Original Article

Significance of Sera Decoy Receptor 3 in Gastric Carcinomas

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ABSTRACT **Introduction and objective:** Decoy receptor 3 (DcR3), a member of the tumor necrosis factor receptor superfamily, is a soluble receptor that binds to the TNF family including Fas ligand, LIGHT and TL1A. DcR3 is expressed in various malignant tumor cells and competitively inhibits the TNF family members. The objective of the present study was to investigate the level and significance of DcR3 in sera of gastric carcinoma (GC) patients. **Material and methods:** The level of DcR3 in sera of GCs (n=34) and healthy controls (n=28) was investigated by ELISA. Statistic analysis was conducted to figure out the correlation between the sera DcR3 levels and clinicopathological features. Five tissue samples from the sera DcR3 positive GC patients were selected to perform immunohistochemistry to confirm the DcR3 protein expression in tumor tissues. **Results:** Sera DcR3 level in GCs was 194.87±98.38pg/ml with the positive rate 73.53%（25/34）, significantly higher than that in the healthy controls (96.69±16.05pg/ml, positive rate 3.57% , P<0.01). In GCs, serum DcR3 values were significantly related to tumor invasion (TNMT)，lymph node metastasis (TNMN) , systemic metastasis (TNMM) and clinical TNM stage (P<0.05). However, the expression of sera DcR3 was not associated with patients' age, gender and differentiation grade. Positive DcR3 expression was observed in all the 5 cases for immunohistochemical staining.

Conclusions: DcR3 is over-expressed in the sera of human GCs and positively correlated with development and metastasis of GCs. DcR3 might serve as an important parameter indicator in diagnosing and predicating the biological behavior of GC patients.

Key Words: DcR3; gastric carcinoma (GC); ELISA; immunohistochemistry (IHC)

Introduction

Decoy receptor 3 (DcR3)/TR6/M68 is a soluble decoy receptor in the tumor necrosis factor receptor superfamily, which consists of 4 members: DcR1, DcR2, DcR3 and osteoprotegerin(1). The gene amplification as well as the over-expression of DcR3 messenger RNA (mRNA) and protein has been demonstrated in lymphoma, glioblastoma, also cancer of the lung, brain, pancreas, liver, renal, esophagus, colon and gastrointestinal tract (2-19). In addition, the DcR3 mRNA level was discovered to be associated with lymph node metastasis and pathological stages using RT-PCR, Northern Blot and in situ hybridization in gastric carcinomas (GCs) (8,11-13). Our group previously also found that DcR3 protein level was related to GCs differentiation, tumor-node-metastasis (TNM) stages, lymph node metastasis and systemic metastasis using Immunohistochemistry (IHC)(19). Moreover, the serum level of DcR3 was significantly elevated in 56.2% of a variety of tumors, including cancers of the digestive system, glioma, renal, thyroid, lung, breast and ovary (6, 11, 14, 20-21). All the evidence suggests that DcR3 could not only become a factor responsible for the early diagnosis and tumor progression, but also serve as an effecter molecule to modulate pathological and physiological functions. The aim of the present study was to investigate the level of DcR3 in the sera of GC patients by enzyme-linked immunosorbent assay (ELISA) analysis. Association of DcR3 sera level with the clinicopathological parameters was also studied.

