Application of serum proteomic patterns by ProteinChip SELDI system in diagnosis of gastric cancer

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Application of serum proteomic patterns by ProteinChip SELDI system in diagnosis of gastric cancer

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Abstract

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Gastric cancer is one of the most common malignancy in the world and one of the leading causes of cancer related death in Korea. Most treatments for advanced gastric cancer have limited efficacy, and the median survival is just 10 months. So early detection of gastric cancer could have profound impact on the successful treatment. While endoscopic evaluation has been the gold standard for screening for gastric cancer, endoscopy is an invasive and expensive procedure. Therefore, development of novel screening methods that reduce costs and risks is critical in impacting mortality rates from gastric cancer. Application of multiple biomarkers may improve the diagnostic prediction to distinguish cancer from non-cancer. ProteinChip Surface-Enhanced Laser Desorption/Ionization Time-of-flight Mass Spectrometry (SELDI-TOF-MS) system is one of the currently used techniques to identify biomarkers for cancers and other diseases. This study was performed to identify whether the serum proteomic patterns by ProteinChip SELDI system can differentiate gastric cancers from non-cancer cohorts.

The protein profiles of 100 serum samples obtained from 60 gastric cancer patients and 40 age-matched healthy individuals were screened by SELDI-TOF-MS system. Protein expression profiles were expressed on CM10 ProteinChip (weak cation exchange) Array and analyzed by Ciphergen PreoteinChip Reader (model PBS II). Peak intensities were normalized by total ion currency and analyzed by the Biomarker Wizard Software to identify the peaks showing significantly different

intensities between normal and cancer groups. Classification analysis and construction of decision trees were done with the Biomarker Pattern Software 5.0.

SELDI-TOF-MS by averaging 50 laser spots collected at a laser intensity setting of 160, a detector sensitivity of 6, and mean mass range of 30 kDa. Seventeen protein peaks shown significant differences between two groups were chosen to make a protein biomarker pattern. The decision tree which gives the highest discrimination for the training set includes four peaks at 5919, 8583, 10286, and 13758 as splitters. The sensitivity and the specificity for classification of the training set with the decision tree giving the highest discrimination were 96.7% (58/60) and 97.5% (39/40), respectively. When the protein biomarker pattern was tested with the blinded test set including 30 gastric cancer patients and 20 healthy individuals and, it yielded a sensitivity of 93.3% (28/30) and a specificity of 90% (18/20). These results suggest that serum-protein profiling pattern by SELDI system may distinguish gastric cancer patients from normal counterparts with relatively high sensitivity and specificity.

Key Words: gastric cancer, protein profiling pattern, SELDI-TOF-MS

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I. INTRODUCTION

Gastric cancer is one of the most common malignancy in the world and one of the leading causes of cancer related death in Korea.[1] Most treatments for advanced gastric cancer have limited efficacy, and the median survival is just 10 months.[2] If the patients are diagnosed as early gastric cancer and the tumor is completely resected, the overall 5-year survival can be expected up to 90%.[3,4,5] So early detection of gastric cancer could have profound impact on the successful treatment. While endoscopic evaluation has been the gold standard for screening for gastric cancer, endoscopy is an invasive procedure, with its own risks of morbidity in 1 in 200 and mortality in 1 in 12000.[6] The considerable expense of endoscopic procedures must be weighed against societal benefit when screening programs for large populations are developed. Therefore, development of novel screening methods that reduce costs and risks is critical in impacting mortality rates from gastric cancer.

Proteomic analysis becomes a valuable tool in determining the presence of biomarkers or in mapping biomarker profiles within different sample groups, for example in healthy and diseased individuals.[7,8,9] The field of proteomics can be defined as the large-scale analysis of the expressed protein complement of the genome. The progression of gastric cancer from premalignant lesions to invasive cancers is characterized by sequential acquired genetic mutations, which may be superimposed on preexisting germline mutations that increase cancer risk. However these mutations will only be selected for if they manifest survival advantages within protein metabolic and signaling pathways and networks. The genes involved in these aberrations include oncogenes, transcriptional factors, tumor suppressor genes, and others, but not all genetic mutations are expressed. Because proteins are the molecules that are functional effectors of cellular processes, analysis of the proteome allows the detection of functionally relevant post-translational modifications, such as phosphorylation, not present at the genome level.[10] The use of molecular techniques to screen for cancer relies on the concept of biomarkers: molecular signals at the genetic or protein level that signal the presence of disease. The human genome contains only about 33,000 genes, but these genes code for more than 200,000 proteins,[11] so proteome is much more complex than the genome.

