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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.

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

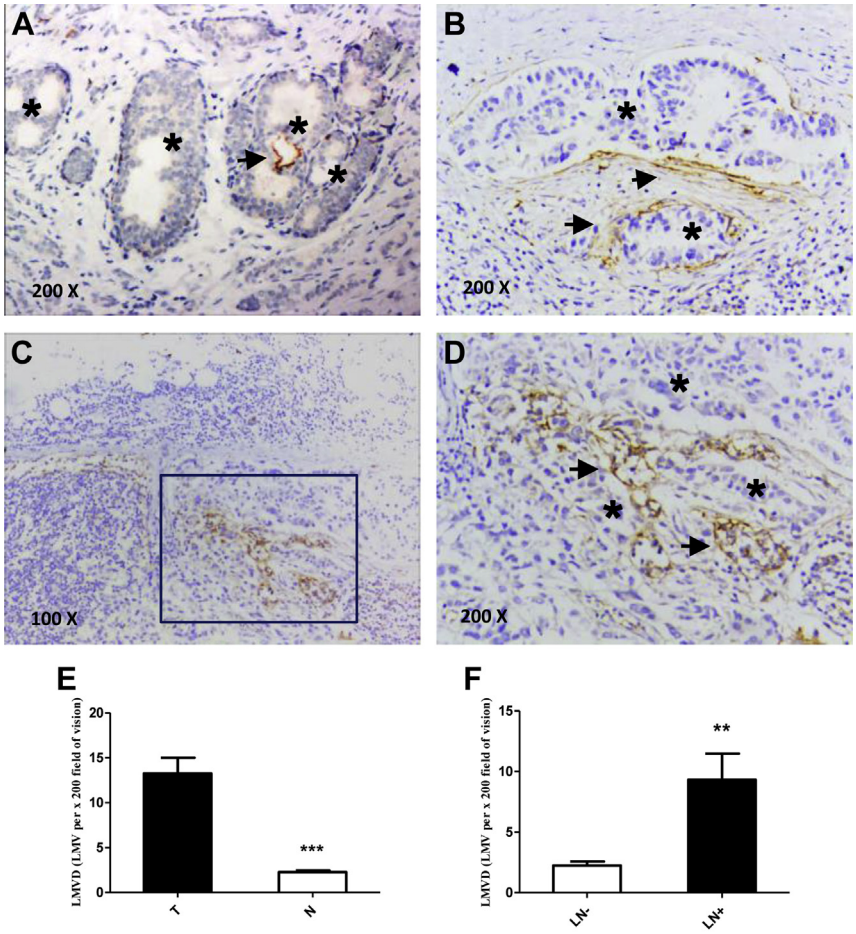

Fig. 1 – LMVD (the number of lymphatic microvessels) is elevated in breast cancer tissue and axillary lymph node with metastasis. (A and B) Representative images of tumor tissue: (A) intratumoral lymphatic vessel is inflated with irregular cell walls; (B) peritumoral lymphatic vessels surrounded tumor sites, which are not stained. (C and D) Representative images of axillary lymph nodes: (C) the box shows the "hot spot"; (D) the magnified "hot spot" in (C). Asterisks represent where tumor cells are. Arrow heads indicate lymphatic microvessels labeled with D2-40 by IHC (EnVision). (E) LMVD increased by approximately 6-fold ($P < 0.001$) in tumor tissue (T), compared with the noncancerous counterparts (N). (F) In the axillary node with metastasis (LN^+), LMVD increased by approximately 3-fold ($P < 0.01$), compared with the node without metastasis (LN^-). (Color version of figure is available online.)

(Santa Cruz) was used in place of the primary antibody. No detectable staining was observed in any of the negative control slides.

Immunohistochemical morphometric analyses were estimated independently by two authors who had no prior knowledge of the patients' clinicopathologic data. LMVD was evaluated by counting the number of immunostained vessels labeled with D2-40 on slides. As reported previously [20], we first identified the area containing the most stained vessels ("hot spot") by scanning the sections at low magnification (×100), then counted the number of positive stained vessels in two high magnification fields (×200) as shown in Figure 1C and D. We defined those vessels as lymphatics if they were lined by a single layer of immunopositive flattened endothelial cells with a vascular lumen without erythrocytes inside [21]. LMVD in tumor sections was determined by averaging the number of total lymphatic vessels in the "hot spot," including intratumoral lymphatic vessels and peritumoral ones. Such representative images were shown in Figure 1A and B. Then the mean LMVD was calculated as the average of four counts (two microscopic fields from each of the two authors). When any discrepancy >10% of the microvessels happened, discordant cases were recounted.

