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Original Article

Proteomic identification of alpha-2-HS-glycoprotein as a plasma biomarker of hypopharyngeal squamous cell carcinoma

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Abstract: Hypopharyngeal squamous cell carcinoma (HSCC) has very poor prognosis compared with other head and neck squamous cell carcinomas. Late-stage diagnosis of HSCC increases mortality. Therefore, more effective biomarkers for early diagnosis of HSCC are necessary. Unfortunately, appropriate biomarkers for clinical diagnosis and prognosis have not been identified yet. However, recent progresses in quantitative proteomics have offered opportunities to identify plasma proteins as biomarkers for HSCC. In the present study, plasma samples were analyzed by two-dimensional differential gel electrophoresis (2D-DIGE), and differentially expressed proteins were identified by matrix assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS). A total of 26 proteins representing 12 unique gene products were identified. The up-regulation proteins were alpha-2-HS-glycoprotein (AHSG), complement C4-B, haptoglobin, C-reactive protein, and ceruloplasmin, whereas the down-regulation proteins were serum albumin, angiotensinogen, alpha-1-antichymotrypsin, Ig gamma-3 chain C region, fibrinogen gamma chain, apolipoprotein A-I, and Ig kappa chain C region. Among all the differentially expressed proteins, AHSG was validated by western blot and ELISA. The results were consistent with the data from 2D-DIGE, further suggesting that AHSG may be employed as a potential biomarker for the early diagnosis of HSCC. In summary, this study was the first to use 2D-DIGE and MALDI-TOF/TOF platform to identify the potential plasma biomarkers for HSCC. The plasma AHSG showed great potential for HSCC screening.

Keywords: Hypopharyngeal squamous cell carcinoma, plasma, biomarker, proteomics, differential in-gel electrophoresis, AHSG

Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) accounts for approximately 5-15% of all head and neck cancers [1]. Over the past decade, the 5-year survival rate of HSCC is estimated to be at 25-40% [2], and it has not been markedly improved despite recent advances in various treatment modalities, including surgery, radiotherapy, and chemotherapy [3]. The poor prognosis of HSCC might be because of the lack of early detection and high rate of metastasis [4]. Detection of HSCC at an earlier stage would be beneficial to patients. Many molecules, such as SIRT1, DBC1 [5], Beclin-1, LC3 [6], and Caveolin-1 [7], have been evaluated as candidate biomarkers for HSCC, but none has been widely used in practice because of the lack of understanding of the molecular mechanisms involved in HSCC development, progression, and treatment response [8]. Therefore, studies of novel and more effective molecular biomarkers for early diagnosis of HSCC are necessary.

Recent progresses in quantitative proteomics have offered promising opportunities to discover biomarkers for HSCC. To date, most studies

on cancer proteomics have used tumor and adjacent non-tumor tissue samples as primary source to search for biomarkers, followed by immortalized cell lines and malignantly transformed counterparts [9, 10]. However, the greatest potential breakthrough will be through the identification of validated biomarkers, ideally in serum and plasma. The use of proteomics methods on peripheral blood plasma to rapidly profile protein markers has gained increasing interest [11]. Liquid chromatography methods such as high performance liquid chromatography and two-dimensional liquid chromatography have been increasingly employed for protein separation, and mass spectroscopy techniques such as matrix assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) have been used for proteins identification [12]. The surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS technique has been investigated to identify serum proteomic profiles of HSCC [13]. Differential in-gel electrophoresis (DIGE), which can co-detect numerous samples in the same two-dimensional gel (2DE) to minimize gel-to-gel variation and compare the protein features across different gels through an internal standard [14], has been widely used to search biomarkers for numerous cancers.

The aim of this study was to use a proteomics-based approach that involved immune-depletion of high-abundance proteins of plasma, DIGE analysis, and subsequent MALDI-TOF/TOF MS identification to search potential biomarkers for HSCC.

Materials and methods

Chemicals and reagents

Generic chemicals and albumin and IgG depletion kit were purchased from Sigma-Aldrich (USA). Reagents for 2D-DIGE were purchased from GE Healthcare (Sweden). All the chemicals and biochemicals used in this study were of analytical grade.

Plasma sample collection and purification

A total of 96 donors from one hospital (Nanfang Hospital, Southern Medical University, Guangdong, China) were enrolled in this study from Jan 2013 to Dec 2013. The subjects were divided into HSCC patients (n=48) and healthy donor group (n=48). Eight cases of early stage HSCC patients and eight cases of healthy donors were enrolled in proteomic and western blot analyses, whereas the other 40 cases of early stage HSCC patients and 40 cases healthy donors were enrolled in ELISA analyses. The criteria for assessing the presence of HSCC were based on the pathological diagnosis and guidelines proposed by the World Health Organization. Healthy individuals were selected with similar age and with no clinical HSCC diagnosis. This study was approved by the Institutional Research Board of Southern Medical University and carried out according to the Helsinki Declaration Principles. Written informed consent was collected from all participating subjects.

To improve the performance of proteomic analysis of the plasma samples, the albumin and immunoglobulin G in the collected plasma samples were depleted using an albumin and IgG removal kit (Sigma, USA) according to the instructions of the manufacturer. The depleted plasma samples were then precipitated with a 2D Clean-up Kit (GE Healthcare, Sweden) according to the protocol of the manufacturer, and then resuspended in Lysis Buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, at pH 8.5). Protein concentrations were determined by 2D Quant Kit (GE Healthcare, Sweden) according to the instructions of the manufacturer. The purified samples were then preserved at -80°C before DIGE.

