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# Serum Proteomic Profiling Reveals Differentially Expressed IGHG3 and A1AG1 as Potential Predictors of Chemotherapeutic Response in Advanced Non-small Cell Lung Cancer

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**Abstract.** *Background: This study aimed to identify differentially expressed proteins in the serum of advanced non-small cell lung cancer (NSCLC) patients responding to carboplatin (CAR) plus paclitaxel (PTX) chemotherapy compared to non-responders. Materials and Methods: Serum from 8 responders and 6 non-responders was subjected to proteomic analysis by label-free liquid chromatography tandem mass spectrometry and validated by western blotting. CAR/PTX-resistant human H1792 and A549 cells were used for evaluating gene expression. Results: Fifty-two proteins were differentially expressed between responders and non-responders. Alpha 1 antitrypsin antibody, alpha 1 acid glycoprotein (A1AG1), afamin, protein S100-A9 and immunoglobulin heavy constant gamma 3 (IGHG3) were validated. IGHG3 was elevated (p=0.037) while A1AG1 was reduced (p=0.003) in responders as compared to non-responders. Gene expression of IGHG3 and ORM1 in resistant cells showed consistent results with the proteomics profiles. Conclusion: Serum expression levels of IGHG3 and A1AG1 proteins may be useful to recruit an NSCLC subpopulation that can benefit from CAR plus PTX standard therapy.*

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Globally, lung cancer is prominent for its aggressive incidence (11.6% of new cancer cases) and mortality (18.4% of deaths) (1). It is categorized into two major types, non-small cell lung cancer (NSCLC, 84% of all lung cancers) and small cell lung cancer (13% of all lung cancers) (2). Moreover, the 5-year survival rate of lung cancer is unsatisfactory, being reduced to 5% in patients at an advanced stage (2). Despite many advanced treatments available, chemotherapy remains the integral part of treatment, since most patients are at an advanced stage of the disease at diagnosis (2). In recent years, doublet chemotherapy containing a combination of platinum agents [cisplatin or carboplatin (CAR)] and one third-generation agent (mainly vinorelbine, gemcitabine or taxanes) has been generally used as first-line chemotherapy (3) in advanced lung cancer, as this shows some benefit with regard to survival, compared to using single-agent chemotherapy (4, 5). Platinum/paclitaxel (PTX)-based chemotherapy is one of these regimens, but this regimen has a response rate of less than 30%, and is associated with a median survival time of 8-10 months (6-8). Hence, many patients suffer from its toxic side effects without any survival benefit. Therefore, identification of predictive biomarkers that may guide selection of treatment is of prime importance.

PTX, a microtubule-stabilizing agent, acts as an inhibitor of the microtubule structure resulting in cancer cell death (9). In 2003, Belani *et al.* reported a large clinical trial in late-stage NSCLC that aimed to evaluate the efficacy of three regimens of weekly CAR plus PTX and found that the highest objective response rate (32% of all cases) was among patients receiving PTX weekly for 3 of 4 weeks with CAR

administered on day 1 (10). Some studies have attempted to improve the response rate using a combination of CAR and nanoparticle albumin–bound PTX (also called nab-PTX). Their results demonstrated that nab-PTX plus CAR had a significantly higher overall response rate than solvent-based CAR plus PTX in patients with the squamous subtype (41% *vs*. 24% response rate); however, no significant difference in response rate among patients with the non-squamous subtype was observed (26% *vs*. 25% response rate) (11). Even though numerous studies have tried to find ways to improve the efficacy of the CAR/PTX treatment, the response rate of this regimen, particularly for the adenocarcinoma subtype, remains low. A contributing factor is the lack of biomarkers, which can be utilized for selecting a regimen. Hence, biomarker-guided chemotherapy is urgently required.

Proteomic analysis is becoming a promising tool in complementing diagnostic capabilities and predicting the treatment outcome of patients with specific therapies (12). It has been used to discover predictive biomarkers for NSCLC over the past decade (13-15). Although studies on serum protein markers of lung cancer are reported frequently, no study, to our knowledge, has used proteomic technology to compare protein expression profiles from the serum of lung cancer patients receiving PTX plus CAR. In this study, we aimed to discover differentially expressed proteins resulting from the response to the CAR/PTX regimen, which are present in the serum of advanced NSCLC patients, using the label-free quantitative proteomics approach. In addition, our *in vitro* results suggest that the candidate protein may be produced directly by cancer cells via the generation of drug-resistant NSCLC cell lines.

## Materials and Methods

*Subject selection*. Specimens from 14 inoperable NSCLC patients were obtained for the study during the 2016-2018 period. The study protocol was approved by the Human Research Ethics Committee, Faculty of Medicine, Prince of Songkla University, Thailand (REC: 59-011-05-1). Written informed consent forms were obtained from all participants. We included patients who were newly diagnosed with pathologically confirmed NSCLC and had completed five or six cycles of first-line chemotherapy treatment using CAR plus PTX. Patients who had undergone any lung cancer-related treatment before receiving chemotherapy, or were lost to follow-up, or died during treatment were excluded from the study. A computed tomography scan of the chest was taken before treatment for staging and after treatment, at the third cycle and onwards, to assess the response to the therapy.

*Treatment evaluation*. Chemotherapeutic efficacy was measured according to the guidance of Response Evaluation Criteria in Solid Tumors (RECIST 1.1) (16). The evaluated response was classified into four categories: complete response (CR) defined as the disappearance of all target lesions, partial response (PR) involving a reduction of at least 30% from the sum total of target lesions, stable disease (SD) when neither lesion reduction nor progression were present, and progressive disease (PD) when a progress of at least 20% among target lesions or the presence of new lesions was observed. CR and PR were considered responders, whereas PD and SD were classified as non-responders.

*Serum preparation*. Blood samples were collected at the time of diagnosis and coagulated for 30 min at room temperature before centrifugation at 3,400 × *g* for 10 min. The serum was filtrated through a polyvinylidene difluoride (PVDF) syringe filter with a pore size of 0.22 mm (Merck Millipore, Darmstadt, Germany), and aliquots were kept at –80˚C until use. On the basis of histology (adenocarcinoma, ADC and squamous cell carcinomas, SCC), individual serum samples were pooled into four pools according to each subtype, wherein two out of the four pools were responders and non-responders, respectively.

*Immunoaffinity depletion*. The top 14 high-abundance proteins in crude serum consisting of albumin, immunoglobulin G, immunoglobulin A, transferrin, haptoglobin, transferrin, fibrinogen, antitrypsin, alpha2-macroglobulin, alpha1-acid glycoprotein, transthyretin, apolipoprotein AI, apolipoprotein AII, and complement C3 were depleted using the Human 14 Multiple Affinity Removal System (MARS) Column with a 4.6×100 mm dimension (Agilent Technologies, Inc., Santa Clara, CA, USA) as described by Verathamjamrus *et al*. (17). Serum was diluted 4-fold with buffer A, and one-tenth of the total volume of the protease inhibitor (Promega, Madison, WI, USA) was added. The ensuing mixture was filtered through a 0.22 μm Spin-X centrifuge tube filter (Corning Inc., New York, NY, USA) at 16,000 × *g* for 1 min at 4 ˚C to remove particles before injection onto the MARS column. The column was equilibrated using 100% buffer A at a flow rate of 0.125 ml/min for 10 min. Then, separation was started by injecting 80 μl of pooled sera into the column, and eluted with buffer A at 0.125 ml/min for 18 min and at 1 ml/min for 2 min. The buffer was then changed to 100% buffer B at a flow rate of 1 ml/min for 7 min to elute the bound fraction. The column was finally eluted with 100% buffer A at a flow rate of 1 ml/min for 11 min and 0.125 ml/min for 1 min to regenerate the column. Absorbance was detected at 280 nm. The collected flow-through fractions were desalted using an Amicon® Ultra-0.5 device, at a 30 kDa cutoff (Merck Millipore), and centrifuged at 15,000 × *g* for 30 min at 4˚C. Samples were kept at –80˚C for further analysis.

