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RESEARCH

Tumor Associated Glycoprotein-72 is a Novel Marker for Poor Survival in Hepatocellular Carcinoma

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Abstract To investigate the relationship of tumor associated glycoprotein-72 (TAG-72) expression with clinicopathological features in hepatocellular carcinoma (HCC) patients. Sixty pairs of HCC and paracarcinomatous (PCLT) tissues, and 10 nomral liver (NL) tissues were collected for Western blot analysis, and 244 pairs of HCC and PCLT tissues were collected for immunohistochemistry analysis. TAG-72 protein expression was elevated significantly in HCC tissues compared with PCLT and NL tissues. Its increased expression was correlated with TNM stage, Edmondson-Steiner grade, vein invasion and multiple tumor nodes. It is noteworthy that the HCC patients with high TAG-72 expression had shorter overall survival and disease-free survival than the patients with low expression. Multivariate Cox regression analysis revealed that TAG-72 expression was an independent prognostic factor for HCC patients. The current study demonstrated for the first time that the increased expression of TAG-72 was correlated with poor survival in patients with HCC, indicating that TAG-72 is a novel prognostic marker for HCC.

Keywords Hepatocellular carcinoma · Tumor associated glycoprotein-72 · Clinicopathology · Prognosis · Disease-free survival · Overall survival

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Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal and aggressive neoplasms. It is the fifth most common cancer and the second leading cause of cancer-related deaths in China [1]. HCC occurs mostly in Southeast Asia and sub-Saharan Africa, but its incidence and mortality in the world is the fastest growing of all cancers in the last years. More seriously, the worldwide incidence of HCC in 2001 was 21 per 100,000 people and the mortality rate was 20.2 per 100,000 individuals [2]. Currently, options for the curative treatment include surgical resection, percutaneous ablation therapy, liver transplantation, etc. Nevertheless, the overall prognosis of HCC is poor, with a 5-year survival rate of 3–5 % after initial diagnosis, which is largely attributed to the aggressive nature of this malignancy, characterized by multicentric development with a high metastatic potential [3]. Therefore, it is urgently needed to explore diagnostic markers, prognostic factors, and treatment targets for improving survival of patients with this deadly tumor.

Tumor associated glycoprotein 72 (TAG-72) is a membrane mucin (MUC1)-like glycoprotein complex of approximately 220–400 kD, which is over-expressed in the majority of human adenocarcinomas occurring within the colon, stomach, esophagus, ovary, pancreas, breast, and lung, but is not expressed in most normal tissues, with the exception of the endometrium during the secretory phase [4–10]. Anti-TAG-72 monoclonal antibodies have been studied in preclinical animal models as well as in humans for cancer detection based on their high specificity against cancer antigens in various solid cancers. Monoclonal antibodies against TAG-72 have been used for tumor detection in radioimmunoguided surgery [11]. Historically, radioimmunoguided surgery combined radioactive-labeled monoclonal antibodies and a handheld gamma probe for the intraoperative detection and resection

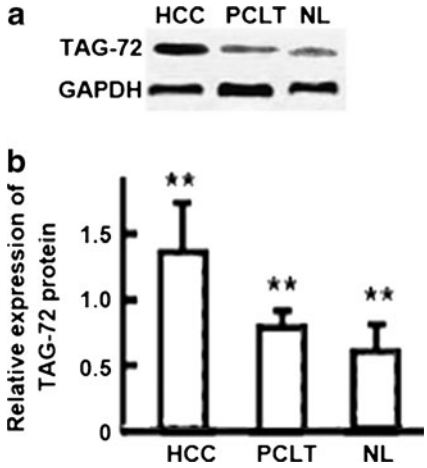

Fig. 1 The detection of tumor associated glycoprotein-72 (TAG-72) protein expression in samples of hepatocellular carcinoma (HCC). **a** TAG-72 protein expression was detected in the same samples of HCC, PCLT, and NL. **b** The *t* test indicated that TAG-72 protein expression levels in HCC were significantly higher than those in PCLT (P=004) and in NL(P=002)

of tumor-bearing tissues in colorectal cancer patients [12]. The successful detection of additional occult disease within regional lymph nodes and the subsequent complete resection of the antibody-bound tissues significantly improved survival rates. In addition, the B72.3 and CC49 antibodies targeted to TAG-72 have been used in the diagnostic imaging of both colorectal and ovarian cancers [13]. A recombinant anti-TAG-72 antibody has recently been engineered, and the expressed proteins have been characterized and evaluated for use in both diagnostic and therapeutic applications. Despite these recent developments, the expression profile and function of TAG-72 in HCC currently remains unknown. In the current study, we sought to determine the expression level of TAG-72 in HCC and analyzed the correlation between TAG-72 expression and clinicopathologic characteristics. Furthermore, the relation between TAG-72 expression and survival in patients with HCC was analyzed.