2D-DIGE and gel image analysis

Before performing 2D-DIGE, plasma samples from 8 early stage HSCC patients and 8 healthy donors were pooled respectively. Approximately 50 μg of protein samples from patients and healthy donors were labeled with 400 pmol of either Cy3 or Cy5 for triplicate comparison on three 2DE gels. To facilitate image matching and cross-gel statistical comparison, 50 μg of pooled proteins (a pool of all samples) was also prepared and labeled with 400 pmol Cy2 as an internal standard for every gel. Thus, the triplicate samples and the internal standard can be run and quantified on multiple 2DE gels. At the same time, a preparative gel using 800 μg of pooled protein sample without labeling was prepared. The plasma sample arrangement for a triplicate 2D-DIGE experiment is shown in

Table 1. Plasma samples arrangement for a triplicate 2D-DIGE experiment

Gel No.	Cy2 (50 μg)	Cy3 (50 μg)	Cy5 (50 μg)
Gel 1	Pool	Healthy Donors	HSCC Patients
Gel 2	Pool	HSCC Patients	Healthy Donors
Gel 3	Pool	Healthy Donors	HSCC Patients

Table 1. The labeling reactions were performed in the dark on ice for 30 min, and then quenched with a 20-fold molar ratio excess of free L-lysine to dye for 10 min. The differentially Cy3- and Cy5-labeled samples were mixed with the Cy2-labeled internal standard and reduced with dithiothreitol for 10 min. The IPG buffer, pH 3-10 nonlinear [2% (v/v), GE Healthcare], was added, and the final volume was adjusted to 450 μL with 2D-lysis buffer for rehydration. The rehydration process was performed with immobilized non-linear pH gradient (IPG) strips (pH 3-10 NL, 24 cm), which were later rehydrated by CyDye-labeled samples in the dark at room temperature overnight (at least 12 h). Isoelectric focusing was then performed using an IPGphor III apparatus (GE Healthcare) for a total of 60 kVh at 20°C. Strips were equilibrated in 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, 100 mM Tris-HCl (pH 8.8), and 65 mM dithiothreitol for 15 min, and then in the same buffer containing 240 mM iodoacetamide for another 15 min. The equilibrated IPG strips were transferred onto 26×20 cm 12.5% polyacrylamide gels casted between low fluorescent glass plates. The strips were overlaid with 0.5% (w/v) low melting point agarose in a running buffer containing bromophenol blue. The gels were run in an Ettan™ DALT Six gel tank (GE Healthcare) at 3 Watt per gel at 10°C until the dye front had completely run off the bottom of the gels.

Afterward, the fluorescence 2DE gels were scanned directly between the low fluorescent glass plates using an Ettan™ DIGE Imager (GE Healthcare). This imager is a charge-coupled device-based instrument that enables scanning at different wavelengths for Cy2-, Cy3-, and Cy5-labeled samples. Gel analysis was performed using DeCyder 2D Differential Analysis Software v7.0 (GE Healthcare) to co-detect, normalize, and quantify the protein features in the images. Features detected from non-protein sources (e.g, dust particles and dirty backgrounds) were filtered out. Spots displaying 1.5 average-fold increase or decrease in abundance with a $P<0.05$ were selected for protein identification.

Protein staining and spot picking

The preparative gel was stained with colloidal Coomassie brilliant blue G-250. Bonded gels were fixed in 40% (v/v) ethanol, 10% (v/v) acetic acid for 1.5 h, washed three times (10 min each time) with ddH_2O, and then incubated in 20% (v/v) methanol, 10% (w/v) ammonium sulfate, 10% (v/v) phosphoric acid, and 0.12% w/v Coomassie brilliant blue G-250 for 5 to 7 days. No destaining step was required. The stained gels were then imaged on an Image Scanner III densitometer (GE Healthcare), which processed the gel images as TIF files. Matched spots of interest were picked automatically from the preparative gel by Ettan™ Spot Picker (GE Healthcare).

In-gel digestion and protein identification

The picked spots were destained with 50% acetonitrile (ACN)/100 mM NH_4HCO_3 for 10 min, dehydrated with 100% ACN for 10 min, and then dried using a centrifugal concentrator (TOMY SEIKO, Tokyo). Afterward, 2 μL of 25 ng/ml trypsin (Promega) diluted in 50 mM NH_4HCO_3 was added to each gel piece and incubated for 30 min at 4°C, and then 30 μL of 50 mM NH_4HCO_3 was added and the spots were incubated overnight at 37°C. Supernatants were collected, peptides were further extracted twice with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile for 15 min, and the supernatants were then pooled. Peptide extracts were vacuumdried and dissolved with 2 μL 50% acetonitrile/0.1% TFA, and aliquots of 0.5 μL were applied on the target and air-dried. Subsequently, 0.5 μL of matrix solution (CHCA saturated in 50% acetonitrile/0.1% TFA) was added to the dried samples and allowed to dry again.

Samples on the MALDI target plates were then analyzed by ABI 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems). A total of 800 shots were accumulated for MS analyses. MS/MS analyses were performed using air at collision energy of 2 KV. MASCOT search engine (version 2.1, Matrix Science) was used to search all of the tandem mass spectra. GPS Explorer™ software (version 3.6.2, Applied Biosystems) was used to create and search files with the MASCOT search for peptide and protein identification. Protein identities were obtained using Mascot searching engine against Swiss-Prot non-redundant sequence databases selected for human taxonomy.

