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Upregulation of plasma C9 protein in gastric cancer patients

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Abstract

Gastric cancer is one of the leading causes of cancer-related deaths worldwide. Current biomarkers used in the clinic do not have sufficient sensitivity for gastric cancer detection. To discover new and better biomarkers, protein profiling on plasma samples from 25 normal, 15 early-stage and 21 late-stage cancer was performed using an iTRAQ-LC-MS/MS approach. The level of C9 protein was found to be significantly higher in gastric cancer compared with normal subjects. Immunoblotting data revealed a congruent trend with iTRAQ results. The discriminatory power of C9 between normal and cancer states was not due to inter-patient variations and was independent from gastritis and *Helicobacter pylori* status of the patients. C9 overexpression could also be detected in a panel of gastric cancer cell lines and their conditioned media compared with normal cells, implying that higher C9 levels in plasma of cancer patients could be attributed to the presence of gastric tumor. A subsequent blind test study on a total of 119 plasma samples showed that the sensitivity of C9 could be as high as 90% at a specificity of 74%. Hence, C9 is a potentially useful biomarker for gastric cancer detection.

Keywords

Biomarker; Biomedicine; C9; Gastric cancer; Plasma

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1 Introduction

Gastric cancer is a leading cause of cancer-related deaths worldwide in which almost one million of new cases are being diagnosed yearly [1]. The global 5-year survival rate is around 20% except for Japan where the rate is close to 60% [2]. There is currently no sensitive biomarker for gastric cancer detection. Common markers such as CA19-9, fetoprotein antigen, pepsinogen I/II and carcinoembryonic antigen (CEA) are prone to high degree of false negatives [1]. Clearly, there is a need to find better molecular markers. The advancement in analytical tools and MS platforms has spurred the quest of biomarker discovery. Proteomic approaches in unearthing biomarkers have shown successes in breast [3], prostate [4], lung [5], ovarian cancer [6] and to a smaller extent in gastric cancer in which potential candidates have been identified from tumor tissue [7], cell lines [8] and gastric juice [9]. Although studies on tumor and gastric juice had provided great insights on the disease, biomarker discovery based on these approaches engage invasive sampling methods and are not ideal from a clinical point of view.

Researchers envision that tumor-specific markers are released from cancerous tissues into body fluids such as the blood and urine. Blood is an ideal substrate for biomarker discovery for two main reasons. First, tumor-specific proteins released into the blood or acute-phase reactants involved in tumor-specific immune response could serve as biomarkers. Second, the blood sampling process is simple, minimally invasive and can be repeated without adverse consequences. A popular approach toward mining the blood for biomarkers is the ProteinChip system, which is based on the SELDI approach [10–12]. Although differences were found in the peptide mass fingerprints, the information is incomplete without knowing their identities. This is especially true in view of the light that reproducing the serum profiles using SELDI is difficult due to various intrinsic and/or extrinsic factors. To resolve this problem, Peng *et al.* had successfully developed a methodology to identify SELDI profile peaks using the ProteinChip coupled with a tandem mass spectrometer [13]. Another study employed conventional 2-DE gel coupled with MS analysis [14]. However, sensitivity remains an issue with the 2-DE approach and detection of low-abundance proteins remains challenging. This problem is further amplified by the fact that proteins in the blood have a wide protein dynamic range spanning over ten orders of magnitude.

This study utilized the iTRAQ approach to profile the levels of proteins in plasma from early and late gastric cancer stages *versus* normal control. The aims of the study were (i) to identify and validate proteins that have not been implicated in gastric cancer and (ii) to ascertain the specificity and sensitivity of these candidates for gastric cancer detection.

2 Materials and methods

2.1 Blood collection

Since January 2006, patients with newly diagnosed gastric cancer at the National University Hospital and Tan Tock Seng Hospital, Singapore, have been prospectively enrolled with informed consent in a research study (Gastric Cancer Biomarker Discovery II, GASCAD II) and blood, paired normal and tumor tissues, and gastric juice samples obtained together with clinical and pathologic annotation. Blood collection was obtained before surgery or chemotherapy. Staging information was determined histopathologically and in combination with all clinical information. There was no evidence of other malignancies. American Joint Committee on Cancer (AJCC) (6th edition) on gastric cancer staging system and Lauren's classification of gastric cancer were used.

The Gastric Cancer Epidemiology and Molecular Genetics Cohort Study enrolls people aged > 50 years who are at high risk of gastric cancer and offers screening by endoscopy with

systematic prospective follow up over a minimum of 5 years [15]. The control subjects used in this study were from the Gastric Cancer Epidemiology and Molecular Genetics Cohort Study. These subjects are largely healthy people with familial history of gastric cancer. The same blood collection protocols were used.

