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ORIGINAL ARTICLE

Interaction of HCCR-1 and Bax in breast cancer

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Summary

Purpose: *To evaluate the effect on breast cancer cell proliferation and apoptosis after silencing the HCCR-1 and to study its mechanism.*

Methods: *HCCR-1 siRNA was transfected into the breast cancer cell line MCF -7, and mRNA and protein level of HCCR-1 and Bax were evaluated by real-time quatitative PCR (qRT-PCR) and Western blotting, respectively. The cell proliferation and apoptosis were studied by MTT assay and flow cytometry.*

Results: *The apoptosis rate in the experimental, control and blank groups were 32.57±2.35%, 3.53±0.60% and 3.15±0.46% respectively. The apoptosis rate of MCF-7 cells was significantly increased in the experimental group, compared with the other two groups ($p<0.05$). The A490 value in the experimental, control and blank groups were: 24h: 0.78±0.06, 1.18±0.05, 1.24±0.05; 48h: 1.09±0.05, 1.48±0.02, 1.54±0.04; 72h: 1.29±0.01, 1.81±0.02, 1.84±0.04. The proliferation of MCF-7 cells was significantly decreased in the experimental group, compared with the other two groups ($p<0.05$). The mRNA levels of HCCR-1 and Bax in the experimental, control and blank groups were: HCCR-1 0.46±0.03, 1.01±0.11, 1.00; and Bax 4.40±0.99, 1.03±0.10, 1.00. The protein levels were: HCCR-1 0.62±0.07, 0.89±0.09, 0.94±0.17; and Bax 0.95±0.22, 0.67±0.19, 0.69±0.11. The expressions of mRNA and protein of HCCR-1 were significantly reduced, however the expressions of mRNA and protein of Bax were significantly increased in the experimental group compared with the other two groups ($p<0.05$).*

Conclusions: *HCCR-1 siRNA transfection causes significant increase in the apoptosis and decreases in the proliferation of MCF-7 cells. These effects are related to the upregulation of the Bax expression in the MCF-7 cells.*

Key words: *breast carcinoma, HCCR-1, Bax, siRNA*

Introduction

In the biological process of malignant tumors, the proliferation of tumor cells is excessively increased, while apoptosis is inhibited, and inhibition of apoptosis is more critical than excessive proliferation. Currently, radiotherapy, chemotherapy and biological therapy in tumors contribute to induction of apoptosis [1]. The Bcl-2 family is an important family of apoptosis-regulating proteins and a key part in the apoptotic signal transduction pathway [2]. Bcl-2 is the most representative apoptosis-suppressing gene in the Bcl-2 family, and Bax is a protein co-precipitated with Bcl-2, discovered by Oltvai in 1993 [3,4]. Bax has partial homology with Bcl-2 protein, belonging to the Bcl-2 family, and is a pro-apoptotic factor [3,4]. Its product binds to Bcl-2 protein as a heterodimer, regulates the

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activity of Bcl-2 [5], and inhibits the anti-apoptotic effect of Bcl-2. Zong et al. found that Bax protein is a key factor to regulate apoptosis in the Bcl-2 family [6]. Studies have shown that wild-type p53 regulates apoptosis in breast carcinoma by its downstream factors Bax, Bcl-2, etc. [7]. At present, a number of studies have shown that HCCR-1 is associated with tumorigenesis [8-11]. In addition to being overexpressed in cervical cancer, it is also found to be highly expressed in breast cancer and may even serve as a marker for early diagnosis of breast cancer [8-11].

The role HCCR-1 and Bax in the occurrence and development of breast cancer, the relationship between them, and whether HCCR-1 promotes the development of breast cancer by affecting Bax expression are main concerns. At present, many studies have found that the expression level of HCCR gene does not affect the expression of p53. It affects the stability of wild-type p53 by modifying the expression of Bax, thereby promoting the occurrence of tumors, but the related research regarding HCCR-2 is relatively rare for HCCR-1 [12-15].

The purpose of this study was to investigate the usefulness of RNAi technology to inhibit the expression of HCCR-1 in MCF-7 cells and its effect on Bax expression, cell proliferation and apoptosis. The possible molecular mechanism of HCCR-1 on the biological characteristics of breast cancer was also explored.