The ideal biomarker should be detectable in a readily accessible body fluid, such as serum, and would reflect not only the presence of disease but the status of a disease process as it changes overtime. The blood proteome changes constantly as a consequence of the perfusion of diseased organ adding, subtracting or modifying the circulating proteome. Use of single-protein biomarkers to screen for malignancy can lead to improvement in disease detection, as has been the case for prostate specific antigen (PSA) in prostate cancer. Only PSA has been discovered to be useful in testing for early cancer.[12] Candidate serum biomarkers for gastric cancer include carcinoembryonic antigen and other proteins such as CA 19-9, CA 50, CA 72-4.[13,14,15] However, none of these markers has sufficient sensitivity and specificity to merit routine clinical use for the early detection of cancer. In general, the validation of single-protein biomarkers one by one from the thousands of candidates in human

proteome is a laborious and often ineffective approach to developing cancer-screening tools. Moreover, because of the genetic heterogeneity among populations, one biomarker might indicate disease in one group but be statistically non significant in another. Thus the proteomic patterns might have a higher level of diagnostic accuracy.[11,16]

ProteinChip surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) is an innovative proteomic technology that enables high throughput analysis of a variety of biological samples for discovery of biomarkers. The ProteinChip SELDI system uses proprietary technology to rapidly profile, detect, and analyze proteins directly from complex biological samples.[17,18] The system consists of three major components including the ProteinChip Array, the ProteinChip reader (SELDI-TOF-MS), and the software.[16,19,20] Crude biological samples including sera or total lysates can be applied directly to the ProteinChip Arrays. Depending on the type of chromatographic matrix used, which is week cation, strong anion or immobilized metal affinity, a subset of the proteins in the sample bind to the surface of the chip. After a short period of incubation, unbound proteins are washed off the surface of the ProteinChip Array. A matrix capable of being photoactivated is then applied, and the chip is dried. A laser irradiates the chip in a vacuum chamber and desorbed proteins are launched as charged ions. The proteins bound to the ProteinChip Array are analyzed in the ProteinChip reader (SELDI-TOF-MS) that allows the molecular weight of the bound proteins to be determined (Fig 1). The spectra obtained consist of many different biomarkers. Comparisons of the protein peak patterns obtained from samples representing different populations are expected to provide detailed diagnostic patterns classifying pathological states.

Recently, the ProteinChip SELDI technology has been used for analyzing protein expression profiles to find and identify biomarkers for diagnosis from body fluids like serum, urine, and pancreatic juice. Some of the biomarkers in this process were identified and further characterized. However, without identifying individual biomarkers, the protein biomarker patterns were successfully used to screen diseases.

The first report using pattern recognition algorithms coupled to high-throughput mass spectrometry for proteomic pattern diagnostics applied to ovarian cancer detection with a sensitivity of 100% and a specificity of 95%.[21] Yet, these results were criticized and the expectations were smoothened in recent publications.[22,23,24] This provoked a debate about future processes of establishing and proving the reliability of novel technologies.[25,26,27] Although, since this initial report, the method has been confirmed in other types of cancer like lung,[28] breast,[29] prostate,[30] and pancreatic cancers.[31] These studies suggest that SELDI protein profiling can distinguish cancer patients from normal subjects with relatively high sensitivity and specificity. Once the best fitting mass-to-charge ratios values are selected, the biomarkers can be used for screening.

This study explored the application of protein patterns obtained from sera using the ProteinChip SELDI system to differentiate gastric cancer patients from non-cancer people.

II. Materials and Methods

1. Preparation of sample

A total of 100 serum samples including 60 pathologically confirmed gastric cancer patients and 40 healthy subjects were collected from the Yonsei Yongdong Cancer Center. Healthy subjects were received comprehensive medical examination including gastroscopy and proved to have no malignancy. The two groups were matched for age. The serum samples were stored at -80℃ until use.

