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Haptoglobin α-Subunit and Hepatocyte Growth Factor can Potentially Serve as Serum Tumor Biomarkers in Small Cell Lung Cancer

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Abstract. *Background: Small cell lung cancer (SCLC) frequently presents as metastatic disease. It would be useful to detect serum tumor biomarkers at an earlier stage in order to improve the overall survival. Materials and Methods: Serum samples from SCLC patients (6 limited disease , 7 extensive disease, 4 relapsed disease, 4 no evidence of disease post-treatment) and 5 normal controls were used to identify tumor biomarkers utilizing proteomics. Serum hepatocyte growth factor was also studied using standard ELISA. Results: Utilizing MALDI-TOF-Mass Spectrometry (MS) based protein identification techniques, a SCLC specific overexpressed protein was identified to be haptoglobin α-subunit, with its serum level correlating with the disease stage. The mean level of α-haptoglobin was increased in SCLC serum as compared to the normal controls. Serum HGF was also studied as potential tumor biomarker and was found to correlate with the disease status. Either serum α-haptoglobin relative level (above 1.9 U), or HGF level (above 500 pg/ml) was associated with a trend towards worse survival. Conclusion: Our current findings suggest that serum levels of α-haptoglobin and HGF may serve as useful serum tumor biomarkers in SCLC. It would now also be useful to determine if these serum biomarkers are altered in response to therapy for this disease.*

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In the United States, there will be greater than 171,900 projected new cases of lung cancer in 2003. It is estimated that there will be 157,200 deaths related to lung cancer for this year (1). The most aggressive form of lung cancer is small cell lung cancer (SCLC), which has a very poor prognosis. SCLC is a small blue cell tumor with a propensity for early metastasis. SCLC occurs as limited disease (LD) approximately 1/3 of the time and as extensive disease (ED) approximately 2/3 of the time. SCLC is invariably associated with smoking. There are a number of genetic alterations that can occur within SCLC – such as alteration and loss of chromosome 3p, p53 mutations, Rb deletions/mutations and overexpression of oncogenes such as myc or Bcl-2 (2). Even though there are extensive genetic changes, we need to be able to understand proteins that are involved in the pathogenesis and the clinical biology of SCLC. Specifically, we should also be able to discern the proteins that are abnormally found in the serum that can serve as tumor biomarkers for SCLC. Finding serum tumor biomarkers that are specific for SCLC would expedite the diagnosis and improve therapy and monitoring of the disease (3). Recent technological advancements in proteomics should help us determine circulating tumor biomarkers for any cancers.

Traditionally, several tumor biomarkers, such as CEA, CA125, CFRA1, neuron-specific enolase (NSE), chromogranin A and others have been utilized, however they may not be specific and/or sensitive in SCLC (3). Serum tumor biomarkers in lung cancer have long been studied in hope of allowing early detection of the disease in asymptomatic individuals, improving diagnosis, as well as in monitoring recurrence after treatments (4). Nonetheless, their clinical usefulness remains limited by the largely retrospective studies and often inconclusive data (5). The

proteomics approach utilizing mass spectroscopy to identify low abundance, differentially expressed proteins is being widely utilized in a variety of disease states (6, 7). Also, with the advent of the hybrid mass spectrometer and automated sample preparation, it is possible to handle a large number of samples in a significantly shorter time; thereby, making the identification of serum tumor biomarkers easier.

In this report, we have identified haptoglobin α-subunit using mass spectrometry based techniques and show that the relative levels of the protein correlated with the stage of SCLC. Also, through our global approach in identifying tumor biomarkers, we have also evaluated the levels of the cytokine hepatocyte growth factor (HGF), the ligand for c-MET, and found that HGF has a potential role as SCLC tumor biomarker.

Materials and Methods

Serum processing. Serum from patients with SCLC or controls were obtained with institutional IRB approved informed consent. Albumin removal kit (Genomic Solutions) was used to remove albumin from serum. The samples were initially diluted with dilution buffer (according to the manufacturer's directions) and protease inhibitors were added to reduce degradation of proteins. Utilizing this method, approximately 60-70% of albumin was removed.

