

## RESEARCH ARTICLE

# Observation of peptide differences between cancer and control in gastric juice

Wei-Chao Chang<sup>1</sup>, Ping-I Hsu<sup>2, 3\*</sup>, Yuan-Yan Chen<sup>1</sup>, Michael Hsiao<sup>1</sup>, Pei-Jung Lu<sup>2, 3</sup> and Chung-Hsuan Chen<sup>1</sup>

<sup>1</sup> Genomics Research Center, Academia Sinica, Taipei, Taiwan

<sup>2</sup> Department of Internal Medicine, Kaohsiung Veterans General Hospital and National Yang-Ming University, Kaohsiung, Taiwan

<sup>3</sup> Department of Education and Research, Kaohsiung Veteran General Hospital and National Yang-Ming University, Kaohsiung, Taiwan

Biomarkers for various diseases have been extensively searched for the past 5 years. Nevertheless, most efforts were focused on the search for protein biomarkers from serum samples. In this work, we tried to look for peptide biomarkers from gastric juice samples with MALDI-TOF-MS. More than 200 gastric juice samples from healthy people, gastric ulcer patients, duodenal ulcer patients, and cancer patients were examined. There were clear pattern differences of mass spectra among samples from healthy people and patients with different gastric diseases. We found five peptides for gastric cancer diagnosis with high sensitivity and specificity. Sequences of these five peptides, including two pepsinogen fragments, leucine zipper protein fragment, albumin fragment, and  $\alpha$ -1-antitrypsin fragment, have been identified by mass spectrometric analysis and immuno-deplete assay with antibodies.

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## 1 Introduction

Gastric cancer (GC) is one of the most common cancers worldwide. Prognosis of cancer patients can dramatically be improved if gastric tumors are detected at early stage [1–4]. Thus, a reliable and convenient screening method of GC is in a critical need. Recently, proteomic analysis has become a valuable tool for novel biomedical research and biotechnology development. In the past, a time-consuming 2-DE followed by MS is typically used for the discovery of biomarkers.

There are thousands of proteins in blood, a complete qualitative and quantitative analysis of all proteins at present is not practical due to the limited sensitivity and laborious work. An alternative analytic technique in terms of the comparison of peptide mass spectrum profile is considered to be feasible. The mass spectra analysis of serum samples from cancer patients and healthy people without detailed protein identification through amino acid sequencing have been pursued for ovarian [5] and prostate cancer [6] diagnosis.

The biological samples such as blood serum and plasma are less invasive and have been used for clinical diagnosis. Nevertheless, the relatively low quantity of cancer-related proteins in blood stream makes it difficult to be measured precisely. Therefore, cancer diagnosis by protein analysis is often confined to cancer patients at late stages [7]. There is an advantage on clinical biomarker study by using body fluid samples in which the concentration of cancer-related pep-

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**Correspondence:** Chung-Hsuan Chen, Genomics Research Center, Academia Sinica, Taipei, Taiwan  
**E-mail:** winschen@gate.sinica.edu.tw  
**Fax:** +886-2-2789-9923

**Abbreviations:** CEA, carcinoembryonic antigen; DU, duodenal ulcer; GC, gastric cancer; GU, gastric ulcer; PNN, plausible neural network; ROC, receiver operating characteristic

\* Both authors contributed equally to this work.

tides/proteins can be higher than that in blood in certain cancers. For stomach cancer diagnosis, gastric juice analysis is a good compromise. It is relatively easy to obtain gastric juice [8, 9] since millions of patients perform endoscopic checkups each year. Therefore, gastric juice analysis can be a convenient way to obtain biomarkers.

For the past few years, proteomic studies for biomarker searches have been extensively pursued for various diseases including Alzheimer's disease, schizophrenia, depression, and cancer [10–13]. Our previous work [14] established that  $\alpha$ 1-antitrypsin precursor in gastric juice is a novel biomarker for gastric cancer and ulcer. In addition to proteomics for disease diagnosis, peptidomics was recently developed [15, 16]. It is much easier to obtain peptide spectra with a high mass resolution directly than protein spectra. Peptide sequences can also be determined by tandem mass analysis without the need of enzymatic digestion. Up to now, only a few peptides with their functions resolved [17, 18]. Peptide measurements have been applied to the diagnosis of neuron-diseases [19] and oral cancer [20] diagnosis. It is a reasonable inference that peptidomic analysis is a more convenient tool than proteomic approach to find biomarkers.

