

RESEARCH COMMUNICATION

Clinical Significance of Expression and Amplification of the DcR3 Gene in Pancreatic Carcinomas

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Abstract

This study aimed to investigate the clinical significance of expression and amplification of decoy receptor 3 (DcR3) in pancreatic carcinomas (PC). mRNA expression was detected by PQ-PCR, and amplification was determined. DcR3 protein expression was detected by immunohistochemistry and ELISA. Correlations between DcR3 expression and clinical pathological factors were analyzed. The relative amount of DcR3 in PC tissues and non-cancerous tissues showed a statistically significant difference, 21 cases displaying more than two fold DcR3 amplification, while no such amplification was found in normal pancreatic tissues. DcR3 positive cell staining was located in the cytoplasm. The positive rate of DcR3 in PC and non-cancerous tissues showed a significant difference. DcR3 mRNA expression was correlated with clinical staging, size of the tumor, lymph node metastasis and histological staging, while protein expression was correlated with clinical data like tumor size. DcR3 gene amplification only correlated with tumor size. The level of DcR3 in serum of the PC resectable group before operation was 72.2 ± 10.2 pg/ml, showing a significant difference compared to gallbladder carcinoma group (GC) or pancreatic benign tumor (PBT) group ($P < 0.01$). In conclusion, DcR3 amplification is correlated with DcR3 expression in PC tissues, especially those clinical pathological factors which reflect tumor progression. Assessment of DcR3 level in sera of PC patients may be helpful for the early diagnosis and prognostic judgement.

Keywords: DcR3 - pancreatic carcinoma - PCR - ELISA - immunohistochemistry

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Introduction

Decoy receptor 3 (DcR3), also named TR6 or M68, is a member of the TNFR (tumor necrosis factor receptor) superfamily. DcR3 is a kind of anti-apoptosis protein. It can competitively bind to Fas ligand (FasL), tumor necrosis factor-like ligand 1A (TL1A) and lymphotoxin analogues (LIGHT) on T cells which can induce expression and compete with herpes simplex virus (HSV) glycoprotein D to bind to HSV invasion mediator, thus, to block cell apoptosis mediated by them (Gill and Hunt JS, 2004; Li et al., 2007; Bamias et al., 2008). Studies have shown that DcR3 expression and amplification are closely correlated with the development of various malignant tumors as well as their immune escape (Ohshima et al., 2000; Chen et al., 2009; Mueller et al., 2009).

However, up to now, seldom reports about DcR3 in PC have been released. Thus, in this study, the expression and amplification of DcR3 in PC tissues and sera were detected by fluorescent quantitative PCR, immunohistochemistry and ELISA with the expectation to explore the correlation between DcR3 amplification and expression as well as its clinical significance.

Materials and Methods

Samples

PC samples were obtained from 50 patients who received resection at No. 1 Affiliated Hospital of Suzhou University between January 2008 and December 2010. Of all the patients, 26 were males and 24 were females with the median age of 58.7 years old (ranging from 41 to 75). According to WHO 2000 tumor differentiation grading criteria, well-differentiated tumor was found in 19 cases, moderate differentiation in 15 cases and poor differentiation in 16 cases. Stage I, II, III and IV were respectively found in 9, 15, 22 and 4 cases based on UICC (2002) tumor staging criteria. Normal pancreatic tissues far away from tumor were taken as the controls (pathologically confirmed). Samples were excised under sterile conditions. One part of the sample was placed in liquid nitrogen immediately, and then kept in a -80 °C fridge. And the other part was kept in 10 % formaldehyde for later use.

Sera for ELISA were respectively obtained from peripheral blood of 50 PC patients before and after operation. Blood samples were EDTA anticoagulated, and

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then centrifuged at 3000 r/min for 15 min. Sera from 20 PC internal drainage patients (patients with unresectable PC) and from 18 patients with pancreatic benign tumor (7 cases of islet cell tumor, 6 cases of solid-pseudopapillary tumor of pancreas, and 5 cases of serous cystic tumor) before and one week later after operation were taken as the controls. In order to minimize the interference upon detection results by open abdomen surgery, sera from 10 patients undergoing cholecystectomy were respectively taken before and after operation as the blank controls. All the patients didn't receive any chemotherapy, radiotherapy or immunotherapy before operation.

