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Expression of Stanniocalcin 2 in Breast Cancer and Its Clinical Significance*

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Summary: This study aims to explore the expression of stanniocalcin 2 (STC2) gene in breast cancer and its clinical significance. Female patients with breast cancer from Zhongnan Hospital of Wuhan University admitted during March 2014 to October 2014 were enrolled in this study. All the tissues used in this experiment included 50 cases of breast cancer tissues and corresponding 50 cases of paracancer normal breast tissues with complete patients' information. The real-time quantitative polymerase chain reaction (qPCR) was applied to detect the expression of STC2 gene in 50 cases of breast cancer and paracancer normal breast tissues. The results showed that the expression level of STC2 gene in 50 cases of breast cancer tissues was significantly higher than that in paracancer normal breast tissues (P<0.001). The expression of STC2 gene was correlated with lymph node metastasis, distant metastasis, TNM stage and histological grade (P<0.001). The expression level of STC2 gene was significantly higher in breast cancer tissues with higher expression of Ki-67 (P<0.001). The expression level of STC2 gene was significantly higher in estrogen receptor (ER) positive breast cancer tissues than in ER negative ones (P<0.001). However, different groups of age, pathological type, tumor size, PR expression and human epidermal growth factor receptor-2 (HER2) expression did not show significant differences in STC2 expression (P>0.05). In conclusion, the abnormal overexpression of STC2 gene may play a role in the development and progression of breast cancer, and it can be used as an independent metastasis and prognostic factor of breast cancer. In addition, STC2 gene probably promotes the development and metastasis of breast cancer by interacting with estrogen and ER, and it may become a new direction for breast cancer endocrine therapy.
Key words: stanniocalcin 2; breast cancer; real-time quantitative polymerase chain reaction; metastasis

The global incidence rate of breast cancer is rising continuously, according to 2012 Global Cancer Epidemiology Statistics (GLOBOCAN)[1]. The estimated new cases and estimated deaths of breast cancer both rank first among all the female cancers worldwide. With the progress in breast cancer treatment technology, the survival rate of breast cancer patients has been improved. But the mortality rate still ranks second among female cancers, just behind the lung cancer[2]. Therefore, we need to find out a biomarker to detect breast cancer early and assist in the precise treatment of breast cancer.

Stanniocalcin (STC) is a kind of glycoprotein hormone discovered by Stannius in a secretory gland of teleost fish in 1839[3]. STC promotes the absorption of phosphate by the kidney, avoids hypercalcemia, and maintains the dynamic balance of calcium and

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phosphorus. In 1995, Chang[4] found that there are similar proteins in humans and mammals. Recent research showed that STC was closely related to tumor[3]. Moreover, previous studies have shown that the STC2 gene interrelates with the development and metastasis of many kinds of cancers[5, 6], and also becomes effective biomarkers in some cancers[7, 8]. We accidentally found abnormal high expression of STC2 in liver metastasis specimens from the patients with breast cancer. Reviewing the previous research, we found that the relationship between STC2 and breast cancer has not been clearly reported. Therefore, we hypothesized that STC2 expression might be related to breast cancer. In this study, we aimed to investigate the expression of STC2 gene in breast cancer and its clinical significance

1.1 MATERIALS AND METHODS

1.1 Samples

All the tissues used in this experiment were from Zhongnan Hospital of Wuhan University with the study protocol approved by ethnics committee of the hospital. Written informed consent was obtained from the patients before surgery, with permission to use the specimens for scientific research purposes as well as clinicopathological studies. None of the patients had received any kind of anticancer treatments before the surgery. A total of 100 cases of samples were collected from March 2014 to October 2014, including 50 cases of breast cancer tissues and corresponding 50 cases of paracancer normal breast tissues with complete patients' information. Once the breast cancer lumps and breast tissues were surgically removed from the bodies, the lesions and their surrounding 5 cm outside normal breast tissues were quickly cut off, stored in cryogenic vials labeled, placed in liquid nitrogen tank and transferred to –70°C ultra-low temperature freezer. All the specimens were confirmed through hematoxylin-eosin staining pathology. Fifty cases of breast cancer were all females aged from 28 to 79 years old (mean 49.62 years old). According to TNM stages of breast cancer, there were 14 cases of stage Ⅰ, 20 cases of stage Ⅱ, 9 cases of stage Ⅲ and 7 cases of stage Ⅳ.

