Expression of survivin and VEGF-C in breast cancer tissue and its relation to lymphatic metastasis

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Summary

Purpose: To study the expression of survivin and VEGF-C in breast cancer tissue and its relation to lymphatic metastasis. *Methods:* The expression level of survivin and VEGF-C in breast cancer tissue is determined by immunohistochemistry and reverse transcriptase polymerase chain reaction (Rt-PCR). *Results:* Among the 60 breast cancer tissues, the percentage of positive expression of survivin or VEGF-C was 85.0% and 78.3%, respectively. The expression rate of VEGF-C was 84.3% (43/51), 44.4% (4/9) in the survivin positive and negative expression group, respectively. Linear correlation analysis showed a correlation coefficient of survivin and VEGF-C in breast cancer was 0.80 ($p = 0.035$), which indicates a positive correlation between the two biomarkers. *Conclusions:* Survivin and VEGF-C are highly expressed in breast cancer tissues with a positive correlation. The regulation of survivin expression can cause excessive expression of VEGF-C, leading to the generation of breast cancer lymphangiogenesis, thus causing lymphatic metastasis of breast cancer.

Key words: Breast cancer; Lymphatic metastasis; Survivin; Vascular endothelial growth factor-C.

Introduction

The major cause of death in breast cancer patients is widely spread metastasis. Metastasis usually first occurs in the axillary lymph node; therefore, lymphatic metastasis is frequently used as one of the main standards to evaluate prognosis and choose treatment methods for breast cancer patients [1]. During the diagnosis and treatment of breast cancer, early detection of a lymphangiogenesis and control of lymphatic metastases are very important in the prevention of the patient dying from breast cancer. Vascular endothelial growth factor-C (VEGF-C) is a new member of the vascular endothelial growth factor family, and is highly expressed in most human tumors. In vivo studies confirm that VEGF-C can induce a generation of tumor lymphatic metastasis and participate in the metastasis of tumor lymphatics through vascular endothelial growth factor receptor-3 (VEGFR-3), which is considered a regulatory factor of specific lymph angiogenesis [2].

Survivin belongs to the inhibitor of apoptosis protein (IAP) family, and is highly expressed in human embryonic tissues. It plays a role in tissue differentiation and organ generation in the course of embryonic development. Survivin expression cannot be detected in mature differentiated adult tissues, but is widely detected in many kinds of human tumors. Studies have shown that survivin not only is expressed in cancer tissues, but also is expressed in many pre-cancer tissues [3]. Here we investigated whether survivin expression is related to the expression of VEGF-C in breast cancer and their roles in the generation of lymphangiogenesis. At present there are no related reports of whether survivin expression is related to VEGF-C expression in breast cancer. This paper focuses on studying the expression of survivin and VEGF-C in breast cancer and their effects and relationship in the course of lymphatic metastases, suggesting potential targets for comprehensive therapy of breast cancer.

Patients and Methods

Patients

Sixty cases of breast cancer postoperative specimens diagnosed by hematoxylin-eosin (HE) staining pathology [4] were collected from female patients with an average age of 43.6 (range 27-66) between January 2007 and December 2009 in a subsidiary hospital, Taishan Medical College. The collection was approved by the Medical Ethics Committee of the subsidiary hospital, Taishan Medical College. Among the 60 cases, there were 54 cases of infiltrative ductal carcinoma, one case of infiltrative lobular carcinoma, four cases of medullary carcinoma, and one case of infiltrative ductal carcinoma with squamous carcinoma. Thirty-two cases had lymph node metastasis while the remaining cases did not. All patients were not given radiotherapy or chemotherapy prior to the surgery. Based on the 7th edition staging of UICC (International Union Against Cancer and AJCC American Joint Committee on Cancer) [5], there were 11 Stage I cases, 38 Stage II cases and 11 Stage III cases of breast cancer. In addition, among the 60 cases we also collected 23 fresh specimens of breast infiltrative ductal carcinoma [4] diagnosed by pathology, with 12 cases having lymph-node metastases and 11 cases free of lymph node metastasis. All of the specimens were excised to about 1.2 cm^3 in size and quickly frozen in a -70°C freezer.