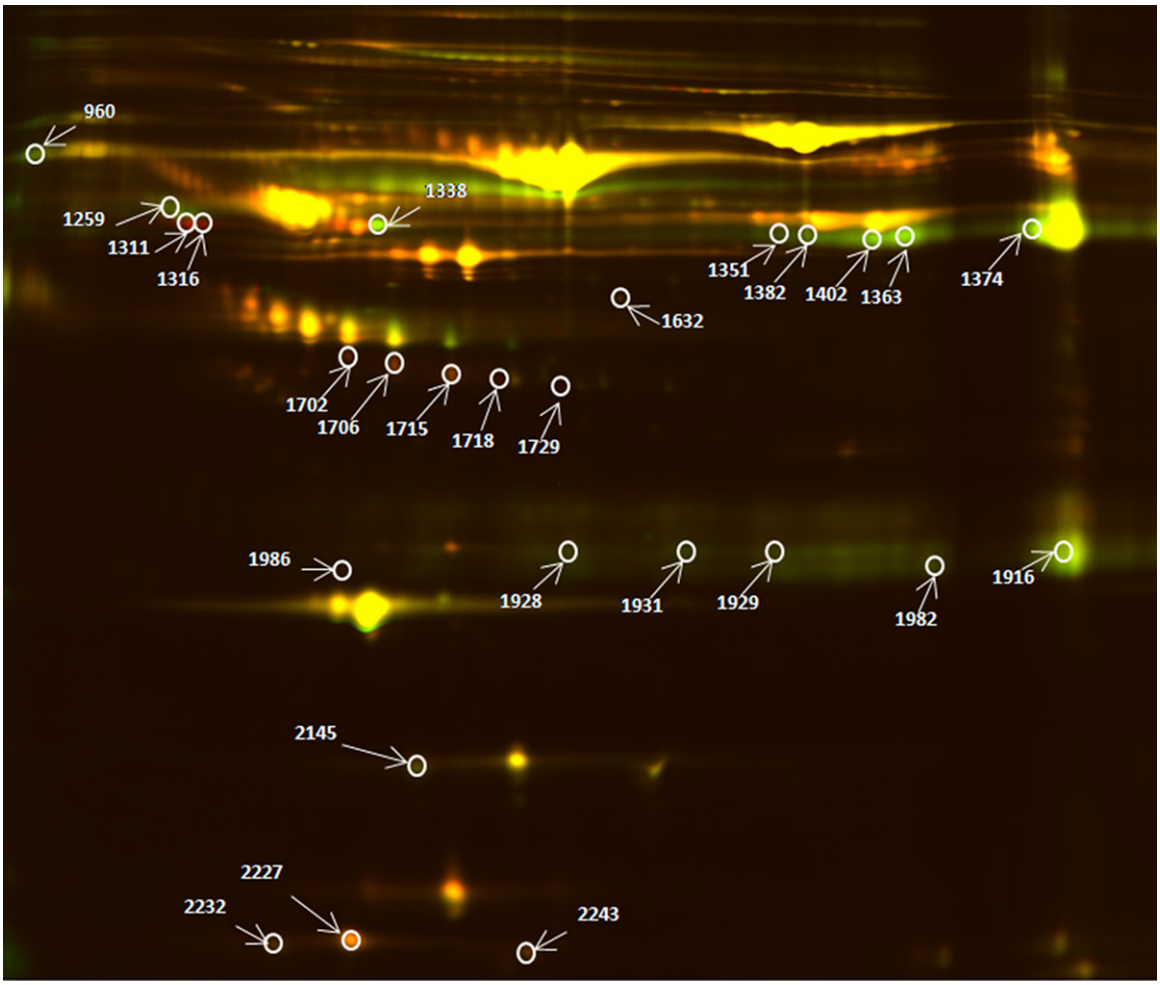


Figure 1. Location Map of Differentially Expressed Protein Spots between HSCC Patients and Healthy Donors. 2D-DIGE images of plasma samples were merged with Image Quant Tool (GE Healthcare). The 26 differentially expressed protein spots corresponded to those in **Table 2**.

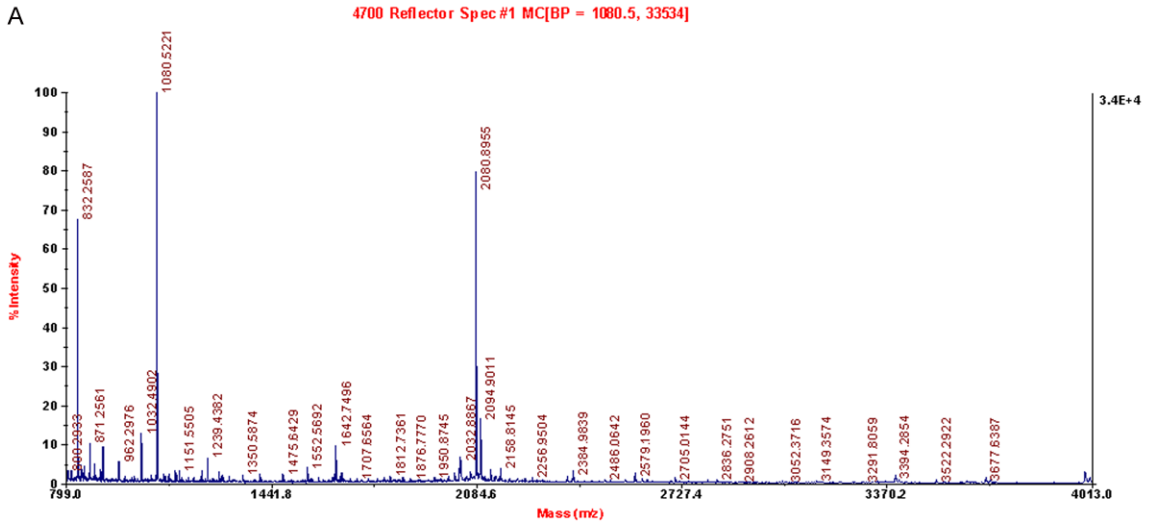
Western blot

Western blot was used to validate the differential abundance of identified proteins by mass spectrometry. Aliquots of 20 μg of plasma proteins were diluted in Laemmli sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue) and separated by SDS-PAGE following standard procedures. After electroblotting, the proteins were transfered onto 0.45 μm PVDF membranes (Millipore), and the membranes were blocked with 5% (w/v) skimmed milk in TBST (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween-20 (v/v)) for 1 h. Membranes were then incubated in primary antibody solution in TBST containing 0.02% (w/v) sodium azide for 2 h. Membranes were washed 3 times in TBST (5 min each time), and then incubated with the appropriate horseradish peroxidase-coupled secondary antibody for 1 h (Santa Cruz, USA). After washing 3 times with TBST, the reaction was detected by chemiluminescence with ECL reagents (Pierce Biotechnology, USA). All membranes were exposed and scanned by Carestream Image Station 4000R (Carestream Health, USA). A semi quantitative analysis based on OD was performed using Quantity One software (Bio-Rad, USA).

