

# Screening and identification of novel B cell epitopes in human heparanase and their anti-invasion property for hepatocellular carcinoma

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## Abstract

**Background** The aim of this study was to screen and identify novel B cell epitopes within the human heparanase protein and to investigate the impact of self-developed anti-heparanase polypeptide antibodies on growth and invasion of HCCLM6 human hepatocellular carcinoma cells in vitro. **Methods** The flexible regions of secondary structure and the B cell epitopes of the human heparanase amino acid sequence were predicted by DNASTar and Bcepred software. The multiple antigenic peptides (MAP) of the epitopes were synthesized in eight-branched form. Rabbits were immunized with the eight-branched MAPs mixed with the universal T-helper epitope human IL-1 $\beta$  peptide (VQGEESNDK, amino acid 163–171). The immunogenicity of the synthesized peptides was evaluated by ELISA, western blot and immunohistochemistry. The impact of the self-developed rabbit anti-heparanase polyclonal antibodies

on growth and invasion ability of HCCMLM6 cells were analyzed in a cell culture model. The cells were first treated with one of the three antibodies, respectively, and then measured by using MTT, flow cytometry, plate clone formation, invasion assay and heparan sulfate degrading enzyme assay.

**Results** The three amino acid sequences 1–15 (MAP1), 279–293 (MAP2), and 175–189 (MAP3) in the large subunit of the human heparanase protein were predicted as its most potential epitopes. ELISA, western blot and immunohistochemistry analysis showed that all three MAPs were capable to induce high titer of serum antibodies. Antibodies induced by MAP1 and MAP2 were high specific. Furthermore, anti-MAP2 antibodies showed the strongest avidity towards liver cancer tissues. Under the treatment with the three anti-heparanase antibodies, respectively, the growth, cell cycle and clone formation of the cells remained unchanged when compared with a treatment with normal rabbit IgG. However, an inhibition of cell invasiveness and heparanase activity could be detected under the treatment with anti-MAP1- or anti-MAP2-antibody (with a terminal concentration of 100  $\mu$ g/ml). The cell invasiveness was decreased by 54 and 38%, respectively, the heparanase activity by 43 and 39%, respectively.

**Conclusion** The multiple antigenic peptides MAP1 (AC 1–15) and MAP2 (AC 279–293) may be the dominant B cell epitopes in the human heparanase protein. The induced polypeptide antibodies can effectively inhibit the heparanase activity of HCCLM6 liver cancer cells and therefore influence their invasion ability, which provides a theoretic basis for the development of anti-heparanase antibodies and their clinical use as vaccine.

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**Keywords** Heparanase · Hepatocellular carcinoma ·  
B cell epitopes · Epitope identification · Immunogenicity

## Introduction

The heparanase gene is an important cancer-metastasis-associated gene, which is mainly distributed in the cells of immune tissues and organs such as the placenta, spleen, thymus and lymph, without expression in the other mature tissues. However, the products of heparanase gene expression are ubiquitously found in the tumor cell lines and metastatic tumor cells. Heparanase is one of the essential enzymes for destroying the extracellular matrix and basement membrane [1]. Recently, it was discovered that heparanase inhibitors can effectively suppress the invasion and metastasis of tumors. Therefore, heparanase is regarded as an important target molecule for anti-metastasis treatment of tumors [2–6]. The heparanase precursor protein has a molecular weight of about 65 kDa. It is a hetero dimer consisting of two subunits, with a molecular weight of 50 and 8 kDa, respectively. The large subunit represents its mature active form [7]. According to the primary structure of heparanase and on the basis of predicting the heparanase B cell epitopes via bioinformatics, we designed and synthesized the multiple antigen peptide (MAP) using the eight-branched polypeptide modus [8, 9]; meanwhile, interleukin-1 $\beta$  163–171 peptide was used as the universal helper epitope peptide (Th) due to its enhancing properties of antigen peptide cellular immunity and humoral immune response [10]. By combining the above two substances, the animals were immunized to induce the polyclonal antibodies. ELISA test, immunoblotting and immunohistochemistry were used to identify the antibody titers, their specificity and the antigen expression in liver cancer tissues and cells; then the cell growth curve, cell cycle analysis, determination of heparanase activity and invasion assay were carried out to study the impact of those MAP-induced antibodies on HCCLM6 hepatocarcinoma cell growth and invasion ability *in vitro*, so as to provide an experimental basis for the heparanase-targeted anti-tumor metastasis research.

## Materials and methods

### Experimental materials

Heparanase protein standards and heparanase degrading enzyme assay kit (Code No. MK412) were purchased from Takara Bio Inc. (Japan). Polyclonal rabbit anti-human heparanase 1 (HPA1) antibody (Catalog No. INS-26-2-0000-11, 0.24 mg/100  $\mu$ l), a protein G affinity purified polyclonal antibody raised against the 50–8 kDa heparanase heterodimer, was used from InSight Biopharmaceuticals Ltd (Israel). Freund's adjuvant complete and incomplete (F588, F5506) were provided by Sigma-Aldrich Corpora-

tion (USA). Peroxidase-conjugated goat anti-rabbit IgG (Catalog No. BA1054) was provided by Boster Biotechnology Co., Ltd (Wuhan, China). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Catalog No. 111-035-470) was obtained from Jackson Immuno Research (USA). Protein marker (Precision Plus Protein Western C Standards, Catalog No. 161-0376) was provided by Bio-Rad Laboratories (USA). General western blot detection kit was purchased from KangChen Bio-Tech Inc. (Shanghai, China). Histostain<sup>TM</sup>-Plus kits (SP-9000) and 3,3'-diaminobenzidine tetrahydrochloride (ZLI-9032) were purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd (Beijing, China). Flow cytometry (Beckman-coulter eplcsxl) was supplied by Beckman Coulter, Inc. (USA). QCM<sup>TM</sup> 24-well cell invasion assay kit (Catalog No. ECM554) was purchased from Chemicon International, Inc. (USA). Human liver cancer cell line HCCLM6 [11, 12] was supplied by the Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). The white-hair-black-eye rabbits, derived from Japanese big-ear white rabbits, were supplied by Animal Experimental Center of Zhejiang Chinese Medical University (Hangzhou, China).

