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ORIGINAL ARTICLE

Correlations of breast cancer FHIT gene with the incidence and prognosis of breast cancer

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Summary

Purpose: *The study aimed to investigate the expression level of fragile histidine triad (FHIT) in breast cancer and analyze its prognostic value.*

Methods: *148 patients admitted and definitely diagnosed with breast cancer in Daqing OilField General Hospital from January 2011 to January 2013 were collected. Breast cancer, cancer-adjacent and normal tissues of the patients were taken and immunohistochemically stained, and the relationship between FHIT and p16 expressions were analyzed at the gene and protein levels. In addition, clinical data of patients were collected, and analyzed if there was a correlation between FHIT and p16 expressions.*

Results: *FHIT and p16 were strongly positive in cancer-adjacent tissues and normal tissues but weakly positive in breast cancer tissues, with statistically significant differences in FHIT and p16 expressions ($p<0.05$). FHIT expression was positively correlated with p16 expression in breast cancer tissues (Spearman's correlation coefficient $r=0.352$, $p=0.026$). There were correlations of FHIT with TNM staging of breast cancer, grade of differentiation, lymph node metastasis and formation of portal vein tumor thrombi ($p<0.05$ in all comparisons). P16 was correlated with tumor size and grade of differentiation ($p<0.05$ in all comparisons). Expressions of both FHIT and p16 genes and proteins in breast cancer tissues were remarkably lower than those in cancer-adjacent and normal tissues ($p<0.05$ in all comparisons). Log-rank analysis showed that the 5-year overall survival of patients with $FHIT^+p16^+$ expressions was significantly longer than that of patients with other phenotypes of expressions ($p<0.0001$).*

Conclusion: *The tumor suppressor gene FHIT is lowly expressed in breast cancer tissues and positively associated with the expression of the multi-tumor suppressor gene p16. The 5-year overall prognosis of patients with $FHIT^+p16^+$ expressions was better and can be used as one of the prognostic indicators for breast cancer patients.*

Key words: *breast cancer, fragile histidine triad, prognosis*

Introduction

Breast cancer, a relatively common malignant tumor, occurs in breast epithelial tissues, and its mortality rate ranks first among all cancers in females [1]. Hence, understanding the pathogenesis of breast cancer is of great significance for clinical treatment and improvement of prognosis evaluation. Fragile histidine triad (FHIT) is a brand-new tumor suppressor gene existing in most normal organ tissues in the body [2]. It has been currently confirmed that FHIT can induce cell apoptosis and retard cell growth cycle, so as to impede tumor proliferation [3]. P16 is a multi-tumor suppressor gene, which is named for its expression products, approximately 16 kD protein molecules [4]. The active function of cyclin-dependent kinases (CDK) is a prerequisite for all cells entering the growth cycle. Besides, inhibiting the biological activity of CDK is one of the functions of p16 protein, which blocks the growth cycle and subsequently exerts an inhibitory effect on growth [5]. In this study,

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immunohistochemistry was applied to detect clinical specimens of 148 breast cancer patients, so as to understand the expression levels of FHIT and P16 proteins in breast cancer tissues, and to explore the relationship between the expressions of the two in breast cancer. The follow-up analysis was carried out.

Methods

Data

A total of 148 patients admitted and definitely diagnosed with breast cancer in Daqing OilField General Hospital from January 2011 to January 2013 were enrolled, including 16 males aged 28-67 years and 132 females aged 28-75 years. Specimens of breast cancer cases were washed, placed in 10% formalin solution, embedded in paraffin and cut into sections . According to the pathological classification, the patients were divided into 54 cases of poor differentiation, 64 cases of moderate differentiation and 30 cases of well differentiation. Based on TNM staging, these patients were divided into 88 cases in stage I+II and 60 cases in stage III+IV, 96 cases with tumor size <5cm and 52 cases with tumor size ≥5cm, 46 cases with lymph node metastasis and 102 cases with no lymph node metastasis, and 38 cases experiencing formation of portal vein tumor emboli and 110 cases not experiencing formation of portal vein tumor emboli. The study was approved by the ethics committee of Daqing OilField General Hospital and all patients and their family members signed informed consent.

