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RESEARCH

Monoclonal Antibody Preparation and Expression Profile Analysis of a Novel Hepatoma Associated Gene

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Abstract Hepatoma associated gene (*HTA*), a gene screened and cloned by our previous research, was specifically expressed in certain kinds of tumors and had a cancer-promoting role in hepatocellular carcinoma (HCC). To further elucidate the mechanism of *HTA* in hepatoma carcinogenesis and its potential role as a cancer biomarker, refolded HTA protein (HTA) was obtained by prokaryotic recombinant expression system and immobilized metal affinity chromatography. Then anti-HTA monoclonal antibody (mAb) was produced by hybridoma technique. Using the high titer anti-HTA mAb with high specificity obtained, the expression profile of HTA was analysed by immunohistochemistry staining. It showed that HTA expressed specifically in some kinds of tumors, and didn't express in almost any of the normal tissues. The positive expression rate and expression quantity of HTA was significantly higher in HCC tissues than in hepatic cirrhosis tissues, hepatic fibrosis tissues and normal hepatic tissues. The expression of HTA was positively correlated with hepatoma carcinogenic process.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive cancer worldwide with poor prognosis due to lack of early diagnosis and its resistance to conventional chemotherapy [1, 2]. Surgical therapy with liver transplantation or resection remains the mainstay of curative therapy for patients with HCC [3]. It is widely accepted that the dynamic expression changes of tumor associated genes are closely related with tumor evolution thus the researches focus on them can provide powerful tools for earlier diagnosis, molecular biological therapy, better prognosis classification of tumors [4, 5]. A number of HCC associated genes have been discovered and applied as diagnostic markers and therapeutic targets in clinic already [6–8]. However, most of them have their disadvantages in diagnosis and therapy to some extent, such as false positive or lack of specificity [9, 10]. So there is an urgent need of discovering some potent novel biomarkers, to facilitate early diagnosis, to illuminate the carcinogenesis mechanism of tumors, and to identify new drug targets for therapeutic interventions.

Hepatoma associated gene (*HTA*) was a novel tumor associated gene screened through bioinformatic approach and identified by our research group. Reverse transcription-polymerase chain reaction (RT-PCR) revealed that *HTA* gene was specifically expressed in certain kinds of tumors and had a high expression rate in HCC. Knockdown of endogenous *HTA* expression in malignant hepatic cell line HepG2 by small interfering RNA(siRNA) can attenuate cell growth, weaken its tumor formation ability in nude mice [11], Overexpression of

HTA gene in hepatic cell line QSG-7701 via stable transfect can promote its proliferation rate and colony forming ability, and change the cell cycle distribution of the cell lines [12]. These results showed that HTA gene might be a potential therapeutic target in HCC.

The full-length sequence of HTA gene was also cloned by 3'RACE and 5'RACE and identified by Northern blot, which was 1414 bp [12]. According to the bioinformatics analysis, *HTA* was composed of 3 exons and 2 introns and was located at 16q22.3, the Open Reading Frame (ORF) of *HTA* gene encoded a 10KD hydrophilic, alkaline protein. To further study the molecular mechanism of *HTA* promoting canceration in HCC, and to verify the clinical significance of *HTA* as a novel tumor associated gene, specific anti-HTA monoclonal antibody (mAb) was produced by hybridoma technique as a powerful molecular tool for future study. Furthermore, the subcellular location and expression profile of HTA was investigated.

Materials and Methods

Bacterial Strains and Cell Lines

Escherichia coli (E.coli) DH5α and *E.coli* BL21 (DE3) strains, prokaryotic expression vector pET21a(+)-MBP, which can express heterologous fusion protein with a maltose-binding protein (MBP), cell lines HepG2, QGY-7703, QGY-7701, HUVEC and SP2/0 were stored by our laboratory. When they are needed, SP2/0 cells were cultured in RPMI 1640 (Hyclone, USA), supplemented with 15 % fetal bovine serum (FBS). The other cell lines were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, USA), supplemented with 10 % FBS. All the cells are maintained at 37 °C in a 5 % CO_2 incubator.

