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Original Article

Use of iTRAQ to discover serum proteins associated with acquired EGFR-TKIs resistance in patients with advanced lung adenocarcinoma

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Abstract: Background & Aim: Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) achieve clinical benefits in patients with lung adenocarcinoma (ADC), but a considerable amount of EGFR-TKI treated patients acquire resistance to TKI during the course of their treatments. The present work aimed to identify serum proteins associated with acquired TKI resistance in patients with advanced lung ADC. Method: Blood of patients with lung ADC (n=21) was collected before and after TKI resistance was acquired. Samples from age-matched healthy subjects (n=21) were also included as the baseline control. Sera prepared from the collected samples were then analyzed using isobaric tags for relative and absolute quantitation (iTRAQ) combined with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Proteins in sera were then identified and quantitated using Mascot 2.3.02. Functional and pathway enrichment analyses were performed using Ingenuity Pathway Analysis (IPA). Selected resistance-associated proteins were then validated using enzyme-linked immunosorbent assay (ELISA). Results: A total of 428 serum proteins were identified, and 47 of them were found associated with acquired TKI resistance in patients with advanced lung ADC. Thirty-three out of 47 resistance-associated proteins were up-regulated before the resistance was acquired, while 14 of 47 resistance-associated proteins were elevated after the resistance was developed. Enrichment analysis of the proteins indicated the potential involvement of resistance-associated proteins in signaling pathways for acute phase response, inflammatory reaction, ERK/MAPK signaling, etc. Serum levels of three selected resistance-associated proteins, ceruloplasmin (CP), apolipoprotein C-1 (APOC1), and alpha-1-acid glycoprotein 1 (ORM1), were validated using ELISA. Conclusion: iTRAQ combined with LC-MS/MS is an accurate, sensitive and high throughput method for the quantitative screening of serum proteins of patients with lung ADC. The identified TKI resistance-associated proteins are potential biomarkers for surveillance of TKI resistance and prognostication of patients, and novel targets for lung cancer treatment.

Keywords: Lung adenocarcinoma, acquired EGFR-TKI resistance, iTRAQ, LC-MS/MS

Introduction

The incidence and mortality rates of lung cancer are increasing worldwide. In many countries including China, lung cancer is the most prevalent malignancy, and is the leading cause of cancer-related mortality.

Non-small cell lung cancer (NSCLC) accounts for 80-90% of total lung cancer cases, and about 30% of NSCLC cases are histologically classified as lung adenocarcinoma (ADC) [1-3]. Epidermal growth factor receptor (EGFR) is a well-known, key oncogenic driver in NSCLC. About 35-40% of Asian patients with advanced NSCLC show mutations in EGFR [4]. EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib, erlotinib and icotinib have become standardized frontline treatment for non-metastatic NSCLC because of their satisfactory therapeutic efficacies [5]. Unfortunately, a considerable amount of cancer patients treated with these EGFR-TKIs ultimately acquire TKI resistance during the course of their treatments. The acquired resistance to TKI greatly limits the clinical benefits of EGFR-TKIs. In this context, early and accurate prediction of acquired TKI resistance, which utilizes biomarkers circulating in blood, is believed essential to the successful treatment of patients with NSCLC.

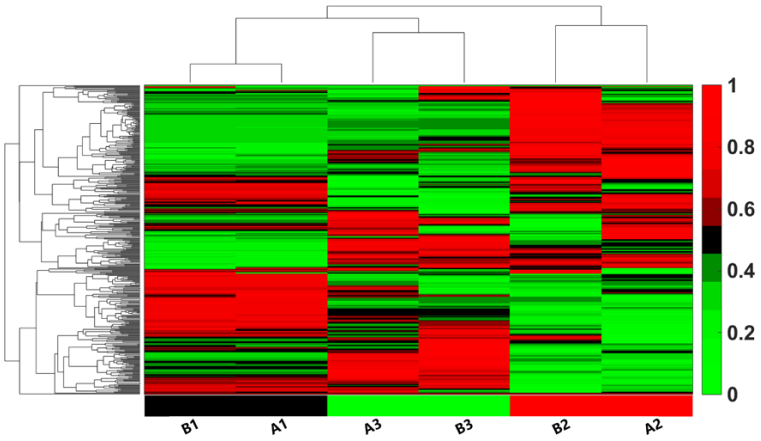

Figure 1. Clustering of serum samples according to the quantitative data of 428 serum proteins identified using iTRAQ combined with LC-MS/MS. The cluster was generated by Multiple Array Viewer. Column, serum samples; row, protein IDs.