Instruments and reagents

The following instruments and reagents were used in this study: microscope (Olympus), FHIT polyclonal antibody (Beijing Dingguo Changsheng Biotechnology Co., Ltd.), p16 polyclonal antibody (Biosciences, Franklin Lakes, NJ, USA), SP9000 kit (Shanghai Ruian Biotechnology Co., Ltd.), 3,3'-diaminobenzidine (DAB) color development reagent (Shanghai X-Y Biotechnology Co., Ltd.), neutral gum (Shanghai Ruian Biotechnology Co., Ltd.), hematoxylin and eosin (Baomanbio, Shanghai), fluorescence real-time quantitative polymerase chain reaction (PCR) kit (Vipotion, Guangzhou, China), TRIGene reagent (GenStar BioSolutions, Beijing), reverse transcription (RT) kit (Shanghai Yuduo Biotechnology Co., Ltd.) and cell total protein extraction kit (Jiangsu KeyGEN BioTECH Co., Ltd., Jiangsu, China).

Immumohistochemical staining

Cancer, cancer-adjacent and normal tissues of breast cancer patients were prepared from paraffin embedded sections and then dewaxed, hydrated and washed with phosphate-buffered saline (PBS), respectively. After blocking for 15 min, the sections were blocked with 10% serum under non-specific condition for 15 min at room temperature, and then primary antibodies (FHIT and p16 monoclonal antibodies) were added for incubation at 4°C overnight. After taking out, the sections were rinsed with PBS, and biotin-labeled secondary antibody was added for incubation at room temperature for 30 min, followed by washing with PBS. Afterward, the sections were incubated in streptomyces antibiotic protein-peroxidase solution at room temperature for 30 min, followed by washing with PBS. Subsequently, DAB was used for color development, followed by rinsing with tap water and counterstaining with hematoxylin. Finally, the sections were mounted with neutral gum and were observed under a microscope.

Determination of results

The fields of view to be observed were randomly selected to count 100 cells, and the average number of cells in the fields of view was calculated as the number of positive cells of the expression protein in tissues. Staining intensity scores: 0-2 points represented no staining, weak staining and strong staining, respectively. Stained cell positive rate scores: 1-4 points showing the percentages of positive cells were 1,25; 26,50; 51,75 and 76,100, respectively. The product of the scores of the above two groups were: ≤2 points for negative, 3-4 points for weakly positive (+), 5-8 points for moderately positive (++), and ≥9 points for strongly positive (+++).

Detection of the expressions of FHIT and p16 genes via RT-PCR

Total RNA was extracted from tissues according to the instructions of the TRIGene kit and spectrophotometer was employed to determine the concentration and purity of the two types of total RNAs. The ratio of absorbance at 260 and 280 nm (A260/A280) was 1.8-2.0. According to the instructions of the RT kit [Reverted Fist Strand Complementary Deoxyribonucleic Acid (cDNA) Synthesis Kit, Thermo (Waltham, MA, USA), K1622], primer sequences were synthesized by Shanghai Jiran Biotechnology Co., Ltd. FHIT forward primer: 5'-AAGAGGAAAACTGAGCCATCTG-3', and reverse primer: 5'-CGGCTAACATCCCACTGATAAT-3'; p16 forward primer: 5'-TGGTTAGAGGCTGCCTGTG-3', and reverse primer: 5'-TGGACAAGACCCTGAAGACA-3'; and β-actin forward primer: 5'-CAGGAAGGAAGGCTGGAAG-3', and reverse primer: 5'-CGGGAAATCGTGCCTGAC-3'. A total volume of 20 μL reaction systems were reversely transcribed on a RT-PCR machine to generate cDNA.

