Original Articles

Expression of decoy receptor 3 in liver tissue microarrays

GANG CHEN, DIANZHONG LUO

ABSTRACT

Background. Decoy receptor 3 (DcR3), a new member of the tumour necrosis factor receptor (TNFR) superfamily, is amplified and overexpressed in various cancers. We investigated the expression of DcR3 protein in liver tissue microarrays and assessed its importance in patients with hepatocellular carcinoma (HCC).

Methods. In this retrospective study, tissue from 120 patients with HCC, 48 with tissue at least 2 cm away from the tumour (juxta-tumour tissue), 62 with cirrhosis and 23 with normal livers were studied as tissue microarrays. Immunohistochemistry was used to detect the expression of DcR3. Statistical analyses were done to assess the association between DcR3 expression and the clinicopathological features of HCC.

Results. The positivity rate of DcR3 in HCC tissue was significantly higher than that in juxta-tumour tissue, cirrhosis and normal liver ($p = 0.017$, $p < 0.0001$, $p < 0.0001$, respectively). The positive rate of DcR3 in juxta-tumour and cirrhotic tissue both increased significantly when compared with normal liver tissue ($p < 0.0001$, $p = 0.005$, respectively). The positivity rate of DcR3 in HCC in clinical TNM stages I and II was significantly lower than that in stages III and IV ($p < 0.0001$). The positivity rate of DcR3 in patients without metastasis within 20 months decreased significantly compared with those with metastasis ($p < 0.0001$). DcR3 expression in patients with alphafoetoprotein levels $\geq 400\ \mu g/L$, portal vein tumour emboli, capsular infiltration and multicentric tumour was significantly higher than in groups without these features ($p = 0.021$, $p < 0.0001$, $p < 0.0001$, $p = 0.002$, respectively).

Conclusion. The overexpression of DcR3 might play an important role in the pathogenesis, progression and metastases of HCC. The DcR3 gene might serve as an important molecular biological indicator in diagnosing and predicting the biological behaviour of patients with HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common tumours worldwide and its incidence has been increasing.[1–3] Although the prognosis of patients with HCC has improved marginally over the past 2 decades, the 5-year survival rate remains low (about 5%).[4] Decoy receptor 3 (DcR3)/TR6/M68 is a soluble receptor belonging to the tumour necrosis factor receptor (TNFR) superfamily that has 4 members—DcR1, DcR2, DcR3 and osteoprotegerin.[5–7] Compared with the other 3 members of the TNFR family, DcR3 has no transmembrane domain and can thus be secreted.[5] The gene amplification, as well as overexpression of DcR3 messenger RNA (mRNA) and protein, has been demonstrated in various tumours including lymphomas and glioblastomas.[5,8–14] DcR3 has been postulated to assist tumour cells to obtain a survival advantage by inhibiting apoptosis and also by interfering with immune surveillance, due to neutralization of the cytotoxic and immunomodulatory effects of Fas ligand (FasL); homologous to lymphotoxins, showing inducible expression, and competing with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes (LIGHT), and TNF-like molecule 1A (TL1A).[5,15,16] The serum level of DcR3 has been shown to be significantly elevated in more than half (56.2%) of the patients with a variety of tumours, including cancers of the digestive system, thyroid, lung, breast and ovary.[17,18] The expression and significance of DcR3 in HCC were studied by Shen *et al.* using reverse transcription–polymerase chain reaction (RT–PCR) and quantitative genomic PCR.[13] However, Shen *et al.* analysed the expression of DcR3 protein in only 5 cases of HCC using immunohistochemistry (IHC).[13] Wu *et al.* showed that in HCC and cirrhosis, the expression of DcR3 could be detected both in the serum and tissue.[17] To date, the association, if any, between DcR3 protein expression and the clinicopathological features of HCC has not been studied in a large number of patients. Also, no information is available about the protein status of DcR3 in liver tissue microarrays (TMAs). Therefore, in this retrospective study, we investigated the expression and clinicopathological importance of DcR3 in patients with HCC using IHC and TMA, and explored its role in the invasion and metastases as well as the relationship between biological behaviour and prognosis of HCC.

