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A Protein Chip System for Parallel Analysis of Multi-tumor Markers and its Application in Cancer Detection

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Abstract. *Background: Tumor markers are routinely measured in clinical oncology. However, their value in cancer detection has been controversial largely because no single tumor marker is sensitive and specific enough to meet strict diagnostic criteria. One strategy to overcome the shortcomings of single tumor markers is to measure a combination of tumor markers to increase sensitivity and look for distinct patterns to increase specificity. This study aimed to develop a system for parallel detection of tumor markers as a tool for tumor detection in both cancer patients and asymptomatic populations at high risk. Materials and Methods: A protein chip was fabricated with twelve monoclonal antibodies against the following tumor markers respectively: CA125, CA15-3, CA19-9, CA242, CEA, AFP, PSA, free-PSA, HGH, β-HCG, NSE and ferritin. Tumor markers were captured after the protein chip was incubated with serum samples. A secondary antibody conjugated with HRP was used to detect the captured tumor markers using chemiluminescence technique. Quantification of the tumor markers was obtained after calibration with standard curves. Results: The chip system showed an overall sensitivity of 68.18% after testing 1147 cancer patients, with high sensitivities for liver, pancreas and ovarian tumors and low sensitivities for gastrointestinal tumors, and a specificity of 97.1% after testing 793 healthy individuals. Application of the chip system in physical checkups of 15,867 individuals resulted in 16 cases that were subsequently confirmed as having cancers. Analysis of the detection results with a Support Vector Machine algorithm considerably increased the specificity of the system as reflected in healthy individuals and hepatitis/cirrhosis patients, but only modestly decreased the sensitivity for cancer patients. Conclusion: This protein chip system is a potential tool for assisting cancer diagnosis and for screening cancer in high-risk populations.*

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Cancer causes about 7 million deaths a year worldwide (1), being second only to cardiovascular diseases. Because of the lack of effective therapy to cure late stage cancers, tumor screening is by far the best way to detect cancers early enough to improve the prognosis of cancer patients. Image-based technologies such as computed tomography (CT) scan are the most commonly used means for cancer detection. However, such devices are too expensive and inconvenient to operate in screening large populations, especially in developing countries. They also require tumors to grow to detectable size. Examination of tumor markers has long been sought as another means for cancer screening because of the non-invasive nature and ease of usage.

Tumor markers refer to substances secreted by tumor cells or other parts of the body in response to the growth of a tumor (2, 3). Several tumor markers, such as α-fetoprotein (AFP), prostate specific antigen (PSA) and cancer antigen 125 (CA125), have been proven to be effective in the screening of liver, prostate and ovarian cancers, respectively. However, those markers are used only for limited tumor types. The main clinical applications of most tumor markers are for monitoring the effectiveness of therapy and for helping physicians in designing treatment.

The two major reasons that most tumor markers are not used for tumor screening are their low sensitivity and specificity, resulting in low detection rates and unacceptable false-positive diagnoses. There have been attempts to do combined measurement of multiple tumor markers for cancer screening with certain success in terms of increasing the sensitivity (4-9). However, they were all done by measuring each tumor marker separately and thus are time-consuming and usually too expensive to be accepted by the public and the healthcare industry for health checkups. Furthermore, combined marker measurement increases false-positive diagnoses.

Recent advances in proteomics and protein chip/microarray technology appear promising for medical diagnosis including cancer screening and early detection (10-16). For example, proteomic studies have discovered

certain patterns of serum proteins in ovarian cancer patients that are different from control samples (17). When combined with proper artificial intelligence algorithms, a specific serum protein pattern reflected the existence of ovarian cancer with high precision. A similar study was also able to differentiate prostate cancer from benign prostate hyperplasia and healthy men (18). However, such methods are still at the research stage and technologically and economically not feasible for clinical applications on a large scale. On the other hand, the high integration and parallel analytical capacity of the protein chip provides a new platform to explore the value of traditional tumor markers in cancer screening.

