

Original Article

Combination of immuno-MS and ELISA: a new strategy for detection of cancer

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Abstract: Objective: Currently, detection method for cancer mainly includes biopsy, ultrasound and blood-based biomarkers detection. Although all of these methods are being employed in the clinical practice, they are often characterized by low sensitivity or/and specificity. In this study, we took the liver cancer as an example and combined immuno-MS and ELISA to verify the potential biomarker role of pep5 for hepatocellular carcinoma detection. Methods: A polypeptide named pep5 was designed and its antibody was used to detect liver cancer by methods of immuno-MS and ELISA. Proteins were determined using immunoprecipitation and Immunohistochemistry. Result: The data showed that combination of immuno-MS and ELISA can largely improve the sensitivity and specificity of the cancer detection. While the sensitivity and specificity are 74.83% and 71.52% (immuno-MS alone), 67.72% and 97.35% (ELISA alone), the combination of immuno-MS and ELISA can lead to a sensitivity and specificity of 72.78% and 100%, respectively. Immuno-MS and ELISA assay showed that pep5 antibody binds with both pep5 and its precursor protein. Conclusion: The combination of immuno-MS and ELISA may be a new strategy of the detection of cancer, and the pep5 also can be a hopeful serum biomarker for liver cancer.

Keywords: pep5, liver cancer, combination detection, HK

Introduction

Cancer, a globally devastating disease, accounts for one in every eight deaths worldwide- more than HIV/AIDS [1]. Especially in developing countries, cancer is the second most common cause of death, comprising 23%-25% of total mortality [2]. The reason for so high mortality is that it is difficult to diagnose cancer during the early step [3]. At present, the detection methods for cancer mainly include biopsy, ultrasound and blood-based biomarkers detection. There are both advantages and disadvantages to all these methods. For example, biopsy is the gold standard for diagnosis of cancer but characterized by invasiveness and restriction for accessibility, reproducibility as well as cost; ultrasound detection is operator dependent, especially for the small

size tumors; now, blood biochemical examination is thought to be the easiest method that can be used for detection of cancer and biomarkers occupy the dominant position during the examination [4-9].

At present, combining multiple serum cancer markers has been given more and more attention in the blood biochemical examination. Zhen Zhang et al. combined CA125II, CA72-4, CA15-3 and macrophage colony stimulating factor (M-CSF) four tumor markers to detect stage I epithelial ovarian cancer in 2007, the results showed that the sensitivity can be improved from 46% (CA125II alone) to 71% for detecting early stage epithelial ovarian cancer at a fixed specificity of 98%, for detecting invasive early stage epithelial ovarian cancer, the sensitivity can be improved from 43% (CA125II alone) to

71% [10]. Eddy S. Leman et al. also found the sensitivity for detection of the combined end point of colorectal cancer and advanced adenoma for CCSA-3 was 89.1% and for CCSA-4 was 84.8% but 91.3% for either marker positive [11]. OVA1, the first in vitro diagnostic multivariate index of proteomic biomarkers cleared by the US FDA, includes five markers-CA125, transthyretin (prealbumin), apolipoprotein A1, beta 2 microglobulin and transferrin, was validated by Dr. Frederick Ueland and the sensitivity for malignancy was 95.7%, compared to only 75.1% without OVA1. Moreover, of the patients identified as low risk by OVA1, 94.7% proved to be benign [12].