We employed standardized protocols for blood collection and plasma preparation used by various centers involved in this study was guided by reports on the Plasma Proteome Project [16]. The details of blood collection are described in Supporting Information Materials and methods. To ensure the consistency in plasma preparation, a quality control measure was implemented where all the blood samples must be processed into plasma in the laboratory within an hour after blood collection in the clinic. The integrity of each plasma sample was further verified by running 1-D SDS-PAGE and stained with SyproRuby fluorescent dye to check for massive protein degradation. Samples that did not satisfy the specified time frame or integrity check would be stored away and would not be used for analysis.

The study was approved by the respective institutional review boards and all patients gave written informed consent.

2.2 Sample preparation, iTRAQ and LC-MS/MS

One milliliter of plasma sample was first subjected to delipidation by centrifugation at $130000 \times g$ at 4°C for 2h. The bottom layer of lipids-free plasma was collected and the total protein was estimated using BCA assay. A total of 10 mg of delipidated plasma sample from each patient were then pooled accordingly to their sample nature *i.e.* normal, early-and late-stage gastric cancer. Following depletion of the seven most abundant proteins using MARS Hu-7 affinity column (Agilent Technologies, USA) according to the manufacturer's instructions, total protein was estimated using BCA assay. Protein samples were then reduced, alkylated, digested and labeled with iTRAQ reagents according to the recommended protocol (Applied Biosystems, Framingham, MA, USA). The samples were labeled as follows: 114 – normal, 115 – early-stage gastric cancer and 116 – late-stage gastric cancer.

The labeled peptides were fractionated into 30 fractions using strong cation exchange using a PolySULFOETHYL™ A Column (PolyLC, Columbia, MD, USA) $5 \mu\text{m}$ of 200mm length $\times$ 4.6 mm id, 200Å pore size. These fractions were cleaned up using a C18 Discovery® DSC-18 SPE column (100 mg capacity, Supelco, Sigma-Aldrich). The dried and cleaned fractions were then analyzed using Agilent 1100 nLC system (Agilent) coupled online to a quadrupole time of flight mass spectrometer (QStar XL, Applied Biosystems), as described recently [17]. Eluent from the reverse phase nLC was directly subjected to positive ion nanoflow electrospray analysis in an information-dependant acquisition mode. A TOF-MS survey scan was acquired (m/z 370–1600, 0.5 s), with the three most intense multiple charged ions (counts>70) sequentially subjected to MS/MS analysis. The time of summation of MS/MS events was set to be 2s in the mass range of m/z 100–1600.

Similarly to the previous study, protein identification and quantification were carried out using ProteinPilot™ software (version 2.0; Applied Biosystems, MDS-Sciex), searching against IPI human database (version 3.41) [17]. The search was performed using Paragon Algorithm™, which is discussed in detail elsewhere [18]. Only those proteins identified with at least 95% confidence were taken into account. All results were then exported into Excel for manual data interpretation. To ensure the reliability of the data, false-positive rate was estimated by searching against a concatenated pseudo-reverse database, created in-house which consists of the forward database and their pseudo-reverse sequences [19]. Using this strategy, the false discovery rate for this data set is estimated to be approximately 1%. Here, we defined false discovery rate as the percentage of decoy proteins identified against the

total protein identification. This insignificant false positive within the data set is acceptable and tolerable.

2.3 Immunoblotting strategy for validation and blind test studies

For validation of iTRAQ results, the pooled depleted plasma samples used for iTRAQ analysis were subjected to immuno-blotting for C9 using mouse monoclonal antibody (Abcam, Cambridge) as described in Supporting Information Materials and methods and according to the previous studies [20, 21]. Triplicate blots were carried out for each sample to ensure robustness of data generated. To profile the expression level of C9 in individual plasma samples, crude plasma sample (without depletion) was used. Prior to this, optimized conditions for immunoblotting of C9 in crude samples were obtained by varying the protein loadings and X-ray film exposure times (data not shown). Consequently, a total of 5 mg of crude plasma protein from each sample were loaded into 1-D SDS-PAGE. Gel strips spanning the desired molecular weight range within which C9 migrated were cut out from various 1-D gels. All the desired strips were then laid onto the same PVDF membrane and Western blotted. Triplicate PVDF membranes transferred from the triplicate runs of each plasma sample were subjected to chemiluminescence detection on a single x-ray film following immunoblotting of C9. For densitometry, images from X-ray film were first captured using Imager Scanner and its corresponding software LabScan version 5.0 (GE Healthcare). The image was then analyzed using ImageQuantTL software v2003.03 (GE Healthcare).