Methods

Experimental reagents

The following reagents were used in this study: HCCR-1 siRNA and its control (Guangzhou Ruibo, China), the target sequence was GGAAATACCATCGTTTCTT. opti-MEM (Gibco, USA), FUGENE 6 Transfection Kit (Promega, USA), M-MLV Reverse Transcriptase (Promega, USA), SunShineBioTM Total RNA Extraction Reagent (Nanjing Sangon Biotech, China), GoTaq® qPCR Master Mix (Promega, USA), dNTP Mix (Promega, USA), Oligo (dT) 15 Primer (Nanjing Sangon Biotech), RNase Inhibitor (Nanjing Sangon Biotech), Pierce BCA Protein Assay Kit (Thermo Scientific Scientific, USA), Supersignal West Pico Trial kit (Pierce, USA), PageRuler Prestained Protein ladder (Fermentas, Canada), HCCR-1/Bri-3 Binding Protein Antibody (Bioss, Beijing), Bax Monoclonal Antibody (Boster Biological technology, Wuhan, China), Annexin V-FITC Apoptosis Detection Kit (Keygentec, Nanjing, China), MTT kit (Boster Biological Technology, Wuhan, China). SP immunohistochemistry kit (Zsbio, Beijing, China), DAB horseradish peroxidase colorimetric kit (Boster Biological technology, Wuhan, China).

Primer design

Primer sequences were designed and synthesized by Nanjing Sangon Biotechnology Co., Ltd. as follows:

HCCR-1 F: TCCACCTTTTGCCAACTACCT
R: AGTTTGTTGTTTTGGGGTCCA
Bax F: GCTTCAGGGTTTCATCCAGG
R: GAGACACTCGCTCAGCTTCTTG
β-actin F: GGAAATCGTGCGTGACATTAAG
R: CGTCAGGCAGCTCGTAGCTC

Research cells

The breast cancer cell line MCF-7 is the most commonly used cell in breast cancer research. It has a fast reproduction rate and is easy to passage. It can express both estrogen and progesterone receptors and express the wild-type p53 gene [7]. In order to make this study consistent with the literature, this cell line was selected in this experimental work. The MCF-7 cells required for this experiment were purchased from Nanjing Sunshine Biotechnology Co., Ltd.

Research sample

A hundred and sixty paraffin-embedded human breast cancer blocks were obtained from patients who were hospitalized in Qilu Hospital of Shandong University from 2012.03 to 2015.06. All of them were identified as primary breast cancer by the Hospital's department of pathology. Of these cases, 92 were ≤50 years, 68 were >50 years and the age range was 27-76 years (median 48). All 160 specimens were obtained from female patients who had not radiotherapy or endocrine therapy performed before surgery.

Research methods

Cell culture

MCF-7 cells were taken out from liquid nitrogen, put in a 38℃ water bath and inoculated into DMEM medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cells were cultured at 37°C in an incubator with 5% CO_2. The cell culture medium was changed every other day, and the cells were digested with 0.25% trypsin when reaching a confluence of 80%. The split ratio was 1:3.

Cell groups

The material was divided into the following three groups: experimental group, control group and blank group. A 6-well culture plate was used for each group. 3×10^5 MCF-7 cells were added into 3 wells of each plate and cultured for 12 h. A hundred μl of FuGENE6/HCCR-1 siRNA transfection complex were added to each well of the experimental group according to the experimental procedure of FUGENE 6 Transfection kit. A hundred μl of FuGENE6/HCCR-1 siRNA control transfection complex were added to each well of the control group; and 100 μl of opti-MEM nutrient solution were added to each well of the blank group. After cell incubation at 37°C under 5% CO_2 for 6 h, nutrient solution was added and cells were incubated at 37°C under 5% CO_2 for 24 h. The cells were collected for detecting the mRNA and protein expression levels of HCCR-1 and Bax respectively, and the apoptosis of the cells after transfection was detected by flow cytometry.

Real-time quantitative PCR (qRT-PCR) detection of the expression of HCCR-1 and Bax mRNA in different groups

The TRIzol method was used for the extraction of total RNA from a cell sample. The interaction of Random Primer and Oligo(dT)15 Primer reverses the complete transcription of mRNA into cDNA. Amplification was performed using the GoTaq® qPCR Master Mix kit. The reaction procedure was as follows: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min for a total of 40 cycles. The data was obtained according to the above experimental method, and the threshold and Ct values were automatically obtained by Bio-Rad iQ5 Real-Time PCR Analysis Software.

Western blotting

Western blotting was used to detect the expression of HCCR-1 and Bax protein before and after transfection: after the transfection was completed, the cells were taken out. 1×RIPA lysate (300 μl/well in a 6-well plate) was added, and the DNA was ultrasonically sheared, centrifuged at 12000 g for 10 min, and the supernatant was collected. The protein concentration was determined by the bicinchoninic acid (BCA) method. The membrane was transferred after electrophoresis separation and washed with Tris-buffered saline with Tween (TBST) 3 times. A suitable solution of horseradish peroxidase (HRP) labeled secondary antibody was added. The film was developed and fixed according to the instructions in the chemiluminescence kit and was scanned and the net optical density value of the target strip was analyzed using a gel image processing system and ImageJ2 software.