*In-solution digestion*. The total protein content was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). For each pool, a total 5 μg of desalted serum protein was resuspended in 50 mM ammonium bicarbonate (Fluka Chemical Corp., New York, NY, USA). The pooled samples were reduced with dithiothreitol (Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 10 mM at 95˚C for 5 min, followed by alkylation with a 1:10 ratio of 200 mM iodoacetamide (Sigma-Aldrich, St Louis, MO, USA) and incubation in the dark for 30 min at room temperature. Sequencing grade trypsin (Promega) with 1:50 w/w was added to digest proteins into peptides overnight at 37˚C. The digested peptide solution was cleaned up with Zip Tip C18 Pipette Tips (Merck Millipore), and the digestion was stopped with a 1% final concentration of formic acid (Sigma-Aldrich). The samples were dried using a SpeedVac Evaporator (Labconco Corp., Kansas, MO, USA).

*Label-free LC-MS/MS analysis*. The concentrated samples were re-dissolved in 0.1% formic acid (Merck Millipore) and analyzed with the Thermo Scientific™ Dionex™ Ultimate™ 3000 RSLC Nano System that was set up with electrospray ionization-ion trap tandem-mass spectrometry (Bruker Corp., Billerica, MA, USA) with a captive-electrospray ion source. Peptide mixtures were injected onto the Acclaim PepMap RSLC C18 column (75 μm × 150 mm dimension) connected with an Acclaim PepMap100 C18 micro-precolumn (300 μm × 5 mm dimension) (Thermo Fisher Scientific). Separation was performed at a flow rate of 0.3 μl/min at 40˚C using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The gradient elution was as follows: mobile phase B, 1-50% at 0-70 min, increased to 90% at 70-75 min, maintained at 90% for 5 min, set back to equilibration at 1% at 90-91 min, and left at 1% for 9 min. During data acquisition, the positive ion mode with a spray voltage of 1,300 V and a heated inlet capillary temperature of 150˚C were used. The mass spectra were acquired at a 400 1,400 m/z value with an ion charge count target of 400,000. The MS scans, ranging from 50-3,000 m/z and optimized at 922 m/z, were automatically recorded for MS/MS analysis using the Bruker Compass 1.4 software (Bruker Corp.).

*Data analysis for protein identification and quantification*. Label-free protein quantification was processed using the Progenesis QI software version 3.1 (Nonlinear Dynamics, Ltd., Newcastle upon Tyne, UK). Triplicate raw spectra files were uploaded to the software. All chromatographs were automatically aligned, and a reference run with a slight difference in MS peaks and retention time was set for all runs. Retention times and intensities were aligned and normalized to the reference values. Default operation used 'relative quantitation using non-conflicting peptides'. All of the MS/MS data were exported as Mascot generic files (mgf) and searched against the Swiss-Prot Database using MASCOT engine database searching algorithms, version 2.4.0 (www.matrixscience.com) in the *Homo sapiens* taxonomy with the following search parameters: (1) enzyme specificity as trypsin; (2) only one missed cleavage; (3) variable modifications as Carbamidomethyl (C), Oxidation (M), Phospho (ST) and Phospho (Y); (4) peptide tolerance as ±1.2; (5) MS/MS tolerance as ±0.6 Da; (6) peptide charge as +2, +3 and +4; and (7) only data with more than one peptide. The database of decoy search for reversed protein sequence was set at a threshold of 1%, allowing a missed cleavage value of 1. Relative protein abundances were normalized using only unique peptides with a MASCOT score of greater than 30. Thereafter, screening ANOVA values in triplicate runs, proteins with $p<0.05$ were considered for subsequent analysis. The expression levels of proteins were observed in three comparisons: in all pools of responders and non-responders, within pools of ADC, and within pools of SCC. Fold change was calculated by the ratio of average intensity in responders to average intensity in non-responders. A plus (+) value denoted up-regulated expression in responders, while a minus (–) value implied a down-regulated expression in responders.

*Bioinformatics analysis*. The distribution of differential proteins commonly or exclusively identified in each subtype was determined using Venn diagrams made available by an online package (http://www.interactivenn.net). The expression level of significant proteins was analyzed utilizing the hierarchical clustering method. The results were graphically presented using a web tool created by Clustvis (https://biit.cs.ut.ee/clustvis/), which was built from Pearson's correlation and average clustering values for rows and columns (18). The attributions of 53 altered proteins were queried through the web-based Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (http://string-db.org) to construct a protein-protein interaction network at medium interaction scores of <0.4 and a false discovery rate (FDR) of <0.05. Nodes signify proteins in the constructed network, edges refer to protein interactions, edge thickness relate to the confidence of data supported and node degrees relate to the average number of interactions.

*Western blotting*. The expression levels of selected proteins in serum were determined by western blotting in individual samples, which were used in the profiling. The serum was diluted using a sample buffer, and protein concentrations were measured via the Bradford assay. Thirty micrograms of protein were denatured by boiling before loading onto a polyacrylamide gel. After resolving with 10% SDS-PAGE using a current of 10 mA for each gel, the proteins were transferred to a 0.20 μm nitrocellulose membrane (Bio-Rad Laboratories) at a constant 100 V for 1 h. Membranes were blocked with 3% bovine serum albumin (Sigma-Aldrich) and incubated overnight at 4˚C with primary antibodies: rabbit monoclonal anti-alpha 1 antitrypsin (1:500, Abcam, Cambridge, UK), rabbit monoclonal anti-alpha 1 acid glycoprotein (1:5,000, Abcam), rabbit monoclonal anti-afamin (1:2,000, Abcam), rabbit monoclonal anti-S100A9 (1:1,000, Abcam), and mouse polyclonal anti-IgG3 (1:5,000, Abcam). The membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies at a dilution of 1:2,000 (Agilent Dako, Santa Clara, CA, USA) at room temperature for 1 h. Bands were detected by WesternBright ECL (Advansta, San Jose, CA, USA), and images were visualized via the ImageQuant™ digital imaging system (GE Healthcare, Chicago, IL, USA). One of the serum samples was used as a loading control to calculate the relative expression of each protein.

*Cell culture and generation of resistant cell lines*. Human NSCLC cell lines of H1792 and A549 obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in a Roswell Park Memorial Institute Medium (RPMI)-1640 containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and incubated in a humidified incubator supplied with 5% $CO_2$ at 37 ˚C to allow for the attachment of cells as a monolayer. The cells were seeded at $1.8\times10^6$ cells in a T-75 flask (Corning Inc.) overnight to achieve a confluence of 50-60%. They were then treated with each drug; PTX (Sigma-Aldrich) at a concentration of 20 nM and CAR (TOKU-E, Bellingham, WA, USA) at a concentration of 15 μM for 3 days. The treated cells were further cultured for another 3 days in the recovery phase and the cycle was repeated by gradually increasing the concentration of each drug over a period of 6 months.

*Evaluation of resistance level*. The sensitivity of the cell lines to chemotherapeutic drugs was determined using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Gibco). In brief, $1\times10^4$ cells were seeded in a 96-well plate and maintained at 37 ˚C in a 5% $CO_2$ incubator overnight. Following incubation for 72 h. with the desired range of drug concentrations, 100 μl of MTT solution at a 5 mg/ml concentration was added, and the mixture was further incubated for 2 h. The MTT solutions were then removed, and 100 μl of dimethyl sulfoxide (DMSO) (Gibco) was added. The absorbance of formazan blue

crystals soluble in DMSO was measured at 570 nm subtracted with background at 650 nm using a Varioskan LUX microplate reader (Thermo Fisher Scientific). Survival curves were generated, and the inhibitory concentrations ($IC_{50}$) for each condition were calculated from a range of drug concentrations; PTX ranging from 3.125 nM to 200 nM, and CAR ranging from 1 nM to 400 nM. The experiments were performed in duplicate for each condition, and the calculation of resistance factors determined as the ratio of $IC_{50}$ values of resistant cells to the $IC_{50}$ values of parental cells.