ELISA analysis

Polystyrene microtitration wells were coated with 50 μg of protein samples and incubated at 37°C for 2 h. The plate was washed for three times with phosphate buffered saline-Tween 20 (PBST) and three times with PBS. After the uncoated space was blocked with 100 μL of 5%

A



B

Matched peptides shown in **Bold Red**

1 MKSLVLLLCL AQLWGCHSAP HGPGLIYRQP NCDDPETEEA ALVAIDYINQ
51 NLPWGYKHTL NQIDEVKVWP QQPSGELFEI EIDTLETTCH VLDPTPVARC
101 SVRQLKEHAV EGDCDFQLLK LDGKFSVVYA KCDSSPDSAE DVRKVCQDCP
151 LLAPLNDTRV VHAAKAALAA FNAQNNGSNF QLEEISRAQL VPLPPSTYVE
201 FTVSGTDCVA KEATEAAKCN LLAEKQYGFC KATLSEKLGG AEVAVTCTVF
251 QTQPVTSQPQ PEGANEAVPT PVVDPDAPPS PPLGAPGLPP AGSPPDSHVL
301 LAAPPGHQLH RAHYDLRHTF MGVVSLGSPS GEVSHPRKTR TVVQPSVGAA
351 AGPVVPPCPG RIRHFKV

Figure 2. MALDI-TOF/TOF MS Analysis of Differential Protein Spot No. 1316. A. The Peptide Mass fingerprinting (PMF) of Spot No. 1316. B. The protein was identified as alpha-2-HS-glycoprotein (AHSG) according to the matched peptides which were shown in bold red.

skimmed milk in PBS at 37°C for 2 h, the plate was washed three times with PBST. Antibody solution was added and incubated at 37°C for 2 h. After washing with PBST and PBS for a total of 10 times, 100 μL of peroxidase-conjugated secondary antibody in PBS was added for incubation at 37°C for 2 h. After 10 washings, 100 μL of 3,3',5,5'-tetramethyl Benzedrine (Pierce) was added. After incubation at room temperature for 30 min, 100 μL of 1 M H_2SO_4 was added to stop the reaction, followed by measurement of absorbance at 450 nm using a Stat Fax 2100 Microtiter Plate Reader (Awareness Technology Inc., FL, USA).

Statistical analysis

Statistical features in DeCyder were used for the evaluation of the DIGE gels. For all protein spots, comparison between HSCC/Control groups was calculated, as change in volume ratios and with Student t-test as selection criteria. Protein spots differentially expressed between groups (at least 50% change of ratios between groups and t-test $P<0.05$) were extracted. For western blot bands and ELISA analyses, two-tailed non-paired Student's t-test was used to determine the mean differences between the two groups, and a $P<0.05$ was used to assess the significance of the differences using SPSS 13.0 software.

Results

Differentially expressed plasma proteins between HSCC patients and healthy donors

According to the DeCyder software analysis, about 1500 protein spots were constantly detected in each gel, which localized in the ranges of pI 3-10 and MW 10-200 kDa. On the basis of the average intensity ratios of protein spots, the spots were quantified, normalized, and inter-gel-matched. A total of 26 protein spots exhibited significant differences occurring between the HSCC patients and the healthy

Table 2. Differentially expressed plasma proteins in hscc patients identified by MALDI-TOF/TOF compared with healthy donors

Spot No.[a]	Protein name	Gene name	Accession No.[b]	MW/pI[c]	Ion score[d]	Fold change[e]
960	Serum albumin	ALB	P02768	71.3/5.92	85	-2.01
1259	Alpha-1-antichymotrypsin	SERPINA3	P01011	47.8/5.33	73	-1.70
1311	Alpha-2-HS-glycoprotein	AHSG	P02765	40.1/5.43	121	2.11
1316	Alpha-2-HS-glycoprotein	AHSG	P02765	40.1/5.43	145	4.23
1338	Angiotensinogen	SERPINA8	P01019	53.4/5.87	81	-1.73
1351	Ig gamma-3 chain C region	IGHG3	P01860	42.3/8.23	164	-1.97
1363	Fibrinogen gamma chain	FGG	P02679	52.1/5.37	103	-2.04
1374	Fibrinogen gamma chain	FGG	P02679	52.1/5.37	98	-2.74
1382	Fibrinogen gamma chain	FGG	P02679	52.1/5.37	215	-2.12
1402	Fibrinogen gamma chain	FGG	P02679	52.1/5.37	176	-1.94
1632	Complement C4-B	C4B	P0C0L5	194.2/6.73	139	1.57
1702	Haptoglobin	HP	P00738	45.9/6.13	157	1.76
1706	Haptoglobin	HP	P00738	45.9/6.13	83	1.88
1715	Haptoglobin	HP	P00738	45.9/6.13	124	1.88
1718	Haptoglobin	HP	P00738	45.9/6.13	115	1.99
1729	Haptoglobin	HP	P00738	45.9/6.13	146	2.16
1916	Apolipoprotein A-I	APOA1	P02647	30.8/5.56	256	-2.02
1928	Ig kappa chain C region	IGKC	P01834	11.8/5.58	88	-1.62
1929	Ig kappa chain C region	IGKC	P01834	11.8/5.58	132	-1.67
1931	Ig kappa chain C region	IGKC	P01834	11.8/5.58	117	-1.54
1982	Ig kappa chain C region	IGKC	P01834	11.8/5.58	122	-1.53
1986	C-reactive protein	CRP	P02741	25.2/5.45	94	1.83
2145	Ceruloplasmin	CP	P00450	122.9/5.44	82	3.08
2227	Haptoglobin	HP	P00738	45.9/6.13	103	1.62
2232	Haptoglobin	HP	P00738	45.9/6.13	258	1.71
2243	Haptoglobin	HP	P00738	45.9/6.13	172	1.68

[a]Spot No. is the unique sample spot protein number that refers to the labels in **Figure 1**; [b]Swiss-Prot database accession number; [c]Theoretical molecular weight (kDa) and theoretical pI; [d]Ion score of more than 66 is significant ($P<0.05$); [e]Fold change of the differentially expressed plasma proteins in HSCC patients compared with healthy donors.

honors, in which 13 protein spots were up-regulated and 13 down-regulated in HSCC patients compared to healthy controls. The differentially expressed protein figures that showed greater than 1.5-fold changes in the expression level were shown in **Figure 1** ($P<0.05$).