Although the combination of multiple markers was popular, there is no relationship among the markers. Our group thinks there will be better result if the markers have some relationships, for example peptide and its precursor protein. Therefore, we made brave attempts to validate our idea. In preliminary experiment of serum peptides spectrum technology, we obtained a tumor-specific polypeptides from hepatic carcinoma patient serum which following was confirmed to be derived from high molecular weight kininogen (HK). This polypeptides was named as pep5 and speculated to be a good biomarker for the detection of cancer. HK is a plasma glycoprotein with a molecular weight of 120 kD and can be divided into six domains. After proteolyzed by plasma kallikrein or other proteases, HK can release the bradykinin of domain 4 and generate a double-stranded region of molecular weight kininogen (HKa). As an important part of the plasma kallikrein/kinin system [13, 14], HKa can be not only combined with endothelial cells inhibits formation of new blood vessels to prevent tumor formation, but also can block the EGFR in combination with uPAR to inhibit tumor migration and invasion [8, 13-17]. 2009, Yuchuan Liu et al. HKa and its domain 5 can inhibit the migration and invasion of human prostate cancer cells via the epidermal growth factor receptor pathway [17]. 2011, Hiroshi Umemura et al. identified a high molecular weight kininogen fragment as a biomarker for the early gastric cancer by serum proteome analysis [8].

Here, we combined immuno-MS and ELISA to detect the pep5 and its precursor protein in the serum of liver cancer patients (n=158) and

healthy controls (n=153). The result showed when the sensitivity and specificity are 74.83% and 71.52% (immuno-MS alone), 67.72% and 97.35% (ELISA alone), the combination of immuno-MS and ELISA can lead to a sensitivity and specificity of 72.78% and 100%, respectively. Therefore, the combination of immuno-MS and ELISA detecting the biomarkers in serum may be a novel strategy for the detection of cancer and some other diseases.

Materials and methods

Reagents and sample collection

Synthetic signature peptide (pep5; sp0105, NLGHGHHKHERDQGHGHQ) with purities of 94% (assessed by high-performance liquid chromatography, HPLC) were obtained from Beijing Scilight Biotechnology Co. Ltd. Acetonitrile (ACN) and α -cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Thermo Fisher Scientific Inc. Protein G Agarose was purchased from Santa Cruz Biotechnology, Inc. Horse Reddish Peroxidase (Roche, Switzerland), 96 microplate well (Costar, USA), TMB coloration kit (Thermo, USA), MK3-Universal microplate spectrophotometer (Thermo, USA), DEM-III-Automatic washing machine (Beijing Tuopu analytical instrument company), All other chemicals were of analytical grade.

Sera specimens were collected after obtaining informed consent and approval from Beijing Cancer Hospital, 302 Military Hospital of China, Air Force General Hospital, PLA. All patients were diagnosed by pathology without chemo/radiotherapy. A total of 327 serum samples were collected, including healthy controls (n=153; median age, 48 \pm 12 SD; range 39-83 years) and liver cancer (n=158; median age, 48 \pm 12 SD; range 39-83 years). Liver cancer tumor and adjacent non-tumor liver tissue samples were collected from liver cancer patients (n=16; median age, 48 \pm 12 SD; range 39-83 years).

Preparation of the monoclonal antibody

BALB/c mice (6-8 weeks, female) were immunized biweekly with 50 μ g of peptide-pep5 emulsified in Freund's adjuvant by intraperitoneal and subcutaneous injections and boosted

intravenously with 25 µg of peptide-KLH without adjuvant 3 days prior to fusion. Mice producing high serum Ab titers against peptide were selected by ELISA, and their spleen lymphocytes were fused with nonsecreting mouse myeloma SP2/0 cells. Hybridomas were selected in medium supplemented with HAT and HT-selective culture, and supernatants screened by ELISA. Hybridomas secreting peptide antibodies were cloned by limiting dilution. Ascites were produced in female BLB/c mice following intraperitoneal injection with 0.5 ml of liquid paraffin and a week later with 5×10^6 hybridoma cells. Ascites fluids were purified on affinity chromatography using Protein G 5 ml column according to the manufacturer's instruction. The titer of monoclonal antibody and the immunoglobulin isotypes for peptide reactive mAbs were determined by ELISA and purity of antibody was determined by SDS-PAGE.