Based on the instructions of the real-time fluorescence quantitative PCR kit (2×RealStar Green Power Mixture, GenStar, Calgary, AB, Canada, A311), there were 25μL reaction systems. Reaction conditions: a total of 40 cycles of reaction at 95°C for 10 min, 95°C for 30s and 59.4°C for 30s, and after 15s of reaction at 95°C, the systems were cooled to 65°C. The fluorescence value was read with β-actin as an internal reference. RT-PCR machine was applied to automatically calculate the relative expressions of FHIT and p16 mRNAs.

Detection of the expressions of FHIT and p16 proteins via Western blotting

According to the instructions of the total protein extraction kit, the total proteins were extracted in each type of tissues. The concentration of the extracted proteins was measured, and the proteins were stored at

-70°C for standby application. Gels at different concentrations were prepared for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and the location of gels where the two kinds of proteins were located was selected according to the marker bands. After transfer onto membranes, the proteins were changed to be blotted with 5% skim milk powder and sealed at 37°C for 90 min. Primary antibodies were used for incubation at 4°C overnight. Then tris-buffered saline with Tween 20 (TBST) solution was added, placed, shaken and washed on a shaker 3 times, 15 min/time. Subsequently, secondary antibodies were added for incubation for 1 hr at 37°C, and TBST solution was added. Thereafter, the sections were placed, shaken and washed on the shaker for 15 min for 3 times. The enhanced chemiluminescence (ECL) liquid was added in a dark room for coloring, followed by exposure, color development and fixation. Finally, ChemiDocTM MP imaging system was applied for scanning. ImageJ professional image analysis software was adopted for image analysis, and the absorbance value was recorded.

Statistics

In this experiment, SPSS 17.0 professional statistical software (provided by Beijing Xinmei Jiahong Technology Co., Ltd.) was used for data analysis. The chi-square test was employed for comparison between positive rates and comparisons of FHIT and p16 with clinicopathological parameters. The correlation between FHIT expression and p16 expression was detected using Spearman's test. α=0.05 was taken as the limit of statistical significance.

Results

FHIT protein expression in three kinds of tissues

FHIT protein mainly existed in the cytoplasm of cells, and yellow or brown-yellow color represented its positive expression (Figure 1).

The positive expression rate of FHIT protein was 39.19% (58/148) in breast cancer tissues, 66.89% (99/148) in cancer-adjacent tissues and 93.24% (138/148) in normal tissues. The differences in the positive expression rate of FHIT protein among the three kinds of tissues were statistically significant ($p<0.05$) (Table 1).

p16 protein expression in three kinds of tissues

p16 protein was mainly expressed in the cytoplasm but less expressed in the nucleus (Figure 2).

Table 1. Positive expression rate of FHIT in breast cancer, cancer-adjacent and normal tissues

Tissues	*n*	*FHIT*		*Positive rate (%)*	x^2	*p*
		- *n*	+~+++ *n*			
Breast cancer tissue	148	90	58	39.19		
Cancer-adjacent tissue	148	49	99	66.89	26.44	0.000
Normal tissue	148	10	138	93.24		