Identification of differentially expressed proteins by MALDI-TOF/TOF MS

To identify the differentially expressed proteins of HSCC plasma, all 26 differentially-expressed protein spots were excised from these 2-DE gels and subjected to in-gel trypsin digestion and subsequent analyzed by MALDI-TOF/TOF MS. A total of 26 proteins representing 12 unique gene products were successfully identified by MALDI-TOF/TOF MS and database search. **Figure 2** shows an example of a MALDI-TOF/TOF peptide mass fingerprint of protein spot No. 1316. The up-regulation proteins included alpha-2-HS-glycoprotein (AHSG), complement C4-B, haptoglobin, C-reactive protein, and ceruloplasmin, whereas the down-regulation proteins are serum albumin, angiotensinogen, alpha-1-antichymotrypsin, Ig gamma-3 chain C region, Ig kappa chain C region, fibrinogen gamma chain, and apolipoprotein A-I. Details of these proteins are listed in **Table 2**.

Validation of AHSG by western blotting

To validate the DIGE/MS-obtained results and further evaluate the nature and importance of some of the identified proteins, western blot analyses were performed. Among the varied proteins, AHSG was validated by western blot. As shown in **Figure 3**, AHSG was over-expressed

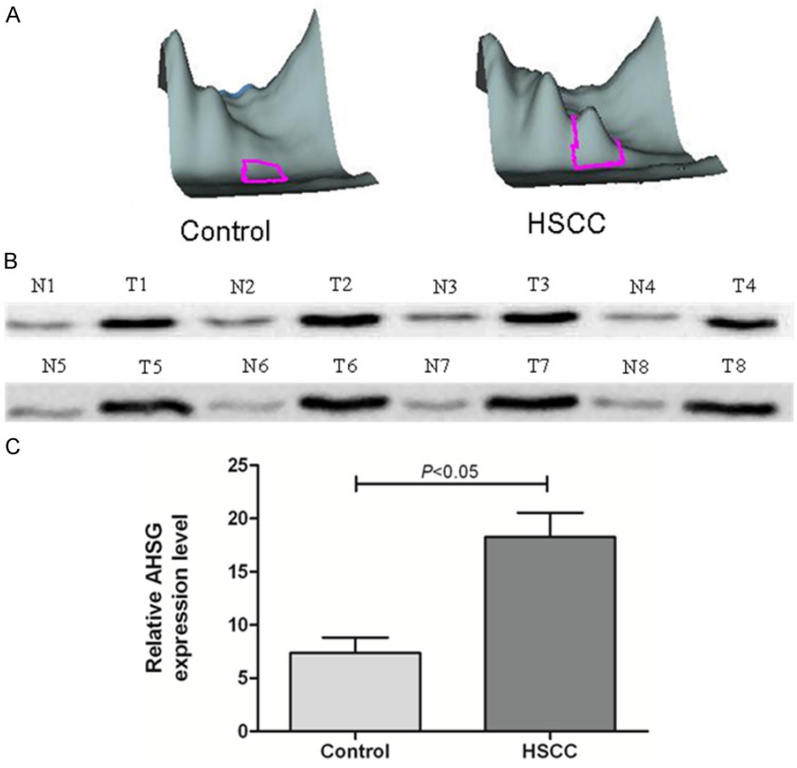


Figure 3. Western Blotting Analysis of AHSG between HSCC patients and healthy donors. A. Three-dimensional images of AHSG in HSCC and control groups. B. Up-regulation of AHSG in HSCC patients compared to healthy donors according to Western Blotting. N, normal donors; T, HSCC patients. C. The relative quantification from WB was shown in the bar graph, error bar revealed standard deviation. The two-tailed non-paired Student's t-test was performed using GraphPad Prism 5.0 (P<0.05). WB results were consistent with the data from 2D-DIGE.

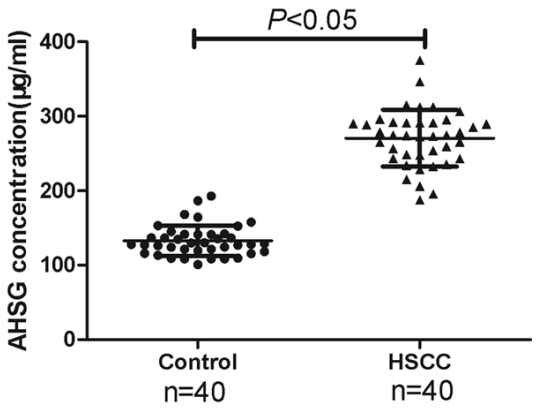


Figure 4. Verification of AHSG Expression Level between HSCC Patients and Healthy Donors by ELISA Analysis. The scatter plot represented AHSG protein expression variation. The two-tailed non-paired Student's t-test was performed using GraphPad Prism 5.0 (P<0.05). The plasma AHSG concentration of control group was 132.8±20.56 μg/ml, but 270.6±38.14 μg/ml in HSCC group. These ELISA results were consistent with the data from 2D-DIGE, which further suggested that AHSG may be employed as a potential biomarker for the early diagnosis of HSCC.

in HSCC patients compared with the controls. These data agreed with the expression changes shown by the DIGE analysis.

Validation of AHSG by ELISA

To verify the abundances of proteins identified from the results of 2D-DIGE and MALDI-TOF/TOF MS, the abundance level of AHSG was investigated by ELISA. AHSGwas significantly increased in the plasma of HSCC patients as shown in **Figure 4**. The plasma AHSG concentration of the control group was 132.8± 20.56 μg/ml, whereas that of HSCC group was 270.6± 38.14 μg/ml. These ELISA results are consistent with the data of the proteomics experiment, and further suggest that AHSG may be employed as a potential biomarker for the early diagnosis of HSCC.

Discussion

Although early diagnosis is established as a mainstay of successful HSCC treatment, no accepted screening test exists for this cancer. Thus, new biomarkers with high sensitivity and specificity to detect HSCC at an early stage are urgently needed. Differential proteomics have been widely used to identify all types of biomarkers in cancer research [15]. Liang Zhou *et al*. reported that a diagnostic pattern consisting of four protein peaks with m/z values of 7796, 4216, 5927, and 5361 Da was established with a sensitivity of 94% and a specificity of 89% by SELDI-TOF-MS [13].

