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Overexpression of heparanase multiple antigenic peptide 2 is associated with poor prognosis in gastric cancer: Potential for therapy

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Abstract. Tumor-associated antigens (TAAs) trigger a TAA-specific immune response, thus they are the crux of antitumor immunosurveillance. A major advance in tumor immunology in the last 20 years was marked by the verification that CTL or B-cell epitopes rather than integral TAAs induce immunoreactivity. Previous studies on the correlation between heparanase (Hpa) expression and clinical or pathological features have generally used commercial antibodies against full-length Hpa protein rather than the functional epitopes, and the antigen determinants of such antibodies have not yet been defined. In our investigation of Hpa peptide expression in gastric cancer tissues and its association with tumor invasion, metastasis and prognosis, we analyzed Hpa expression in the tissues of 132 patients with gastric cancer using tissue microarray (TMA) technology and immunohistochemical staining.Three self-developed rabbit polyclonal antibodies against Hpa multiple antigenic peptides (MAP) and one commercial polyclonal rabbit antibody against the 50-8 kDa Hpa heterodimer were used. Clinical and pathological significance was evaluated using the Chi-square test and Kaplan-Meier survival curve analysis. The results demonstrated that the positivity rates using the antibody against MAP2 and the commercial antibody were 60.6% (80/132) and 65.2% (86/132), respectively. No expression of either MAP1 or MAP3 was noted in the cancer tissues of the 132 cases. MAP2 behaved in a similar manner to the commercial antibody in that a higher Hpa expression was observed in the cancer tissues with vessel invasion, serosal involvement, distant metastasis, poor differentiation and TNM stages III and IV. Moreover, the patients with a positive Hpa expression had a far poorer prognosis, with lower one-year and five-year survival rates. Our results demonstrate that in a similar manner to full-length Hpa proteins, MAP2 expression is closely associated with the invasion, metastasis and prognosis of gastric cancer. This finding may be of potential use in clinical therapy and in estimating the prognosis of a tumor.

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Introduction

To date, heparanase (Hpa) is the only endogenous endoglycosidase found that degrades the heparan sulfate proteoglycans (HSPGs) in the extracellular matrix (ECM) and the basement membrane (BM). Hpa is highly expressed in most mammalian malignant tumors, and its expression has been significantly linked to the formation of microvessels and lymphatic vessels, tumor invasion, metastasis and angiogenesis (1). Previous studies have focused on the correlation between Hpa expression in cancer tissues and clinical and pathological features, which demonstrate that increased Hpa levels are most often associated with increased tumor metastasis, high microvessel density and reduced patient survival time following surgery (2-6). However, the antibodies used to recognize and combine Hpa protein are typically commercial, and the antigen determinants of such antibodies have not yet been defined.

Gastric cancer remains one of the most lethal malignancies in the world. Surgical resection, chemotherapy and radiotherapy have been the most common forms of management used in treating such malignancies. Recently, immunotherapy has been proposed as a new modality for cancer treatment due to its reduced side effects and easy applicability. It utilizes the immune system to recognize and specifically eradicate tumor cells and has shown encouraging results in human clinical trials (7). Tumor-associated antigens (TAAs) presented by dendritic cells trigger a TAA-specific immune response, thus they are the crux of antitumor immunotherapy. A major advance in tumor immunology in the last 20 years was marked by the verification that CTL or B-cell epitopes rather than integral TAAs induce immunoreactivity (8). The first group of immunogenic epitopes in Hpa was identified by Sommerfeldt *et al* using the SYFPEITHI algorithm to identify nonapeptides of the Hpa amino acid sequence (9). In our previous study, according to the primary structure of Hpa and on the basis of predicting the Hpa B-cell epitopes

via bioinformatics, we designed and synthesized multiple antigenic peptides (MAPs) using the eight-branched polypeptide modus (10,11). We also found that each of the three MAPs was capable of inducing the production of antibodies (antisera) of high titer (12).

The aim of the present study was to determine the expression of Hpa MAP presented with explicit epitopes in gastric cancer and its correlation with clinical and pathological features of gastric malignancies, using tissue chip technology and immunohistochemical staining. The antibodies we harnessed were three self-developed rabbit polyclonal antibodies against Hpa MAP, as mentioned above, and one commercial polyclonal rabbit antibody against the 50-8 kDa Hpa heterodimer. In addition, the role of Hpa MAP in the pathogenesis and aggressiveness of gastric cancer and its prognostic value were explored.

