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BASIC RESEARCH

Discovering differential protein expression caused by CagA-induced ERK pathway activation in AGS cells using the SELDI-ProteinChip platform

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Abstract

AIM: To identify the protein expression differences related to the CagA-induced ERK pathway activation in AGS cells.

METHODS: Human AGS cells transfected with *cagA* and blank vector were treated with specific mitogen-activated protein kinase kinase (MEK) inhibitor. Total cell proteins were combined by strong anion exchange (SAX2) and weak cation exchange (CM10) ProteinChip arrays and analyzed using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) proteomics technology. Protein expression profiles were compared with those of inhibitor-untreated *cagA* transfectants. SwissProt/TrEMBL database searching for differentially expressed proteins was carried out using the TagIdent tool with the pI and mass information.

RESULTS: When a total of 16 proteins that showed expression differences in inhibitor-untreated *cagA* transfectants were compared with vector transfectants, three proteins with m/z 4229, 8162 and 9084 were found to have no expression differences after treatment with MEK inhibitor, while the other 13 maintained the same expression differences after inhibitor treatment. Seven pieces of meaningful matching information for the three proteins were obtained from database searching.

CONCLUSION: Biomarkers with m/z 4229, 8162 and 9084 are ERK1/2 phosphorylation dependent, and therefore are the downstream molecules of ERK1/2 in the ERK/MAPK signaling pathway. The three biomarkers may be important cancer-associated proteins according to SwissProt/TrEMBL database information.

Key words: CagA; ERK pathway; SELDI-TOF-MS; ProteinChip

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INTRODUCTION

H pylori infection, a primary etiological cause leading to gastritis and peptic ulcer[1], is also a high risk factor for gastric cancer, and is classified as a class I carcinogen by the International Research Agency on Cancer (IRAC)[2-6]. Cytotoxin-associated gene A (CagA) protein, which is encoded by cytotoxin-associated gene pathogenicity island, is the major virulence factor of type I *H pylori*. Epidemiological studies indicate that CagA plays an important role in the development of *H pylori*-induced disease, especially in gastric cancer. The CagA antibody titer in gastric cancer patients is higher than in healthy individuals. Similarly, CagA antibody titer is elevated in high-risk populations[7]. Compared with *cagA*-negative *H pylori* strains, *cagA*-positive strains dramatically increased the occurrence of severe gastritis and gastric adenocarcinoma[8-12]. Experimental studies reveal that CagA is translocated into host cells through a type Ⅳ secretion system of *H pylori*[13]. After being tyrosine-phosphorylated at the C-terminal repeat sequence(s) by c-src/Lyn kinase[14,15], CagA strongly stimulates the mitogen-activated protein kinase (MAPK) signaling pathway in host cells, through interaction with SHP-2, a tyrosine phosphatase containing an SH2 homolog, and

results in cell proliferation and differentiation[16]. As one of the major three members of the MAPKs, extracellular signal-regulated kinase (ERK) has been reported to be a key molecule in cell carcinogenesis in various cell types[17-19]. Higashi *et al*[20] have found that ERK is activated by CagA *via* SHP-2 recruitment and activation in AGS cells (human gastric adenocarcinoma epithelial cell line). Our previous research further proved that transformation of immortalized gastric epithelial cells by CagA takes place through the ERK/MAPK pathway[21]. However, the precise mechanism of the interactions between CagA and host cells in associated pathogenesis has not been fully elucidated.

Recently, gene microarrays have been used to establish the global pattern of gene expression in *cagA* gene-transfected AGS cells[22]. Nevertheless, gene and protein expression levels cannot easily be correlated or equated since proteins can exist in different posttranslational functional states. Therefore, it is important to gain an overall view of host response to CagA interference at the protein level. However, to date, few studies that exploit the global protein-expression pattern that can reflect host-cell response to CagA have been reported. Based on surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology, the ProteinChip platform has recently been shown to be valuable in establishing protein expression profiles and discovering new biomarkers[23-25]. ProteinChips, on which proteins from biological samples are selectively retained according to their biochemical properties, are analyzed directly by MS to form peak patterns as protein-expression profiles. The location and intensity of every peak in the pattern reflect the molecular weight and abundance of the corresponding protein. Therefore, the patterns can be used to analyze the protein expression differences under different conditions. This innovative technique has certain advantages over other traditional methods such as two-dimensional gel electrophoresis in its small sample volume, high throughput capability, subfemtomole range sensitivity, high resolution at low mass range, and easy operation, thus it has been successfully applied in new biomarker discovery for early diagnosis of cancer and basic research into disease mechanisms[26-29].

The proteome of AGS cells transfected with *cagA* gene was analyzed using a SELDI-ProteinChip platform in our previous study. Sixteen biomarker proteins involved in CagA induced pathogenesis were identified[30]. In this study, we further investigated the relationship of these protein expression differences and activation of the ERK/MAPK signaling pathway by CagA. Three more pivotal disease-related biomarkers among the 16 were further figured out.

MATERIALS AND METHODS

Expression vectors

Wild-type (WT) *cagA*/pcDNA3.1(+) plasmid (WT-*cagA*) and phosphorylation site mutant *cagA*/pcDNA3.1(+) plasmid (MT-*cagA*) were constructed as previously described[31,32]. Briefly, WT-*cagA* was constructed for encoding CagA proteins containing three tandem Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, and one repeated EPIYA motif sequence with Asp-Phe-Asp (DFD) in the D2 region. MT-*cagA* was generated from WT-*cagA* by substituting a phenylalanine codon for a tyrosine codon in the repeated EPIYA motif.