Main reagents

Rabbit anti-human polyclonal antibody against survivin and VEGF-C were purchased from Beijing Boaosen Biotechnology Limited Company (Beijing). Streptavidin-horseradish peroxidase, S-P kit (sp-9001), and enrichment-type 3,3N-diaminobenzidine tetrahydrochloride (DAB) chromomeric kit were obtained from Beijing Zhongshanjinqiao Biotechnology Co.

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Ltd. (Beijing, China). One-step RT-PCR kit was purchased from Beijing Tiangen Biochemical Technology Co. Ltd. (Beijing, China); Trizol reagent was purchased from Invitrogen Corp. (Shanghai); RT-PCR primers were synthesized by Invitrogen (Shanghai). Chemical luminescent agent was purchased from Santa Cruz Biotechnology, Company (Santa Cruz, USA) and pre-stained protein marker was from Solarbio Company (Beijing, China).

Experimental Methods

Detection of survivin and VEGF-C by immunohistochemistry

Paraffin-embedded specimens were deparaffinized and then embedded again to restore antigenicity. Specimens were cut into sections of 4 μm in thickness and the immunohistochemical S-P method was used following the manufacturer's protocol. The sections of known VEGF-C and survivin served as positive controls in each experiment while phosphate buffered saline (PBS) was used as the negative control. All sections were stained with DAB and read under light microscope within 48 hours.

Results determination: The positive expression of VEGF-C was localized in the cytoplasm with a distribution of brownish yellow or sepia colored particles. Survivin was localized in the cytoplasm with yellow to brownish yellow particles. Two expert pathologists evaluated the experimental results using the double blind method. Three visual fields were randomly chosen in each tissue section and the proportion of positive cells was counted out of 100 cells of every visual field under the microscope (×400). The average percentage of the three visual fields was reported as the result. The evaluation standard was adjusted properly according to the reference [6, 7] with the scores ranked as follows: score 0 for those with less than 5% of total cell count, score 1 for 5% to 25% total cell count, score 2 for 25% to 50% total cell count, score 3 for 51% to 75% total cell count and score 4 for greater than 75% total cell count. Staining strength: score 0 without color, score 1 with light yellow, score 2 with yellow and score 3 with brownish yellow. Immunohistochemical results were evaluated by the number of positive stained cells and staining strength, respectively. Based on the product of the above two, negative (-) had a score of 0-1, positive (+to++) had a score of 2-3, and strong positive (+++) had a score of 4.

Detection of survivin and VEGF-C mRNA expression by RT-PCR

Total RNA was extracted from 23 fresh specimens by the TRIzol method; 2 μg total RNA was used to perform reverse transcriptase polymerase chain reaction in each case. At the same time, B_2 M (beta-2 microglobulin) was amplified as an internal reference. Every specimen was repeated at least three times. Internal reference primer sequence F: GGCTATCCAGCGTACTCCAAA, R:CGGCAGGCATACTCATCTTTT, the length of PCR product was 246bp;VEGF-C Primer sequence F: CACGGCTTATGCAAGCAAAGA, R:TCCTTTCCTTAGCTGACACTTGT, the length of PCR product was 121bp; survivin primer sequence:F: 5'-CCCTGCCTGGCAGCCCTTTC-3', R: 5'-CTGGCTCCCAGCCTTCCA-3' the fragment of amplified product was 188 bp. cDNA was first synthesized by reverse transcription in 50°C for 50 min, then the PCR reaction began after pre-degeneration in 95°C for 15 min followed by 35 cycles of amplification at 94°C for 45 sec, 52°C ~58°C for 45 sec, 72°C for 1 min and a final 10 min extension at 72°C. The amplification product was separated on 2.0% agarose gel by electrophoresis. Gel pictures were taken under ultraviolet light after staining with ethidium bromide. The graphical analysis software of BandScan Version 4.3 was used to analyze the densities of the electrophoresis bands. The ratio of density values between the target bands and internal reference bands was the relative expression quantity of survivin and VEGF-C mRNA.

