ORIGINAL ARTICLE

Expression and significance of Shh, Gli1 and β-catenin in triple-negative breast cancer tissues

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ABSTRACT

Objective: To detect the expression of Shh, Gli1 and β-catenin in triple negative breast cancer tissues and paracancer breast tissues by qRT-PCR and immunohistochemistry and to analyze their correlation with clinicopathological features.
Methods: (1) qRT-PCR was used to detect the mRNA expression of Shh, Gli1 and β-catenin in 30 cases of triple negative breast cancer and their paracancer breast tissues, and the correlation among them was analyzed. (2) The expression of Shh, Gli1 and β-catenin proteins in 30 triple negative breast cancer and their paracancer breast tissues was detected by immunohistochemistry, and their correlation with clinicopathological features was analyzed.
Results: (1) Shh mRNA expression (1.2334 ± 0.27867), Gli1 mRNA expression (1.2135 ± 0.20636) and β-catenin mRNA expression (1.1421 ± 0.32330) in triple negative breast cancer tissues were higher than that in paracancer breast tissues, i.e., Shh mRNA expression (1.0022 ± 0.06721), Gli1 mRNA expression (1.0003 ± 0.02420) and β-catenin mRNA expression (1.0033 ± 0.07920) were significantly different ($p < .05$). There was a significantly positive correlation between the mRNA expression of Shh and Gli1 ($r = .989$, $p < .001$), and between the mRNA expression of Shh and β-catenin ($r = .868$, $p < .001$). There was a significantly positive correlation between the mRNA expression of Gli1 and β-catenin expression ($r = .869$, $p < .001$). (2) The positive expression rates of Shh, Gli1 and β-catenin in triple negative breast cancer tissues were 93.3% (28/30), 96.7% (29/30) and 93.3% (28/30), respectively, which were higher than those in paracancer tissues 60% (18/30), 73.3% (22/30) and 73.3% (22/30), the differences were statistically significant ($p < 0.05$). There was a significantly positive correlation between the mRNA expression of Shh and Gli1 ($r = .958$, $p < .001$), and between the mRNA expression of Shh and β-catenin ($r = .952$, $p < .001$). There was a significantly positive correlation between the mRNA expression of Gli1 and β-catenin expression ($r = .927$, $p < .001$). The expression of Shh, Gli1 and β-catenin protein in triple negative breast cancer was not correlated with age and tumor size ($p > .05$), but Shh was positively correlated with histological grade (G) ($r = .774$, $p < .001$). Furthermore, Gli1 was positively correlated with histological grade ($r = .757$, $p < .001$). β-catenin was positively correlated with histological grade ($r = .739$, $p < .001$). Shh was positively correlated with TNM staging ($r = .460$, $p = .010$). Gli1 was positively correlated with TNM staging ($r = .414$, $p = .023$). β-catenin was positively correlated with TNM staging ($r = .404$, $p = .027$). Shh was positively correlated with lymph node metastasis ($r = .540$, $p = .002$). Gli1 was positively correlated with lymph node metastasis ($r = .515$, $p = .004$). β-catenin was positively correlated with lymph node metastasis ($r = .559$, $p = .001$).
Conclusions: (1) The up-regulated expression of Shh, Gli1 and β-catenin proteins in triple negative breast cancer suggests that Shh, Gli1 and β-catenin proteins are involved in tumor genesis. The combined detection of the three proteins may provide a theoretical basis for the diagnosis and prognosis evaluation of triple negative breast cancer. (2) Shh was positively correlated with Gli1 protein expression and β-catenin protein expression. Gli1 was positively correlated with β-catenin protein expression, suggesting that the three types of proteins play a synergistic role in the occurrence and development of TNBC. There may be

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crosstalk in the Wnt/β-catenin and Hedgehog signaling pathways in TNBC, which may provide a new approach for the treatment of TNBC. (3) The expression of Shh, Gli1 and β-catenin proteins was correlated with the degree of differentiation, TNM staging and lymph node metastasis of triple negative breast cancer, but not correlated with age and tumor size. Therefore, it was predicted that the three types of proteins were related to the invasion, metastasis and prognosis of TNBC.

Key Words: Triple negative breast cancer, Shh, Gli1, β-catenin

1. INTRODUCTION

With the rapid development of society, the protection of human health has become an urgent problem to be solved. At present, cancer is still a challenging problem that human beings are overcoming. It is necessary to further explore the mechanism of tumor occurrence, development, metastasis and invasion and related signaling pathways in order to improve the effectiveness and safety of treatment methods. Breast cancer has become one of the most common cancers in women worldwide, accounting for about one-third of female cancers, with an average of 1 in 8 to 10 women diagnosed with breast cancer.[1] The incidence of breast cancer has been continuously increasing, which must be paid great attention.[2] Breast cancer can be classified into three main types, namely hormone receptor (HR) positive breast cancer, human epidermal growth factor receptor 2 (HER-2) positive breast cancer and triple-negative breast cancer. HR-positive and HER-2-positive breast cancers can be treated with corresponding targeted therapies. Triple-negative breast cancer (TNBC) is defined as tumors that lack HR expression and do not have HER-2 amplification,[3] which accounts for about 15%-20% of overall breast cancer. Unlike other types, TNBC shows high heterogeneity, patients are at high risk of recurrence with a worse prognosis, and TNBC is currently treated with chemotherapy.[4] In 2016, on the basis of sequencing analysis data from the transcription group. Professor Shao Zhimin classified TNBC into 4 types: LAR, IM, BLIS and MES.[5] With the proposal of TNBC molecular typing and the deepening of molecular mechanism exploration, some targeted therapies such as androgen receptor (AR) inhibitors, phosphoinositide 3-kinase (PI3K) inhibitors, AKT inhibitors and epidermal growth factor receptor inhibitors are in the clinical research stage. Currently, only PARP inhibitors have been approved for BRCA1/2 mutated HER-2 negative metastatic breast cancer.[6] At the same time, immunotherapy, i.e., the combination of immune checkpoint inhibitors (anti-programmed cell death protein 1 (Anti-PD-1) and anti-programmed death ligand 1 (Anti-PD-L1) with chemotherapy, shows a particular prospect.[7] Despite the above studies, the treatment of TNBC is still a significant difficulty that remains to be resolved. It is necessary to study the mechanism of TNBC in order to provide more possibilities for the diagnosis and treatment of TNBC.