MTT assay for cell proliferation

Log phase cells were collected, the cell suspension concentration was adjusted to 2x104 cells/ml, and 200 μl of cell suspension were added to each well of a 96-well plate. The experiment was divided into the experimental group, the control group and the blank group, and each group was supported with 4 duplicate wells. Transfection was observed for 24, 48, and 72 h under a microscope. Twenty μl of MTT staining solution were added to each well and the incubation continued for 4 h. A hundred and fifty μl of Formazan solution were added to each well and mixed gently by pipetting. The mixture was placed in a cell culture incubator for incubation. The degree of dissolution was monitored under a light microscope until complete dissolution of Formazan was observed and detected by a microplate reader. The detection wavelength was 490 nm. The data were recorded for statistical analysis.

Apoptosis was detected by Annexin-V FITC/PI double staining method

After transfection, the cells were digested with trypsin without EDTA, centrifuged at 4°C, 2000 r/min for 5 min and the cells were prepared and counted. Each centrifuge tube with not less than $5x10^5$ cells was resuspended in 100 μl of 1xBinding Buffer. 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) were added and mixed gently. Four hundred μl of 1×Binding Buffer were added and mixed gently, and tested by flow cytometry within 60 min. Flow cytometry was zeroed with unstained cells, and Annexin V-FITC single stained tubes and PI single stained tubes were used as references, respectively. Data of each sample tube was measured, recorded and analyzed using CellQuest 3.0 to calculate the percentage of apoptosis.

Construction of a breast cancer tissue microarray

A two-dimensional array map was constructed, and the rows and columns were represented by A1, 2, 3...and B1, 2,3...respectively. Each chip was set with 42 dots, which were divided into 6 horizontal rows and 7 vertical rows, which were represented by A1-6 and B1-7, respectively. To avoid confusion, the two starting horizontal points were set to be blank. Two puncture points were selected for each sample so that each tissue chip could hold a total of 20 samples.

Immunohistochemistry

Phosphate buffered saline (PBS) was used instead of primary antibody as a negative control. The specific procedure was performed according to the instructions of SP immunohistochemistry kit. The score was based on the degree of cytoplasmic staining and the percentage of stained cells. Dyeing degree: grade 0: basically not colored, grade 1: light yellow, grade 2: yellow, grade 3: brown. Percentage of positive staining cells: The percentage of stained positive cells in the counted cells was < 5% for grade 0, 5% to 25% for grade 1; > 25% to 50% for grade 2; and > 50% for grade 3. The cytoplasmic staining degree was multiplied by the percentage of stained cells, and the product was ≥4 for positive expression and 0 to 3 for negative expression.

Statistics

Data analysis was performed using the statistical software SPSS19.0. Data are expressed as mean ± SD. T-test was used for comparison between the two groups of data, and x^2 test was used for comparison of positive rates between groups. P<0.05 indicated that the difference was statistically significant.

Results

Changes in HCCR-1 mRNA and Bax mRNA between different groups (Table 1)

Compared with the blank group (1.00) and the control group (1.01±0.11), the HCCR-1 mRNA mean

Table 1. Changes in HCCR-1 mRNA and Bax mRNA between different groups (x ±s)

Group	*Bax mRNA*	*HCCR-1 mRNA*
Blank	1.00	1.00
Control	1.03±0.10	1.01±0.11
Experimental	4.40±1.00*	0.47±0.03*

Compared with the blank and control group, *p<0.05

Table 2. Changes in HCCR-1 and Bax proteins between different groups (x ±s)

Group	*HCCR-1*	*Bax*
Blank	0.94±0.17	0.69±0.11
Control	0.89±0.09	0.67±0.19
Experimental	0.62±0.07*	0.95±0.22*

Compared with blank and control group, *p<0.05

Table 3. Apoptosis rate detection by flow cytometry

Group	*Apoptosis rate*
Blank	3.15±0.46
Control	3.53±0.60
Experimental	32.57±2.35*

Compared with blank and control group, *p<0.05

expression (0.46±0.03) in the experimental group was significantly down-regulated, and the difference was statistically significant (p<0.05), while the blank group HCCR-1 mRNA expression was not significantly different from the control group (p>0.05).

Compared with the blank group (1.00) and the control group (1.03±0.10), the mean expression of Bax mRNA in the experimental group (4.40±0.99) was significantly increased, and the difference was statistically significant (p<0.05). The expression of Bax mRNA was not significantly different compared with that in the control group (p>0.05).