*RNA isolation and quantitative RT-qPCR*. Total RNA from parental and resistant cells was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) as stated in the manufacturer's instructions. The quantity of extracted RNA was verified using a NanoDropND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) at an optical density (OD) ratio of 260/280 nm and 260/230 nm. Subsequently, the purified RNA was converted to cDNA by a Thermal Cycler (Bio-Rad Laboratories) using 4 μl of iScript™ supermix (Bio-rad Laboratories) in a 20 μl final volume. The cDNA products were used to compare the relative quantification of *IGHG3* and *ORM1* using *GAPDH* as a reference gene. Primer sequences are shown in Table I. The SsoFast EvaGreen supermix (Bio-Rad Laboratories) was used, and the PCR cycling parameters were as follows: 95˚C for 15 sec, followed by 40 cycles at 60˚C for 30 sec and at 70˚C for 60 sec. A BioRad CFX96 qPCR System (Bio-Rad Laboratories) was used for the analysis. Expression was presented as cycle threshold (Ct) values, and the experiments were performed in duplicate. Each gene was quantified using the $2^{-(\Delta\Delta Ct)}$ method (19), and the relative expression level was calculated as the ratio of each targeted gene to the *GAPDH* gene.

*Statistical analysis*. The Ct values are presented as mean±standard deviation (SD) of two independent experiments. Statistical measurements employed the unpaired *t*-test and were reported as significant when the *p*-value was <0.05 using the GraphPad Prism, Version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

*Patient characteristics*. The study subjects comprised 14 patients (11 males and 3 females) with NSCLC. All patients had stage IV disease. Nine patients had ADC, and the other five had SCC. Their median age was 63.0 years, ranging from 53 to 80 years. According to the chemotherapeutic response, two patients had CR, six patients achieved PR, while the remaining six patients had PD.

*Differentially expressed serum proteins between responders and non-responders*. The Human 14 Multiple Affinity Removal Column was used together with the comparative label-free proteomic techniques to discover predictive biomarkers for NSCLC. A total of 124 proteins were identified in all cases and analyzed for differential expression between responders and non-responders and for histological type (ADC and SCC) using the Progenesis QI software. The statistical analysis revealed 52 proteins differentially expressed in the serum of responders compared to those of non-responders (Table II). The expression of alpha-1-antitrypsin (A1AT) and alpha-1-acid glycoprotein (A1AG1) was decreased by 2.23-fold to 4.35-fold in responders of all histological types as compared to non-responders, while the expression of alpha-1-acid glycoprotein 2 (A1AG2) was 11.2-fold lower in ADC responders compared to ADC non-responders. In contrast, immunoglobulin heavy constant gamma 3 (IGHG3), immunoglobulin lambda constant 3 (IGLC3), complement C3 (C3), and heparin cofactor 2 (SERPIND1) were expressed at significantly higher proportions in responders of all histological types compared to non-responders.

Table I. *Primer sequences of IGHG3, ORM1 and GAPDH for qRT-PCR.*

| Gene | Primer sequence ('5 to 3') | Length (bases) | Product size (bp) |
|---|---|---|---|
| *IGHG3* | Forward: CCAGAGCCCAAATCTTGTGA | 20 | 321 |
| | Reverse: CTTGGCATTATGCACCTCCA | 20 | |
| *ORM1* | Forward: CTTGCTTTTGACGTGAACGA | 20 | 209 |
| | Reverse: CCCTCCTCCTGTTTCCTCTC | 20 | |
| *GAPDH* | Forward: ACCACAGTCCATGCCATCAC | 20 | 452 |
| | Reverse: TCCACCACCCTGTTGCTGTA | 20 | |

*Functional and pathway bioanalysis*. To understand the protein-protein interactions of altered serum proteins, the interaction network was analyzed using STRING (Figure 1A). The web-based Database Annotation Visualization Integrated Discovery (DAVID) platform was utilized to extract biological explanations in terms of three main GO processes as well as for the pathway analysis. The results of the most enriched list are reported in bubble plots (Figure 1B-E). According to the biological process, molecular function, and cellular component, the most changes were observed in the serum proteins involved in the negative regulation of endopeptidase activity, platelet degranulation, and acute-phase response. Notably, the complement and coagulation cascades were determined to be the most enriched.

*Validation of protein expression in serum*. Five proteins – IGHG3, A1AT, A1AG1, S10A9 and AFAM – were selected for study by immunoblotting to confirm differential expression. Compared to non-responders, the level of IGHG3 expression was significantly increased among responders of all cases ($p$=0.037) and those with ADC ($p$=0.013), while the A1AG1 expression was significantly lower among patients with SCC ($p$=0.003) (Figure 2). No significant differential expression of A1AT, S10A9, and AFAM was observed in any responder vs non-responder group.

*Expression of IGHG3 and ORM1 in CAR- and PTX-resistant cell lines*. To demonstrate whether the candidate proteins (IGHG3 and A1AG1) were related to a resistant status, we