Cell culture and transfection

Human gastric adenocarcinoma epithelial AGS cells (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Gibco) in six-well plates. For ERK-associated analysis, cells were transiently transfected with WT-*cagA* or blank vectors. For each well, 4 μg plasmids were transfected into 5.5×10^5 cells by using 6 μL Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. WT-*cagA* transfectants, vector transfectants and untransfected cells were divided into three groups that were respectively treated with the specific mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (Sigma, St. Louis, MO, USA) at 10 μmol/L, DMSO vehicle, or were not treated. Cells were collected after 48 h for protein expression analysis. The transfection efficiency was at least 37%, as determined by the cells transfected with EGFP/pcDNA3.1(+). The expression of *cagA* gene was confirmed by RT-PCR.

Western blot analysis

WT-*cagA* transfectants, vector transfectants and untransfected AGS cells were incubated in 10% FCS for 0, 1 or 3 h following a 24-h serum-starvation period. After washing three times with PBS, cells were harvested and treated with lysis buffer (20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 10 mmol/L EDTA, 10 mL/L Triton X-100, 40 g/L SDS, and 100 mL/L glycerol) at 100℃ for 5 min and then centrifuged at 15000 × *g* for 5 min. Western blot analysis was performed with standard techniques, using anti-total ERK1/2 antibody (1:1000; Cell Signaling, Beverly, MA, USA), anti-phospho-ERK1/2 antibody (1:1000; Cell Signaling), followed by an anti-mouse secondary antibody (Beijing Zhongshan Biotechnological, China). Samples were separated on 10% sulfate-polyacrylamide gel and electrotransferred onto nitrocellulose membranes (Amersham Biosciences, USA). Immunoreactive bands were visualized with the ECL detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Kodak X-ray film.

Preparation of total cell lysates

AGS cells of each group were harvested after PBS washing and resuspended in lysis buffer (8 mol/L urea, 40 g/L CHAPS, 50 mmol/L DTT, 40 mmol/L Tris-HCl) containing protease inhibitor cocktail (Sigma). After 30 min lysis on ice, total cell lysates were obtained by centrifugation at 20000 × *g* for 30 min to remove insoluble debris. Protein concentration was measured using BioRad DC protein assay kit (BioRad, Hercules, CA, USA) and then adjusted to 5 mg/mL for further protein expression analysis.

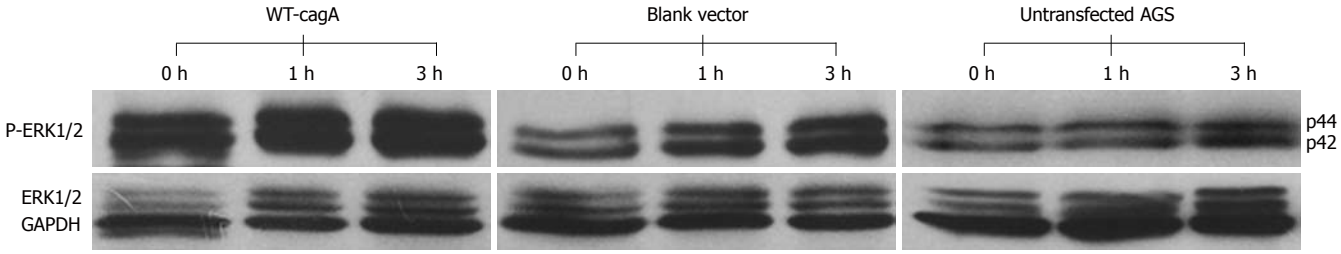

Figure 1 Detection of phosphorylated ERK1/2 in transfected AGS cells. Cells transfected with WT-*cagA* or blank vectors were stimulated with serum by adding fresh medium containing 10% FCS for 0, 1 and 3 h, after 24 h serum starvation, and then harvested for Western blot analysis of total and phosphorylated ERK1/2 (P-ERK1/2). The level of P-ERK1/2 in WT-*cagA* transfectants was significantly higher than that in blank vector transfectants and untransfected AGS cells at all three time points of serum stimulation. No obvious increases in total ERK among the three groups were found. GAPDH level was used as a loading control.

SELDI protein profiling

Expression profiles of each group were obtained by strong anion exchange (SAX2) and weak cation exchange (CM10) ProteinChip Arrays (Ciphergen Biosystems, Fremont, CA, USA) using an "*in situ*" protocol provided by the manual. Before sample loading, each spot of SAX2 and CM10 arrays were equilibrated with 3 μL of binding buffer (50 mmol/L Tris-HCL, pH 9.0 for SAX2 and 50 mmol/L sodium acetate, pH 4.0 for CM10). After 5 min incubation for three consecutive periods, 5 μL total cell lysate (diluted to 1.5 mg/mL in binding buffer) was added for chip binding. All arrays were incubated in a humidity chamber for 1 h and each spot was washed three times with 3 μL binding buffer. After rinsing quickly with HPLC water twice, the arrays were air-dried. One microliter of saturated sinapinic acid (Ciphergen Biosystems) solution in 500 mL/L acetonitrile, in water containing 5 mL/L trifluoroacetic acid, was applied twice, followed by air-drying. Mass accuracy was calibrated using the All-in-1 peptide and All-in-1 protein molecular mass standards (Ciphergen Biosystems). MS analysis of each sample was performed using the ProteinChip Biology System Reader (Model PBS Ⅱ; Ciphergen Biosystems) at a laser intensity of 180 and a detector sensitivity of 7.