Sample Collection

All the 356 paraffin sections (173 cancerous tissues and 183 non-cancerous tissues) were acquired from the pathology department of second affiliated hospital of Xiangya medical school of Central South university. All the paraffin sections of cancerous tissues and non-cancerous tissues were pathologically confirmed and collected with clinical information.

Construction of Expression Vectors

Total RNA was extracted from HepG2 cells by homogenization in Trizol reagent (Invitrogen, USA), and first-strand cDNAs were synthesized from 2 μg of DNase I (fermentas, USA)-treated total RNA using First Strand cDNA Synthesis Kit (fermentas, USA) by way of a two-step system. gene-specific polymerase chain reaction (PCR) primers for *HTA* ORF amplification were: 5′-CGCGGATCCATGCTC TGTGTGCCCTTCCT-3′ (sense) and 5′- CCGCTCG AGGCCCAAGATGAAGACAAGGC-3′ (antisense) and introduced with *BamHI* site (sense) and an *Xho I* site (antisense) respectively (underlined). The PCR products of *HTA* gene were purified by 1 % agarose gel electrophoresis then double digested and ligated into the prokaryotic expression vector pET21a(+)-MBP. The recombinated plasmid was transformed into *E.coli* DH5α then identified by PCR, double endonuclease digestion and DNA sequencing.

Expression, Purification and Renaturation of Recombinant Protein

The recombinant plasmid pET21a (+)-MBP-HTA and plasmid pET21a (+)-MBP were transformed into *E.coli* BL21 (DE3) respectively with standard procedures. Isopropyl-b-D- thiogalactopyranoside (IPTG) was added to induce protein expression at 30 °C when the culture's optical density (OD) at 600 nm reached 0.6. To check the expression of MBP-HTA and MBP protein, *E. coli* BL21 (DE3) was induced at different final concentrations of IPTG and different duration. The cells were harvested by centrifugation at 12000 rpm for 2 min and the pellet was resuspended in 50 mmol/L sodium phosphate, with 0.3 mol/L NaCl, and pH8.0. The resuspended cells were lysed by sonication. The cell lyses were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The protein MBP-HTA and MBP was further purified by immobilized metal affinity chromatography (MagExtractor® His-tag protein purification kit, TOYOBO, Japan). The denatured target protein obtained was then refolded in renatured buffer [25 mmol/L Tris-Cl,0.1 mol/L NaCl,10 % glycerol,0.4 mol/L arginine,1 mmol/L glutathione (GSH)/ 0.5 mmol/L glutathione disulfide (GSSG)], let the concentration of urea decrease as grads 1/2, 1/4, 1/8 at intervals of 2 h, then after overnight dialysis in 1×phosphate buffered saline (PBS) in 4 °C, the protein was condensed and stored at −20 °C.

Production of Mouse Monoclonal Antibodies Against HTA

Refolded fusion HTA protein (100 μg) was emulsified with equal volume of Freund's complete adjuvant, and the complex was used to immunize 6–8 weeks old *BALB/c* female mice by intraperitoneally injection. 2 weeks later, the animals were injected with half dose of refolded fusion HTA proteins (50 μg) and equal volume of Freund's incomplete

adjuvant complex. When the titer of mice serum were higher than $1:10^4$, the mice were given a final booster with refolded fusion HTA protein (100 μg) three days before fusing program.

Splenocytes (1×10^8) were fused with 1×10^7 SP2/0 myeloma cells in 45 % polyethylene glycol (PEG). Hybridoma clones were screened by HAT medium (20 % FBS RPMI1640, 10 mM sodium hypoxanthanine, 40 mM aminopterin, 1.6 mM thymidine). Two weeks later, when the clones were visible, indirect enzyme-linked immune-sorbent assay (ELISA) was performed to test all wells. MBP protein negative wells and MBP-HTA protein positive wells were selected as the positive wells. The positive wells were then subcloned twice by way of limiting dilution. Then high concentration anti-HTA monoclonal antibodies were prepared from ascitic fluid of *BALB/c* mice and its isotype was identified by Monoclonal Antibody Isotyping Kits(Zymed, USA) and its sensitivity and specificity were tested by ELISA and western blot.

