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RESEARCH ARTICLE

Decoy receptor 3 expression in esophageal squamous cell carcinoma: correlation with tumour invasion and metastasis

Gang Xiong[1]*, Hong Guo[2]*,Xiaodong Ge[3], Xueqing Xu[2], Xiaoya Yang[2], Kang Yang[1], Yaoguang Jiang[4]**, Yun Bai[2]**

[1]Dr. Gang Xiong, Prof. Kang Yang, Department of Thoracic and Cardiac Surgery, Southwest Hospital, Third Military Medical University, Chongqing 400038, P. R. China, [2]Dr. Hong Guo, Dr. Xueqing Xu, Dr. Xiaoya Yang, Prof. Yun Bai, Department of Medical Genetics, College of Basic Medical Sciences, Third Military Medical University, Chongqing 400038, P. R. China, [3]Dr. Xiaodong Ge, Department of Pathology, Southwest Hospital, Third Military Medical University, Chongqing 400038, P. R. China, and [4]Prof. Yaoguang Jiang, Department of Thoracic Surgery, Daping Hospital, Third Military Medical University, Chongqing 400042, P. R. China

Abstract

Objective: Decoy receptor 3 (DcR3) is a soluble receptor, which can bind to and inactivate the apoptosis-inducing ligands. We studied a possible association between DcR3 expression and clinicopathologic features in patients with esophageal squamous cell carcinoma (ESCC).

Methods: The mRNA expression of DcR3 was examined by RT-PCR in 109 primary ESCC patients. For the 52 pairs of DcR3 positive tissues, the protein expression was determined by immunohistochemistry.

Results: There was a strong correlation among DcR3 mRNA expression and tumor invasion (P = 0.01) and lymph node metastasis (P = 0.036). We also found that there was a correlation between DcR3 overexpression with lymph node metastasis (P = 0.014) in 52 pairs of DCR3 mRNA positive tissues.

Conclusions: Our finding suggested that the overexpression of DcR3 is significantly related with ESCC clinical staging. DcR3 might be a candidate as a tumor specific biomarker for ESCC.

Keywords: *Decoy receptor 3 (DcR3); esophageal squamous cell carcinoma (ESCC); metastasis; invasion*

Introduction

Esophageal carcinoma is regarded as the eighth most common malignant cancer around the world (Kamangar et al. 2006). Esophageal squamous cell carcinoma (ESCC) is the most common pathologic type in the developing nations, particularly in China (Brown et al. 2001, Pickens et al. 2003, Stoner et al. 2001). Despite many improvements in its detection, surgical resection and adjuvant therapy, the overall survival rate of esophageal cancer remains lower than other solid tumors (Enzinger et al. 2003, Jemal et al. 2008). The 2-year and 5-year survival rate of esophageal cancer is 35%–42% and 15%–24%, respectively (Enzinger et al. 2003). Currently, the pathogenesis of ESCC remains unclear and no biologic markers can be routinely recommended for determination of prognosis.

Decoy receptor 3 (DcR3), which is a recently discovered member of the tumor necrosis factor receptor superfamily, binds to three ligands——FasL (CD95L), LIGHT and TNF-like molecule 1A (TL1A) (Pitti et al. 1998, Yu et al. 1999, Migone et al. 2002). DcR3 is a secreted protein, lacking transmembrane and intracellular regions in its peptide (Zhang et al. 2001). So DcR3 can interfere with

The first two authors contributed equally to this work.

Address for Correspondence: Yun Bai, Phone: +86 23 68752258; Fax: +86 23 68752224; E-mail: baiyungene@gmail.com. Or Yaoguang Jiang, Phone: +86 23 68757261; Fax: +86 23 68816736, E-mail: zlxg@sina.com.cn.

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the binding of these ligands to their respective receptors, i.e. FasL with Fas, LIGHT with HVEM and LTβR and TL1A with DR3 (Pitti et al. 1998, Migone et al. 2002, Zhang et al. 2001, Bai et al. 2000). These receptors are all capable of inducing cell apoptosis, so DcR3 can prevent cell death and may play an important role in the pathogenesis of many malignancies. In addition, it also can inhibit LIGHT triggered T cell costimulation via HVEM and modulate immune cell interaction by down-regulating functions of macrophages and T cells (Chang et al. 2006, Wan et al. 2003, Chang et al. 2004). All the functions of DcR3 can help tumor cells to gain survival advantage and escape immune surveillance. Some studies reported that DcR3 was overexpressed in many types of malignancies (Pitti et al. 1998, Bai et al. 2000, Ohshima et al. 2000, Tsuji et al. 2003). Recently, Wu *et al*. (Wu et al. 2008) found that gastric cancer patients with high DcR3 expression presented more advanced pN2-3 staging than those with low expression. Moreover, Macher-Goeppinger *et al*. (Macher-Goeppinger et al. 2008) reported that high DcR3 expression was associated with the risk of death in the patients with renal cell carcinomas (RCCs) and serum DcR3 levels were significantly elevated in patients with high-stage compared to controls. Accordingly, the expression of DcR3 has been suggested as a potential tumor marker for the early detection or determination of prognosis of RCC, gastric carcinoma and ovarian cancer (Wu et al. 2008, Macher-Goeppinger et al. 2008, Simon et al. 2007).