METHODS

Patients

This retrospective study included 120 patients (115 men, 5 women) with HCC, 48 patients in whom tissue at least 2 cm away from the tumour (juxta-tumour tissue) was obtained, 62 patients with cirrhosis and 23 normal controls in whom tissue was obtained from a site adjacent to liver cavernous haemangioma tissue. The age range of patients with HCC was 23–81 years (mean 49 years). Among the 120 patients, 73 were positive for alphafoetoprotein (AFP) while

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47 were negative. In 48 patients the juxta-tumour tissue was obtained at least 2 cm from the site of HCC. Thirty-six of these patients had cirrhosis while 12 did not. All tissue used were from initial hepatectomies so as to avoid the secondary changes of healing. Sixty-two cirrhotic samples were from explant patients (41 men, 21 women). The age range was 15–74 years (mean 43 years). The control group had 16 men and 7 women with a mean age of 42 (range 18–72) years. All patients underwent hepatectomy at the First Affiliated Hospital, Guangxi Medical University, China between May 2002 and December 2005. Written informed consent to use the samples for research was obtained from the patients and clinicians.

The histopathological diagnoses were made according to the WHO International Histological Classification of HCC: 9 well differentiated, 73 moderately differentiated and 38 poorly differentiated tumours. According to the TNM classification, 15 were stage I, 53 stage II, 23 stage III and 29 stage IV. Clinical information was obtained from the medical records of the patients. Among the 120 patients with HCC, 64 were followed up for 20 months. Thirty had metastasis while 34 did not. The diagnosis and classification were based on histology or cytology by the same pathologist.

Tissue microarrays

A representative area of each tumour was identified by microscopy and the corresponding area was marked on the tissue block according to previously reported methods.[19,20] A single 0.6 mm core of tissue was used to assemble the arrays. The 3 tissue array blocks contained tissue from 120 HCCs, 48 juxta-tumour tissue and 85 non-tumorous (62 cirrhosis of liver and 23 normal livers) specimens. The blocks were heated at 42 °C for 3 minutes and then sectioned at 4 μ thickness.

Immunohistochemistry

Monoclonal antibody anti-DcR3 (Santa Cruz) was used as described elsewhere.[14] Sections of tissue from carcinoma of the stomach were used as positive controls for DcR3. The primary antibody was replaced by phosphate buffer solution (PBS) for negative controls. The positive signal for DcR3 appeared as yellow–brown in the cytoplasm of the cells. One hundred cells from 5 representative areas from each case were counted. The staining results were evaluated according to the immunodetection of stain intensity and number of positive cells. If there was a difference of opinion between us, we discussed the case to reach 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

0: No staining
1: Focal or fine granular, weak staining
2: Linear or cluster, strong staining
3: Diffuse, intense staining

The positive cells in the observed tissue ranged from 0 to 3 in percentage

0: No staining
1: <30%
2: 30%–70%
3: >70%

The samples were categorized as positive and negative based on the sum of the scores as follows:

0–1: Negative
2–3: Positive (+)
4: Positive (++)
5–6: Positive (+++)

Any score ≥2 was considered as positive expression.[14]

Statistical analyses

The Fisher exact test was used to compare overexpression of DcR3 among different groups with SPSS 13.0 for Windows. A value of $p<0.05$ was considered statistically significant. Multivariate analysis was used to identify the relationship of DcR3 overexpression with patients' age and gender, and clinical and pathological parameters. A value of $p<0.05$ (for one-sided) or $p<0.025$ (for two-sided) was considered statistically significant.

RESULTS

Quality of tissue array

The haematoxylin and eosin stained TMAs were highly representative of the tissue. All the samples in the TMAs were suitable in this study.

Expression of DcR3 in different liver tissue

Absent or weak DcR3 signals could be detected in normal, cirrhotic and juxta-tumour tissue sections. In contrast, moderate-to-strong DcR3 immunostaining was seen within the cytoplasm of tumour cells (Figs 1a and 1b). The rate of DcR3 positivity in patients with HCC was significantly higher than that in juxta-tumour, cirrhotic and normal liver tissues (Table I). The expression of DcR3 in juxta-tumour and cirrhotic tissue was significantly