In this report, we described a protein chip system for multi-tumor marker detection, which is based on the principle of sandwich immunoassay and uses the sensitive chemiluminescence detection method. This protein chip system quantitatively measured 12 common tumor markers including CA125, cancer antigen 15-3 (CA15-3), cancer antigen 19-9 (CA19-9), cancer antigen 242 (CA242), carcinoembryonic antigen (CEA), AFP, PSA, free-prostate specific antigen (f-PSA), human growth hormone (HGH), β-human chorionic gonadotropin (β-HCG), neuron-specific enolase (NSE) and ferritin in the serum and was tested in clinically confirmed cancer patients and apparent healthy individuals. The value of this system in cancer screening of apparently healthy populations and in other clinical applications for cancer patients was discussed.

Materials and Methods

Antigens, antibodies and reagents. Antigens and antibodies were either produced in our laboratory or obtained from commercial sources. Horseradish peroxidase (HRP) and chemiluminescence substrates (SuperSignal Femto maximum sensitivity) were purchased from Pierce (Rockford, IL, USA). Antibody conjugation was according to Nakane and Kawaio (19). Single tumor marker detection kits were purchased from Roche (Basel, Switzerland), Abbott Laboratories (Abbott Park, IL, USA), Bayer (Bayerwerk, Germany) and CanAg (Gothenburg, Sweden). Nitrocellulose membrane was purchased from Millipore (Billerica, MA, USA). CCD camera was purchased from Roper (Duluth, GA, USA). Other reagents were all analytical grade. Serum samples were collected from local hospitals with consent from patients for testing the sensitivity and specificity of the protein chip system.

Chip fabrication. Twenty nL of each of the twelve capture antibodies, with an average concentration of 1 mg/mL, were arrayed in duplicates on a 1x1cm nitrocellulose membrane using the Cartesian ink jet printer, GT5000 Gantry System (Irvine, CA, USA). The array consisted of a 5x5 matrix with 12 pairs of antibody spots and one blank spot as control (Figure 1). After spotting, the membrane was mounted with a plastic mold and blocked with 10% BSA. Each kit held 48 microarrays, of which 5 were used for standard curve construction and one for quality control. Therefore, each kit could detect 12 tumor markers for 42 samples in parallel.

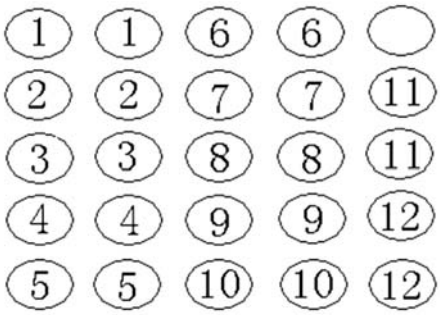

Figure 1. *Arrangement of monoclonal antibodies against respective tumor markers in the protein chip. 1, CA15-3; 2, CA242; 3, CEA; 4, NSE; 5, CA19-9; 6, CA125; 7, PSA; 8, free-PSA; 9, AFP; 10, β-HCG; 11, ferritin; 12, HGH. Each antibody was arrayed in duplicates.*

Processing of the protein chip. Each protein chip was incubated with 100 μL of serum, standard tumor markers, or quality controls for 30 min at 37˚C, rinsed and washed 2x10 min with TBST (8.5 g NaCl, 0.1 mol Tris-HCl, 1 mL Tween20, pH 7.6). The chip was then incubated with 100 μL of HRP-labeled antibodies for 30 min at 37˚C and again rinsed and washed 2x10 min with TBST. Chemiluminescence substrates were added for one min. Light signals were captured with the self-built ChipReader based on a CCD camera and controlled with a computer system using self-developed software.

Data analysis. For calculation of the concentrations of serum tumor markers, a standard curve was obtained for each marker. For method comparison between the protein chip and single marker detection kits, Spearman rank correlation test (2-tailed) was performed. The Support Vector Machine (SVM) analysis used the mySVM software (http://www.support-vector.net/software.html). Three groups of samples from healthy people, hepatitis/cirrhosis patients and liver cancer patients, 100 for each group, were selected for the analysis. Each group was randomly divided into 2 sets of 50 samples and used for training and testing, respectively. All 12 tumor markers plus age and sex were weighted in the training and testing process. After training, the machine was used to evaluate the testing samples.