Patients and Methods

Patients and Tissue Samples

The study was approved by the Research Ethics Committee of Xiangya Hospital, China. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Specimens from 60 patients with HCC and 10 normal liver samples from patients with liver cavernous hemangioma (controls) were immediately frozen in liquid nitrogen and stored at −80 after hepatectomy. In addition, specimens from 244 patients with HCC were collected for immunohistochemistry analysis, which included 210 men and 34 women with a median age of 46 years (range, 22–73 years). All specimens were obtained from patients who underwent hepatectomy at the Department of Surgery, Xiangya Hospital, between February 2006 and March 2008. Specimens were paraffin embedded and stained with hematoxylin and eosin. Clinicopathologic parameters, included sex, age, greatest tumor dimension, the number of tumor nodes, tumor capsule, Edmondson-Steiner grade, vein invasion, and TNM classification (according to the International Union Against Cancer, 2003). The diagnoses were confirmed by histopathologic study.

Western Blot Analysis

To demonstrate the specificity of TAG-72 antibody and the expression level of TAG-72 protein, Western blot analysis was perfromed. Total protein was extracted from HCC, PCLT and NL tissues with lysis buffer using an SDT Tissumizer (Kimble, Vineland, NJ, USA). Lysates were centrifuged at 13,000 rpm at 4°C for 3 min. Supernatants were stored at −80°C, and subsequently analyzed in batch fashion. We determined protein concentration in the supernatants using a modified bicinchoninic acid (BCA) protein assay kit (Dingduo, Beijing, China),

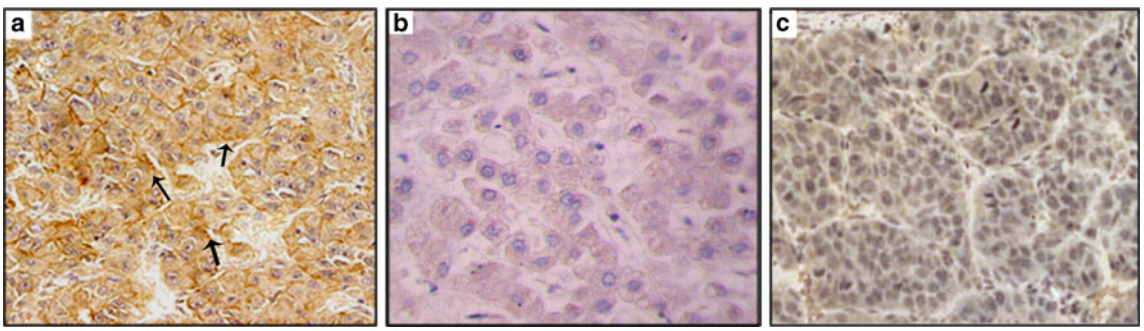

Fig. 2 Representative staining detected by immunohistochemical staining (original magnification: 400×). **a** TAG-72 positive staining in the cytoplam and membrane of tumor cells in hepatocellular carcinoma tissues; **b** TAG-72 negative staining in paired noncancerous liver tissues; **c**: the primary antibody was replaced with pre-immuned normal mouse IgG

employing bovine serum albumin as a standard. Aliquots of 30 μg of total tissue protein were subjected to SDS-polyacrylamide gel electrophoresis on 4–12 % Novex Bis-Tris precasting gels (Invitrogen, Carlsbad, CA, USA). Gels were run at 100 V for 70mins. Separated protein was then transferred onto nitrocellulose membranes with a 6.5-min iBlot Dry Blotting System (Invitrogen, Carlsbad, CA, USA). Membranes 7 were kept for 1 h in blocking buffer (3 % BSA in TBST). After blocking, the blots were incubated with anti-TAG-72 MAb (CC49) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:5000. After washing (TBST), the membranes were incubated with a 1:20000 dilution of horseradish peroxidase conjugated mouse anti-goat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was also determined by using the specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a loading control. After washing, the blots were developed using the ECL Plus chemiluminescent detection system (Thermo, Rockford, IL, USA). We performed densitometric analysis of the immunoblots to quantify the amounts of TAG-72 protein. TAG-72 specific signals were quantified from X-ray films using a scanner with BandScan 4.30 densitometry software, and expressed as integrated intensity units relative to the GAPDH signal. To assess the reproducibility of the assay, all samples were tested in triplicate, and TAG-72 concentration was calculated as the mean of triplicate determinations for each protein extraction from tissue samples. There was <10 % variation in sample-to-sample analysis.