Isobaric tags for relative and absolute quantitation (iTRAQ) is an efficient method for quantitative proteomics. The use of iTRAQ in cancer biomarker discovery from clinical samples has gained wide acceptance. This study was aimed to identify serum proteins that are associated with acquired TKI resistance in patients with lung ADC. As such, we collected blood from the patients before and after TKI was developed, and then employed iTRAQ combined with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify serum proteins that were differentially present before and after patients with lung ADC became resistant to TKI. The findings should not only provide insights into the underlying mechanisms of drug resistance, but also pave the way for the development of serum protein-based tests for early assessment of EGFR-TKI resistance in patients with lung cancer.

Materials and methods

Patient recruitment and blood collection

Blood was collected from patients with advanced lung ADC (i.e. stage IIIB or IV) harboring EGFR mutations, who were admitted to The First Hospital of Jiaxing, Zhejiang and Hangzhou Tumor Hospital from March 2014 to September 2015. Primary lung cancer cases and metastatic foci were confirmed by pathological examination. Patients with any disorders in cardiovascular, neurological or other systems were not included in this study. All patients received for the first time gefitinib, erlotinib or icotinib in their lifetimes. Blood collections were done before and after the patients acquired TKI resistance. The criteria to define resistance to EGFR-TKI were as published by Jackman et al [6]. A total of 21 samples were collected before TKI resistance appeared, while 21 samples were received after the resistance was confirmed. In addition to patients with advanced lung ADC, 21 age-matched healthy subjects having routine medical check up in our hospitals were also recruited. All subjects were fasted overnight before the collection of 4 mL peripheral blood at the next morning. Blood was placed at 4°C for one hour, and immediately followed by a centrifugation at 3000 rpm at 4°C. The sera obtained were stored in -80°C until use. The collection and use of human blood were approved by the ethics committee of our institute. From all patients with advanced lung ADC and healthy subjects informed consents for the study were obtained.

Protein extraction

Equal volume of serum from each subject in the same group was pooled to give a final volume of 1 mL. High abundance proteins in these samples were removed, while rare proteins were collected using ProteoMiner (Bio-Rad, Hercules, CA). The protein concentration was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Protein digestion and iTRAQ labeling

For each sample, 100 μg of protein was reduced at 60°C for 1 hour, followed by cysteine blocking at room temperature for 10 minutes. Five volumes of cold acetone were then added to one volume of sample for precipitation. After centrifugation, acetone was decanted, and 20 μL of dissolution buffer was added to dissolve the precipitated pellet. To each sample tube, trypsin (Sigma, Saint Louis, MO) was added in a final trypsin: protein ratio of 1:20 (w:w). The tubes were incubated at 37°C for overnight digestion. For labeling, each iTRAQ reagent was dissolved in 70 ìL of ethanol, and the mixture was added to the respective sample (label 113: healthy subjects; label 114: before drug resis-

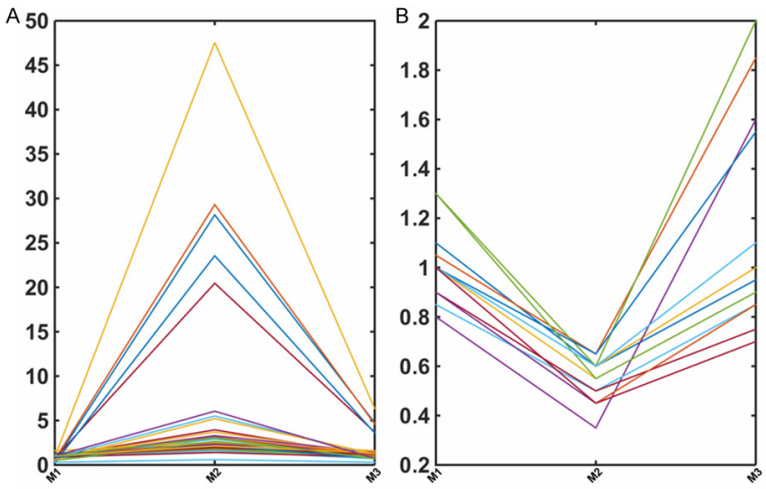

Figure 2. Graphs showing the expression patterns of acquired TKI resistance-associated proteins in samples of healthy subject (M1), samples of patients with lung ADC before TKI resistance was acquired (M2), and samples of patients with ADC after TKI resistance was developed (M3). A. Thirty-three resistance-associated proteins were elevated in M2 comparing to M1 and M3. B. Fourteen resistance-associated proteins were up-regulated in M3 comparing to M1 and M3. Each data point represents an average value of biological duplicates.

tance; label 115: after drug resistance). The tubes were then incubated for 1 hour.