Data analysis

Data of every spectrum were collected and analyzed by ProteinChip Software version 3.0 (Ciphergen Biosystems) with integrated Biomarker Wizard software (Ciphergen Biosystems). In brief, baselines were subtracted and normalization was performed by total ion current normalization function, following the software instructions. Biomarker Wizard was then used to identify corresponding peaks in the spectra. Significant differences ($P < 0.05$) in peak intensity of particular proteins between two sample groups were calculated by the software using a Mann-Whitney non-parametrical test.

RESULTS

ERK activity in transfected cells

Western blot results showed that the phosphorylation level of ERK1/2 in WT-*cagA* transfectants was significantly higher than that in vector transfectants and untransfected AGS cells, after serum stimulation at all time points (Figure 1), which indicates that phosphorylated CagA can strongly enhance the phosphorylation level of ERK1/2 in host cells.

Profiling of cell lysates

Protein profiles of cell lysates from each group were obtained from SAX2 (strong anion exchange) and CM10 (weak cation exchange) ProteinChip Arrays. Each type of array surface retains different groups of proteins depending on the surface properties (SAX2 captures proteins with low pIs, while CM10 captures proteins with high pIs), therefore assuring that more host proteins are analyzed. MS analysis was carried out under a laser intensity of 180 and a detector sensitivity of 7.0, which brought out the highest resolution determined by previous experiments. Approximately 200 separate protein peaks with a signal to noise ratio > 5.0 and intensity > 1.0 were obtained in the mass range of 2000-20 000 m/z (mass/charge) in every spectrum, using either CM10 or SAX2 chips.

Biomarker proteins dependent on activation of ERK/MAPK signaling pathway detected by SELDI-TOF-MS

In our previous study[30], we transfected AGS cells with WT-*cagA*, phosphorylation site mutant *cagA*, and blank vector. Protein-expression profiles were analyzed using a SELDI-ProteinChip platform, in order to gain an insight into the effect of CagA and CagA phosphorylation on host protein expression. A total of 16 proteins in AGS cells were found have expression differences under the effect of CagA, and 12 of these were CagA phosphorylation dependent (Figure 2, Table 1). These biomarkers may contribute to the host response to CagA and may be involved in CagA-induced pathogenesis.

According to the previous results, most changes in expression after *cagA* transfection are related to CagA phosphorylation. Phosphorylated CagA can interfere with signal transduction of the ERK/MAPK pathway that plays an important role in CagA-induced cell proliferation and transformation. Various downstream molecules involved in the pathway need to be identified. Thus, our next question is which biomarkers among the 16 are caused by CagA-induced activation of the ERK/MAPK signaling pathway. The results may help us to focus on the most important proteins involved in the host response to CagA. After confirming by Western blotting that WT CagA can significantly increase the activation of ERK1/2, we treated AGS cells with MEK