Results of DcR3 immunohistochemistry (IHC) staining were determined according to Wu study [22]. The percentage of cell staining was shown on a graduated percentage (0%–100%): (+) represented that 10%–30% of cells were positive staining; (++) represented that 30%–60% of cells were positive; (+++) represented that 60%–100% of cells were positive. For analysis as a dichotomous variable, sections with <10% of stained cells were classified as negative staining of DcR3.

2.4. *Statistics*

SPSS (version 17.0; SPSS Inc, Chicago) software was used. Data, which were normally distributed, were expressed as

Table 1 – Correlation between DcR3 expression, LMVD, and clinicopathologic parameters.

Clinicopathologic parameters	N	DcR3 protein		P	DcR3 mRNA	P	LMVD (per ×200 field)	P
		–	+					
Age (y)				1.000		0.268		0.691
<60	45	4	41		4.02 ± 1.41		13.67 ± 2.18	
≥60	18	1	17		7.17 ± 2.67		11.00 ± 2.59	
Histologic grade*				0.648		0.555		0.156
I + II	26	3	23		5.72 ± 2.04		9.75 ± 2.15	
III + IV	32	2	30		4.75 ± 2.04		16.30 ± 2.90	
Tumor size				0.609		0.127		0.483
T1	22	1	21		5.44 ± 2.07		8.73 ± 1.09	
T2	35	3	32		2.88 ± 0.55		16.22 ± 2.82	
T3	6	1	5		23.29 ± 16.5		9.00 ± 4.04	
Axillary nodal involved				0.900		0.775		0.408
N0	33	3	30		6.61 ± 2.23		11.05 ± 1.97	
N1	18	1	17		2.54 ± 0.70		15.75 ± 3.81	
N2	9	1	8		3.67 ± 1.88		17.40 ± 6.49	
N3	3	0	3		3.79 ± 3.24		6.00 ± 2.00	
Estrogen receptor status				0.083		0.881		0.287
–	15	3	12		6.23 ± 4.21		14.50 ± 3.18	
+ ~ +++	48	2	46		4.52 ± 1.10		12.45 ± 2.07	
Progesterone receptor				0.055		0.621		0.217
–	13	3	10		8.23 ± 5.35		16.75 ± 4.01	
+ ~ +++	50	2	48		4.19 ± 1.03		12.03 ± 1.02	
HER-2/neu score				0.649		0.318		0.244
–(0–1)	20	2	18		3.81 ± 1.67		11.54 ± 3.40	
+(2–3)	43	3	40		5.46 ± 1.71		13.61 ± 2.02	

"+", "++," and "+++" for DcR3 immunochemistry staining was all grouped together as "+."
* Only indicating the 58 patients with invasive ductal breast cancer.

mean ± standard error of the mean. Statistical evaluation was performed using Spearman correlation test to analyze the rank data and Mann–Whitney *U*-test to differentiate nonparametric means of different groups. Chi-square test, Yates' correction, or Fisher's exact test was used to analyze qualitative independent variables. All statistical tests were two sided. *P* value <0.05 was considered statistically significant.

3. Results

3.1. LMVD assessment

D2-40 positive stainings were primarily detected in the cytoplasm and membrane of lymphatic endothelial cells, whereas not in the tumor cells or the blood vessel endothelial cells (Fig. 1). The average LMVD in the breast cancer tissue from the 63 patients was 13.25 ± 1.75 (range 1.17–42.83) lymphatic microvessels per ×200 field (LMV per ×200 field). The LMVD in the noncancerous counterparts was 2.28 ± 0.18 (range 1.00–5.73) LMV per ×200 field. Thus, the LMVD was significantly different between the cancer tissue and the counterparts (Mann–Whitney test, $P < 0.001$; Fig. 1E). Furthermore, according to that whether the axillary lymph node had metastasis, patients were divided into two groups: metastatic (30 cases) and nonmetastatic (33 cases). We found that the LMVD in the axillary node with metastasis was greater than that without metastasis (Mann–Whitney test, $P = 0.005$; Fig. 1F). Then the relevance between LMVD and the clinicopathologic characteristics was also evaluated. However, the LMVD was not correlated with patients' age, histologic grade, tumor size, axillary lymph node involvement, ER or PR status, or Her-2/neu score ($P > 0.05$, respectively; Table 1).