The Hedgehog (Hh) signaling pathway plays an important role in controlling cell proliferation, differentiation and homeostasis of stem cells, and Shh and Gli1 are initiators and essential transcription proteins in the signaling pathway, respectively. Shh (Sonic Hedgehog) is one of the protein ligands of the human Hh signaling pathway. When Shh aggregates, it can bind to Ptch1, resulting in the fact that Ptch1 cannot bind to Smo, and Smo is activated, further promoting the nuclear transcription factor Gli of the Hh signaling pathway into the nucleus and activating the transcription of downstream target genes. SHH plays a crucial role in the growth and development of human organs.

Gli1 (containing 1106 amino acids) was first discovered as an amplification gene product in malignant gliomas.[8] Gli1 has a activation function, and Gli1 proteins are present in the nucleus and cytoplasm. When the upstream pathway activates Gli proteins, Gli1 will enter the nucleus to activate the expression of downstream target genes. Gli1 is one of the core members of the Hedgehog pathway. The abnormal expression of Gli1 has significant implications for DNA damage repair, cell cycle, carcinogenesis, and multidrug resistance (MDR) in organisms through classical or non-classical signaling environments.[9]

The Wnt signaling pathway is crucial for the continuous renewal of cells, the regeneration process, and tissue homeostasis. β-catenin is a core member of the Wnt/β-catenin classical pathway, β-catenin leads to the opening of the Wnt pathway after aggregation in the nucleus, and its abnormal overexpression can cause many types of diseases, including cancer.[10]

The occurrence and development of breast cancer is a multistage process involving many genes and signaling pathways. Shh, Gli1 and β-catenin are critical proteins in the two signaling pathways. There are currently studies of the Wnt pathway in breast cancer, but only few studies have been conducted on the Hedgehog pathway. There are few reports on the influence of the relationship between the two pathways on breast cancer, and even fewer reports on the pathogenesis of triple-negative breast cancer. Therefore, in this study, the mRNA expression levels of Shh Gli1 and β-catenin in

TNBC tissues and corresponding paracancer tissues were detected to analyze the correlation between the three indicators. At the same time, the expression of the three proteins in cancer and paracancer tissues was detected, and the correlation among the three proteins and the clinicopathological characteristics of patients were analyzed. We hope that the molecular pathways involved in the progression of TNBC can be more deeply understood through this study, which will provide new ideas for the targeted therapy of TNBC.

2. MATERIALS AND METHODS

2.1 Research subjects

2.1.1 *qRT-PCR specimens*

30 patients with the same wax blocks as immunohistochemical samples were selected for the subsequent experiments in qRT-PCR samples.

2.1.2 *Immunohistochemical specimens*

The tissue samples were obtained from the paraffin block library of the Department of Pathology in our hospital, and 30 cases of breast cancer patients who were treated in Baogang Hospital in Inner Mongolia from January 2019 to December 2021 were selected and included in this study. After breast cancer resection, 30 cases of samples were taken from the cancerous tissues and the corresponding paracancer tissues (2 cm from the cancerous tissues). The samples were prepared and made into paraffin blocks, with the histopathological results showing breast invasive ductal carcinoma. The selected cases were not treated with anti-tumor therapies (e.g., chemotherapy, radiotherapy etc.) before surgery. Main experimental reagents and instruments and equipments were shown in Tables 1-2.

Table 1. Main experimental reagents

Experimental Reagents	Resource
Rabbit anti-human Shh polyclonal antibody	Proteintech Group, Inc
Goat serum for blocking	Proteintech Group, Inc
Mouse anti-human Gli1 monoclonal antibody	Proteintech Group, Inc
PBS buffer (10×)	Sangon Biotech
PCR primers	Proteintech Group, Inc
immunohistochemistry kit (universal for rabbits and mice)	Proteintech Group, Inc
Rabbit anti-human β-catenin polyclonal antibody	Proteintech Group, Inc
EDTA Antigen Retrieval solution (50×)	Proteintech Group, Inc
FastQuant RT Kit (With gDNase)	Tiangen
SuperReal PreMix Plus (SYBR Green)	Tiangen
Paraffin-embedded tissue section total RNA extraction kit	Tiangen
Rnase free deionized water;	Tiangen

2.2 Experimental methods

2.2.1 *The application of qRT-PCR to the measurement of the mRNA expression of Shh, Gli1 and β-catenin*

(1) **Paraffin-embedded tissue section total RNA extraction**

Reagent preparation

DNase I working solution: Place 10 μl of DNase I solution in a enzyme-free tube, add 70 μl of RDD buffer, and mix invertibly.