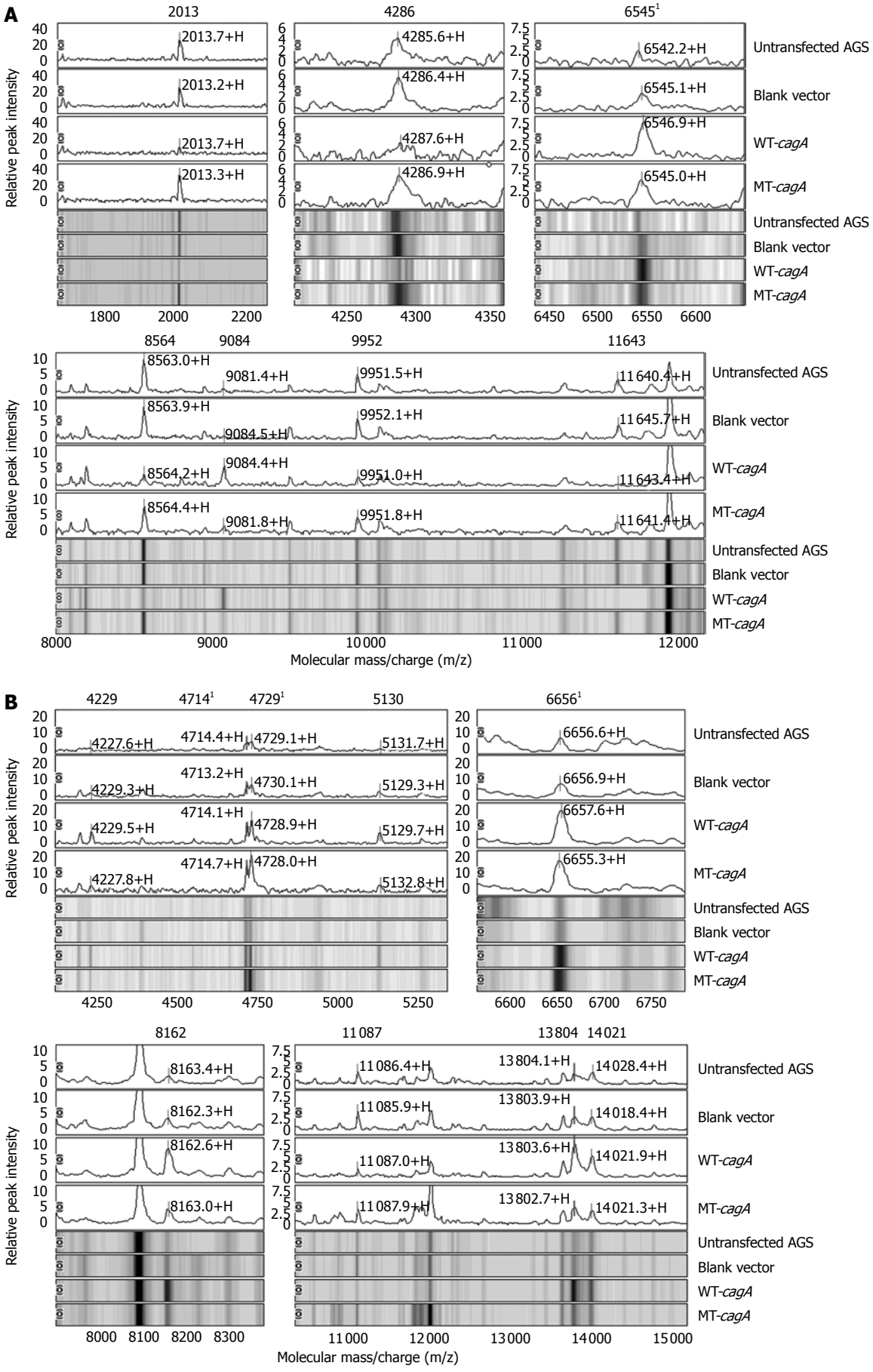

Figure 2 Biomarker proteins with different expression level in WT-*cagA*, phosphorylation site mutant *cagA* (MT-*cagA*) and blank-vector-transfected AGS cells detected by ProteinChip arrays. Cell lysates of each group were analyzed on SAX2 and CM10 Chip surfaces under laser intensity of 180 and sensitivity of 7. One representative spectrum was selected from a quadruplicate set of samples for each group. The top panel in each figure is the spectral view, and the lower panel is the gel view. Peaks with different expression level were marked with m/z values. **A**: Biomarkers detected by SAX2; **B**: Biomarkers detected by CM10. [1]Indicates CagA tyrosine-phosphorylation-independent proteins, which can be induced by both WT CagA and MT CagA (adopted from *Chinese J Cell Biology* 2006; 28: 603-610).

Table 1 Overview of biomarkers' expression level in the 2000-20000 m/z mass range in wild-type *cagA*, phosphorylation site mutant *cagA* and blank vector transfected AGS cells

Array type	m/z	Peak intensity (mean ± SD)			Fold change		Comments
		V	W	M	W *vs* V	M *vs* V	
SAX2	2013	30.39 ± 7.19	8.82 ± 5.37	29.92 ± 9.53	-3.45[a]	-1.02	CagA phosphorylation dependent
	4286	5.08 ± 0.85	2.25 ± 0.70	4.44 ± 0.58	-2.26[a]	-1.14	
	8564	9.73 ± 1.88	3.72 ± 0.42	10.86 ± 1.77	-2.62[a]	1.12	
	9084	2.61 ± 1.35	6.13 ± 1.74	2.75 ± 0.60	2.35[a]	1.05	
	9952	5.22 ± 2.07	2.51 ± 0.95	5.19 ± 1.30	-2.08[a]	-1.01	
	11643	3.39 ± 0.98	1.32 ± 0.86	3.70 ± 1.10	-2.57[a]	1.09	
CM10	4229	4.29 ± 0.57	9.36 ± 0.32	6.13 ± 0.78	2.18[a]	1.43	
	5130	3.59 ± 0.72	6.93 ± 0.61	4.58 ± 0.46	1.93[a]	1.28	
	8162	4.26 ± 0.38	8.88 ± 1.06	4.97 ± 0.24	2.08[a]	1.17	
	11087	3.37 ± 0.75	1.94 ± 0.46	3.19 ± 0.39	-1.74[a]	-1.06	
	13804	2.97 ± 0.79	8.66 ± 0.48	4.14 ± 1.23	2.92[a]	1.39	
	14021	3.06 ± 0.24	5.59 ± 0.41	3.66 ± 1.04	1.83[a]	1.20	
SAX2	6545	3.08 ± 0.88	6.95 ± 1.34	5.53 ± 0.69	2.26[a]	1.80[a]	CagA phosphorylation independent
CM10	4714	10.16 ± 1.26	17.95 ± 3.61	17.31 ± 1.41	1.77[a]	1.70[a]	
	4729	11.11 ± 1.75	20.51 ± 4.02	21.92 ± 2.58	1.80[a]	1.97[a]	
	6656	9.67 ± 2.45	22.83 ± 4.27	21.46 ± 1.92	2.36[a]	2.22[a]	

Biomarkers were selected by these criteria: Statistical *P*-value among sample groups less than 0.05; signal to noise ratio higher than 5.0; peak intensity higher than 1.0. The particular fold change in average peak intensity of wild-type *cagA* transfectants (W) or phosphorylation site mutant *cagA* transfectants (M) was calculated compared with the intensity of blank vector transfectants (V). [a]$P < 0.05$. Statistical *P*-value was calculated with a non-parametrical Mann-Whitney test ($n = 4$).