SDS-PAGE. Once albumin was removed, acetone was added to the serum sample and incubated at -20˚C for 2 hours for protein precipitation. The precipitates were recovered by centrifugation and reconstituted in phosphate buffer. Protein concentration was determined by the Bradford method and equal amounts of protein (40 μg) were processed on SDS-PAGE gel. The gel was then stained with silver stain using Silver Stain Plus Kit (Bio-Rad, CA, USA).

Sample preparation for analysis by MALDI-TOF-MS. Differentially expressed proteins from the silver-stained gel were excised and processed for in-gel digestion as described (8, 9). Briefly, the gels were cut into small but uniform pieces. The gel pieces were dehydrated by acetonitrile and then rehydrated by 100 mM ammonium bicarbonate. To protect peptides from oxidation, DTT was added in 100 mM ammonium bicarbonate and incubated at 56˚C for one hour. For protecting the amino terminus blocking, the gel was then treated with 10 mM iodoacetamide in 100 mM ammonium bicarbonate. The gel pieces were washed with ammonium bicarbonate and dried by acetonitrile. These last two steps were repeated twice. After completely dehydrating with acetonitrile, the gel pieces were suspended in 12.5 ng/μl trypsin in 50 mM ammonium bicarbonate. The in-gel digestion was carried out at 37˚C for 10-12 hours. The peptides were extracted from the gel in 50% acetonitrile and 5% formic acid. The extract was concentrated under reduced pressure and finally desalted by Zip-Tip (Millipore, MA, USA). The C-18 bound peptides were eluted and plated on MALDI-plate with α-cyano-4-hydroxycinnamic acid (alpha cyanol) in 70% acetonitrile. Peptides were analyzed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using a Voyager DE-PRO (Perceptive Biosystem Inc, Framingham, MA, USA). The proteins were identified by mass fingerprinting using Matrix Science and Protein Prospector (MS-Fit) database. Selected peptides were further analyzed by sequencing using post source decay (PSD).

Immunoblotting with α-haptoglobin. Utilizing serum samples from SCLC patients and controls, we processed proteins for immunoblotting (10). Anti-haptoglobin antibody that recognizes the α-subunit of haptoglobin was utilized as the primary antibody at 1:1000 dilution (Sigma). Densitometric scans were performed for signal quantitation with statistical analysis performed using two-tailed Student's *t*-test.

ELISA assay for HGF. ELISA assay for HGF was performed using the Quantikine human HGF ELISA Kits (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Recombinant human HGF was used for standard calibrations.

Statistical analysis. Student's *t*-test was used in the analysis of the serum levels of α-haptoglobin and HGF. For these analyses, the differences observed between various patient subgroups are considered to be statistically significant at a two-tailed nominal p value of less than 0.05. Log rank analysis was performed for the Kaplan-Meier survival analysis and p values less than 0.05 are considered statistically significant.

Results

Identification of novel serum tumor biomarkers through SDS-PAGE and MALDI-TOF-Mass spectrometry. In order to identify any proteins that may be elevated in SCLC patients' serum, we utilized SDS-PAGE gels and determined if any proteins are differentially expressed in SCLC as compared to the controls. There was a high expression of approximately 35-40 kDa protein in serum samples from LD and ED SCLC. This was specific to SCLC and not controls. The protein was identified as α-haptoglobin by MALDI-TOF-MS analysis, both by mass fingerprinting followed by PSD (post source decay) analysis to sequence selected peptides (Figure 1).

Through gel excision of the protein band and identification on MALDI-TOF-MS as described above, we identified this to be haptoglobin α-subunit (Figure 1). Utilizing antibody against α-haptoglobin, we further show that α-haptoglobin was expressed three-fold higher in serum of patients with SCLC (mean relative level of α-haptoglobin 2.98±0.47) than that observed in the normal controls (mean relative level 1.0±0.31), and was 2.06-fold higher than in patients with no evidence of disease post-therapy (mean relative level 1.45±0.32). The levels of α-haptoglobin in serum from patients with LD-SCLC and ED-SCLC were 2.25±0.69 ($p<0.005$) and 3.61±0.57 ($p<0.001$) respectively, both highly statistically significant when compared to the healthy subject control (Figures 2 and 3). Also, mean serum level of α-haptoglobin was 1.61-fold higher in the ED-SCLC serum than the LD-SCLC serum.