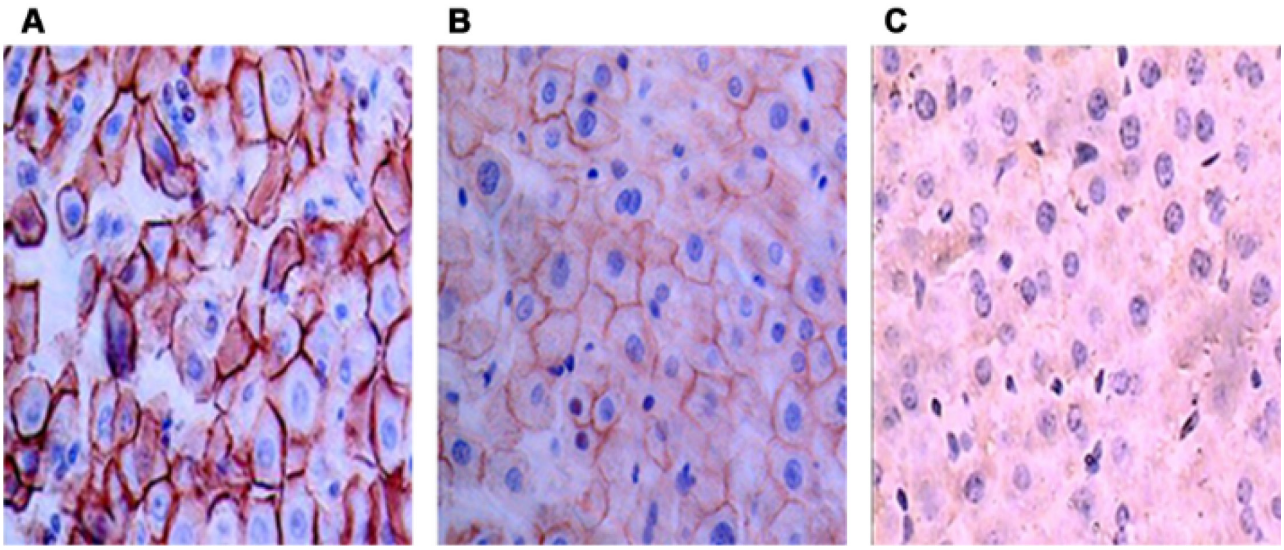

Figure 1. FHIT expression in cancer, cancer-adjacent and normal tissues of breast cancer patients (×100). **A:** The positive expression rate of FHIT protein in breast cancer tissues was 39.19% (58/148). **B:** The positive expression rate of FHIT protein in cancer-adjacent tissues was 66.87% (99/148). **C:** The positive expression rate of FHIT protein in normal tissues was 93.24% (138/148). The positive expression rate of FHIT protein was significantly different among the 3 tissues ($p<0.05$).

The positive expression rate of p16 protein was 41.22% (61/148) in breast cancer tissues, 73.65% (109/148) in cancer-adjacent tissues and 97.97% (145/148) in normal tissues, displaying statistically significant differences in the positive expression rate of p16 protein among the three kinds of tissues (p<0.05) (Table 2).

Correlation between FHIT and p16 protein expressions

In 148 cases of breast cancer tissues, FHIT protein was positively expressed in 58 cases, 50 cases of which also had positively expressed p16 protein at a rate of 86.21%. Statistical analysis revealed that the expressions of the two types of proteins exhibited a positive correlation (Spearman's correlation coefficient r=0.352, p=0.026) (Table 3).

Correlations of the positive expressions of FHIT and p16 in breast cancer tissues with clinicopathological factors

Analysis revealed that the positive expression of FHIT was not correlated with the patient's age, gender and tumor size (p>0.05 in all comparisons), but it was related to the TNM staging of breast can-

Table 2. Positive expression rate of p16 in breast cancer, cancer-adjacent and normal tissues

Tissues	*n*	*p16*		*Positive rate (%)*	x^2	*p*
		- *n*	+~+++ *n*			
Breast cancer tissue	148	87	61	41.22		
Cancer-adjacent tissue	148	39	109	73.65	19.36	0.005
Normal tissue	148	3	145	97.97		