Preparation of protein from fractionation for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis

The labeled samples from the three groups were mixed, and the first dimension separation of the proteins was carried out using ultra performance liquid chromatography (UPLC) (Waters Corp, Milford, MA) with C18 column (2.1 mm × 50 mm, 1.7 μm) (Waters). The UPLC analysis utilized flow rate of 600 μL/min, and the column was eluted with a linear gradient of phase B (nitrile) 5-35% and phase A of formic acid at pH 10. The gradient duration was 10 min. UV detection was carried out at 214 nm, and a total of 15 fractions were collected.

LC-MS/MS analysis

Peptides were separated in the second dimension using nano high performance liquid chromatography (nano-HPLC) with C18 reversed-phase column (75 μm × 150 mm, 3 μm) (Eksigent, Dublin, CA). A linear gradient 5-35% of Phase B (98% nitrile, 0.1% formic acid) was used, with the flow rate maintained at 300 nl/min and duration of 5-100 minutes. LC-MS/MS analysis was performed using Q Exactive spectrometer (Thermo Scientific, Rockford, IL), applying 250 V spray voltage. The mass-to-charge (m/z) ratio was 350-1600 with acumulative time of 250 s. The 10 strongest precursor ions per sample were chosen for MS/MS analysis, and the time window for dynamic exclusion was 30 seconds. Protein identification was performed twice for each sample.

Proteomics data analysis

Identification of proteins from mass spectrometry data was done using Mascot version 2.3.02 (Matrix Science, London, UK) with database SP_Human_20150825. Proteomic profiles were analyzed using Scaffold, with false positive rate (FDR) defined as < 1%. Differentially detected proteins were identified by comparisons between groups with fold change > 1.5 and $P < 0.05$. P values were determined using T-test. Functional and pathway enrichments of the identified proteins were then done using Ingenuity Pathway Analysis (IPA).

Enzyme-linked immunosorbent assay

Serum ceruloplasmin (CP) and apolipoprotein C-1 (APOC1) levels were determined using ELISA kits from Abnova (Walnut, CA), while that of alpha-1-acid glycoprotein 1 was measured using ELISA kit from R&D Systems (Minneapolis, MN). All ELISAs were done following manufacturers' instructions.

Statistical analysis

Quantitative measurements from ELISAs were analyzed using SPSS version 19.0, and were presented as mean ± S.D. Pairwise comparisons between mean values were done using Student's t test. $P < 0.05$ indicated statistically significance.

Results

Serum proteins associated with TKI resistance

In this study we identified totally 428 serum proteins. We used Multiple Array Viewer to cluster these proteins according to their quantita-