To the best of our knowledge, no research using 2D-DIGE/MALDI-TOF/TOF to identify plasma biomarkers of HSCC has been reported yet. Therefore, this study was the first to utilize this platform to look for potential plasma biomarkers for HSCC. Our proteomics analysis revealed 26 differentially expressed protein spots (13

up-regulated and 13 down-regulated) corresponding to 12 unique plasma proteins. These proteins have not been reported as plasma biomarkers for HSCC in previous studies. Accordingly, the combination of these identified proteins may be further evaluated as HSCC specific biomarkers.

Up-regulated plasma proteins of HSCC

AHSG, also known as human fetuin-A, is a major serum glycoprotein synthesized and secreted by the liver. A number of studies have suggested that AHSG is a multifunctional protein involved in calcium homeostasis, bone development, and insulin sensitivity [16]. In addition, AHSG has the characteristics of a negative acute-phase protein, that is, the serum concentrations decrease significantly after major surgical procedures, trauma, burns, and severe inflammation [17]. Interestingly, AHSG can inhibit intestinal tumor progression by blocking TGF-β signal transduction [18]. In our results, AHSG was up-regulated in HSCC plasma, and validated by western blot and ELISA. Therefore, the up-regulation of plasma AHSG showed great potential diagnosis value in the identification HSCC from controls. The results suggested that AHSG may be a useful biomarker for HSCC.

Complement C4-B (C4B) is an isotype of complement C4. C4B functions more in the propagation of the activation pathways that lead to the formation of the membrane attack complex in attacking foreign antigens [19, 20]. Several studies, however, have reported that serum complement proteins may include efficient biomarkers related to cancers, such as ovarian cancer, breast cancer, and bladder cancer [21]. C4B was increased in our study, which might play an important role in the elimination of damaged tumor cells.

Haptoglobin (HP) is an acute phase protein synthesized by liver parenchymal cells, which is widely existing among serums and other body fluids in human and many other mammals, its main function is to combine the free hemoglobin so as to prevent the kidneys and vasculature from oxidative damage induced by free hemoglobin, and promote the cycle of heme iron [22]. Moreover, HP has important roles in the regulation of epidermal cell transformation, immune suppression in cancer, and angiogenesis. Studies have pointed out that HP has higher expressions in lung cancer, leukemia, breast cancer, prostate cancer, ovarian cancer, and gastric cancer [23-25], indicating that HP has potential clinical application value in tumor diagnosis. In this study, we found an increased change of HP between HSCC and controls. However, whether plasma HP can be a potential biomarker for screening HSCC requires further validations.

C-reactive protein (CRP), generally produced by hepatocytes, is an acute-phase protein, which will be increased in various inflammatory conditions [26]. CRP has been reported as a predictor of survival in different malignancies, including pancreatic, ovarian, pulmonary, and colorectal cancers [27]. The plasma level of CRP increased in the HSCC group, which suggested that CRP can be a predictor of HSCC.

Ceruloplasmin (CP) is the major copper-carrying protein in the blood and has a role in iron metabolism, exerting the copper-dependent activity of ferroxidase [28]. The Elevated levels of CP were identified in the serum and tissue of several malignancies and found associated with poor prognosis of cancers [29]. In our study, we found that CP expression was up-regulated in the plasma of HSCC, which was consistent to the previous reports.

Down-regulated plasma proteins of HSCC

Serum albumin (ALB), the main protein of plasma, is synthesized and secreted by the liver. ALB is generally used to assess the nutritional status, severity of disease, and disease progression and prognosis. In the hospital setting, many reports have related ALB level to in-hospital mortality, length of stay, and nosocomial infection. ALB has also been described as an independent prognosticator of survival in various cancers, such as lung, pancreatic, gastric, colorectal, and breast cancers. Low serum albumin has also been shown to be an independent indicator for prognosis in cancer patients with unknown primaries [30]. Our study revealed that ALB was down-regulated in HSCC plasma, indicating that malnutrition and inflammation of HSCC may suppress ALB synthesis.

Angiotensinogen (AGT), mainly synthesized in the liver, is the only known precursor of angio-

tensin I. Recombinant human AGT inhibits human endothelial cell proliferation, migration, and angiogenesis in vitro [31]. A study demonstrated that an adenovirus encoding human AGT could inhibit tumor progression through an antiangiogenic effect in different mice models and represent a promising new strategy to block primary tumor growth and prevent metastasis [32, 33]. We interpreted that the down-regulated AGT in HSCC patients may play an important role in tumor growth.

Alpha-1 antichymotrypsin (ACT), is a protease inhibitor thought to limit tissue damage produced by excessive inflammation-associated proteolysis [34]. In addition, ACT has been recognized as a biomarker for certain cancers including colorectal adenocarcinoma [35], breast cancer [36], and cervical carcinoma [37]. In our experiment, ACT was down-regulated in the HSCC plasma. Thus, we speculated that ACT may be as a specific biomarker for HSCC.

Ig gamma-3 chain C region (IGHG3) and Ig kappa chain C region (IGKC) belong to the major immunoglobulin class in body secretions. High blood Igs and circulating immune complex values in patients with cancer have been repeatedly reported and suggested as tumor markers with varying regulation patterns in numerous cancers [38]. In our study, however, the decreased plasma levels of IGHG3 and IGKC were contrary to the previous reports about other cancers, revealing their potential as biomarkers for HSCC.

Fibrinogen gamma chain (FGG) is one of the fibrinogen degradation products generated during fibrinolysis. FGG has a COOH-terminal globular domain to which several integrin cell adhesion receptors bind. In addition, fibrinogen and its gamma chain contain many specific binding sites and can regulate cellular adhesion and invasion. Recently, the correlation of fibrinogen and its degradation products with carcinogenesis was reported in oral squamous carcinoma [39]. From our current DIGE results, the down-regulated FGG plasma level might be related to tumor thrombosis, which was suggested to be a useful predictor for HSCC.