Immuno-MS analysis

Firstly, our laboratory previously established immuno-MS used to detect synthetic peptide standard were optimized, including serum dilution ratio, incubation time, and the choice of the reference. Using the optimized immuno-MS, all of 311 sera were detected. The detection procedure is as follows: (1) 20 µl of ProteinG Agarose was mixed with 10 µl of anti-pep5 antibody and incubated at 37°C for 15 min; (2) remove the supernatant after the solid-liquid separation and wash the agarose three times with 1×PBS; (3) 10 µl of serum (diluted 1:4 with 1×PBS) was mixed with the washed agarose and incubated at 4°C for 8 h; (4) after repeating the step (2), wash the agarose twice with 50 µl of NH_4HCO_3 ; (5) remove the supernatant after the solid-liquid separation and join 10 µl of 70% ACN containing 0.1% TFA, incubate for 1 min; (6) drawing the supernatant to a new PCR tube and mixed with 2 µl of another peptide solution at a concentration of 0.048 µg/ml; (7) the mixed solution was used to mass spectrometry analysis.

MALDI-TOF mass spectrometry analysis was performed on a 4700 MALDI-TOF/TOF MS (Applied Biosystems, Framingham, MA). The instrument was equipped with a delayed ion-extraction device and a pulsed nitrogen laser operated at 337 nm; its available accelerating potential is in the range of ± 20 kV. Internal mass calibration was achieved by using two

kinds of standard peptides (m/z2022.3183, m/z3159.5238). The mixture of eluent and CCA was deposited onto MALDI plate, each sample for 3 spots. Spectra were acquired in positive linear mode, laser energy of 2000, 2000 shots for one spectrum and 5 spectra per spot. Baseline correction, noise filter, and peak detection were carried out before the statistical analysis.

Direct enzyme linked immunosorbent assay (ELISA) analysis

Our laboratory previously established Direct ELISA used to detect synthetic peptide standard were optimized, including the enzyme labeled antibody working concentration, the choice of blocking agents and blocking times, the choice of coloration time, the dilution ratio of serum samples. Using the optimized direct ELISA, all of 311 sera were detected. The detection procedure is as follows: (1) Coating the synthesis peptide of pep5 or serum sample (with the dilution of 1:20) on designed micro-plate well, for 100 µL solution each well. Use the carbonate buffer solution as blank control. Place the coated plate in the refrigerator overnight. (2) Washing the coated well 5 times with 250 µL each well by automatic washing machine, then pat dry each well. (3) Adding 300 µL 5% skimmed milk in each wells and put it in 37°C for 2 hours. (4) Clear and dry the well as described earlier. (5) Putting 100 µL HRP labeling antibody and use the blocking buffer as blank control. Then incubate for 1 hour under 37°C. (6) Clear and dry the well as described earlier. (7) Then with the addition of 100 µL TMB substrate in every well, keep in dark place for 15 minutes in 37°C. (8) Finally, adding 50 µL 2 mol/L sulfuric acid in well to stop the coloration reaction and detecting the OD450 value.

Co-immunoprecipitation analysis

65 µl of serum (diluted 1:1 with 1×PBS) 60 µl was incubated with ProteinG Agarose at 4°C for 8 h. The supernatant was obtained and divided into two aliquots, one mixed with 10 µl of anti-pep5 antibody (3rd in **Figure 3A**) and the other mixed with 10 µl of 1×PBS (4th in **Figure 3A**). The mixtures were separately incubated at 4°C for 4 h. After incubation, the reaction solution was added with 20 µl of ProteinG Agarose and incubated at 4°C for 8 h. The reaction tube was removed supernatant and added with 20 µl of