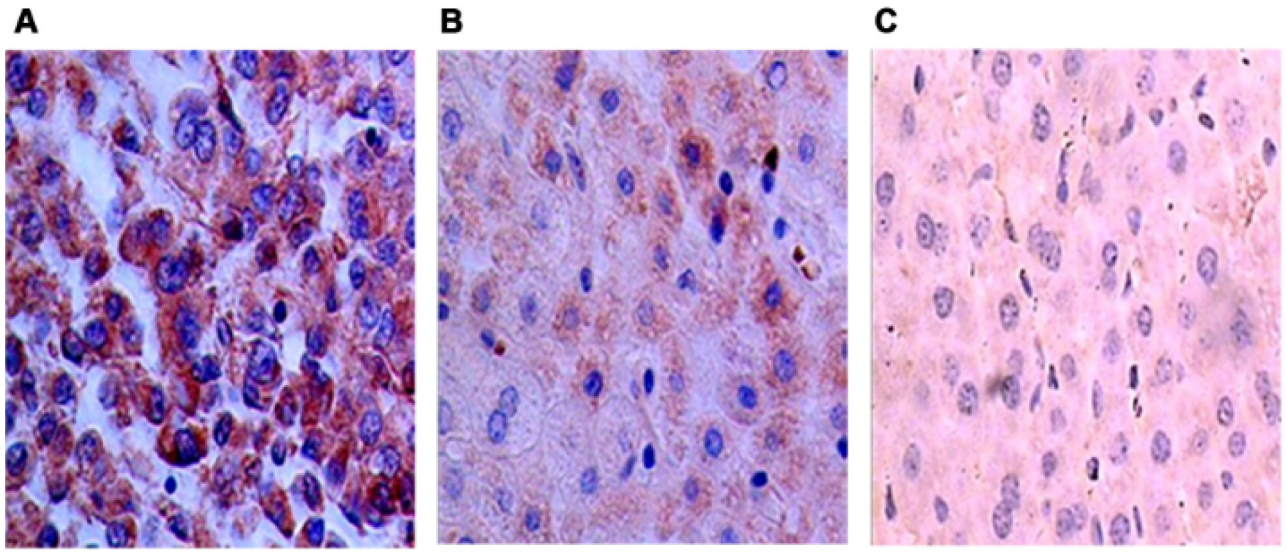

Figure 2. Expression of p16 in cancer, cancer-adjacent and normal tissues of breast cancer patients (×100). **A:** The positive expression rate of p16 protein in breast cancer tissue was 41.22% (61/148). **B:** The positive expression rate of p16 protein in cancer-adjacent tissues was 73.65% (109/148). **C:** The positive expression rate of p16 protein in normal tissues was 97.97% (145/148). The positive expression rate of p16 protein was significantly different among the 3 tissues (p<0.05).

Table 3. Correlation between FHIT and p16 protein expressions in breast cancer tissues

		FHIT		*Total n*	*r (Spearman)*	*p*
		- *n*	+~+++ *n*			
p16	+	50	11	61		
	-	8	79	87	0.352	0.026
Total		58	90	148		

cer, grade of differentiation, lymph node metastasis and formation of portal vein tumor thrombi (p<0.05 in all comparisons). The positive expression of p16 protein had no correlations with gender, age, TNM staging, lymph node metastasis and formation of portal vein tumor emboli (p>0.05 in all comparisons), but it had relationships with tumor size and grade of differentiation (p<0.05 in all comparisons) (Table 4).

Expressions of FHIT and p16 mRNAs in tissues

The relative expression levels of FHIT mRNA in breast cancer, cancer-adjacent and normal tissues were 1.38±0.14, 7.23±1.03 and 10.19±1.26, respectively, showing statistically significant differences (p<0.05). The relative expression levels of p16 mRNA in breast cancer, cancer-adjacent and normal tissues were 6.48±0.96, 14.92±1.83 and 17.49±2.06, respectively, and the differences were statistically significant (p<0.05) (Table 5).

Expressions of FHIT and p16 proteins in tissues

The expression levels of FHIT protein in breast cancer, cancer-adjacent and normal tissues were 7.25±0.97, 19.47±3.32 and 25.93±5.06, respectively, with statistically significant differences (p<0.05). The expression levels of p16 protein were 12.49±3.06, 49.82±4.79 and 58.94±6.03, and comparisons demonstrated that the differences were statistically significant (p<0.05) (Table 6).