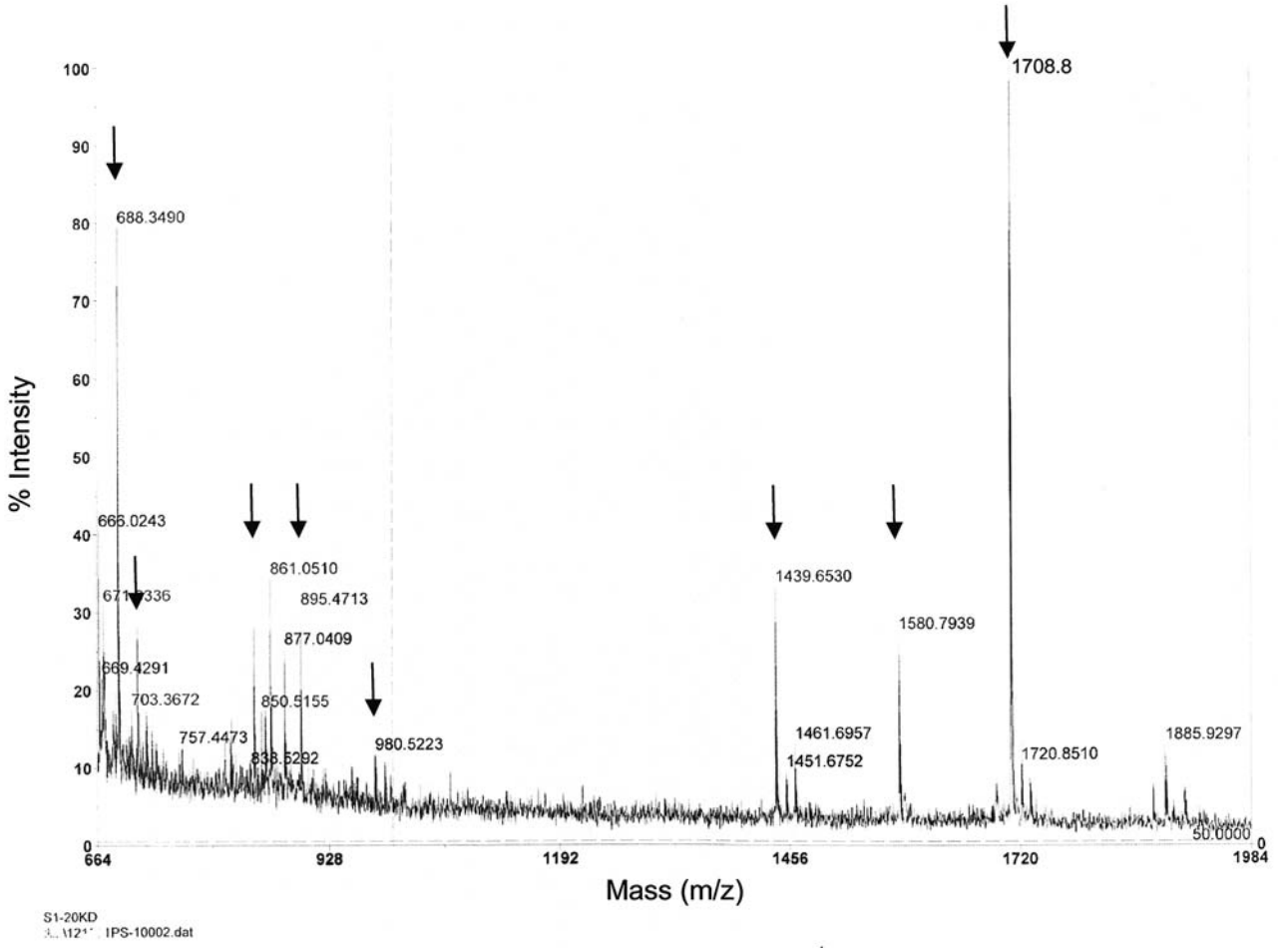


Figure 1. *Identification of α-haptoglobin by MALDI-TOF-MS analysis. Mass spectrum generated by the 40 kDa protein after in-gel trypsin digestion was identified as human α-haptoglobin by mass fingerprinting. The arrow indicates the tryptic peptides representing α-haptoglobin. Matrix Science (Mascot Search) and MS-Fit search both confirmed this as α-haptoglobin with very high probability. Arrows at the mass spectrum peaks (688.34; 703.3; 816.4; 895.4; 980.5; 1439.6; 1580.7; 1708.8) represent peptides from α-haptoglobin.*