In this work, we apply a peptidomic approach to search for gastric cancer biomarkers. In general, a mass spectrum of a gastric juice sample by MALDI-TOF-MS was obtained within a few seconds so that complete mass spectra from many hundreds of samples were obtained in this work. A neural-network program was developed to analyze the large number of the obtained data and to compare the differences of mass pattern profiles. The sequences for these peptides which showed major differences in mass spectra were subsequently resolved *via* tandem mass analysis.

## 2 Materials and methods

### 2.1 Samples collection

Gastric juice samples from 106 asymptomatic subjects (AS), 38 patients with gastric ulcer (GU), 38 patients with duodenal ulcer (DU), and 34 patients with GC were obtained for this study to obtain potential peptide biomarkers. Seven patients were in their early stage (stage I or II) and 27 in advanced stage (stage III or IV) of GC. The demographic information is shown in Table 1. The AS recruited from our health examination clinics had no clinical history of serious gastrointestinal diseases, and their endoscopic findings are either normal or with mild gastritis. The diagnoses of GU and DU were confirmed by endoscopic examination. An ulcer was defined as a circumscribed mucosal wound of 5 mm or more in diameter and a well-defined ulcer crater [21]. Gastric cancer was confirmed by histology and classified as intestinal, diffuse, or mixed type according to Lauren's classification [22]. This study was approved by the Medical Ethics Committee of the Kaohsiung Veterans General Hospital. All patients and control groups gave their consents to this study.

**Table 1.** Demographic information of gastric juice samples

Group	Sex		Age	
	Male	Female	Male	Female
Normal (106)	54	52	51.4	47.7
Duodenal ulcer (38)	27	11	55.3	52.2
Gastric ulcer (38)	19	19	55.6	58.1
Gastric cancer (34)	24	10	68.5	64.8

All the gastric juice samples were collected by endoscopic method and there were no any surgical procedures performed prior to collection of the gastric juice in gastric cancer patients. Endoscopies were performed with the Olympus GIF XV10 and GIF XQ200 (Olympus, Tokyo, Japan) after patients had fasted overnight. Immediately after insertion of the scope into the stomach, 5 mL of gastric fluid was aspirated through the suction channel of the endoscope and collected in a sterile trap placed in the suction line. Gastric juice was then centrifuged at 10 000  $\times$  g for 10 min at 4°C for removal of cell debris and other contaminants within 5 min after the sample collection. Aliquots of the supernatant were stored at  $-70^{\circ}\text{C}$  until assay. All samples were analyzed by mass spectrometric analysis within 2 months after sample collection. Since the gastric juice samples were stored at  $-70^{\circ}\text{C}$ , and the activity of pepsin was expected to be completely lost in such a low temperature, the degradation of our samples could be avoided.

### 2.2 Sample preparation for mass spectrometric analysis

All samples were randomized before the analysis. Signal amplitudes and peak positions were normalized by time normalization and calibration with standard samples periodically. Since the instrument used was reasonably stable, this normalization process did not change the major conclusion of this work. All gastric samples were centrifuged at 10 000 rpm for 10 min at 4°C. The supernatant 10  $\mu\text{L}$  was neutralized with 2  $\mu\text{L}$  of 150 mM ammonium hydroxide before purification by magnetic beads such as C18 or Cu beads (ClinProt™; Bruker Daltonics Company). The binding incubation was hold at room temperature for 1 min. According to the manufacturer's protocol, the bound peptides/proteins were eluted using 10  $\mu\text{L}$  of 50% ACN after binding and washing. Average protein concentrations for samples from cancer patients and healthy individuals after magnetic bead extraction were also measured [14].