ELISA

The titers of selected mAb were detected by indirect ELISA. Protein MBP-HTA (10 μg/ml) was added to microtiter plates, incubated overnight at 4 °C. After washed three times, plates were blocked with 2%BSA for 2 h at 37 °C and then incubated with various dilutions of mice immunized serum or anti-HTA monoclonal antibody. After the plates were incubated with conjugated horseradish peroxidase (HRP) anti-murine antibody (KPL, USA) with 1:2000 dilution at 37 °C for 1 h and incubated with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) as substrate for 30 min at 37 °C, the absorbance of 405 nm (A_{405nm})was measured.

SDS-PAGE and Western Blot

QSG-7701 cell line and QSG-7701 cell line stably overexpress HTA gene (constructed and selected in our previous work [12]) was used to detect the specificity of anti-HTA-mAb. Bacterial extracts containing fusion protein and cell extracts containing natural HTA protein and exogenous HTA protein were separated by SDS-PAGE on a 15 % polyacrylamide gel and then transferred onto NC membrane (Millipore, USA) by electroblotting. The membranes were blocked with 5 % non-fat milk and then incubated with 1:2000 dilution of anti-his-tag antibody (Santa Cruz, USA), 1:2000 dilution of anti-β-actin antibody (Santa Cruz, USA) or 1:500 dilution of anti-HTA mAb as primary antibody overnight at 4 °C, then washed with phosphate buffered saline Tween-20(PBST) for three times of 10 min and incubated for 1 h at room temperature (RT) with a secondary anti-murine antibody conjugated HRP. After washing three times with PBST, the membranes were detected with enhanced chemiluminescence (ECL) system (Pierce, USA).

Immunocytochemistry

Appropriate amounts of cells were cultured on sterile glass slides for overnight, after the cell were adherent, the slides were took out and the cells were fixed with 4 % paraformaldehyde for 15 min then permeabilized with 0.5 % Triton X-100 for 10 min. Endogenous peroxidase activity was blocked by incubation in 3 % hydrogen peroxide at RT for 10 min. Non-specific binding was blocked with 2 % bull serum albumin (BSA) at 37 °C for 2 h. 50ul diluted Anti-HTA mAb, anti-β-actin murine mAb (Santa Cruz, USA) as a positive control, and 1×PBS as a negative control was added to three different separated areas on each slide and incubated at 4 °C overnight. Following three washes, slides were incubated with a secondary anti-murine antibody conjugated HRP for 40 min at RT. Diaminobenzidine (DAB) was used as a chromogenic agent. Sections were counterstained with hematoxylin, dehydrated, and mounted. Evaluation of immunohistochemical slides was done with a Nikon Eclipse E800 microscope at×100, ×200 and×400 magnifications.

Immunohistochemistry

Paraffin sections were deparaffinized with xylene and rehydrated in graded alcohol. Then the paraffin sections were submerged in 0.01 M Sodium Citrate (pH6.0) and microwave antigen retrieval were performed. Then the immunohistochemistry experiment was operated according to the same procedures as immuno-cytochemistry part (therein anti-HBxAg murine mAb was provide by Santa Cruz, USA). The intensity of the staining was scored on a scale of − to +++ where −, +, ++ and + + + represented no, weak, moderate and strong staining, respectively. The mean staining scores for tissues and the mean folds of alteration in protein expression were calculated.

Statistical Analysis

All of the values were presented as the mean±standard deviation (SD) for three or more individual experiments. Data were tested with SPSS software (version 13.0, SPSS inc.) for significance. The Pearson correlation coefficient was used to evaluate the correlation. The ratio or constituent ratios were compared using *chi square* test. $\chi 2$ test and symmetric measures was used for paired enumeration data.

