European Review for Medical and Pharmacological Sciences 2017; 21: 3626-3633

Functional impact of Galectin-3 and TRAIL expression in breast cancer cells

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Abstract. – OBJECTIVE: To examine the expression of Galectin-3 and TRAIL in breast cancer tissue and their effects on the proliferation and apoptosis of breast cancer cells.

PATIENTS AND METHODS: Breast cancer and normal adjacent tissue were collected from 120 patients pathologically diagnosed with breast cancer who underwent a modified radical mastectomy. SP method of immunohistochemistry was used to detect the expression levels of Galectin-3 and TRAIL in breast cancer tissues and normal adjacent tissues. The correlation between the expressions of Galectin-3 and TRAIL, and clinical prognosis of breast cancer were analyzed. Breast cancer cells were transfected with Galectin-3 siRNA and TRAIL overexpression constructs. Cell proliferation was measured by XTT method, and apoptosis was detected by flow cytometry.

RESULTS: Higher Galectin-3 level and lower TRAIL level were found in breast cancer tissues compared with those in normal adjacent tissues ($p < 0.001$). High expression level of Galectin-3 and low expression level of TRAIL were found to be positively correlated with the shorter median survival time and overall survival time. Galectin-3 silencing by siRNA interference and TRAIL overexpression significantly decreased cell viability of MDA-MB-231 and increased the number of apoptotic cells.

CONCLUSIONS: The expression level of Galectin-3 in breast cancer tissues was significantly increased compared with that in normal tissues, while the level of TRAIL protein was significantly decreased in cancer tissue. The biological role of these two proteins seems to be synergistic in inhibiting apoptosis of cancer cells. Therefore, the evaluation method that combined both Galectin-3 and TRAIL is of great clinical value in the evaluation of clinical prognosis of patients with breast cancer.

Key Words:
Breast cancer, Galectin-3, TRAIL, Prognosis.

Introduction

Breast cancer is one of the most common malignancies in females all over the world. In China, breast cancer accounts for 7-10% of all malignancies. In recent years, the incidence of breast cancer gradually increased. Breast cancer has become one of the major diseases threatening women's health[1,2]. In the past 20 years, the diagnosis and treatment of breast cancer have been remarkably improved, and the development of new surgical treatment has been proved to be effective. Meanwhile, the biological behavior of breast cancer has been further elucidated, and the occurrence and development of tumors can be investigated by modern molecular biology techniques. Because of the poor prognosis of breast cancer, especially for the advanced breast cancer patients with invasion and metastasis, therefore, the mechanisms mediating invasion and metastasis has become a research hotspot. It is well known that invasion and metastasis of malignancies is a multi-factorial and multi-step process. However, the concrete mechanisms mediating the occurrence and development of breast cancer are still unclear[3-5].

Five-year survival rate of patients with breast cancer is approximately 50-60%. Recurrence and metastasis occur in about 50% of patients after treatment. Moreover, the average survival time of patients with late breast cancer is 18-30 months. Although many treatment methods have been developed to treat breast cancer, however, almost all the treatment methods were proved to be with poor prognosis and serious side effects. Besides, breast cancer tends to metastasize outside the breast; therefore, it is one of the most difficult tumors to treat[6]. Traditional therapies can only kill tumor cells during cell division. But once the treatment is completed, the proliferation and

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differentiation of tumor stem cells will continue, resulting in recurrence and metastasis. This is the so-called "dandelion phenomenon"[7].

As a newly discovered member of the IL-10 cytokine family, human melanoma differentiation associated gene-7/interleukin-24 (*MDA-7/IL-24*) is a novel tumor suppressor gene with cytokine-like properties[8,9]. *MDA-7/IL-24* encodes secreted protein products (secreted MDA-7/IL-24, SMDA-7/IL-24) that can inhibit tumor growth and specifically induce tumor cell apoptosis. IL-24 can induce and enhance the activity of tumor necrosis factor related apoptosis inducing ligand (TRAIL) and promote cell apoptosis. TRAIL can induce the transformation of Jurkat cells and EB virus, and induce apoptosis of tumor cells and virus from different sources[10-12]. Also, TRAIL is not toxic for normal tissue cells and does not induce cell death. In recent years, a few studies have demonstrated that proliferation and apoptosis of tumor cells are affected by the inhibited expression of Galectin-3. Galectin-3 is a galactose-binding protein of the lectin family that plays an important role in cell-cell and cell-matrix interaction, cell growth, cell-cycle regulation, apoptosis, cell damage and repair, malignant transformation, and metastasis. Recently, reports detected Galectin-3 overexpression in human tumors[13,14]. Galectin-3 seems to play a pivotal role in the development of large-cell lymphoma, colorectal cancer, breast cancer, liver cancer, brain tumor, melanoma, and thyroid cancer. However, the effects and mechanisms of function of TRAIL and Galectin-3 in breast cancer are still unclear. Based on existing studies, we further explored the role of Galectin-3 and TRAIL in proliferation and survival of breast cancer cells, and discussed the application of Galectin-3 and TRAIL in clinical diagnosis, classification, prognosis, and treatment.