### 2.3 Analysis of MALDI-TOF-MS profile

For mass spectrum analysis, we typically mixed 1  $\mu\text{L}$  of eluted sample with 1  $\mu\text{L}$  matrix solution consisting of 2,5-dihydroxybenzoic acid (50 nmol/ $\mu\text{L}$  in 50% ACN) and phos-

phoric acid. Then 1  $\mu\text{L}$  of the resulting mixture was spotted onto the MALDI stainless steel sample plate and allowed to air dry at room temperature. The peptide/protein mass profiles of eluted samples were generated by a MALDI TOF mass spectrometer modified from an ABI Voyager mass spectrometer (Foster City, CA, USA). The mass resolution ( $M/\Delta M$ ) was  $\sim 400$  for mass range below 10 000 Da. The specific feature of this equipment is the capability of adjusting the gain of the microchannel plate and the measurement of laser energy. Laser energy *per* pulse was measured by a Laser Technik power meter (PEM 101, Laser Technik, Berlin, Germany). Most spectra were obtained in the range of  $m/z$  from 1000 to 10 000. A typical mass spectrum was obtained with the laser fluence at  $\sim 150 \text{ mJ}/\text{cm}^2$  and the accumulation of 100 laser shots. The criterion of S/N for a peak detection was  $\geq 5$ . Samples from healthy people and cancer patients were performed under the same laser fluence. Other matrices suitable for producing protein ions such as sinapinic acid and CHCA have also been attempted and similar results were obtained.

## 2.4 Reverse phase HPLC purification

RP HPLC was performed using Agilent 1100 Series HPLC instrument (Agilent Technologies, Palo Alto, CA). The peptides/proteins mixtures from gastric juice samples were separated using a 4.6 mm  $\times$  15 cm Extend-C18 column (Agilent).

## 2.5 Identification of peptide sequence by tandem mass spectrometric analysis

Two types of mass spectrometer were used for tandem mass (MS/MS) experiments.

(i) MALDI-TOF/TOF MS/MS: MALDI-TOF/TOF MS/MS experiments were pursued using a 4700 Proteomics Analyzer (MALDI-TOF/TOF, Applied Biosystems). During MS/MS data acquisition, a maximum of 20 000 shots (accumulated from 125 Hz laser shot) were accumulated for each spectrum. For antibody check, a MALDI-TOF/TOF Ultraflex mass spectrometer was used (Bruker; USA). The mass resolution ( $M/\Delta M$ ) was  $\sim 600$ .

(ii) ESI-MS/MS: LC-MS/MS experiments were pursued on a LTQ FTICR (Linear quadrupole IT Fourier transform ICR) mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospray ion source (New Objective), an Agilent 1100 Series binary HPLC pump (Agilent Technologies), and a Famos autosampler (LC Packings, San Francisco, CA). The sample solution was injected (1  $\mu\text{L}$ ) at 8  $\mu\text{L}/\text{min}$  flow rate on to a self packed precolumn (150  $\mu\text{m}$  id  $\times$  15 mm, 5  $\mu\text{m}$ , 100  $\text{\AA}$ ). Chromatographic separation was performed on a self-packed RP C18 nanocolumn (75  $\mu\text{m}$  id  $\times$  300 mm, 5  $\mu\text{m}$ , 100  $\text{\AA}$ ). Full-scan MS spectra ( $m/z$  350–1800) were acquired in the FT-ICR with mass resolution of 100 000 at  $m/z$  equal to 400. The ten most intense ions were sequentially isolated for MS/MS by LTQ.

## 2.6 Statistical method

Each spectrum obtained from MALDI TOF-MS was output as a text file, then compiled and analyzed by plausible neural network (PNN) pattern recognition software [23, 24]. PNN is an intelligent self-organized neural networks system. PNN performs associative memories, clustering, classification, function approximation, and belief judgment in a single network architecture. Differences between spectra are identified based on the features selection module. Peptide mass peaks with significant differences between samples from cancer patients and noncancer people were then identified. One specific advantage of PNN is the capability to deal with multiple biomarkers simultaneously. Results from PNN analysis indicate it can distinguish cancer from normal without the process of protein identification.