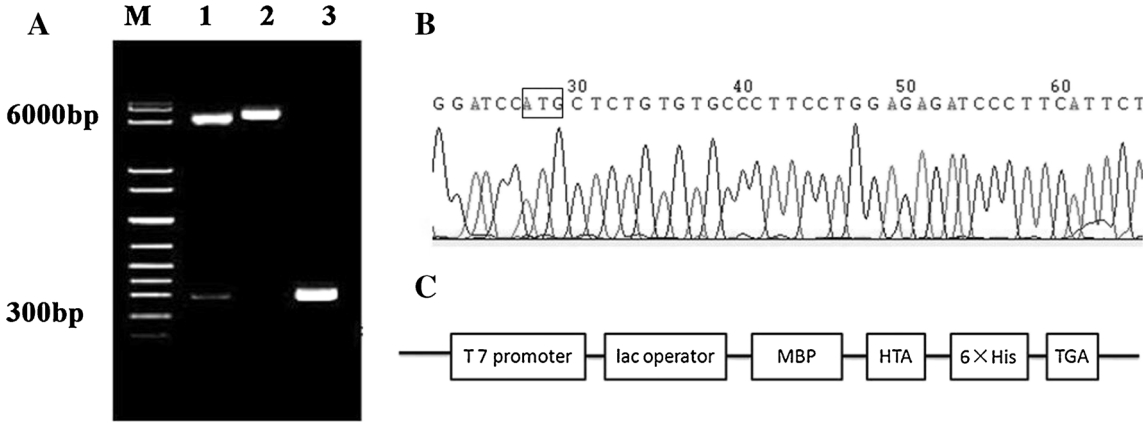

Fig. 1 Construction of plasmid pET21a(+)-MBP-HTA (**a**) Identification of recombinant plasmid pET21a(+)-MBP-HTA by double enzyme digestion and bacterial colony PCR. M: DNA ladder; 1: Double digestion with BamHIand XhoI; 2: pET21a (+)-MBP-HTA without digestion; 3: PCR product of positive bacterial colony. (**b**) Part sequence of the 5′ terminal of pET21a (+)-MBP-HTA plasmid. The square shows the initial codon of *HTA*. (**c**) The schematic diagram of recombinant prokaryotic expression plasmid pET21a (+)-MBP-HTA

When p values<0.05, the differences were considered statistically significant.

Results

Construction of pET21a (+)-MBP-HTA

The recombinant prokaryotic expression plasmid pET21a(+)-MBP-HTA was successfully constructed and verified by bacterial colony PCR, restriction enzyme digestions (Fig. 1a) and complete sequencing (Fig. 1b). The recombinant HTA protein obtained from this plasmid carries an N-terminal MBP-tag and a C-terminal 6×his-tag (Fig. 1c).

Expression and Purification of pET21a (+)-MBP-HTA Recombinant Protein

The expression and purification were identified by running the crude lysate and the elution fractions respectively on a 15 % polyacrylamide gel and subsequently staining with coomassie brilliant blue R250. The molecular weight of MBP-HTA protein obtained was 52kD (Fig. 2a), which was conforming to bioinformatic prediction. Additionally, the cells should be harvested 6 h after induction, as the largest amount of the MBP-HTA protein was produced at this time and which was not influenced by IPTG concentration. The protein was further purified by immobilized metal affinity chromatography (Fig. 2a) and confirmed by western blot using anti-his-tag antibody (Fig. 2b).