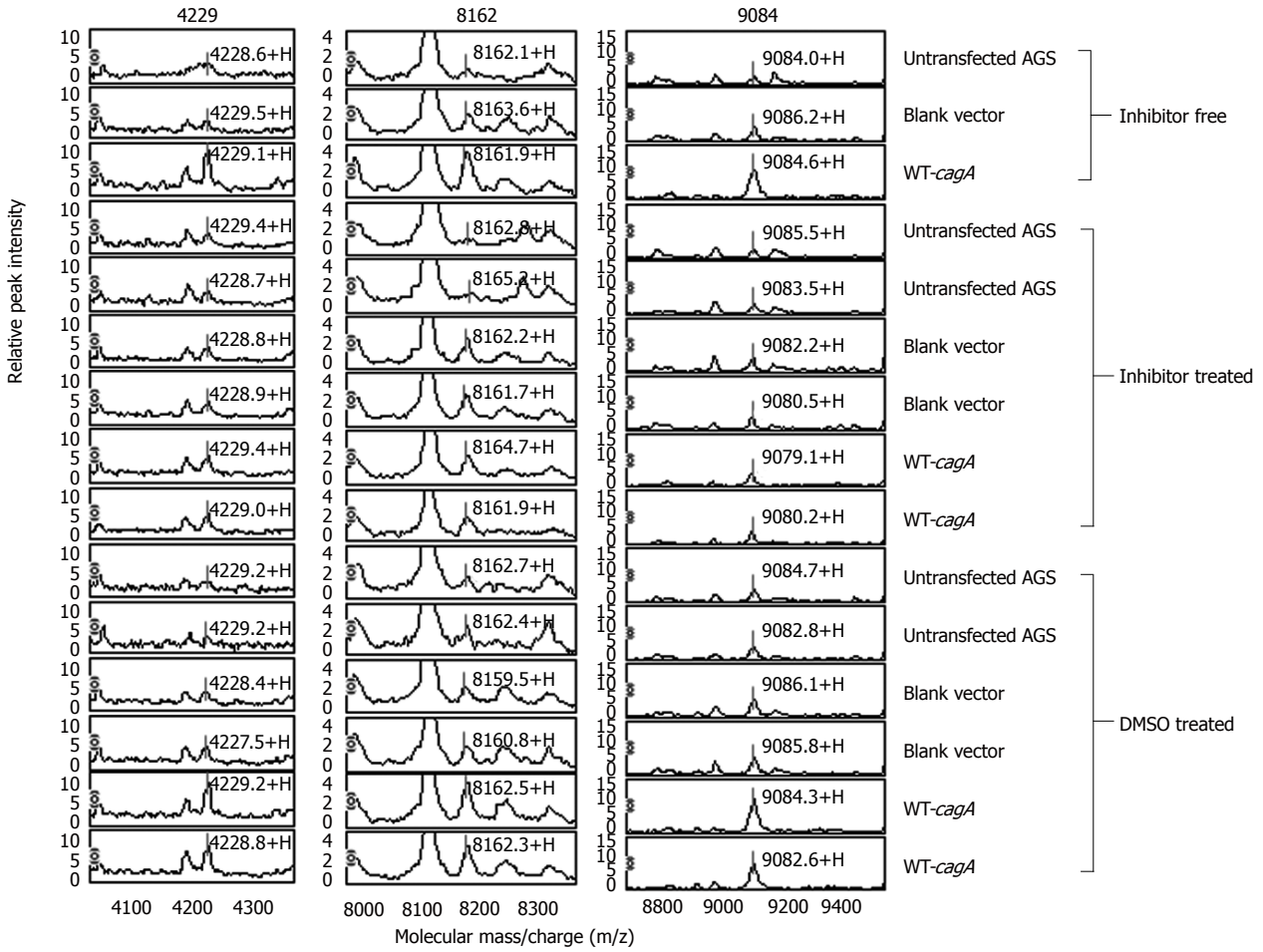

Figure 3 Expression differences of biomarker proteins caused by ERK pathway activation. Cells were transfected with WT-*cagA* or blank vectors with treatment of MEK inhibitor U0126, or its vehicle DMSO. Expression differences of three biomarkers with m/z 4229 (CM10), 8162 (CM10) and 9084 (SAX2) between WT-*cagA* transfectants and vector transfectants appeared under both MEK-inhibitor-free and DMSO-treated conditions, but disappeared under inhibitor-treated conditions.

inhibitor simultaneously to transfection to investigate if the changes in expression of the 16 biomarkers induced by WT-*cagA* disappeared when ERK1/2 was deactivated. The expression levels of three biomarkers with m/z 4229, 8162 and 9084 were found up-regulated significantly in WT-*cagA* transfectants with 2.25-, 1.71- and 1.64-fold changes