2. Protein expression profiling with ProteinChip -SELDI-TOF-MS

Twenty μl of serum was mixed with 30 μl of U9 buffer (9M Urea and 2% CHAPS(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid)) and the mixed sample was further diluted 10 fold with the buffer containing 50mM sodium acetate pH 4 and 0.1% Triton X-100. Protein expression profiling was processed as described by the manufacturer (Ciphergen Biosystems, Fremont, CA, USA). First, a CM10 ProteinChip (weak cation exchange) Array was pretreated with the binding and washing buffer (50 mM sodium acetate pH 4, 0.1% Triton X-100). Then 50 μl of the diluted sample were applied to a CM10 ProteinChip Array. After 30 min incubation with vigorous agitation, the ProteinChip Array was washed 3 times with excess volume of the binding and washing buffer to remove the unbound proteins and other contaminants. The ProteinChip Array was dried on air and an energy absorbing material, SPA(sinapinic acid) in 50% acetonitrile, 0.5% TFA(trifluoroacetic acid) was added to each spot on the ProteinChip Array.

3. ProteinChip analysis by SELDI -TOF-MS

The chips were analyzed by the Ciphergen PreoteinChip Reader (model PBS II) (Fig 1). SELDI-TOF-MS by averaging 50 laser spots collected at a laser intensity setting of 160, a detector sensitivity of 6, and mean mass range of 30 kDa.

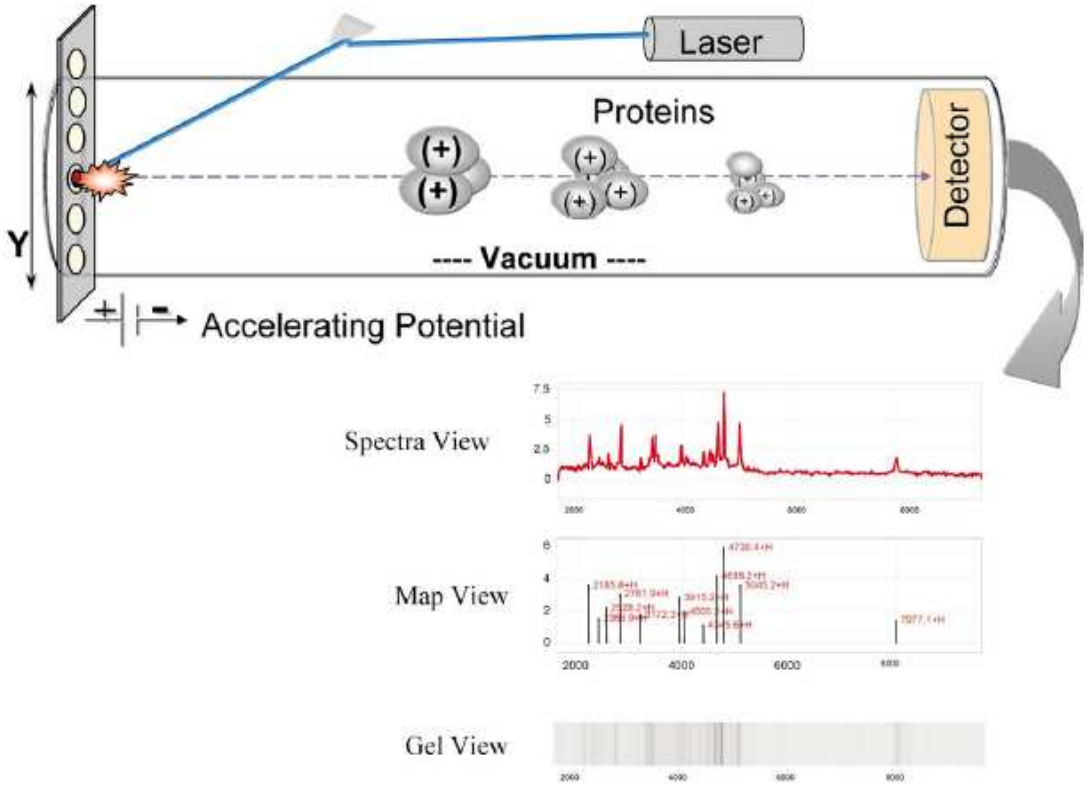

Figure 1. Schematic diagram of the SELDI mass spectrometer. After sample preparation the ProteinChip arrays are analyzed by a laser desorption ionization time-of-flight mass spectrometer (TOF MS). The TOF MS measures the molecular weights of the various proteins that are retained on the array. For comparison purposes, the software associated with the SELDI instrument is capable of displaying the resultant data as either a spectra, map or gel view.