### Predication of B cell epitopes and polypeptide synthesis

DNAS<sup>TM</sup> software and Bcepred online predication tool were used to analyze the active form of the human heparanase protein, i.e. a 386 amino acid long sequence from the C-terminus region of the large subunit, by also taking into account the physical and chemical properties such as hydrophilicity, accessibility and plasticity, which means ability to change or deform, etc. of the large subunit. Thus, the antigenicity of a high hydrophilic region within the heparanase protein was assessed. By combining these two analytic methods, the overlap region of the predication results was regarded as the most probable B cell dominant epitope of the heparanase protein. Based on the selected amino acid sequences, Chinese Peptide Company (Hangzhou) was entrusted to synthesize the polypeptides with the Solid-Phase Peptide Synthesizer by adopting eight-branched peptide design. The purity of the polypeptides can reach 97% or more by purification through HPLC.

### Binding affinity between MAP and commercialized heparanase antibody

Ninety-six well ELISA plates were coated with different MAPs with a final concentration of 1  $\mu$ g/100  $\mu$ l per well, respectively, overnight at 4°C. After blocking with cattle serum protein, 0.06  $\mu$ g/100  $\mu$ l per well of the commercialized heparanase antibodies were added. As control,

normal rabbit serum in a dilution of 1:4,000 was used. The results were denoted by the average OD value of double wells.

#### Animal immunization

Eight 6-month-old male white-hair–black-eye rabbits, each weighing 2.5 kg, were randomly divided into four groups (MAP1, MAP2, MAP3, and control group), each group including two rabbits. Eight-branched peptide MAP1, MAP2, and MAP3 were used to immunize the rabbits of the group MAP1, MAP2, and MAP3, respectively, by four times injection with an interval of once every 2 weeks. Freund's complete adjuvant was used for the first immunization and Freund's incomplete adjuvant was used for strengthening the immunization, with a dose of 1 mg MAP/rabbit mixed with an equal amount of Th linear peptide and dissolved in 0.5 ml PBS solution. After emulsified with 0.5 ml Freund's adjuvant, multi-point hypodermic injection was performed in the back region of the animals. For the control group, 0.5 ml PBS was completely emulsified with equal amount of Freund's adjuvant, and then injected.

#### Serum isolation and antibody detection

Blood samples were taken before the first immunization and 10 days after each immunization, five times in total. The samples were taken from the central artery of the animals' ears for the first four times and taken from the carotid artery for the final time. The blood was collected and the serum was separated. A standardized indirect ELISA was performed to determine the antibody titers in the serum. The 96-well ELISA plates were coated with MAP at concentration of 10 µg/ml and the Th peptide, respectively, according to 100 µl/well, overnight at 4°C. After blocked with cattle serum protein, the multiple-diluted animal immunized serum was used as the primary antibody, the pre-immunized serum was used as the control. A ratio (sample OD value/OD value of negative control) > 2.1 was taken as the positive standard. The maximum dilution still showing a positive reaction was defined as the antibody titer of the sample.

#### Protein electrophoresis and western blot

The HCCLM6 hepatocarcinoma cells ( $3 \times 10^7$ ) of passage culture were lysed, subsequently, the cell proteins were extracted, and an SDS-PAGE was performed. The primary antibody was either one of the self-made three types of rabbit anti-serum with a dilution of 1:3,000, or the commercialized heparanase antibody with a dilution of 1:200. The pre-immune rabbit serum was used as negative control.

Immunoreactive bands were detected using chemiluminescence (KangChen Bio-Tech Inc.).

#### Immunohistochemistry analysis

Paraffin sections of positive staining with the commercialized heparanase antibody from 31 cases of hepatocellular carcinoma from 1999 to 2007 in the hospital were selected (14 cases of hepatoma without metastasis, 10 cases of hepatoma accompanied by metastasis and 7 cases of hepatoma metastasis loci). In addition, the paraffin sections of four cases with normal liver tissue were used as control.

The sera of the MAP1, MAP2, and MAP3 immunized rabbits were used as primary antibody. The commercialized heparanase antibody was taken as positive control, while normal rabbit serum and PBS were used as negative control instead of the primary antibody. The scoring standard of immunohistochemical results were based on the coloring of the cancer cells and on the percentage of positive cells: I. color: no staining: 0, light yellow: 1, brown: 2, dark brown: 3. II. percentage: negative: 0, the percentage of positive cells  $\leq 10\%$ : 1, 11–50%: 2,  $\geq 51\%$ : 3. The intensity of heparanase expression was denoted by the product of cancer cells staining intensity and positive cell percentage: 0–2 scores: –, 3–4 scores: +, 5–7 scores: ++, and 8–9 scores: +++.