Table 1. List of proteins differentially expressed

Accession	Protein Name	Gene Name	114:113	*p*-value (114:113)	115:113	*p*-value (115:113)	115:114	*p*-value (115:114)	*Mode
CO3_HUMAN	Complement C3	C3	-0.87	0.00	-0.21	0.10	0.66	0.02	-1
FIBA_HUMAN	Fibrinogen alpha chain	FGA	6.14	0.01	3.57	0.00	-2.57	0.01	1
FIBB_HUMAN	Fibrinogen beta chain	FGB	5.61	0.00	2.94	0.00	-2.67	0.00	1
C9JEU5_HUMAN	Fibrinogen gamma chain	FGG	5.72	0.00	2.82	0.00	-2.90	0.00	1
C4BPA_HUMAN	C4b-binding protein alpha chain	C4BPA	0.88	0.01	0.25	0.04	-0.63	0.02	1
CERU_HUMAN	Ceruloplasmin	CP	0.75	0.03	-0.44	0.15	-1.19	0.02	1
A0A087X232_HUMAN	Complement C1s subcomponent	C1S	1.86	0.00	0.32	0.00	-1.54	0.00	1
CO5_HUMAN	Complement C5	C5	-0.69	0.03	0.82	0.04	1.51	0.02	-1
PON1_HUMAN	Serum paraoxonase/arylesterase 1	PON1	2.06	0.01	0.07	0.42	-1.98	0.01	1
B4DPQ0_HUMAN	Complement C1r subcomponent	C1R	1.36	0.01	0.25	0.10	-1.11	0.02	1
HRG_HUMAN	Histidine-rich glycoprotein	HRG	0.68	0.02	-0.18	0.10	-0.86	0.01	1
C9JV77_HUMAN	Alpha-2-HS-glycoprotein	AHSG	2.04	0.01	0.42	0.00	-1.62	0.01	1
APOC1_HUMAN	Apolipoprotein C-I	APOC1	-0.74	0.00	0.00		0.74	0.00	-1
PROS_HUMAN	Vitamin K-dependent protein S	PROS1	1.87	0.00	0.64	0.04	-1.24	0.01	1
C4BPB_HUMAN	C4b-binding protein beta chain	C4BPB	1.18	0.00	0.42	0.04	-0.77	0.00	1
CO6_HUMAN	Complement component C6	C6	-1.19	0.01	1.00	0.02	2.19	0.01	-1
J3KRP0_HUMAN	Beta-Ala-His dipeptidase	CNDP1	0.75	0.01	-0.44	0.04	-1.19	0.00	1
TRFE_HUMAN	Serotransferrin	TF	0.64	0.00	0.00	1.00	-0.64	0.04	1
PHLD_HUMAN	Phosphatidylinositol-glycan-specific phospholipase D	GPLD1	0.58	0.01	-0.06	0.42	-0.64	0.01	1
CO7_HUMAN	Complement component C7	C7	-1.12	0.00	0.62	0.00	1.74	0.00	-1
FCN2_HUMAN	Ficolin-2	FCN2	-0.77	0.02	0.00	1.00	0.77	0.02	-1
CFAI_HUMAN	Complement factor I	CFI	1.32	0.00	0.43	0.02	-0.89	0.01	1
H0YAC1_HUMAN	Plasma kallikrein heavy chain (Fragment)	KLKB1	0.67	0.02	0.00		-0.67	0.02	1
HEMO_HUMAN	Hemopexin	HPX	1.35	0.01	0.29	0.00	-1.06	0.01	1
K2C71_HUMAN	Keratin, type II cytoskeletal 71	KRT71	2.41	0.03	1.00	0.00	-1.41	0.04	1
F13B_HUMAN	Coagulation factor XIII B chain	F13B	2.69	0.00	0.44	0.05	-2.26	0.00	1
C9JPQ9_HUMAN	Fibrinogen gamma chain (Fragment)	FGG	4.77	0.03	2.30	0.04	-2.47	0.04	1
K0753_HUMAN	Uncharacterized protein KIAA0753	KIAA0753	6.56	0.02	3.85	0.04	-2.71	0.02	1
COF1_HUMAN	Cofilin-1	CFL1	-0.85	0.00	-0.26	0.10	0.58	0.04	-1
F5H8B0_HUMAN	Coagulation factor VII	F7	0.85	0.00	0.07	0.42	-0.78	0.00	1
H7BZ17_HUMAN	Sulfotransferase (Fragment)	SULT1C2	3.12	0.01	1.06	0.05	-2.06	0.01	1
H31T_HUMAN	Histone H3.1t	HIST3H3	-0.62	0.02	0.63	0.01	1.25	0.01	-1
C9JY79_HUMAN	Non-erythrocytic beta-spectrin 4	SPTBN4	-1.15	0.01	-0.23	0.10	0.92	0.03	-1
COL11_HUMAN	Collectin-11	COLEC11	1.98	0.00	0	1	-1.98	0.01	1
E7ERK6_HUMAN	Clusterin beta chain (Fragment)	CLU	-0.86	0.01	-0.15	0.00	0.71	0.02	-1