Table II. *List of differentially expressed proteins.*

| Accession | Gene symbol | Protein name | Unique peptide | Score | Anova (p) | Fold Change | | | 2 gr-*p*-Value |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ADC | SCC | All | |
| IGHG3_HUMAN | IGHG3 | Immunoglobulin heavy constant gamma 3 | 7 | 823.7 | 1.41E-11 | +1.36 | +1.87 | +1.70 | *a,b,c* |
| IGKC_HUMAN | IGKC | Immunoglobulin kappa constant | 3 | 178.6 | 2.30E-10 | +1.05 | +2.00 | +1.59 | *b,c* |
| KNG1_HUMAN | KNG1 | Kininogen-1 | 22 | 1674.2 | 1.19E-09 | +1.03 | +1.88 | +1.33 | *b,c* |
| HRG_HUMAN | HRG | Histidine-rich glycoprotein | 9 | 914.5 | 2.00E-09 | –1.43 | +3.12 | +1.19 | *a,b* |
| IGLC3_HUMAN | IGLC3 | Immunoglobulin lambda constant 3 | 2 | 283.7 | 7.65E-08 | +1.41 | +1.95 | +1.74 | *a,b,c* |
| CO8B_HUMAN | C8B | Complement component C8 beta chain | 9 | 741.2 | 3.89E-07 | +1.03 | +1.62 | +1.28 | *b,c* |
| APBB3_HUMAN | APBB3 | Amyloid-beta A4 precursor protein-binding family B member 3 | 1 | 48.0 | 5.12E-07 | +1.09 | +1.57 | +1.35 | *b,c* |
| APOB_HUMAN | APOB | Apolipoprotein B-100 | 95 | 7424.1 | 8.46E-07 | –1.17 | +2.46 | +1.35 | *b,c* |
| GELS_HUMAN | GSN | Gelsolin | 13 | 1151.1 | 1.88E-06 | +1.07 | +2.68 | +1.58 | *b,c* |
| PGRP2_HUMAN | PGLYRP2 | N-acetylmuramoyl-L-alanine amidase | 8 | 534.5 | 3.45E-06 | –1.13 | +1.81 | +1.23 | *b,c* |
| THBG_HUMAN | SERPINA7 | Thyroxine-binding globulin | 7 | 467.5 | 3.89E-06 | –1.40 | +2.00 | +1.14 | *a,b* |
| PCYOX_HUMAN | PCYOX1 | Prenylcysteine oxidase 1 | 1 | 48.5 | 4.05E-06 | +1.25 | +2.20 | +1.58 | *b,c* |
| ALS_HUMAN | IGFALS | Insulin-like growth factor-binding protein complex acid labile subunit | 10 | 754.8 | 9.34E-06 | –1.15 | +2.31 | +1.35 | *b,c* |
| APOH_HUMAN | APOH | Beta-2-glycoprotein 1 | 14 | 1425.2 | 1.06E-05 | +1.11 | +1.64 | +1.30 | *b,c* |
| CO3_HUMAN | C3 | Complement C3 | 16 | 1246.1 | 2.04E-05 | +1.39 | +1.56 | +1.45 | *a,b,c* |
| PSA_HUMAN | NPEPPS | Puromycin-sensitive aminopeptidase | 1 | 52.2 | 4.73E-05 | +2.22 | –1.92 | +1.03 | *a,b* |
| FINC_HUMAN | FN1 | Fibronectin | 31 | 2680.3 | 4.94E-05 | –1.52 | +1.24 | –1.26 | – |
| PRG4_HUMAN | PRG4 | Proteoglycan 4 | 1 | 59.8 | 5.21E-05 | –1.91 | +2.16 | –1.25 | *a* |
| FETUA_HUMAN | AHSG | Alpha-2-HS-glycoprotein | 9 | 939.7 | 5.39E-05 | +1.14 | +2.35 | +1.55 | *b,c* |
| AFAM_HUMAN | AFM | Afamin | 19 | 1321.3 | 1.27E-04 | –1.08 | +1.62 | +1.13 | *b* |
| VTNC_HUMAN | VTN | Vitronectin | 7 | 561.6 | 1.37E-04 | –1.31 | +1.55 | +1.04 | *a,b* |
| QSOX1_HUMAN | QSOX1 | Sulfhydryl oxidase 1 | 1 | 52.9 | 2.09E-04 | –1.93 | +2.11 | –1.12 | *a,b* |
| K2C1_HUMAN | KRT1 | Keratin, type II cytoskeletal 1 | 3 | 427.7 | 3.00E-04 | +1.61 | +1.57 | +1.59 | *a,b,c* |
| KAIN_HUMAN | SERPINA4 | Kallistatin | 7 | 459.1 | 3.43E-04 | –1.08 | +2.14 | +1.35 | *b,c* |
| CO4B_HUMAN | C4B | Complement C4-B | 2 | 4862.6 | 4.17E-04 | –2.54 | +2.74 | –1.31 | *a,b* |
| CO8G_HUMAN | C8G | Complement component C8 gamma chain | 7 | 615.0 | 4.78E-04 | +1.01 | +1.53 | +1.24 | *b,c* |
| C4BPA_HUMAN | C4BPA | C4b-binding protein alpha chain | 5 | 440.8 | 5.67E-04 | +1.92 | –1.04 | +1.28 | *a* |
| ECM1_HUMAN | ECM1 | Extracellular matrix protein 1 | 4 | 162.2 | 1.18E-03 | –1.86 | –1.01 | –1.38 | *a,c* |
| THRB_HUMAN | F2 | Prothrombin | 18 | 1704.5 | 1.18E-03 | +1.01 | +1.57 | +1.23 | *b,c* |
| HEP2_HUMAN | SERPIND1 | Heparin cofactor 2 | 12 | 833.8 | 1.22E-03 | +1.13 | +1.74 | +1.38 | *a,b,c* |
| KLKB1_HUMAN | KLKB1 | Plasma kallikrein | 10 | 777.1 | 1.29E-03 | –1.24 | +1.67 | +1.12 | *b* |
| ZPI_HUMAN | SERPINA10 | Protein Z-dependent protease inhibitor | 2 | 121.3 | 1.58E-03 | –1.25 | +1.68 | +1.10 | *a,b* |
| KI18A_HUMAN | KIF18A | Kinesin-like protein KIF18A | 1 | 49.1 | 1.98E-03 | –1.18 | –2.44 | –1.79 | *b,c* |
| FUBP1_HUMAN | FUBP1 | Far upstream element-binding protein 1 | 1 | 48.3 | 2.33E-03 | +1.28 | +2.21 | +1.60 | *b,c* |
| A1AG2_HUMAN | ORM2 | Alpha-1-acid glycoprotein 2 | 1 | 135.6 | 3.86E-03 | –11.22 | +1.09 | –5.53 | – |
| CNDP1_HUMAN | CNDP1 | Beta-Ala-His dipeptidase | 1 | 72.6 | 4.81E-03 | –1.42 | +1.81 | +1.05 | *a,b* |
| S10A9_HUMAN | S100A9 | Protein S100-A9 | 1 | 53.9 | 6.10E-03 | –1.52 | +1.38 | –1.14 | – |
| ITIH2_HUMAN | ITIH2 | Inter-alpha-trypsin inhibitor heavy chain H2 | 21 | 1790.7 | 6.19E-03 | –1.10 | +1.67 | +1.19 | *b* |
| VASN_HUMAN | VASN | Vasorin | 1 | 70.7 | 7.93E-03 | –1.51 | +1.61 | –1.04 | *a* |
| CHLE_HUMAN | BCHE | Cholinesterase | 2 | 148.7 | 9.39E-03 | +1.08 | +1.83 | +1.38 | *b,c* |
| ITIH1_HUMAN | ITIH1 | Inter-alpha-trypsin inhibitor heavy chain H1 | 14 | 1439.6 | 1.11E-02 | –1.05 | +1.63 | +1.21 | *b* |
| FIBA_HUMAN | FGA | Fibrinogen alpha chain | 3 | 208.7 | 1.46E-02 | +1.46 | +1.70 | +1.60 | *c* |
| B2MG_HUMAN | B2M | Beta-2-microglobulin | 2 | 89.7 | 1.49E-02 | –2.63 | +1.42 | –1.46 | *a* |
| HPT_HUMAN | HP | Haptoglobin | 19 | 1510.7 | 1.76E-02 | –1.11 | –2.95 | –2.28 | – |
| FA10_HUMAN | F10 | Coagulation factor X | 4 | 186.4 | 1.83E-02 | –1.18 | –1.63 | –1.43 | *a* |
| CO4A_HUMAN | C4A | Complement C4-A | 4 | 4946.9 | 1.83E-02 | –1.93 | +1.99 | –1.02 | *a* |
| A1AG1_HUMAN | ORM1 | Alpha-1-acid glycoprotein 1 | 3 | 315.9 | 1.86E-02 | –3.54 | –4.35 | –3.97 | *c* |
| SAA2_HUMAN | SAA2 | Serum amyloid A-2 protein | 2 | 150.8 | 1.92E-02 | –1.56 | +1.15 | –1.18 | *a* |
| PLF4_HUMAN | PF4 | Platelet factor 4 | 1 | 66.9 | 2.15E-02 | +1.10 | +1.70 | +1.36 | *b,c* |
| A1AT_HUMAN | SERPINA1 | Alpha-1-antitrypsin | 8 | 610.9 | 2.45E-02 | –2.23 | –3.24 | –2.76 | *c* |
| ANT3_HUMAN | SERPINC1 | Antithrombin-III | 12 | 1102.7 | 2.72E-02 | +1.14 | +1.67 | +1.36 | *b,c* |
| SEPP1_HUMAN | SELENOP | Selenoprotein P | 1 | 74.6 | 4.12E-02 | –1.23 | +1.71 | +1.19 | *b* |

ADC, adenocarcinoma; SCC, squamous cell carcinoma; **p*-value from ANOVA testing of differences between all pooled samples; **comparison group showing significant differential expression using the independent *t*-test: *a* in ADC pools, *b* in SCC pools, *c* in all pools; (+) denotes up-regulation and (–) denotes down-regulation.