#### Cell growth and cell cycle

An MTT was performed for the cell growth curve. The HCCLM6 cells in the logarithmic phase were made into single-cell suspension after digested with trypsin, inoculated in a 96-well plate according to a density of 2,000 cells/200 µl/well and then cultured at 37°C, 5% CO<sub>2</sub> and condition of saturated humidity. The MAP1-, MAP2- and MAP3-derived antibodies were added till the final concentration of 0 (i.e. the control group), 10, 50, 100 µg/ml, respectively, or 100 µg/ml normal rabbit IgG in the culture solution was reached. Afterwards the cells were cultured for 8 days. MTT and DMSO were added every 24 h. Three wells were established for each group of cells. The absorbance value of each well was determined at 490 nm. The cell cycle was determined by flow cytometry, three batches of samples for each group.

#### Plate colony formation

The different groups of HCCLM6 cells were treated with the MAP1-, MAP2- and MAP3-derived antibodies with a final concentration of 0, 10, 50, 100 µg/ml or normal rabbit IgG of 100 µg/ml for 48 h. Then, the single-cell suspension of HCCLM6 was prepared and inoculated in 24-well plates with a density of  $5 \times 10^2$  cells per well and 4 wells per group and cultured for 12 days. Afterwards, Giemsa

staining was performed and the number of colonies with more than 50 cells was counted under the microscope.

#### Heparanase activity assay

This assay is based on the principle of detecting the amount of heparan sulfate substrate remaining after digestion of enzyme substrate reaction. The heparanase activity was calculated by measuring the absorbance at 450 nm, which reflects the content of heparan sulfate not being degraded by heparanase, and by referring the value to a standard curve. The methods and procedures were followed as indicated in the instruction: 0.5 U/well of a standard heparanase product was added to 96-well plate and then incubated with a final concentration of either 0, 10, 50, 100 µg/ml of MAP1-, MAP2-, and MAP3-derived antibodies or 100 µg/ml of normal rabbit IgG as control in the culture medium for 1 h at 37°C. Three wells were established for each group to measure the heparanase activity, so as to understand the inhibition of the activity of the heparanase standard product by these antibodies. Furthermore, HCCLM6 cells of a density of  $5 \times 10^4$ /well were inoculated in a 24-well plate and after cultured for 48 h, treated with the final concentration of 0, 10, 50, 100 µg/ml of anti-MAP1, -MAP2, and -MAP3 peptide antibodies or 100 µg/ml of normal rabbit IgG as control in culture medium for 1 h at 37°C. The supernatant of the culture medium was fetched to determine the heparanase activity so as to understand the inhibition of the polypeptide antibodies on the heparanase activity of HCCLM6 cells.

#### Cell invasion assay

Transwell invasion chamber was used to perform the test, which is similar to the methods reported previously [13]. The culture medium containing 10% of fetal cattle serum was added to the bottom part, and  $1.25 \times 10^5$ /well HCCLM6 cells were plated in the upper part. For each group a triple measurement was realized. Under the condition of 37°C, 5% CO<sub>2</sub> and saturated humidity, the cells were cultured with a final concentration of either 0, 10, 50, 100 µg/ml of MAP1-, MAP2-, and MAP3-derived antibodies, respectively, or 100 µg/ml of normal rabbit IgG for 48 h and afterwards stained by Wright–Giemsa. Five 200-multiple microscopic visions were randomly selected to calculate the total count of the cells. The relative number of cells having penetrated the artificial basement membrane was used to denote the invasion ability of the tumor cells.

#### Statistics

SPSS 10.0 and SigmaPlot 10.0 statistical software were used for data processing. There was a statistically significant difference if  $P < 0.05$ .

## Results

#### B cell epitope prediction and polypeptide synthesis

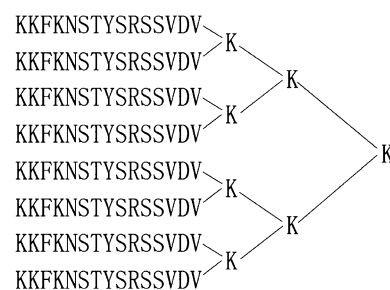
Bcepred analysis predicted that the possible B cell epitopes of the large heparanase subunit are the peptide fragments of the sites 4–12, 52–58, 82–88, 111–117, 144–152, 174–181, 267–273, and 279–291 of the amino acid sequence, while the DNASTar software predicted the peptide fragments of the sites 1–15, 279–293, 175–189, 185–194, and 54–70. The obtained results are basically the same. Combining the results of these two analytic methods, it is assumed that the peptide fragments 1–15, 279–293, and 175–189 of the large heparanase subunit are most probably the dominant B cell epitopes. These three fragments are located in the extensive structure or non-regular coils of the protein and their hydrophilicity, accessibility and plasticity are in favor of this assumption. Therefore, we synthesized three-eight-branched peptide MAPs according to the amino acid sequence sites 1–15 (KKFKNSTYSRSSVDV, MAP1), 279–293 (HCTNTDNPRYKEGDL, MAP2), and 175–189 (STRPGKKVWLGETSS, MAP3) of the large heparanase subunit, respectively (Fig. 1).

#### Binding affinity between synthesized MAP and commercialized heparanase antibody

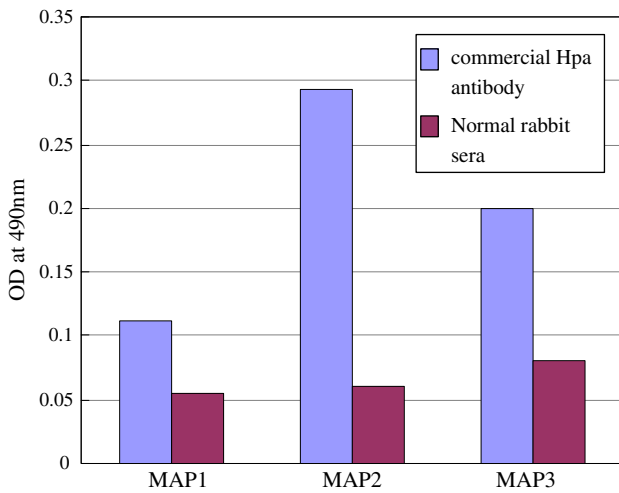
An indirect ELISA test was used to detect the binding affinity of the commercialized heparanase antibody towards the three synthesized MAP polypeptides. The coated MAP polypeptides reacted with the commercialized heparanase antibody (1:4,000 dilution) or the normal rabbit serum (1:4,000 dilution), respectively. The results showed that the binding affinity between the MAP2 polypeptide and commercialized heparanase antibody was significantly stronger than that between MAP1 and MAP3 and the antibody (Fig. 2).