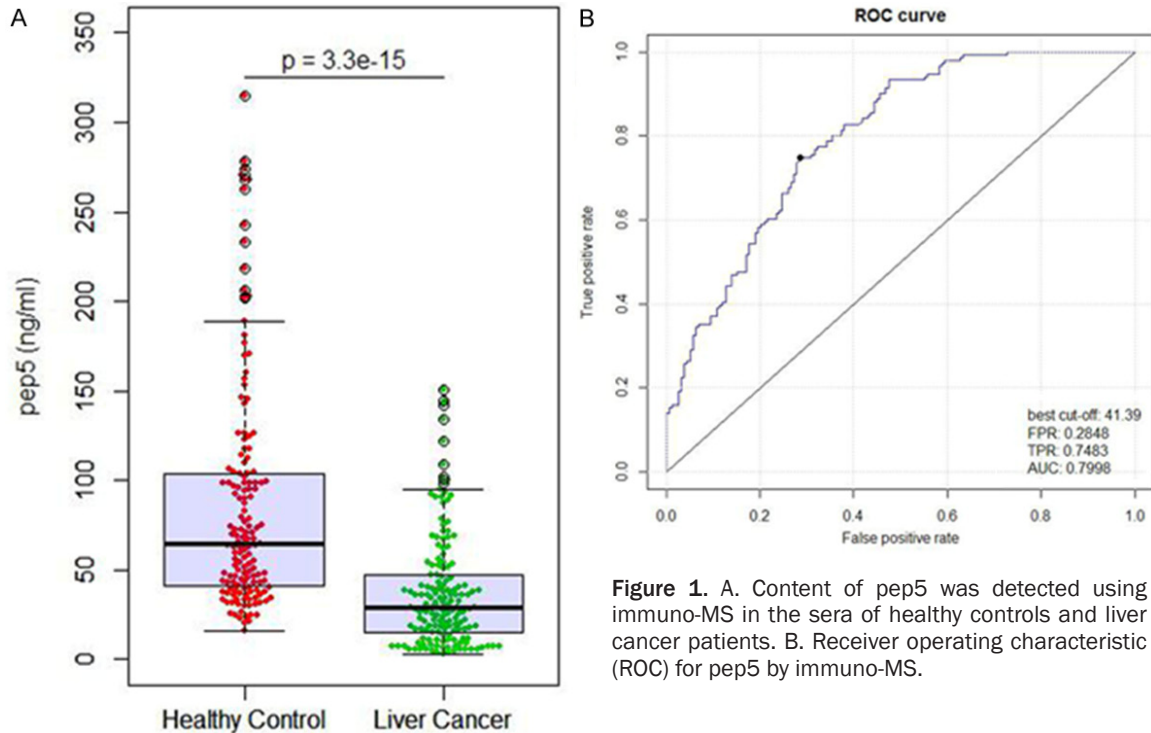


Figure 1. A. Content of pep5 was detected using immuno-MS in the sera of healthy controls and liver cancer patients. B. Receiver operating characteristic (ROC) for pep5 by immuno-MS.

1×LB to the agarose following boiled for 5 min. The supernatant was acquired and separated by SDS-PAGE. For chose the targeted band for in-gel digestion, the step of SDS-PAGE and in-gel digestion are as described previously. Finally, the product of in-gel digestion was analyzed by LC-MS.

Immunohistochemistry

After deparaffinage and hydration, the paraffin section was washed three times with PBS (pH 7.4), three minutes for once followed by incubating for 10 minutes with 50 µl of peroxidase blocking solution. The sample was washed three times with PBS in three minutes for once and then incubated with 50 µl of non-immune animal serum. After 10 minutes, the serum was removed and 50 µl primary antibody to the section was added. The primary antibody was incubated for 1 h and then the 50 µl second antibody to the section incubated for 10 minutes. DAB reagent was used to develop color of samples. The samples were finally vitrified by dimethylbenzene and sealed by resinene.

Statistical analysis

We used SPSS software 18.0 (SPSS Inc., IL) to calculate all statistical comparisons. The data

were represented as mean ± SD. A P-value less than 0.05 was considered to be statistically significant.

Results

Monoclonal antibody was prepared

Booster immunization mouse and its immune sera antibody titer attain to 1:10000, which suggested the pep5 antibodies incitation. Through cell fusion and cell cloning, 7 hybridoma strains that stably secreted monoclonal anti-pep5 antibody have been screened. And we choose three cell lines (2F6, 4F6, 7G7) that have highest antigen sensitivity (can be low at 10 ng/mL) for cell supernatant detection. The cell lines were identified by IgG antibody subtype and 2F6 and 7G7 are the IgG2b while 4F6 is the IgG1. The light chains of three lines are as same as lambda (λ). Each ascites antibody were purified by Protein G affinity and identified by using SDS-PAGE. The purified antibody had no obvious miscellaneous protein and the purity is well. The antibody titer had no significant decline after the purification processing.