The specificity of C9 antibody was tested by performing competition experiments in which immunoblotting of C9 was carried out in the presence of purified C9 protein (Fitzgerald, USA) or albumin as a control, both in ten molar excess of the antibodies used. No C9 band was detected in the immunoblot when C9 antibody was preincubated with the purified C9 protein, as shown in Supporting Information Fig. 1.

2.4 Immunohistochemistry

For immunohistochemistry (IHC), matched malignant and adjacent normal gastric tissues were requested from the Tissue Repositories of National University Hospital, following approval from Institutional Review Board from the National University of Singapore. IHC was carried out as described previously [22, 23]. Briefly, frozen tissues were freshly prepared for IHC by fixing them in 10% neutral-buffered formalin (Sigma) for 16h at 41C, subject to Ther-moShandon tissue processor and embedded in paraffin. Sections were warmed in a 60°C oven and dewaxed in three changes of Histo-Clear solution and passaged through graded alcohols. Antigen retrieval was performed using the Target Retrieval Solution (Dakocytomation, Denmark) at 95°C for 40min. After quenching of endogenous peroxidase activity with 3% H₂O₂ for 10min and blocking with BSA for 30 min, sections were incubated at 4°C for overnight with mouse monoclonal antibodies against C9 (Abcam) at 1:1000 dilution. Detection was achieved with the Envision+/HRP system (Dakocytomation). All slides were counterstained with Gill's hematoxylin for 1min, dehydrated and mounted for light microscopic evaluation. Competition studies demonstrated specificity of C9 antibody during IHC (Supporting Information Fig. 1).

2.5 CEA screening

The CEA measurements were done in a medical diagnostic laboratory in the Department of Medicine Laboratory, National University Hospital, Singapore. This laboratory is accredited by Ministry of Health, Singapore, to perform clinical samples screening for hospitals. CEA analyses were routinely performed using ADVIA Centaur CEA assay kit (Siemens Healthcare Diagnostics), a two-site sandwich immunoassay using direct chemiluminometric technology, according to the manufacturer's instructions. The CEA reference range used in

this diagnostic lab is 0–5 µg/L. Any reading that falls within this reference range is considered normal.

2.6 Statistical analysis

Statistical analyses were carried out using both asymmetric and nonasymmetric analysis. All these analyses were performed at 5% significant using statistical software SPSS 16.0 for Windows. ANOVA was carried out to investigate statistically differences in C9 expression level obtained *via* immunoblot in normal plasma compared with cancer plasma. To investigate whether *Helicobacter pylori* infection (HP + /—) or gastritis infection (+/—) status had any statistical correlation with C9 expression in the normal and cancer samples, two-sample *t*-test and ANOVA analysis were employed. On the other hand, correlation between C9 expressions level with Lauren classification was calculated using ANOVA. Receiver-operating characteristics (ROC) curve was also generated to estimate the sensitivity and specificity of C9 using Stata 10.0 software package (Stata, TX, USA).

3 Results

3.1 Identification and validation of C9 as a biomarker for gastric cancer

To identify potential biomarkers, we analyzed plasma samples from 15 early-stage and 21 late-stage gastric cancer patients. We defined early-stage gastric cancer as those diagnosed as stages I and II based on AJCC staging system, whereas late-stage gastric cancer were those in stages III and IV. Twenty-five plasma samples from noncancer subjects were used as controls. The control and test samples were matched by age and gender. The detailed clinical data of these samples used are summarized in Table 1A.

The plasma samples were pooled together according to their classification (*i.e.* normal, early-gastric cancer and late-gastric cancer) prior to depletion of high-abundance proteins. Plasma proteins were then profiled using iTRAQ coupled to LC-MS/MS-based approaches. Similar to other studies, proteins with iTRAQ ratio > 1.30 were considered to be overexpressed, whereas those with ratio < 0.77 were considered as underexpressed [22, 24, 25]. This cutoff was applied since we found that the technical variation for this study is less than 30% (Supporting Information Table 1). The complete list of proteins and peptides identified in the iTRAQ experiment is provided in Supporting Information List 1. The total protein identified in the study is rather low but is comparable to another study, in which less than 200 plasma proteins were identified [26]. Although depletion of high-abundance protein was carried out (three times *per* sample) to resolve this issue, the affinity-based depletion of abundant proteins probably is well known to remove other proteins due to their association with the high-abundance ones (*e.g.* albumin). A more intensive fractionation, ideally a combination of protein and peptide fractionation to reduce sample complexity will increase the proteome coverage as proven by other studies [27, 28]. However, protein fractionation is not feasible in this study since iTRAQ labeling is performed at the peptide level.