#### Dynamic changes of rabbit antibody titers

Specific antibodies can be detected 10 days after the first immunization. The average maximum antibody titers of the rabbits after MAP1, MAP2 or MAP3 immunization were



**Fig. 1** Schematic structure of heparanase-1-15 (MAP1) eight-branched peptide

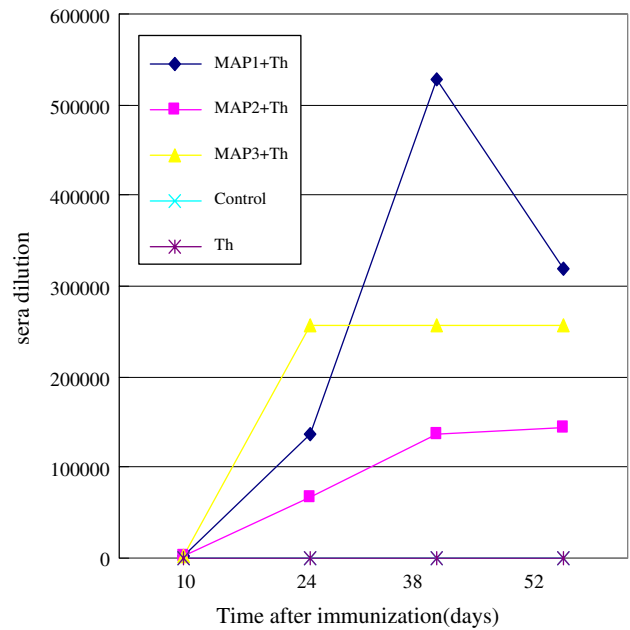


**Fig. 2** Binding affinity of different MAPs with the commercialized rabbit anti-heparanase (Hpa) antibody and control rabbit sera 96-well ELISA plates were coated with different MAPs with a final concentration of 1 µg/100 µl per well, respectively, overnight at 4°C. After blocking with cattle serum protein, 0.06 µg/100 µl per well of the commercialized heparanase antibodies were added. As control, normal rabbit serum in a dilution of 1:4,000 was used. The results were denoted by the average OD value of double wells. The results showed that the binding affinity between the MAP2 polypeptide and commercialized heparanase antibody was significantly stronger than that between MAP1 or MAP3 and the antibody

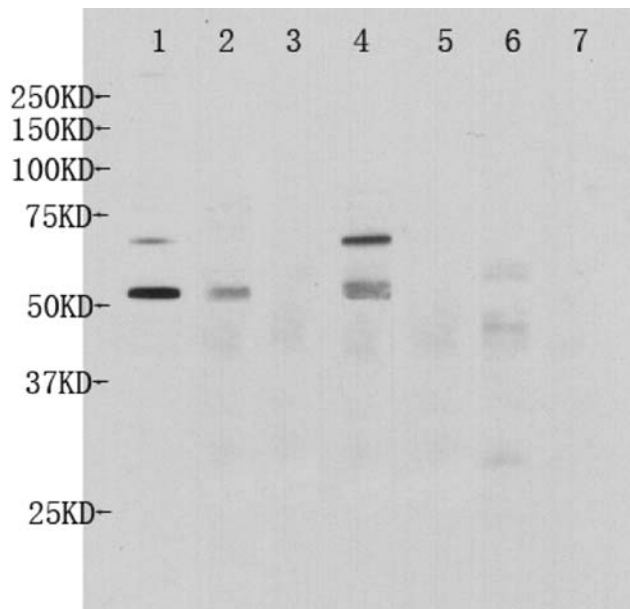
1:520,000, 1:140,000, and 1:250,000, respectively. The antibody titer of MAP1 immunized animals reached a peak after the third immunization, while the antibody titer of MAP2 and MAP3 immunized rabbits reached a platform stage after the third or second immunization alternatively. No antibodies were produced in the normal group, and no reaction was performed between the Th peptide and the three kinds of antisera (Fig. 3).

**Protein electrophoresis and western blot**

The total protein extract of the HCCLM6 cells was used as sample to react with the three kinds of *anti-MAP* sera. The commercialized heparanase antibody was used as positive control and the corresponding rabbit pre-immunized serum was used as negative control. A very clear band of about 50 kDa and a clear band of about 65 kDa were shown in the commercialized antibody group. In the MAP1 antiserum group, only 50 kDa protein band was found; whereas in the MAP2 antiserum group, both 65 and 50 kDa bands were detected. Based on the commercialized antibody instructions, the protein of 65 kDa is the precursor of heparanase protein, whereas 50 kDa protein corresponds to the large subunit of heparanase protein. In contrast, no band was found in the expected locations for the MAP3 antiserum group and for the corresponding negative controls (Fig. 4).