Results

Selection of tumor markers. Twelve tumor markers were selected for the fabrication of the protein chip with the goal of detecting most common tumor types. Some of the tumor markers, such as AFP, PSA and CA125, have been used routinely for screening liver, prostate and ovarian cancers, respectively. CA15-3 has been proven to be a valuable marker for breast cancer diagnosis and monitoring. CA19-9 and CA242 were included mainly to increase the detection sensitivity of gastrointestinal tumors. The inclusion of NSE was mainly intended for lung cancer detection. Two hormonal tumor markers, β-HCG and HGH, and two less specific tumor markers, CEA and ferritin, were included to

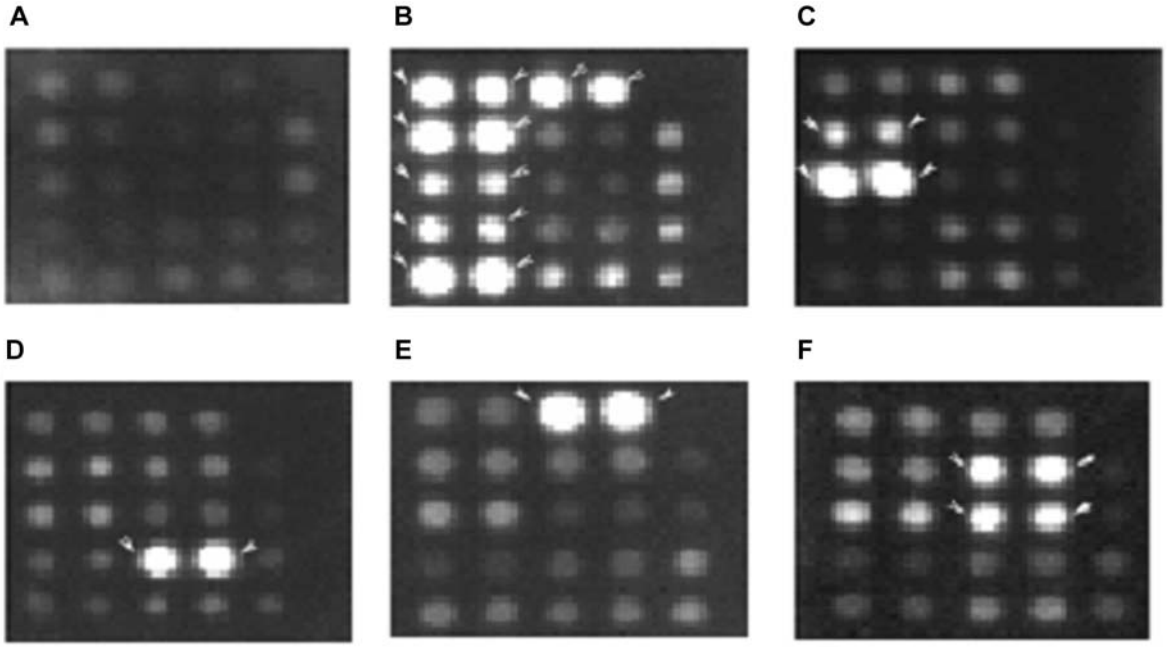

Figure 2. *Reaction patterns of the protein chip with serum samples from cancer patients. A. healthy control, B. pancreas cancer, C. esophagus cancer, D. liver cancer, E. ovarian cancer F. prostate cancer. Arrowheads indicate positive reactions.*

increase the overall detection rate. Cut-off values for the markers were: CA19-9, 35 μ/mL; NSE, 13 ng/mL; CEA, 5 ng/mL; CA242, 20 ng/mL; ferritin, 322 ng/mL; β-HCG, 3 ng/mL; AFP, 20 ng/mL; f-PSA, 1 ng/mL; PSA, 5 ng/mL; CA125, 35 μ/mL; HGH, 7.5 ng/mL; CA15-3, 35 μ/mL.