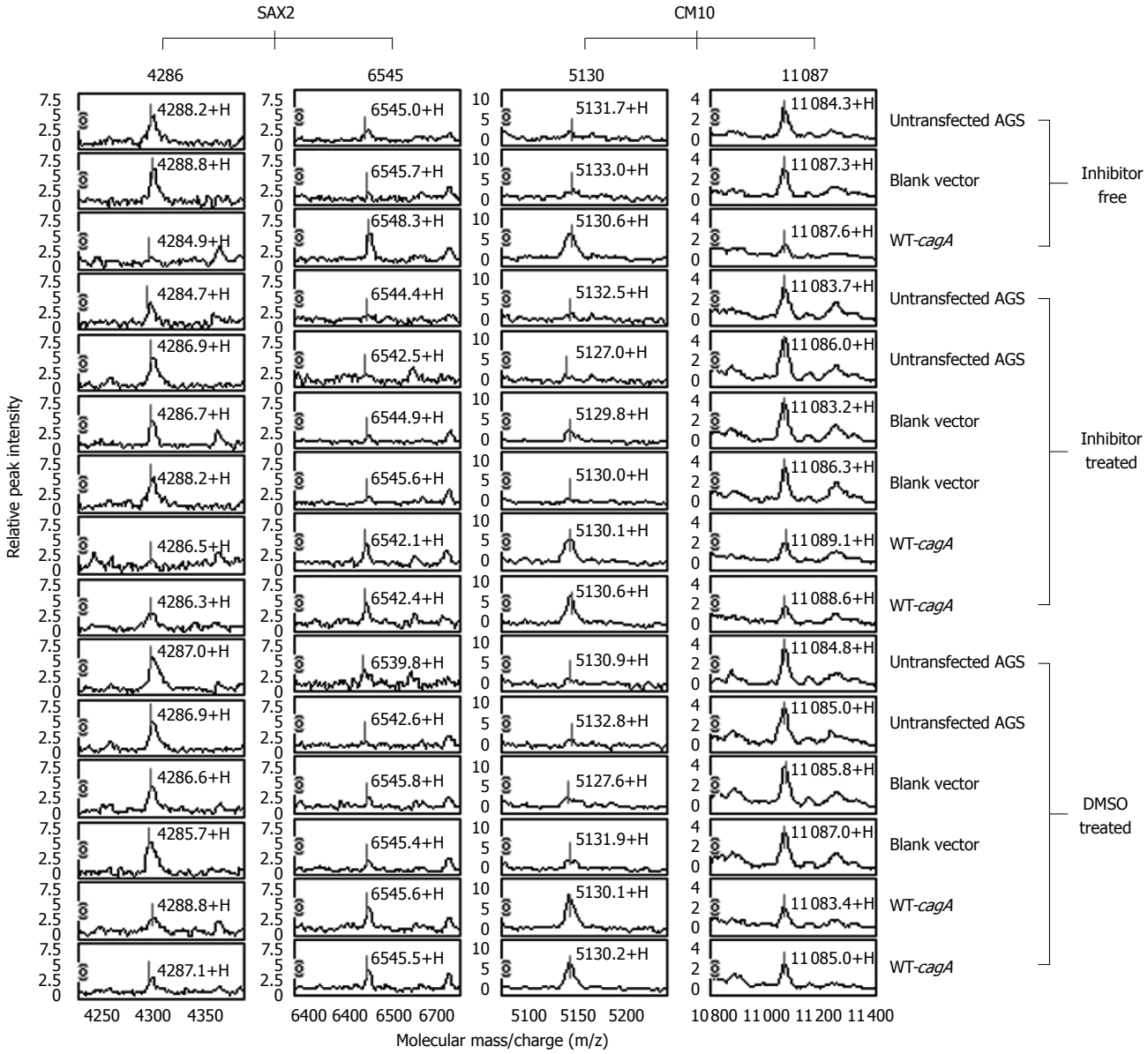

Figure 4 Expression differences of biomarker proteins not caused by ERK pathway activation. Expression differences of the other 13 biomarker proteins between WT-*cagA* transfectants and vector transfectants appeared not only under MEK-inhibitor-free and DMSO-treated conditions, but also under MEK-inhibitor-treated conditions. Spectra of typical biomarker proteins with m/z 4286 (SAX2), 6545 (SAX2), 5130 (CM10) and 11 087 (CM10) are shown in the figure.

compared with vector transfectants under inhibitor-free conditions (Figure 3, panels 1-3). The fold changes were very similar to those in our previous experiment (Figure 2, panels 1-3). In DMSO-treated conditions, they displayed the same expression pattern (Figure 3, panels 10-15) as under inhibitor-free conditions. However, when treated with MEK inhibitor during transfection, the peak intensity of these three proteins in WT-*cagA* transfectants was found not to be significantly different ($P > 0.05$) from that of vector transfectants (Figure 3, panels 4-9). This indicates that the expression differences of biomarkers with m/z 4229, 8162 and 9084 were caused by activation of the ERK/MAPK signaling pathway. The expression differences for the other 13 proteins existed in both ERK1/2-active and -inactive conditions (Figure 4, Table 2). Thus, they were independent of ERK1/2 activation. The biomarkers with m/z 4229, 8162 and 9084 that were identified as ERK1/2 downstream molecules involved in CagA-induced pathogenesis are worthy of further investigation.