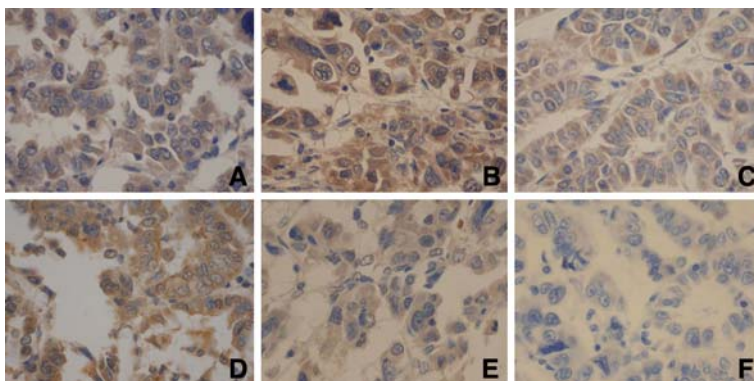


**Fig. 3** Dynamic curve of anti-MAP antibody titers in rabbit sera The specific antibody can be detected ten days after the first immunization. The average maximum antibody titers of the two rabbits after MAP1, MAP2 or MAP3 immunization were 1:520,000, 1:140,000 and 1:250,000, respectively. No antibody was produced in the normal group, and no reaction was performed between the Th peptide and three kinds of antisera



**Fig. 4** Specificity of the rabbit antisera against MAPs measured by western blotting. 1 Commercialized heparanase antibody (1:200); 2, 4 and 6 rabbit MAP1-, MAP2- and MAP3-antisera, respectively (1:3,000); 3, 5 and 7 the corresponding pre-immunized rabbit serum negative control, respectively (1:3,000)

**Fig. 5** Expression of heparanase MAP antigens in the hepatocellular carcinoma. Six pictures were from the sections of the same paraffin block. **a** MAP1 antisera, **b** MAP2 antisera, **c** MAP3 antisera, **d** commercialized heparanase antibody, **e** normal rabbit sera, **f** PBS. SP  $\times$  400



**Table 1** Impact of anti-MAP antibodies on cell cycle and clone formation of HCCLM6 cells

Groups	G0/G1 (%)	S (%)	G2/M (%)	Clones
MAP1 antisera	65.1 $\pm$ 6.13*	27.4 $\pm$ 4.19*	7.3 $\pm$ 1.24*	36.02 $\pm$ 1.23**
MAP2 antisera	67.7 $\pm$ 5.97*	25.3 $\pm$ 3.48*	7.8 $\pm$ 2.05*	40.30 $\pm$ 2.34**
MAP3 antisera	66.3 $\pm$ 3.96*	26.3 $\pm$ 7.49*	8.2 $\pm$ 1.07*	39.30 $\pm$ 3.45**
Normal rabbit IgG	67.9 $\pm$ 7.43	23.4 $\pm$ 5.08	8.1 $\pm$ 1.37	38.87 $\pm$ 3.42
Blank control	68.4 $\pm$ 8.32	22.1 $\pm$ 3.61	8.0 $\pm$ 2.33	42.24 $\pm$ 2.56

\*  $P > 0.05$ : cell cycles, MAPs (100  $\mu$ g/ml) as compared with of normal rabbit IgG (100  $\mu$ g/ml) or blank control group

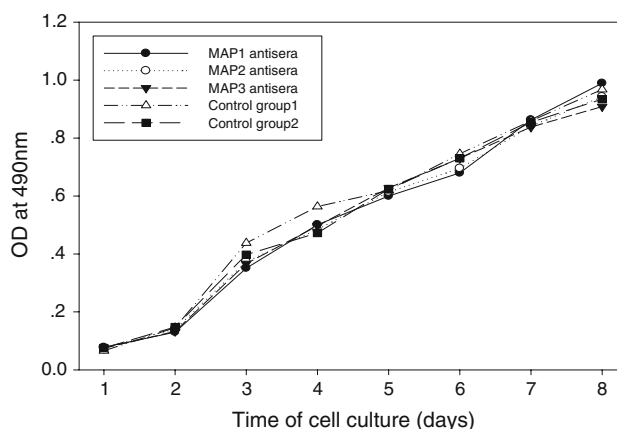
\*\*  $P > 0.05$ : clone numbers, MAPs (100  $\mu$ g/ml) as compared with of normal rabbit IgG (100  $\mu$ g/ml) or blank control group

#### Expression of polypeptide antigen in the hepatoma tissues

Immunohistochemical staining was obtained by using commercialized heparanase antibody and MAP2 antiserum as primary antibody. Both antibodies stained the cell plasma of hepatoma tissue dark brown, while the cell plasma was stained light yellow when MAP1 or MAP3 antiserum and the normal rabbit serum were used as the primary antibody. No staining of the cell plasma was detected in the negative control group with PBS (Fig. 5). According to the scoring standard of the immunohistochemistry staining, the results were positive for all 31 cases of hepatoma tissue sections for the commercialized antibody and MAP2 antiserum. The corresponding antigens were located in the cancer cell plasma, and the expression intensity grew between hepatocellular carcinoma without metastasis, hepatocellular carcinoma with metastasis and hepatocellular carcinoma metastasis loci; the staining with MAP1 and MAP3 antiserum were both negative. However, the four cases of normal liver tissues were all negative by staining with commercialized heparanase antibody as well as all three kinds of MAP antisera.