Establishment of the high throughput protein chip system. Figure 2 shows the CCD-based chip scanner captured images of the protein chip after reaction with control and cancer samples. With the normal sample (Figure 2A), only background reaction was observed with the 12 tumor markers. In the case of liver (Figure 2D) and ovarian (Figure 2E) cancers, elevation of a single tumor marker, AFP or CA125, was respectively observed in each sample (see arrowheads). However, two or more tumor markers were apparently elevated in the pancreas (Figure 2B), esophagus (Figure 2C) and prostate (Figure 2F) cancer samples (see arrowheads).

Quantification of each tumor marker was achieved by calibration with standard curves of individual markers in each reaction. Figure 3 shows the calibration curves of CA19-9 and AFP as examples. Tumor marker measurements obtained with the protein chip were also compared with those obtained with ELISA single marker detection systems from different manufacturers including Roche, Abbott and CanAg. Using Spearman's rank correlation test, the measurement results from the protein system and single marker detection methods showed fairly good correlation (Table I). Therefore, the protein chip system for multi-tumor marker detection is a reliable system for quantitative measurement of tumor markers in serum.

The protein chip system has high throughput screening capacity. Reactions of the 12 tumor markers with respective antibodies and subsequent visualization with reagents generating chemiluminescent signals were completed within 2 hours. Data collection and analysis required 1.5 to 2 hours. On a 6x8 format kit, 42 samples could be simultaneously analyzed together with 6 calibrating and control samples. One technician could perform the reactions of 3 kits at one time and thus finish parallel analysis of 12 tumor markers from 126 patient samples in half a day.

Sensitivity and specificity of the protein chip system. To determine if the protein chip is suitable for clinical applications, its sensitivity and specificity were tested using blood samples from clinically confirmed cancer patients and healthy people. Table II shows overall sensitivity for cancer detection and sensitivity for each cancer type. The protein chip had an overall sensitivity of 68.18% (782 out of a total of 1147 samples) and a specificity of 97.10% (770/793). It had the highest detection rate for liver cancer (92.05%), followed by pancreas (81.50%) and ovarian (81.30%) cancers. The detection rates for breast, cervical, ovarian and endometrium cancers, which were all female-specific, were modestly high, ranging from 62.50% to 81.30%. However,

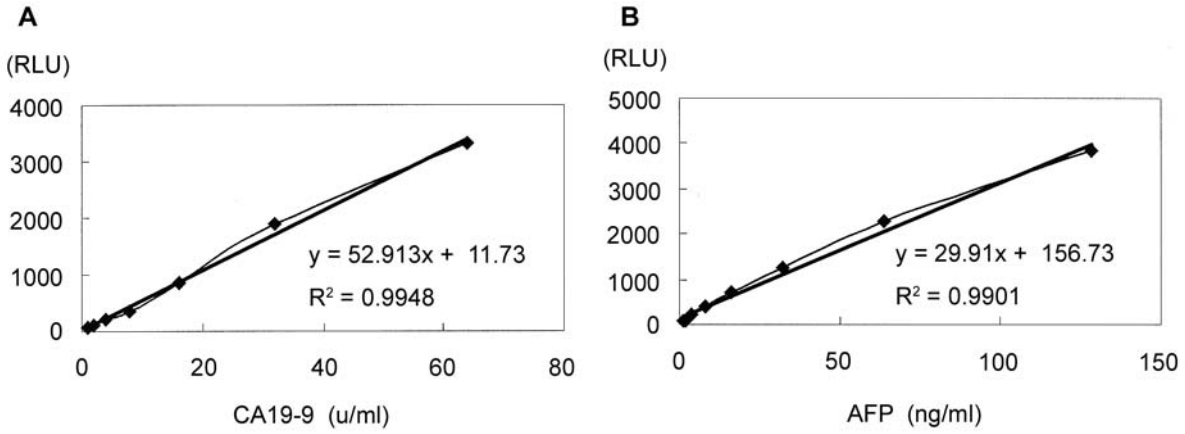

Figure 3. *Standard curves of the protein chip system. The standard curves of CA19-9 (A) and AFP (B) are shown as examples. The X axis represents concentration of the antigen and the Y axis represents relative light unit (RLU) as captured with the ChipReader. The cut-off reference values of CA19-9 (35 u/mL) and AFP (20 ng/mL) are within the linear range of the standard curves. The equation and the coefficient of determination are indicated for each curve. Concentrations of tumor markers were calculated according to respective standard curves in each assay.*