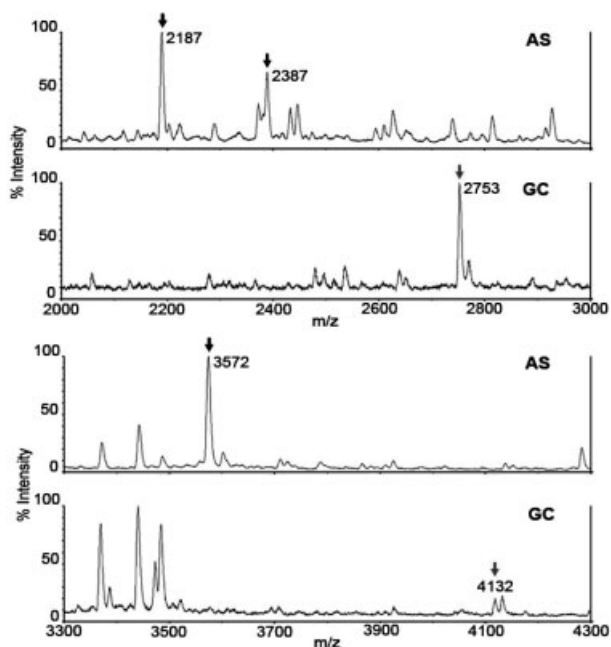
## 2.7 Immuno-depleted assay

The multiple antigen peptide (MAP) of pepsinogen fragment (FLKKHLNPARKYFPQW) was immunized to mice for obtaining polyclonal antibody. Two microliters of antibody were incubated with 20  $\mu\text{L}$  gastric juice on ice for 30 min. The protein A-sepharose (Amersham Pharmacia Biotech) was washed and changed to PBS solution, and then 10  $\mu\text{L}$  were added to gastric juice-antibody solution for 30 min. After centrifugation, the supernatant was subjected to C18 purification and MALDI-TOF mass analysis. As a negative control, the gastric juice without the specific antibody was incubated without and with protein A-sepharose for 30 min on ice.

## 3 Results

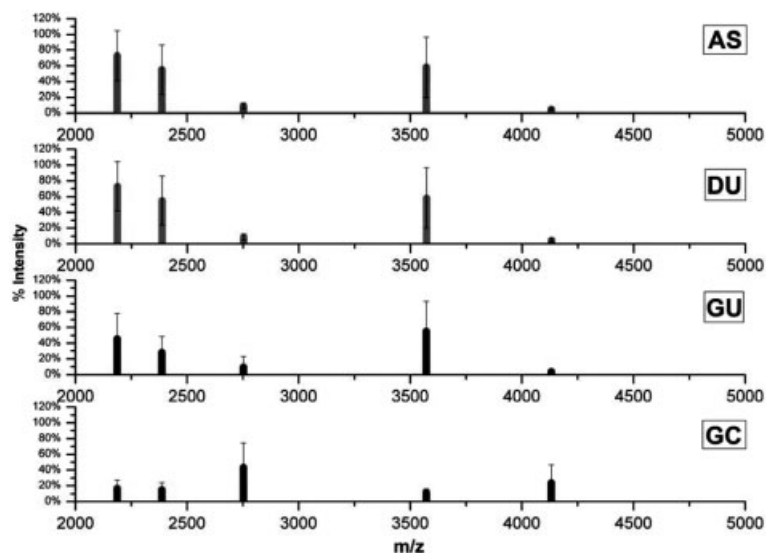
Mass spectra from all samples from GC, GU, DU patients, and AS were obtained. All of the signals with an S/N ratio  $\geq 5$  in a mass range of 1000–10 000 were defined and the number of peaks was around  $200 \pm 35$ . Peaks from samples of GC and GU were observed to be fewer than those of DU and AS. In general, the distribution of proteomic data is usually too wide to fit normal distribution well. It turns out to be that the conventional approach with mean value and SD often leads to an inaccurate interpretation on available data as far as the quantitative aspect is concerned. This dilemma is, however, conquered by PNN since it is based on a nonparametric estimation of data distribution. The data of mass spectra from lots of samples are quickly compiled and analyzed by PNN pattern recognition software.

Some of the peak differences of mass spectra between healthy subjects and cancer patients identified by PNN were easily observed by the naked eye. We considered the peptides related to these mass peaks could be reliable biomarkers for stomach cancer diagnosis. We found five mass peaks correlated with stomach cancer with  $m/z$  at 2187, 2387, and 3572 showed down-regulation but 2753 and 4132 showed up-regulation. The comparative spectra are shown in Fig. 1. These



**Figure 1.** Comparisons of mass spectra of gastric juices between asymptomatic subjects and gastric cancer group. Major different mass peaks of gastric juices between AS and GC groups are shown by arrows. Data were obtained from ABI Voyager mass spectrometer. The mass resolution is  $\sim 400$ . All data obtained to compare the differences between samples from cancer and non-cancer were obtained by the same instrument in this work.

five peaks were also chosen by PNN among the top list of differential masses. Mass spectra from all samples with the average of peak amplitudes and their fluctuations from AS, GU, DU, and GC are shown in Fig. 2. The expression levels of these molecules are obviously different between cancer and noncancer groups.