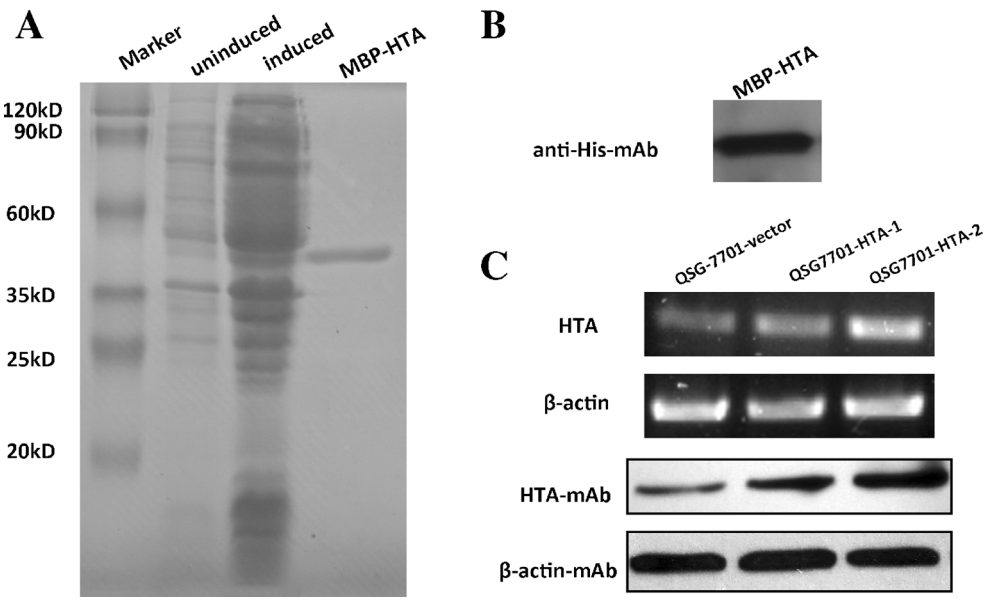

Fig. 2 Purification of MBP-HTA recombinant protein and preparation of anti-HTA mAb (**a**) Expression and purification of MBP-HTA fusion protein. (**b**) Western blot detecting the purified MBP-HTA protein by His-tag antibody. (**c**) Western blot detecting the natural HTA protein in QSG-7701 cell line and HTA overexpress cell lines

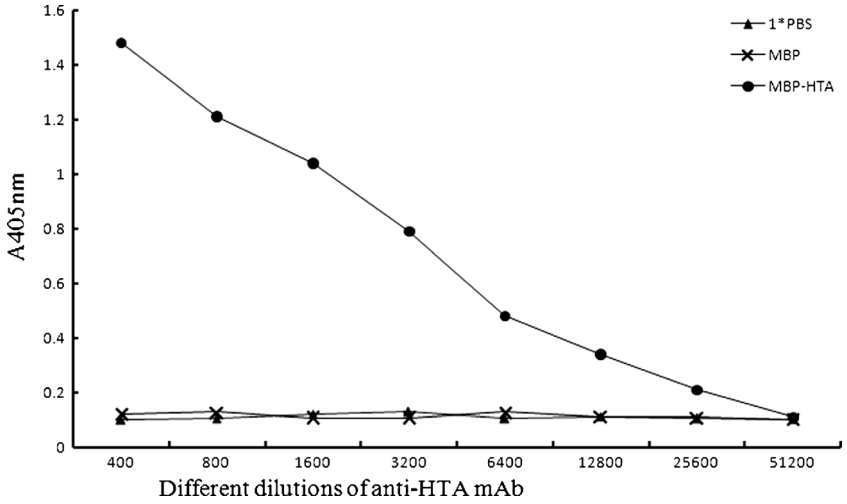

Fig. 3 Titer of anti-HTA mAb detected by indirect ELISA

Preparation of Murine Monoclonal Antibodies Against HTA

Through the procedures of immunization, fusion and clone selection, a hybridoma strain that can stably produce anti-HTA mAb and with a 1:12800 ascitic fluid titer (Fig. 3), was identified as IgG1, λ chain. Western blot assay revealed that the anti-HTA mAb can recognize the natural HTA protein in QSG-7701 cell lines and had a bolder band in QSG-7701 cell lines with HTA gene overexpression (Fig. 2c), which also proved its specificity.

Subcellular Location and Expression Profile of HTA Protein

The result of immunocytochemistry demonstrate that the HTA protein was negative stain in QSG-7701 and HUVEC cell lines and positive stain in HepG2 and QSG-7703 cell lines. The expression of HTA was distributed in the cytoplasm (Fig 4a).