Table I. *Comparison of measurement results of the protein chip system with ELISA/EIA. Spearman correlation test (two-tailed) was performed.*

Tumor marker (manufacturer)	Number of samples, n	Spearman, r^s	*p*
CA125 (Roche)	32	0.8106	<0.01
CEA (Abbott)	25	0.9425	<0.01
PSA (Abbott)	20	0.9162	<0.01
f-PSA (Abbott)	11	0.7886	<0.01
CA242 (CanAg)	7	0.8143	<0.01
CA15-3 (Roche)	10	0.8364	<0.01
NSE (Roche)	16	0.5669	<0.01
AFP (Abbott)	16	0.7265	<0.01
CA19-9 (Roche)	34	0.9812	<0.01

Table II. *Detection rates of individual cancer types with the protein chip system.*

Cancer type	Positive/Total, n	Positive rate (%)
Lung	118/154	76.62
Breast	90/144	62.50
Liver	139/151	92.05
Cervix	17/22	77.27
Colon	45/78	57.69
Rectum	31/80	38.75
Stomach	51/88	57.95
Ovary	48/59	81.30
Esophagus	31/72	43.05
Prostate	22/33	66.67
Pancreas	59/65	81.50
Endometrium	23/30	76.67
Others	108/171	53.19
* Total cancers	782/1147	68.18
# Normal control	23/793	2.90

the detection rate for the gastrointestinal cancers was low (from 38.75% for rectum cancer to 57.95% for stomach cancer), consistent with the fact that there is a lack of better tumor markers for this group of cancers. Nevertheless, the overall detection rate of the protein chip system was comparable to or higher than most existing methods.

The protein chip system improved cancer detection rate with tumor markers. Table III shows the detection rate of individual tumor markers for each cancer type. There were several things worth noting. First, for each cancer type multiple tumor markers were elevated, though not necessarily altogether in the same patient. For example, CA125 was elevated in 49.01% of liver cancer patients, while HGH was elevated in only 13% of them. In the case of lung, liver, ovarian and pancreas cancers, all 12 markers showed elevated levels, either individually or in different combinations, depending on individual patients. Thus, the 12 markers all contributed to the detection rate. Second, there was no so-called tumor-specific marker. For example, AFP has been considered as the best liver cancer-specific marker. However, it was detected in only 56.29% of all liver cancer samples. On the other hand, it was found to be elevated in all other cancer types examined with considerable sensitivity (for example, 22.03% for ovarian cancer). Another example was PSA, a marker considered specific for prostate cancer or other benign prostate conditions. While it was mainly detected in prostate cancer samples (61.11%), it was still found in several other cancer

Table III. *Positive rate (%) of individual tumor marker for each cancer type with the protein chip system.*

Cancer Type	CA19-9	CEA	CA242	Ferritin	NSE	β-HCG	AFP	f-PSA	PSA	CA125	HGH	CA15-3
Lung	33.12	44.81	33.77	54.43	12.99	17.53	15.70	0.65	7.79	41.56	6.49	24.68
Breast	41.60	37.50	37.50	41.11	0	0	4.17	0	0.69	50.00	4.17	41.70
Liver	45.03	25.17	33.11	45.36	7.28	20.53	56.29	4.64	6.62	49.01	13.00	25.83
Cervix	27.27	40.91	36.36	44.28	0	0	18.18	2.00	2.00	50.00	9.09	45.45
Colon	33.08	35.90	23.08	35.23	8.97	14.58	6.25	0	0	33.33	2.08	18.75
Rectum	14.78	19.56	11.28	12.32	3.74	9.23	4.56	0	0	18.65	3.24	10.49
Stomach	21.59	20.45	25.00	13.73	5.68	7.95	10.23	0	0	25.00	7.95	7.95
Ovary	22.03	30.51	25.42	47.37	6.78	35.59	22.03	1.69	6.78	62.71	5.08	16.95
Esophagus	15.28	28.50	15.28	30.00	0	1.38	12.50	0	0	16.70	2.77	2.77
Prostate	12.50	33.33	12.50	62.79	9.09	0	16.67	61.11	61.11	33.33	8.33	8.33
Pancreas	56.15	41.54	47.69	34.14	29.00	9.70	12.90	9.70	16.10	52.30	25.80	23.70
Endometrium	23.30	36.70	20.00	29.89	0	33.30	16.70	0	0	46.70	23.30	6.70
Others	23.40	30.57	19.86	34.25	3.64	13.48	7.80	0.71	5.67	20.57	9.93	11.35