Table 1. — *Relation of the expression of survivin, VEGF-C and clinical pathology of breast cancer examples (%).*

Clinical pathology	n	Survivin			VEGF-C		
		Positive	χ^2	*p*	Positive	χ^2	*p*
Tumor size (cm)							
≤ 2	21	17 (81.0)	0.07	0.79	14 (82.4)	1.64	0.2
> 2	39	34 (87.2)			33 (84.6)		
Histological typing							
Invasive ductal carcinoma	54	46 (85.2)		1.00	43 (79.6)	0.04	0.83
Other cancer	6	5 (83.3)			4 (66.7)		
Lymph node metastasis							
Yes	32	31 (96.9)	5.72	0.02	29 (90.6)	6.10	0.01
No	38	20 (71.4)			18 (64.3)		
TNM staging							
Stage I~II	49	41 (83.7)	0.02	0.89	36 (73.5)	2.33	0.13
Stage III	11	10 (90.9)			11 (100)		

VEGF-C: Vascular endothelial growth factor-C.

Table 2. — *Correlation of survivin and VEGF-C expression in breast cancer.*

Survivin	VEGF-C			
	–	+	++	+++
–	3	3	2	1
+	7	1	2	1
++	2	3	6	9
+++	1	5	6	9

VEGF-C: Vascular endothelial growth factor-C.

Table 3. — *Expression of survivin and VEGF-C mRNA in breast cancer tissue.*

Pathological factors	Survivin mRNA				VEGF-C mRNA			
	Positive	Negative	u	*p*	Positive	Negative	u	*p*
Lymph node metastasis	11	1	-2.87	0.00	12	0	-2.28	0.02
No lymph node metastasis	8	3			9	2		

VEGF-C: Vascular endothelial growth factor-C.

Statistical analysis

Applying SPSS 13.0 statistics analysis software, experimental data was analyzed using χ^2 and relative linear regression analysis. Ranks table χ^2 inspection and the four-layer table χ^2 inspection, Spearman correlation analysis, independent sample t inspection and Wilcox two-sample comparison were performed according to the type of information and different statistical purposes. A *p* value less than 0.05 was considered statistically significant.

Results

Expression of survivin and VEGF-C protein in breast cancer tissue

In 60 cases of breast cancer specimens, the positive expression rate of survivin was 85.0% (51/60) and 78.3% (47/60) for VEGF-C. The expression rate of survivin in the lymph node positive group was 96.9% (31/32), which

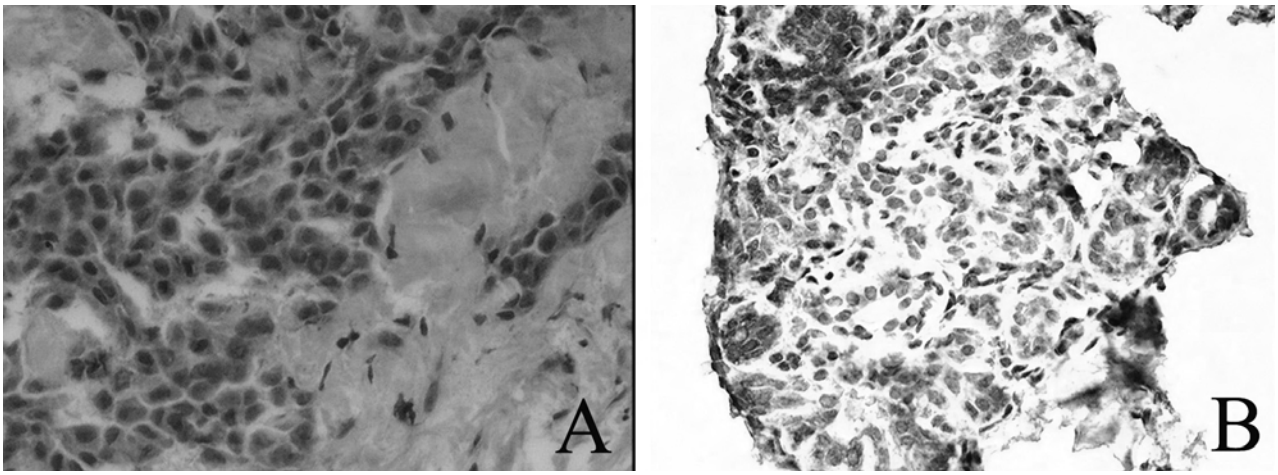

Figure 1. — Expression of survivin and VEGF-C in breast invasive ductal carcinoma. A) Survivin. B) VEGF-C.
VEGF-C: Vascular endothelial growth factor-C.