Materials and methods

Clinical Materials

The studied population consisted of 34 patients who were

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diagnosed with operable GCs from September 2006 to September 2007 in the First Initiated Hospital to Guangxi Medical University, P.R.China. Informed consent was obtained and the study was approved by the institutional review committee. All the guidelines for experimental investigation with human subjects required by the institution were followed. The preoperative peripheral blood was harvested from 34 cases of GCs and 28 cases of healthy blood donors as controls. Patients' demographics were obtained and included age, gender, differentiation grade, depth of tumor invasion(TNMT), lymph node metastasis (TNMN) , distant metastasis (TNMM) and TNM stage (Table 1). The patients were 23 males and 11 females whose ages were ranged from 23 to 77 years (mean, 54.7±13.5 years). Carcinomas were classified according to the histological classification of World Health Organization (WHO) criteria. Of 34 GCs, 1 case was histologically graded as G1, 9 cases as G2, 20 cases as G3 and 4 cases as G4, respectively. The clinical tumor-node-metastasis (TNM) stage was processed according to the pathology TNM classification of WHO criteria. For "T" category, there were 2 cases in T1, 19 cases in T2, 4 cases in T3 and 9 cases in T4, respectively. For "N" category, 12 patients were divided into N0, 10 were N1, 9 were N2 and 3 were N3, respectively. Whereas 16 cases were M0 and 18 cases were M1 for "M" category. Thus, of 34 GC s, 8 were stage I, 5 stage II, 3 stage III and 18 stage IV with the TNM stages. The 34 GC patients had never received any radiation therapy or chemotherapy.

ELISA

The human DcR3 ELISA kit (Bender Med Systems, Vienna, Austria) was applied to measure DcR3 level in serum according to the manufacturer's protocol. Briefly, 100 µl of each sample were incubated in duplicate in microplates coated with anti-DcR3 monoclonal antibody for 2h. Following incubation, biotin-conjugate anti-DcR3 monoclonal antibody was added and incubated as a primary antibody for 2h. After the microplate had been washed, streptavidin-horseradish peroxidase (HRP) was added and incubated for 1h. After washing off any unbound streptavidin-HRP, tetramethylbenzidine was added as a substrate and the absorbance was measured at 450 nm after 20min in a microplate reader (BioTek Instruments, Burlington, VT, USA). The absorbance of each sample was plotted against a standard curve produced by serial dilutions of recombinant human DcR3-Fc in duplicate. The concentrations of DcR3 in serum were calculated by logarithmic analysis. All samples with an absorbance of less than zero according to the standard curve were discarded from the final analysis. The level higher than 122.22pg/ml was regarded as positive according to the DcR3 average level in the normal control group in our study.

Immunohistochemistry (IHC)

Five tissue samples from the sera DcR3 positive GC patients were selected to perform immunohistochemistry to confirm the DcR3 protein expression in tumor tissues. The procedure of IHC was done as described previously (18, 19). One hundred cells from 5 representative areas in each lesion were counted. The staining results were evaluated according to the immunodetection of stain intensity and amounts of positive cells by two pathologists, who discussed each case until they reached a consensus. Stain intensity was up to the standard of the relative stain intensity of most cells. The degree of staining was subdivided as follows: the stain intensity could be from 0 to 3 (0, no staining; 1, focal or fine granular, weak staining; 2, linear or cluster, strong staining; and 3, diffuse, intense staining); and the positive cells in the observed gastric mucosa ranged from 0 to 3 in percentage (0, no staining; 1, < 30%; 2, 30%-70%; and 3, > 70%). The samples were scored by their summation: 0-1 (-); 2-3 (+); 4 (++); 5-6 (+++). Any staining score ≥ 2 (+) was considered as positive expression.

Statistical analysis

Statistical significance was evaluated using SPSS 17.0 software for Windows (Munich, Germany), difference of $P<0.05$ was considered statistically significant.

Results

Sera DcR3 level in GCs was 194.87±98.38pg/ml with the positive rate 73.53%(25/34), significantly higher than the healthy controls (96.69±16.05pg/ml, positive rate 3.57% , $P<0.01$) (Figure1A, Table 1). In GCs, sera DcR3 values were significantly related to tumor invasion (TNMT) ,lymph node metastasis (TNMN) , distant metastasis (TNMM) and TNM stages($P<0.05$, Figure 1B, 1C, 1D, 1E). However, the expression of sera DcR3 was not associated with patients' age, gender and differentiation grades (Table 1). Positive immunostaining for DcR3 was observed in the cytoplasm of cancer cells and positive DcR3 expression was found in all the 5 cases for immunohistochemical staining (2 cases +, 3 cases ++, Figure 1F).