Table 4. Correlations of the positive expressions of FHIT and p16 in breast cancer tissues with clinicopathological factors

Characteristics	*Total n*	*FHIT*			*p16*		
		Positive	x^2	*p*	*Positive*	x^2	*p*
Age (years)							
<40	44	16	0.68	0.914	12	0.045	0.861
≥40	104	38			44		
Gender							
Male	102	34	0.917	0.626	42	1.375	0.472
Female	46	12			18		
Tumor size (cm)							
<5	96	64	1.385	0.744	54	5.026	0.035
≥5	52	24			30		
TNM staging							
I-II	88	58	8.494	0.025	40	2.195	0.218
III-IV	60	18			18		
Edmondson staging							
High differentiation	30	26	15.027	0.001	22	12.941	0.003
Moderate differentiation	64	30			26		
Low differentiation	54	14			16		
Lymph node metastasis							
Yes	46	18	7.492	0.024	24	1.492	0.174
No	102	54			46		
Formation of portal vein tumor emboli							
Yes	38	10	9.621	0.004	18	3.285	0.376

Table 5. The relative expression levels of FHIT and p16 mRNAs in three kinds of tissues

Tissues	*FHIT*	*p16*
Breast cancer tissue	1.38±0.14	6.48±0.96
Cancer-adjacent tissue	7.23±1.03*	14.92±1.83*
Normal tissue	10.19±1.26*#	17.49±2.06*#

*p<0.05 vs. breast cancer tissues, and #p<0.05 vs. cancer-adjacent tissues

Table 6. Expressions of FHIT and p16 proteins in three kinds of tissues

Tissues	*FHIT*	*p16*
Breast cancer tissue	7.25±0.97	12.49±3.06
Cancer-adjacent tissue	19.47±3.32*	49.82±4.79*
Normal tissue	25.93±5.06*#	58.94±6.03*#

*p<0.05 vs. breast cancer tissues, and #p<0.05 vs. cancer-adjacent tissues

Prognosis analysis

Log-rank analysis was conducted for the prognosis of FHIT$^+$ p16$^+$ and other types of patients. The results illustrated that the 5-year overall survival of patients with FHIT$^+$ p16$^+$ was significantly higher than that of patients with other types, displaying statistically significant differences (p<0.0001) (Figure 3).

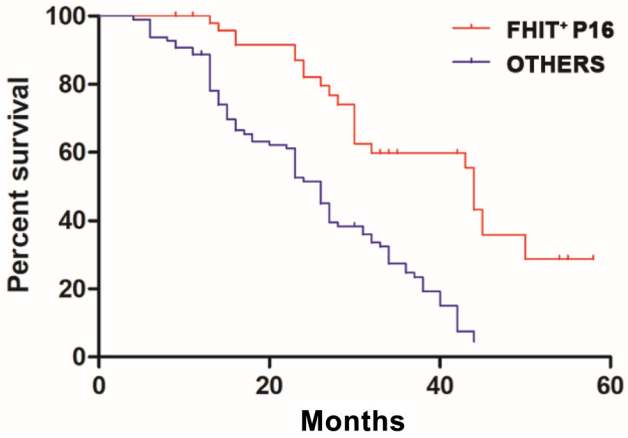

Figure 3. Prognostic analysis. OTHERS represent FHIT$^+$p16$^-$, FHIT$^-$p16$^+$ and FHIT$^-$ p16$^-$. The 1-5 year survival rates of FHIT$^+$ p16$^+$ breast cancer patients were 98.1%, 82.5%, 58.4%, 37.2% and 29.3%, respectively. The median survival was 43.67 months. The 1-3 year survival rates of breast cancer patients with OTHERS were 88.7%, 59.0%, 24.6%, 6.3% and 3.9%, respectively, and the median survival was 22.87 months. Significant difference was noted between the two groups (p<0.05).

Discussion

Breast cancer is a malignant tumor with the highest incidence rate in females and the main cause of cancer-related deaths, accounting for over 15% of cancer patients according to the latest global cancer statistics [6]. Breast cancer is a tumor with high heterogeneity. At present, the occurrence and development processes of breast cancer are not yet fully clear. However, it is widely accepted in academia that the pathogenesis of breast cancer is a process involving multiple factors under the crossover effects of multiple steps, including overexpression of oncogenes and loss of expression of tumor suppressor genes [7].