- The paraffin blocks were sliced (5 μm, 8 slices)
- The sections were quickly placed in 1.5 ml enzyme-free tube, added with 1 mL xylene and vortexed for 10 sec
- The temperature was set at 25°C, with the rotation speed of 12,000 rpm, and the centrifugation time was 2 min
- A pipette was used to remove the supernatant while avoiding suction precipitation
- 1 ml of absolute ethanol was added and mixed well
- The temperature was set at 25°C, with the rotation speed of 12,000 rpm, and the centrifugation time was 2 min
- A pipette was used to remove the supernatant while avoiding suction precipitation
- The residual ethanol was completely volatilized by sitting at 25°C for 10 min
- Lysate RF was added to the precipitation with an amount of 200 μl, and proteinase K was added with an amount of 10 μl, and the mixture was mixed together and uniformly

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- The incubation was performed at 55°C for 15 min, followed by continued incubation at 80°C for another 15 min
- At 25°C, the supernatant was centrifuged at 12,000 rpm for 5min, then transferred with a pipette and placed in a new enzyme-free tube
- The buffer RB was added with an amount of 220 μl and mixed well
- The absolute ethanol was added with an amount of 660 μl and mixed well
- 700 μl of solution in step 13 was transferred into the adsorption column CR3, centrifuged at 12,000 rpm for 1 min, with the waste in the collection tube discarded
- Step 14 was repeated so that all the solution and the precipitation could pass through the adsorption column CR3
- DNase I working solution was added in the center of the adsorption column CR3 with an amount of 80 μl and allowed to sit at a temperature of 25°C for 15 min

Table 2. Main instruments and equipments

Instruments and Equipments	Resource
Specimen freezing table	LEICA (Germany)
Microtome	LEICA (Germany)
Water Bath-Slide Drier	Taiva (Hubei)
Adhesion slides	Mevid (Jiangsu)
Pipette	Gilson (French)
Micro-wave oven	Supor (Hangzhou)
Ultra low temperature freezer	Panasonic (Japan)
Optical microscope	OLYMPUS(Japan)
Vortex mixer	Thermo (UK)
Cryogenic high-speed centrifuge	Bio Science (Shanghai)
Electronic analytical balance	Sartorius (Germany)
Vertical Flow Clean Bench	Zhicheng (Shanghai)
Real-time fluorescence quantitative PCR instrument	BIO-RAD (USA)
PCR instrument	ABI (USA)
Enzyme oscillator	DRAGON (Finland)
Microplate reader	Perlong (Beijing)
Micro-spectrophotometer	Aosens (Beijing)
Ice machine	GRANT(Germany)
Tip	Axygen (USA)

(2) **Reverse transcription reactions**

- The mixture was prepared according to the system in Table 3 (Operation was made on ice)
- The mixture was centrifuged, incubated at a temperature of 42°C for 3 min, and then set aside on ice
- The mixture was prepared according to the system in Table 4
- The mixture was incubated at a temperature of 42°C for 15 min
- It was incubated at a temperature of 95°C for 3 min and then set aside on ice. The cDNA obtained could continue to be used in subsequent experiments. If it was not used temporarily, it needed to be stored at -20°C.

Table 3. gDNA removal reaction system

Ingredient	Usage
5×gDNA Buffer	2 µl
Total RNA	-
RNase-Free ddH_2O	Made up to 10 µl

Table 4. Reverse transcription reactions

Ingredient	Usage
10×King RT Buffer	2 µl
FastKing RT Enzyme Mix	1 µl
FQ-RT Primer Mix	2 µl
RNase-Free ddH_2O	Made up to 10 µl

(3) **Real Time PCR reaction**

The cDNA obtained by the above operation will act as a template in subsequent PCR reactions.

- Primer design and synthesis of Real Time PCR
  The design and synthesis of primers were completed by Sangon Biotech, and GAPDH was selected as the internal reference, and the sequence was shown in Table 5 below.
- The Real Time PCR reaction system and its conditions were shown in Tables 6 and 7
- After the reaction, the ct value was used to further calculate the relative expression of the three indicators

2.2.2 *Detection of the expression of Shh, Gli1 and β-catenin proteins by SP method*

Reagent Preparation:

EDTA antigen retrieval solution (50×) was prepared by 1 ml EDTA + 50 ml distilled water, i.e., working solution made after diluting 50 times.

DAB working solution was prepared as follows: DAB stocking solution: DAB diluent = 1:50. It was kept in dark place and used right after it was ready.

- Dewaxing: the slice was placed in xylene for 10 minutes. The step was repeated twice. 100%, 95%, 80% and 60% ethanol need to sit for 5 minutes. It was followed by the soaking operation with distilled water and repeated three times, each lasting 3 min.

- Antigen retrieval: EDTA working solution was added. Subsequently, the mixture was placed in the microwave oven and retrieved with medium fire twice, each lasting 5 min. It was cooled down naturally when finished. The mixture was rinsed with PBS three times, each lasting 1 min.
- 3% H_2O_2 was added, and the mixture was set aside at 25°C for 10 min. The mixture was rinsed with PBS three times, each lasting 1 min.
- Blocking: the mixture was added dropwise with 5% blocking serum and set aside at 25°C for 10 min.
- The primary antibody was incubated as follows: it was diluted and added dropwise. Finally, it was incubated overnight at 4°C. It was rinsed with PBS three times, each lasting 1 min.
- The secondary antibody was incubated as follows: it was added dropwise with 70 μl of HRP labeled anti mouse/rabbit polymer, incubated at 25°C for 30 min, and then rinsed with PBS three times, each lasting 1 min.
- DAB dying: 70 μl of DAB working solution was added dropwise, and the mixture was incubated at 25°C for 10 min. Finally, it was rinsed with distilled water.
- The mixture was re-dyed for 3 min and rinsed with distilled water.
- The mixture was dehydrated with graded ethanol, each grade lasting 5 min. Xylene was set aside for 10 minutes. The step was repeated twice. Finally, the slide was treated with the mounting medium and observed by the microscope.