1.2 Reagents and Instruments

The following reagents and instruments were applied: SV Total RNA Extraction Kit (Promega Company, USA), Revert Aid First Strand cDNA Synthesis Kit (Thermo Company, USA), SYBR Green PCR MASTER MIX (ABI Company, USA); StepOne Plus Real-time PCR instrument (ABI Company, USA).

1.3 RNA Extraction of Breast Cancer and Paracancer Normal Breast Tissues

A total of 30 mg frozen tissue blocks were cut off by a sterile blade without enzyme, then total RNA was extracted according to the instructions of SV Total RNA Extraction Kit. Finally, the RNA was dissolved in 40 μL nuclease-free water. The ultraviolet spectrophotometer was used to measure the concentration and purity of RNA, and the RNA samples with absorbance at wavelengths of 260/280 between 1.9 and 2.1 were of better quality. The agarose gel electrophoresis was conducted to confirm the integrity of RNA and the results were detected by gel electrophoresis imager. If the brightness of 28S strip was two times that of 18S strip, it was indicated that the integrity of the samples was good. The RNA samples were labeled and stored in –70°C ultra-low temperature freezer.

1.4 cDNA Synthesis

We took 3 μg RNA as standard and then created 20 μL reaction system referring to Revert Aid First Strand cDNA Synthesis Kit instructions. Finally, the reverse transcription reaction process was carried out by PCR instrument. The reaction procedure was set as 25°C for 10 min, 42°C for 90 min and 70°C for 15 min. When the reverse transcription was completed, the synthesized products of cDNA were labeled and stored in –70°C ultra-low temperature freezer.

1.5 Detection of Expression of STC2 Gene by qPCR

In the 20 μL reaction system, 8.5 μL nuclease-free water, 0.5 μL upstream, 0.5 μL downstream primers, 0.5 μL cDNA, and 10.0 μL Taqman Universal Master Mix (2×) were included. The amplification reaction was carried out on StepOne Plus real-time PCR instrument with a cycle parameter of 94°C for 15 min, 58°C for 15 min, and 72°C for 15 min, a total of 45 cycles.

1.6 Data Processing of qPCR Results

The cycle threshold (Ct) of each sample was obtained after qPCR, and there was a linear relationship between the Ct value and the logarithm of the initial RNA concentration of each sample. To ensure the accuracy of quantitative analysis, all the Ct values were controlled from 15 to 35. β-actin[9] was selected as reference gene to control the consistency of all the samples. The purpose of the experiment was to detect the expression of the target gene STC2, and the ΔΔCt method was applied to process data. Finally, the relative mRNA expression levels of target gene STC2 was presented as $2^{-\Delta\Delta Ct}$ ($\Delta Ct=Ct_{STC2}-Ct_{\beta\text{-actin}}$, $\Delta\Delta Ct=\Delta Ct_{cancer}-\Delta Ct_{paracancer}$).

1.7 Statistical Analysis

SPSS 19.0 software was applied for statistical analysis. The normality of each set of data was tested by One-Sample Kolmogorov-Smirnov (K-S) test. If the data were normally distributed, *T* test was adopted. If the data were non-normally distributed, the non-parametric test, Mann-Whitney *U* test was adopted. In addition, Kruskal-Wallis *H* test was used to test more than two sets of data. All the quantitative data were expressed as $\bar{x}\pm s$ and $P<0.05$ was set as the cut-off for statistical significance.

2 RESULTS

2.1 Expression of STC2 in Breast Cancer and Paracancer Normal Breast Tissues

In 50 pairs of samples, breast cancer and paracancer normal breast tissues were compared pairwise and all the data met $2^{-\Delta\Delta Ct}>2$, which initially suggested that the STC2 gene expression in breast cancer tissues was significantly higher than in paracancer normal breast tissues. Statistics showed that $2^{-\Delta\Delta Ct}$ mean=29.2677±24.2404 in breast cancer, and the data didn't meet the normal distribution through K-S test [Z=1.671, Sig.(2-tailed)=0.008<0.05]. And $2^{-\Delta\Delta Ct}$ mean=1.2601±1.0539 in paracancer normal breast tissues, and the data also didn't meet the normal distribution through K-S test [Z=1.530, Sig.(2-tailed)=0.019<0.05, table 1]. Ultimately, Mann-Whitney U test was used to compare the relative mRNA expression of STC2 gene ($2^{-\Delta\Delta Ct}$) in 50 pairs of samples, and the difference was statistically significant (P<0.001; table 1 and fig. 1).