However, the study on DcR3 expression in ESCC is rarely to date. In this study, we investigated the relationship between DcR3 expression and clinicopathologic features in a consecutive series of 109 primary ESCC patients.

Materials and methods

Patients and samples

Tissue samples were collected from 109 patients who underwent curative transthoracic esophagectomy for primary ESCC. The consecutive series of cases were recruited between October 2006 and February 2008 at the Southwest Hospital, Chongqing, China. There were 84 men and 25 women with a mean age of 57.9 years (range, 38–79 years). The pathological stage of ESCC was classified according to the TNM staging system of carcinoma of the esophagus from the American Joint Committee on Cancer (AJCC)/ International Union Against Cancer (UICC).[20] Paired tumor and tumor-adjacent normal tissue samples of 5 mm in diameter were obtained at the time of surgical resection, frozen in liquid nitrogen immediately and then stored at -80°C until use. Tumor-adjacent normal tissues were located at esophagus mucosa at least 5 centimeter away from tumor. The tumor and tumor-adjacent normal tissues were confirmed by hisopathologic examination. Histopathologic classifications of the cancerous tissues were based on the postoperative histopathologic examination. Informed consent was obtained from the subjects and the study was performed with the approval of the ethical committee of Third Military Medical University.

Semiquantitative RT-PCR

Total RNAs were extracted from tumor and corresponding normal tissues for 109 ESCC patients using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), in conformity with the manufacturer's instructions. One microgram (μg) of RNA was treated with DNase and reverse transcribed in a 20-μL reaction volume into first-strand cDNA. The first-strand cDNA (1 μL) was then used for PCR. The primer pair composed of a forward primer, 5'-TTCTGCTTGGAGCACGCATCG-3', and a reverse primer, 5'-TGTCCTGGAAAGCCACAAAGTCG-3', with PCR product of 298 bp. In all experiments, β-actin as a constitutively expressed gene was used as an internal positive control. RT-PCR for β-actin was performed by using the primers 5'-CATCATGAAGTGTGACGTGGA-3' and 5'-ACATCTGCTGGAAGGTGGAC-3', respectively, with expected product size of 224 bp. Conditions for PCR were: an initial denaturation step for 5 minutes at 95°C, then 20 seconds at 95°C, 20 seconds at 65°C (DcR3) or 60°C (β-actin), followed by a step for 20 seconds at 72°C for 30 cycles (DcR3) or 26 cycles (β-actin), then a final extension for 5 min at 72°C. PCR products were run on 2% agarose gel and visualised by ethidium bromide staining and UV transillumination. The signal intensity of PCR products was analyzed by a gel documentation system (GEL DOC 2000, Bio-rad, Milan, Italy). β-actin mRNA expression levels were used to normalise the mRNA levels of DcR3, calculating the ratios as relative mRNA levels.

Immunohistochemistry

The immunohistochemical analysis was performed on formalinfixed paraffin-embedded tissue sections from 52 DcR3 mRNA-positive ESCC patients. The sections were deparaffinized in multiple fresh xylene, rehydrated in descending ethanol changes and soaked with 3% H_2O_2 to eliminate endogenous peroxidase activity. They were then submerged in 0.01 M citrate buffer and boiled at 94–98 °C in a microwave oven. Next, they were rinsed with PBS with 0.3% Tween-20 and blocked with 10% normal goat serum. Subsequently, the blocker was removed and the sections were incubated overnight at 4 °C with the anti-DcR3 polyclonal antibody (3H5/ DcR3, Santa Cruz Biotechnology, CA, USA) at a 1:100 dilution. After washing, the slides were incubated with

a secondary biotinylated goat-anti-mouse antibody at a 1:100 dilution. DcR3 signal was revealed in brown by streptavidin-conjugated peroxidase using liquid diaminobenzidine (DAB) as a substrate according to instructions from the Histostain™- Plus kits (SP-9002, ZSGB-BIO, Beijing, P.R.China) and showed cytoplasmic staining. Finally, the slides were sealed with Crystal/Mount TM Mounting Media (BI-M02, Sigma, LA, USA). As negative controls, the primary antibodies were substituted with non-immune normal mouse IgGs.