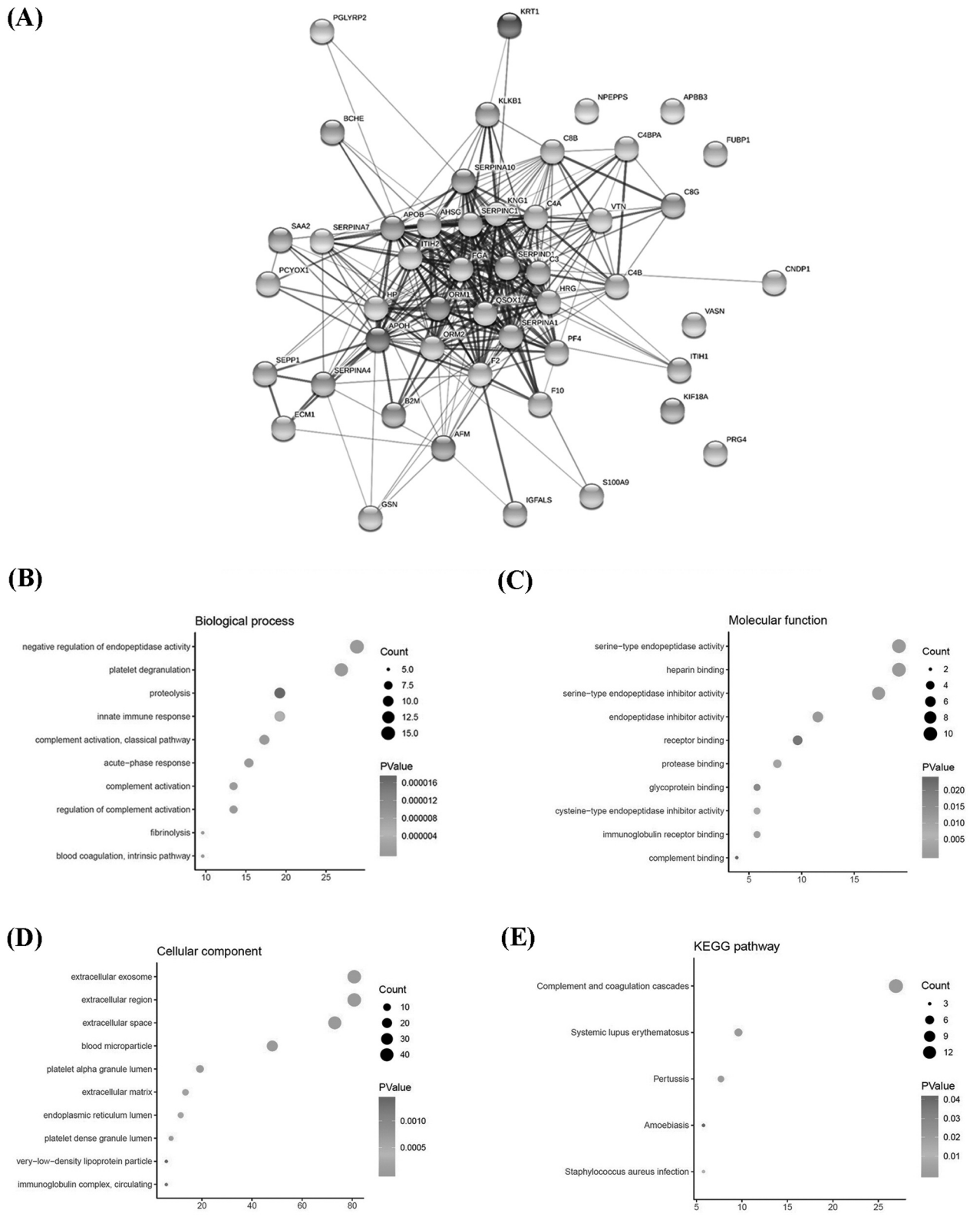


Figure 1. *Bioinformatics analysis of differentially expressed proteins. A) STRING network analysis of 52 proteins found significantly more often in responders compared to non-responders. GO enrichment plots in terms of (B) biological process, (C) molecular function, and (D) cellular component. E) Enrichment analysis via the KEGG pathway.*

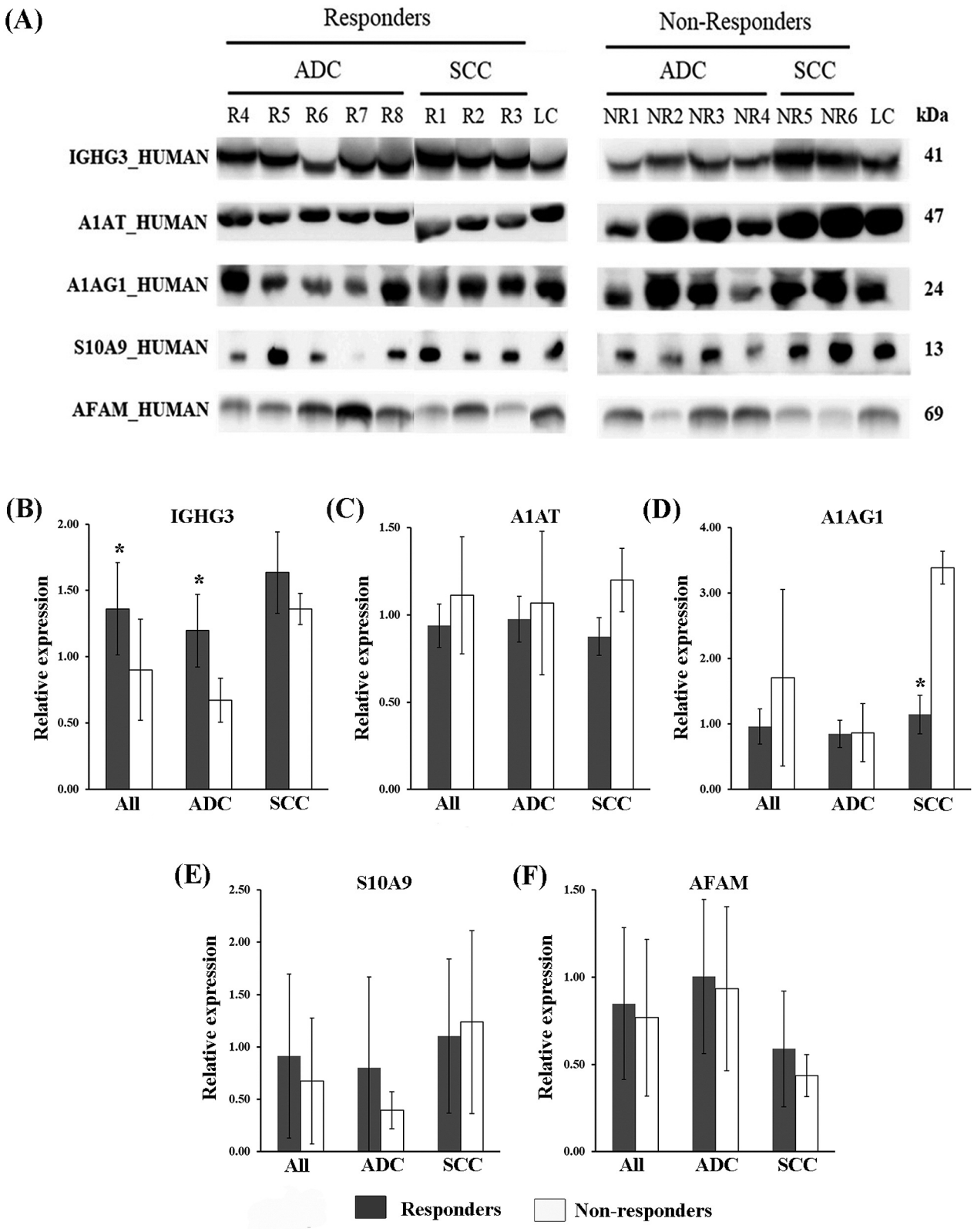


Figure 2. *Western blotting to validate the expression level of selected candidate proteins in serum. A) Band intensities of proteins of interest. Relative quantitation of selected proteins by radiometric analysis with loading controls: B) IGHG3, C) A1AT, D) A1AG1, E) S10A9, and F) AFAM. *indicates a significant p-Value of <0.05.*

examined the expression levels of their coding genes (*IGHG3* and *ORM1*, respectively) in our generated drug-resistant cells. After a total of six months, the H1792 and A549 cells were 5.2- and >2-fold more resistant to PTX, respectively (Figure 3A and C) and 2.3- and 1.5-fold more resistant to CAR, respectively than their parental cells (Figure 3B and D).

The *IGHG3* expression level was significantly lower in H1792/CAR ($p$=0.003), A549/PTX ($p$<0.001), and A549/CAR ($p$<0.001) cells compared with their parental cells (Figure 3E). In contrast, the expression level of *ORM1* was significantly higher in A549/CAR ($p$=0.002) cells compared with A549 cells (Figure 3F).

## Discussion

High-abundance protein depletion is a major challenge in the investigation of serum biomarkers (17, 20, 21). Here, the high-abundance serum proteins from advanced NSCLC patients in different response groups to CAR/PTX treatment were removed, and the resulting proteins analyzed using the label-free quantitative proteomics approach. We found 52 proteins differentially expressed between responders and non-responders among all patients and patients representing the ADC and SCC subtypes. Some of these proteins have been reported to be related to chemotherapeutic response in previous proteomic profiles of patients with lung cancer; they include gelsolin, complement C3, alpha-2-HS-glycoprotein, vitronectin, prothrombin, fibrinogen alpha chain, haptoglobin, alpha-1-acid glycoprotein 1, and alpha-1-antitrypsin (15, 22). However, the list of proteins is inconsistent among the studies. This may be due to the use of various chemotherapeutic regimens, histological subtypes, sample sources, sample preparations, and proteomic platforms during the investigation. Six proteins were significantly altered in all subtypes of NSCLC, namely A1AT, A1AG1, IGHG3, IGLC3, C3, and SERPIND1. Among the 6 candidate proteins, the differential expression of IGHG3 and A1AG1 was confirmed using western blotting. The levels of IGHG3 and A1AG1 proteins showed consistent results with the proteomics profiles. However, the regulation of IGHG3 was significantly high in responders of ADC and all histological types. In contrast, a decrease in the expression of A1AG1 was seen in SCC responders compared to non-responders. Interestingly, both proteins are involved in the immunoinflammatory process. In addition, a consistent level of gene expression of both *IGHG3* and *ORM1* was found in our generated CAR/PTX-resistant human H1792 and A549 cells.