Materials and methods

Patients and tissue samples. Tissue specimens were obtained from 165 gastric cancer patients and embedded in paraffin. The clinical data of these cases were reviewed and analyzed retrospectively. Paired samples of normal gastric tissue were obtained from 39 patients during surgery. However, on account of the tissues being worn down through use of tissue chip technology and immunohistochemical staining, only 132 samples (35 females and 97 males, aged 17-80 years, average age 58±11 years) of gastric carcinoma tissue and 30 paired normal samples were selected for use in this study. None of the selected patients had been treated with radiotherapy or chemotherapy prior to the surgery, and clinical and follow-up data were available in all cases. All 132 patients were treated at Department of General Surgery, Zhejiang Provincial People's Hospital, China, between January 1998 and December 2004. The cancers were analyzed for histological type, depth of invasion, lymphatic involvement and venous infiltration according to the definitions used in clinical and pathological studies on gastric cancer. According to the World Health Organization (WHO) gastric cancer typing criteria of 2002, the lesion diameter was <5 cm in 90 cases and ≥5 cm in the remaining 42 cases. Highly or moderately differentiated adenocarcinomas were found in 40 cases while poorly or undifferentiated carcinomas were found in the remaining 92 cases. The number of patients with and without regional lymph node metastasis, distant metastasis and vessel invasion were 90 (68.2%) and 42 (31.8%), 25 (18.9%) and 107 (81.1%), and 42 (31.8%) and 90 (68.2%), respectively. According to the gastric cancer TNM staging criteria revised by the International Union against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) in 2003, there were 52 cases in stages I and II, and 80 cases in stages III and IV. Excluding the cases of mortality, the shortest follow-up time was 4 years while the longest was 10 years, and the cut-off date was January 2008.

This study was approved by the ethics committee of Zhejiang Provincial People's Hospital. Consent was obtained from all patients.

Materials and reagents. Polyclonal rabbit antibody against full-length human Hpa was purchased from InSight Biopharmaceuticals Co., Ltd., Israel. Histostain™-Plus kits (SP-9000) and the DAB chromogenic kit were purchased from Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., China. Rabbit polyclonal antibodies to Hpa MAP were self-designed as described previously (12-14) and were synthesized and purified by Chinese Peptide Company, Ltd., Hangzhou, China. The amino acid sequences of MAP1, MAP2 and MAP3 were KKFKNSTYSRSSVDV (1-15), HCTNTDNPRYKEGDL (279-293) and STRPGKKVWLGETSS (175-189), respectively, at the large-subunit locus of Hpa. Three different antibodies against human Hpa were obtained following the immunization of white-hair-black-eye rabbits with the three MAPs, respectively. The specificity, immunogenicity and anti-tumor activity of the antiserum were evaluated as described previously (12-14).

Tissue microarray (TMA) construction and immunohistochemistry. TMA for the gastric cancer tissues and compared normal tissues was constructed using a semiautomatic tissue arrayer (Beecher Instruments, Woodland, TX, USA). Areas involving malignant and normal tissues were marked on hematoxylin and eosin-stained sections. Cylindrical cores 0.6 mm in diameter were then punched out of the corresponding paraffin-embedded block and inserted into a recipient block which was receptor-free and punched with the same diameter. This procedure was repeated until all the specimens were implanted into the paraffin block. After successive slicing and laminating, TMAs were constructed and tissue chips were obtained.

The labelled streptavidin-biotin technique (SP method) was used for immunohistochemical staining. The sera of the MAP1, MAP2, MAP3 and commercial Hpa antibody were used as the primary antibody. The positive-stained gastric cancer section was used as the positive control, while normal rabbit serum and phosphate-buffered saline (PBS) were used as the negative control instead of the primary antibody. Immunohistochemical staining was performed following the instruction manual of the kit.

Determination of results. The experimental results were analyzed in a blinded manner by senior pathologists. The analysis was initially performed under a low-power lens to select the densely stained areas, then the pathologists counted 1,000 tumor cells in five randomly selected areas at x200 magnification. The scoring used in the immunohistochemical staining results was based on the coloring of the cancer cells (0, no staining; 1, light yellow; 2, brown; 3, dark brown) and on the percentage of positive cells (0, negative; 1, ≤10% positive cells; 2, 11-50% positive cells; 3, ≥51% positive cells). The intensity of Hpa expression was calculated as the product of the staining intensity score and the positive cell percentage score: score 0-2, -; score 3-4, +; score 5-7, ++; and score 8-9, +++.

Statistical analysis. SPSS 18.0 (SPSS Inc., Chicago, IL, USA) statistical software was used for data processing. The correlations between the expression of the four different Hpa antigens (Hpa full-length protein, MAP1, MAP2 and MAP3) and clinical or pathological characteristics were evaluated using the Chi-square test. Survival rates were evaluated by the Kaplan-Meier curve analysis. $P<0.05$ was considered to indicate a statistically significant result.

Table I. Correlation between Hpa expression and clinicopathological characteristics.