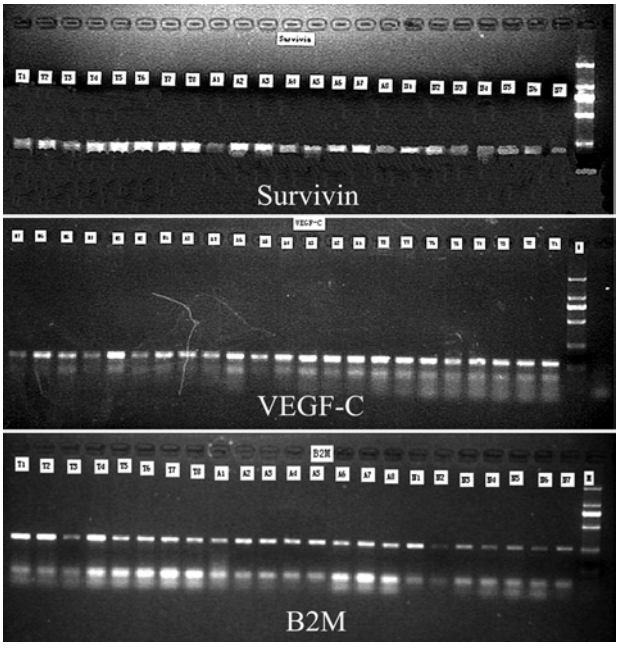

Figure 2. — Expression of survivin and VEGF-C mRNA in breast invasive ductal carcinoma tissue.
B2M, bet-2microglobulin. VEGF-C: Vascular endothelial growth factor-C.

was significantly higher than that of the lymph node negative group (71.4% (20/28), $\chi^2 = 5.72$, $p = 0.02$. The expression rate of VEGF-C in the lymph node positive group was 90.6% (29/32), which was significantly higher than that of the lymph node negative group (64.3% (18/28), $\chi^2 = 6.10$, $p = 0.01$). The expression of survivin and VEGF-C was irrelevant to the size of breast cancer tumor and cancer type ($p > 0.05$), (Table 1, Figure 1).

Correlation of survivin and VDGF-C protein expression in breast cancer

Data analysis showed the expression of VEGF-C in the survivin positive expression group to be 84.3% (43/51) and 44.4% (4/9) in the survivin negative expression group. After liner analysis, correlation coefficient (r) between expression of the two biomarkers in breast cancer was 0.80, positive correlation = 0.035 (Table 2).

Expression of survivin and VEGF-C mRNA in breast cancer tissues

In 23 cases of fresh breast cancer specimens, the expression of survivin mRNA in breast invasive ductal carcinoma tissue with lymph node metastasis was higher than that without lymph node metastasis. The difference between these two groups was statistically significant (u = –2.869, $p = 0.0041$). The difference of VGEF-C expression in the two groups of breast invasive ductal carcinoma tissues was also statistically significant (u = –2.2794, $p = 0.0226$) Table 3, Figure 2.

Discussion

In recent years, with the discovery of lymphatic growth factor and some specific lymphatic endothelial markers, the study of lymphatic formation and lymphatic metastasis has gradually become a new hotspot [8]. Among lymphatic formation factors, VEGF-C, first discovered as a member of the vascular endothelial growth factor (VEGF) family, is a specific lymphatic formation growth factor. The affinity of VEGF-C to VEGFR-3 is three times stronger than that of VEGFR-2. VEGF-C activates VEGFR-3 on lymphatic endothelial cells, induces phosphatidylinositol 3-kinase signaling pathways and subsequently activates p42/p44 mitogen activated protein kinase and kinase B signal pathways. These cascade reactions protect the lymphatic endothelial cells from serum induced apoptosis, promote proliferation and migration of lymphatic endothelial cells, and promote the generation of lymphangiogenesis [9]. At the same time, the adhesions between lymphatic endothelial cells are reduced, improving permeability of lymphatic endothelial cells. Improved permeability of the lymphatic endothelial cells allows cancer cells to infiltrate more