Detection of DcR3 mRNA by PQ-PCR

Total RNA was extracted by Trizol (Invitrogen, USA), CDNA was reversely transcribed into by random primers (Sangon Biotech, Shanghai), and PCR amplification was carried out using Taqman technique. β -actin was taken as the internal standard. For each amplification, ddH₂O replaced the template for blank control. The PCR primers of DcR3 were 5'-CTCTTCCTCCCATGACAC-3' (upstream) and 5'-CTGGAAAGCCACAAAGTC-3' (downstream), and the probe was 5'-FAM-CTCCTCAGCTCCTGGTACCCT-TAMRA-3'. The primers of β -actin were 5'-CGTGAAAAGATGACCCAGATCA-3' (upstream) and 5'-CAGCCTGGATGGCTACGTACA-3' (downstream), and the probe was 5'-FAM-TTGAGACCTTCAACACCCAGCC-TAMRA-3'. The amplification segments of DcR3 and β -actin were 112bp and 96bp, respectively. In the amplification conditions, an initial pre-denaturation was done at 95 °C for 10 min. This was followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The final exposure was implemented at 72 °C for 10 min. The reaction system was 20 μ l. Δ Ct relative quantitative method was adopted for quantitative calculation. The expression difference between PC tissues and non-cancerous tissues was presented as 2^{- Δ Ct}. All samples were repeated in triplicate, and mean expression values were used. PCR products underwent 2% agarose gel electrophoresis (100 V) and photos were taken. For the verification of correctness of target products, three PC samples were taken randomly and sequence analyses of PCR amplification products of DcR3 were performed.

Detection of DcR3 amplification

DcR3 amplification times in PC tissues and normal pancreatic tissues were detected by quantitative PCR (Taqman) method. DNA was extracted by QIAamp DNA kit (Qiagen). β -globin was taken as the internal standard. Two primers were designed on the same intervening sequence in order to avoid RNA amplification. The primers of DcR3 were 5'-CCAGCACGGCTCACTGC-3' (upstream) and 5'-TTTCTGGGCCCCACTCG-3' (downstream), and the probe was 5'-FAM-CAGGGATTTCTCTCTCCTGCAAACCC-TAMRA-3'. The primers of β -globin were 5'-ACCCTTAGGCTGCTGGTGG-3' (upstream) and 5'-GGAGTGGACAGATCCCCAAA-3', and the probe was 5'-FAM-CTACCCTTGACCCAGAGGTTCTTTGAGTC-

TAMRA-3'. DNA extracted from peripheral blood lymphocytes of 10 normal people was used as the blank control. The reaction system and amplification conditions were the same as the preceding procedure. All samples were repeated in triplicate, and mean values were used.

Detection of DcR3 protein expression by immunohistochemistry

Samples were fixed by 10% formaldehyde for 24 h and then imbedded by paraffin. Serial sections at a thickness of 4 μ m were made. S-P immunohistochemical staining was carried out according to the instructions of S-P kit (Maxin). DcR3 monoclonal antibody (ABCAM) was diluted according to a ratio of 1:50. Known positive tissue sections were taken as positive controls. PBS replaced the primary antibody for negative control. Under light microscope, cells with obvious buffy granules in cytoplasm were positive cells. Couple score semi-quantitative method was adopted: the positive rate of target cells \leq 5% was scored 0, 6% - 25% was scored 1, 25% - 50% was scored 2, 51% - 75% was scored 3 and > 75% was scored 4; for coloration, no staining was scored 0, light buffy was scored 1, buffy was scored 2 and brown was scored 3; after the above two scores multiplied, 0 was presented by -, 1-4 was presented by +, 5-8 by ++ and \geq 9 by +++.

Detection of DcR3 expression in serum by ELISA

Enzyme linked immunosorbent assay kit (R&D) was used in double antibody single step sandwich technique. OD values were detected by multiproportionally diluted standard preparation and standard curves were drawn. 50 μ l of sample was pipetted into each well and the plate was incubated at 37°C for 120 min. 100 μ l of HRP-labeled detecting antibody was added into each well, mixed well, and then the reaction plate was placed into a 37°C incubator for 60 min. The plate was washed five times. 100 μ l of substrate working solution was added into each well, and the plate was incubated at 37°C for 15 min. 50 μ l of stop buffer was added into each well and mixed well. OD values at 450nm were detected by enzyme-labeled meter. ELISA sensitivity was less than 1.0 pg/ml.

Statistical analysis

Data were presented by $\bar{x} \pm s$. Statistical analysis was carried out by SPSS13.0 statistical software. A Students t test was performed for pairwise comparison of mean values between groups. One-way ANOVA and SNK test were performed for comparisons of mean values among samples. χ^2 test was carried out for enumeration data as well as the analyses of the correlations among clinical pathological factors. $P < 0.05$ was considered statistically significant.