A total of 32 proteins were found to be differentially expressed either in early- or in late-stage cancers compared with normal state (Table 2). Next, an intensive literature search was carried out against Pubmed database to identify proteins that have never been previously associated with gastric cancer detection. C9 was found to be a suitable candidate and was selected for further investigation. A representative MS/MS spectrum belonging to one of the C9 peptides detected is illustrated in Supporting Information Fig. 2A, which shows that the intensities of reporter ions belonging to early- and late-gastric cancer samples (*m/z* 115 and 116, respectively) were higher compared with the normal control sample (*m/z* 114). The pooled samples used for iTRAQ were subsequently probed for C9 expression level *via*

immunoblotting. C9 was found to be overexpressed in the plasma of cancer compared with normal subjects by 1.37- to 1.60-fold. These results were obtained from triplicate immunoblot readings (Supporting Information Fig. 2B) and equal loadings in these experiments were checked (Supporting Information Fig. 2C). The C9 expression trend observed in the immunoblots was congruent to the trend obtained by iTRAQ approach. Attaining consistent observations from two independent approaches (iTRAQ and immunoblotting) authenticated our findings.

3.2 C9 expression across individual plasma samples

As the initial iTRAQ analyses were performed on pooled samples, we conducted immunoblotting of C9 in individual plasma samples in order to obtain a better resolution on the expression of C9 in individual patient. All the 61 samples comprising 25 normal, 15 early-stage and 21 late-stage gastric cancer were subjected to immunoblotting with C9 antibodies. Figure 1A shows an example of the triplicate blots generated for profiling C9 expression in individual plasma samples. The samples were grouped according to the nature of the samples, i.e. normal, early-gastric cancer and late-gastric cancer groups. The average densitometry values from triplicate data points for each plasma sample were used to calculate the average intensity for each sample group. As shown in Fig. 1A, the mean values of C9 expression level were 42 750 and 57 767 for early-gastric cancer and late-gastric cancer, respectively. These mean values were about twofold higher compared with normal control group (21 577). ANOVA statistical analysis was performed to determine whether the observed difference was statistically significant. The box plot in Fig. 1B shows that the difference in C9 expression was significant (p -value<0.05) when comparing early cancer and late cancer to normal group. Although a statistically significant higher level of C9 expression was observed in early-stage gastric cancer compared with normal group, we caution that the sample size for early-stage cancer was small. Furthermore, as shown in Fig. 1A, only 6 out of 15 early-stage plasma samples showed considerably high C9 expression level. It is conceivable that these “outliers” may skew the outcome of the analysis. Nonetheless, a larger sample size screening for early-stage gastric cancer should clarify this in future.

3.3 C9 expression in a panel of gastric cell lines

At this juncture, we asked the question of whether the elevated C9 level detected in the plasma of cancer patients might be attributed to gastric cancer cells. To this end, we examined the expression of C9 across a panel of normal and gastric cancer cell lines. This investigation was also warranted since the expression of C9 between normal and gastric cancer cell lines has never been studied. Figure 2A shows the expression of intracellular C9. As observed, C9 expression was apparent in 19/19 gastric cancer cell lines, whereas HFE145 normal gastric epithelial cells had no detectable level of C9. Figure 2B shows the expression of C9 in the conditioned media (*i.e.* extracellular C9) of the panel of cell lines used. C9 was detected in the conditioned media of 10/14 gastric cancer cell lines but not in normal HFE145 cells. The above data suggest that C9 was upregulated in gastric cancer. However, there was only one “normal” cell line employed in this study. Hence, the data generated *in vitro* might not be representative. To ascertain whether C9 expression is indeed higher in cancer compared with normal cells, we further conducted IHC on ten matched pairs of normal and gastric cancer tissues. Six out of ten matched cases showed higher C9 expression level in cancer tissue compared with the adjacent normal tissue (Fig. 2C). The remaining four pairs showed no difference in C9 expression level. These data imply that the observed elevated level of C9 in the plasma of gastric cancer patients could have been attributed to gastric cancer cells. Further studies would be needed to support this hypothesis.