It was detected that HTA expressed in more than half of the HCC and moderately expressed in few tumors such as colon cancer, stomach cancer and lung cancer, but the expression of

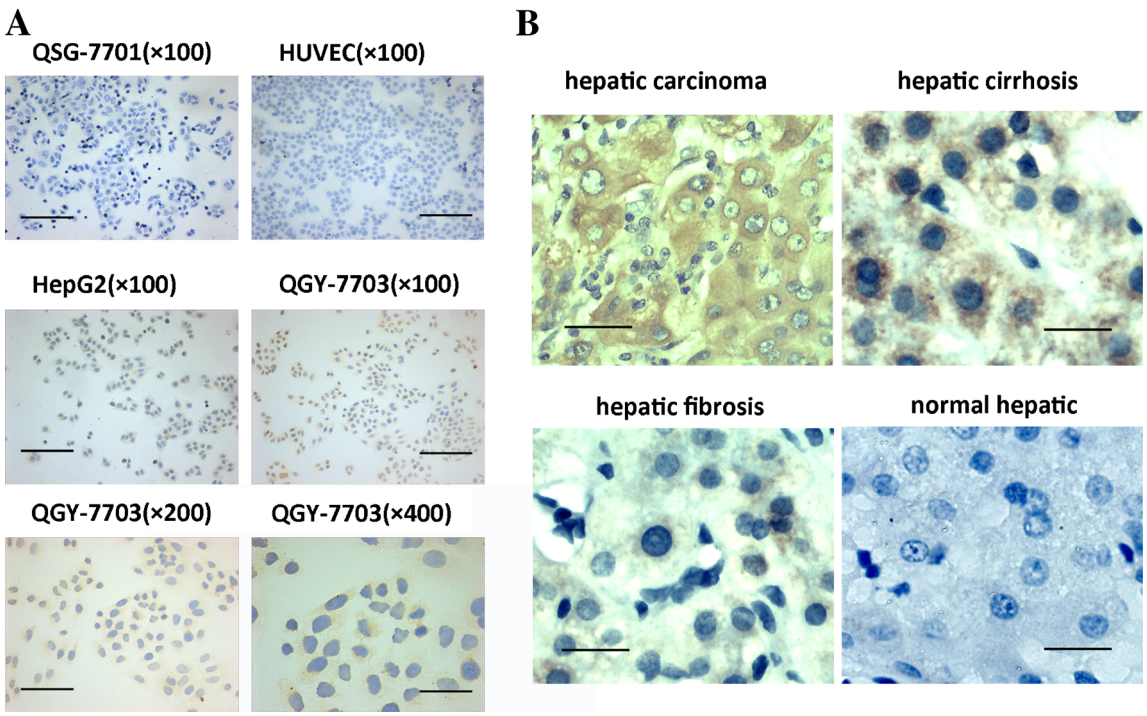

Fig. 4 The expression of HTA protein in different cell lines and different hepatic tissues (**a**) Immunocytochemistry shows expression of HTA protein in different cell lines and subcellular location of HTA. (Bar, ×100:80 μm; ×200: 40 μm; ×400: 20 μm) (**b**) Immunohistochemistry shows the expression of HTA in different hepatic tissues (Bar, ×400: 20 μm)

Table 1 Expression of HTA protein in different carcinoma tissues

Cancer	Total	−	+	++	+++	Positive (rate, %)
Hepatocellular carcinoma	44	21	3	9	11	52.27 %
Colon carcinoma	15	11	2	2	0	26.67 %
Rectal cancer	10	8	0	2	0	20.00 %
Gastric carcinoma	10	8	2	0	0	20.00 %
Lung cancer	28	24	3	1	0	14.29 %
Cervical cancer	13	10	2	1	0	23.08 %
Breast cancer	10	10	0	0	0	0.00 %
Esophageal cancer	9	9	0	0	0	0.00 %
Endometrialcarcinoma	11	11	0	0	0	0.00 %
Bladder cancer	6	6	0	0	0	0.00 %
Renal cancer	5	5	0	0	0	0.00 %
Nasopharyngeal cancer	12	12	0	0	0	0.00 %
Total	173	135	12	15	11	21.97 %

HTA was not detected in almost any of the normal tissues. The specificity of HTA as a tumor marker was 91.2 % (Table 2).