Results

The expression of DcR3 mRNA

The expression of DcR3 mRNA in PC tissues was 4.95 times high of that in the normal pancreatic tissues, showing a significant difference ($P < 0.01$) (Table 1). Gel electrophoresis of RQ-PCR amplification products showed that the amplification segments of DcR3 and

Table 1. The Expression of DcR3 Protein by Immunohistochemistry

Groups	cases	Relative amount	T value	P value	Expression times
PC	50	0.94±0.22	24.02	<0.01	4.95
Normal	50	0.19±0.06			

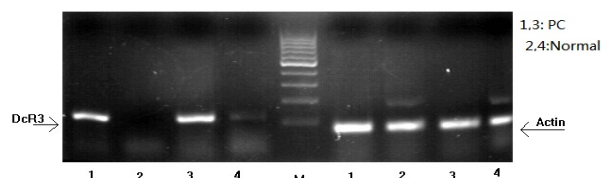
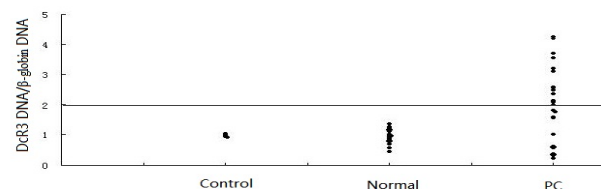
Table 2. Expression of DcR3 Protein by Immunohistochemistry

Groups	Cases	DcR3				Positive rate	x ² value	P value
		-	+	++	+++			
PC	50	11	14	13	12	78%	29.17	<0.005
Normal	50	38	12	0	0	24%		

Table 3. Correlations Between DcR3 Amplification and Its mRNA and Protein Expressions

Groups (IHC)	Cases	DcR3 mRNA	DcR3 protein	
			Positive	Negative
Amplification	21	0.82±0.19*	19**	2
Non-amplification	29	0.34±0.11	20	9

*P<0.001, **P<0.005

**Figure 1. Electrophoretogram of PCR Amplification Products of DcR3.** 1 and 3 were for PC tissues, and 2 and 4 for normal pancreatic tissues**Figure 2. Diagram of DcR3 Amplification Fold Values in Different Tissues**

the internal standard were 112bp and 96bp, satisfying what had been expected (Figure 1). Sequencing result of PCR amplification products showed that the sequence of the amplified DcR3 gene was homologous to the source sequence.

DcR3 amplification times

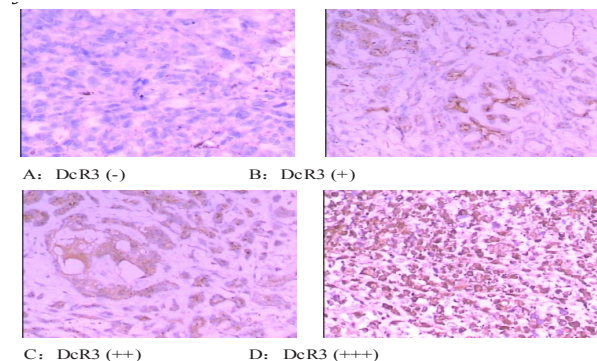
DNA was extracted from peripheral blood lymphocytes of 10 normal people for PCR amplification, and the detection result was 0.99±0.17, satisfying what had been expected. DcR3 DNA/β-globin DNA > 2 was taken as the positive criterion. The amplification times of DcR3 in 50 PC patients ranged from 0.21 to 4.18 with the average of 2.33±0.53, among which > 2 was found in 21 cases, accounting for 42% (21/50).

There was no case with such a positive amplification times in normal pancreatic tissues. A significant difference in DcR3 amplification times was found between PC tissues and normal tissues (P < 0.01) (Figure 2).

Table 4. Correlations Between the Amplification and Expression of DcR3 and Clinical Pathological Factors

Influencing factors	Cases	DcR3 amplification times	DcR3 mRNA	DcR3 protein
Tissues				
PC	50	21	42%	0.94±0.22
normal	50	0	0%	0.19±0.06
age				
<60 years	23	9	39.10%	0.90±0.18
≥ 60 years	27	12	44.40%	0.98±0.21
Sex				
male	26	11	42.30%	0.92±0.12
female	24	10	41.70%	0.98±0.15
location				
Head and neck of pancreas	39	14	35.90%	0.96±0.26
Body and lower part	11	7	63.60%	0.91±0.20
Histological staging				
G1	19	8	42.10%	0.86±0.11
G2/3	31	13	42%	0.99±0.26*
Clinical staging				
I/II	24	7	29.20%	0.81±0.11
III/IV	26	14	53.80%	1.07±0.24**
Tumor size				
<2cm	22	5	22.70%	0.85±0.12
≥2cm	28	16*	57.10%	1.04±0.17**
Lymph node metastasis				
Non-metastasis	29	10	34.50%	0.86±0.14
metastasis	21	11	52.40%	1.02±0.19**