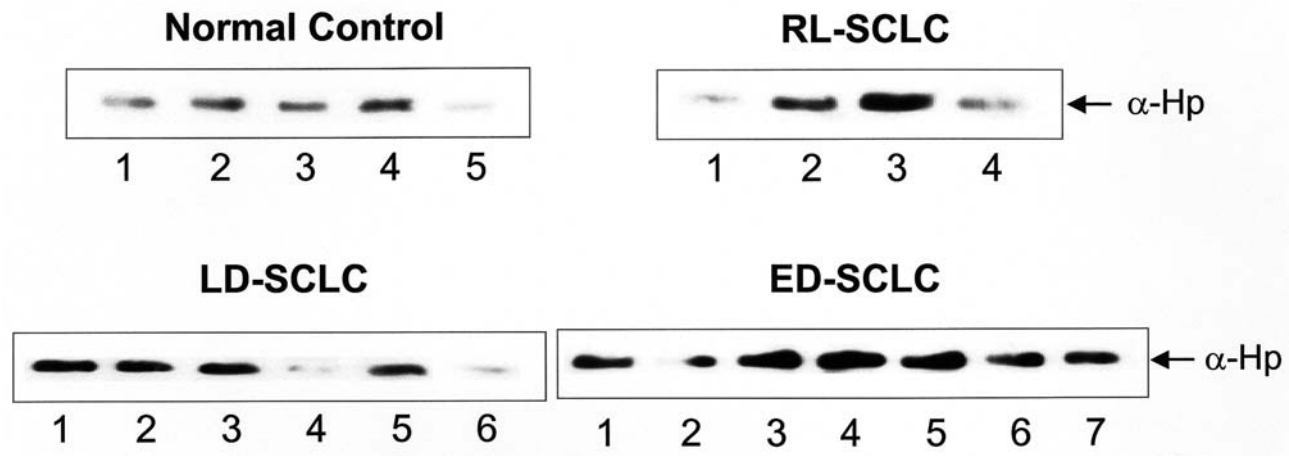


Figure 2. *Immunoblotting of SCLC patient serum samples against α-haptoglobin. Serum samples from patients with SCLC were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane for immunoblotting using an antibody that recognizes α-haptoglobin. Serum samples from normal subjects were used as controls.*

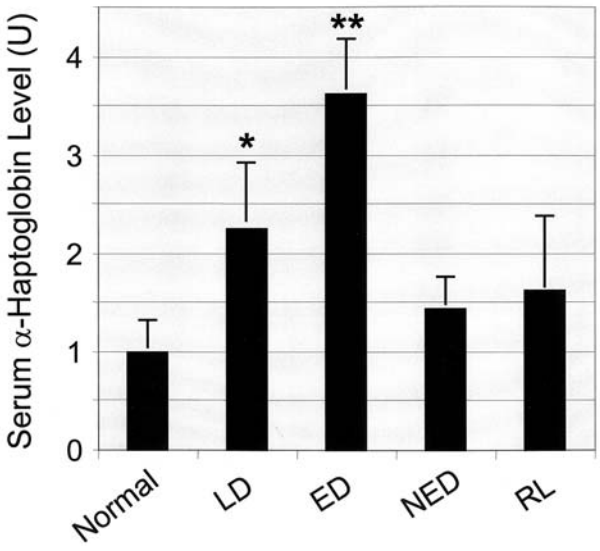


Figure 3. *Elevated serum levels of α-haptoglobin in SCLC patients. The relative serum levels of α-haptoglobin in SCLC patient serum samples were determined by quantitative densitometric scanning of the immunoblots as shown in Figure 2. There was higher level of mean serum α-haptoglobin in the serum samples from extensive stage SCLC (ED) than limited stage SCLC (LD) (p<0.005). The mean serum levels of α-haptoglobin from patients with active disease (both ED and LD) were also higher than that either of the normal subjects (ED, p=0.005; and LD, p=0.001) or of the patients with no evidence of disease post-therapy (NED). Error bars represent the S.E.M.*