Three kinds of anti-pep5 monoclonal antibodies were labeled with Horseradish Peroxidase (HRP) and the antibody titer was both detected

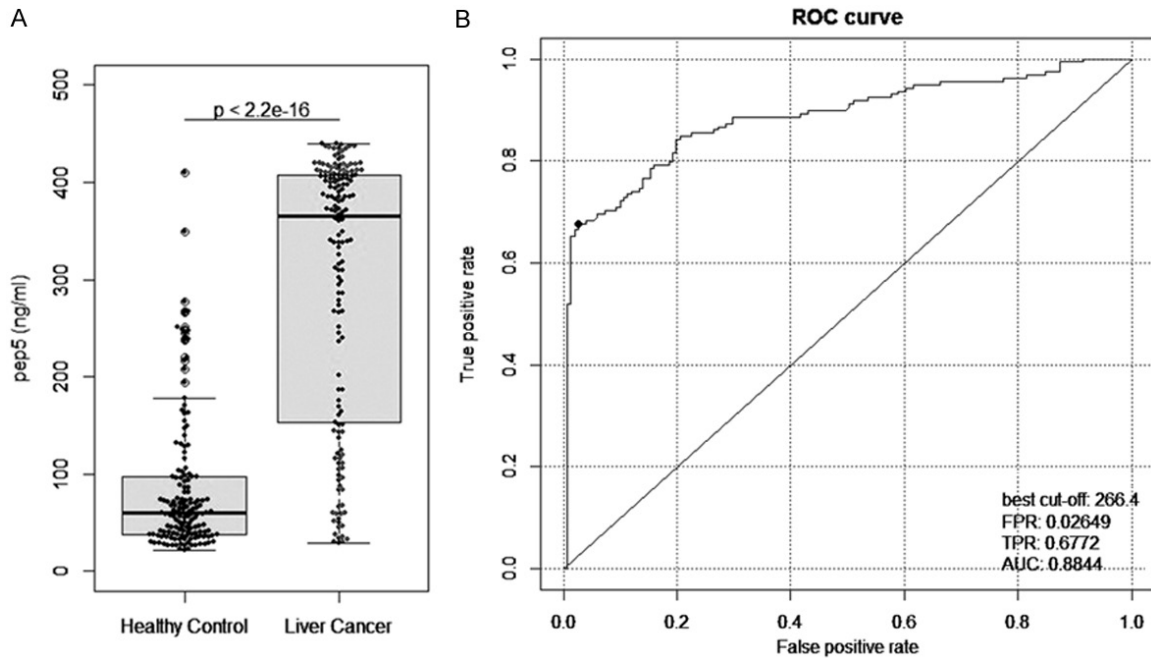


Figure 2. A. Content of pep5 was evaluated by using ELISA in the sera of healthy controls and liver cancer patients. B. Receiver operating characteristic (ROC) for pep5 by ELISA.

before and after labeling 2F6 antibody titer is 1 ng/mL before and after marker. 4F6 antibody titer is 10 ng/mL before labeling, after marked the titer is 100 ng/mL. 7G7 is 1 ng/mL before labeling and to 10 ng/mL after marked. For the no difference of antibody titer through HRP labeling, the HRP-2F6 was optimized as the direct ELISA detection antibody.