Database searching for the three ERK-related biomarkers

A search in the SwissProt/TrEMBL database using the TagIdent tool, along with the pI and mass information was performed to get matching proteins for the three biomarkers with m/z 9084, 4229 and 8162. All together, 22 pieces of matching information were obtained from the database. Seven of these were shown to be proteins related to apoptosis, antimicrobial defense, chemotaxis, proliferation, differentiation and carcinogenesis, which may be closely associated with the ERK/MAPK signaling pathway (Table 3). The existence of these proteins was validated at the protein or transcript level in a series of studies. The other 14 were predicted to be proteins or proteins lacking the references providing insightful

Table 2 Comparison of biomarkers' expression level under mitogen-activated protein kinase kinase (MEK) inhibitor free, treated and DMSO treated conditions

Array type	m/z	Inhibitor free		Inhibitor treated		DMSO treated		Comments
		Fold change (W *vs* V)	*P*-value	Fold change (W+I *vs* V+I)	*P*-value	Fold change (W+D *vs* V+D)	*P*-value	
SAX2	9084	1.64	0.021	1.00	0.827	2.21	0.021	Caused by ERK1/2
CM10	4229	2.25	0.021	1.07	1.000	2.44	0.021	activation
	8162	1.71	0.021	-1.15	0.149	1.81	0.021	
SAX2	2013	-1.99	0.021	1.16	0.149	1.03	0.773	Have no relationship
	4286	-3.50	0.021	-2.14	0.021	-1.95	0.021	with ERK1/2
	6545	2.86	0.021	2.39	0.021	2.00	0.021	activation
	8564	-3.05	0.021	-2.96	0.021	-1.85	0.021	
	9952	-3.35	0.021	-2.87	0.021	-2.62	0.021	
	11643	-1.71	0.021	-2.49	0.021	-2.38	0.021	
CM10	4714	1.89	0.021	1.68	0.043	1.74	0.021	
	4729	1.99	0.021	1.72	0.021	1.86	0.021	
	5130	1.67	0.021	1.66	0.021	2.91	0.021	
	6656	2.12	0.021	2.31	0.021	2.44	0.021	
	11087	-1.72	0.021	-1.85	0.021	-1.52	0.021	
	13804	2.23	0.021	2.56	0.021	2.60	0.021	
	14021	1.98	0.021	1.79	0.021	1.61	0.021	

Biomarkers listed in top three lanes were identified as biomarkers induced by extracellular signal-regulated kinase 1/2 (ERK1/2) activation. Columns 3, 5, 7 give fold changes in peak intensity in wild-type cagA transfectants (W) compared with vector transfectants (V) as reference under MEK inhibitor U0126 free, MEK inhibitor U0126 treated (I) and DMSO treated (D) conditions. Statistical *P*-value were calculated using a non-parametrical Mann-Whitney test (n = 4).

Table 3 Swiss-Prot and TrEMBL database searching results of the three ERK related biomarkers

Biomarker (Mw)	Meaningful matching information in Swiss-Prot and TrEMBL database			Comments
	Protein Name	Mw	pI	
9084 (SAX2)	BIM-alpha3 (BCL2-like 11 transcript variant 10, chain 1-79)	9081	5.42	Over-expression of BIM-alpha3 results in cell apoptosis
	EP2E (chain 1-80)	9091	7.55	Part of the protein sequences homologous to β-defensins, a family of antimicrobial peptides.
	UPF0197 protein (C11orf10 chain 1-79)	9079	5.58	Expressed in CD34+ hematopoietic stem/progenitor cells
4229 (CM10)	Chromogranin-A Precursor (ER-37, chain 420-456)	4233	4.59	Neuroendocrine and endocrine secretory granules, considered as biomarkers in some human cancers
8162 (CM10)	Small-inducible cytokine B6 Precursor (chain 40-114)	8162	9.74	Functional characterization as chemokines, chemotactic for neutrophil granulocytes
	Putative microRNA host gene protein 1 (chain 1-70)	8163	9.25	Highly expressed in B-cell lymphoma and lung cancer and enhanced cell proliferation
	PNAS-145 (Chain: 1-68)	8161	11.24	Human acute promyelocytic leukemia cell line NB4's apoptosis/differentiation related genes

Searching was carried out using TagIdent tool with mass and the pI information (0-9.0 for SAX2, 4.0-14.0 for CM10).

functional information. Therefore, the three biomarkers with m/z 9084, 4229 and 8162 have great potential to be identified as cancer-associated proteins in future research.

DISCUSSION

Translocation of CagA protein into gastric epithelial cells through the type Ⅳ secretion system is considered to be one of the most important processes in the progression of *H pylori* diseases. In this host-bacterial interaction period, many host proteins and signaling pathways are implicated[33]. In order to gain an overall view into the host response to CagA, the innovative proteomic technology SELDI-ProteinChip platform was employed to investigate the expression changes in gastric epithelial AGS cells. The strongpoint of this technology is that it can detect proteins in fmol quantities, and it allows fast, high-throughput analysis, especially in the low-molecular-weight range, which is essential for biomedical research, and cannot be easily achieved by conventional methods. Following this method, 16 biomarkers have been identified in our previous study as being involved in CagA-host cell interactions, and 12 of these are dependent on CagA phosphorylation. This result provides a primary view of CagA-induced pathological changes at the protein level.