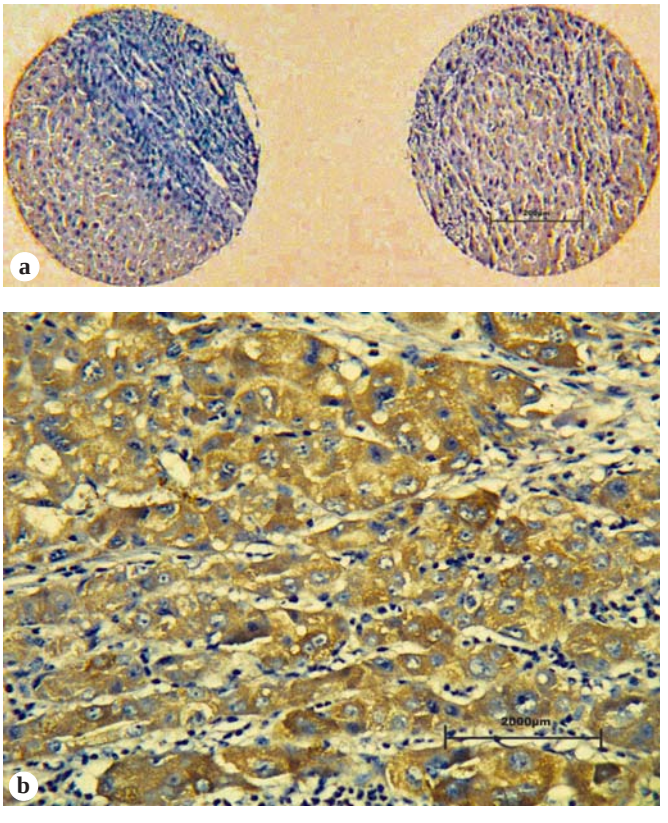

FIG 1. Expression patterns of DcR3 in hepatocellular carcinoma. DcR3 protein was detected by immunostaining in HCC tissue microarray. The signal was localized within the cytoplasm of tumour cells in HCC.

TABLE I. Expression of DcR3 in different types of liver tissue

Liver tissue	Expression of DcR3 *n* (%)	Cirrhosis		Juxta-tumour		HCC	
		χ^2	p	χ^2	p	χ^2	p
Normal (*n*=23)	2 (8.7)	2.78	0.005	3.82	<0.0001	6.10	<0.0001
Cirrhotic (*n*=62)	23 (37.1)	—	—	1.64	0.102	4.54	<0.0001
Juxta-tumour (*n*=48)	26 (54.2)	—	—	—	—	2.40	0.017
HCC (*n*=120)	88 (73.3)	—	—	—	—	—	—

HCC hepatocellular carcinoma

TABLE II. Relationship between DcR3 expression and clinicopathological features in hepatocellular carcinoma

Feature		*n*	DcR3 expression			
			+(*n*)	Rate (%)	χ^2	p
Clinical stage	I–II	68	42	61.8	10.739	<0.0001
	III–IV	52	46	88.5		
Metastasis and recurrence	Yes	30	29	96.7	19.858	<0.0001
	No	34	20	58.8		
AFP (μg/L)	≥400	73	59	80.8	5.345	0.021
	<400	47	29	61.7		
Portal vein tumour embolus	Yes	46	42	91.3	12.319	<0.0001
	No	74	46	62.2		
Tumour capsular infiltration	Yes	83	70	84.3	16.668	<0.0001
	No	37	8	48.7		
Tumour	Multicentric	46	41	89.1	9.519	0.002
	Solitary	74	47	63.5		

AFP alphafoetoprotein

higher than that in normal liver tissue. However, there was no significant difference in the DcR3 expression between juxta-tumour and cirrhotic tissue.

Relationship between DcR3 expression and clinical TNM stage

The expression of DcR3 in HCC in TNM stages I and II was significantly lower than that in stages III and IV (Table II).

Relationship between DcR3 expression and metastasis

DcR3 expression in cases without metastasis within 20 months was significantly lower than that in those with metastasis (Table II).

Relationship between DcR3 expression and other parameters

The percentage of DcR3-positive expression in cases with AFP levels ≥400 μg/L, portal vein tumour embolus, tumour capsular infiltration and multicentric tumours was significantly higher than in those with AFP levels <400 μg/L, without tumour embolus, without tumour capsule infiltration and solitary tumours (Table II). Multivariate analysis revealed that there was no association between DcR3 overexpression in HCC and age, sex, histological classification, tumour diameter or cirrhosis (data not shown).