3.2. DcR3 expression

As shown in Table 2, the DcR3 mRNA expression was elevated approximately four times in the cancer tissue compared with

Table 2 – DcR3 mRNA and protein expression in breast cancer and the noncancerous counterparts.

Tissue type	DcR3 mRNA	U	Staining grades of DcR3				Positive rate (%)	χ^2
			–	+	++	+++		
Tumor specimen	4.910 ± 1.262		5	24	25	9	92.1	
Noncancerous tissue	1.347 ± 0.237	435.0**	29	25	7	2	54.0	31.54***

"+", "++," and "+++" for DcR3 immunochemistry staining are grouped together as "+."
**$P < 0.01$ and ***$P < 0.001$ are considered statistically significant.

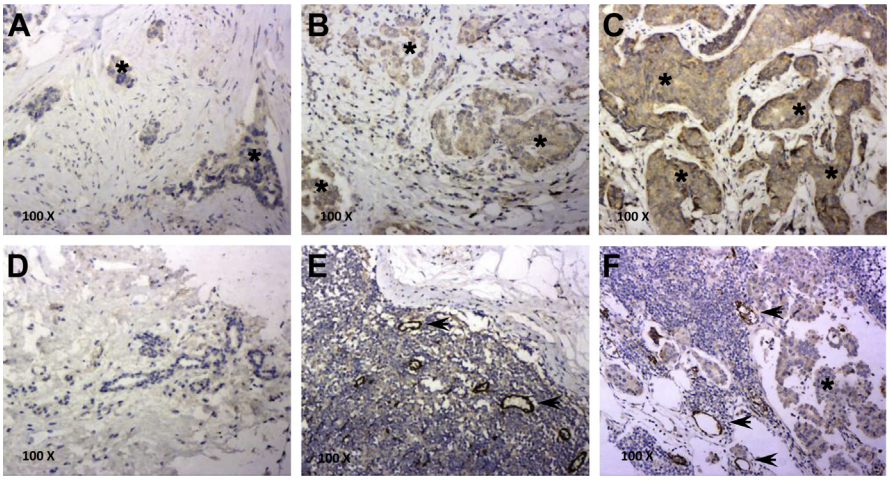

Fig. 2 – Representative IHC staining of DcR3 (EnVision). (A–C) breast cancer tissue, (D) the noncancerous counterparts, (E) lymph node without metastasis, (F) lymph node with metastasis. (D) and (E) negative for DcR3 staining, (A) and (F) + for DcR3 staining, (B) + + for DcR3 staining, (C) + + + for DcR3 staining. Asterisks represent the areas where tumor cells are; Long arrow heads indicate the vessel-like structure, which was detected by DcR3 in lymph node. (Color version of figure is available online.)

the counterparts (Mann–Whitney test, $P < 0.01$). The frequency of DcR3 protein expression in cancer tissue was 92.1% (58 of 63 surgical specimens), which was significantly greater than that in the noncancerous counterparts (54.0%, 34/63; $\chi^2 = 31.54$, $P < 0.001$; Table 2).

As presented in Figure 2A–D, DcR3 positive staining was observed in the tumor nest with faint or even no staining in the surrounding matrix. Besides, in the axillary lymph node, DcR3 was specifically expressed on some endothelial cells, which were arranged like a vessel–lumen structure among lymphatic cells (Fig. 2E and F). When performed IHC staining using either DcR3 or D2-40 antibodies on the serial sections, we found that the endothelial cells which were labeled by DcR3 had negative staining of D2-40 in the matched region of the serial sections (Fig. 3C,D,H, and G), suggesting that the vessel-like structure specifically labeled with DcR3 in lymph node was the blood microvessel, which did not express D2-40. To avoid the interference by blood vessel with the results of DcR3 staining in lymph node, the staining of endothelial cells was excluded when assessing the protein expression level in lymph node.

3.3. *Aberrant expression of DcR3 is associated with LMVD*

As showed in the representative images in Figure 3, the tumor specimen with DcR3 overexpression had higher LMVD. The LMVD was significantly different between breast cancers with and without DcR3 expression (Mann–Whitney test, $P < 0.05$; Fig. 3I); and similarly, the lymph node with DcR3 expression had greater LMVD than that without DcR3 expression (Mann–Whitney test, $P < 0.05$; Fig. 3K). A positive correlation was further established between DcR3 expression and LMVD in both breast cancer tissue and lymph node (Spearman correlation coefficient 0.326 and 0.399, respectively). No significant correlations were observed between DcR3 expression and patients' age, histologic grade, tumor size, axillary node involvement, ER or PR status, or Her-2/neu score, however ($P > 0.05$; Table 1).

