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Aberrant expression of decoy receptor 3 in human breast cancer: relevance to lymphangiogenesis

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ABSTRACT

Background: Decoy receptor 3 (DcR3), a decoy receptor against Fas ligand belonging to the tumor necrosis factor receptor superfamily, is overexpressed in some forms of cancer. It was recently reported that DcR3 could protect endothelial cells from apoptosis, implying a potential role in the development of vessels, whereas its role in the lymphangiogenesis remains unclear. In the present study, we studied the DcR3 expression and its relationship with the lymphatic microvessel density (LMVD) to investigate if it played a role in the lymph metastasis of human breast cancer.

Materials and methods: Real-time polymerase chain reaction and immunohistochemistry were performed to measure the messenger RNA and protein expression of DcR3 in the breast cancer tissues, noncancerous counterparts, and axillary lymph node from 63 patients. LMVD in these specimens was assessed by counting the D2-40 labeled–microvessels. Furthermore, the correlations between DcR3 expression and LMVD and other clinicopathologic parameters were analyzed.

Results: DcR3 was overexpressed in the breast cancer tissue of 58 patients (92.1%) and was also expressed in vascular endothelial cells and tumor cells in the lymph nodes. LMVD in cancer tissue and lymph nodes were both positively correlated to the aberrant expression of DcR3.

Conclusions: The relevance between DcR3 overexpression and LMVD revealed the existence of possible links between DcR3 and lymphangiogenesis. Based on these findings, it is important to further explore the regulation of lymphangiogenesis operated by the reverse tumor necrosis factor signaling of DcR3.

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1. Introduction

It is well known that resistance to apoptosis is an important feature of tumor cells, which favors proliferation and enhances their immune escape. One of the smartest ways known to inhibit death ligand–induced apoptosis is to switch off the signal *via* decoy receptors (DcRs). DcR3, secreted as a soluble molecule, shows high homology to the tumor necrosis factor (TNF) receptor superfamily [1,2]. It recognizes three TNF-superfamily members, such as Fas ligand (FasL/CD95L/TNFSF6) [1], LIGHT (TNFSF14) [2], and TNF-like molecule 1A (TL1A/VEGI-L/TNFSF15) [3], and competes with their

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respective signaling receptors; through which DcR3 defends against FasL-induced apoptotic cell death and chemotaxis and protects tumor cells from immune surveillance by neutralization of LIGHT-mediated tumor apoptosis and T-cell stimulation [4,5]. Moreover, it neutralizes TL1A, the angiostatic factor in endothelial cells, thus, induces angiogenesis [6,7]. DcR3 overexpression has been observed in various malignant tumors arising from esophagus, stomach, glioma, lung, colon, and rectum [1,8–12] and was found to correlate with local lymph node and systemic metastasis [12]. Because regional lymph node metastasis is one of the most important prognostic parameters for cancer patients, DcR3 was considered to be a useful biomarker in some types of cancers [11,13].

Breast cancer was reported to be the most frequently diagnosed cancer and the leading cause of cancer death among female accounting for almost a quarter of the total cancer cases in the developing countries [14]. Metastasis or recurrence happens in approximately 30% of breast cancer patients despite the advances in early detection and understanding of the molecular bases of tumor biology. Lymphatic metastasis is known as a direct approach in dissemination for breast cancer cells because lymphatic microvessel is constituted of unilaminar and discontinuous basement membrane while lacking tight interendothelial junctions [15,16]. Furthermore, mounting clinical and experimental data suggest that tumor cells facilitated lymphatic metastasis by selecting lymphangiogenic factors and promoting the generation of new lymphatic vessels from preexisting lymphatics [17,18] or lymphatic endothelial progenitors [19].