In particular, the expression of HTA was detected in HCC tissues, hepatic cirrhosis tissues, hepatic fibrosis tissues, colon cancer tissues, rectal cancer tissues, gastric carcinoma tissues, lung cancer tissues, cervical cancer tissues, normal hepatic tissues and normal colon tissues, but not in other experimented tissues (Table 1 and 2). The positive expression rate of HTA was especially high in HCC tissues(52.27 %) and the expression quantity of HTA in HCC tissues were significantly higher than that in hepatic cirrhosis tissues, hepatic fibrosis tissues and normal hepatic tissues($p=0.000$). The correlation between the pathological process of HCC and HTA expression was valuated, the Pearson correlation coefficient was 0.429, which

Table 2 Expression of HTA protein in different normal tissues

Tissue-type	Total	−	+	++	+++	Negative (rate, %)
Hepatic	85	71	11	3	0	83.53 %
Colon	11	9	2	0	0	81.82 %
Rectal	7	7	0	0	0	100.00 %
Stomach	9	9	0	0	0	100.00 %
Lung	28	28	0	0	0	100.00 %
Cervix	8	8	0	0	0	100.00 %
Breast	9	9	0	0	0	100.00 %
Esophago	5	5	0	0	0	100.00 %
Endometrium	10	10	0	0	0	100.00 %
Bladder	4	4	0	0	0	100.00 %
Nephridial	2	2	0	0	0	100.00 %
Nasopharynx	5	5	0	0	0	100.00 %
Total	183	167	13	3	0	91.26 %

Table 3 The expression of HTA protein in hepatic tissues

Hepatic tissues	Total	−	+	++	+++	Positive (rate, %)
Hepatocellular carcinoma	44	21	3	9	11	52.27 %
Hepatic cirrhosis	26	22	2	2	0	15.40 %
Hepatic fibrosis	27	22	4	1	0	18.50 %
Normal hepatic	32	27	5	0	0	15.60 %

$\chi^2=38.287$, $P=0.000$, Pearson's R=0.429

suggest the expression of HTA protein was positively correlated with pathological process of HCC (Table 3) (Fig. 4b). Correlation between HTA expression and clinical-pathologic variables in HCC was also analysed, the result showed that the expression of HTA was correlated with the grade and metastasis situation of HCC, but not correlated with sex, age and smoking history of the patients (Table 4). The expression between HTA and HBxAg was also positive relevant according to different hepatic cases test (Table 5). Using $\chi 2$ test and symmetric measures for paired enumeration data, the *Chi-Square* test revealed that there is no difference between HTA and HBxAg expression ($p=1.000$) and the value of $\kappa=0.645$ ($p<0.001$) for *Measure of Agreement*, which means

Table 4 Correlation between HTA protein expression and clinical-pathologic variables in HCC

Variable	*n*	−/+	++/+++	*P* value
Gender				0.124
Male	30	14	16	
Female	14	10	4	
Age				0.911
<50	18	10	8	
≥50	26	14	12	
Smoking history				0.757
Yes	32	17	15	
No	12	7	5	
Grade				0.020
I-II	7	6	1	
II-III	18	12	6	
III-IV	19	6	13	
metastasis				0.008
Yes	19	6	13	
No	25	18	7	

"*n*" represents total sample number for each category, Chi-square test was performed to compare the HTA differential expression levels in different categories of HCCs

Grade I-II: grade I and grade I-II

Grade II-III: grade II, grade III and grade II-III

Grade III-IV: grade III-IV and grade IV

Table 5 The expression relevance of HTA and HBxAg in hepatic tissues

	HBxAg(−/+)	HBxAg (++/+++)	sum
HTA (−/+)	19	4	23
HTA (++/+++)	3	14	17
sum	22	18	40

$p = 1.000$ for $\chi 2$ test, $\kappa = 0.645$ ($p < 0.001$) for *Measure of Agreement*

there was a strong relevance between HTA and HBxAg expression.