Changes in HCCR-1 and Bax proteins between different groups (Figure 1, Table 2)

Compared with those in the blank group (0.94±0.17) and the control group (0.89±0.09), the

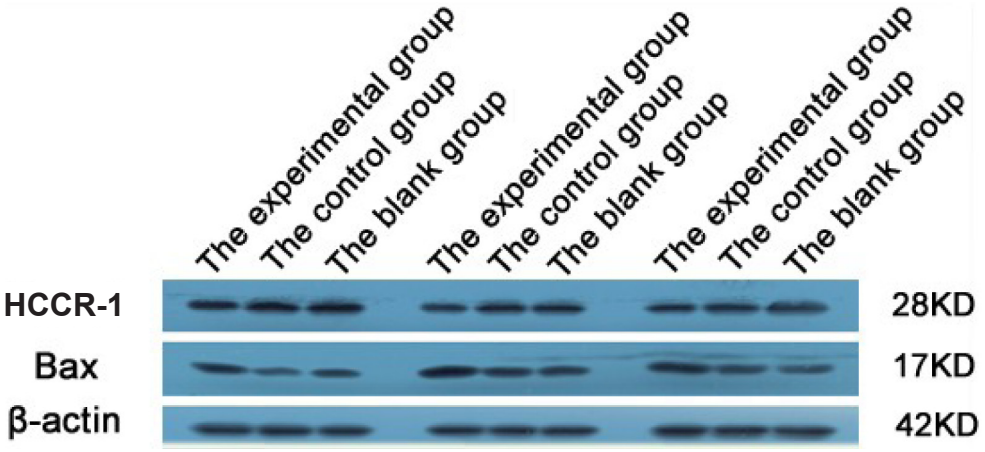

Figure 1. Changes in HCCR-1 and Bax proteins between different groups by western blotting. The expression of HCCR-1 protein in the experimental group was significantly downregulated compared with the blank group and the control group; the expression of HCCR-1 protein in the blank group was not different from that in the control group. The expression of Bax protein in the experimental group was significantly higher than that in the blank group and the control group; the expression of Bax protein in the blank group was not different from that in the control group.

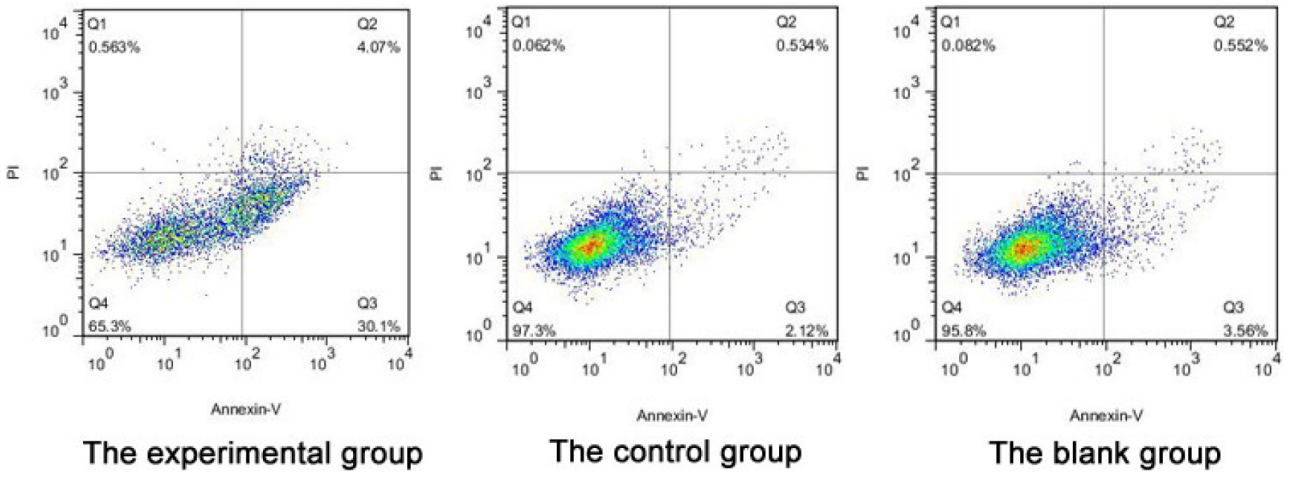

Figure 2. Apoptosis rate detection by flow cytometry. The results of flow cytometry showed that the apoptosis rate of the experimental group was higher than that of the control group, and the apoptosis rate of the control group was not different from that of the blank group.

expression of HCCR-1 protein in the experimental group (0.62±0.07) was significantly down-regulated, and the difference was statistically significant ($p<0.05$). There was no significant difference in HCCR-1 protein expression between the blank and the control groups ($p>0.05$).

Compared with those in the blank group (0.69±0.11) and the control group (0.67±0.19), the expression of Bax protein in the experimental group (0.95±0.22) increased significantly, and the difference was statistically significant ($p<0.05$), while there was no significant difference in expression of Bax protein between the blank and the control groups ($p>0.05$).

Changes in apoptosis in different groups (Figure 2, Table 3)

The apoptotic rate was 32.57±2.35% in the experimental group, 3.53±0.60% in the control group, and 3.15±0.46% in the blank group. The experimental group was statistically different from the control group ($p<0.05$), but there was no difference between the control group and the blank group ($p>0.05$).