Table 5. The sequence of primers

Genes	Forward primer (5'-3')	Forward primer (5'-3')
Shh	TGTCTGCTGCTAGTCCTCGTCTC	GTGCCTCCTCTTCCCGAACCC
Gli1	ATGAAACTGACTGCCGTTGGGATG	TGGATGTGCTCGCTGTTGATGTG
β-catenin	GGCTCTTGTGCGTACTGTCCTTC	GCTTCTTGGTGTCGGCTGGTC
GAPDH	CCTCAACGACCACTTTGTCA	TTACTCCTTGGAGGCCATGT

Table 6. Real time PCR reaction system

Ingredient	Usage
2×SuperReal PreMix Plus	10 μl
Forward primer (10 μm)	0.6 μl
Reverse primer (10 μm)	0.6 μl
cDNA template	2 μl
RNase-Free ddH_2O	Made up to 20 μl

2.3 Result calculation and interpretation

2.3.1 *mRNA result calculation*

ΔCT = CT $_{TargetGene}$ - CT $_{InternalReference}$; the arithmetic mean of ΔCT was calculated, and $\Delta\Delta$CT is equal to ΔCT minus the arithmetic mean of ΔCT ($\Delta\Delta$CT = ΔCT - the arithmetic mean of ΔCT); the expression level of the target gene in each sample of each group was calculated as $2^{-\Delta\Delta CT}$.

2.3.2 *Immunohistochemical result interpretation*

Shh and β-catenin staining positive signals can be localized to cell membranes and the cytoplasm, and Gli1 staining positive signals can be localized to the cytoplasm and the nucleus, generally showing yellow (brownish-yellow) granules; the results were interpreted on the basis of the semi-quantitative integration method: (1) the rating of staining intensity was as follows: no staining was scored as 0, light yellow was scored as 1, brownish-yellow was scored as 2, brown was scored as 3. (2) the rating can also be made on the proportion of the positive cells in the whole: the proportion $\leq$ 5% was scored as 0, the proportion of 6%-25% was scored as 1; the proportion of 26%-50% was scored as 2; the proportion of 51%-75% was scored as 3, the proportion > 75% was scored as 4. (3) the final score was the product of the scores in (1) and (2): negativity: (-): 0-1; weak positivity (+): 2-4; positivity (++): 5-8; strong positivity (+++): 9-12. Whereas, (-) was considered as the negative expression; (+), (++) and (+++) were considered as the positive expression. The biopsy scoring was done independently by two pathologists using the double-blind method and uniform standards.

2.4 Statistical treatment

SPSS26.0 and GraphPad Prism 8 software were used to analyze and plot the experimental data. The comparison of the measurement data between the two groups was made by use of t test; the comparison of the ranked data was made by χ^2 test. Pearson correlation analysis was used to analyze the correlation of the mRNA expression of Shh, Gli-1 and β-catenin. Spearman correlation analysis was used to analyze the correlation of the protein expression of Shh, Gli-1 and β-catenin. Besides, Spearman correlation was used to analyze the correlation of the protein expression of Shh, Gli-1 and β-catenin with clinicopathological features of triple-negative breast cancer. The difference (p < .05) was of statistical significance.

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Table 7. Real time PCR reaction conditions

Stage	Cycle	Temperature	Time	Content	Fluorescence Signal Acquisition
Pre-denaturation	1×	95℃	15 min	Pre-denaturation	No
		95℃	10 s	Denaturation	No
PCR Reaction	40×	50-60℃	20 s	Annealing	No
		72℃	20-32 s	Extension	Yes

3. RESULTS

3.1 The expression of Shh, Gli1 and β-catenin in triple-negative breast cancer tissues and paracancer tissues

3.1.1 *qRT-PCR results*

(1) The mRNA expression of Shh in triple-negative breast cancer tissues (1.2334 $\pm$ 0.27867) was higher than that in the paracancer tissues (1.0022 $\pm$ 0.06721) (t = 4.419, p < .001). See Figure 1 and Table 8 for details.

(2) The mRNA expression of Gli1 in triple-negative breast cancer tissues (1.2135 $\pm$ 0.20636) was higher than that in the paracancer tissues (1.0003 $\pm$ 0.02420) (t = 5.620, p < .001). See Figure 2 and Table 8 for details.

(3) The mRNA expression of β-catenin in triple-negative breast cancer tissues (1.1421 $\pm$ 0.32330) was higher than that in the paracancer tissues (1.0033 $\pm$ 0.07920) (t = 2.283, p = .026). See Figure 3 and Table 8 for details.

3.1.2 *Immunohistochemical results*

(1) The protein expression of Shh can be localized to cell membranes and the cytoplasm, and the positive part showed yellow and brownish-yellow granules. The positive expression rates of Shh in triple-negative breast cancer tissues and the paracancer tissues were 93.3% (28/30) and 60% (18/30) respectively. The difference was of statistical significance (χ^2 = 9.317, p = .002). See Figure 4 and Table 9 for details.

Table 8. The mRNA expression of Shh Tissue Gli1 and β-catenin in triple-negative breast cancer tissues and paracancer tissues

	Sample Type	n	$\bar{X}\pm$ s	*t*	*p*
Shh	Cancer Tissues	30	1.2334±0.27867	4.419	< .001
	Paracancer Tissues	30	1.0022±0.06721		
Gli1	Cancer Tissues	30	1.2135±0.20636	5.620	< .001
	Paracancer Tissues	30	1.0003±0.02420		
β-catenin	Cancer Tissues	30	1.1421±0.32330	2.283	.026
	Paracancer Tissues	30	1.0033±0.07920		

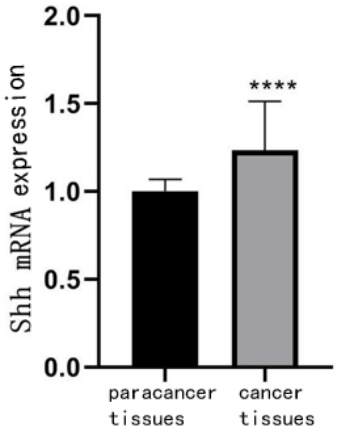

Figure 1. The mRNA expression of Shh in triple-negative breast cancer tissues and paracancer tissues