Discussion

Human DcR3, mostly being over-expressed in many different classes of tumor cells (1), can combine with the TNF family members FasL, LIGHT and TL1A (1,2), thus to block their

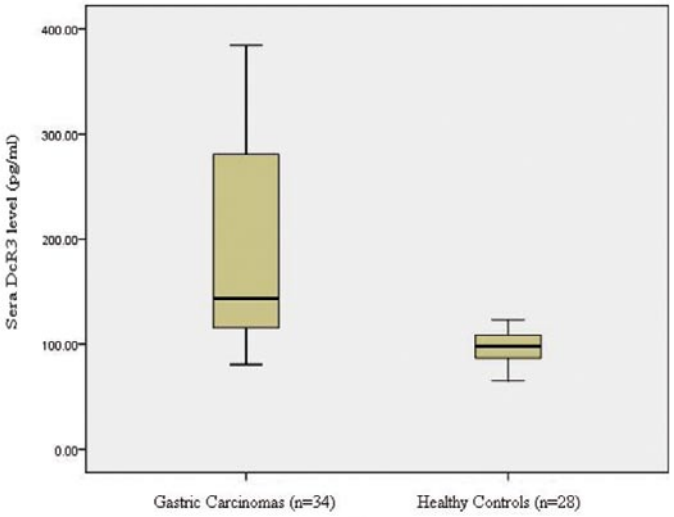

Figure 1A

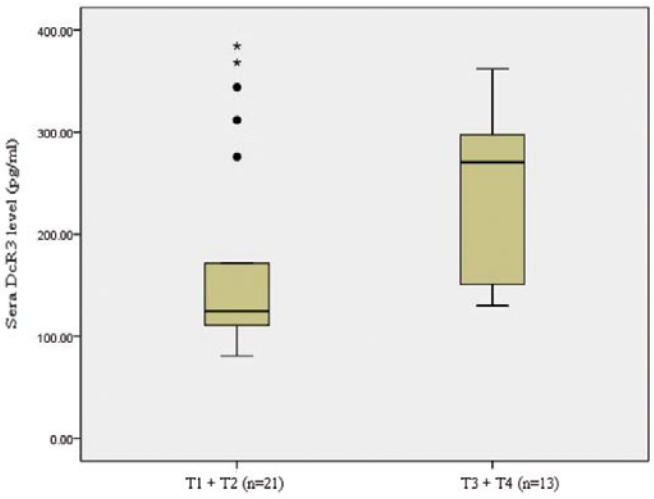

Figure 1B

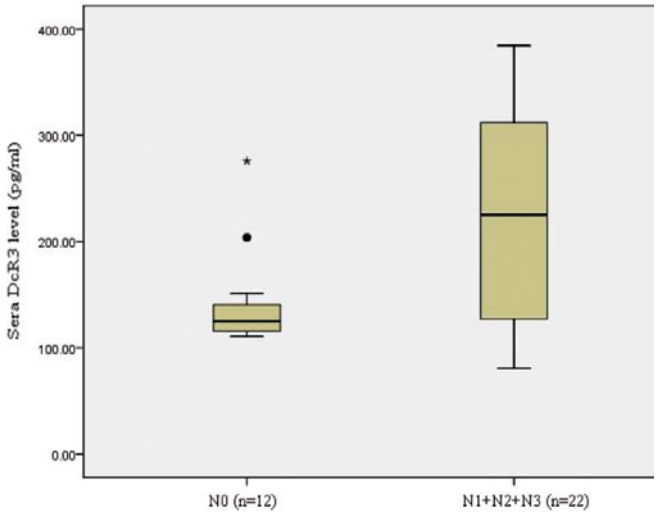

Figure 1C

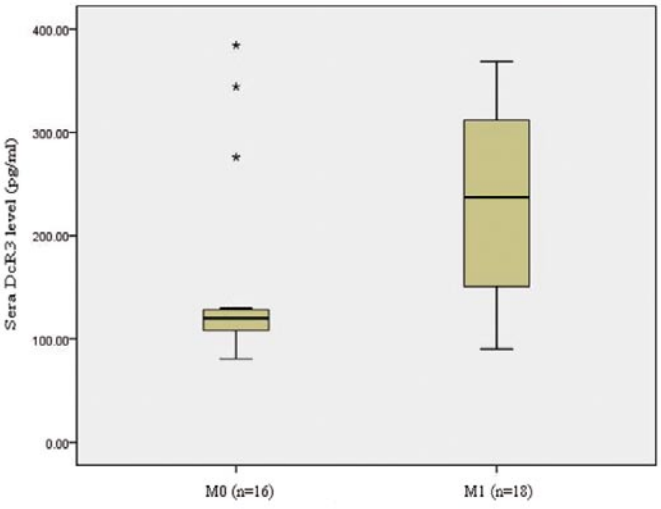

Figure 1D

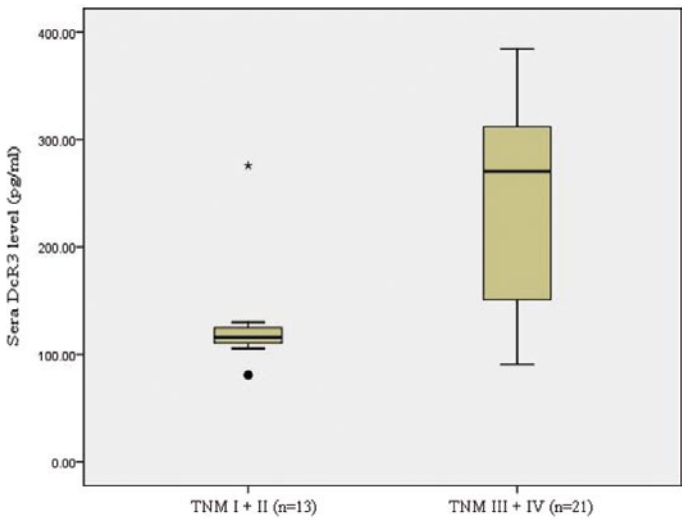

Figure 1E

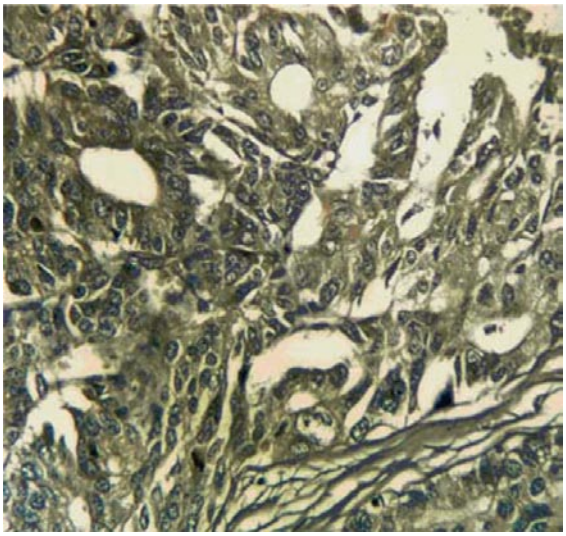

Figure 1F

Figure1: Comparison between the sera DcR3 level and clinicopathological features of gastric carcinomas (GCs).

Patient sera were collected before operation and sera DcR3 was assessed by ELISA in duplicated samples.Figures of Boxplot were drawn using SPSS 17.0. Median DcR3 level of each group is indicated by the solid line. The number of patients tested (n) is shown. Data of sera DcR3 levels in different group were shown in Table 1. Comparison of sera DcR3 level between GCs and healthy controls (Figure 1A). Comparison of tumor invasion depth (Figure 1B), lymph node metastasis (Figure 1C), symantic metastasis (Figure 1D) and TNM stage (Figure 1E). Figure 1F: Positive immunostaining for DcR3 was observed in the cytoplasm of gastric cancer cells with immunohistochemistry. This case was determined as ++ (DAB X400).

Table 1 Relationship between the sera DcR3 levels and clinicopathological parameters of gastric carcinomas (GCs).