Patients and Methods

Clinical Case Collection

We collected breast cancer tissues and adjacent tissues from 120 breast cancer patients after modified radical mastectomy in our hospital from January 2010 to July 2012. All the samples were pathologically confirmed as breast cancer tissues. The study was approved by the Ethics Committee of Xiangyang No. 1 People's Hospital, Hubei University of Medicine. The breast cancer patients received no anticancer therapy before surgery. According to the WHO classification of breast tumors and histological grading method, we selected 120 cases of patients with invasive ductal carcinoma – the most common type of breast cancer. There were 20 cases in G1 phase, 88 in G2 phase, and 12 in G3 phase. All the patients were female with age ranged from 26 to 80 years old, and the median age is 42 years. The tumor diameters ranged from 1.0 to 8.0 cm. 60 cases presented with axillary lymph node metastasis and the other 60 cases showed no axillary lymph node metastasis. The distances between tumors and normal tissues adjacent to tumors were at least 2 cm.

Reagents

Mouse anti-human Galectin-3 monoclonal antibody (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China); rabbit anti-human TRAIL polyclonal antibody (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China); two-step universal anti-rabbit and anti-mouse immunohistochemistry detection kit (ChemMate Envision Detection Kit, Peroxidase/DAB, Rabbit/Mouse) (DAKO, Glostrup, Denmark); 0.01 M citrate buffer (Gibco Co, Grand Island, NY, USA); Mayer's hematoxylin (Gene, Hongkong); paraffin and microscope slides (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China); xylene, absolute ethanol and 30% hydrogen peroxide (Guangzhou Chemical Reagent Factory, Guangzhou, China); Pap Pen (Shanghai Gene Tech); non-immune goat serum (Fuzhou Maixin Biotech); ethylene diamine tetraacetic acid (EDTA), citric acid and trisodium citrate (Guangzhou Chemical Reagent Factory).

Instruments

Medical work table (Suzhou Purification Equipment).

Immunohistochemical Staining

After fixation in formaldehyde solution, cancer tissues and tissues adjacent to tumor were embedded in paraffin and cut into sections. After washing with phosphate buffer saline (PBS) three times, the sections were incubated in 3% hydrogen peroxide at room temperature for 20 min. After washing with PBS three time and adding primary antibodies (Galectin and TRAIL), the sections were incubated overnight at 4°C. Then, the tissues were washed three times with PBS, and enzyme-labeled secondary antibody was added, the sections were incubated at room temperature for 20 min. After that, the sections were

washed three times in PBS, and were stained with diaminobenzidine (DAB) for 3-5 min and the staining was monitored under a microscope. The sections were then washed with distilled water three times and immersed in Mayer's hematoxylin solution for 10-20 sec for differentiation and nuclear staining. After dehydration with a gradient of ethanol and clarification in xylene, the sections were sealed with neutral resin. The results were observed and photographed under an inverted light microscope (Guangzhou Liss Optical Instruments). Yellow-brown granules represent the positive signals.

Scoring of Results

The criteria for positive expressions of Galectin-3 and TRAIL were: positive signal appeared as yellow-brown granules in the cytoplasm and/or nucleus. Five fields (×400) were detected for each section. "-" represented a number of positive cells of less than 10%, "+" positive cells from 10% to 50%, and "++" positive cells more than 50%.

Culture of MDA-MB-231 Cells

MDA-MB-231 cells were cultured in an incubator (37°C, 5% CO_2). Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium containing 10% of fetal bovine serum, 100 Units/ml of penicillin and 100 mg/l of streptomycin was used. The cells were sub-cultured according to their growth state.

Construction of Galectin-3 siRNA

Galectin-3 siRNA was designed and synthesized by Guangzhou RiboBio starting at position 518. 518-Fw: 5'-GCAAUACAAAG-CUGGAUAAdTdT-3' and 5'-UUAUCCAGCU-UU-GUAUUGCdTdT-3'.