4. Statistical analysis and construction of decision trees

Peak intensities were normalized by total ion currency and analyzed by the Biomarker Wizard Software(Ciphergen Biosystems, Fremont, CA, USA) to identify the peaks showing significantly different intensities between normal and cancer groups. The Mann-Whitney's U test was used for statistical analyses of differences between normal group and cancer one. The *p*-value for each peak was shown in Table 1. Classification analysis and construction of "CART" decision trees were done with the Biomarker Pattern Software 5.0 (Ciphergen Biosystems, Fremont, CA, USA). A discriminatory pattern that distinguished normal from gastric cancer was developed from a training set of mass spectra, this diagnostic pattern was then applied to a blinded set of samples from both cancer patients and healthy subjects.

III. RESULTS

1. Peak reproducibility

The reproducibility of the ProteinChip SELDI assays using the pooled sera from 40 control samples was determined. The peaks were analyzed in the mass range of 4,000 Da to 30,000 Da and 23 peaks showing the value of signal to noise ratio higher than 5 were randomly selected. The inter-assay (between chips) coefficient of variance(CV) for normalized intensities (peak heights or relative concentrations) of 23 peaks was 21.5% (Fig 2).

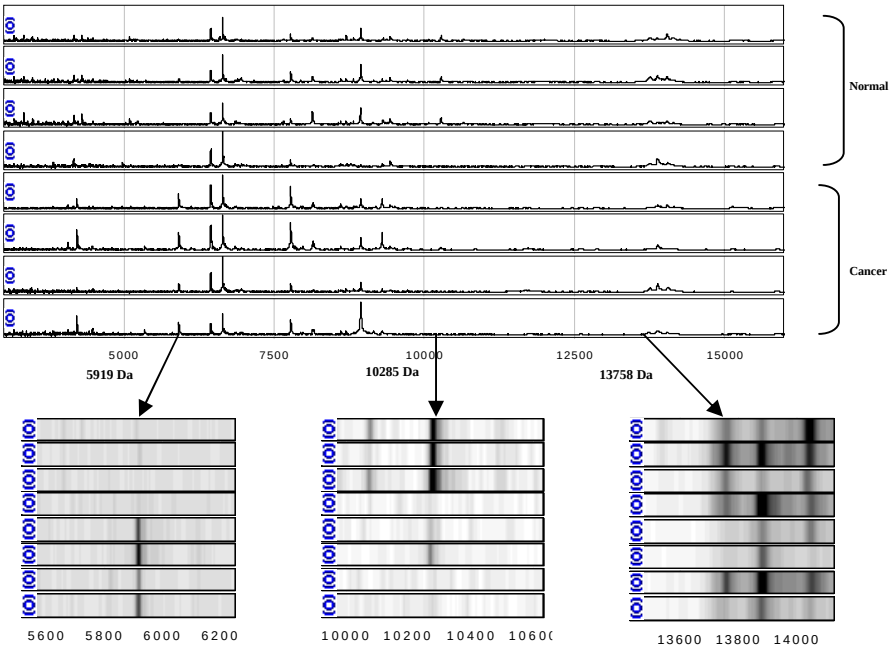

Figure 2. The reproducibility of ProteinChip SELDI assays

2. Serum SELDI profiles of gastric cancer versus healthy controls

We next analyzed the protein profiling spectra of the training set which includes 60 gastric cancer patient samples and 40 control samples and tried to find protein peaks or peak patterns with which we can separate gastric cancer patients from non-cancer cohorts. Peaks were detected by automatic peak detection using Biomarker Wizard software followed by baseline subtraction and normalization with total ion currency (Fig 3).