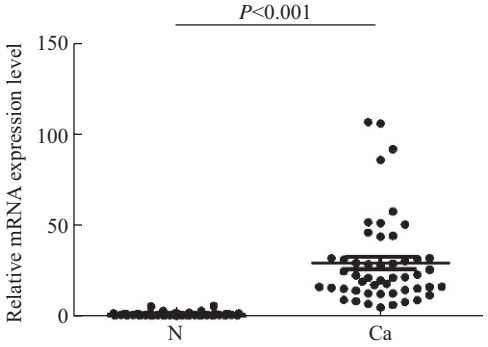

Fig. 1 The distribution of the gene relative mRNA expression level of STC2 in breast cancer tissues (Ca) and paracancer normal breast tissues (N)

2.2 Relationship between Expression of STC2 Gene and Clinicopathological Features of Breast Cancer

The expression of STC2 was correlated with lymph node metastasis, distant metastasis, TNM stages, histological grades and Ki-67 expression (P<0.001). In addition, the expression level of STC2 gene was significantly higher in estrogen receptor (ER) positive breast cancer tissues than in ER negative ones (P<0.001; tables 2–4 and fig. 2).

However, among 50 cases of breast cancer tissues, different groups of age, pathological type, tumor size, progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) expression did not show significant differences in STC2 expression

Table 1 Statistical analysis of STC2 gene expression in breast cancer and paracancer normal breast tissues

Tissue	n	$2^{-\Delta\Delta Ct}$ ($\bar{x}$±s)	K-S test		Mann-Whitney U test
			Z value	Sig.(2-tailed)	P value
Cancer	50	29.2677±24.2404	1.671	0.008	0.000<0.001
Paracancer	50	1.2601±1.0539	1.530	0.019	

Table 2 Relationship between STC2 gene expression and lymph node (LN) metastasis, distant metastasis or TNM stages

Items		n	$2^{-\Delta\Delta Ct}$ ($\bar{x}$±s)	K-S test		T test	
				Z value	Sig.(2-tailed)	T value	P value
LN metastasis	Yes	27	3.4116±2.0680	1.325	0.060	5.772	<0.001
	No	23	1.0663±0.3931	0.570	0.901		
Distant metastasis	Yes	7	4.1444±1.3047	0.613	0.846	6.037	<0.001
	No	43	1.1183±0.5892	0.742	0.641		
TNM stages	Ⅰ–Ⅱ	34	1.1157±0.4585	0.526	0.945	–5.732	<0.001
	Ⅲ–Ⅳ	16	3.6991±1.7750	0.990	0.281		

Table 3 Relationship between STC2 gene expression and histological grades

Histological grades	n	$2^{-\Delta\Delta Ct}$ ($\bar{x}$±s)	K-S test		Kruskal-Wallis H test	
			Z value	Sig.(2-tailed)	χ^2	P value
WHO Ⅰ	5	0.5426±0.1100	0.363	0.999	33.038	<0.001
WHO Ⅱ	34	1.6278±0.5696	0.766	0.601		
WHO Ⅲ	11	5.3253±2.0180	0.926	0.357		

Table 4 Relationship between STC2 gene expression and ER or Ki-67

Items		Cases	$2^{-\Delta\Delta Ct}$ ($\bar{x}$±s)	K-S test		Mann-Whitney U test
				Z value	Sig.(2-tailed)	P value
Ki-67	<14%	15	0.9273±0.3513	0.494	0.968	<0.001
	≥14%	35	2.9700±1.9893	1.599	0.012	
ER	(+)	34	3.0194±1.9971	1.577	0.014	<0.001
	(–)	16	0.8736±0.2827	0.610	0.851	

(P>0.05; tables 5–7).

3 DISCUSSION

STC is a kind of glycoprotein hormone first discovered in bony fish. The recent studies have found that there are similar proteins in humans and mammals[10]. With the further research of the STC, more and more studies have shown that the STC expression is closely related to the development of human cancer[11].