Immunohistochemistry Analysis

The specimens were fixed in 10 % neutral buffered formalin and subsequently embedded in paraffin. The paraffin-embedded tissues were cut at 3 μm and stained following being dried on ProbeOn Plus (Fisher Scientific International, Hampton, NH, USA). Staining was done using avidin- biotin complex with a microprobe manual stainer (Fisher Scientific International). The slide to which a paraffin section was attached went through deparaffinization and hydration, and was then treated with a solution of Peroxidase-blocking reagent (Dako, Glostrup, Denmark) to exhaust endogenous peroxidase activity. It was put in citric acid solution and heated for 10 min in a microwave and then left at room temperature for 20 min to expose antigen hidden inside the tissue due to formalin fixation, and the process was repeated three times. To inhibit non-specific antigen-antibody reactions possible in immunohistochemical staining, reaction was done using a protein blocker (Research Genetics, Huntsville, AL, USA) for 5 min and the slide was washed thoroughly with water. The slides were incubated overnight with anti-TAG-72 mouse monoclonal antibody (CC49) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at 4°C. The specificity of the two antibodies has been validated by the study of Ponnusamy et al.[14] and Ouyang et al. [15]. Secondary antibody for the detection of primary antibody was reacted for 10 min using anti-mouse IgG (Sigma, St. Louis, MO, USA) to which biotin was attached, and then washed with buffer solution and reacted with horseradish peroxidase for 10 min. It was washed

Table 1 Correlations between TAG-72 protein expression levels and clinicopathologic characteristics of 244 patients with hepatocellular carcinoma

Characteristics	No. of cases	TAG-72 expression		P
		Negative	Positive	
Gender				
Male	210	22	188	0.328
Female	34	4	30	
Age (year)				
≤60	194	20	174	0.871
>60	50	6	44	
Cirrhosis				
Presence	188	18	170	0.380
Absence	56	8	48	
Tumor size (cm)				
≤5	66	8	58	0.362
>5	178	18	160	
No. of tumor nodes				
Multiple	96	6	90	0.029
Single	148	20	128	
Capsular formation				
Presence	120	20	100	0.338
Absence	124	6	118	
Edmondson-Steiner grade				
1–2	86	16	70	<0.001
3–4	158	10	148	
Vein invasion				
Presence	190	14	176	<0.001
Absence	54	12	42	

Table 2 Correlations between TAG-72 protein expression levels and tumor-lymph node-metastasis classification in 244 patients with hepatocellular carcinoma

TNM classification	No. of cases	TAG-72 expression		P
		Negative	Positive	
Stage I	44	12	32	0.089[1]
Stage II	92	10	82	0.005[2]
Stage III	108	4	104	0.026[3]

[1] TNM stage I compared with TNM stage II

[2] TNM stage I compared with TNM stage III

[3] TNM stage II compared with TNM stage III

thoroughly with buffer solution; chromogen AEC (3-amino-9-ethylcarvazole; Zymed, San Francisco, CA, USA) was then applied and reddish brown response was examined. After hematoxylin contrast staining, the slide was enclosed with Universal Mount (Research Genetics) and examined. In each immunohistochemistry run, negative controls were carried out by omitting the primary antibody, whereas TAG-72 overexpression confirmed by Western blotting was used as positive controls. Additionally, to further demonstrate the specificity of TAG-72 antibody, the primary antibody was replaced with pre-immuned normal mouse IgG.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The numbers of TAG-72-positive cells that showed immunoreactivity on the cell membrane and cytoplasm in 10 representative microscopic fields were counted and the percentages of positive cells were calculated. The criteria used for assessment were as previously reported [15], where: - (negative, <10 %), + (positive, >10 %) of the tumor cells stained.

Statistical Analysis

SPSS13.0 software for Windows (SPSS Inc, USA) was used for statistical analysis. The independent sample *t* test was used to compare the TAG-72 protein expression levels between HCC and PCLT tissues or NL tissue. A correlation between TAG-72 expression levels and clinicopathologic characteristics in patients with HCC was examined. The chi-square test was used to analyze the expression of TAG-72 protein levels in 244 patients with HCC between tumor tissues and PCLT, and Spearman rank-correlation analysis was used to analyze the correlation between TAG-72 expression levels and clinicopathologic characteristics in patients with HCC. Survival curves were plotted using the Kaplan-Meier method and were analyzed using the log-rank test. The Cox proportional hazards regression model was used to identify factors that were associated independently with survival. All tests were 2-tailed. The p values of less than 0.05 were considered to be statistically significant.