types, though at low percentages. Thirdly, there was no absolute gender-specific tumor marker. For example, PSA was considered as a male-specific marker. However, it was also found with above cut-off concentrations in small percentages of ovarian (6.78%), cervical (2%) and breast (0.69%) cancer patients. On the other hand, β-HCG was found to be elevated in some male patients (data not shown). Fourthly, NSE showed elevation in a very small percentage of lung cancer patients but had modest value for pancreas detection. Finally, CA125, CA15-3, CA19-9, CA242 and CEA were most useful for increasing the overall detection rate for every type of cancer.

The protein chip system was also used for cancer screening in physical checkups for over 15,867 individuals. There were 436 positive detections in total, of which 138 were followed-up. Among the 138 positive cases, 16 were later clinically confirmed as cancer patients. Forty-one of them showed non-malignant conditions which could be the causes for the elevation of tumor markers in the serum, while 81 of them showed no symptoms of any illness and are still under monitoring.

Artificial intelligence analysis of the protein chip results. One of the problems of using known tumor markers in cancer screening and diagnosis is their low specificity, resulting in numerous false-positive detections. In the case of liver cancer, it is an especially important issue as non-malignant liver conditions, such as hepatitis and cirrhosis, also cause AFP, the most often used marker for liver cancer screening and diagnosis, and other markers, to rise in the blood. To test if the use of artificial intelligence was effective in improving cancer detection accuracy with the protein chip system, the SVM was applied to analyze the tumor marker detection results from blood samples of normal persons, hepatitis/cirrhosis patients as well as liver cancer patients. One hundred samples were evenly divided into two age-matched groups. One group was used for the SVM model training, while the other group was used for testing. As shown in Table IV, the detection rates for normal control, disease control and liver cancer groups were 2%, 32% and 60%, respectively with AFP alone. When all 12 markers were applied, the detection rates for all 3 groups increased to 4%, 36% and 92%, respectively. Therefore, the combined use of the 12 markers did increase detection sensitivity. However, it also increased the false-positive rates at the same time, which was evident in the normal (4%) and disease control (36%) groups. With SVM analysis, the detection rates for the 3 groups were 0%, 16% and 88%, respectively. The specificity improved considerably as shown in the control groups, while the detection rate for the cancer group decreased only moderately (4%). Therefore, SVM is a useful tool in interpretation of results from the protein chip system.

Table IV. *Positive rates of three groups of subjects detected with AFP alone, with the protein chip system and with the protein chip system and SVM analysis.*

	AFP alone	Protein chip	Protein chip/SVM
Normal Control	2%	4%	0%
Hepatitis/Cirrhosis	32%	36%	16%
Liver Cancer	60%	92%	88%

Discussion

The protein chip performs parallel measurement of 12 tumor markers in a single reaction and thus avoids the "matrix effect" that exists in ELISA-based assays (20); therefore, the result is more reliable than the measurement obtained with

the single marker methods where the detection of each marker is a separate assay. The measurement of 12 tumor markers increases detection sensitivity compared to single marker measurement, while the biochip platform makes it possible to perform high throughput handling of samples with reduced cost compared to other current methods. Since sensitivity and cost are crucial for any successful screening method, the protein chip system has the potential to be applied for cancer screening in health checkups to globally monitor the occurrence of major tumor types. Indeed, some cancer cases were found with the protein chip in physical checkups of people who showed no signs of malignant illness. For example, there was one breast cancer patient who had elevated serum levels of multiple tumor markers but no lesion apparent with routine CT scans (data not shown). Multiple tiny tumor lesions were finally diagnosed pathologically only after small shadows were discovered with extra care with a third CT scan, further illustrating the value of the protein chip system in cancer screening.