Apolipoprotein A-I is a component of the high density lipoprotein responsible for the cholesterol transport into the liver. In numerous cancers, such as bladder cancer, oesophageal cancer, cholangiocarcinoma, ovarian cancer, and colon cancer, the apolipoprotein A-I level is up-regulated [40]; however, the reduced plasma apolipoprotein A-I level is associated with gastric tumor [41]. Our 2D-DIGE analysis revealed that the apolipoprotein A-I level was down-regulated in HSCC plasma. These previous reports and our results revealed that the regulation of the apolipoprotein A-I level during tumorigenesis was complicated and needed further clarification.

In conclusion, this study adopted the quantitative proteomics approach to screen the plasma biomarkers for HSCC, and 12 potential biomarkers were identified, in which AHSG might be a putative biomarker that has potential to serve as a useful tool for HSCC diagnosing and monitoring. However, the potential of utilizing these markers for screening and treating of HSCC still needs much more further investigations.

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Disclosure of conflict of interest

None.

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References

[1] Gupta T, Chopra S, Agarwal JP, Laskar SG, D'Cruz AK, Shrivastava SK and Dinshaw KA. Squamous cell carcinoma of the hypopharynx: single-institution outcome analysis of a large cohort of patients treated with primary non-surgical approaches. Acta Oncol 2009; 48: 541-548.

[2] Hall SF, Groome PA, Irish J and O'Sullivan B. The natural history of patients with squamous cell carcinoma of the hypopharynx. Laryngoscope 2008; 118: 1362-1371.

[3] Wang JX, Zhang YY, Yu XM, Jin T and Pan XL. Role of centromere protein H and Ki67 in relapse-free survival of patients after primary surgery for hypopharyngeal cancer. Asian Pac J Cancer Prev 2012; 13: 821-825.
[4] Wycliffe ND, Grover RS, Kim PD and Simental A Jr. Hypopharyngeal cancer. Top Magn Reson Imaging 2007; 18: 243-258.
[5] Yu XM, Liu Y, Jin T, Liu J, Wang J, Ma C and Pan XL. The Expression of SIRT1 and DBC1 in Laryngeal and Hypopharyngeal Carcinomas. PLoS One 2013; 8: e66975.
[6] Wang J, Pan XL, Ding LJ, Liu DY, Da-Peng L and Jin T. Aberrant expression of Beclin-1 and LC3 correlates with poor prognosis of human hypopharyngeal squamous cell carcinoma. PLoS One 2013; 8: e69038.
[7] Zhao X, Ma C, Cai X, Lei D, Liu D, Xu F, Jin T, Liu J and Pan X. RNA interference of caveolin-1 via lentiviral vector inhibits growth of hypopharyngeal squamous cell carcinoma FaDu cells In Vitro and In Vivo. Asian Pac J Cancer Prev 2011; 12: 397-401.
[8] Zhou J, Yu X, Wang J, Li T, Jin T, Lei D and Pan X. Aberrant expression of PHLPP1 and PHLPP2 correlates with poor prognosis in patients with hypopharyngeal squamous cell carcinoma. PLoS One 2015; 10: e0119405.
[9] Pawar H, Kashyap MK, Sahasrabuddhe NA, Renuse S, Harsha HC, Kumar P, Sharma J, Kandasamy K, Marimuthu A, Nair B, Rajagopalan S, Maharudraiah J, Premalatha CS, Kumar KV, Vijayakumar M, Chaerkady R, Prasad TS, Kumar RV and Pandey A. Quantitative tissue proteomics of esophageal squamous cell carcinoma for novel biomarker discovery. Cancer Biol Ther 2011; 12: 510-522.
[10] Weinberger PM, Merkley M, Lee JR, Adam BL, Gourin CG, Podolsky RH, Haffty BG, Papadavid E, Sasaki C, Psyrri A and Dynan WS. Use of combination proteomic analysis to demonstrate molecular similarity of head and neck squamous cell carcinoma arising from different subsites. Arch Otolaryngol Head Neck Surg 2009; 135: 694-703.
[11] Smith L, Lind MJ, Welham KJ, Cawkwell L; Cancer Biology Proteomics Group. Cancer proteomics and its application to discovery of therapy response markers in human cancer. Cancer 2006; 107: 232-241.
[12] Colantonio DA and Chan DW. The clinical application of proteomics. Clin Chim Acta 2005; 357: 151-158.
[13] Zhou L, Cheng L, Tao L, Jia X, Lu Y and Liao P. Detection of hypopharyngeal squamous cell carcinoma using serum proteomics. Acta Otolaryngol 2006; 126: 853-860.
[14] Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S and Currie I. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 2003; 3: 36-44.
[15] Luo L, Dong LY, Yan QG, Cao SJ, Wen XT, Huang Y, Huang XB, Wu R and Ma XP. Research progress in applying proteomics technology to explore early diagnosis biomarkers of breast cancer, lung cancer and ovarian cancer. Asian Pac J Cancer Prev 2014; 15: 8529-8538.
[16] Dabrowska AM, Tarach JS, Wojtysiak-Duma B and Duma D. Fetuin-A (AHSG) and its usefulness in clinical practice-review of the literature. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2015; [Epub ahead of print].
[17] Kawakami T, Hoshida Y, Kanai F, Tanaka Y, Tateishi K, Ikenoue T, Obi S, Sato S, Teratani T, Shiina S, Kawabe T, Suzuki T, Hatano N, Taniguchi H and Omata M. Proteomic analysis of sera from hepatocellular carcinoma patients after radiofrequency ablation treatment. Proteomics 2005; 5: 4287-4295.
[18] Swallow CJ, Partridge EA, Macmillan JC, Tajirian T, DiGuglielmo GM, Hay K, Szweras M, Jahnen-Dechent W, Wrana JL, Redston M, Gallinger S and Dennis JW. alpha2HS-glycoprotein, an antagonist of transforming growth factor beta in vivo, inhibits intestinal tumor progression. Cancer Res 2004; 64: 6402-6409.
[19] Schifferli JA, Ng YC and Peters DK. The role of complement and its receptor in the elimination of immune complexes. N Engl J Med 1986; 315: 488-495.
[20] Gatenby PA, Barbosa JE and Lachmann PJ. Differences between C4A and C4B in the handling of immune complexes: the enhancement of CR1 binding is more important than the inhibition of immunoprecipitation. Clin Exp Immunol 1990; 79: 158-163.
[21] Liu W, Liu B, Xin L, Zhang Y, Chen X, Zhu Z and Lin Y. Down-regulated expression of complement factor I: a potential suppressive protein for gastric cancer identified by serum proteome analysis. Clin Chim Acta 2007; 377: 119-126.
[22] Wang B, He YJ, Tian YX, Yang RN, Zhu YR and Qiu H. Clinical utility of haptoglobin in combination with CEA, NSE and CYFRA21-1 for diagnosis of lung cancer. Asian Pac J Cancer Prev 2014; 15: 9611-9614.
[23] Pang X, Tashiro K, Eguchi R, Komatsu N, Sasada T, Itoh K and Kuhara S. Haptoglobin proved a prognostic biomarker in peripheral blood of patients with personalized peptide vaccinations for advanced castration-resistant prostate cancer. Biosci Biotechnol Biochem 2013; 77: 766-770.
[24] Kim YW, Bae SM, Kim IW, Liu HB, Bang HJ, Chaturvedi PK, Battogtokh G, Lim H and Ahn WS. Multiplexed bead-based immunoassay of