Results

Overexpression of TAG-72 Protein in HCC Tissues

The expression of TAG-72 protein was detected in all of 60 fresh HCC tissues, PCLTs, and NL tissues by Western blot analysis. At first, the single band of TAG-72 protein in Fig. 1 demonstrated the specificity of TAG-72 antibody used in this study. Additionally, HCC tissues expressed significantly higher protein levels of TAG-72 than PCLTs (0.82±0.27 vs 0.66±0.29; P=0.004) and NL tissues (0.82±0.27 vs 0.18±0.11; P=0.002) (Fig. 1). Further Western blot analysis performed in the same samples confirmed that TAG-72 protein in HCC was significantly higher than that in PCLTs (P<0.001) and NL tissues (P<0.001) (Fig. 1).

On the other hand, 244 pairs of HCC and PCLT tissues were analyzed by Immunohistochemical staining. As shown in Fig. 2a, the strong positive staining of TAG-72 was localized in the cytoplasm and membrane of tumor cells, whereas its stainings in the paired PCLT tissues were weak or negative (Fig. 2b). Additionally, the specificity of TAG-72 expression in HCC tissues was also demonstrated by replacing the primary antibody with pre-immuned normal mouse IgG (Fig. 2c). Furthermore, the TAG-72 protein was detected in

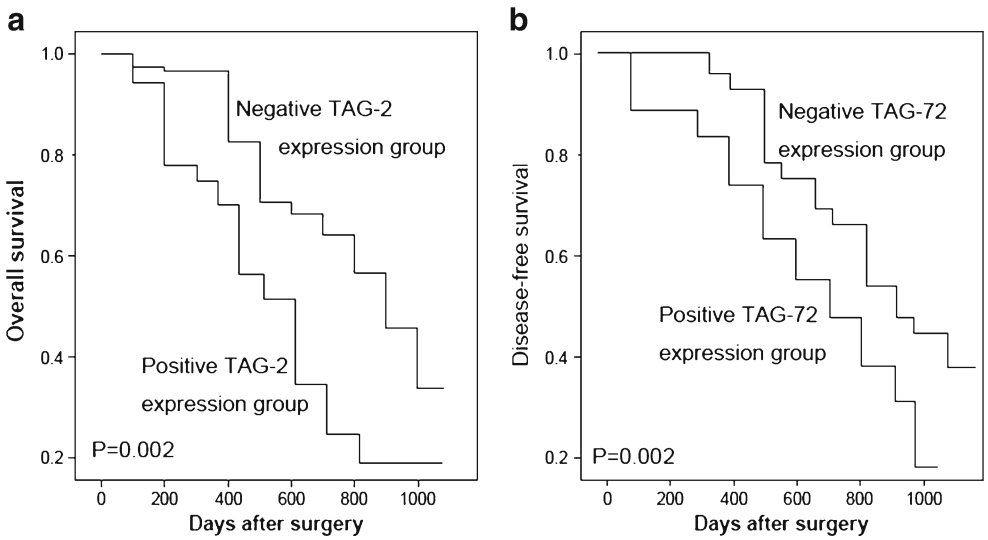

Fig. 3 Kaplan-Meier survival curves of **a** overall survival and **b** disease-free survival in patients with hepatocellular carcinoma (HCC) in patients with positive TAG-72 protein expression and in patients with negative TAG-72 expression based on results from immunohistochemistry. Log-rank tests indicted that patients in the positive TAG-72 protein expression group had lower overall survival and disease-free survival than patients in the negative TAG-72 expression group

218 of 244 HCC tissues and in 66 of 244 PCLTs. The positive expression rate of TAG-72 was significantly higher in HCC tissues than that in PCLTs (89.34 % vs 27.05 %; $P<0.001$).

Correlations between TAG-72 Expression Levels and Clinicopathologic Parameters in HCC Tissues

As shown in Tables 1 and 2, the immunohistochemical staining results indicated that the up-regulation of TAG-72 protein expression was correlated strongly with TNM classification, Edmondson-Steiner grade, multiple tumor nodes and vein invasion. The TAG-72 protein expression levels in tumors at TNM stage III were significantly higher than those in tumors at TNM stage I ($P=0.005$) or TNM stage II ($P=0.026$). Although the expression levels of TAG-72 in tumor tissues from patients with TNM stage I disease were lower than the levels in tissues from patients with TNM stage II disease, the difference was not statistically different ($P=0.089$). There was no significant association between TAG-72 expression and the other clinicopathologic parameters.