Changes in cell proliferation between different groups (Table 4)

Compared with the blank group (24h: 1.24±0.05; 48h: 1.54±0.04; 72h: 1.84±0.04) and the

Table 4. OD value of cell proliferation detected by MTT assay

Time, hours	*Experimental group*	*Control group*	*Blank group*
24	0.78±0.06*	1.18±0.05	1.24±0.05
48	1.09±0.05*	1.48±0.02	1.54±0.04
72	1.29±0.01*	1.80±0.02	1.84±0.04

Compared with blank and control group, *p<0.01

Table 5. Basic pathological data in 136 breast cancer cases

Pathological data	*Number of samples*	*%*
Age, years		
≤50	82	60.3
>50	54	39.7
Tumor size, cm		
≤2	72	52.9
2-5	58	42.7
>5	6	4.4
Tissue type		
Invasive ductal carcinoma (IDC)	98	72.1
Invasive lobular carcinoma (ILC)	9	6.7
Intraductal carcinoma (DCIS)	22	16.2
Mucinous adenocarcinoma	6	4.4
Medullary carcinoma	1	0.7
Estrogen receptor		
Positive	98	72.1
Negative	23	16.9
Unknown	15	11.0
Progesterone receptor		
Positive	86	63.2
Negative	27	19.9
Unknown	23	16.9
Lymph node metastasis		
Positive	70	51.5
Negative	66	48.5
Histological grading		
I	13	9.6
II	87	64.0
III	27	19.9
Unknown	9	6.5
pTNM staging		
I	51	37.5
II	61	44.9
III	23	16.9
IV	1	0.7

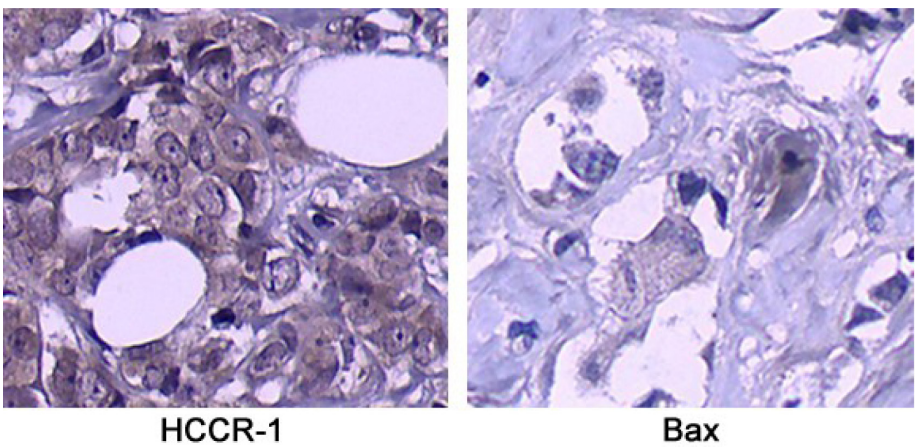

Figure 3. HCCR-1 and Bax expression in breast cancer tissue chips (streptavidin-peroxidase×400).

control group (24h: 1.18±0.05; 48h: 1.48±0.02; 72h: 1.81±0.02), the cell proliferation in the experimental group was significantly inhibited 24 h (0.78 ± 0.06), 48 h (1.09 ± 0.05), 72 h (1.29 ± 0.01) after transfection, and the difference was statistically significant ($p<0.05$), while there was no difference between the blank group and the control group ($p>0.05$).

Results for tissue microarray

A total of 8 tissue chip wax blocks were produced, totaling 336 points, of which each wax block contained 2 blank spots. Each wax block was homogeneous, and there was no bubble or cracking inside. The microarray was evenly arranged and the spacing was moderate. The tissue microarray after immunohistochemical staining was randomly interpreted by two pathologists, and there were some stripping, displacement, and ineffective tissue. The actual effective cases were 136. The relevant clinical basic pathological data are shown in Table 5.

Clinical pathology and histological staging of breast cancer: clinical pathological staging was based on the sixth edition of the AJCC (the American Joint Committee on Cancer) TNM staging criteria [16]. Histology staging was based on Bloom-Richardson System Nottingham Improvement Program Histology Grading Criteria [17].

Expression of HCCR-1 and Bax in 136 breast cancer tissues (Figure 3)

The positive expression of HCCR-1 and Bax protein immunohistochemistry was localized in the cytoplasm of the cells and the staining was mostly brown.