Construction of TRAIL Plasmid Vector

TRAIL primers were designed and chemically synthesized by Guangzhou RiboBio. TRAIL-Fw 5'-ACGCGTCGACATGAGAGTAG-CAGCTCACATAACTGGG-3, and TRAIL-Rv 5-ATAAGAATGCGGCCGCTTAGCCAACTA-AAAAGGCCC-3'.

Cell Proliferation Using XTT Method

Pancreatin was used to digest cells 24 hours after transfection. Cells were then resuspended in 1640 culture medium at 1000 rpm and the cell number was counted. Cell suspensions were appropriately diluted and placed into 96-well plate (4000 cells/well). Each well contained 50 μl of culture medium. Three replicates were set for each group. Cells were cultured in an incubator (37°C, 5% CO_2). 50 μl of XTT solution was added every day at the same time point. Three hours after incubation, ELISA was used to measure the optical density (OD) value of each well of each plate after the staining. Microplate reader was used to measure the OD value of each well at 24, 48, 72, and 96 hours after interference.

Detection of Apoptosis Using Flow Cytometry

Cell culture medium was discarded, and 1 ml of phosphate buffered saline (PBS) was added and removed to remove the remaining culture medium. 1 ml of pancreatin was added to digest cells for 3 min. Cells were shaken after removing pancreatin and adding PBS. Cell suspensions were collected in 1.5 ml tubes and centrifuged at 1000 rpm for 5 min. 20 μl Annexin-V-Flour labeling regent and 20 μl Propidium Iodide Solution were added to 1 ml of Incubation Buffer, and then 100 μl of Incubation Buffer was added to each tube. After spiral oscillation, uniform cell suspensions were stained at room temperature in the dark for 20 min. Cell suspensions were then centrifuged at 1000 rpm for 5 min. After removing supernatant and adding 1 ml of PBS, cells were resuspended, and uniform cell suspensions were centrifuged at 1000 rpm for 5 min. Then, the supernatant was removed, and 20 μl of PBS was added, cells were mixed, and uniform cell suspensions were placed in BD flow cytometry. Control group, negative group, and Galectin-3 siRNA group were set. Samples were analyzed using Cell Quest. All the detections were performed three times. AnnexinV-FITC(-)/PI(-) represented normal cells, AnnexinV-FITC(+)/PI(-) early apoptotic cells, AnnexinV-FITC(+)/PI(+) late apoptotic cells and Annexin-FITC(-) / Pl(+) necrotic cells.

Statistical Analysis

Experimental data were analyzed with SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Chi-square test was used to analyze the correlation between protein expressions of Galectin-3 and TRAIL and clinico-pathological data (including age, gender, tumor classification and TNM staging). Pearson contingency coefficient (C) and Chi-square for paired data were used for validation. One-way ANOVA was used to comparison among multiple groups. The homogeneity of variances was tested. LSD method was used for inter-group comparison if the group variances were statis-

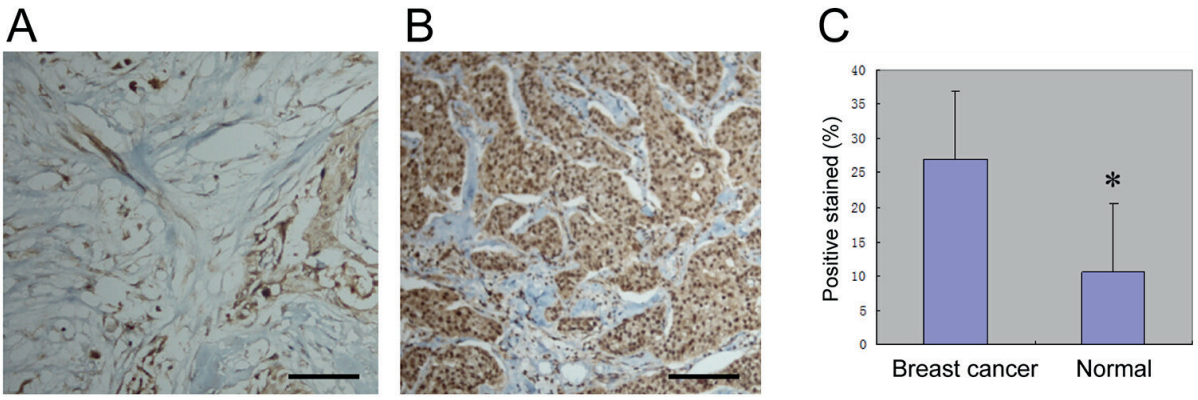

Figure 1. The expression levels of Galectin-2 protein in tumor tissue ***(A)*** and tissue adjacent to tumor ***(B)*** under magnification 200×. Scale bar = 50 μm. ***(C)***. The expression of Galectin-3 in cancerous tissues was higher than that in normal breast tissues, and the difference was statistically significant ($p < 0.05$).