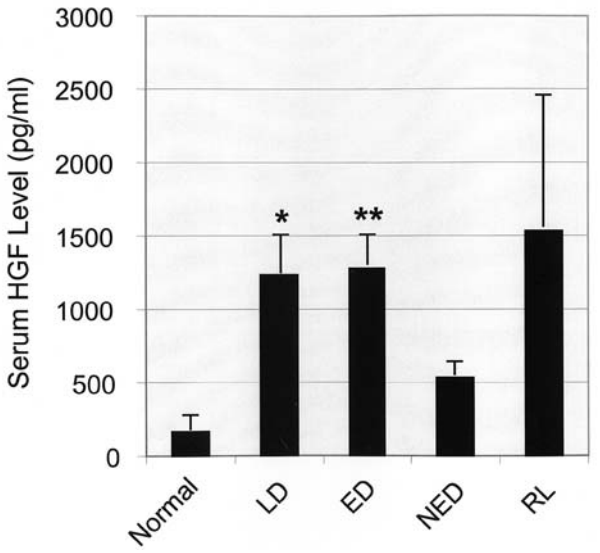
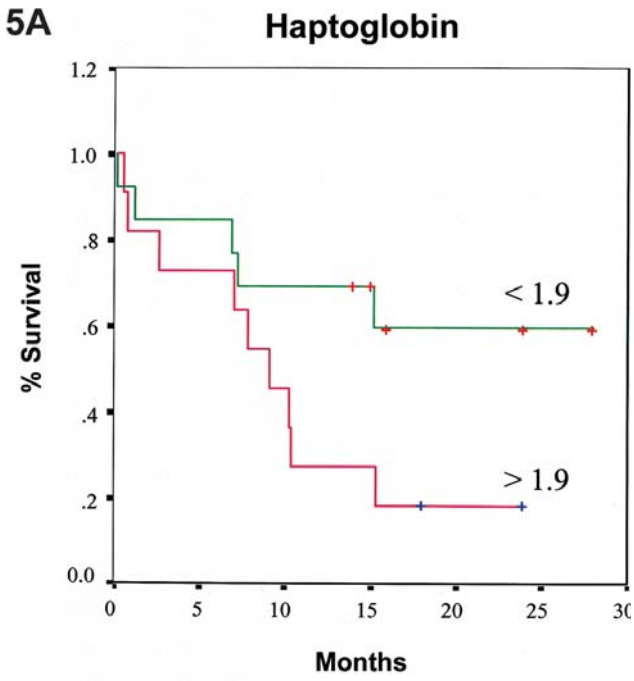


Figure 4. *Serum levels of HGF are higher in SCLC patients. The serum levels of HGF in SCLC patient serum samples were determined by quantitative HGF ELISA (R & D Systems, MN, USA) according to the manufacturer's instructions. There were comparable levels of mean serum HGF between the serum samples from extensive stage SCLC (ED) and from limited stage SCLC (LD). The mean serum levels of haptoglobin from patients with active disease (both ED and LD) were also higher than that of either the normal subjects (ED, p= 0.002; and LD, p=0.010) or of the patients with no evidence of disease post-therapy (NED) (ED, p<0.020; LD, p=0.054). Error bars represent the S.E.M.*



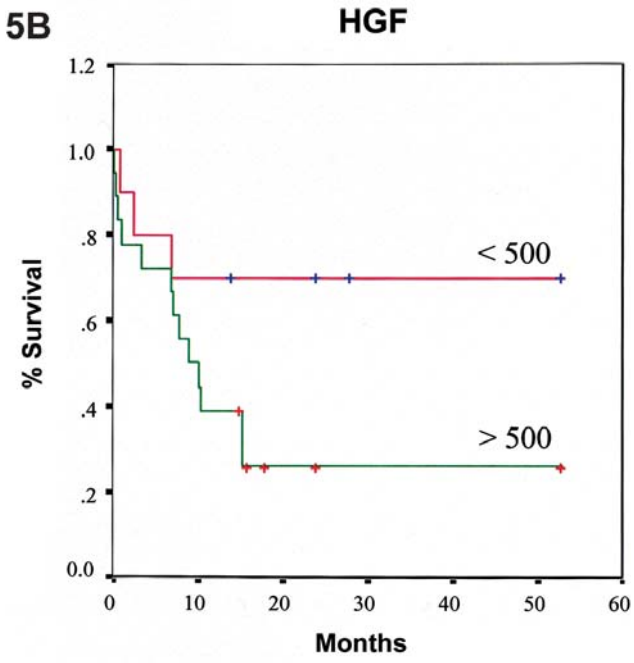


Figure 5. *Kaplan Meier survival analyses. Kaplan-Meier analyses of survival according to serum α-haptoglobin level (A), as well as HGF level (B) are shown. For α-haptoglobin, two groups of subjects were defined using the cut-off relative α-haptoglobin level of 1.9. For the analysis using serum HGF level, the cut-off HGF level was 500 pg/ml. Statistical tests were performed using log-rank statistics.*