4. Discussion

Increasing evidence has shown that DcR3, a decoy receptor belonging to the TNF receptor superfamily, suppressed endothelial cell apoptosis by inhibiting the TL1A-death receptor 3 interaction [7,23], while whether it also plays a role in the lymphangiogenesis is still unknown. In this study, we demonstrated a positive correlation between DcR3 expression and LMVD, not only in breast cancer tissue but also in lymph node, thus provided a new insight into the relationship between DcR3 and lymphangiogenesis. It is possible that the aberrant expression of DcR3 triggers "reverse signaling" for endothelial cell survival, thus facilitates lymphangiogenesis and further lymphatic metastasis for tumor cells.

LMVD is the measurement of lymphatic microvessel growth in and around a tumor and is frequently used as a clinical indicator of lymphangiogenesis [24]. In the present study, LMVD was elevated in breast cancer tissue compared with its noncancerous counterparts, supporting the opinion that tumor cells foster a favorite microenvironment for growth and metastasis by facilitating lymphangiogenesis. Increased LMVD was associated with increasing incidence of metastasis in breast cancer patients [25]. It is unsurprised that the booming lymphatic microvessels create more opportunities for tumor cells to spread. Previous studies showed that lymphangiogenic growth factors derived from primary tumor–induced lymphangiogenesis in sentinel lymph node before the first arrival of metastatic cells [26], suggesting that tumor cells prepared the "soil" in lymph node beforehand to