tically equal. Brown-Forsythe test was used for correction analysis if the group variances were not equal. Factor analysis was used to determine the interaction between grouping and time points. $p < 0.05$ was considered to be statistically significant.

Results

Expression Levels of Galectin-3 and TRAIL in Breast Cancer Tissue

The expression rate of Galectin-3 was significantly higher in breast cancer tissue than that in the adjacent normal tissue ($X2 = 109.71$, $p < 0.001$) (Figure 1). In contrast, expression of TRAIL was significantly lower in breast cancer tissue than that in the adjacent normal tissue ($X2 = 110.46$, $p < 0.001$) (Figure 2). These results suggested that elevated Galectin-3 level and reduced TRAIL level promote breast cancer growth.

Correlation Between Galectin-3 and TRAIL, and Clinical Prognosis

The correlation between the levels of Galectin-3 and TRAIL, and clinical prognosis were analyzed using the Kaplan-Meier method. Survival rate of patients with different expressions analyzed by Kaplan-Meier method. The survival rate of patients with negative expression of Galectin-3 was significantly higher than that of patients with Galectin-3 + /TRAIL - and that of patients with TRAIL + ($p < 0.05$) (Figure 3).

Cell Viability After Galetin-3 Silencing and TRAIL Overexpression

After detecting the expression of Galectin-3 and TRAIL in breast cancer and the correlation

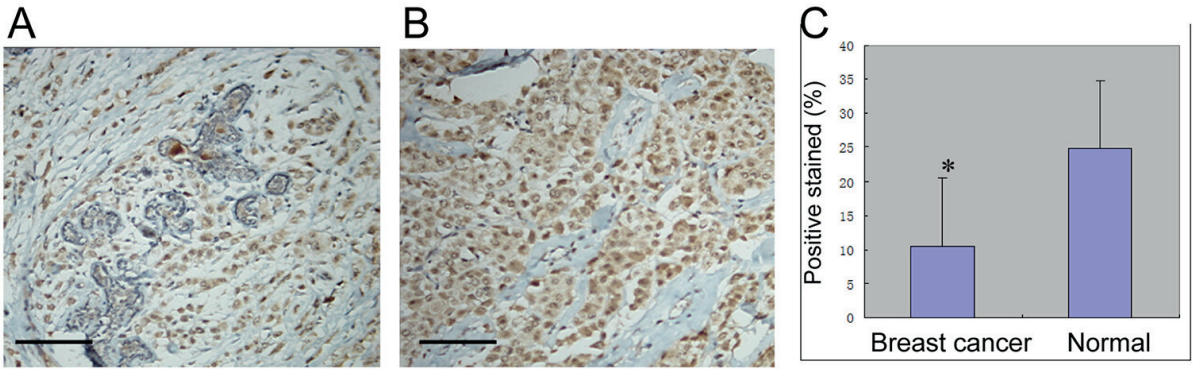

Figure 2. The expression levels of TRAIL protein in tumor tissues ***(A)*** and tissues adjacent to tumor ***(B)*** in the same patient. With immunohistochemical staining for breast cancer (ductal carcinoma) tissues (Magnification 200×), it can be seen that the expression level of TRAIL protein in breast cancer tissues was remarkably lower than that in tissues adjacent to tumor, and the difference was statistically significant ($p < 0.05$).