Clinicopathological parameters		n	Sera DcR3 level (pg/ml)	t	P
Different diseases	GC	34	Sera DcR3 level (pg/ml)		
	Normal controls	28	96.69±16.05	5.728	0.000
Age	≤60	23	201.75±98.42		
	>60	11	175.58±105.53	0.709	0.483
Gender	Male	23	177.85±89.62		
	Female	11	225.57±116.59	1.317	0.197
Differentiation grade	G1+G2	10	222.57±112.50		
	G3+G4	24	181.09±94.06	1.106	0.277
Invasion depth (TNMT)	T1+T2	21	166.91±101.83		
	T3+T4	13	235.89±83.65	2.085	0.046
lymph node metastasis (TNMN)	N0	12	143.50±48.77		
	N1+N2+N3	22	220.44±110.72	2.407	0.022
distant metastasis (TNMM)	M0	16	150.72±95.03		
	M1	18	231.13±90.53	2.441	0.021
TNM	Ⅰ+Ⅱ	13	121.32±50.10		
	Ⅲ+Ⅳ	21	237.84±97.58	3.858	0.001

interaction with their respective receptors. A study of Takahama et al demonstrated that DcR3 mRNA was over-expressed in 22 (26%) primary GC in 84 gastric carcinomas compared with each noncancerous tissue by Northern blot analysis (8). Our previous study also showed that DcR3 was expressed higher in GC compared with the noncancerous gastric tissues (19). Furthermore, the positive immunostaining rate of DcR3 protein was reproduced with 5 samples in the present study. Our data together with the study of Takahama et al proved the over-expression of DcR3 in GCs, both of mRNA and protein levels, which indicated that DcR3 might be involved in the process of gastric carcinogenesis.

DcR3 might serve as an index to monitor the regression or progression of cancer because its serum level dramatically decreased after the removal of tumors. Wu et al (11) established an ELISA to measure sera DcR3 level in 31 GC patients and found 22 patients (70.9%) were positive. The data in the present study with an ELISA kit from Bender Med Systems was consistent with Wu et al. The positive sera DcR3 rate was detected 73.53%, which was significantly higher than the healthy control. The results may provide better understanding of the GC pathogenesis and suggest its possible role as a biomarker for the early non-invasive diagnoses of GCs.

Our previous study with IHC in the GC tissues showed that the expression of DcR3 directly correlated with the differentiable condition of GCs. The DcR3 positive immunostaining rate in well differentiated GCs was lower than that in poorly differentiated GCs (19). This conclusion differed from Takahama et al, which declared no significant differences between the histological type, differentiation and the DcR3 expression level (8). In the present study, no correlation between the sera DcR3 level and the differentiation grades was detected. We believe that variations in technique, materials and methods may partly explain this

distinction. A study with a larger amount of samples will be further needed.

Our findings of the relationship between the sera DcR3 expression and the clinicopathological parameters also supported the study of Takahama et al (8), Wu et al (12, 13) at mRNA level and our previous IHC work at protein level, which showed that both mRNA and protein DcR3 expression were correlated with GC clinical TNM stages, lymph node metastasis and systemic metastasis. Wu et al (11) also analyzed the relationship between sera DcR3 level and clinical parameters with ELISA. The authors implied that lymph nodes metastasis, distant systemic metastasis and TNM stages in GCs were correlated to sera DcR3 level, which is accordant with our finding. However, Wu et al (11) found the no significant difference of sera DcR3 level between T1+T2 and T3+T4, which was contradictory with our data. We observed that the sera DcR3 level was significantly higher in T3+T4 than that in T1+T2, which indicated that the depth of tumor invasion into the gastric wall is related with sera DcR3 level. Hence, the high coincidental level of sera DcR3 might be an important event to enhance GCs and a useful biomarker to assess risk for the development and aggressiveness of GCs.

In conclusion, evidence was shown in our study that the high sera DcR3 level is positively associated with the development of gastric lesions in our present study. Therefore, sera DcR3 might be used as a valuable biomarker in the diagnosis and prognosis of GCs, and also serve as a potential therapeutic target for gene therapy for GCs in the future.

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