Downregulation of pep5 in liver cancer samples from result of immuno-MS

Because of the complexity of serum samples and their different biological environments with synthetic peptide, the choice of the reference peak, the ratio of serum dilution and serum incubation time were optimized before the clinical sera were really detected. The results showed that the detection can be reached best when the serum dilution ratio was 1:4 and the incubation time is 8 h. The reference is a synthetic peptide of molecular weight 1200 Da. By employing the optimized immuno-MS, all of 311 samples were detected. The content of pep5 in human serum can be calculated by the content of added another standard peptide and the formula is as $X=1.2R$ ng/ml (X: the content of pep5; R: (the peak height of pep5/the peak height of reference)*100). As shown in **Figure 1A**, the content of pep5 in liver cancer serum

was significantly lower than that in healthy control ($P<0.05$). The sensitivity and specificity of serum pep5 were represented by ROC curves (**Figure 1B**). For the liver cancer, the optimal cutoff value was 41.39 ng/ml, which led to a sensitivity and specificity of 74.83% and 71.52%, respectively, and the AUC was 0.78.

Direct ELISA detection of clinical serum

The content of pep5 in human samples serum was evaluated by ELISA, and the results showed a higher serum pep5 level in liver cancer compared to that in healthy control ($P<0.05$, **Figure 2A**). As normal group and liver cancer group for the ROC curve analysis, it was concluded that the AUC (area under the curve) is 0.88. When the pep5 serum concentration is more than 266.4 ng/mL as the diagnostic value, the sensitivity of the direct ELISA is 67.72% and specificity is 97.35% (**Figure 2B**).

Antibody can capture pep5 and its precursor protein at the same time

As demonstrated in above, we observed certain difference between the two methods in the analysis of serum pep5 level of healthy control and liver cancer patients. Thus, we tried to find the reason for this difference by co-immuno-