We counted 100 cells from 5 representative areas in each lesion and normal esophageal mucosa. For the immunohistochemical assessment of DcR3 expression, the staining intensity and quantity of immunoreactive tumor cells was calculated based on the following scoring system: the intensity ranged from 0 = negative, 1 = low, 2 = medium to 3 = high; the quantity comprised 0 = no expression or positive in less than 5%, 1 = positive in 5% to 25%, 2 = positive in 25% to 50%, 3 = positive in 50% to 70%, and 4 = positive in more than 70%. The final immunohistochemical score (IHS; ranging from 0 to 12) is obtained by sum of the intensity score and the quantity score. Overexpression of DcR3 was defined as an IHS ≥ 6. The slides were independently analyzed by two authors who blinded to tissue annotations and patient demographic characteristics.

Statistical Analysis

All statistical calculations were carried out using SPSS 13.0 for Windows (Chicago, IL, USA). The semi-quantitative values of DcR3 expression in different groups were analyzed by *t*-test. The χ^2 test and Fisher exact test were used to assess the correlation between clinicopathologic features and DcR3 expression. P value of less than 0.05 was considered statistically significant.

Results

Expression of DcR3 Gene Assessed by Semiquantitative RT-PCR

The specimens were considered as DcR3 positive if visible bands could be observed after RT-PCR. Representative results of the RT-PCR analysis were shown in Figure 1. The DcR3 mRNA expression was detected in 80 of 109 (73.4%) tumor tissues and in 52 of 109 (47.7%) corresponding normal tissues ($P < 0.01$). Furthermore, the DcR3 expression in the 52 normal tissues (0.547 ± 0.135) was lower than that of the matched tumor tissues (0.907 ± 0.125) ($P < 0.01$) (Figure 2). The relationship between the DcR3 mRNA expression and the clinicopathologic features was analyzed. The correlation of DcR3 expression and gender, tumor location and differentiation status, revealed no significant difference between DcR3-positive and DcR3-negative ESCC patients. DcR3 mRNA-positive expression in primary tumors displayed a statistically significant correlation with the invasion extent of the tumor (pT stage) ($P = 0.01$) and regional lymph node metastasis (pN stage) ($P = 0.036$) (shown in Table 1).

DcR3 Overexpression Assessed by Immunohistochemistry

Overexpression of DcR3 (defined as an IHS ≥ 6) was observed in 15 of 52 patients (28.8%). The IHC scoring of DcR3 protein expression in paraffin-embedded sections from ESCC tumor tissues were shown in Figure 3. Using the χ^2 tests, overexpression of DcR3 were not significantly associated with gender, tumor location and differentiation status. No significant association was obtained in the subgroup of patients with the invasion extent of the tumor (pT stages), possibly due to the limited number of

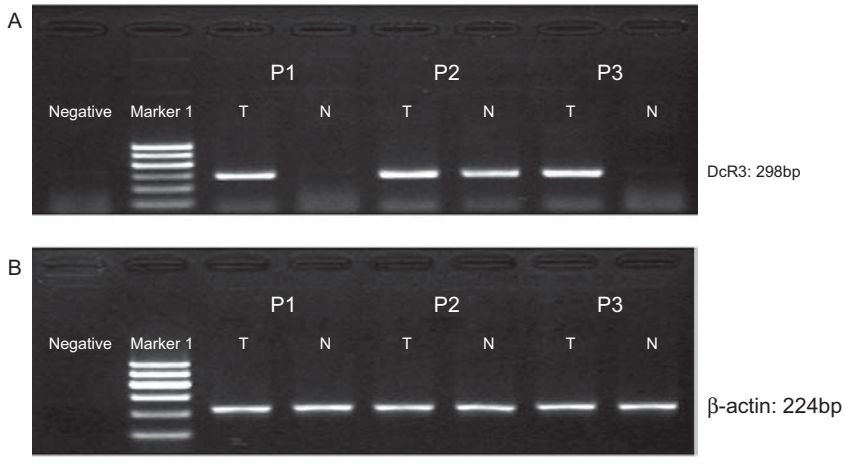

Figure 1. Amplification of DcR3 gene in representative primary lesions of ESCC tissues (lanes T) and corresponding normal mucosa tissues far away from tumor (lanes N). A, Agarose gel electrophoresis of RT-PCR amplified DcR3 cDNA; B, Agarose gel electrophoresis of RT-PCR amplified β-actin cDNA; DNA Marker 1: 600 /500 /400 /300 /200 /100 bp.