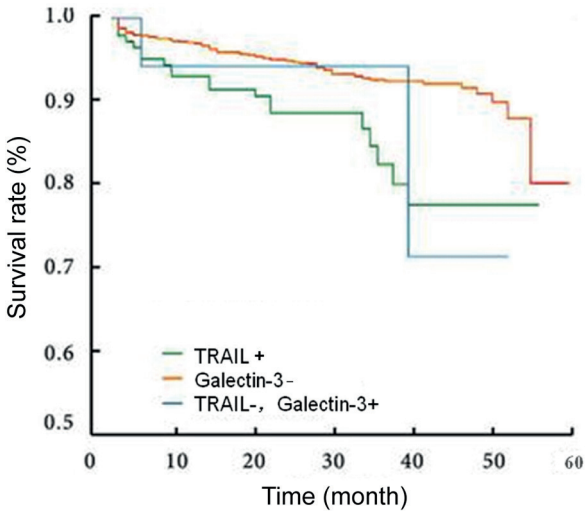

Figure 3. Survival rate of patients with different expressions analyzed by Kaplan-Meier method. The survival rate of patients with negative expression of Galectin-3 was significantly higher than that of patients with Galectin-3 + / TRAIL - and that of patients with TRAIL + ($p < 0.05$). Log-rank test: X2 = 7.69, $p = 0.006$.

with the survival rate, we examined the function Galectin-3 and TRAIL in cancer. The combined treatment with Galectin-3 siRNA and TRAIL overexpression resulted in a more significant reduction in cell viability ($p < 0.05$) (Figure 4).

Apoptosis After Manipulating Galectin-3 and TRAIL

To further understand the physiological role of Galectin-3 and TRAIL in cancer cell survival, we next detected the apoptosis after transfection using flow cytometry. The number of apoptotic cells in the Galectin-3 siRNA group increased significantly compared with the control siRNA group (Figure 5). The number of late apoptotic cells in the TRAIL overexpression group was significantly higher than that of the empty vector group (Figure 5). These results are consistent with the cell viability experiments and support the physiological role for Galectin-3 and TRAIL in breast cancer growth.

Discussion

Approximately 1.2 million females are diagnosed with breast cancer every year all over the world, accounting for 18% of all female tumors. The biological behavior of breast cancer includes strong invasive capacity, early recurrence, fast development, and short survival time[4]. Traditional tumor therapies (surgery, radiotherapy, and chemotherapy) are still the main methods in the tumor treatment. However, the proliferation of residual tumor stem cells

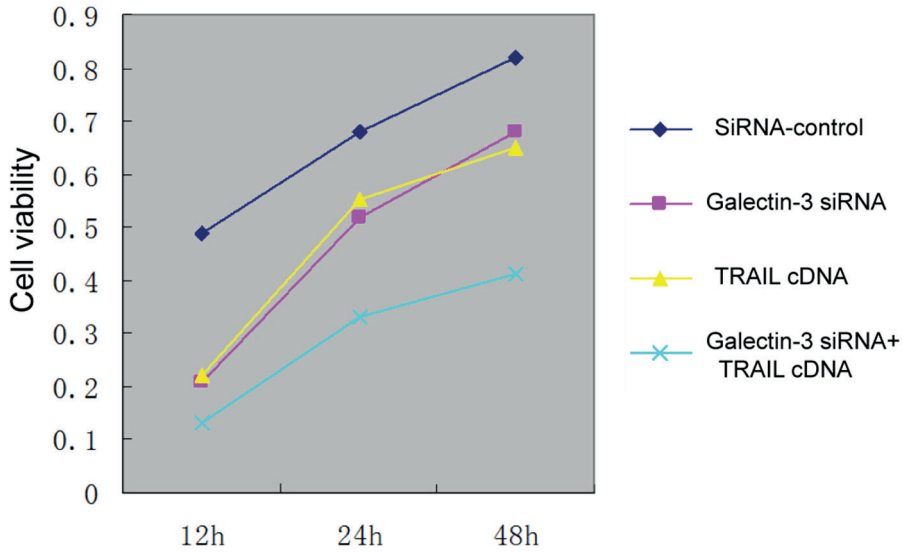

Figure 4. Cell viability detected by XTT method, compared with Galectin-3siRNA group and TRAILcDNA group, the cell viability was significantly decreased in Galectin-3siRNA and TRAILcDNA group, but was significantly higher than that in TRAIL cDNA Galectin-3 group. The differences were statistically significant ($p < 0.05$).