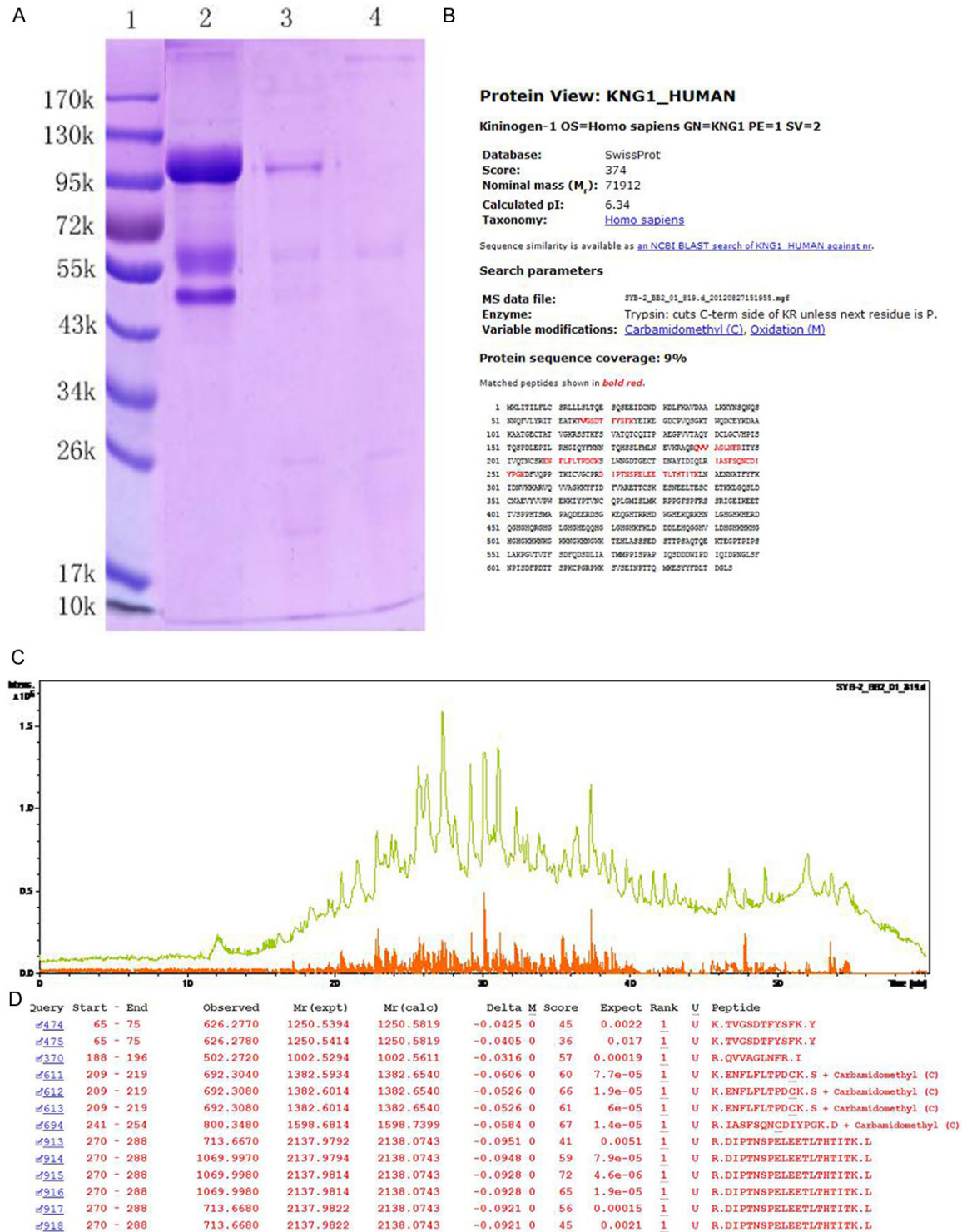


Figure 3. A. Representation of SDS-PAGE for co-immunoprecipitation (1st lane: Marker; 2nd lane: HMW Kininogen Standard with pep5; 3rd lane: serum with pep5 antibody; 4th lane: serum with PBS). B. The total ion current chromatogram of the top band in the 3rd lane. C. MASCOT result of the top band in the 3rd lane. D. The matched peptides of identified protein Kininogen-1.

precipitation experiments. From the result of SDS-PAGE (Figure 3A), we can find that there were some bands in the 3rd lane, but no binds in

the 4th lane that acted as control. This data indicated that something was captured by pep5 antibody in the 3rd lane. Based on the bands at

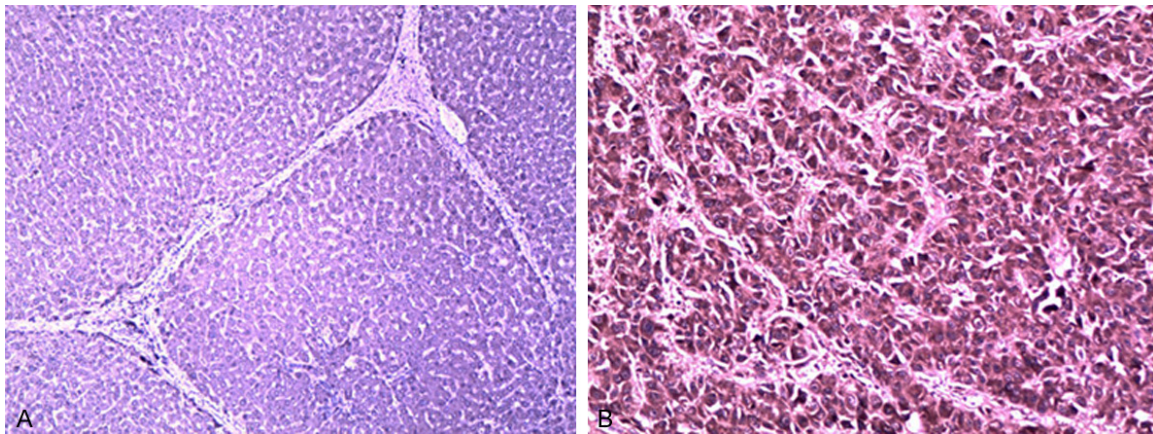


Figure 4. Immunohistochemistry for the different liver parts of the same patient (A. For the part of liver cirrhosis, $\times 10$, S-P immunohistochemistry; B. Immunohistochemistry for the part of liver tumor, $\times 20$, S-P immunohistochemistry).

the same position of 2nd lane, we can determine the three bands of 3rd lane are precursor protein, heavy chain of precursor protein and light chain of precursor protein, respectively.