easily, causing lymphatic metastasis of the tumor [10]. By studying post-transformation nude mice implanted with MDA-MB-435 breast cancer cells expressing VEGF-C, Skobe *et al*. [11] found the transformation rate of nude mice sentinel lymphangiogenesis in VEGF-C overexpression group was greater than 60% higher than that in the matched group, suggesting that VEGF-C can promote the generation of breast cancer lymphangiogenesis, increase the metastasis of local lymphangiogenesis and distant metastasis (lung). Their results also suggest that newly formed lymphangiogenesis exists in malignant tumors. By activating the receptor VEGFR-3 on lymphatic endothelial cells, VEGF-C can promote the generation of lymphangiogenesis in tumors, and thus promote lymphatic metastasis. Our research showed that the positive expression rate of VEGF-C is 78.3% (47/60), the expression rate in the lymph node-positive group [90.6%, (29/32)] was significantly higher than that of lymph node-negative group [64.3% (18/28)] ($\chi^2 = 6.10$, $p = 0.01$), which suggests that the positive expression of VEGF-C is related to breast cancer lymphatic metastasis, indicating VEGF-C plays an important role during the generation and metastasis of lymphatics in breast cancer. Our research results also coincide with those of Nakamura *et al*. [12]. There was no difference on VEGF-C expression between invasive ductal carcinoma and other pathological breast cancer types and between each TNM staging, which suggests that VEGF-C expression is not related to breast cancer size and tumor type.

Survivin is a member of inhibitors of the apoptosis protein family, and participates in controlling apoptosis and cell division with dual functions of inhibiting apoptosis and regulating cell proliferation [13]. The expression of survivin has high specificity; there is no expression or low expression in normal tissue, but highly specific expression in tumor tissue, related to the loss of apoptosis of tumor cells and the generation of tumors. In addition, the expression of survivin is always positively related to the grade of malignancy of breast tumors and poor prognosis [14]. A previous study by Tan *et al*. [15] showed that survivin can effectively maintain micro blood vessel structure, promote VEGF protection effects on endothelial cells, and resist apoptosis of endothelial cells induced by chemotherapy drugs.

In all studied 60 cases of breast cancer specimens, the positive expression rate of survivin was 85.0% (51/60), and the expression rate of the lymph node-positive group [96.9% (31/32)] was significantly higher than that of the lymph node-negative group [71. 4% (20/28)] ($\chi^2 = 5.72$, $p = 0.02$), suggesting that survivin may play an important role during the generation and development of breast cancer by inhibiting apoptosis of cancer cells. In addition, we found that the expression rate of survivin in breast cancer tissue with lymphatic metastasis was significantly higher than that of breast cancer tissue without lymphatic metastasis. There was no obvious difference in survivin expression between invasive ductal carcinoma and other pathological breast cancer types nor between each TNM staging, which suggests that VEGF-C expression is not related to breast cancer size and tumor tissue typing. These results indicate survivin may play a promoting role in the activity of breast cancer cell metastasis and invasion. The presence of lymphatic metastasis was an independent index related to breast cancer therapy and prognosis. The expression of survivin mRNA and VEGF-C mRNA in breast invasive ductal carcinoma with lymphatic metastasis was higher than that without lymphatic metastasis. Survivin mRNA and VEGF-C mRNA were positively related to lymphatic metastasis, indicating survivin and VEGF-C jointly promote lymphatic metastasis. Further research results showed a positive relation between the expression of survivin and VEGF-C in breast cancer tissues and that they were closely related to lymphatic metastasis of breast cancer. Therefore, we conclude that elevation of the expression of survivin can upregulate the expression of VEGF-C in breast cancer, induce lymphangiogenesis via VEGFR-3 action on endothelial cells, consequently promoting lymphatic metastasis.

We also conclude that survivin and VEGF-C play a common role during lymphatic metastasis of breast invasive ductal carcinoma. It is likely that survivin inhibits the apoptosis of breast cancer cells, directly or indirectly promotes the expression of VEGF-C protein, and thus promotes lymphatic endothelial cell growth and lymphatic metastasis of breast cancer. However, due to limited number of specimens in this study, it is still not clear how survivin promotes the expression of VEGF-C, which needs to be confirmed by relevant in vivo studies.

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