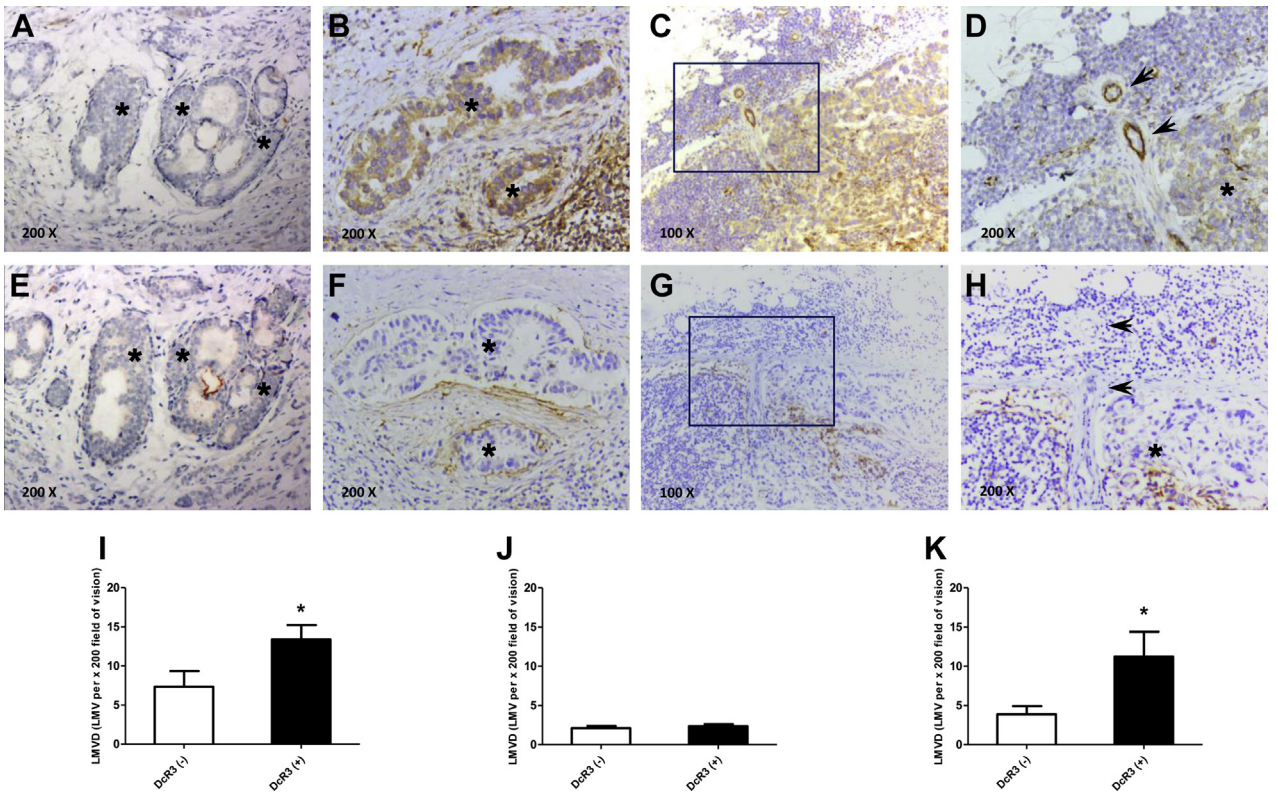

Fig. 3 – DcR3 overexpression is correlated to higher LMVD (EnVision) in tumor tissue and lymph node. (A–D) Representative IHC staining of DcR3, (A) negative for DcR3 staining, (B) and (C) + + for DcR3 staining, (D) + for DcR3 staining; (E–H) representative images of lymphatic microvessels labeled by D2-40. (A and E), (B and F), (C and G), and (D and H) represent the same specimen, respectively; (A), (B), (E), and (F) are breast cancer tissue; (C), (D), (G), and (H) are axillary lymph nodes. Asterisks represent the areas where tumor cells are. (D and H) respectively show the magnified "hot spot" in (C and G). Long arrow heads indicate the vessel-like structure which was labeled by DcR3 in lymph node. (I) and (K) LMVD in the tumor tissue and lymph node with DcR3 positive expression (DcR3 +) are greater than that in the corresponding tissue without DcR3 expression (DcR3–) ($P < 0.05$, respectively). (J) Difference in LMVD in the noncancerous counterparts between these two groups is not statistically significant, however ($P > 0.05$). (Color version of figure is available online.)

render the microenvironment more hospitable for secondary tumor formation [27].

Several clinical studies reported that elevated DcR3 protein expression in tumor specimen was positively correlated with high-grade of tumor or the incidence of lymph node metastasis, including esophageal cancer [28], renal cell cancer [13] and gastric cancer [11]. We found here that the mRNA and protein expression of DcR3 were both elevated in the human breast cancer tissue, and the aberrant expression of DcR3 was closely correlated to the number of lymphatic microvessels, indicating that DcR3 had a close relationship with tumor infiltration and lymphatic metastasis. Thus, it is reasonable to speculate that as a lymphangiogenic factor secreted by tumor cells, DcR3 might be used as a molecular marker predicting the potential of tumor invasion and metastasis. Certainly, intensive study is needed.

Previous studies showed that DcR3 was overexpressed in various malignant tumors [1,8–13,28], whereas rare studies focused on the DcR3 expression pattern in normal human lymph nodes. Bai *et al.* [8] found that the DcR3 mRNA was expressed at a low level in normal lymph node, whereas they did not detect the protein expression further. To the best of our knowledge, our work is the first study that demonstrates the vessel-like structure detected by DcR3 in lymph node is the blood microvessel rather than the lymph microvessel. It is known that TL1A, a ligand for DcR3, is predominantly expressed in endothelial cells and functions as an autocrine cytokine, inhibiting angiogenesis, cell proliferation, and tumor growth [29]. As a soluble molecular, DcR3 fraudulently bound to TL1A and trapped on endothelial cells, which might act as an angiogenic factor *via* blocking the negative regulator TL1A [7]. From this aspect, DcR3 may serve as a biomarker of blood vessel in lymph node.

5. Conclusions

We found here that DcR3 was aberrantly overexpressed in human breast cancer tissue. In addition to tumor cells, the protein expression was also detected specific in blood vessels in the axillary lymph node. LMVD was elevated in the cancer tissue and lymph node with metastasis. The relevance

between DcR3 expression and LMVD reveals that DcR3 is closely related to the lymphangiogenesis. Based on these findings, it is promising for us to further explore the possible regulation of lymphangiogenesis operated by the reverse TNF signaling of DcR3.

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Author contributions: Q.W.W. and Y.H.Z. performed most of the experiments. D.H.C participated in analysis of the IHC data, statistical analyses and discussion of the results. X.H.L. and C.H.L. participated in the IHC staining experiments and specimen collection. Z.M.Z. designed and participated in all stages of the study. All authors read and approved the final manuscript.

Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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