*P<0.05, **P<0.01

**Figure 3. The Expression of DcR3 Protein by Immunohistochemistry: (-) - (+++), 10×20**

Expression of DcR3 protein by immunohistochemistry

The staining of DcR3 positive cells mainly located in cytoplasm, exhibiting buffy granules or flakes in shape (see Figure 3). Positive cells were seen scattered, in nests or diffused. The positive rate of DcR3 in PC tissues was 78% (39/50), among which overexpression (++ - +++) accounted for 25% (25/50) while the positive rate of DcR3 in normal pancreatic tissues was 24% (12/50), showing a significant difference (P < 0.01).

Correlations between DcR3 amplification and expression of DcR3 mRNA and protein

Based on the criterion of gene amplification times > 2 or ≤ 2, two groups were divided. DcR3 amplification was found in 21 cases with an mRNA level of 0.82±0.19 while non-amplification was found in 29 cases with an mRNA

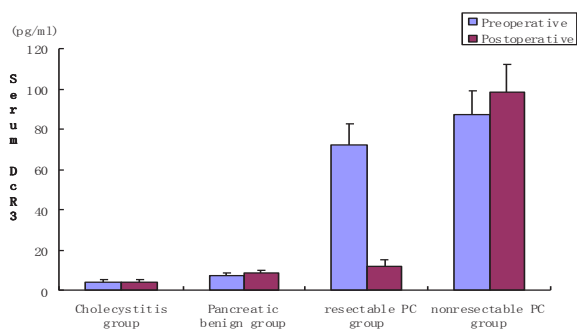


Figure 4. Expression of DcR3 in Serum by ELISA

level of 0.34 ± 0.11 , showing a significant difference. Meanwhile, results showed that the positive rates of DcR3 protein in two groups were 90.5% and 68.9%, respectively, showing a significant difference.

Correlations between the amplification and expressions of DcR3 and clinical pathological factors

The clinical pathological data of 50 PC patients were analyzed for the correlations between the amplification and expressions of DcR3 and clinical pathological factors. Results showed that the expression of DcR3 mRNA was correlated with clinical PC staging, size of the tumor, lymph node metastasis and histological staging, but it had no correlation with age, sex or the location of the tumor. The expression of DcR3 protein was correlated with clinical PC staging and size of the tumor, but it had no correlation with age, sex, tumor location, histological staging or lymph node metastasis. DcR3 amplification was only correlated with tumor size.

The expression of DcR3 in serum by ELISA

The expression of DcR3 before operation in the resectable PC group was 72.23 ± 10.18 pg/ml, which is significantly higher than that in the gallbladder carcinoma group (GC) or pancreatic benign tumor group ($P < 0.01$) (see Figure 4). The expression of DcR3 before operation in the unresectable PC group was 87.18 ± 11.72 pg/ml, which was significantly higher than that in any other group. One week later after operation, DcR3 levels in serum of different groups were detected. There was no significant difference before and after operation in GC or pancreatic benign tumor group whereas the DcR3 level in the resectable PC group decreased significantly after operation, showing a significant difference compared to that before operation ($P < 0.01$).

Discussion

It has been known that the genesis of tumor is caused by the unbalance between cell proliferation and apoptosis (Longley et al., 2004). The induction of tumor cell apoptosis is a decisive step for a biotherapy to achieve its therapeutic effect, and the tumor cell-killing mechanism is mediated by the interaction between ligand and receptor which are respectively responsible for up- and down- regulation. Thus, apoptosis-inducing ligand and its receptor of TNF family remain the hotspots of research. DcR3 is a newly-discovered member of the soluble tumor necrosis factor receptor (STNFR) superfamily. In 1988,

Pitti et al. discovered an EST with high homology to TNFR superfamily when he was searching EST database, and he denominated it DcR3. DcR3 can inhibit cell apoptosis and promote immune escape of tumor cells, and it is one of the most powerful anti-apoptosis factors which have been found till now (Hsu et al., 2005). DcR3 can competitively bind to FasL, TL1A and LIGHT, thus, to block cell apoptosis mediated by receptors. In addition, it also plays important roles in immunological regulation of T cells and DC cells, angiogenesis of tumors, etc (Shi et al., 2003; Yang et al., 2004; You et al., 2008).