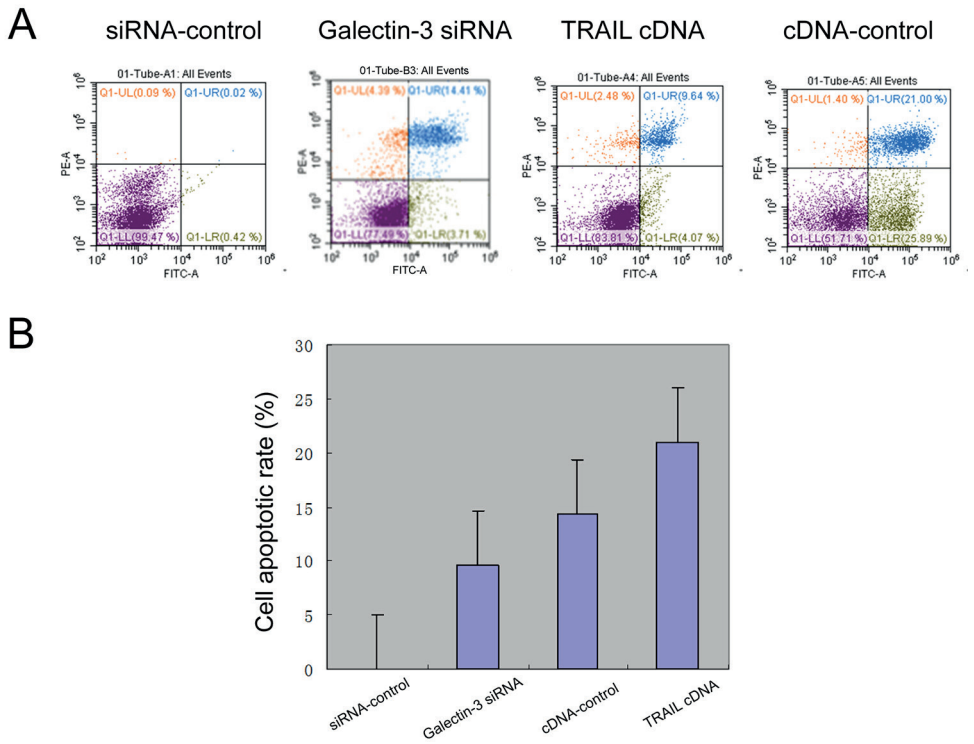

Figure 5. ***A,*** Number of apoptotic cells detected by flow cytometry; ***B,*** Number of apoptotic cells in transfection of Galectin-3 siRNA group remarkably increased compared with that in transfection of control siRNA group, and the difference was statistically significant ($p < 0.05$). The number of late apoptotic cells in transfection of TRAIL vector group was remarkably higher than that in empty vector group, and the difference was statistically significant ($p < 0.05$).

after treatment can cause tumor recurrence and metastasis. The surface markers of breast cancer stem cells are CD44, ABC protein, MDR, and ABCG2. At present, the study of targeted therapy for breast cancer stem cells mainly includes: (1) inhibitors of DNA damage and drugs can repair DNA damage such as poly ADP-ribose polymerase-1 (PARP-1); (2) cell surface receptors such as epidermal growth factor receptor (EGFR) and C-Kit; (3) vascular endothelial growth factor receptor (VEGFR) inhibitor; (4) Src kinase inhibitor; (5) mammalian target of rapamycin (mTOR); (6) Interleukin (IL); and (7) TRAIL[5-7].

Although IL-24 has the advantage of pleiotropic effects, selectivity, overlapping function, cooperativity, and antagonistic functional duality, IL-24 still could not completely eliminate the tumors implanted in nude mice[15,16].

In this study, we found that the expression level of Galectin-3 was significantly increased in breast cancer tissue compared with normal breast tissue adjacent to the tumor. This finding is consistent with the results of existing studies that the increase of expression level of Galectin-3 implies the exuberant proliferation of breast cancer cells. Current studies showed that TRAIL is activated mainly by the combination of death receptors DR4 and DR5 on cellular membrane. Intracellular death domain (DD) conducts apoptotic signals and activates FADD-caspase, mitochondrial-dependent and mitochondrial-independent pathways, which mediates the occurrence of apoptosis[17-19]. Here, we found that the expression level of TRAIL was remarkably decreased in breast cancer tissues compared with that in normal breast tissues adjacent to the tumor. This finding may be related to the inhibition of TRAIL expression in breast cancer cells. Therefore, IL-24 cannot inhibit cell proliferation by activating TRAIL.

Conclusions

The synergistic effect of increased Galectin-3 level and decreased TRAIL level in breast

cancer tissues plays an important role in the occurrence and development of breast cancer by inhibiting the apoptosis of cancer cells. Therefore, the evaluation method that combined both Galectin-3 and TRAIL is of great clinical value in the evaluation of clinical prognosis of patients with breast cancer.

Acknowledgements

This work was supported by the Hubei Province Health and Family Planning Scientific Reasearch Project (WJ2017M230 and WJ2017Q038).

Conflict of interest

The authors declare no conflicts of interest.

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