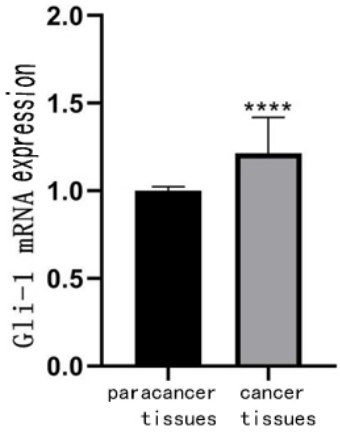

Figure 2. The mRNA expression of Gli1 in triple-negative breast cancer tissues and paracancer tissues

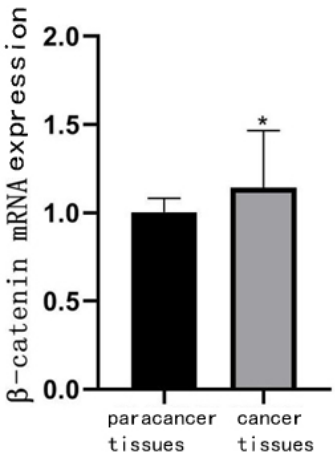

Figure 3. The mRNA expression of β-catenin in triple-negative breast cancer tissues and paracancer tissues

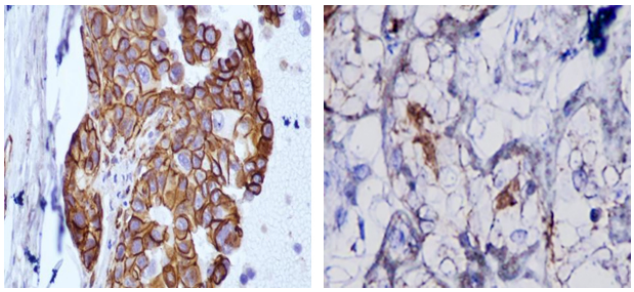

Figure 4. The immunohistochemical results of Shh in triple-negative breast cancer tissues and paracancer tissues ($\times$400)

(2) The protein expression of Gli1 can be localized to the nucleus and the cytoplasm, and the positive part showed yellow and brownish-yellow granules. The positive expression rates of Gli1 in triple-negative breast cancer tissues and the paracancer tissues were 96.7% (29/30) and 73.3% (22/30) respectively. The difference was of statistical significance (χ^2 = 6.405, p = .011). See Figure 5 and Table 9 for details.

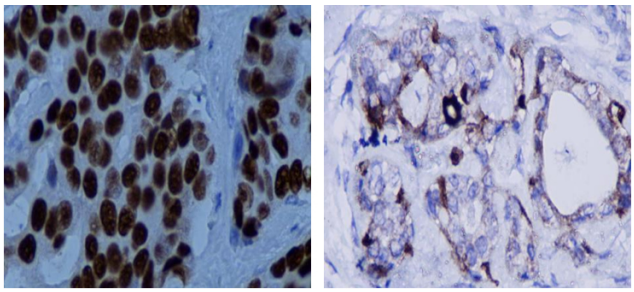

Figure 5. The immunohistochemical results of Gli1 in triple-negative breast cancer tissues and paracancer tissues ($\times$400)

(3) The protein expression of β-catenin can be localized to cell membranes and the cytoplasm, and the positive part showed yellow and brownish-yellow granules. The positive expression rates of β-catenin in triple-negative breast cancer tissues and the paracancer tissues were 93.3% (28/30) and 73.3% (22/30) respectively. The difference was of statistical significance (χ^2 = 4.320, p = .038). See Figure 6 and Table 9 for details.

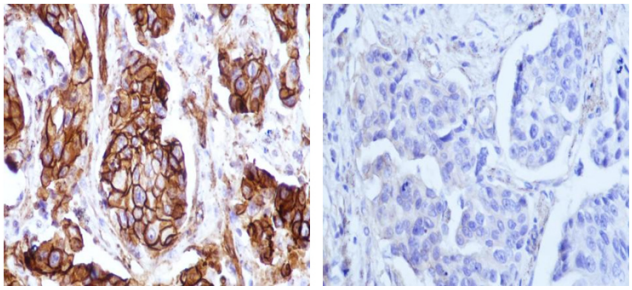

Figure 6. The immunohistochemical results of β-catenin in triple-negative breast cancer tissues and paracancer tissues ($\times$400)

3.2 The correlation of Shh, Gli1 and β-catenin in triple-negative breast cancer tissues

3.2.1 *qRT-PCR results*

(1) The expression levels of Shh and Gli1 were both high in triple-negative breast cancer tissues. Pearson correlation analysis showed that the mRNA expression of Shh was positively correlated with that of Gli1 (r = .989, p < .001). See Figure 7 for details.

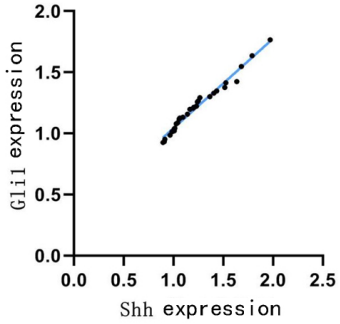

Figure 7. The mRNA expression correlation of Shh and Gli1 in triple-negative breast cancer tissues

(2) The expression levels of Shh and β-catenin were both high in triple-negative breast cancer tissues. Pearson correlation analysis showed that the mRNA expression of Shh was positively correlated with that of β-catenin (r = .868, p < .001). See Figure 8 for details.

(3) The expression levels of Gli1 and β-catenin were both high in triple-negative breast cancer tissues. Pearson correlation analysis showed that the mRNA expression of Gli1 was positively correlated with that of β-catenin (r = .869, p < .001). See Figure 9 for details.