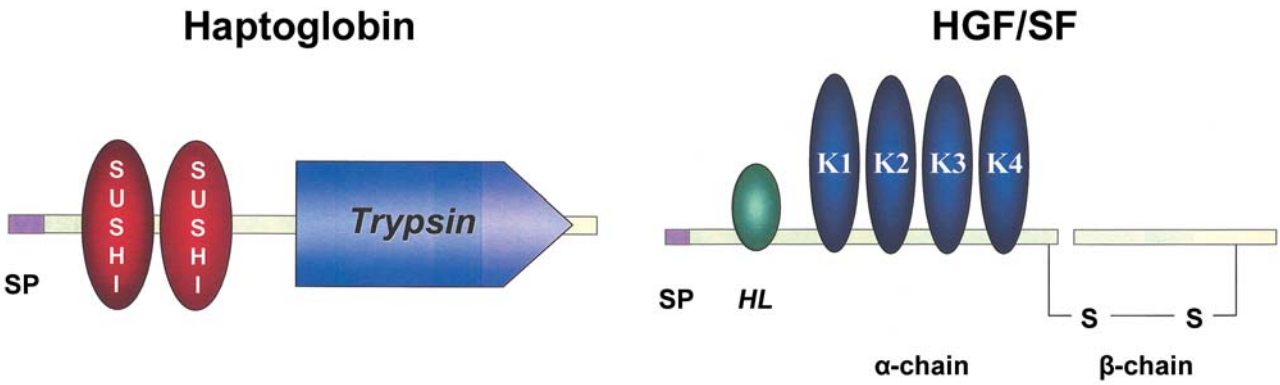


Figure 6. *Predicted functional domains of haptoglobin and HGF/SF. A, Haptoglobin has a gene map locus 16q22.1 and contains two "Sushi" domains from amino acid (a.a.) residues 33-86, and from 92-145; followed by a Trypsin domain from a.a. 161-399. It has protein-protein interactions to CD163, SIGLEC2, integrin β2, hemoglobin β-chain, CD11b and interleukin-6. SP, signal peptide. B, HGF/SF maps to the gene locus 7q21.1 and contains an N-terminal hairpin-loop domain (HL) consisting of about 27 amino acids followed by four canonical kringle domains (K1-K4), which are 80-amino-acid double-looped structures stabilized by three internal disulfide bridges. Kringle domains are important for protein-protein interaction. The first kringle domain contains the high-affinity binding domain for the HGF receptor (c-Met) while the hairpin loop and second kringle domain form a low-affinity binding site for membrane-associated heparan sulfate proteoglycans. The mature HGF heterodimer is formed by proteolytic cleavage at a specific dibasic arginine-valine (R-V) site, giving rise to the α- and β-chains linked by a disulfide bond. SP, signal peptide.*

Serum expression levels of HGF in SCLC. Also, as a general proteomic approach toward identification of serum proteins in SCLC, we have determined the levels of the cytokine HGF. We have previously shown that c-Met (receptor for HGF) is expressed, functional and sometimes mutated in SCLC (11). Based on the receptor expression profiles, we determined if the respective ligand is detectable in the serum of patients with SCLC. The levels of serum HGF in the various patient samples are shown in Figure 4. It is appreciated that the mean serum levels of HGF was higher in SCLC (ED > LD) as compared to controls.

The mean HGF level of healthy subject controls and the NED patients were 168.11±108.14 pg/ml, and 533.29±112.54 pg/ml respectively; while that of the LD-SCLC patients is 1237.67±277.57 pg/ml and that of the ED-SCLC patients is 1278.14±232.53 pg/ml. When compared to the healthy subjects, the increase of the HGF levels seen in both the LD-SCLC and ED-SCLC patients are highly statistically significant ($p=0.010$ and $p=0.002$, respectively) (Figure 4). Similar observations were made when compared to the NED patients (LD-SCLC, $p<0.054$; ED-SCLC, $p<0.020$) (Figure 4).