DISCUSSION

TMA is a new technique with numerous advantages including quick analysis, reducing the requirement for time and reagents, enhances laboratory efficiency and provides uniform results. Many studies have shown that TMA is a powerful tool for pathological and clinicopathological oncology research.[21–25] We used 3 TMAs consisting of 120 samples from HCC, 48 samples from juxta-tumour liver tissue and 85 non-cancerous liver tissue to detect the expression of DcR3.

Human DcR3, a newly identified member of the TNFR superfamily, maps to chromosome 20q13.3, a region known to be associated with gene amplification and rearrangement in human cancer.[8,9] DcR3 is a soluble receptor that is expressed in many different classes of tumour cells and competitively inhibits binding of TNF to TNFRs.[5,8–14] DcR3 can bind the TNF family members FasL, 1 LIGHT[8,26] and TL1A,[27] as a result blocking interaction with their respective receptors, i.e. Fas, herpes virus entry mediator (HVEM) and the death domain-containing receptor DR3.[8,26,28]

Shen *et al*. showed, using RT–PCR analysis, that DcR3 mRNA was overexpressed in 29 of 48 patients with HCC (60.4%). No positive expression was detected in the adjacent normal tissue.[13] However, they analysed the expression of DcR3 protein using IHC in only 5 cases with HCC and an obvious positive stain was seen in 3 of the 5 cases analysed.[13] Our results showed that absent or weak DcR3 signals could be detected in normal, cirrhotic and juxta-tumour tissue. In contrast, moderate-to-strong DcR3 immunostaining was seen within the cytoplasm of tumour cells. This is consistent with the results of Wu *et al*.[17] To the best of our knowledge, no study of overexpression of DcR3 in HCC by IHC and TMA has been reported. We found higher DcR3 expression (73.3% *v.* 60.4%) in HCC compared with the results of Shen *et al*.[13] This difference may be due to the larger number of samples (120 *v.* 48) or differences in the methodology (IHC *v.* RT–PCR) used by us. The significant overexpression of DcR3 in HCC in our study suggests that it may have a role to play as a biomarker for the diagnosis of HCC.

It is known that HCC can result from a dysplasia–carcinoma sequence, i.e. cirrhosis to HCC.[29] However, the molecular mechanisms in such a process are not known. Our finding of increasing expression of DcR3 in normal, cirrhotic, juxta-tumour and tumour tissue suggests that increased expression of DcR3 might be an important molecular event involved in the process of hepatocarcinogenesis. This conclusion is consistent with that of Shen *et al*.[13] and Wu *et al*.[17] who suggest that measurement of DcR3 activity might be a valuable clinical marker in the progression of dysplasia and subsequent development of HCC.

Another interesting observation was that the expression of DcR3 was related to the clinical and pathological features. In our study, 29 of 30 patients (96.7%) with metastasis and recurrence had positive DcR3 protein expression, whereas only 20 of 34 patients (58.8%) without metastasis and recurrence had positive DcR3 expression. Shen *et al*.[13] had similar findings. Also, DcR3 expression was more in stages III and IV. DcR3 expression was also associated with AFP, portal vein tumour emboli, capsular infiltration and multicentricity of the tumour. Shen *et al*.[13] found that DcR3 mRNA expression in HCC was associated with the size of the mass but not with capsular infiltration, tumour emboli and multicentricity. The variations in techniques used in the study by Shen *et al*. and us may partly explain these differences. Our results also suggest that the expression of DcR3 is associated with a higher frequency of tumour progression and postoperative metastasis and recurrence.

The prognosis of patients with HCC has improved only marginally over the past 2 decades.[4] This is in part due to the late presentation and delay in diagnosis. Patients at risk for developing HCC are therefore kept on surveillance programmes that use ultrasound with or without measurement of AFP levels.[30] This enables detection of tumours at a stage still eligible for treatment.[31]

AFP is the only widely used serum marker for HCC. However, given its low positive predictive value, it has limited use in a population at average risk.[32] DcR3 might be a better marker to monitor the progression of cancer because its serum level decreases dramatically after removal of the tumour.[17,18] Further studies on serum detection of DcR3 may be needed to determine its role in predicting the prognosis and early diagnosis of HCC.

In conclusion, our study suggests that DcR3 expression has a role in the carcinogenesis of HCC, and a relationship with the development and aggressiveness of HCC. DcR3 protein expression might be useful for clinical prognostication of HCC.

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