Discussion

HTA was a novel tumor associated gene screened and cloned by our research group. There is few study work focus on *HTA* gene so far. Specific expression characteristic and the cancer-promoting effect revealed by knock down and overexpression experiment suggested that *HTA* may play a role in HCC development. In order to detect its expression profile and to explore the role of *HTA* in depth, using recombinated MBP-HTA protein expressed by prokaryotic system, we produced anti-HTA mAb that can be applied in immunohistochemistry and western blot, which can be favorable in the further research of *HTA*.

The *E. coli* heterologous protein expression system was chosen to express HTA protein. The molecular weight of HTA protein was about 10 kD, which was too small to expression alone conveniently, so the prokaryotic expression vector pET21a(+)-MBP was chosen to express the MBP-tagged HTA protein. MBP is a 42.5KDa tropina of *E. coil*, which has no biological activity and is widely used in molecular cloning expression. MBP protein can prevent the target protein from being degradated by endoproteinase and can provide the ligand binding sites for the affinity purification [13, 14]. The recombinant protein MBP and MBP-HTA with 6× his-tag can be expressed in abundance in the form of inclusion body [15–17] and purified by immobilized metal affinity chromatography in under degeneration condition [17].

With the advantage of high purity, high specificity and good reproducibility, monoclonal antibody technology was a potent tool in research [18, 19]. Because the molecular weight of HTA protein was only about 10 KD, and it was predicted that the HTA protein had 4 potential epitopes by bioinformatic software antigic [20–22], the whole protein of HTA with a MBP-tag was used as immune antigen. The anti-HTA mAb obtained from ascitic fluid with the titer of 1:12800 was favorable for following study. However, according to our experiment result, HTA was not a secretory protein and it barely had positive reaction towards the blood serum of HCC patients, so it was regretful that HTA could not be an immune-serological biomarker for early stage HCC screening.

Immunocytochemistry revealed that the HTA protein had specific positive stain in hepatic cancer cell lines rather than normal cell lines, which was according to our estimation. The primary investigation of its subcellular location suggested that HTA protein was distributed in the cytoplasm. However, more accurate detect method was acquired to identify this conclusion. Immunohistochemistry staining showed that HTA protein expressed in some kinds of tumor tissues as well as HCC, and didn't express in almost any of the normal tissues. All the results suggested that HTA had a high specificity as a tumor marker, and a relative higher sensitivity in HCC than other tumors. The expression quantity of HTA was positively correlated with pathological process of HCC (including normal hepatic tissues, hepatic fibrosis tissues, hepatic cirrhosis tissues and HCC), which called attention to the speculation that *HTA* gene might play a role in the early development of HCC. Correlation between HTA protein expression with the grade and metastasis situation of HCC also implied its role in the pathological progress of HCC. Moreover, the expression between HTA and HBxAg was positive relevant according to different hepatic cases test, which suggested that just as HBxAg [23], HTA might have a specific role in malignant evolution of HCC.

Compared to the former study focus on *HTA*, this piece of work promoted the investigation of *HTA* from mRNA level to protein level, and revealed the protein expression profile of HTA for the first time. The expression profile analysed by immunohistochemistry was generally agreed to RT-PCR [11]. The results showed that *HTA* was a potential tumor associated gene, and played a role in HCC progress whose up-regulated mechanism and specific function still need more experiment evidences. Follow-up study of the biological function and molecular mechanism of *HTA* gene by *in vitro* and *in vivo* approaches were underway. High titer anti-HTA mAb obtained in this study can be a useful tool in subsequent thorough study of *HTA*.

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Conflict of Interest Statement We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, *"Monoclonal antibody preparation and expression profile analysis of a novel hepatoma associated gene"*.

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