Prognostic values of α-haptoglobin and HGF levels in SCLC. Kaplan-Meier analyses of survival according to serum α-haptoglobin levels as well as HGF levels are shown in Figure 5A and 5B. For α-haptoglobin, the two groups of subjects were defined as having relative α-haptoglobin level either > 1.9 or < 1.9. As shown in Figure 5A, a tendency of increased survival was observed in the group with α-haptoglobin < 1.9, although it did not reach statistical significance (log rank statistics $p=0.069$). Similarly, a difference in survival was observed between the two groups of subjects with serum HGF levels < 500 pg/ml or > 500 pg/ml (Figure 5B). Log-rank statistics, in this case, also did not reach statistic significance ($p=0.074$).

Discussion

In this pilot study of potential serum biomarkers for SCLC, we have identified both α-haptoglobin as well as HGF as useful candidate biomarkers for this disease. SCLC is an aggressive cancer that is marked by a poor overall prognosis despite current standard cytotoxic chemotherapy (2). Despite the fact that patients with SCLC often show initial response to chemotherapy, most of them would eventually succumb to the disease with relapse relatively soon (12). Moreover, many patients present with metastatic disease in SCLC. Hence, identification of useful serum tumor biomarkers for SCLC may be clinically useful in early diagnosis, monitoring of progression, prognostication as well as prediction of treatment response.

Haptoglobin is an acute phase reactant tetrameric ($\alpha_2\beta_2$) glycoprotein that functions as a hemoglobin-scavenger following hemolysis, thereby preventing iron loss, renal damage and tissue destruction (13). This protein has been known for long and, until recently, its potential role as a tumor biomarker was not well recognized. Javid (1967) described a genetic variant of the haptoglobin β-polypeptide chain and proposed the locus to be called Bp, for "binding

peptide", since it binds to hemoglobin; while the longer known locus of α-chain to be called Hp (14, 15). Haptoglobin is known to have at least 3 major haptoglobin phenotypes, Hp(1-1), Hp(2-1) and Hp(2-2), which are each associated with a variety of medical illnesses (13). Its serum level has also been shown to be decreased, with statistical significance, among other serum hormones and proteins, by the use of adjuvant tamoxifen therapy in breast cancer patients (16). Since haptoglobin is also an anti-oxidant, haptoglobin-hemoglobin complexes formed in the serum of patients may serve to neutralize superoxidative products (13). In addition, haptoglobin contains two "Sushi" domains in the N-terminus (Figure 6A), which is based on a structure of a β-sandwich arrangement, existing in a wide variety of complement and adhesion proteins such as CD21 (Epstein Barr virus receptor). A Sushi domain is a characteristic domain composed of two staggered disulfide bridges, first identified in the plasma membrane β2 glycoprotein I (β2-GPI) (17). It has also been found in many proteins involved in mammalian complement systems (18), as well as in cell-cell adhesion proteins of the invertebrate cellular slime mold, *Polysphondylium pallidum* (19). Interestingly, haptoglobin has also been known to interact with a number of proteins, including integrin β2 and SLGLEC2 (Sialic acid binding immunoglobulin like lectin 2; also named as B-cell antigen CD22), both are adhesion molecules participating in tumor biology modulations and immunity (see Human Protein Reference Database, website: www.hprd.org). There has been a report showing secretion by human lung cancer lines of a family of high Mr plasminogen activators (PAs) which are disulfide-bonded multiprotein oligomeric complexes, functionally and immunologically related to the human urinary PA (uPA). Further analysis showed that the covalent oligomeric complexes contain also neural cell adhesion molecule (NCAM) and both the α- and β-chains of haptoglobin. These oligomers may have a selective advantage for such cancer cells in the focalization of proteolytic activity through the interaction of the NCAM domain with the extracellular matrix (ECM) and in immunosuppression of lymphocytes by the haptoglobin portion of the complex (20).