Our previous studies have shown that STC2 gene was overexpressed in liver metastasis specimens of colorectal cancer by microarray technology[12]. In addition, abnormal high expression of STC2 was accidentally detected in liver metastasis specimens

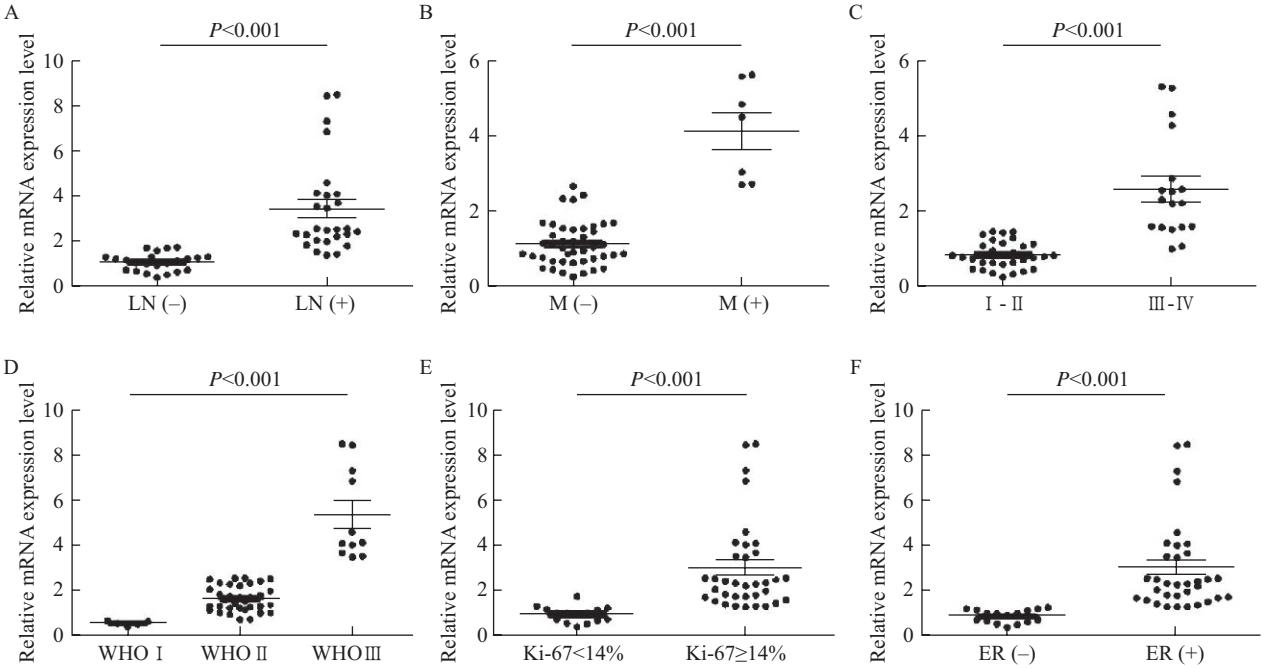

Fig. 2 The distribution of STC2 gene relative mRNA expression level in different groups of breast cancer tissues
A: with and without lymph node (LN) metastasis groups; B: with and without distant metastasis (M) groups; C: Ⅰ–Ⅱ stage and Ⅲ–Ⅳ stage groups; D: WHO Ⅰ, WHO Ⅱ and WHO Ⅲ groups; E: Ki-67 <14% and Ki-67 ≥14% groups; F: ER (+) and ER (–) groups. The difference of STC2 gene relative mRNA expression in different groups was statistically significant.

Table 5 Relationship between STC2 gene expression and age or HER2

Items		n	$2^{-\Delta\Delta Ct}$ ($\bar{x}$±s)	K-S test		T test	
				Z value	Sig.(2-tailed)	T value	P value
Age	≤50	26	1.9333±1.4583	1.115	0.167	–0.041	0.967
	>50	24	1.9523±1.7894	1.296	0.069		
HER2	(+)	13	1.9090±1.3394	1.024	0.246	–0.086	0.932
	(–)	37	1.9541±1.7100	1.328	0.059		

Table 6 Relationship between STC2 gene expression and pathological types or PR

Items		n	$2^{-\Delta\Delta Ct}$ ($\bar{x}$±s)	K-S test		Mann-Whitney U test
				Z value	Sig.(2-tailed)	P value
Pathological types	Ductal	47	1.9768±1.6511	1.588	0.013	0.791
	Lobular	3	1.4035±0.4951	0.418	0.995	
PR	(+)	25	1.7015±0.5115	0.636	0.813	0.093
	(–)	25	2.1892±2.2106	1.663	0.008	