To further investigate the role of CagA-phosphorylation-associated functions in gastric carcinogenesis, we focused our interest on the role of the ERK signaling pathway. It has been verified by clinical pathological tests that the level of phosphorylated ERK in gastric cancer tissue is higher than that in the gastric mucous membrane. The level is also higher in cancer tissue of *H pylori*-positive gastric cancer patients than that in *H pylori*-negative patients[34]. Besides, it has been reported that *cagA*-positive *H pylori* is more powerful than *cagA*-negative strains for ERK activation[35]. Thus, ERK/MAPK pathway activation and subsequent cell proliferation, differentiation, as well as morphological changes, are among the most important pathogenic factors

in CagA-induced diseases. However, this complicated process includes multi-level regulation that has rarely been understood. In the present study, we tried to investigate if the expression changes of the 16 host proteins induced by CagA have some relationship with the activation of the ERK pathway. The phosphorylation level of ERK1/2 in WT-*cagA* transfectants was confirmed to be much higher than that in vector transfectants and blank control cells. MEK inhibitor U0126 that specifically inhibits ERK1/2 phosphorylation was employed to eliminate the changes induced by ERK activation during the transfection of WT-*cagA*. The relationship of the 16 biomarkers and ERK pathway can be determined by comparing their expression levels under ERK-inhibited and ERK-activated conditions. On the other hand, protein profiles indicated that protein expression levels were influenced by DMSO as the solvent of U0126. Therefore, we first compared profiles of WT-*cagA* transfectants and vector transfectants under U0126-free conditions, U0126 treatment, and DMSO treatment. Only the proteins exhibited similar expression differences between WT-*cagA* transfectants and vector transfectants under both U0126 free and DMSO treated conditions, but they exhibited no expression differences under the U0126 treated condition, and thus were considered to be biomarkers that related to ERK activation. In this manner, among the 16 biomarkers, three with m/z 4229, 8162 and 9084 were identified as ERK pathway downstream proteins related to CagA pathogenesis. These three biomarkers will be characterized in our future research.

Most biomarkers discovered so far are peptides or small proteins with low abundance. They are probably metabolic products, abnormally cleaved proteins, modified proteins, neurokinins or cytokines that exist in disease states. The biomarkers detected in our study are also likely small molecules of this kind, which can hardly be detected by traditional methods, but are essential for associated research. While the SELDI-ProteinChip platform is very rapid for protein profiling, it does not allow direct purification and functional identification. A search in the SwissProt/TrEMBL database using the TagIdent tool with pI and mass information was performed to obtain matched information for each biomarker. The search results suggested that these three biomarkers are probably peptides of the following type: β-defensin homologs, which might be involved in host defense to bacterial infection; BCL2-like protein, which might function as an apoptosis inhibitory protein; chemokines; and tumor-related proteins, which might be responsible for cell proliferation and differentiation induced by ERK/MAPK pathway activation, and contribute to CagA-induced carcinogenesis in the gastric epithelium. The matching information provides helpful references for obtaining corresponding antibody which will be employed in future biomarker identification. The biochemical properties provided by the selective ProteinChip surfaces and SELDI-TOF-MS for each biomarker are also useful for the purification process. Further purification and identification will elucidate the function of related small molecules in CagA pathogenesis, and may therefore discover new therapeutic targets.

COMMENTS

Background

H pylori infection is an important causative factor of gastritis, peptic ulcer and gastric cancer. CagA, the major virulence factor of type I *H pylori*, is injected by the bacterium into gastric epithelial cells, and has been shown by epidemiological and experimental studies to be closely associated with the development of *H pylori*-induced diseases, especially gastric cancer. However, the precise role of CagA in cell function after *H pylori* infection remains unclear.

Research frontiers

CagA is translocated into host cells through a type Ⅳ secretion system of *H pylori*. After tyrosine phosphorylation at the C terminus, CagA strongly interferes with signal transduction, which results in cell proliferation and differentiation. Various studies have indicated a relationship between CagA, ERK/MAPK pathway activation and gastric cancer. In this study, a proteomic approach was employed to detect host cell proteins related to CagA-induced ERK pathway activation.

Innovations and breakthroughs

A new proteomic approach based on ProteinChip array and SELDI-TOF-MS was employed to investigate the global protein expression changes in gastric epithelial cells induced by CagA. Three biomarkers were further identified as downstream molecules of ERK1/2 in the ERK/MAPK signaling pathway. Due to the high sensitivity and resolution in the low-molecular-weight range of SELDI-ProteinChip technology, biomarkers discovered in this study are mainly low-mass and/or low-abundance disease-related proteins, which are difficult to detect by traditional methods.

Applications

These results provide new targets for further understanding the biological function and pathogenesis of CagA, as well as new therapeutic targets.

Terminology

CagA: cytotoxin-associated gene A protein encoded by cytotoxin-associated gene pathogenicity island is the major virulence factor of type I *H pylori*. ERK: extracellular signal-regulated kinase. ERK pathway is one of the major three members of the MAPK signaling pathways. ERK has been reported as a key molecule in carcinogenesis in various cell types. ProteinChip array: the surface of array selectively retains proteins from biological samples according to their biochemical properties, and can be analyzed directly by MS. SELDI-TOF-MS: surface-enhanced laser desorption/ionization time-of-flight MS detects proteins in fmol quantities, and allows fast, high-throughput analysis, especially in the low-molecular-weight range.

Peer review

This is a state-of-the-art, well-written paper that has used a proteomic approach to understand CagA protein biology as it relates to gastric cancer.

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