**Figure 2.** Expression profiles of five specific markers in gastric juices. The statistics of relative intensity and SD of each marker from asymptomatic healthy subjects and various types of gastric disease patients are presented. The percentage of intensity of marker was normalized by highest peak in each spectrum.

Protein molecules with similar molecular weight in body fluid sometimes are difficult to be distinguished due to the limited resolution of MS. Hence, the relatively low mass resolution from MALDI-TOF-MS is usually not high enough to distinguish the corresponding molecules from these mass peaks. We used HPLC to separate and purify gastric juice samples. With each fraction from HPLC, we further did MALDI-TOF-MS analysis to verify which zone contained material showing mass peaks of interest. HPLC fractions with interest were further concentrated and then applied to MS for tandem mass analysis. We carried out tandem mass analysis directly from the eluted samples by ABI 4700 mass spectrometer. MS/MS spectra of these molecules with 2187, 2387, and 2753 Da were obtained. MS/MS spectra of peptides with  $m/z$  at 3572 and 4132 were obtained by LTQ FTICR mass spectrometer. The MS/MS spectra obtained from MALDI-TOF-MS (ABI 4700) and LTQ FT-ICR (Thermo) were resolved with MASCOT [25] search engine to identify the sequences of these peptides.

In the above procedures, we have identified down-regulated peptides as pepsinogen fragments with the mass of 2187 Da and sequence of FLKKHNLNPARKYFPQW as well as 2387 Da with the sequence of FLKKHNLNPARKYFPQWEA. Other down-regulated peptide with mass of 3572 Da was identified as a leucine zipper protein fragment with the sequence of ETKKTEDRFVSSSKSEGKKSREQPSVLSRY. For up-regulated peptide with mass at 2753 Da in stomach cancer, it was identified as an albumin fragment with sequence of DAHKSEVAHRFKDLGEENFKALVL. For up-regulated peptide with 4132 Da, it was identified as a C-terminal fragment of  $\alpha$ -1-antitrypsin with sequence of SIPPEVKFNKPFVFLMIEQNTKSPLFMGKVVNPTQK. According to the result, it suggests the existence of excessive amount of albumin or  $\alpha$ -1-antitrypsin in gastric juice can be served as a warning sign for a thorough check to prevent the progression to the stomach cancer.

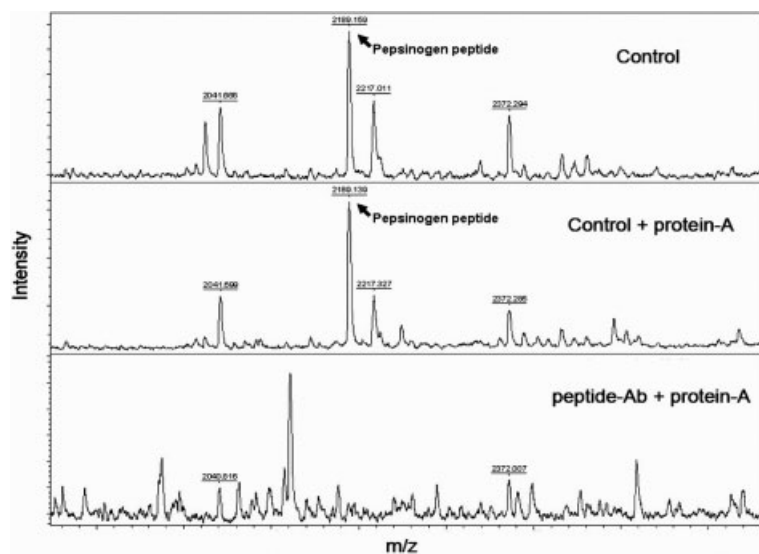
We utilized immuno-deplete assay to confirm the sequences of the above biomarkers. For example, polyclonal antibody against FLKKHLNPARKYFPQW peptide (2187 Da) was manufactured to confirm the peak in mass spectrum indeed has the same sequence as identified. After the binding of antibody with the specific peptide from gastric juice samples, protein A-sepharose bead was used to remove the binding complex. Thus, the mass spectra of gastric samples we obtained were lack of mass 2187 Da (Fig. 3). It clearly indicates the sequences previously identified should be reliable. Antibodies derived from these peptides can be a convenient item for stomach cancer screening or diagnosis in the future.