Table 7 Relationship between STC2 gene expression and tumor size

Size	n	$2^{-\Delta\Delta Ct}$ ($\bar{x}$±s)	K-S test		Kruskal-Wallis H test	
			Z value	Sig.(2-tailed)	χ^2	P value
≤2 cm	20	1.8295±1.4075	0.927	0.357	0.036	0.982
>2 cm<size≤5 cm	25	2.0802±1.8845	1.459	0.028		
>5 cm	5	1.7045±0.8553	0.436	0.991		

from the patients with breast cancer, thereby we guessed that STC2 expression might be related to breast cancer.

The results of this study showed that the STC2 expression in breast cancer was significantly higher than that in paracancer normal breast tissues, and the abnormal high expression in breast cancer showed certain tumor specificity. In addition, it has been confirmed that the lymph node metastasis[13], distant metastasis[14], clinical stage[15] and histological grades[16] are closely related to prognosis. During this study, WHO histological grades were adopted. The higher the grade is, the poorer the differentiation is, and it often comes to the poorer prognosis. According to the results, the expression of STC2 gene was significantly higher in those breast cancer tissues with lymph node metastasis, distant metastasis, higher clinical stages and higher histological grades (P<0.001), which infers that STC2 may be involved in the metastasis of breast cancer and the high expression of STC2 predicts a poor prognosis. Moreover, Ki-67 is a kind of nucleoprotein, which is often used in clinicopathological immunohistochemistry and it prompts the level of activity of cell proliferation. Previous research has shown that 14% is the reasonable cut-off for Ki-67 expression[17]. If the positive rate of Ki-67 <14%, it means lower expression. Conversely, if the positive rate of Ki-67 ≥14%, it means higher proliferation of breast cancer cells. According to the results, the expression of STC2 gene was significantly higher in those breast cancer tissues with higher expression of Ki-67 (P<0.001), which infers that STC2 may affect the proliferation of tumor cells of breast cancer and the high expression of STC2 indicates that tumor cells proliferate actively.

Besides, a large number of clinical epidemiology surveys showed that the rising endogenous estrogen or exogenous estrogen replacement therapy could significantly increase the incidence of breast cancer[18]. Additionally, the *in vitro* studies also showed that the estrogen could accelerate breast cells' transformation and promote ER-positive breast tumor cells' proliferation and invasion[19]. So studies have shown that estrogen plays an important role in the ER-positive breast cancers, and ER-positive is an important indicator of breast cancer endocrine therapy[20]. The results of this study showed that the STC2 expression levels were significantly higher in ER-positive breast cancer tissues than those in ER-negative breast cancer tissues (P<0.001), indicating relationship between STC2 and estrogen. But how STC2 gene, estrogen and estrogen receptor interact with each other remains to be further studied.

Breast cancer is a comprehensive disease and the prognosis is affected by multiple factors. So molecular subtypes of breast cancer are becoming more and more important in clinical research[21]. Among them, subtype luminal A breast cancer refers to ER-positive, PR-positive, HER2-negative and Ki-67<14%. Moreover, a large number of research results showed that patients with luminal A breast cancer[22] were sensitive to endocrine therapy and have a good prognosis, which seems to contradict our experimental results. We reflect on our experimental results and there are several possible reasons. First of all, the clinical stage of the ER-positive breast cancer patients enrolled was higher. Secondly, in some breast cancer patients with ER-positive, the PR was negative. Furthermore, it is possible that this result is influenced by a combination of other indicators such as Ki-67, age and HER-2 expression etc. If we want to solve these problems, we need to expand the sample size of the study and refine grouping of experiments.

In conclusion, the STC2 gene is probably involved in the development and metastasis of breast cancer, and it may become a biomarker to judge the prognosis of patients with breast cancer. But its specific role and how the three factors: STC2 gene, estrogen and estrogen receptor interact with each other remains to be further studied. Furthermore, STC2 gene may become a new direction for the researcher of endocrine therapy in breast cancer.

Conflict of Interest Statement

The authors declare no potential conflicts of interest.

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