FHIT is a tumor suppressor gene, whose expressed protein is located in the cytoplasm. A study has revealed that FHIT protein mainly plays its role in the specific structure of mRNA and interferes with the normal translation function of mRNA, resulting in loss of the expression functions of the target gene. The accumulation of these lost functions to a certain degree will induce the occurrence and development of tumors ultimately [8]. Koc et al. [9] reported that tissues in more than half of gastric cancer patients experience abnormal transcription of FHIT gene, and nearly 70% of patients undergo loss of expression of FHIT gene. However, Cardoso et al. [10] found that abnormal transcription of FHIT gene is present in tissues of 87% of gastric cancer patients. PCR detection conducted by Simonavice E et al. [11] showed that abnormal transcription and expression of FHIT gene are found in both gastric and colorectal cancers, suggesting that FHIT gene is usually absent in tumors of the digestive system such as gastric cancer. According to previous studies, the expression of FHIT gene is decreased and FHIT protein is depleted in 40% of tumor cells, and lymph node metastasis occur in most patients [12], indicating that the reduced expression of FHIT protein may be related to lymph node metastasis and poor prognosis of tumor patients. This study revealed that FHIT was abnormally expressed in breast cancer tissues, whose expression level was significantly lower than those in cancer-adjacent and normal tissues (p<0.05). Besides, the positive expression of FHIT was not associated with age, gender and tumor size of the patients (p>0.05 in all comparisons) but correlated with TNM staging of breast cancer, grade of differentiation, lymph node metastasis and formation of portal vein tumor thrombi (p<0.05 in all comparisons). Hence, it was speculated that detecting FHIT gene expression could be used as a reference for the diagnosis, treatment and metastasis of breast cancer, thus providing important clinical significance.

P16 gene is a multi-tumor suppressor gene and an anti-oncogene mainly acting on the cell cycle [13]. Studies have demonstrated that p16 protein mainly competes with cyclin D1 to bind to CDK4/CDK6, thus arresting cells at G1 phase and ultimately negatively regulating cell proliferation [13,14]. Qiu et al. [15] found that p16 protein can suppress the proliferation and metastasis of tumor cells. However, Zhang et al. [16] revealed that the methylation level of p16 gene in serum of patients after gastric cancer surgery notably declines, while the level of the normal expression product of p16 is remarkably increased. The results of this study illustrated that p16 protein was abnormally expressed in breast cancer tissues, whose expression level was significantly lower than those in cancer-adjacent and normal tissues (p<0.05), and p16 expression was correlated with tumor size and grade of differentiation (p<0.05).

Studies have shown that FHIT and p16 loss of expression exists in the occurrence process of lung cancer, and absence of the FHIT gene is a highly frequent incident in the early disease stage [17,18].

However, Bianchi et al. [19] found that loss of expression of p16 gene occurs in a later stage after the occurrence of breast tumors, and patients with both FHIT and p16 expression losses have worse prognosis. It was also reported in this study that there was an obvious positive correlation between the FHIT and p16 protein expressions in breast cancer tissues, indicating that the expression losses of both are involved in the process of breast cancer and jointly exert an inhibitory effect. However, specifically speaking, the mechanism of the joint inhibition is not yet understood and needs further studies. This study also revealed that the 5-year overall survival of patients with $FHIT^+$ $p16^+$ expressions was significantly higher than those of patients with other phenotypes of expressions, displaying statistically significant differences ($p<0.0001$), which is consistent with the findings of Czarnecka et al. [20]. This suggests that the expression loss(es) of FHIT and/or p16 in the tissues may lead to poor prognosis of breast cancer patients, thus providing a reference for the clinical evaluation of the prognosis of breast cancer.

In summary, the tumor suppressor gene FHIT is lowly expressed in breast cancer tissues, and it has a positive correlation with the expression of the multi-tumor suppressor gene p16. The prognosis of 5-year overall survival of patients with $FHIT^+$ $p16^+$ expression is relatively good, and so they can be used as indicators for prediction of prognosis of breast cancer patients.

Authors' contributions

YF and XS collected and analyzed the general data. YF and WS performed PCR. KX helped with immumohistochemical staining. CJ and QZ were responsible for western blot.

Conflict of interests

The authors declare no conflict of interests.

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