For each peptide marker, the averages of S/N for both cancer and noncancer groups were calculated. The cut-off values were determined with the optimization of the multiplication of sensitivity and specificity. We did not use SD [20] to define the cut-off values for sensitivity and specificity determination due to the relatively broad amplitude distribution of mass spectra obtained. Once the cut-off value was determined, sensitivity was thus obtained with the percentage of correct prediction from cancer samples only. The cut-off value of specificity is defined as a similar way with the percentage of correct prediction from noncancer samples. Summary on the regulation agreement for samples from AS,

DU, GU, and GC for all five mass peaks are listed in Table 2. It is worthy to note that only two duodenal ulcer samples were misdiagnosed as a gastric cancer even with one peptide as biomarker.

Figure 4 shows results from the receiver operating characteristic (ROC) analysis and specificity and selectivity based on the number of peptides in agreement with the predictions on the regulation for GC. These five biomarkers possess good diagnostic power with the value of area under the curve (AUC) is 0.87. From ROC analysis, it is found that the optimum combination for both sensitivity and specificity is when three of the peptides are used for cancer prediction. The overall sensitivity for gastric cancer was 79%, and specificity was 92%. This judgment can be accomplished by observation of mass spectra with naked-eye, without PNN bioinformatics analysis.

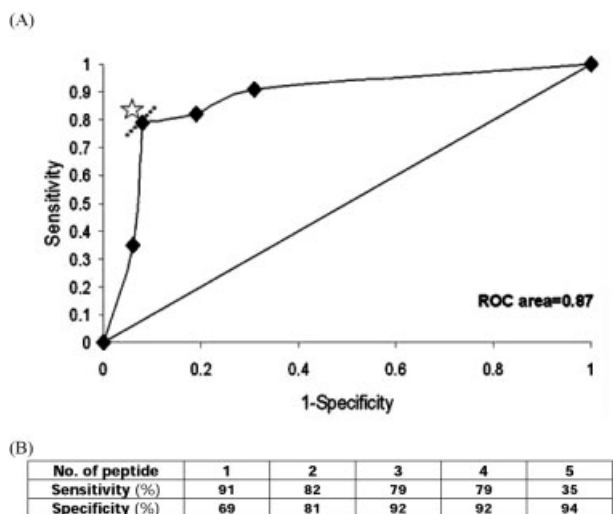
The drawback of using a cut-off level is that it treats each biomarker with equal weight; however, some biomarkers may be more important than others and should have a stronger weight. Also, when a biomarker is near the cut-off line it does not provide strong differential capability toward the final diagnosis and thus should be represented as null information. In order to use the weight information of the biomarkers more sensibly, we utilize the theory of PNN



**Figure 3.** Immuno-deplete assay of gastric juice. A peak (indicated by arrow) representing pepsinogen peptide was detectable in control (normal gastric juice) (spectrum at the top) and negative control (normal gastric juice added protein A) (spectrum in the middle), but not in the corresponding depleted antibody (spectrum at the bottom). The mass resolution obtained is ~600.

**Table 2.** Sensitivity and specificity of the five specific gastric cancer markers

Signal	2187 m/z		2387 m/z		2753 m/z		3572 m/z		4132 m/z	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Normal	89	17	83	23	7	99	88	18	6	100
Duodenal ulcer	37	1	38	0	0	38	37	1	0	38
Gastric ulcer	26	12	22	16	7	31	23	15	9	28
Cancer	5	29	4	30	23	11	7	27	16	18
Sensitivity (%)	85		88		68		79		47	
Specificity (%)	84		79		92		81		91	



**Figure 4.** ROC curve analysis of arbitrary combination from five specific gastric cancer markers. (A) The AUS is 0.87. The star sign, optimal Youden index, shows the point of contact of ROC curve with the tangent of slope 1. (B) Sensitivity and specificity based on the number of peptides meet the prediction of gastric cancer.

analysis [23, 24], which combines the neural networks model and evidential reasoning. Each biomarker provides a part of the weighted diagnostic evidence; the network aggregates them together and then makes a final differential diagnosis. In this particular PNN analysis, we used 15 hidden neurons for training, which corresponds to clustering the five dimensional  $m/z$  data into 15 up- and down-regulation patterns. The clusters were then linked to the diagnosis categories. Specificities obtained for AS, DU, and GU are 99, 100, and 92%, respectively. Our results from PNN calculation indicate the sensitivity of gastric cancer diagnosis was 82% and specificity for all noncancer was 98% (Table 3). If we take the average of the results by PNN analysis and naked-eye observation, the sensitivity and specificity were obtained as 80 and 95%, respectively.