Clinicopathological characteristics	No. of cases	Expression of MAP2		Positivity rate (%)	Expression of Hpa protein		Positivity rate (%)
		Positive	Negative		Positive	Negative	
Gender							
Male	97	32	65	67.0	35	60	63.2
Female	35	14	21	60.0	17	20	54.1
Tumor diameter							
<5 cm	90	33	57	63.3	35	53	60.2
≥5 cm	42	13	29	69.0	17	27	61.4
Tissue type							
Intestinal	95	27	68	71.6	34	59	63.4
Diffuse	37	19	18	48.6	18	21	53.8
Differentiation							
Well or moderate	40	8	32	80.0	9	26	74.3
Poor or undifferentiated	92	38	54	58.7[a]	43	54	55.7[a]
Lymphatic metastasis							
Negative	42	24	18	42.9	25	16	39.0
Positive	90	22	68	75.6[a]	27	64	70.3[a]
Distant metastasis							
Negative	107	46	61	57.0	47	60	56.1
Positive	25	0	25	100[a]	5	20	80.0[a]
Vessel invasion							
Negative	90	41	49	54.4	40	49	55.1
Positive	42	5	37	88.1[a]	12	31	72.1[a]
Serosal infiltration							
Negative	39	16	23	59.0	17	21	55.3
Positive	93	30	63	67.7[a]	35	59	62.8[a]
TNM staging							
I-II	52	30	22	42.3	29	22	43.1
III-IV	80	16	64	80.0[a]	23	58	71.6[a]

[a]P<0.05, compared with the control group.

Results

Expression of Hpa antigens. Immunohistochemical staining was achieved when the commercial Hpa antibody and MAP2 antiserum were used as primary antibodies. The two antibodies stained the gastric cancer cells equally dark brown, and their positivity rates were similar. The positive Hpa staining rates using the commercial antibody in gastric cancer and normal tissue were 60.6% (80/132) and 3.3% (1/30), respectively, while the rates of positive MAP2 expression in gastric cancer and normal tissue were 65.2% (86/132) and 3.3% (1/30), respectively; the positive cell percentage was much higher in gastric cancer tissue than in normal tissue in both cases (P<0.05). The two antigens were mainly located in the cell plasma and cell membranes in cancer tissues, and were restricted to the glandular epithelium of mucosal cells in normal gastric tissues. The expression intensity of the antigens in the tumor tissues (scores: ++ to +++) was much greater than that in the normal gastric tissues (scores: + to ++). In addition, positive staining was detected in a few vascular endothelial cells and smooth muscle cells. Staining using MAP1 and MAP3 antiserum was negative for both, compared to PBS.

Correlation between Hpa expression and clinicopathological characteristics. The expression of MAP2 polypeptide was similar to that of Hpa protein: a markedly higher expression was detected in the malignant tissues with vessel invasion, serosal involvement, distant metastasis, poor differentiation and TNM stages III and IV (P<0.01; Table I). However, no significant correlation was found between Hpa expression and patient gender, tumor diameter and histological type.

Correlation between Hpa expression and prognosis. Follow-up data were available for all patients. The prognosis of patients with positive expression of MAP2 antigen was far poorer than

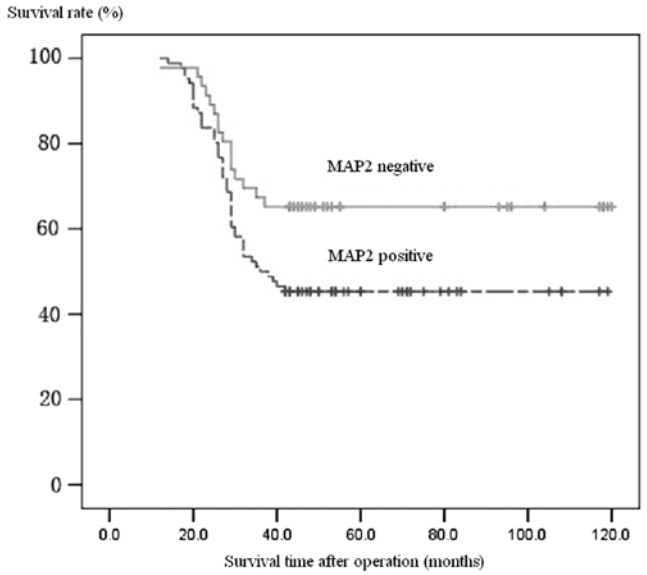

Figure 1. Kaplan-Meier survival curve of patients with and without MAP2 expression.

that of patients with a negative expression, which was similar to that of Hpa protein using the McNemar matched Chi-square test (P>0.05). Patients with Hpa expression demonstrated one- and five-year postoperative survival rates of 80 and 44%, respectively, compared to 92 and 63% in those without Hpa protein expression. Similarly, patients with positive MAP2 staining had one- and five-year postoperative survival rates of 84 and 45%, respectively, compared to 91 and 65% in those with negative MAP2 staining (Fig. 1).