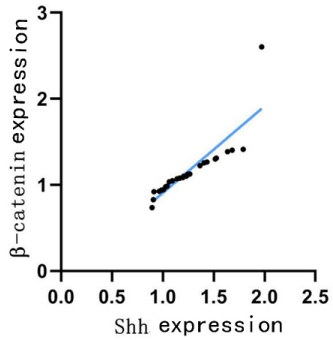

Figure 8. The mRNA expression correlation of Shh and β-catenin in triple-negative breast cancer tissues

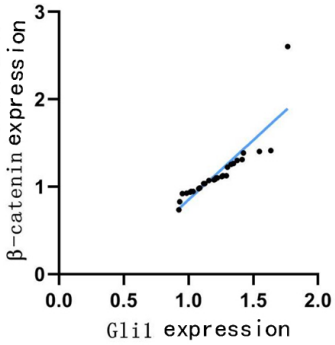

Figure 9. The mRNA expression correlation of Gli1 and β-catenin in triple-negative breast cancer tissues

Table 9. The protein expression of Shh, Gli1 and β-catenin in triple-negative breast cancer tissues and paracancer tissues [n (%)]

Group	n	Shh	Gli1	β-catenin
Cancer Tissues	30	28 (93.3)	29 (96.7)	28 (93.3)
Paracancer Tissues	30	18 (60)	22 (73.3)	22 (73.3)
χ^2		9.317	6.405	4.320
p		.002	.011	.038

3.2.2 *Immunohistochemical results*

(1) The expression levels of Shh and Gli1 were both high in triple-negative breast cancer tissues. Spearman correlation analysis showed that the protein expression of Shh was positively correlated with that of Gli1 (r = .958, p < .001). See Table 10 for details.

(2) The expression levels of Shh and β-catenin were both high in triple-negative breast cancer tissues. Spearman correlation analysis showed that the protein expression of Shh was positively correlated with that of β-catenin (r = .952, p < .001). See Table 10 for details.

(3) The expression levels of Gli1 and β-catenin were both high in triple-negative breast cancer tissues. Spearman correlation analysis showed that the protein expression of Gli1 was positively correlated with that of β-catenin (r = .927, p < .001). See Table 10 for details.

3.3 The correlation of the expression of Shh, Gli1 and β-catenin with the clinicopathological characteristics in triple-negative breast cancer tissues

(1) The expression of Shh in triple-negative breast cancer tissues was not associated with age and tumor size, but positively correlated with histological grade (r = .774, p < .001), TNM staging (r = .460, p = .010) and lymph node metastasis (r = .540, p = .002). The expression of Shh was down-regulated in the groups with low histological grade, with earlier TNM stage and without lymph node metastasis, while the expression was up-regulated in the groups with high histological grade, with later TNM stage, and with lymph node metastasis. See Table 11 for details.

Table 10. The protein expression correlation of Shh, Gli1 and β-catenin in triple-negative breast cancer tissues

Indexes	Shh	Gli1	β-catenin
Shh	1		
Gli1	.958**	1	
β-catenin	.952**	.927**	1

Note. ** at 0.01 level, the correlation was significant

(2) The expression of Gli1 in triple-negative breast cancer tissues was not associated with age and tumor size, but positively correlated with histological grade (r = .757, p < .001), TNM staging (r = .414, p = .023) and lymph node metastasis (r = .515, p = .004). The expression of Gli1 was down-regulated in the groups with low histological grade, with earlier TNM stage and without lymph node metastasis, while the expression was up-regulated in the groups with high histological grade, with later TNM stage, and with lymph node metastasis. See Table 12 for details.

(3) The expression of β-catenin in triple-negative breast cancer tissues was not associated with age and tumor size, but positively correlated with histological grade (r = .739, p < .001), TNM staging (r = .404, p = .027) and lymph node metastasis (r = .559, p = .001). The expression of β-catenin

was down-regulated in the groups with low histological grade, with earlier TNM stage and without lymph node metastasis, while the expression was up-regulated in the groups with high histological grade, with later TNM stage, and with lymph node metastasis. See Table 13 for details.

Table 11. The correlation of the expression of Shh with the clinicopathological characteristics

Indexes	Shh	Age	Tumor Size	TNM Staging	Histological Grade	Lymph Node Metastasis
Shh	1					
Age	0.103	1				
Tumor Size	-0.026	-0.257	1			
TNM Staging	.460*	0.120	-0.039	1		
Histological Grade	.774**	-0.008	0.038	.386*	1	
Lymph Node Metastasis	.540**	0.055	0.043	-.485**	-.583**	1

Note. * at 0.05 level, the correlation was significant; ** at 0.01 level, the correlation was significant

Table 12. The correlation of the expression of Gli1 with the clinicopathological characteristics

Indexes	Gli1	Age	Tumor Size	TNM Staging	Histological Grade	Lymph Node Metastasis
Gli1	1					
Age	0.040	1				
Tumor Size	0.047	-0.257	1			
TNM Staging	.414*	0.120	-0.039	1		
Histological Grade	.757**	-0.008	0.038	.386*	1	
Lymph Node Metastasis	.515**	0.055	0.043	-.485**	-.583**	1

Note. * at 0.05 level, the correlation was significant; ** at 0.01 level, the correlation was significant

Table 13. The correlation of the expression of β-catenin with the clinicopathological characteristics

Indexes	β-catenin	Age	Tumor Size	TNM Staging	Histological Grade	Lymph Node Metastasis
β-catenin	1					
Age	-0.057	1				
Tumor Size	-0.045	-0.257	1			
TNM Staging	.404*	0.120	-0.039	1		
Histological Grade	.739**	-0.008	0.038	.386*	1	
Lymph Node Metastasis	.559**	0.055	0.043	-.485**	-.583**	1

Note. * at 0.05 level, the correlation was significant; ** at 0.01 level, the correlation was significant

4. DISCUSSION

According to the latest statistical analysis, breast cancer ranks first in the aspect of incidence for women. Particularly, TNBC often happens to young women in the premenopausal period, and 30%-40% of TNBC can develop metastatic breast cancer. With the rapid development of gene detection technology in the era of precision medicine, it is an urgent problem for us to find and determine specific target genes that can make effective early diagnostic, prognostic markers and new therapeutic targets. TNBC is highly heterogeneous, which inspires us a lot. It is unrealistic to cure TNBC in one way, and we need to find more specific targets according to the mechanism of TNBC occurrence and development. In this study, the relevant protein factors in TNBC tissues may participate in the occurrence and development of TNBC by regulating the Hedgehog and Wnt pathways from the protein and gene levels. They can also be used to explore the relationship between the three indicators. It is helpful to provide a direction for early diagnosis and treatment of TNBC, as well as the target for targeted intervention at a later stage.