four serum biomarkers for diagnosis of ovarian cancer. Oncol Rep 2012; 28: 585-591.
[25] Penno MA, Klingler-Hoffmann M, Brazzatti JA, Boussioutas A, Putoczki T, Ernst M and Hoffmann P. 2D-DIGE analysis of sera from transgenic mouse models reveals novel candidate protein biomarkers for human gastric cancer. J Proteomics 2012; 77: 40-58.
[26] Mantovani A, Allavena P, Sica A and Balkwill F. Cancer-related inflammation. Nature 2008; 454: 436-444.
[27] Song ZB, Lin BC, Li B, He CX, Zhang BB, Shao L and Zhang YP. Preoperative elevation of serum C-reactive protein as an indicator of poor prognosis for early-stage esophageal squamous cell carcinoma. Kaohsiung J Med Sci 2013; 29: 662-666.
[28] Cairo G, Conte D, Bianchi L, Fraquelli M and Recalcati S. Reduced serum ceruloplasmin levels in hereditary haemochromatosis. Br J Haematol 2001; 114: 226-229.
[29] Doustjalali SR, Yusof R, Govindasamy GK, Bustam AZ, Pillay B and Hashim OH. Patients with nasopharyngeal carcinoma demonstrate enhanced serum and tissue ceruloplasmin expression. J Med Invest 2006; 53: 20-28.
[30] Gupta D and Lis CG. Pretreatment serum albumin as a predictor of cancer survival: a systematic review of the epidemiological literature. Nutr J 2010; 9: 69.
[31] Celerier J, Cruz A, Lamande N, Gasc JM and Corvol P. Angiotensinogen and its cleaved derivatives inhibit angiogenesis. Hypertension 2002; 39: 224-228.
[32] Bouquet C, Lamande N, Brand M, Gasc JM, Jullienne B, Faure G, Griscelli F, Opolon P, Connault E, Perricaudet M and Corvol P. Suppression of angiogenesis, tumor growth, and metastasis by adenovirus-mediated gene transfer of human angiotensinogen. Mol Ther 2006; 14: 175-182.
[33] Vincent F, Bonnin P, Clemessy M, Contreres JO, Lamande N, Gasc JM, Vilar J, Hainaud P, Tobelem G, Corvol P and Dupuy E. Angiotensinogen delays angiogenesis and tumor growth of hepatocarcinoma in transgenic mice. Cancer Res 2009; 69: 2853-2860.
[34] Santamaria M, Pardo-Saganta A, Alvarez-Asiain L, Di Scala M, Qian C, Prieto J and Avila MA. Nuclear alpha1-antichymotrypsin promotes chromatin condensation and inhibits proliferation of human hepatocellular carcinoma cells. Gastroenterology 2013; 144: 818-828 e814.
[35] Dimberg J, Strom K, Lofgren S, Zar N, Hugander A and Matussek A. Expression of the serine protease inhibitor serpinA3 in human colorectal adenocarcinomas. Oncol Lett 2011; 2: 413-418.
[36] Sano H, Wada S, Eguchi H, Osaki A, Saeki T and Nishiyama M. Quantitative prediction of tumor response to neoadjuvant chemotherapy in breast cancer: novel marker genes and prediction model using the expression levels. Breast Cancer 2012; 19: 37-45.
[37] Kloth JN, Gorter A, Fleuren GJ, Oosting J, Uljee S, ter Haar N, Dreef EJ, Kenter GG and Jordanova ES. Elevated expression of SerpinA1 and SerpinA3 in HLA-positive cervical carcinoma. J Pathol 2008; 215: 222-230.
[38] Choi JW, Liu H, Shin DH, Yu GI, Hwang JS, Kim ES and Yun JW. Proteomic and cytokine plasma biomarkers for predicting progression from colorectal adenoma to carcinoma in human patients. Proteomics 2013; 13: 2361-2374.
[39] Tung CL, Lin ST, Chou HC, Chen YW, Lin HC, Huang KJ, Chen YJ, Lee YR and Chan HL. Proteomics-based identification of plasma biomarkers in oral squamous cell carcinoma. J Pharm Biomed Anal 2013; 75: 7-17.
[40] Van Hemelrijck M, Walldius G, Jungner I, Hammar N, Garmo H, Binda E, Hayday A, Lambe M and Holmberg L. Low levels of apolipoprotein A-I and HDL are associated with risk of prostate cancer in the Swedish AMORIS study. Cancer Causes Control 2011; 22: 1011-1019.
[41] Chong PK, Lee H, Zhou J, Liu SC, Loh MC, So JB, Lim KH, Yeoh KG and Lim YP. Reduced plasma APOA1 level is associated with gastric tumor growth in MKN45 mouse xenograft model. J Proteomics 2010; 73: 1632-1640.