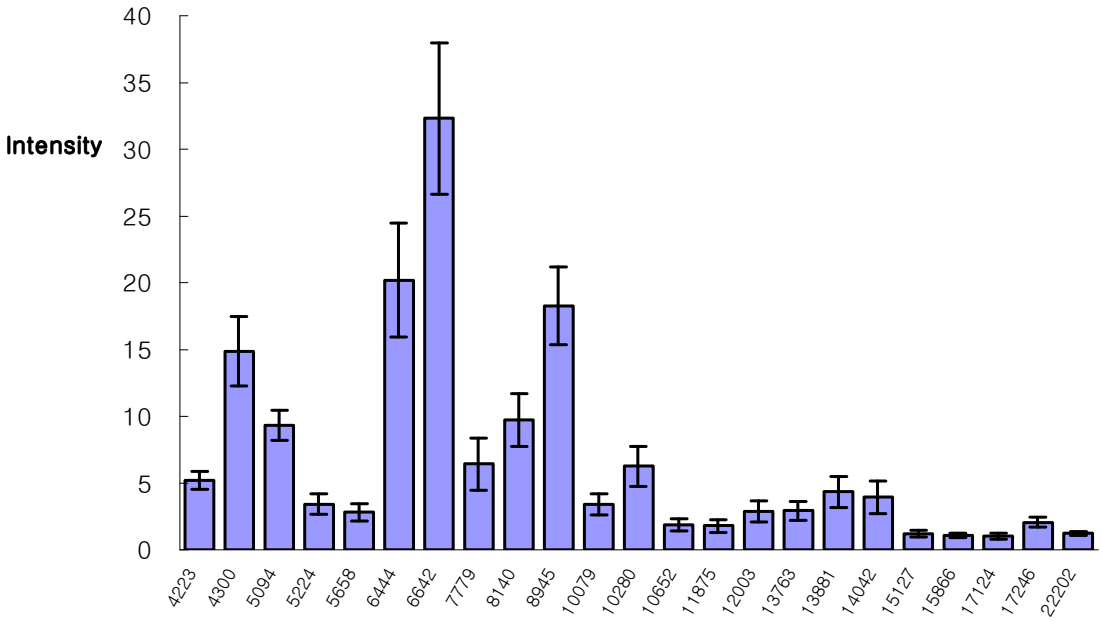

Figure 3. Representative protein profiles of sera from gastric cancer patients and healthy individuals

3. Decision tree classification

Decision trees were constructed using 17 peaks that gives *p*-value <0.01 in Mann-Whitney U test in the differences between cancer patient group and control one by Biomarker Wizard software (Table 1). The decision tree which gives the highest discrimination for the training set includes four peaks at 5919, 8583, 10286, and 13758 as splitters (Fig 4). One peak at a time was used as a splitter: the left node included the cases with peak intensity lower than or equal to specific value and the right node contained the remaining ones with peak intensity higher than the value. The 5919 Da peak was used as the root node in the classification tree to divide the samples into two groups. The cases in each branch node were then reclassified at the next layer following the same process with another peak and a specific value as a splitter. The splitting process continues until terminal nodes have no gain by further splitting.

Table 1. P value for each peak

M/Z	*p*	Mean – Cancer	SD – Cancer	Mean – Normal	SD – Normal
5919	0.000001	4.79	4.30	1.10	0.84
11738	0.000013	1.36	1.16	0.63	0.42
4484	0.000035	2.91	1.38	1.79	1.44
13758	0.000046	2.85	2.24	4.55	2.80
4218	0.000051	5.32	5.51	1.36	1.31
10843	0.000086	0.77	0.70	0.34	0.33
8776	0.000149	1.20	0.88	1.90	0.99
25641	0.000167	0.19	0.05	0.15	0.05
10286	0.000371	0.79	1.30	2.56	3.03
21021	0.000627	0.11	0.06	0.16	0.07
4976	0.000967	0.73	0.63	4.46	5.71
3488	0.007044	0.56	1.14	1.67	2.39
25434	0.007347	0.17	0.09	0.13	0.07
8583	0.008156	1.04	0.75	1.47	0.79
9435	0.009045	3.09	1.67	3.96	1.75
17137	0.009233	0.63	0.48	0.86	0.48
17255	0.009817	1.38	0.74	1.75	0.90

