) method, which was used to predict the recurrence risk of breast cancer patients who treated with tamoxifen. They learnt that these 21 genes had high correlation with ER positive, recurrence risk of lymph node negative breast cancer. In this study, we found that the gene mutations of breast cancer were not related to the histological classification and which had no statistical significance ($\boldsymbol{P} > 0.05$) (Table 3), this result we got was not inconsistent with previous studies results. This phenomenon might account for the genes we picked up were different from those genes in previous study; and the occurrence and development of breast cancer was a complicated process that related to multiple factors. Based on our results, we believed that different mutate genes had different effects on the different types and stages of breast cancer.

Further, from our study, we found that the gene with the highest mutate detection rate was *PIK3CA*. *PIK3CA* (OMIM 171834) is located at 3q26.3 and contains 20 exons [15]. Some studies reported that *PIK3CA* gene had no significant correlation with patients' ages, pathologic lymph node status, tumor diameter and local invasion, degree of differentiation of tumor cells, but related to the molecular classification of different breast cancer patients [16]. It has also been reported that this gene mutation is related to lymph node metastasis and histological classification [17–19] Here we found that there existed correlation between *PIK3CA* gene mutations and histological grading. *PIK3CA* mutation rate of grade I was obviously higher than that of grade II ~ III histological grading ($\boldsymbol{P} < 0.05$) (Table 4), which prompted that *PIK3CA* gene mutations might play a key role in the development of the early stage of breast cancer. *PIK3CA* mutations not only could inhibit apoptosis but also promote infiltration, improve activity of its downstream kinase PI3Ks. These mechanisms had the effect of promoting the early occurrence and development of breast cancer so that tumor cells spread to the deep tissue at the early stage [20]. No significant correlation was found between *PIK3CA* gene and other clinic pathological features here. The average age of breast cancer patients with *PIK3CA* mutations in this study was 49.6 years, which was higher than the average age of 46 breast cancer patients. Liang, X. et al. [21] found that the mutation of *PIK3CA* gene was more frequent in older patients with earlier clinical stages. We had the same result here. In addition, Liedtke, C. et al. [22] found that the mutation of *PIK3CA* gene was also common in older patients, but he believed that this correlation was influenced by negative lymph node. According to the above research results and

related reports, the relationship between *PIK3CA* gene mutations and the clinical pathological features of breast cancer also need to be further studied. These correlations may be affected by many factors, such as the number of cases, mean age, race, and pathology type and so on.

Moreover, mutations in *TP53*, *AKT1*, *APC*, *BRAF*, *CDKN2A*, *KRAS* and *PTEN* genes were also detected in this study. *TP53* (OMIM 191170) is a cancer suppressor gene whose mutations are associated with an increased risk of breast cancer. Early studies suggested that *TP53* mutations play a weak, or even less than 1%, role in the genetic susceptibility to breast cancer [23]. However, in recent years, foreign studies have found that *TP53* germ line mutations may play a more pathogenic role in family or hereditary breast cancer, especially in patients with early onset breast cancer [24]. Lee, D. S. et al. [25] reported that the detection rate of *TP53* gene mutation in breast cancer patients under the age of 35 was 5% in Asian populations. The average age of breast cancer patients with TP53 mutation gene in this study was 42.66 years old, less than the average age of the overall sample of 47.22 years old. *TP53* mutations can be either embryo mutation or somatic mutation, and this gene mutation was more common in young patients with breast cancer; it is recommended that young breast cancer patients with family history or *BRCA1/2* gene mutation negative should detect mutation situation of *TP53* gene.

Akt1, *PTEN* gene influenced the activity of the *PI3K/AKT* signal transduction pathway, not only influence the tumor cell proliferation, apoptosis, but also invasion and metastasis of tumor. Studies [26, 27] confirmed that *Akt1*, *PTEN* gene mutation was associated with lymph node metastasis, tumor stage, histological grade.

ACP gene [28] inhibits the Wnt signaling pathway, and Clevers, H. et al. [29] proved that the mutation of *ACP* gene was related to tumor distant metastasis. As for *KRAS*, Sanchez-Munoz, A. et al. [30] found that *KRAS* gene mutation was associated with basal breast cancer, and *KRAS* was preferentially activated in basal breast cancer cells compared with tubular breast cancer. *BRAF* gene mutation [31] can continuously activated the Raf-MEK-ERK signaling pathway, and existing studies have found that the mutation of this gene is related to the occurrence and development of breast cancer. *CDKN2A* gene [32] have influence on cell cycle regulation function, the genetic mutation leads to cells out of control and become a malignant tumor, Debniak, T. et al. [33] found *CDKN2A* gene mutations in the highest risk is less than 30 years old, that the gene carcinogenicity has some correlation with age.

In a word, the development of breast cancer was regulated by various cancer related genes. Therefore early prevention of breast cancer and provide certain theoretical basis for clinical treatment and prognosis evaluation through detection of breast cancer related gene mutations is very important.

Conclusion

To sum up, we used SSP high throughput technology that of higher detection sensitivity and specific degree to analyze 207 hot spot mutation region of 50 cancer related genes in 46 breast cancer patients in the present study, and found that there were 33 gene locus mutations of 8 genes including *AKT1*, *APC*, *BRAF*, *CDKN2A*, *KRAS*, *PTEN*, *PIK3CA* and *TP53*, but difference was not statistically significant ($P > 0.05$) when compared these gene mutations (except for *PIK3CA*) in each groups according to the histological classification of breast cancer and the ER/PR/HER2 classification. *PIK3CA* mutation rate of grade I was obviously higher than that of grade II~III histological grading in breast cancer patients ($P < 0.05$), which meant *PIK3CA* may play a key role in the development of the early stage of breast cancer. Last, SSP technology used in our study could be an effective and sensitive method for detection of gene mutation spectrum in breast cancer.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

Ethics Approval This study was approved by the Ethics Committee of Dongguan Maternal and Child Health Care Hospital.

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