Relation Between the TAG-72 Expression Level and Prognosis

To examine the correlation between TAG-72 expression levels and prognosis, patients with HCC were divided into the negative TAG-72 expression group and the positive TAG-72 expression group. The TAG-72 expression level and the prognosis of patients with HCC were analyzed by using the Kaplan-Meier method. The results indicated that patients who had positive TAG-72 expression had a shorter overall survival and disease-free survival than patients who had negative TAG-72 expression (median survival, 282 days vs 376 days; $P=0.002$; median disease-free survival, 218 days vs 316 days; $P=0.002$) (Fig. 3). The multivariate Cox regression analysis indicated that positive TAG-72 expression (relative risk [RR], 1.628; $P=0.008$) and multiple tumor nodes (RR, 1.226; $P=.023$) were independent prognostic factors for survival. The other clinicopathologic parameters did not add any independent prognostic information (Table 3).

Discussion

The roles of TAG-72 in tumor cell invasion and metastasis have been demonstrated immunohistochemically in colon carcinomas, non-small cell lung carcinomas, ovarian epithelial carcinomas, and in the majority of carcinomas of the pancreas, stomach, and esophagus [4–10]. These studies either utilized a lower affinity antibody B72.3 or a second generation, higher affinity monoclonal antibody CC49. It has demonstrated an overexpression of TAG-72 in majority of malignant epithelial cells compared to normal or benign lesions. Due to the high specificity and strong immunoreactivity to target antigen, CC49 antibody has entered in clinical trials for the imaging and treatment of various carcinomas [16]. However, the correlation between TAG-72 expression and HCC patient prognosis has not been documented, and the expression profile and function of TAG-72 in HCC currently remain unknown. We hypothesized that overexpression of TAG-72 plays a potential role in the development of cancer, and it may be a novel prognostic marker for HCC. Thus, we performed the current study to investigate the correlation between TAG-72 expression and prognosis in patients with HCC after surgery. In this study. first, we examined TAG-72 expression profiles in HCC and the correlations between its expression and clinicopathologic parameters and prognosis. Our data revealed that TAG-72 protein levels were significantly higher in HCC tissues than in the corresponding PCLTs and NL tissues.

Table 3 Cox regression analyses of overall survival, TAG-72 protein expression levels, and clinicopathologic parameters

Factors	Univariate analysis			Multivariate analysis		
	P	Relative risk	95 % confidence interval	P	Relative risk	95 % confidence interval
Gender	0.466	1.191	0.960~2.763	0.656	1.062	0.662~1.589
Age	0.389	0.867	0.339~1.398	0.285	0.731	0.386~1.302
Cirrhosis	0.817	0.986	0.558~1.387	0.752	0.778	0.359~1.330
Tumor size	0.057	1.053	0.528~1.903	0.055	1.151	0.548~2.162
No. of tumor nodes	0.012	1.477	1.280~2.156	0.023	1.226	1.08~2.33
Capsular formation	0.066	1.025	0.517~1.958	0.069	1.022	0.252~1.039
Edmondson-Steiner grade	0.028	1.349	1.329~3.710	0.058	1.053	0.261~1.501
TNM stage	0.022	1.218	1.166~3.521	0.055	1.096	0.298~1.327
Vein invasion	0.529	0.739	0.521~1.001	0.507	0.718	0.149~1.403
TAG-72 expression	0.002	1.921	1.058~3.952	0.008	1.628	0.968~3.369

The surgical therapeutic regimen could be determined by the prediction of recurrence, metastasis, and prognosis in patients with HCC after surgery. In this study, we observed that increased expression of TAG-72 in HCC was correlated positively with TNM classification, Edmondson-Steiner grade, tumor vein invasion and multiple tumor nodes, which were well known to be highly correlated with invasion and metastasis and with a poor prognosis in patients who have HCC. Our current study indicates that the up-regulated expression of TAG-72 is correlated with poor survival for patients with HCC. It is noteworthy that, whereas our univariate analysis indicated that positive TAG-72 expression, multiple tumor nodes, Edmondson-Steiner grade and TNM classification were risk factors for prognosis in patients with HCC, our multivariate Cox regression analysis indicated that positive TAG-72 expression and multiple tumor nodes were the only independent risk factors of prognosis for patients with HCC. Thus, our data indicate that TAG-72 expression can serve as a prognostic marker for patients with HCC.

In conclusion, the current study demonstrated for the first time that the increased expression of TAG-72 was correlated with poor survival in patients with HCC, indicating that TAG-72 is a novel prognostic marker for HCC.

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