4.1 Expression and significance of Shh in triple-negative breast cancer tissues

SHH plays an important role in the development of various tissues and organs, and it is closely related to the maintenance and renewal of stem cells in many adult tissues. Researchers discovered a highly conserved glycoprotein en-

coded by Hedgehog in fruit flies in 1980, and the Hedgehog signaling pathway is one of the important pathways for normal embryonic development in mammals. Researchers found homologous genes for Hedgehog in vertebrates (mice, chickens, humans, etc.), including Sonic Hedgehog (Shh), India Hedgehog (Ihh), and Desert Hedgehog (Dhh). Of the three, Shh is the most widely studied and closely associated with breast cancer.[11]

It has been found that there is an abnormally high expression of Shh in lung cancer,[12] bladder cancer,[13] colorectal cancer,[14] pancreatic cancer,[15] prostatic cancer[16] and other tumors. It is confirmed that the abnormal expression of SHH plays a key role in the occurrence and development of breast cancer.[17] In this study, the mRNA and protein expression of Shh in triple-negative breast cancer tissues and corresponding paracancer tissues can be detected by qRT-PCR and immunohistochemistry. The final results showed that the relative mRNA expression of Shh in triple-negative breast cancer tissues was significantly higher than that of paracancer tissues, and the positive expression rate of Shh protein was significantly higher than that of paracancer tissues, and the conclusions were consistent at the gene and protein levels. TAO Y et al. believed that,[18] the expression level of Shh was moderate in TNBC, but significantly increased in TNBC samples. It is indicated that the abnormally high expression of Shh has a specific promoting effect on the occurrence of TNBC.

Our results suggest that the expression of Shh is upregulated in cases with high histological grade, positive lymph nodes, and late clinical stage. Studies have shown that[19] the expression of Shh is increased in patients with high histological grade in TNBC, and there is also a significant correlation between Shh protein expression and the tumor stage. Therefore, we believe that the high expression of Shh promotes the occurrence, invasion and metastasis of triple-negative breast cancer.

4.2 Expression and significance of Gli1 in triple-negative breast cancer tissues

The human Gli1 gene, located on chromosome 12, was first discovered due to a more than 50-fold gene amplification in glioblastoma multiforme.[20] The Gli1 protein is a major Hh signaling pathway effector, activating downstream target genes.[21] Gli1 plays an important role in tumor transformation, apoptosis evasion, invasion, metastasis and angiogenesis.[22] Gli1 is also closely associated with a variety of signal transduction pathways.[23] The experimental results showed that the relative mRNA expression of Gli1 in triple-negative breast cancer tissues was significantly higher than that in paracancer tissues. The immunohistochemical results showed that the positive expression rate of Gli1 in triple-negative breast cancer tissues was significantly higher than that in paracancer tissues. Some scholars have reported[18] that Gli1 is not expressed in normal breast tissues, while it is significantly increased in TNBC. Studies have also confirmed that Gli1 is highly expressed in gastric cancer,[24] lung cancer,[25] bladder cancer,[26] melanoma,[27] gallbladder cancer[28] and other tumors. In conclusion, Gli1 is positively correlated with the malignancy of tumors, and its expression will promote the occurrence of tumors.

In addition, higher Gli1 expression was found in patients with high histological grade, positive lymph nodes and later clinical stage in comparison with patients with low histological grade, negative lymph nodes and earlier clinical stage, which could also be confirmed by this study. NOMAN A S et al.[19] studied the relationship between breast cancer tissues and clinicopathological features, and the results showed that the elevated levels of Gli1 in breast cancer lead to higher tumor staging and positive lymph node status. All these suggest that the high expression of Gli1 promotes the metastasis and invasion of triple-negative breast cancer.

4.3 Expression and significance of β-catenin in triple-negative breast cancer tissues

β-catenin is an adhesion factor that binds to E-cadherin on the cell membranes to form a compound, and it can ensure the stability of the cytoskeleton and play a role in intercellular adhesion. β-catenin also plays an important role in cellular epithelial-mesenchymal transformation (EMT), which promotes tumor invasion and metastasis. It is also an essential transcriptional activator of the Wnt pathway. Studies[29] have shown that the increased expression level of β-catenin has been observed in various subtypes of human breast cancers, and they are higher than in normal breast tissues. Besides, it is more evident in TNBC. This study showed that the positive expression rates of β-catenin in triple-negative breast cancer and paracancer tissues in 30 patients were 93.3% and 73.3%, respectively. At the same time, qRT-PCR was used to detect the relative mRNA expression level of the triple negative breast cancer group was significantly higher than that of the paracancer tissue group, which was consistent with the immunohistochemical results.

In addition to the above results, it is also found that as the histological grade increases, lymph node metastasis occurs and the clinical stage increases, the protein expression of β-catenin will also be up-regulated. This was consistent with the result of Liu Man et al.[30] that the expression of β-catenin in G3 group was up-regulated in triple-negative breast cancer. Therefore, we can believe that the abnormal expression of β-catenin promotes cell proliferation, diffusion

and metastasis of triple-negative breast cancer.