Utilizing ProteinChip arrays and a combination of Surface Enhanced Laser Desorption/Ionization (SELDI)-Mass Spectrometry (MS) and Liquid Chromatography (LC), serum α-haptoglobin (Hp-α) has recently been identified as a more sensitive biomarker than total haptoglobin for ovarian cancer early detection (21). ELISA assay indicated that haptoglobin α-subunit was ≤ 2-fold higher in ovarian cancer serum compared to normal, benign tumor and other gynecological cancers ($p<0.05$). Also, Hp-α alone has the sensitivity and specificity reported to be 64% and 90%, respectively, in 80 ovarian cancer cases and 91 normal controls in the ELISA screening test using the purified polyclonal antibody against haptoglobin α-1. When combined with CA125, the sensitivity was increased to 91% and specificity to 95% (21). In the present study, we demonstrated that the serum α-haptoglobin level correlated with the disease status and disease stage of SCLC and has potential utility in prognostication. However, the mechanism of action of this biomarker in SCLC patient's serum remains to be elucidated.

Hepatocyte growth factor/scatter factor (HGF/SF) is a heterodimer composed of a 4-kringle-containing 62 kDa α-chain linked to a serine protease-like 32/34 kDa β-chain by a disulfide bond (Figure 6B). HGF was first identified as a molecule that stimulates hepatocyte proliferation (22) and later found to be identical as the scatter factor (SF), a fibroblast-secreted protein that promotes motility and matrix invasion of epithelial cells (23). HGF is the natural ligand for the transmembrane receptor tyrosine kinase c-MET, which stimulates the invasive growth of carcinoma cells, is tumorigenic and overexpressed in many solid tumor cells including SCLC (24, 25). Clinical studies have found an association between the level of HGF in serum or tumor tissues and the progression of disease in various cancer types, including gastric (26), breast (27, 28), myeloma (29), bladder (30), synovial sarcoma (31) and oral squamous cell carcinoma (32). Moreover, an elevated tumor tissue level of HGF has been implicated in more aggressive biology with poor prognosis in NSCLC (33, 34). Here we demonstrated that HGF is correlated with the disease status of SCLC patients also allowing for potential use as a novel tumor biomarker in this aggressive disease.

Our current findings suggest that the serum level of α-haptoglobin and HGF can serve to facilitate diagnosis of SCLC patients. α-haptoglobin may potentially be helpful in staging of SCLC patients at the time of diagnosis as well. Furthermore, both α-haptoglobin and HGF may also be clinically useful in monitoring response to therapy and recurrence. Since most of the SCLC patients with initial response to chemotherapy would ultimately relapse and succumb to the disease within two years, better novel therapy for the disease need to be developed urgently. In addition, improved serum biomarkers for disease monitoring are also much needed. In the Kaplan-Meier survival analyses in this pilot study, we found that both α-haptoglobin and HGF can potentially be a prognostic indicator for survival (Figure 5A, B). However, further studies with larger numbers of patient samples would be warranted to validate this finding.

With advances in global gene expression analysis (genomics), the search for genes that are highly expressed in SCLC cells has been pursued intensely using techniques such as oligonucleotide microarrays (35). Pedersen *et al.* recently have reported the transcriptional gene expression profiling of 21 SCLC cell lines and 8 xenograft tumors cell lines to compare to the expression profiles of 17 normal adult tissues (35). This represents a promising global genomic approach to identify novel potential therapeutic targets or diagnostic

markers in SCLC. Nonetheless, the extreme difficulties in obtaining sufficient tumor tissues for genomics analysis would be a practical limitation of the utility in this approach. The proteomics approach, on the other hand, allows the analysis of potential serum protein biomarkers which can be readily collected *via* simple serial peripheral venous phlebotomy. It has been shown in past studies that it is relatively difficult to achieve high sensitivity and specificity with single protein biomarkers (3). To further improve the sensitivity and specificity of SCLC serum biomarkers, future directions would involve the combined use of multiple-biomarkers, such as α-haptoglobin and HGF together. On the other hand, identification of "cancer proteomics signature-profiling" may also be useful. With this approach, it may not be necessary to establish the identity of "individual protein biomarkers". Nonetheless, knowledge of identity of individual biomarkers, in our opinion, would be of paramount importance to eventually improve our understanding of the tumor biology. Utilization of high throughput technology platform of proteomics, such as MALDI-TOF-/TOF and protein-biochip SELDI/Mass Spectrometry (MS), to detect novel serum protein tumor biomarkers certainly would greatly facilitate to achieve the goal of arriving at clinically useful serum biomarkers/biomarker-signatures for SCLC.

Acknowledgements

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