The relationship between HCCR-1 expression and clinicopathological features of breast cancer (Table 6)

Among 136 human breast cancer tissues in 54 patients aged >50 years, 34 were positive for HCCR-1, with positive rate 63.0% (34/54); 82 cases were aged ≤50 years and HCCR-1 expression of 55 cases was positive, the positive rate been 67.1% (55/82), and there was no correlation between HCCR-1 protein expression and age ($x^2=0.24$, $p>0.05$). Ninety-eight cases were ER-positive, in which 70 cases were HCCR-1 positive, with a positive rate 71.4% (70/98); 23 cases were ER-negative, in which 9

Table 6. The relationship between HCCR-1 expression and clinicopathological features of breast cancer

Clinicopathological features	*Cases (n)*	*HCCR expression*		x^2	*p*
		Positive	*Negative*		
Age, years				0.24	>0.05
≤50	82	55	27		
>50	54	34	20		
pTNM staging				6.27	<0.05
I-II	112	68	44		
III-IV	24	21	3		
Histological grading				6.35	<0.05
I	13	6	7		
II	87	54	33		
III	27	23	4		
Tumor size, cm				1.36	>0.05
≤ 2	72	44	28		
>2, ≤ 5	58	41	17		
> 5	6	4	2	6.73	<0.05
Lymph node metastasis					
Yes	70	53	17		
No	66	36	30		
Estrogen receptor				8.57	<0.05
Positive	98	70	28		
Negative	23	9	14		
Progesterone receptor				3.48	>0.05
Positive	86	64	22		
Negative	27	15	12		

There were 9 cases with unknown histological grade, 15 cases with unknown ER expression and 23 cases with unknown PR expression

cases were HCCR-1 positive (positive rate 39.1%, 9/23), HCCR-1 protein expression was positively correlated with ER expression (x^2=8.57, p<0.05). There were 86 cases with PR positive, in which 64 cases with positive HCCR-1 expression, (positive rate 74.4%, 64/86); 27 cases with PR negative in which 15 cases with positive HCCR-1 expression (positive rate 55.6%, 15/27), HCCR-1 protein expression was not correlated with PR expression (x^2=3.48, p>0.05). There were 72 cases with tumor ≤2cm, in which 44 cases had positive expression of HCCR-1 (positive rate 61.1%, 44/72); 58 cases had tumors >2cm and ≤5cm, in which 41 cases were positive for HCCR-1 expression (positive rate 70.7%, 41/58); there were 6 cases with tumor >5cm, in which 4 cases with positive HCCR-1 expression (positive rate 66.7%, 4/6); HCCR-1 protein expression was not correlated with tumor size (x^2=1.36, p>0.05). Among 70 patients with lymph node metastasis, 53 were positive for HCCR-1 expression (positive rate 75.7%, 53/70). Among 66 patients without lymph node metastasis, 36 were positive for HCCR-1 expression. The positive rate was 54.5% (36/66), and the expression of HCCR-1 protein was positively correlated with lymph node metastasis (x^2=6.73, p<0.05). In 24 patients with pTNM stage III-IV, the positive rate of HCCR-1 expression (87.5%, 21/24), was higher than that of 112 patients with stage I-II (60.7%, 68/112), and the HCCR-1 protein expression was positively correlated with pTNM staging (x^2=6.27, p<0.05). In 87 patients with histological grade II breast cancer, the positive rate of HCCR-1 expression (62.1%, 54/87) was higher than that of the 13 patients with grade I (46.2%, 6/13), and lower than the positive rate of the 27 patients with grade III (85.2%, 23/27); there was a positive correlation between HCCR-1 protein expression and histological grade (x^2=6.35, p<0.05).

Relationship between Bax expression and clinicopathological features of breast cancer (Table 7)

Among 136 human breast cancer tissues, there were 54 patients aged >50 years, including 26 patients with positive Bax expression (positive rate 48.1%, 26/54), and 82 patients aged ≤50 years, including 35 patients with positive Bax expression (positive rate 42.7%, 35/82). There was no corre-

Table 7. Relationship between Bax expression and clinicopathological features of breast cancer

Clinicopathological features	*Cases (n)*	*HCCR expression*		x^2	*p*
		Positive	*Negative*		
Age, years					
≤50	82	35	47	0.39	>0.05
>50	54	26	38		
pTNM staging					
I-II	112	54	58	2.90	>0.05
III-IV	24	7	17		
Histological grade					
I	13	8	5		
II	87	45	42		
III	27	1.27	>0.05		
Tumor size, cm				6.53	<0.05
≤ 2	72	40	32		
>2, ≤ 5	58	20	38		
> 5	6	1	5		
Lymph node metastasis				6.51	<0.05
Yes	70	24	46		
No	66	37	29		
Estrogen receptor					
Positive	98	47	51	0.54	>0.05
Negative	23	13	10	1.87	>0.05
Progesterone receptor					
Positive	86	38	48		
Negative	27	16	11		