Another major concern in using currently available tumor markers for cancer screening is their low specificity. The combined use of multiple tumor markers further lowers detection specificity while increasing sensitivity, resulting in even more false-positive diagnoses. However, recent developments in artificial intelligence algorithms provide another means to optimally interpret experimental results of multivariate measurements through machine training and pattern recognition (8,9, 17, 18, 21-29). It is known, for example, that gastrointestinal cancers are difficult to detect with commonly used tumor markers alone and accuracy is usually low. However, concomitant measurement of CEA, CA19-9, cancer antigen 72-4 (CA72-4) and β-HCG combined with logistic regression analysis improves the diagnostic accuracy (8, 9). Serum protein fingerprinting has proved to be highly valuable in differentiating prostate cancer from benign prostate hyperplasia and healthy men with pattern-matching algorithm analysis (18). Proteomic patterns in serum can also be used to accurately detect early stage ovarian cancer when an interactive searching algorithm is applied to identify the pattern that completely discriminates cancer from non-cancer (17), or when a neural network algorithm is used to interpret the results (26). Here the SVM algorithm was used to interpret results obtained with the protein chip system with regard to liver cancer and resulted in higher accuracy of cancer detection as reflected in the considerable decrease of false-positives in the control groups but only moderate decrease in sensitivity in the cancer group (Table IV). Therefore, proper interpretation of the result from the protein chip with an artificial intelligence method may further increase the value of multi-tumor marker detection in cancer screening.

In addition to cancer screening, the protein chip can also be used in other clinical applications. One such application is to efficiently select the proper tumor marker for monitoring the effectiveness of cancer treatment. Instead of using single marker detection kits one by one to search for the proper marker in the cancer patient, the protein chip system can accomplish the selection in one assay. After the proper marker(s) is determined with the protein chip before therapy, changes can then be monitored periodically after the start of the therapy or surgery with a method that measures only that particular marker. The change of the serum level of the marker can then be used as an indicator to assess the effectiveness of the treatment and the possibility of metastasis. The protein chip can also be used to assist physicians in the diagnosis of suspected cancer patients.

Carcinogenesis is a very complex process with diverse mechanisms still to be elucidated. Our results of tumor marker detection with the protein chip system underlie this complexity. For example, even the best tumor markers, namely AFP and PSA, are not as sensitive and specific as desired (Table III). All cancer types express elevated levels of multiple tumor markers. In fact, there are many other potential markers that are over-expressed in liver cancer samples as evidenced by expression profiling using cDNA arrays (Gengxi Hu, unpublished results). There is no so-called organ- or gender-specific tumor marker, either. An elevated serum level of AFP, for example, was found in considerable percentages of various types of cancer, and PSA was found in female subjects while β-HCG was found in male subjects. Therefore, it is important to interpret tumor marker detection results in context.

An ideal tumor marker should have 100% sensitivity and specificity, and be tissue- and organ- specific. Its serum levels should correlate with the size or stage of the tumor, the effectiveness of cancer therapy and prognosis. However, no ideal tumor marker is currently available. While the search for such ideal tumor markers has been extensively pursued, it is also a meaningful practice to fully explore the value of commonly used tumor markers. One way to do that is to utilize new advances in biotechnology to detect those markers with higher sensitivity, specificity and efficiency, and to interpret the results with novel mathematical tools. In this report, the protein chip platform was employed to measure 12 tumor markers in parallel for high throughput handling of serum samples. This approach proved to have a value for cancer screening, for the selection of proper markers for cancer therapy monitoring and for assisting physicians in cancer diagnosis. Interpretation of the protein chip results with SVM theory further showed the potential usefulness of this system in such applications.

Future studies will focus on optimizing combinations of tumor markers for global monitoring of cancers or for the detection of distinct types of cancers with the protein chip platform and on developing an artificial intelligence algorithm that can be routinely used in clinical settings. Newly discovered markers may also be integrated into future protein chips to improve the detection rate and

accuracy, especially for gastrointestinal cancers that had a low detection rate with the current protein chip system. The results from the protein chip system for multi-tumor marker detection also validate the value of the global approach strategy to monitor other diseases or physiological states by using parallel quantification of specific protein targets.

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