3.4 Use of C9 expression to discriminate between normal and cancer states in blind test study

Since there was statistical significance in the difference in expression of C9 between normal and cancer subjects, we proceeded to conduct a blind test study to determine whether plasma C9 levels could be used to distinguish between normal and cancer states. In total, 119 plasma samples were analyzed and the clinical data for these samples are summarized in Table 1B. The test was performed by an operator who had no prior knowledge of the samples' nature. The immunoblotting approach used for blind testing was the same as that used in the validation step described above except that an additional reference sample comprising 5 µg of a known late-gastric cancer from the validation set was spiked into the gels containing samples for blind test, acting as an internal control for densitometry scan normalization between- and within-test/ validation blots. Similar to the validation process, the average densitometry reading for each sample was calculated from triplicate data points. The sample was then predicted to be either normal, early-gastric cancer or late-gastric cancer based on the mean C9 densitometry reading cutoff values determined during the validation step. Samples with values below 21 577 were classified as normal, between 21 577 and 42 750 were classified as early cancer and above 42 750 were classified as late cancer. A representative blot is shown in Supporting Information Fig. 3. Table 3 summarizes the performance of C9 in the blind test study. C9 specificity was estimated to be approximately 74%. C9 was less sensitive for early-gastric cancer where its sensitivity was found to be 38% compared with late-stage gastric cancer at 77%. If cancer stages were not taken into account, C9 was able to detect cancer in general with a sensitivity of 83%. This illustrates that C9 has considerable specificity and sensitivity in discriminating between plasma from normal subjects and (i) cancer patients and (ii) advanced stage cancer patients.

Since *H. pylori* infection is one of the risk factors associated with gastric cancer [29] while gastritis is not uncommon in gastric cancer patients, we investigated whether the predictive function of C9 was influenced by *H. pylori* infection and gastritis inflammation. Statistical analyses showed that there was no statistically significant correlation between C9 expression and *H. pylori* or gastritis status the samples used (Supporting Information Table 2). This indicates that the diagnostic value of C9 in either normal or gastric cancer plasma was not affected by inflammation or infection status of the subjects tested.

3.5 Performance of CEA in blind test study

In a clinical setting, CEA is routinely used as marker for gastrointestinal carcinoma [30]. To compare the performance of C9 with CEA, 115 samples (all the 61 samples from validation set and 54 randomly selected samples from blind test set) were subjected to blind test study. First, the samples were analyzed for CEA levels in an accredited diagnostic laboratory as described in Section 2. The CEA values were then used to predict samples as normal or cancer according to the reference range practiced by the hospital laboratory. As summarized in Table 4, CEA specificity was 100%, a similar observation reported previously [31]. However, CEA sensitivity was poor at approximately 9% for early-stage and 24% for late-stage gastric cancer. With all stages of gastric cancer combined, the sensitivity was around 18%. This sensitivity range obtained is consistent with existing figures [32].

It is an attractive notion to combine markers in order to improve predictive performance in clinical practice. Hence, we conducted blind test using a combination of C9 and CEA levels for diagnosing the same test set samples used above. Samples were predicted to be cancer when the CEA value is >5 µg/L and the average densitometry reading of C9 expression is >21 577. Any sample that only fulfilled either C9 or CEA cutoff was considered as normal sample. Using this marker combination, high specificity (100%) was achieved but there was no significant improvement in sensitivity (20%) compared with CEA (18%) alone (Table 4).

The sensitivity of CEA and C9 marker combination was limited by the high specificity of CEA, suggesting that the combination of C9 with the CEA biomarker was not synergistic in gastric cancer diagnosis. We have also investigated the sensitivity and specificity when either C9 or CEA cutoff were applied on these samples. In this case, samples were predicted to be cancer if either their C9 or CEA level was above the cutoff value. The sensitivity and specificity for this approach were 90 and 74%, respectively. These values were not significantly improved compared with when only C9 was used (Table 4).

3.6 Post-blind test analysis

Following the discovery and blind test studies, we collated the expression profiles of C9 of all the samples used in this study and constructed an ROC curve to estimate C9 specificity and sensitivity. As shown in Fig. 3, at a confidence interval of 95% and an average densitometry reading cutoff of 27757, the area under ROC curve was estimated to be 0.86, giving C9 a specificity and sensitivity in detecting gastric cancer at 85 and 73%, respectively. These values were similar to that obtained in the original blind test analysis – reiterating the superior performance of C9 over conventional cancer markers. Approximately, 84% (79/94) of all cancer samples displayed high expression level of C9 compared with the average normal value. When subgrouped further, C9 level was elevated in 77% (30/39) and 89% (49/55) in early- and late-stage gastric cancers, respectively.

Classification of gastric cancer had been attempted many decades ago and Lauren classification is probably the most successful and widely used today [33]. Based on Lauren classification, gastric cancer can be classified into two main cancer pathogenesis: (i) diffuse (DGCA) and (ii) intestinal (IGCA) subtypes, which show significant differences in epidemiological and prognostic features [34]. Our study showed 95% (19/20) and 77% (27/35) of the diffuse and intestinal type gastric cancer, respectively, had higher C9 expression level in plasma sample compared with the average value in noncancer subjects. ANOVA statistical analysis revealed an interesting and statistically significant difference (p -value= 0.04) between the C9 expression levels in different histology types of gastric cancers (Supporting Information Table 2). This might have clinical implications as the symptoms of diffuse gastric cancer are rather nonspecific and hence the diagnosis is difficult and often late, compared with the intestinal type [35].