Recently, it was found that DcR3 could induce a proangiogenic phenotype by deceiving binding to TL1A in human endothelial cells [7], while whether it plays a role in the generation of lymph microvessels or tumor lymphangiogenesis is still unknown. The goal of this study was to investigate whether DcR3 is relevant to lymphangiogenesis and whether this protein could be used as a biomarker predicting lymphatic metastasis in breast cancer. We will use the lymphatic microvessel density (LMVD) in the slides of specimens to assess the number of lymph vessels, detect the gene and protein expression of DcR3 in breast cancer tissue, noncancerous counterparts, and matched lymph node from 63 patients and evaluate their relationship with clinicopathologic parameters.

2. Materials and methods

2.1. Patients and specimens

This study was in compliance with the Helsinki Declaration and approved by the Institutional Research Board at Xiamen University. Written informed consent was obtained from all patients. Institutional Ethics Committee approval for this project was provided before the commencement of the study.

A total of 189 samples were obtained from 63 randomly selected female patients who underwent mastectomy at the First Affiliated Hospital of Xiamen University from February 2009 to February 2011. Each patient contributed three types of specimen, including breast cancer tissue, noncancerous counterparts (located more than 5 cm away from the tumor margins) and one of the suspicious metastatic lymph nodes from the same side of the armpit. Each specimen was microdissected immediately after mastectomy and divided into two parts: one part was snap-frozen in liquid nitrogen and the other was fixed for immunostaining. Patients with metastases breast cancer or who had received preoperative treatment, including radiotherapy or chemotherapy, were excluded. Histologic type, tumor size, and histologic grade of tumors were evaluated by routine pathologic examination. The status of lymph node metastasis, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2/neu) score were evaluated according to the American Joint Committee on Cancer (seventh edition).

2.2. Real-time polymerase chain reaction

Total RNA was extracted from frozen materials by Trizol reagent according to the manufacturer's protocol (Invitrogen). Reverse transcription of total RNA into complementary DNA was conducted using TaKaRa Reverse Transcription Reagents (Takara Bio Inc, Japan) at 37°C for 15 min followed by 85°C for 5 sec. Primers were designed using Primer Premier 5.0 software (Premier, Canada) and synthesized by Invitrogen. DcR3 messenger RNA(mRNA) sequence–specific primers used (GenBank Accession No. NM 032945.2) were the following sequences: forward: 5′-CACGCTGGTTTCTGCTTGGA-3′; and reverse: 5′-CGATGACGGCACGCTCACA-3′. The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a reference: forward: 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse: 5′-GAAGATGGTGATGGGATTTC-3′. Real-time quantitative polymerase chain reaction was performed using the Takara SYBRR Premix Ex Taq II PCR kit (Takara Bio Inc) in a Roche Lightcycler 480 instrument (Roche, Switzerland). Reactions were performed in 10 μL volumes with denaturation at 95°C for 5 sec, annealing at 58°C for 15 sec, and extension at 72°C for 20 sec, more than 40 cycles. To determine the fold change in expression and to normalize DcR3 expression level, triplicates of cycle threshold for the target gene were averaged and divided by the average of the triplicate obtained from glyceraldehyde 3-phosphate dehydrogenase in the same specimen.

2.3. Immunohistochemistry staining and evaluation

Sections of formalin-fixed, paraffin-embedded tissues were deparaffinized, stepwise rehydrated, and the endogenous peroxide was blocked. For D2-40 staining, slides were processed with antigen retrieval by boiling the slides in citrate buffer (pH 6.0) for 1.5 min. For DcR3 staining, slides were boiled in an ethylene diamine tetraacetic acid solution for 20 min. Nonspecific binding was blocked using 10% nonimmune goat serum (Santa Cruz) for 10 min. Sections were then incubated for 120 min at room temperature with anti-DcR3 antibody (clone SC-05; Abcam, UK) at a 1: 350 dilution or with D2-40 antibody (clone D2-40; Abcam) at a 1:40 dilution. After rinsing and incubating in the second antibody, sections were incubated with the EnVision Detection System (Dako, Denmark), counterstained with hematoxylin, dehydrated, and mounted. Negative controls were processed using the same procedure, except that 10% nonimmune mouse–rabbit serum

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