IGHG3 is a member of the human immunoglobulin G (IgG) proteins and is encoded by the *IGHG3* gene (21). Upon binding to specific antigens, IgG triggers important responsiveness events to eliminate antigens such as the activation of the complement cascade, antibody-facilitated phagocytosis, and cytotoxicity (22). Normally, IgG is produced and released by B lymphocytes. However, increasing evidence suggests that various cancer cell types, such as colon cancer (23), pancreatic carcinoma (24), and esophageal squamous cell carcinoma (25) are capable of producing IgG. Our *in vitro* experiments using cell lines also agree with the suggestion that cancer cells can express IGHG3, and that this is related to the chemoresistance status. Our findings are consistent with those of Hsu *et al*. who reported that the levels of two immunoglobulins (IGHG1 and IGHG3) were >3-fold higher in tumors of triple-negative breast cancer patients with a pathological complete response compared to the non-responsive patients (26). Nevertheless, there is little information in the literature concerning the biological function of IgG expression in cancer cells. The existing evidence indicates that the IgG secreted by cancer cells has the capacity to promote growth and survival of cancer cells (23, 27, 28). However, the specific biological function of cancer-derived IgG in relation to chemotherapeutic response/resistance is not known.

alpha-1-acid glycoprotein 1 (A1AG1) or orosomucoid 1 (ORM1), which is encoded by the *ORM1* gene, is a glycosylated serum protein that can bind and transport molecules in the bloodstream (29). It plays an essential role in the immune response and the anti-inflammatory process in the acute phase, and is classified as an acute phase protein (30). It has been suggested that acute-phase reactants such as A1AG1 may contribute to the 'immune escape' of tumor cells. Many studies have shown higher serum levels of A1AG1 in cancer patients compared to non-cancerous patients or healthy persons, as found in various cancer types, *e.g*., pancreatic (31), cervical (32), liver (33), bladder (34), as well as lung cancer (35, 36). In addition, A1AG1 expression has recently been reported to be a potential diagnostic marker. Using the transcriptome sequencing of peripheral leukocytes in early-stage NSCLCs compared to healthy individuals, Ye *et al*. found a total of 358 immune-related genes were differentially expressed, and *ORM1* was identified as a potential diagnostic marker, having an area under the curve (AUC) of 0.862 (37).

Few studies have documented the potential role of A1AG1 expression in predicting chemotherapeutic responsiveness. In our study, an elevated A1AG1 expression was found to be associated with chemotherapeutic resistance. Our result is consistent with those of two other proteomics studies on breast cancer and NK/T cell lymphoma. In 2011, Hyung *et al*. performed the serum proteomic profiling of N-glycosylated proteins in 10 patients with advanced breast cancer sensitive to and 5 patients resistant to neoadjuvant chemotherapy (38). They found that A1AG1 expression level was significantly higher in chemotherapeutic resistant patients compared to the chemo-sensitive ones. Likewise, Zhou *et al*. found A1AG1 to be up-regulated among non-responders compared to

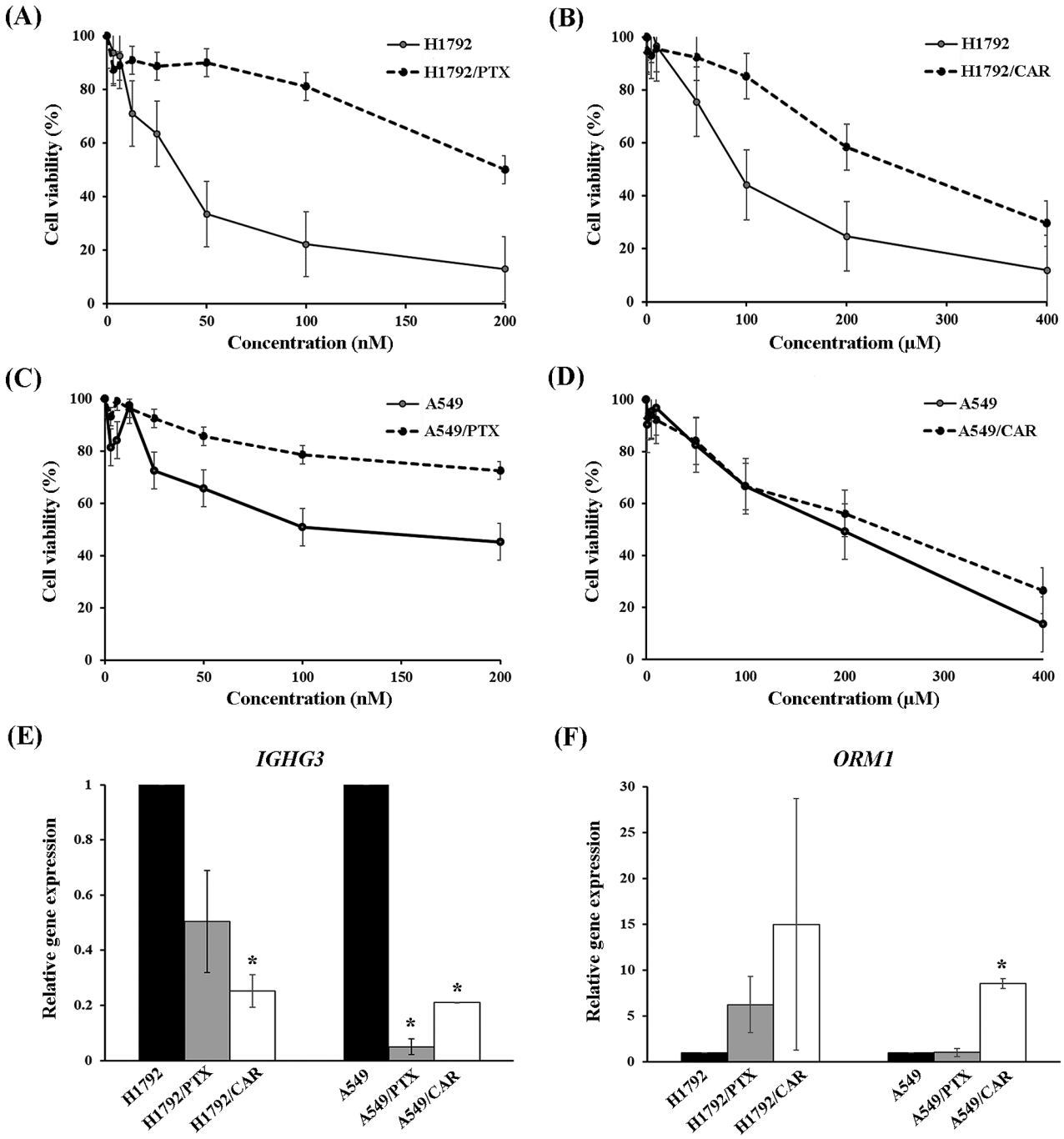


Figure 3. *Survival curves of H1792 and A549 after treatment with CAR (A and C) and PTX (B and D). The relative expression values of IGHG3 (E) and ORM1 (F) among parental cells and PTX- and CAR-resistant cells in H1792 and A549 cell lines. *indicates a significant p-Value of <0.05.*

responders with advanced extranodal NK/T cell lymphoma (39). In addition, both of these studies demonstrated that A1AG1 expression is associated with a poor treatment outcome. However, neither A1AG1 nor IGHG3 have been documented in proteomic profiling studies of lung cancer in relation to their role in chemotherapeutic responsiveness.