The overexpression of DcR3 can be found in multiple human malignant tumors such as gastric carcinoma (Takahama et al., 2002), colon carcinoma (Wu et al., 2003), esophageal carcinoma (Li et al., 2005), lung carcinoma (Pitti et al., 1998), hepatic carcinoma (Chen et al., 2007; Chen and Luo, 2008), spongioblastoma (Arakawa et al., 2005), oophoroma (Connor and Felder, 2008; Anderson et al., 2010), etc. Research has found that both the gene level and protein level of DcR3 in tumor tissues are over-expressed whereas those in normal tissues surrounding tumor are hardly. In this study, the expressions in 50 PC tissue samples as well as 50 non-cancerous tissue samples were respectively detected using PCR method and immunohistochemistry, and results showed that both DcR3 mRNA level and protein level in PC tissues were notably higher than those in non-cancerous tissues, suggesting that the abnormal expressions of DcR3 may play critical roles in the genesis of PC. In addition, the correlations between DcR3 expressions and clinical pathological factors were analyzed. Our results showed that the expressions of DcR3 mRNA and protein in clinical III/IV groups and the group with tumor > 2 cm were significantly higher than those in I/II groups and the group with tumor < 2 cm, suggesting that DcR3 is correlated with the clinical pathological factors which can present the development of tumor. However, DcR3 mRNA and protein expressions were not always in agreement when their correlations with clinical pathological factors were analyzed. Our result showed that the expression of DcR3 mRNA was correlated with lymph node metastasis, but the expression of DcR3 protein was not. The possible reason may come from the fact that there may still be multiple regulatory steps to translation after gene transcription, which may lead to the disagreement between DcR3 transcription and protein expression levels. To better understand it, further studies will be necessitated in the future.

DcR3 coding gene was located at human chromosome 20q13.3, which is also a site prone to gene amplification and rearrangement (Schwendel et al., 1998). However, whether the overexpression of DcR3 in tumor tissues is dependent on gene amplification is still ambiguous. Pitti et al. thought that DcR3 amplification was the molecular basis of DcR3 overexpression in lung carcinoma and colon carcinoma. Shen et al. also found that there was DcR3 amplification in primary hepatic carcinoma and there were great differences in DcR3 mRNA expression among patients with different amplification times (Shen et al., 2005). But Bai discovered that the overexpression of DcR3 in gastric carcinoma occurred earlier than amplification, which indicated that the overexpression of DcR3 might

be an early event of primary tumor gene activation (Bai et al., 2000). In our study, DcR3 amplification times in PC tissues were detected by fluorescent quantitative PCR method. Our results showed that there was gene amplification in PC tissues and the expressions of DcR3 mRNA and protein in PC tissues with gene amplification were higher than those without amplification, indicating that there is DcR3 amplification and overexpression in PC tissues and DcR3 amplification may be one of the factors of DcR3 overexpression.

As DcR3 lacks a transmembrane structure in its amino acid sequence, it belongs to a kind of secretory protein. DcR3 is not expressed or only slightly expressed in normal tissues and serum, but highly expressed in malignant tumor tissues. Thus, the detection of DcR3 level in serum of tumor patients will be of great help for early diagnosis of tumor and prognostic judgement. Macher et al detected DcR3 in the serum of renal carcinoma patients, and found that DcR3 level was closely correlated with TNM staging and the height of DcR3 expression in the serum was identical to that obtained by immunohistochemistry (Macher-Goeppinger et al., 2008). Wu detected DcR3 expression in patients with tumor, acute infection and cirrhosis as well as that in normal people, and he found that DcR3 negative rate in the sera of patients with acute infection and normal people was 96.9% while DcR3 positive rate of malignant tumor patients was 55% (Li et al., 2005). Our study also showed that DcR3 level in the serum of PC group was obviously higher than that of the normal group and benign tumor group before operation. And DcR3 level decreased significantly after tumor resection while DcR3 level in the unresectable group increased. Our results indicated that the expression of DcR3 may stem from PC cells. It is feasible to detect DcR3 levels in the serum for PC clinical diagnosis and prognosis monitoring.

To sum up, the overexpression and amplification of DcR3 play important roles in the initiation and development of PC. The detection of DcR3 expression and amplification is of great significance for PC biological behavior as well as prognostic judgement, which may also provide a potential target in PC treatment.

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