4.4 Expression and significance of Shh, Gli1 and β-catenin in triple-negative breast cancer tissues

Shh is one of the important protein ligands of the human Hh signaling pathway. Gli1 transcription is the most reliable marker of pathway activation.[31] This study showed that the expression of Shh and Gli1 in TNBC was positively correlated. Some scholars confirmed[32] that the expression of Shh was positively correlated with the expression of Gli1 in the cytoplasm. This conclusion is obviously consistent with the final results of this study. It was shown that Shh and Gli1 were able to activate the Hedgehog signaling pathway jointly and lead to malignant transformation in many tissues.

The results also suggest that Shh and β-catenin are positively correlated. Previous studies have demonstrated[33–35] that Shh activates the expression of the proto-oncogene N-myc, a member of the Myc transcription factor family, in the developing cerebellar and medulloblastomas. When N-myc coding genes are silenced, the expression of β-catenin is reduced, suggesting that inhibition of N-myc expression leads to β-catenin degradation. Conversely, when the N-myc gene is activated, the expression of β-catenin will be up-regulated. In conclusion, the expression of N-myc is regulated by Shh, and the change of N-myc will affect the expression level of β-catenin, and then regulate the transcription of downstream target genes.

Besides, the results also suggest that Shh and β-catenin are positively correlated. Li et al. have reported that[36] Gli1 induces the activation of Wnt2b, Wnt4 and Wnt7b (all of which are ligands of the Wnt pathway), improving the stability of β-catenin, thereby triggering Wnt signaling. Li Wenling et al. have confirmed that[37] Gli1 promotes cancer cell proliferation by up-regulating the expression of intracellular C-myc (C-myc is another member of the Myc transcription factor family), and the increased expression of β-catenin protein is detected when over-expression of Gli1 is detected. SONG L et al. have confirmed that[38] Gli3R (Gli3R is a repressor produced by hydrolysis of Gli3 protein in the Gli protein family) will physically interact with the C-terminal domain of β-catenin, thereby reducing Wnt-mediated transcriptional activity, but at the same time, the over-expression of Gli1 can inhibit Gli3R, increase the expression of β-catenin, and then enhance the transcriptional activity of the Wnt pathway.

The Wnt signaling pathway is an essential and complex signaling pathway involved in a variety of physiological processes, including tissue transformation, cell migration, EMT, and stem cell maintenance.[39–41] New tumor markers and anti-tumor targeting molecules can be further explored by studying the Wnt/β-catenin signaling pathway. In the Wnt/β-catenin signaling pathway, when Wnt is activated, Wnt binds to the corresponding receptor. At this time, the intracellular β accumulates and translocates to the nucleus, initiating the transcription process of downstream target genes, and Wnt signals drive β-catenin-mediated transcription, β-catenin is therefore considered as an indicator of Wnt activation.[42–44] Shh and Gli1 are of great significance to the Hedgehog signaling pathway and have an indispensable and important effect.

It has been suggested that[45] Gli1 binds to SuFu (serine threonine kinase Fused inhibitor) and is inhibited by SuFu. At the same time, SuFu can also inhibit the newly synthesized Gli1. After ligand Shh increases and binds to the receptor, Smo is activated, promoting the dissociation of the SuFu-Gli1 complex to make Gli1 active. Meanwhile, Michael D Taylor et al.[46] believed that SuFu could bind β-catenin and export it from the nucleus, thus inhibiting the activation of Wnt signaling pathway. SuFu is a negative regulator of both the Wnt/β-catenin signaling pathway and the Hedgehog signaling pathway, which can bind Gli1 protein and β-catenin protein at the same time. Some scholars have shown that[38] Wnt/β-catenin signaling pathway may be the downstream signaling pathway of Shh. When the expression of ligand Shh is increased, an increase of Gli1 is observed in the activation of the Hedgehog signaling pathway, while the Wnt/β-catenin signaling pathway is also activated, with the expression of β-catenin protein increased. Therefore, we speculate that there may be an interactive dialogue between Wnt and Hedgehog signaling pathways during the occurrence and development of triple-negative breast cancer, which is more consistent with the final results of this study. In triple-negative breast cancer, there may be a synergistic relationship between Shh, Gli1, β-catenin and the two signaling pathways in which they are located, and the crosstalk between them plays an important role in the recurrence, invasion and metastasis of triple-negative breast cancer. This conclusion may have some enlightenment for treating triple-negative breast cancer, and the combination of inhibitors that interfere with Wnt/β-catenin or Hedgehog/Gli signaling respectively, may be a feasible way to treat triple-negative breast cancer.

In summary, this study preliminarily explored the interaction between three key proteins in Hedgehog and Wnt in TNBC. The occurrence and development of TNBC is an extremely complex process, and the relationship between signaling pathways is intricate. The sample size of this topic is small, and the sample source is limited. It is expected that large-scale experiments will further elucidate the molecular mechanism of the two pathways in TNBC to develop selective inhibitors based on Hedgehog/Gli and Wnt/β-catenin

signaling crosstalk, which may become a new way to block disease progression and treat triple-negative breast cancer.

5. CONCLUSION

The up-regulated expression of Shh, Gli1 and β-catenin proteins in triple negative breast cancer suggests that Shh, Gli1 and β-catenin proteins are involved in tumor genesis. The combined detection of the three proteins may provide a theoretical basis for the diagnosis and prognosis evaluation of triple negative breast cancer.

Shh was positively correlated with Gli1 protein expression and β-catenin protein expression. Gli1 was positively correlated with β-catenin protein expression, suggesting that the three types of proteins play a synergistic role in the occurrence and development of TNBC. There may be crosstalk in the Wnt/β-catenin and Hedgehog signaling pathways in TNBC, which may provide a new approach for treating TNBC.

The expression of Shh, Gli1 and β-catenin proteins was correlated with the degree of differentiation, TNM staging and lymph node metastasis of triple negative breast cancer, but not correlated with age and tumor size. Therefore, it was predicted that the three types of proteins were related to the invasion, metastasis and prognosis of TNBC.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare they have no conflicts of interest.

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