Discussion

Hpa protein is an endo-β-glucuronidase that cleaves heparan sulfate side chains, presumably at sites of low sulfation, releasing saccharide products with appreciable size (4-7 kDa) that still associate with protein ligands and facilitate their biological potency. Hpa has been detected at relatively low levels in mammalian lymphoid organs, placenta and platelets and has been found to be highly expressed in most mammalian malignant tumors, while it is either not expressed or expressed at extremely low levels in other normal tissue. In conditions of injury or inflammation, certain immunocytes (i.e., lymphocytes, macrophages and neutrophils) secrete Hpa, which has the ability of breaking down HSPGs and helping cells to migrate, gather at lesions and fulfill their anti-inflammatory or repairing responsibilities (1). The activation of Hpa enables tumor cells to break through the ECM and BM barriers. Furthermore, Hpa releases multiple cytokine types, including VEGF and bFGF. These cytokines are crucial in promoting cell movement, enhancing tumor cell invasion and promoting tumor angiogenesis. They are therefore considered to be closely associated with invasion, metastasis and prognosis in multiple malignant tumor types (12-14).

TMA was proposed in 1998 as a new biochip technology and has recently been of high interest, particularly in the areas of human genomes, protein research and drug development. TMA is envisioned to be available for routine biomedical and diagnostic applications provided that the ongoing technological developments are successful in improving sensitivity and specificity, and in reducing costs. Compared with traditional immunohistochemical staining, TMA has many advantages including TMA has many advantages, including lower volume of tissue required, reduced error rate and greater provision of information to researchers. TMA is useful for parallel testing of antibody specificities on a broad range of histological specimens in a single slide, and improves the reliability and the accuracy of study outcomes by ensuring the coherence of experimental parameters.

In this study, we found that the Hpa protein and polypeptide were highly expressed in most advanced-stage gastric cancer tissues, which were mainly located within the cytoplasm and cytomembrane. In addition, they were weakly expressed in a few vascular endothelial cells and smooth muscle cells. However, no expression of Hpa protein or MAP was detected in normal gastric mucosal glands. These findings were consistent with previously published studies (15). According to the standard scoring used in immunohistochemical staining, the expression of the three different MAPs in gastric cancer tissues had clear discrepancies: i) a distinct higher expression of MAP2, and ii) no expression of MAP1 and MAP3, which was similar to the findings of our previous study which focused on hepatocellular carcinoma tissues (12). Such results may be explained by the different affinity of the three antibodies against MAP combining with antigenic determinants of Hpa. As the different epitopes in the Hpa large subunit had different spatial locations, it can be hypothesized that the epitopes of MAP2 are dominant, whereas those of MAP1 and MAP3 are recessive (12).

Certain studies using commercial antibodies against Hpa whole protein have demonstrated that the expression of Hpa protein was closely correlated with the invasion and metastasis of gastrointestinal tumors (16,17). Our study revealed that the immunohistochemical staining with MAP2 antiserum was similar to that observed using the commercial antibody, as both had a strong expression in the cell plasma and cell membrane. Furthermore, a markedly increased expression of Hpa protein or MAP antigen was detected in cases with lymphatic metastasis, vessel invasion, distant metastasis, serosal infiltration, poorly differentiated or undifferentiated cancers and TNM stage III or IV. The survival curve also showed that the one- and five-year survival rates of patients without Hpa expression were significantly higher than those with Hpa expression. Therefore, Hpa MAP2 antibody is a promising tool in the diagnosis of gastric cancer phenotypes.

Hpa inhibitors such as polypeptide antibodies block Hpa, reduce the content of Hpa or lower the enzymatic activity, and thus prevent the degradation of HS, reduce the release of active substances and maintain the stability of the ECM. The antitumor role of Hpa antibodies has been reported in several experimental documents (18,19). Our previous study also suggested that Hpa MAP2 antibodies are neutralizing, and are capable of blocking the hepatocellular cancer cell invasion by inhibiting Hpa activity (14). However, the clinical significance of Hpa polypeptides in gastric cancer has not been fully explored thus far, possibly due to the lack of long-term follow-up data. In this study, we found for the first time that

the overexpression of Hpa MAP2 is positively correlated with tumor invasion and metastasis and negatively correlated with prognosis. Thus, it can be hypothesized that antibodies targeting Hpa MAP2 elicit a potential immunotherapeutic effect in gastric malignancies, and that downregulation of Hpa MAP2 expression is likely to improve the prognosis of gastric cancer patients.

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