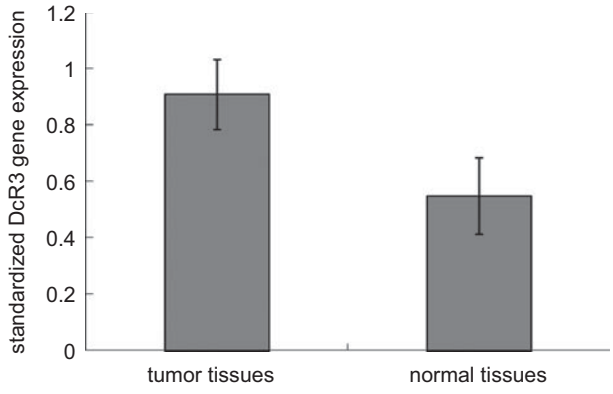

Figure 2. Correlation on standardized DcR3 gene expression ratio in ESCC tissues and corresponding normal mucosa tissues.

patients (n = 52). The correlations of DcR3 overexpression and regional lymph node metastasis, revealed significant difference in ESCC patients. When stratified by pN stages, patients with pN1 disease (n = 18) had much higher ratio of DcR3 overexpression than those with pN0 disease (n = 34) (50% vs. 17.6%, P = 0.014). (shown in Table 2).

Discussion

In this study, we detected the mRNA and protein expression levels of DcR3 in different tumor and tumour-adjacent normal tissues. We found both mRNA and protein expression levels of DcR3 increased in tumor group compared with tumour-adjacent group. Besides, overexpression of DcR3 is correlated with patient's lymph node metastasis.

In tumor group, positive ratio of DcR3 mRNA was significantly higher than the tumour-adjacent group (73.4% vs. 47.7%, P < 0.05). And the relative quantity of DcR3 mRNA was also much higher (P < 0.01). Some reports found the expression of DcR3 mRNA was only detected in tumor group (Pitti et al. 1998, Bai et al. 2000), which was different from our findings. As shown above, we found DcR3 mRNA can be detected in some tumor-adjacent samples. In consistent with Wu's report (Wu et al. 1998), they showed that the positive expression ratio of DcR3 mRNA in gastric cancer was 54.8%, while 24.2% in control. The reson may lie in the heterogeneity of different cancer cells and different biological roles. Based on our results, we think it is worth to further investigate if there is atypical hyperplasia or precancerous lesion of the esophageal cancer around the oncogenesis region in ESCC.

So far, DcR3 has been shown to be related with many different kinds of cancer. Our work in ESCC demonstrated that the expression of DcR3 mRNA was not related with the age, gender, areas of involvement or the tissue differential degree. In contrast, it was highly related with cancer invasion (pT stage) and the metastatic status of

Table 1. Correlation on DcR3 mRNA expression with clinicopathologic features in 109 ESCC patients.

Features	No. of Case (N)	DcR3-Positive Cases[N (%)]	*P*-value
Age at surgery (yr)			0.186
< 60	60	41(68.3)	
≥ 60	49	39(79.6)	
Gender			0.737
Male	84	61(72.6)	
Female	25	19(76.0)	
Tumor location			0.365
Upper	8	7(87.5)	
Middle	65	50(76.9)	
Low	32	20(62.5)	
Multiple	4	3(75.0)	
Depth of invasion			0.010
pT1	6	3(50.0)	
pT2	37	21(52.6)	
pT3	61	52(86.2)	
pT4	5	4(80.0)	
Regional lymph node metastasis			0.036
pN0	82	56(68.3)	
pN1	27	24(88.9)	
Histological type			0.225
Well differentiated	63	47(74.6)	
Moderately differentiated	41	29(70.7)	
Poorly differentiated	5	5(100)	

regional lymph nodes (pN stage). Meanwhile, protein expression of DcR3 in the patients with regional lymph nodes metastasis (pN1) were much higher than that in the patients without regional lymph nodes metastasizes (pN0) (P < 0.05), shown in immunohistochemical staining. When comparing the invasion of cancer, the DcR3 protein was higher in pT3/T4 than in pT1/T2 (35.1% vs. 13.3%, but it was not significant (P = 0.116)). Our data suggested the overexpression of DcR3 indicate a high possibility of regional lymph nodes metastasis and a later clinical stage. However, it's not confirmed that the overexpression of DcR3 indicated a later T stage and rapid prognosis.