4 Discussion

Conventional tumor markers such as CA19-9, CA72-4 and CEA are not adequately sensitive for gastric cancer detection. A recent review summarized the tumor marker sensitivity in gastric cancer detection including CEA at 16–63%, CA19-9 at 20–56% and CA72-4 at 18–51% [32]. The specificity of these markers was not defined. M2-pyruvate kinase (M2-PK), described as tumor-associated metabolic marker, had also been evaluated for gastric cancer detection, with the sensitivity and specificity ranging from 57 to 67% and 89 to 95%, respectively [36–38]. Two conclusions could be derived from these reports: (i) the current tumor marker candidates have a sensitivity of less than 67% for gastric cancer and (ii) most, if not all, are not specific for any cancer type. With reference to the first point, our blind test studies from >100 samples indicate that C9 has a sensitivity range of 83–90%, which is higher than existing markers in gastric cancer detection. C9's specificity for gastric cancer was at 74%, which is lower compared with CEA. With respect to the second point, while C9 is a potentially novel biomarker for gastric cancer detection, its use is not limited to gastric cancer. Increased C9 level has also been reported in sera samples of patients with acute leukemia and sarcoma [39], whereas another group has detected upregulation of C9 gene expression in esophageal adenocarcinoma compared with normal epithelial cells [40]. Therefore, it seems logical that C9 as a biomarker should be used within the context of specialty clinics such as in gastroenterology where high-risk gastric cancer patients could be

subjected to closer monitoring using C9. Alternatively, C9 could be used to monitor relapse when recurrent tumors are expected to result in higher level of plasma C9. However, the use of C9 for cancer detection/monitoring in general should be taken with care since high-sera C9 level in patients with immune response diseases as such rheumatoid arthritis [41] and autoimmune disease [42] has been observed. As a contraindication, the use of C9 as for gastric cancer detection should take into account the medical history/background of patients.

Given the heterogeneous nature of cancer, it is not surprising that combination of different markers may be necessary for improved cancer detection. For example, one study had reported a new and better sensitivity of 82% for gastric cancer detection when M2-PK (sensitivity of 67%) was complemented with CA72-4 (sensitivity of 41%), despite that the specificity remained at 89% [38]. Even, higher specificity and sensitivity of 89 and 96% were obtained for pancreatic cancer when tumor M2-PK was used in conjunction with CA19-9 marker [38, 43]. Our attempt to combine C9 with CEA did not prove to be helpful in increasing the diagnostic performance. Although it is possible to increase the performance of C9 in gastric cancer detection by combining other markers such as M2-PK, CA72-4 and CA19-9 with C9, it is not practical with our current method since it is semi-quantitative. As ELISA kits for C9 are not commercially available, we are planning to develop this assay which is high throughput, consistent and quantitative in nature. This should facilitate future studies involving larger sample sizes to evaluate the clinical utility of C9 in gastric cancer detection and monitoring, alone or in combination with other markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AJCC	American Joint Committee on Cancer
CEA	carcinoembryonic antigen
IHC	immunohistochemistry
M2-PK	M2-pyruvate kinase
ROC	receiver-operating characteristics

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Figure 1.

(A) A representative validation blot showing C9 expression in the plasma samples of individual patients used in iTRAQ experiments. The average densitometry readings for each sample category (normal, early-stage and late-stage cancer) were calculated from triplicate data points from C9 immunoblots. (B) A box plot showing the distribution of C9 expression within each sample category. ANOVA was performed using the average C9 densitometry reading for each category of samples. Significant difference (p -value < 0.05) in C9 expression level was observed between normal *versus* cancer groups (early-and late-stage gastric cancer).

Figure 2.

C9 expression (A) in cell lysates of a panel of gastric cancer cell lines and (B) in conditioned media of gastric cancer cell lines compared with normal gastric cell line, HFE145. Dotted line in the figures highlighted C9 expression level in normal gastric cell line HFE145. (C) Immunohistochemical staining of C9 on six out of ten matched normal and tumor gastric cancer tissues, showing the expression of C9 is upregulated in tumor tissues compared with the normal tissues.

Figure 3.

The ROC curve using C9 average densitometry reading for all individual plasma samples (180) screened in the study. C9 sensitivity and specificity in detecting gastric cancer were estimated to be 73 and 85% at the cutoff point of average densitometry reading of 27 757.