Serum proteins are emerging as useful biomarkers for cancer. There is some evidence that numerous proteins can be directly released from cancer cells into the bloodstream (40). Furthermore, the levels of several secreted proteins are mainly due to the alternative mRNA synthesis, and some of these also show a positive correlation with the mRNA level

(41, 42). Our results showed that H1792 and A549 cells, which were resistant to CAR and PTX, had lower expression levels of *IGHG3* and a higher expression level of *ORM1* than their parental cells. The results of gene expression level in resistant cells were also consistent with the results found in clinical samples. Hence, we suggest that IGHG3 and A1AG1 may be directly produced from cancer cells and secreted into the bloodstream. However, these proteins should be further investigated in conditioned media in order to provide better insight.

In summary, the present study demonstrates that serum IGHG3 and A1AG1 are potentially useful for the identification of patients who would benefit from the CAR-PTX treatment. However, further studies are required to evaluate the clinical usefulness of this finding.

## Conflicts of Interest

The Authors declare no conflicts of interest.

## Authors' Contributions

P.R. C.S., and P.T. designed the research. P.R., M.M.M., D.C., K.W., C.W., and K.M. performed the experiment, and interpreted all results. P.R., M.M.M., C.S., C.W., J.S., and P.T. wrote the paper and approved the final manuscript.

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## References

1 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin *68(6)*: 394-424, 2018. PMID: 30207593. DOI: 10.3322/caac.21492

2 SEER Cancer Statistics Review, 1975-2017, National Cancer Institute. Bethesda, MD, [Internet]. Available from at: https://seer.cancer.gov/csr/1975_2017/, based on November 2019 SEER data submission, posted to the SEER web site, April 2020. [Last accessed on February 21, 2021]

3 Spira A and Ettinger DS: Multidisciplinary management of lung cancer. N Engl J Med *350(4)*: 379-392, 2004. PMID: 14736930. DOI: 10.1056/NEJMra035536

4 Delbaldo C, Michiels S, Syz N, Soria JC, Le Chevalier T and Pignon JP: Benefits of adding a drug to a single-agent or a 2-agent chemotherapy regimen in advanced non-small-cell lung cancer: a meta-analysis. JAMA *292(4)*: 470-484, 2004. PMID: 15280345. DOI: 10.1001/jama.292.4.470

5 Dranitsaris G, Cottrell W and Evans WK: Cost-effectiveness of chemotherapy for nonsmall-cell lung cancer. Curr Opin Oncol *14(4)*: 375-383, 2002. PMID: 12130919. DOI: 10.1097/00001622-200207000-00001

6 Kelly K, Crowley J, Bunn PA Jr, Presant CA, Grevstad PK, Moinpour CM, Ramsey SD, Wozniak AJ, Weiss GR, Moore DF, Israel VK, Livingston RB and Gandara DR: Randomized phase III trial of paclitaxel plus carboplatin versus vinorelbine plus cisplatin in the treatment of patients with advanced non—small-cell lung cancer: A Southwest Oncology Group trial. J Clin Oncol *19(13)*: 3210-3218, 2001. PMID: 11432888. DOI: 10.1200/JCO.2001.19.13.3210

7 Schiller J, Harrington D, Belani C, Langer C, Sandler A, Krook J, Zhu J and Johnson D: Comparison of four chemotherapy regimens for advanced non–small-cell lung cancer. New England Journal of Medicine *346(2)*: 92-98, 2020. PMID: 11784875 DOI: 10.1056/NEJMoa011954

8 Kelly K, Chansky K, Mack PC, Lara PN Jr, Hirsch FR, Franklin WA, Wozniak AJ, Edelman MJ, Williamson SK and Gandara DR: Chemotherapy outcomes by histologic subtypes of non-small-cell lung cancer: analysis of the southwest oncology group database for antimicrotubule-platinum therapy. Clin Lung Cancer *14(6)*: 627-635, 2013. PMID: 23910067. DOI: 10.1016/j.cllc.2013.06.010

9 Weaver BA: How Taxol/paclitaxel kills cancer cells. Mol Biol Cell *25(18)*: 2677-2681, 2014. PMID: 25213191. DOI: 10.1091/mbc.E14-04-0916

10 Belani CP, Barstis J, Perry MC, La Rocca RV, Nattam SR, Rinaldi D, Clark R and Mills GM: Multicenter, randomized trial for stage IIIB or IV non-small-cell lung cancer using weekly paclitaxel and carboplatin followed by maintenance weekly paclitaxel or observation. J Clin Oncol *21(15)*: 2933-2939, 2003. PMID: 12885812. DOI: 10.1200/JCO.2003.02.563

11 Socinski MA, Bondarenko I, Karaseva NA, Makhson AM, Vynnychenko I, Okamoto I, Hon JK, Hirsh V, Bhar P, Zhang H, Iglesias JL and Renschler MF: Weekly nab-paclitaxel in combination with carboplatin versus solvent-based paclitaxel plus carboplatin as first-line therapy in patients with advanced non-small-cell lung cancer: Final results of a phase III trial. J Clin Oncol *30(17)*: 2055-2062, 2012. PMID: 22547591. DOI: 10.1200/JCO.2011.39.5848

12 Kisluk J, Ciborowski M, Niemira M, Kretowski A and Niklinski J: Proteomics biomarkers for non-small cell lung cancer. J Pharm Biomed Anal *101*: 40-49, 2014. PMID: 25175018. DOI: 10.1016/j.jpba.2014.07.038

13 Shen H, Fang XF, Yuan Y, Yang J and Zheng S: Serum protein pattern could predict the therapeutic effect of first-line pemetrexed/cisplatin chemotherapy in patients with lung adenocarcinoma. World J Oncol *6(1)*: 292-296, 2015. PMID: 29147418. DOI: 10.14740/wjon901w

14 Han M, Liu Q, Yu J and Zheng S: Identification of candidate molecular markers predicting chemotherapy resistance in non-small cell lung cancer. Clin Chem Lab Med *48(6)*: 863-867, 2010. PMID: 20345233. DOI: 10.1515/CCLM.2010.169

15 Huang W, Ding X, Li B, Fan M, Zhou T, Sun H, Yi Y and Zhang J: Serum biomarkers analyzed by LC-MS/MS as predictors for short outcome of non-small cell lung cancer patients treated with chemoradiotherapy. Neoplasma *60(1)*: 11-18, 2013. PMID: 23067211. DOI: 10.4149/neo_2013_002

16 Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D and Verweij J: New response evaluation criteria in solid tumours: Revised RECIST

guideline (version 1.1). Eur J Cancer *45(2)*: 228-247, 2009. PMID: 19097774. DOI: 10.1016/j.ejca.2008.10.026

17 Verathamjamras C, Weeraphan C, Chokchaichamnankit D, Watcharatanyatip K, Subhasitanont P, Diskul-Na-Ayudthaya P, Mingkwan K, Luevisadpaibul V, Chutipongtanate S, Champattanachai V, Svasti J and Srisomsap C: Secretomic profiling of cells from hollow fiber bioreactor reveals PSMA3 as a potential cholangiocarcinoma biomarker. Int J Oncol *51(1)*: 269-280, 2017. PMID: 28560424. DOI: 10.3892/ijo.2017.4024

18 Metsalu T and Vilo J: ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res *43(W1)*: W566-W570, 2015. PMID: 25969447. DOI: 10.1093/nar/gkv468

19 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(–Delta Delta C(T)) Method. Methods *25(4)*: 402-408, 2001. PMID: 11846609. DOI: 10.1006/meth.2001.1262

20 Weeraphan C, Diskul-Na-Ayudthaya P, Chiablaem K, Khongmanee A, Chokchaichamnankit D, Subhasitanont P, Svasti J and Srisomsap C: Effective enrichment of cholangiocarcinoma secretomes using the hollow fiber bioreactor culture system. Talanta *99*: 294-301, 2012. PMID: 22967555. DOI: 10.1016/j.talanta.2012.05.054

21 Keeratichamroen S, Subhasitanont P, Chokchaichamnankit D, Weeraphan C, Saharat K, Sritana N, Kantathavorn N, Wiriyaukaradecha K, Sricharunrat T, Paricharttanakul NM, Auewarakul C, Svasti J and Srisomsap C: Identification of potential cervical cancer serum biomarkers in Thai patients. Oncol Lett *19(6)*: 3815-3826, 2020. PMID: 32391095. DOI: 10.3892/ol.2020.11519