There were 9 cases with unknown histological grade, 15 cases with unknown ER expression and 23 cases with unknown PR expression

lation between Bax protein expression and age (x^2=0.39, p>0.05). There were 98 cases with ER positive, including 47 cases with positive Bax expression (positive rate 48.0%, 47/98) and 23 cases with ER negative, including 13 cases with positive Bax (positive rate 56.5%, 13/23). There was no correlation between Bax protein expression and ER expression (x^2=0.54, p>0.05). There were 86 cases with PR positive, including 38 cases with positive Bax expression (positive rate 44.2%, 38/86) and 27 cases with PR negative, including 16 cases with positive Bax (positive rate 59.3%, 16/27. There was no correlation between Bax protein expression and PR expression (x^2=1.87, p>0.05). There were 72 cases with tumor size ≤ 2cm, including 40 cases with positive Bax expression (positive rate 55.6%, 40/72), 58 cases with tumor size >2cm and ≤5cm, including 20 cases with positive Bax expression (positive rate 34.5%, 20/58), 6 cases with tumor size >5cm, including 1 case with positive Bax expression (positive rate 16.7%, 1/6). The positive rate of Bax was negatively correlated with tumor size (x^2=6.53, p<0.05). Among 70 patients with lymph node metastasis, 24 were positive for Bax (positive rate 34.3%, 24/70) and among 66 patients without lymph node metastasis, 37 were positive for Bax (positive rate 56.1%, 37/66). Bax protein expression was negatively correlated with lymph node metastasis (x^2=6.51, p<0.05).

There was no statistical difference in the positive rate of Bax expression between the 24 patients with pTNM stage III-IV (29.2%, 7/24) and the 112 patients with pTNM stage I-II (48.2%, 54/112) (x^2=2.90, p>0.05), indicating that Bax protein expression was not associated with breast cancer pTNM stage. The positive rate of Bax expression was 51.7% (45/87) in 87 patients with histological grade II breast cancer, the positive rate was 61.5% (8/13) in 13 patients with grade I, and the positive rate was 40.7% (11/27)] in the 27 grade III patients. No correlation between the positive rate of Bax and histological grade was noted (x^2=1.27, p>0.05).

Correlation analysis between HCCR-1 and Bax expression (Table 8)

In 136 human breast cancer tissues, the positive rate of Bax was 44.9% (61/136) and the positive rate of HCCR-1 was 65.4% (89/136), showing statistically significant difference (x^2=5.83, p<0.05). Among the 89 cases with positive expression of HCCR-1, Bax was positively expressed in 25 cases; among the 61 cases with positive Bax expression, 36 cases showed negative expression of HCCR-1, and there was a negative correlation between positive expression of Bax and HCCR-1 (p<0.01, γ=-0.464).

Table 8. Correlation analysis between HCCR-1 and Bax expression in breast cancer

HCCR-1	*Bax expression*		*Total*
	Positive	Negative	
Positive	25	64	89
Negative	36	11	47
Total	61	75	136

p<0.01, correlation coefficient γ= -0.464

Discussion

P53 is a tumor suppressor gene, and when its coding sequence changes, it will affect the structural changes of the entire gene, and the corresponding protein will also show abnormal expression [18]. Deletion, mutation and inactivation of P53 protein will directly affect the biological process of tumors [19]. Related studies have shown that [9,10], HCCRs inhibit the activity of P53 gene through a variety of pathways, making its expression decreased or even inactivated. Bax protein is one of the most widely studied pro-apoptotic factors. It is also a downstream factor of P53, and it is a key protein regulating apoptosis of Bcl-2 family [6]. Guo J et al. found that the change of HCCR-2 expression in HepG2 cells did not affect the expression of P53 mRNA, but affected the expression of downstream gene Bax [12]. Ko also confirmed that HCCR-2 gene expression level does not affect the expression of P53, it affects the stability of wild-type P53 by affecting the expression of Bax, thereby promoting tumorigenesis [10,15]. Jiang Lin et al. found that silencing HCCR-2 gene can decrease the expression of Bcl-2 in pancreatic cancer cell line PANC-1, and increase the expression of Bax, inhibit cell proliferation and promote cell apoptosis [13]. Gui Chun et al. found that up-regulation of HCCR-2 gene expression can promote the proliferation of breast cancer cells MCF-7, inhibit the apoptosis, and reduce the expression of Bax protein [14]. The study of Liu in esophageal cancer confirmed that targeted inhibition of HCCR-1 can increase the expression of Bax and promote the apoptosis of esophageal cancer EC1 cells [11]. However, there is no literature reported whether reducing the expression of HCCR-1 in breast cancer cells can cause the same effects. In order to further elucidate the biological effects of HCCR-1 in the development of breast cancer, we used RNAi to reduce the expression of HCCR-1 in breast cancer cell line MCF-7, and detected the expression of HCCR-1 and Bax in the experimental group, control group and blank

group by RT-qPCR and Western Blot. At the same time, the changes of proliferation and apoptosis of MCF-7 were detected. The results showed that the expression of HCCR-1 mRNA and protein in the experimental group was significantly lower than that in the control group, indicating that the purpose of inhibiting the expression of HCCR-1 from the translation and transcription levels by RNAi was achieved. The transfection in the experimental group was effective. The cell proliferation of MCF-7 in the experimental group was lower than that in the control group, and the apoptosis was increased. At the same time, the expression of Bax in the control group was significantly increased than that in the experimental group, indicating that decreasing the expression of HCCR-1 led to an increase in the expression of Bax, suggesting that HCCR-1 is involved in the metabolic process of Bax in breast cancer cells MCF-7. It also suggested that the decrease of proliferation of MCF-7 in breast cancer cells and the increase in apoptosis following decreased HCCR-1 expression may be caused by up-regulation of Bax expression.