Characteristics and clinical data of samples used for (A) discovery phase through iTRAQ approach and (B) blind test study for further result validation

Table 1

Description	(A) Samples for iTRAQ approach			(B) Blind test cohort		
	Normal ^{a)}	Gastric cancer patients	Total	Normal ^{a)}	Gastric cancer patients	Total
AJCC Staging	N/A	I/II	III/IV	N/A	I/II	III/IV
Sample size	25	15	21	61	24	34
						119
Clinical data						
(i) Gender	19M6F	8M7F	17M4F	44M17F	15M9F	24M10F
						80M37F
(ii) Age (median)	67	67	73	70	69	66
						65
(iii) Ethnicity ^{c)}						
Chinese	N/A	13	21	34	23	28
						51
Malay	N/A	1	0	1	0	3
						3
Indian	N/A	1	0	1	0	1
						1
Others	N/A	0	0	0	1	2
						3
(iv) Lauren Classification						
Diffused type	N/A	1	8	9	3	8
						11
Intestinal type	N/A	8	5	13	11	11
						22
Mixed type	N/A	1	4	5	0	5
						5
NOS	N/A	0	0	0	2	1
						3
Unknown	N/A	5	4	9	8	9
						17
(v) Gastritis						
Positive	25	15	21	61	16	12
						62
Negative	0	0	0	0	7	20
						44
Unknown	0	0	0	0	1	2
						13
(vi) <i>H. pylori</i>						
Positive	16	6	4	26	8	3
						26
Negative	9	9	17	35	14	27
						83
Unknown	0	0	0	0	2	4
						10

^{a)} The cancer-free gastric cancer plasma samples were used as normal control. These samples were matched against gastric cancer samples based on the gender and age of patients.

^{b)} There were four normal samples collected with no specified gender given in the clinical data.

^{c)} Ethnicity for normal controls is not available from clinical data.

Table 2

The list of differentially expressed proteins identified in early- or/and late-stage of gastric cancer compared with normal control

Gene symbol	Name	Early GC:normal (115:114)	Late GC:normal (116:114)	Early GC:normal (PVal 115:114)	Late GC:normal (PVal 116:114)	Early GC:normal (EF 115:114)	Late GC:normal (EF 116:114)	No. of unique peptide
(i) Underexpressed proteins								
APCS	Serum amyloid P-component precursor	0.70	0.93	0.01	0.54	1.29	1.26	7
PGLYRP2	Isoform 2 of N-acetylmuramoyl-L-alanine amidase precursor	0.75	0.90	0.04	0.18	1.32	1.16	10
GC	Vitamin D-binding protein precursor	0.78	0.96	0.00	0.35	1.15	1.08	32
GSN	Isoform 1 of gelsolin precursor	0.79	0.68	0.00	0.00	1.17	1.13	17
IGFALS	Insulin-like growth factor-binding protein complex acid labile chain precursor	0.85	0.68	0.47	0.02	1.57	1.38	8
LUM	Lumican precursor	0.88	0.68	0.43	0.02	1.40	1.37	8
-	13kDa protein	0.89	0.70	0.20	0.00	1.20	1.12	9
BTD	Uncharacterized protein BTD (fragment)	0.94	0.69	0.88	0.03	2.66	1.33	3
APOA4	Apolipoprotein A-IV precursor	1.09	0.65	0.38	0.00	1.21	1.12	38
-	Transferrin	0.28	0.30	0.00	0.00	1.20	1.13	15
HBB	Hemoglobin subunit β	0.41	0.66	0.00	0.00	1.25	1.19	9
HBA2;HBA1	Hemoglobin subunit α	0.42	0.60	0.00	0.01	1.42	1.31	3
APOC1	Apolipoprotein C-I precursor	0.46	0.60	0.01	0.00	1.83	1.32	15
CA1	Carbonic anhydrase 1	0.55	0.59	0.00	0.01	1.32	1.44	4
APOA2	Apolipoprotein A-II precursor	0.57	0.66	0.00	0.00	1.19	1.09	23
AZGP1	α -2-Glycoprotein 1, zinc	0.60	0.57	0.05	0.01	1.68	1.40	7
IGHM	IGHM protein	0.68	0.76	0.00	0.00	1.17	1.15	7
(ii) Over-expressed proteins								
AMBP	AMBP protein precursor	1.32	1.21	0.00	0.01	1.17	1.15	9
A2M	α -2-Macroglobulin precursor	1.33	1.14	0.00	0.00	1.06	1.04	124
SERPIND1	Serpin peptidase inhibitor, clade D (heparin cofactor), member 1	1.39	1.18	0.00	0.05	1.23	1.17	12
SERPINA6	Corticosteroid-binding globulin precursor	1.58	1.20	0.01	0.07	1.32	1.23	5
SERPINA1	Isoform 1 of α -1-antitrypsin precursor	0.88	5.68	0.05	0.00	1.13	1.09	33
FGA	Isoform 1 of Fibrinogen α chain precursor	0.97	1.51	0.82	0.00	1.35	1.27	7
PLG	Plasminogen precursor	0.97	1.34	0.83	0.00	1.36	1.18	15