22 Ciereszko A, Dietrich MA, Słowińska M, Nynca J, Ciborowski M, Kisluk J, Michalska-Falkowska A, Reszec J, Sierko E and Nikliński J: Identification of protein changes in the blood plasma of lung cancer patients subjected to chemotherapy using a 2D-DIGE approach. PLoS One *14(10)*: e0223840, 2019. PMID: 31622403. DOI: 10.1371/journal.pone.0223840

23 Niu N, Zhang J, Huang T, Sun Y, Chen Z, Yi W, Korteweg C, Wang J and Gu J: IgG expression in human colorectal cancer and its relationship to cancer cell behaviors. PLoS One *7(11)*: e47362, 2012. PMID: 23133595. DOI: 10.1371/journal.pone.0047362

24 Wan X, Lei Y, Li Z, Wang J, Chen Z, McNutt M, Lin D, Zhao C, Jiang C, Li J, Pu Q, Su M, Wang Y and Gu J: Pancreatic expression of immunoglobulin *g* in human pancreatic cancer and associated diabetes. Pancreas *44(8)*: 1304-1313, 2015. PMID: 26390427. DOI: 10.1097/MPA.0000000000000544

25 Zhang L, Hu S, Korteweg C, Chen Z, Qiu Y, Su M and Gu J: Expression of immunoglobulin G in esophageal squamous cell carcinomas and its association with tumor grade and Ki67. Hum Pathol *43(3)*: 423-434, 2012. PMID: 21855109. DOI: 10.1016/j.humpath.2011.05.020

26 Hsu HM, Chu CM, Chang YJ, Yu JC, Chen CT, Jian CE, Lee CY, Chiang YT, Chang CW and Chang YT: Six novel immunoglobulin genes as biomarkers for better prognosis in triple-negative breast cancer by gene co-expression network analysis. Sci Rep *9(1)*: 4484, 2019. PMID: 30872752. DOI: 10.1038/s41598-019-40826-w

27 Qiu X, Zhu X, Zhang L, Mao Y, Zhang J, Hao P, Li G, Lv P, Li Z, Sun X, Wu L, Zheng J, Deng Y, Hou C, Tang P, Zhang S and Zhang Y: Human epithelial cancers secrete immunoglobulin *g* with unidentified specificity to promote growth and survival of tumor cells. Cancer Res *63(19)*: 6488-6495, 2003. PMID: 14559841.

28 Zheng H, Li M, Liu H, Ren W, Hu DS, Shi Y, Tang M and Cao Y: Immunoglobulin alpha heavy chain derived from human epithelial cancer cells promotes the access of S phase and growth of cancer cells. Cell Biol Int *31(1)*: 82-87, 2007. PMID: 17074514. DOI: 10.1016/j.cellbi.2006.09.009

29 Bteich M: An overview of albumin and alpha-1-acid glycoprotein main characteristics: highlighting the roles of amino acids in binding kinetics and molecular interactions. Heliyon *5(11)*: e02879, 2019. PMID: 31844752. DOI: 10.1016/j.heliyon.2019.e02879

30 Hochepied T, Berger FG, Baumann H and Libert C: Alpha(1)-acid glycoprotein: An acute phase protein with inflammatory and immunomodulating properties. Cytokine Growth Factor Rev *14(1)*: 25-34, 2003. PMID: 12485617. DOI: 10.1016/s1359-6101(02)00054-0

31 Zhou Q, Andersson R, Hu D, Bauden M, Sasor A, Bygott T, PawŁowski K, Pla I, Marko-Varga G and Ansari D: Alpha-1-acid glycoprotein 1 is upregulated in pancreatic ductal adenocarcinoma and confers a poor prognosis. Transl Res *212*: 67-79, 2019. PMID: 31295437. DOI: 10.1016/j.trsl.2019.06.003

32 Boichenko AP, Govorukhina N, Klip HG, van der Zee AG, Güzel C, Luider TM and Bischoff R: A panel of regulated proteins in serum from patients with cervical intraepithelial neoplasia and cervical cancer. J Proteome Res *13(11)*: 4995-5007, 2014. PMID: 25232869. DOI: 10.1021/pr500601w

33 Zhu HZ, Zhou WJ, Wan YF, Ge K, Lu J and Jia CK: Downregulation of orosomucoid 2 acts as a prognostic factor associated with cancer-promoting pathways in liver cancer. World J Gastroenterol *26(8)*: 804-817, 2020. PMID: 32148378. DOI: 10.3748/wjg.v26.i8.804

34 Li F, Yu Z, Chen P, Lin G, Li T, Hou L, Du Y, Tan W. The increased excretion of urinary orosomucoid 1 as a useful biomarker for bladder cancer. Am J Cancer Res. 2016 Jan 15;6(2):331-40. PMID: 27186407

35 Wang Y, Song G, Wang Y, Qiu L, Qin X, Liu H, Li F, Wang X, Li F, Guo S, Zhang Y and Li Z: Elevated serum levels of circulating immunoinflammation-related protein complexes are associated with cancer. Journal of Proteome Research *13(2)*: 710-719, 2019. DOI: 10.1021/pr4008255

36 Ayyub A, Saleem M, Fatima I, Tariq A, Hashmi N and Musharraf SG: Glycosylated Alpha-1-acid glycoprotein 1 as a potential lung cancer serum biomarker. Int J Biochem Cell Biol *70*: 68-75, 2016. PMID: 26563422. DOI: 10.1016/j.biocel.2015.11.006

37 Ye X, Zhang N, Jin Y, Xu B, Guo C, Wang X, Su Y, Yang Q, Song J, Yu W, Cheng P, Cheng L, Gong Y, Fu X and Sun H: Dramatically changed immune-related molecules as early diagnostic biomarkers of non-small cell lung cancer. FEBS J *287(4)*: 783-799, 2020. PMID: 31482685. DOI: 10.1111/febs.15051

38 Hyung SW, Lee MY, Yu JH, Shin B, Jung HJ, Park JM, Han W, Lee KM, Moon HG, Zhang H, Aebersold R, Hwang D, Lee SW, Yu MH and Noh DY: A serum protein profile predictive of the resistance to neoadjuvant chemotherapy in advanced breast cancers. Mol Cell Proteomics *10(10)*: M111.011023, 2011. PMID: 21799047. DOI: 10.1074/mcp.M111.011023

39 Zhou Z, Li Z, Sun Z, Zhang X, Lu L, Wang Y and Zhang M: S100A9 and ORM1 serve as predictors of therapeutic response and prognostic factors in advanced extranodal NK/T cell lymphoma patients treated with pegaspargase/gemcitabine. Sci Rep *6*: 23695, 2016. PMID: 27021626. DOI: 10.1038/srep23695

40 Fijneman RJ, de Wit M, Pourghiasian M, Piersma SR, Pham TV, Warmoes MO, Lavaei M, Piso C, Smit F, Delis-van Diemen PM, van Turenhout ST, Terhaar sive Droste JS, Mulder CJ, Blankenstein MA, Robanus-Maandag EC, Smits R, Fodde R, van Hinsbergh VW, Meijer GA and Jimenez CR: Proximal fluid proteome profiling of mouse colon tumors reveals biomarkers for early diagnosis of human colorectal cancer. Clin Cancer Res *18(9)*: 2613-2624, 2012. PMID: 22351690. DOI: 10.1158/1078-0432.CCR-11-1937

41 Deschoolmeester V, Baay M, Specenier P, Lardon F and Vermorken JB: A review of the most promising biomarkers in colorectal cancer: One step closer to targeted therapy. Oncologist *15(7)*: 699-731, 2010. PMID: 20584808. DOI: 10.1634/theoncologist.2010-0025

42 Guo Y, Xiao P, Lei S, Deng F, Xiao GG, Liu Y, Chen X, Li L, Wu S, Chen Y, Jiang H, Tan L, Xie J, Zhu X, Liang S and Deng H: How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochim Biophys Sin (Shanghai) *40(5)*: 426-436, 2008. PMID: 18465028. DOI: 10.1111/j.1745-7270.2008.00418.x

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