#### 4. Discussion

Current available serum markers are limited in sensitivity for the detection of gastric cancer. In clinical practice, carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9) are the most commonly used serological biomarkers for gastric cancer. The sensitivity of serum CEA and CA19-9 for detection of gastric cancer are less than 30% [26–30]. Recently, serum p53 antibody and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) have been applied to detect gastric cancer (26, 28) but with poor sensitivities (15 and 17%, respectively). In this work, we demonstrated that the sensitivity and specificity of peptidomic analysis of gastric juice for diagnosis of GC were, respectively, up to 80 and 95% which are obtained from the average of results by

**Table 3.** Sensitivity and specificity obtained from samples with various types of gastric disease, using PNN analysis and five biomarkers as predictors

Status	Prediction		Diagnosis (%)	
	Noncancer	Cancer	Sensitivity (%)	Specificity (%)
Normal	105	1	–	99
Duodenal ulcer	38	0	–	100
Gastric ulcer	35	3	–	92
Cancer	6	28	82	–

naked-eye observation from Table 2 and PNN analysis from Table 3. Our results indicate that the sensitivity and specificity of even a single peptide as a biomarker for gastric cancer detection are still significantly higher than those of CEA and CA19-9. For two up-regulated peptides with mass at 2753 and 4132 Da, the sensitivities are 47 and 68%, respectively (see Table 2). For down-regulated peaks with mass 2187, 2387, and 3572  $m/z$ , sensitivities are 85, 88, and 79%, respectively. These results indicate that a non-invasive method to obtain gastric juice followed by peptidomic analysis may serve as a new tool to screen gastric malignancies.

With peptidomic analysis, we have found that down-regulation of a pepsinogen fragment and a leucine zipper protein fragment in gastric juices from GC patients. It is known that pepsinogen is secreted by chief cells of the stomach. Gastric carcinogenesis progresses through the stage of gastric atrophy, which leads to a decreased number of chief cells. Therefore, it is reasonable to expect the down-regulation of pepsinogen in gastric juices from gastric cancer patients. Leucine zipper protein was found as a potential tumor suppressor [31] and its expression is down-regulated in human gastric and pancreatic cancer cell lines [32–34]. Furthermore, the expression of leucine zipper protein is also reduced in oral squamous cell carcinoma [35] and bladder cancer [36]. The decreased gastric juice level of leucine zipper protein fragments in GC patients seems to be reasonable.

In this study, we also observed significant number of albumin peptide fragments in gastric juices from GC patients. Since occult bleeding from ulcerated tumor is a common phenomenon for GC, the albumin may shed into the gastric juice in the process of tumor bleeding. Therefore, an increase of albumin levels in GC samples is reasonable. However, abundant albumin fragment in gastric juice may not be cancer-specific. In this work, some GU patients also had abundant albumin in gastric juices. Therefore, an increase of albumin fragment levels and a decrease of pepsinogen and leucine zipper protein levels in gastric juice are very common in GC patients. In a separate work, we also found  $\alpha$ -1-antitrypsin precursor in gastric juice could be a biomarker for gastric cancer [14].

Therefore, it is not surprising that a peptide segment of albumin and/or  $\alpha$ -1-antitrypsin could serve as biomarkers for gastric cancer. These five specific peptides do not necessarily reflect the coexistence of the corresponding proteins. In our experiments, the peptides we measured exist in gastric juice without any additional enzymatic reaction.

In conclusion, this work verifies that the peptidomic pattern of gastric juices from various gastric diseases were of significant difference. The decreased levels of pepsinogen and leucine zipper protein fragments and increased levels of albumin and  $\alpha$ -1-antitrypsin fragments will be valuable for the detection of GC. Sequences of these peptides can be used to develop antibodies for rapid GC screening.

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