To further demonstrate the proteins, we carried out the in-gel digestion experiment to the three bands of the 2nd lane, and the products were analyzed by LC-MS. Digested the three bands were analyzed by LC-MS/MS (**Figure 3B**). The resulted MGF file were searched online through the Swiss-Prot and NCBI database by the MASCOT search engine (<http://www.matrixscience.com>) with a peptide mass tolerance of ± 0.2 Da, a fragment mass tolerance of ± 0.6 Da and two missed cleavage. We identified IgG and the protein Kininogen-1 (KNG1_HUMAN), which scored 374 and had a coverage of 9% (**Figure 3C**). The matched peptides are showed in **Figure 3D**. These data showed that the captured protein is kininogen-1.

Pep5 expression in liver tumor tissue

Next, we evaluated pep5 expression in liver tumor tissue and adjacent non-tumor liver tissue (liver cirrhosis) using immunohistochemistry. From the **Figure 4A**, we can see the normal liver lobular structure disappeared and fiber-bridged between the portal areas. There was false lobules in the divided liver tissues, however, liver cells was immunohistochemically negative. While in tumor tissue, liver cells are arranged in cords and its structure was disordered. Most of the liver cells were positive in the immunohistochemical reaction. The positive mark points were indicated as brown pel-

lets filled with cytoplasm and overwrite the nucleus (**Figure 4B**).

Discussion

Despite recent efforts focusing on developing new tumor biomarkers and combining multiple biomarkers for cancer detection, no evidence verified a peptide associated with its precursor protein as effective markers for cancer. In this study, we combined immuno-MS and ELISA to evaluate the detection index of pep5 and its precursor protein in liver cancer respectively and obtained good results. Our results are summarized as follows: (1) pep5 and its precursor protein can be the biomarkers for the detection of liver cancer; (2) the combination of immuno-MS and ELISA can improve the sensitivity and specificity and may be a good strategy for the detection of cancer.

On the first point, the data of the ROC showed that combination detection improved the sensitivity and specificity (72.78% and 100%, respectively). Therefore, we reached the conclusion that combination can be a better strategy for the detection of cancer. On the second point, the different comparison showed that both the content of pep5 and its precursor protein are significantly difference between the healthy controls and liver cancer patients. Otherwise, compared with AFP (sensitivity: 40%~65%, specificity: 76%~76%), the current gold standard for detection of liver cancer, both the sensitivity and specificity of pep5 and its precursor protein are better [18]. From the result of immunohistochemistry, although the tissues were from

the same patient, we only observed obviously positive reaction in the liver tumor tissue but not in the liver cirrhosis tissue. From the results above, we considered that pep5 and its precursor protein were specific for liver cancer compared with healthy controls or liver cirrhosis.

From results of analysis, we find a surprising phenomenon that the content of biomarker detected by immuno-MS and ELISA are opposite, the result of immuno-MS shows that the content of biomarker is higher in controls than that in liver cancer patients, while the result of immuno-MS shows content of biomarker is higher in liver cancer. To explain this problem, we designed co-immunoprecipitation experiments and the result showed that, in ELISA assay, both pep5 and its precursor protein were captured whereas immuno-MS only detected serum pep5. Our preliminary experiment showed that pep5 is derived from cleavage of domain 5 of HMW kininogen (data not shown). HMW kininogen and its domain 5 can inhibit the vascularization and the migration and invasion of cancer cell [17, 19]. Therefore, we can think that in response of the human-self to tumor, the production of HMW kininogen and the proteolysis of HMW kininogen decreased lead to the content of HMW kininogen increases and the pep5 decreases. However, further validation for this viewpoint is in progress.

In conclusion, our findings show that combination pep5 and HMW kininogen can be good strategies for biomarkers detection of liver cancer; and the combination of immuno-MS and ELISA might be a novel strategy for the detection of cancer through further validation.

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Disclosure of conflict of interest

None.

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