#### Impact of anti-MAP antibodies on the hepatoma cell growth and clone formation

The different concentrations of anti-MAP1, -MAP2, and -MAP3 antibodies all showed no significant impact on the

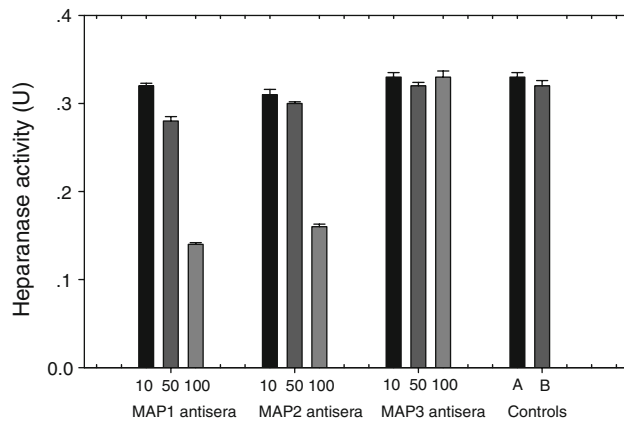


**Fig. 6** Growth curves of HCCLM6 cells under treatment with anti-MAP antibodies Anti-MAP1, -MAP2 and -MAP3 antibodies (100  $\mu$ g/ml) had no significant impact on the cell growth curves as compared with normal rabbit IgG (100  $\mu$ g/ml) or blank control group

cell growth curve, cell cycle and the plate clone formation ability of the HCCLM6 liver cancer cells (Table 1; Fig. 6).

#### Inhibition of the heparanase activity by anti-MAP antibodies

After adding anti-MAP1, -MAP2, and -MAP3 antibodies, respectively, to the cell culture medium containing heparanase standard product and after an incubation time of 1 h at 37°C, the heparanase activity decreased by 58 and 52%,



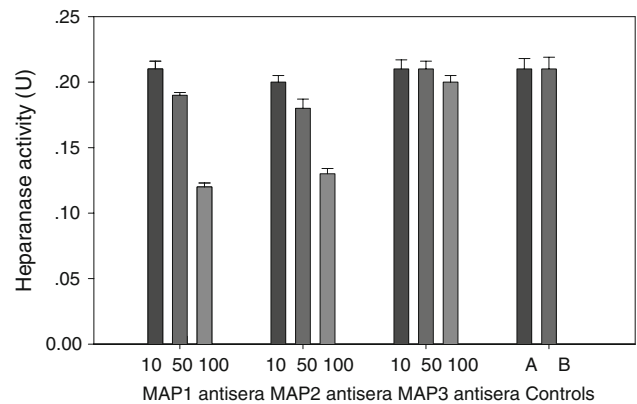
**Fig. 7** Inhibition of anti-MAP antibodies on heparanase enzyme activity. After adding anti-MAP1, -MAP2 and -MAP3 antibodies, respectively, to the cell culture medium containing heparanase standard product as commercial enzyme and after an incubation time of 1 h at 37°C, the heparanase activity decreased by 58 and 52%, respectively, after treatment with anti-MAP1 or -MAP2 antibodies with a final concentration of 100 µg/ml comparing to the blank control and the normal rabbit IgG control group ( $P < 0.01$ )

respectively, after treatment with anti-MAP1 or -MAP2 antibodies with a final concentration of 100 µg/ml comparing to the blank control group and the normal rabbit IgG control group ( $P < 0.01$ ). When treated with a final concentration of 50 µg/ml, a decrease of 16% was detected for anti-MAP1 and of 10% for anti-MAP2 ( $P < 0.05$ ). No decrease of the heparanase activity could be found under treatment with a final antibody concentration of 10 µg/ml. An effect of anti-MAP3 antibodies could not be measured at any concentration (Fig. 7).

Anti-MAP1, -MAP2, and -MAP3 antibodies were added to the cell culture medium and HCCLM6 cells were cultured for 1 h under this condition. The heparanase activity of the culture supernatant, compared with the blank control group and the control group after adding normal rabbit IgG, was decreased by 43 and 39% ( $P < 0.01$ ), respectively, after incubation with anti-MAP1 or -MAP2 antibodies of a final concentration of 100 µg/ml; under treatment with anti-MAP1 or -MAP2 antibodies of a final concentration of 50 µg/ml, a decrease by 10 and 5% ( $P < 0.05$ ) could be measured; no decrease of the heparanase activity could be found when anti-MAP1 or -MAP2 antibodies of a final concentration of 10 µg/ml or anti-MAP3 antibodies of a final concentration of 10–100 µg/ml were added (Fig. 8).

#### Inhibition of the invasion of hepatoma cells by anti-MAP antibodies in vitro

Anti-MAP1, -MAP2, and -MAP3 antibodies with a final concentration of 0, 10, 50, 100 or 100 µg/ml of normal rabbit IgG were added to the upper part of the invasion chamber. After HCCLM6 cells were cultured for 48 h with



**Fig. 8** Inhibition by anti-MAP antibodies on the activity of heparanase in HCCLM6 culture medium. Anti-MAP1, -MAP2 and -MAP3 antibodies were added to the cell culture medium and HCCLM6 cells were cultured for 1 h under this condition. The heparanase activity of the culture supernatant, compared with the blank control group and the control group after adding normal rabbit IgG, was decreased by 43 and 39% ( $P < 0.01$ ), respectively, after incubation with anti-MAP1 or -MAP2 antibodies of a final concentration of 100 µg/ml

a final concentration of 100 µg/ml anti-MAP1 or -MAP2 antibodies, the amount of penetrated cells was significantly decreased within 48 h in the transwell invasion assay with an inhibition rate of 54 and 38%, respectively, comparing to the normal rabbit IgG control group and the blank control group ( $P < 0.05$ ); the inhibition rate was 14 and 7%, respectively, by using a final concentration of 50 µg/ml anti-MAP1 or -MAP2 antibodies ( $P < 0.05$ ). The amount of penetrated cells was not decreased under treatment with a final concentration of 10 µg/ml anti-MAP1 or -MAP2 antibodies. Concentrations of 10–100 µg/ml of anti-MAP3 antibodies showed no influence on the penetration rate (Table 2; Fig. 9).