To further analyze the differential expression of HCCR-1 and Bax in breast cancer tissues, we selected 30 breast cancer tissues from recent operations, and selected 120 human breast cancer specimens prepared earlier to make tissue chips. The expression of HCCR-1 and Bax was examined at the protein level by immunohistochemistry, and the relationship between its expression and clinicopathological factors of breast cancer was further analyzed. This study showed that the positive rate of HCCR-1 expression in patients aged ≤50 years was 67.1% higher than that in patients >50 years old, the difference was not statistically significant (x^2=0.24, p>0.05), which confirmed that the age of onset is reduced year by year in epidemiological investigations [15], but it is also related to the enhancement of people's awareness of disease prevention and the improvement of early diagnosis. The positive expression rate of HCCR-1 in the pTNM clinical pathological stage of breast cancer was significantly higher in stage III-IV than in stage I-II (x^2=6.27, p<0.05). Since the clinical pathological stage of breast cancer represents the degree of invasion and metastasis of breast cancer, the positive expression of HCCR-1 is correlated with invasion and metastasis of breast cancer.

The histological grade of breast cancer has important significance for prognosis. This study showed that the positive rate of HCCR-1 increased gradually with the increase of histological grade (x^2=6.35, p<0.05). This study also found that HCCR-1 positive expression was not correlated with tumor size (x^2=1.36, p>0.05). Whether lymph node metastasis occurred is the main indicator for evaluating the prognosis of breast cancer, the results showed that the positive expression of HCCR-1 in breast cancer tissues was related to the peripheral lymph node metastasis (x^2=6.73, p<0.05), if we can compare the prognosis of HCCR-1 positive and negative patients with lymph node metastasis, it is possible to analyze whether HCCR-1 can be an independent indicator for judging the prognosis of breast cancer. The expression of ER and PR is related to the prognosis of breast cancer and has a guiding role in endocrine therapy of breast cancer. According to the analysis of the results, the positive expression of HCCR-1 in breast cancer tissues was correlated with ER expression (x^2=8.57, p<0.05), which was consistent with the study of Ha SA [20], but the positive expression of HCCR-1 was not related to PR (x^2=3.48, p>0.05). The data analysis found that although HCCR-1 positive rate was not related to PR significantly, the statistical result P=0.06 was close to the result of Ha SA [20], the reason may be that the number of samples we selected was small. The positive expression of Bax in breast cancer tissues was correlated with tumor size and peripheral lymph node metastasis, which was consistent with the literature [21-24]. However, the positive expression of Bax was not correlated with age, tumor pTNM staging, histological grade and estrogen and progesterone expression, which was consistent with the study of Kapranos [25]. Seeing from its appearance, there was contradiction that Bax expression was associated with lymph node metastasis but not correlated with histological grade and pTNM staging. However, by analyzing the statistical data, we found that the positive rate in pTNM stage I-II is 48.2%, which was higher than the 29.2% positive rate in stage III-IV, the positive rates of histology grades I, II, and III were 61.5%, 52.7%, and 40.7%, respectively, showing a decreasing trend. Whether this contradiction was due to the limitation of the number of samples leading to a statistically significant difference between Bax positive expression and the above factors, further research was needed. There were 25 cases of Bax expression positive in 89 cases of breast cancer with positive HCCR-1 expression, and 36 cases of HCCR-1 negative in 61 cases of Bax positive. It is suggested that there is a certain degree of negative correlation between HCCR-1 and Bax in the development of breast cancer (p<0.01, correlation coefficient γ=-0.464), which confirms the results of transfection experiments and previous related research reports [8,12-14].

HCCR-1 is an oncogene that has been studied in recent years, although some of the biological

function of HCCR-1 has been revealed: in addition to the P53 signaling pathway, EGF/PI3K/AKT/mTOR, TCF/β-catetin, Ras/RafMEK signaling pathways are also associated with expression of HCCR [11,19,26-28], but there are still many key issues to be studied, such as how HCCR-1 is activated, its signaling mechanism that promotes tumor growth, and the other interacting molecules besides Bax that interacts with HCCR-1.

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Conflict of interests

The authors declare no conflict of interests.

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