Gene symbol	Name	Early GC:normal (115:114)	Late GC:normal (116:114)	Early GC:normal (PVal 115:114)	Late GC:normal (PVal 116:114)	Early GC:normal (EF 115:114)	Late GC:normal (EF 116:114)	No. of unique peptide
AGT	Angiotensinogen precursor	1.00	1.34	0.97	0.04	1.24	1.31	15
CD14	Monocyte differentiation antigen CD14 precursor	1.21	1.38	0.13	0.03	1.31	1.31	3
C9	Complement component C9 precursor	1.31	1.74	0.08	0.00	1.36	1.28	9
LRG1	Leucine-rich α -2-glycoprotein precursor	1.40	2.13	0.31	0.00	1.98	1.46	8
SERPINA3	α -1-Antichymotrypsin precursor	1.36	1.80	0.00	0.00	1.15	1.11	22
C7	Complement component C7 precursor	1.56	1.43	0.00	0.01	1.28	1.28	9
ORM1	α -1-Acid glycoprotein 1 precursor	1.58	1.74	0.00	0.00	1.10	1.07	9
ITIH3	Isoform 1 of Inter- α -trypsin inhibitor heavy chain H3 precursor	1.73	2.18	0.00	0.00	1.15	1.14	7

Those proteins were considered to be underexpressed when their ratio <0.77 and highly expressed when ratio >1.3, with their p -value<0.05. GC, gastric cancer; PVal, p -value and EF, error factor.

Use of C9 expression level for blind test study to estimate C9 sensitivity and specificity in detecting gastric cancer

Table 3

Plasma samples	Sample prediction based on average densitometry cutoff		Total sample prediction	Percentage of right prediction (%)	Percentage of wrong prediction (%)	C9 sensitivity (%)	C9 Specificity (%)
	No. of samples correctly predicted	No. of Samples wrongly predicted					
Normal ^{a)}	45	16	61	73.8	26.2	N/A	73.8
Early GC (stages I and II) ^{b)}	9	15	24	37.5	62.5	37.5	
Late GC (stages III and IV) ^{c)}	26	8	34	76.5	23.5	76.5	
Gastric cancer (stages I–IV) ^{d)}	48	10	58	82.8	17.2	82.8	

^{a)} Sample is predicted as normal when its average densitometry reading is <21 577.

^{b)} Sample is predicted as early-gastric cancer (stages I and II) when its average densitometry reading is >21 577 and >42 750.

^{c)} Sample is predicted as late-gastric cancer (stages III and IV) when its average densitometry reading is >42 750.

^{d)} Sample (either early or late stage) is predicted to be gastric cancer (in general, regardless to the staging) when its average densitometry reading is >21 577.

Table 4

In comparison, CEA levels were measured for 115 plasma samples, including those samples used in validation set and 55 randomly selected samples from blind test cohort

Samples screened for	Plasma samples	No. of samples correctly predicted	No. of samples wrongly predicted	Total sample prediction	Percentage of right prediction (%)	Percentage of wrong prediction (%)	Sensitivity (%)	Specificity (%)
CEA only	Normal	54	0	54	100	0	18	100
	EarlyGC (stages I and II)	2	21	23	9	91		
	Late GC (stages III and IV)	9	29	38	24	76		
	Gastric cancer (stages I–IV)	11	50	61	18	82		
C9 only	Normal	40	14	54	74	26	90	74
	Gastric cancer (stages I–IV)	55	6	61	90	10		
Either C9 or CEA ^{a)}	Normal	40	14	54	74	26	90	74
	Gastric cancer (stages I–IV)	55	6	61	90	10		
Both C9 and CEA ^{b)}	Normal	54	0	54	100	0	20	100
	Gastric cancer (stages I–IV)	12	49	61	20	80		

The sensitivity and specificity of CEA for gastric cancer prediction were then calculated. Using the same data set sent for CEA screening, the sensitivity and specificity of marker combination (CEA and C9) were also estimated.

^{a)} Samples were predicted to be cancer when the CEA value is 45mg/L or the average densitometry reading of C9 expression is 421577.

^{b)} Samples were predicted to be cancer when the CEA value is 45mg/L and the average densitometry reading of C9 expression is 421 577, otherwise samples were predicted to be normal.