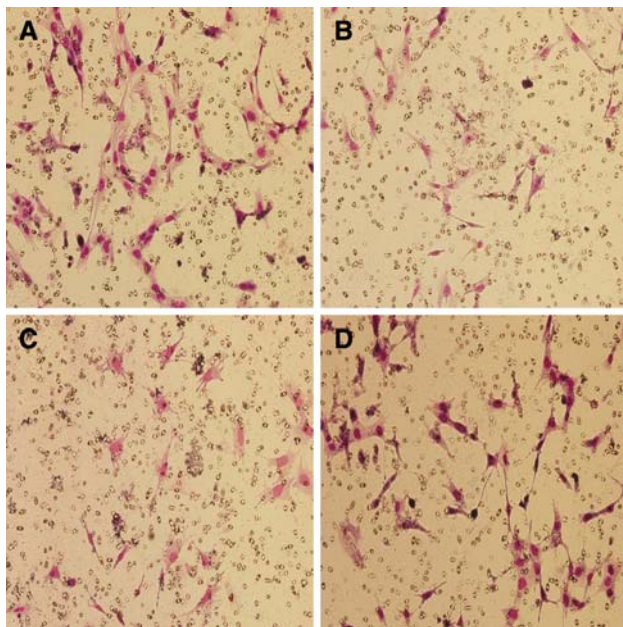
#### Discussion

Development of anti-cancer vaccines by application of tumor-associated antigens is expected to be promising [14, 15]. For tumor immunotherapy, main focus lies on epitope peptide vaccines, which have advantages like consisting of simple components, having a strong pertinency for inducing immune response, etc. But due to its small molecular weight and single structure, the immunogenicity is weak and thus an ideal immune response in the body cannot be induced. Traditionally, the epitope peptide crosslinking carrier protein method was used to improve the immunogenicity of epitope peptides [16], but since the carrier proteins are foreign antigenic macromolecules, the induced antibodies are often against the carrier proteins instead of against the target epitopes. In recent years, a MAP design plan was proposed. Lysine core matrix of small molecular weight and weak immunogenicity was taken to couple a number of

**Table 2** Inhibition of the invasion of HCCML6 cells by anti-MAP antibodies in vitro

Groups	Numbers of penetrated cells in transwell invasion assay			
	0 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$
MAP1 antisera		40.6 $\pm$ 3.1	34.7 $\pm$ 2.6*	18.8 $\pm$ 1.8*
MAP2 antisera		39.7 $\pm$ 3.4	37.5 $\pm$ 2.4*	25.1 $\pm$ 1.9*
MAP3 antisera		41.3 $\pm$ 4.6	40.9 $\pm$ 3.2	40.4 $\pm$ 2.9
Normal rabbit IgG				39.6 $\pm$ 3.6
Blank group	40.2 $\pm$ 4.3			

\*  $P < 0.05$ , numbers of the penetrated cells as compared with that in normal rabbit IgG (100  $\mu\text{g/ml}$ ) or blank control group



**Fig. 9** Penetrated HCCML6 cells (red) in transwell invasion assay (Wright–Giemsa staining,  $\times 200$ ). **a** No inhibition of HCCML6 cell invasion by normal rabbit IgG (100  $\mu\text{g/ml}$ ). **b** HCCML6 cell invasion inhibited by MAP1 antisera (100  $\mu\text{g/ml}$ ). **c** HCCML6 cell invasion inhibited by MAP2 antisera (100  $\mu\text{g/ml}$ ). **d** No inhibition of HCCML6 cell invasion by MAP3 antisera (100  $\mu\text{g/ml}$ )

monomeric peptides and to form a dendritic structure. Such a design model can simulate the natural epitope conformation to induce the formation of specific antibodies [17, 18], but the induced antibody titers were still not high. Recently, some researchers proposed that a combined use of MAP and Th epitope peptide can induce an ideal antibody titer [10]. This has also been confirmed in our research.

Bioinformatics is extensively used in the vaccine research and development. In this study, DNASTar software and Bcepred online prediction tool were used to obtain three heparanase B cell epitope peptides. Considering that most B cell epitopes are the conformational epitopes, only the epitopes located at consecutive peptide fragments can be used for antigen peptide design. According to the protein structural characteristics and the 3D structural model of heparanase, three epitopes are found: peptide fragment

1–15 is located at the N-terminus of the heparanase large subunit and peptide fragment 279–293 peptide is located at the C-terminus of the heparanase large subunit, while fragment 175–189 is located at the active zone of the heparanase protein [19]. Only these peptide fragments form a relatively independent of the 3D structure, therefore, which can be used for the design of B cell epitope peptide.