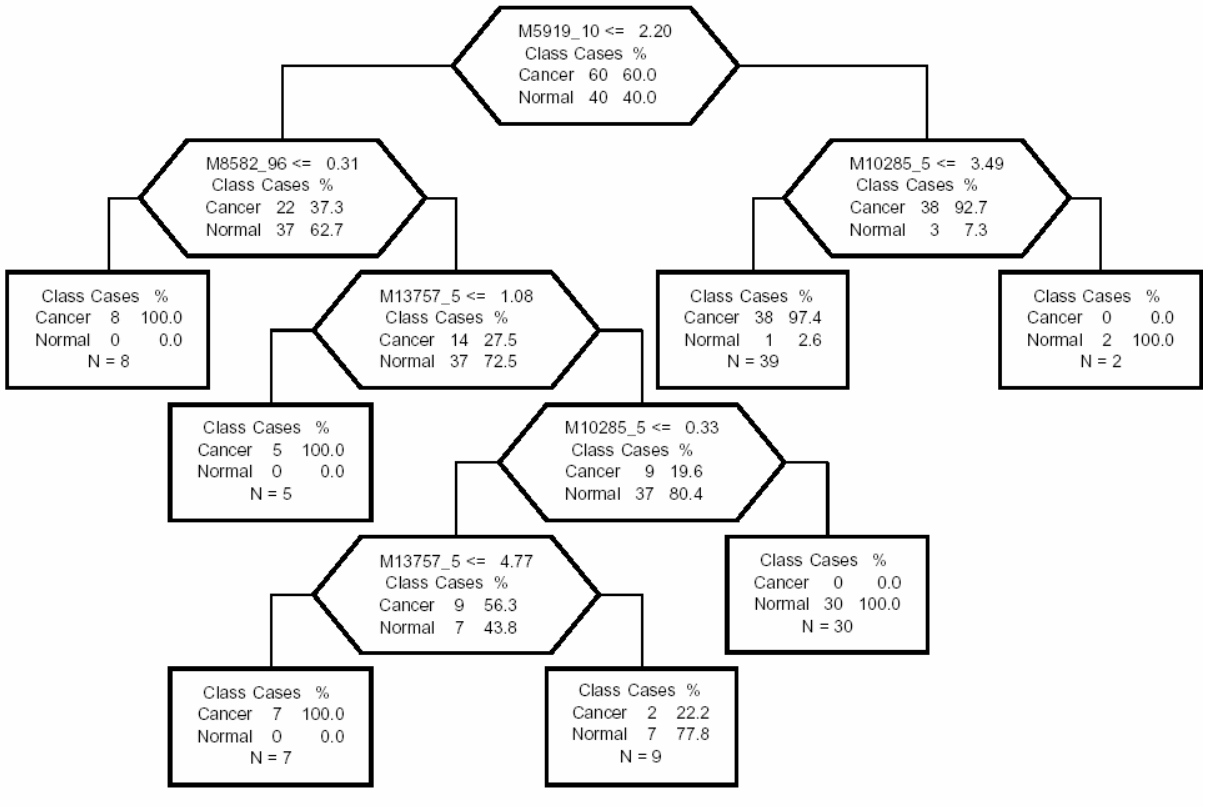

Figure 4. Decision tree that gives the highest discrimination

4. Testing the classifier

The reliability of the decision tree with the training set (including 60 gastric cancer samples and 40 control ones) was validated, and the sensitivity and the specificity for classification of the training set with the decision tree giving the highest discrimination were 96.7% (58/60) and 97.5% (39/40), respectively. Then decision tree was used to classify the blinded set including 30 gastric cancer samples and 20 control samples. The sensitivity and the specificity for the blinded set were 93.3% (28/30) and 90% (18/20) (Table 2). This results indicate that protein biomarker patterns obtained with ProteinChip SELDI system can be used to distinguish gastric cancer patients from normal subjects with relatively high sensitivity and specificity and that the ProteinChip SELDI system can be applied as a useful tool for screening gastric cancer.

Table 2. Classification results by CART

	Training set	Classification results of test set
Sensitivity(%)	58/60 (96.7)	28/30 (93.3)
Specificity(%)	39/40 (97.5)	18/20 (90)

IV. DISCUSSION

It is very important to develop a convenient and non-invasive diagnostic method for routine screening and thereby to increase early diagnosis of cancers, which may lead to increased recovery and reduced mortality of cancer patients. Recently, the diagnostic technique to detect proteins originated from tumor cells in the serum has been developed. However, at present, there are no satisfactory diagnostic biomarkers and methods for gastric cancer. There are several obstacles to identify cancer serum biomarkers. Many potentially valuable biomarkers are expressed at very low levels and are difficult to detect. Also protein concentrations are unfixed, changeable to stress, disease or treatment. Proteins can be modified by cleavage or by addition of new functional groups.