The overexpression of DcR3 was reported to be related with the tumor size, invasion level, regional lymph nodes metastasis and the differentiation degree of cancer cells (Takahama et al. 2002, Wu et al. 2003, Roth et al. 2001). Although the mechanism is not very clear, the expression of DcR3 may promote the malignant phenotype of cancer cells through two pathways. First, the expression of DcR3 can inhibit the anti-tumor effect of its three ligands (FasL, LIGHT and TLIA) (Pitti et al. 1998, Migone et al. 2002, Zhang et al. 2001, Bai et al. 2000). Second, DcR3 can inhibit the maturation and differentiation of DCs (Hsu et al. 2002) and suppress the chemotaxis to T cells (Wan et al. 2003, Shi et al. 2003), which can interfere the

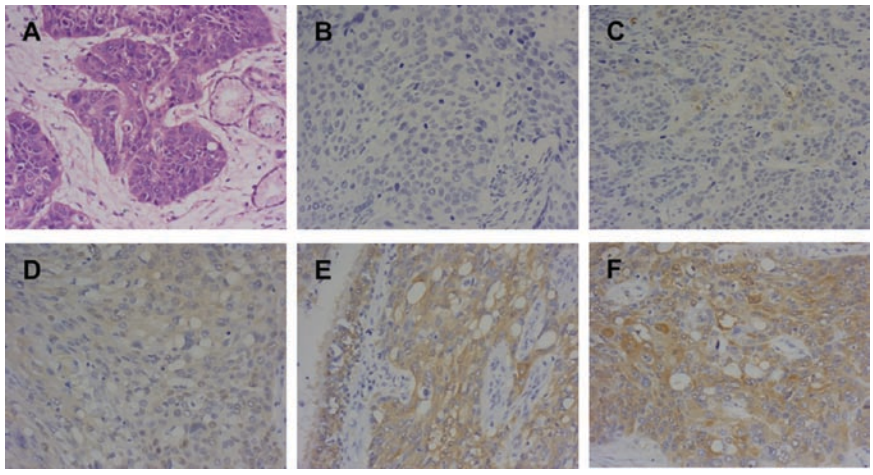

Figure 3. IHC scoring of DcR3 protein expression in paraffin-embedded sections from ESCC tissues (original magnification,×200). (A: H&E staining, which shows the tumor cells in ESCC tissues. B-F: IHC scoring of DcR3 protein expression in different cases. Blue spots shows the cell nucleus and the brown pieces shows the expression of DcR3 in the cytoplasm and intercellular substance. The IHS scores were as follows: B = 0, no expression; C = 3, low expression; D = 6, medium expression; E = 9, overexpression; F = 12, extremely overexpression.)

Table 2. Comparison of DcR3 expression levels and clinicopathologic features in 52 ESCC patients.

		IHS		
Features	No. of Cases(n)	<6 (score value) n(%)	≥6 (score value) n(%)	*p*-value
Age at surgery (yr)				0.358
< 60	26	20(76.9)	6(23.1)	
≥ 60	26	17(65.4)	9(34.6)	
Gender				0.473
Male	38	26(68.4)	12(31.6)	
Female	14	11(78.6)	3(21.4)	
Depth of invasion				0.116
pT1/ T2	15	13(86.7)	2(13.3)	
pT3/ T4	37	24(64.9)	13(35.1)	
Regional lymph node metastasis				0. 014
pN0	34	28(82.4)	6(17.6)	
pN1	18	9(50.0)	9(50.0)	
Histological type				0.100
Well differentiated	30	23(76.7)	7(23.3)	
Moderately differentiated	18	13(72.2)	5(27.8)	
Poorly differentiated	4	1(25.0)	3(75.0)	

immune surveillance of host to tumor cells and facilitate the immunologic escape.

As a secretory protein, DcR3 may be a good candidate as a tumor specific biomarker. It is easy to detect DcR3 expression level in serum. Reports shown that detecting the DcR3 level in the serum was a good way to do early diagnosis, clinical classification, prognosis estimation and treatment determination of patients (Wu et al. 2008, Macher-Goeppinger et al. 2008, Simon et al. 2007). Study the mRNA and protein expression levels in ESCC patients in large scale are important to verify and establish DcR3 as a tumor specific marker, providing evidence for the early diagnosis and clinical treatment.

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Declaration of interest

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