To validate whether the predicted epitopes have specific immunogenicity, we synthesized the corresponding MAPs and immunized rabbits under co-stimulation with the Th epitope peptide, followed by isolation of the polyclonal sera. In our study, MAP1 (KKFKNSTYSRSSVDV) has one more amino acid than the reported sequence KKFKNSTYSRSSVD (158–171) by Zetser et al. [20]; and MAP2 (HCTNTDNPYKEGDL) is close to the sequence 411–432 mentioned by Levy-Adam et al. [21], which has seven amino acids in common with the reported sequence KLRVYLHCTNTDN (430–422) by Dempsey et al. [22]; but the MAP3 sequence has not yet been reported. ELISA showed that all three MAPs can induce the production of antibodies of high titers. Western blot results showed one clear band when using MAP1 antiserum appearing at about 50 kDa and two clear bands for MAP2 antiserum located at about 65 and 50 kDa. No bands appeared at the target site for MAP3 antiserum. These results indicate that the antibodies induced by MAP1 can only bind to the large subunit of heparanase, but not to the heparanase precursor protein. MAP1 is probably located at the site where the large subunit of heparanase connects with the peptides of large and small subunits. It is a dominant epitope in the mature heparanase protein, but since this epitope is covered in the heparanase precursor protein, MAP1 antibodies cannot bind to the precursor protein. MAP2 antibodies can bind to heparanase precursor proteins as well as to the large subunit of heparanase, which indicates that this epitope is a dominant epitope of both, the heparanase precursor protein and the large subunit monomer. Similarly, the MAP3 epitope of antiserum antibody is not a dominant epitope on the heparanase precursor protein and the subunits. Meanwhile, immunohistochemical staining was performed with MAP1, MAP2, and MAP3 antisera showing that hepatoma tissues



have heparanase expression. The immunohistochemical staining with MAP2 antiserum is similar to that using the commercialized antibody, both having a strong expression in the hepatoma cell plasma. However, the results of MAP1 and MAP3 antisera are similar to that of normal rabbit serum without a positive heparanase expression. ELISA test was used to detect the binding affinity of these three kinds of MAPs towards the commercialized heparanase antibody; the results were similar to those of western blot and immunohistochemistry. To sum all these results up, we can say that the binding affinity of MAP2 is the strongest.

Metastases are the main reason for clinical manifestation of malignant advanced tumors and they are also the main cause of death through cancers, since it is still almost impossible to cure them. The key and necessary step in the tumor metastasis is that the tumor cells pass through natural barriers composed of basement membrane and extracellular matrix, and invade the periphery tissues and blood vessels. The barriers mainly compose of two ingredients: structural proteins and glycosaminoglycan with its main component heparan sulfate proteoglycan (HSPG). There are 17 kinds of matrix metalloproteinase (MMPs) that can degrade the extracellular matrix proteins. Therefore, it is difficult to develop metalloproteinase inhibitors for anti-metastasis treatment. However, heparanase is the sole endo-glycosidase to split the HS chain of the glycosaminoglycan, allowing it to degrade the extracellular matrix and basement membrane. Furthermore, it is able to release multiple kinds of cytokines such as VEGF, bFGF, etc. These cytokines play a key role in promoting the cell movement, in enhancing tumor cell invasion and in promoting tumor angiogenesis. So they are considered to be closely related to the invasion, metastasis and prognosis of multiple kinds of malignant tumors [1–4, 23]. Therefore, heparanase is regarded as a promising important target molecule for anti-metastasis treatment at present. Studying and screening for its inhibitors become the direction for people to discover new drugs for tumor treatment.

Heparanase inhibitors include polysaccharides, siRNA, antisense heparanase gene and polypeptide antibodies, etc. PI-88, the representative for polysaccharides, have just entered stage III of clinical trials as an adjuvant therapy of post-operative hepatocellular carcinoma [6]. Heparanase polypeptide antibodies can block HPSE, reduce the content of HPSE or lower the enzymatic activity, and thus prevent the degradation of HS, reduce the release of active substances and maintain the stability of ECM. Therefore, they can inhibit tumor cell invasion and metastasis. The anti-tumor role of heparanase heparin/heparan sulfate binding domain and heparanase antibodies have been reported in several documents. Levy-Adam et al. [21] discovered that a binding domain exists in the N-terminus of the 50 kDa large subunit of the heparanase. Its amino acid sequence is

located at Lys (158)-Asp (171) region. These region-derived antibodies can inhibit the heparanase activity. He et al. [24] reported that antibodies against recombinant heparanase protein derived from full-length human heparanase cDNA can inhibit the activity of the heparanase and block the invasion of ovarian cancer. Gingis-Velitski et al. [25] prepared multiple kinds of monoclonal antibodies, which can inhibit the activity of heparanase. But also one monoclonal antibody was prepared which can enhance the activity of heparanase and promote tumor invasion and wound healing. However, no researches on heparanase polypeptide antibodies blocking the hepatoma cell invasion and metastasis have been reported till now.

The antibody bioactivity and function assay indicated that, although these polypeptide antibodies have no obvious impact on the cell growth and cell cycle of HCCLM6, anti-MAP1 and -MAP2 antibodies can significantly inhibit the invasion capability of the HCC cells showing dose-dependency. Anti-MAP1 and -MAP2 polypeptide antibodies of final concentration of 50–100 µg/ml can significantly inhibit the invasion of HCCLM6 cells, while a treatment with the concentration of 10 µg/ml shows no effect. Moreover, the heparanase activity has been inhibited when the invasion inhibition occurred, and vice versa, suggesting that MAP1 and MAP2 are neutralizing antibodies, with the function of blocking the tumor cell invasion, which is realized by inhibiting the heparanase activity. The three kinds of polypeptide antibodies have different impacts on the heparanase activity and the HCC cell invasion capability, which may relate to the amino acid sequence and special position of different epitope peptides in the heparanase protein.

In conclusion, the peptide fragments 1–15 and 279–293 of the large subunit in heparanase are a newly discovered dominant B cell epitope. Peptide fragment 1–15 seems to only induce antibodies against the mature heparanase protein, but peptide fragment 279–293 is able to induce antibodies against both, heparanase precursors and mature protein. The anti-MAP1 and -MAP2 antibodies corresponding to the above peptide fragments can significantly inhibit the heparanase activity and invasion capability of HCCLM6 cells, showing certain dose dependence. This provides a valuable theoretic and experimental basis for the development of heparanase polypeptide antibodies and tumor vaccines.

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