The enzyme-linked, immunosorbent assay(ELISA) represents the most reliable, sensitive and widely available protein-based testing platform for the detection and monitoring of cancer. But ELISA is laborious and time-consuming, low-throughput proteomics approaches. The two-demensional polyacrylamide gel electrophoresis (2D-PAGE) is a well-established technology for the detection of serum biomarkers through changes in serum protein concentrations. The method is also labor intensive and requires large amounts of samples, and cannot be reliably used to detect low-abundance proteins. Therefore it is not suitable for large-scale screening or clinical setting.[19,20] The technology that has revolutionized the proteomic screening of biological samples such as serum is high-throughput mass spectrometry, specifically utilizing surface-enhanced laser desorption ionization time–of–flight (SELDI-TOF). In SELDI-TOF, a proteomic profile of the sample can be created from as little as one microliter of serum, representing tens of thousands of data points. Based on these proteomic profiles, novel bioinformatic approaches to pattern recognition using artificial intelligence-based learning algorithms can discriminate between 2 groups of samples (eg, cancer versus no cancer) or identify new subsets within a population cluster and may represent a novel clinically relevant entity.

This study analyzed protein expression patterns of sera obtained from gastric cancer patients and normal cohorts using the CM10 (weak cation exchange) ProteinChip Arrays and constructed a decision tree for differentiating gastric cancer patients from normal individuals. Four peaks were used to discriminate the two groups and it is worthy of notice that three peaks were decreased in gastric cancer patients. This is different from other serum based molecular markers which are tumor-derived proteins secreted into the bloodstream. From this fact, it can be sensed that decreased proteins in cancer patient sera should be approached for the identification of serum markers. The decision tree distinguished gastric cancer patients from non-cancer people with relatively high sensitivity (93.3%) and specificity (90%), when tested with the blinded sample set. Jang et al [32] identified protein alterations in 18 gastric cancer tissues compared with normal controls, comprising elevated levels of eight proteins. Five proteins were decreased. The fact that they analyzed the proteins of gastric tissues and used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) shows difference to our method. Qian et al [33] analyzed 70 serum samples of gastric cancer patients and 60 serum samples of healthy adults by SELDI-TOF-MS. Sixteen mass peaks were found to be potential biomarkers with significant level of $p<0.01$. Among them, nine mass peaks showed increased expression and the expression of other seven mass peaks decreased in patients with gastric cancer. Two peaks were chosen to make the model tree, then the sensitivity and specificity of the model were 90% (36/40) and 86.7% (26/30), respectively in gastric cancer detecting. They used WCX2 (weak cation exchange) chips similar to our study and the result were comparable to ours. Ebert et al [34] made training set with 41 samples of gastric cancer patients and 49 samples of people without gastric cancer. 71 peaks were identified and 28 of them were used to construct 50 different decision trees. Each decision tree consisted of 3 to 5 masses. The most promising decision tree of 3-masses complexity distinguished gastric cancer patients from non-cancer people with 92.7% sensitivity and 94.1% specificity. A classifier ensemble, consisting of 50 decision trees, correctly classified all gastric cancers and all controls of training set with

100% sensitivity, 100% specificity. Although this method is more accurate than one promising decision tree, it is more laborious. And eight of 9 stage I gastric cancers were correctly classified with 88.9% sensitivity. These results demonstrate that SELDI-TOF-MS would be effective method to seek for serum biomarkers of gastric cancer.

But, there are several limitations. Due to the relatively fewer sample size, our results require more samples to broaden and improve its diagnostic value. Further studies to find the difference of proteins among stages should be performed. Especially, it must be proved that early gastric cancer patients and healthy subjects manifest different serum protein profiles. Also, it is possible that using other chips such as hydrophobic, IMAC-Cu and anion exchange Proteinchip except CM10 Proteinchip manifest another results.[20]

For the practical use of SELDI-TOF-MS, there are several considerations. In the clinic, the early diagnosis of cancer as health checkup is not for only one cancer. Prevalent cancers such as lung cancer, colorectal cancer, gastric cancer and breast cancer have to be screened at a time. Therefore the database about the protein expression patterns of each cancer must be established. The reproducibility is also important. The results of the protein expression patterns must be constant independently of time and place. It is a task of proteomics to develop the method that predict treatment response and disease prognosis. The targeted therapy can be developed by identifying each protein and its function in tumorigenesis. Many of the substances in the new generation of cancer drugs are designed to interfere with specific molecular targets, which have a critical role in tumor growth by regulating key signaling pathways. This requires the development of superior detection techniques and it would meet the demand.

V. CONCLUSION

In conclusion, although this study has several limitations to detect serum biomarkers for gastric cancer, we can show the potential that serum biomarkers and protein patterns to predict gastric cancer could be detected by SELDI-TOF-MS with relatively high sensitivity and specificity.

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