COX15_HUMAN	Cytochrome c oxidase assembly protein COX15 homolog	COX15	0.89	0.00	-0.07	0.42	-0.96	0.01	1
COPB_HUMAN	Coatomer subunit beta	COPB1	1.00	0.00	0.00		-1	0.00	1
PROP_HUMAN	Properdin	CFP	-1.00	0.01	-0.36	0.00	0.64	0.04	-1
A0A087X1C7_HUMAN	Ig gamma-1 chain C region	IGHG1	0.96	0.00	0.2	0.00	-0.70	0.00	1
H0YHH7_HUMAN	ATP-dependent RNA helicase DDX54 (Fragment)	DDX54	-1.24	0.00	-0.53	0.00	0.71	0.02	-1
FA11_HUMAN	Coagulation factor XI	F11	1.29	0.01	-0.26	0.42	-1.55	0.02	1
TETN_HUMAN	Tetranectin	CLEC3B	1.63	0.02	0.20	0.10	-1.43	0.02	1
S100P_HUMAN	Protein S100-P	S100P	-0.74	0.00	0.14	0.00	0.87	0	-1
A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	ORM1	1.10	0.02	0.43	0.02	-0.67	0.04	1
K2C5_HUMAN	Keratin, type II cytoskeletal 5	KRT5	-1.15	0.01	-0.51	0.00	0.64	0.04	-1
B7Z278_HUMAN	Rap guanine nucleotide exchange factor 4	RAPGEF4	2.60	0.01	-0.42	0.04	-3.01	0.01	1
PGM2_HUMAN	Phosphoglucomutase-2	PGM2	1.61	0.04	-0.51	0.10	-2.12	0.04	1

113, 114, and 115 represents protein level of healthy subject, patients before TKI resistance was acquired, and patients after TKI resistance was acquired, respectively. (*Mode: “1” represents higher expression of the protein in patients before drug resistance, when compared to healthy volunteer, while the protein had lower expression in patients after drug resistance, when compared to patients before drug resistance. “-1” is vice versa.).

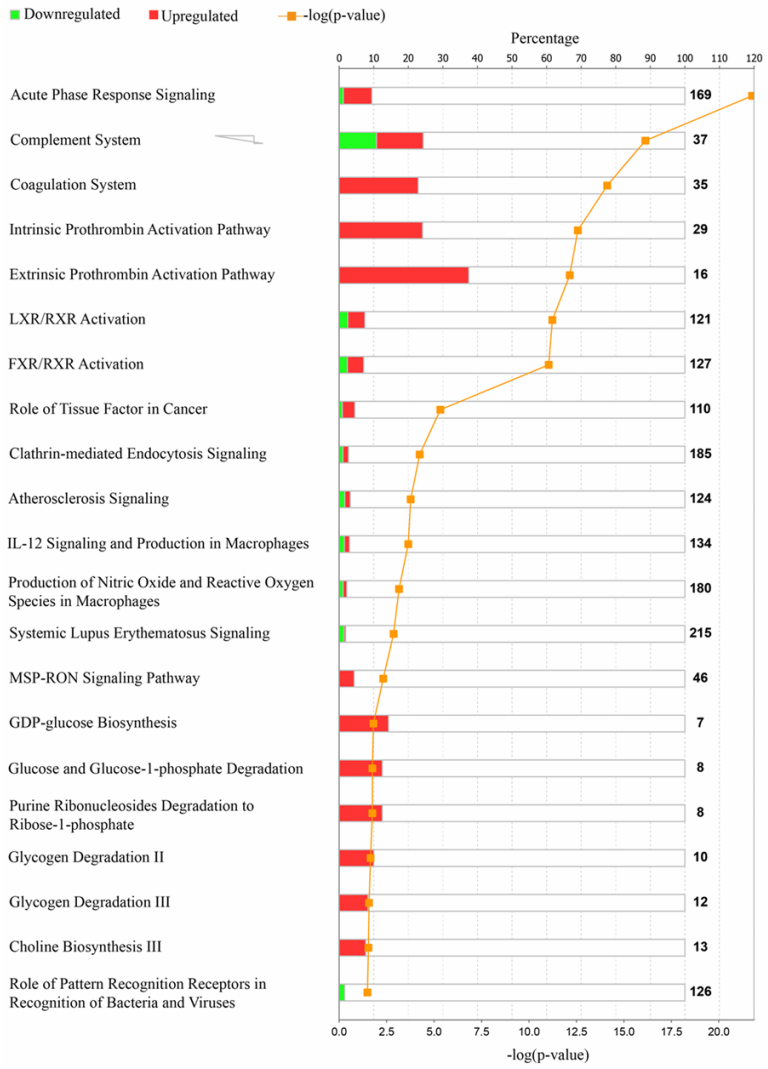

Figure 3. Enrichment of acquired TKI resistance-associated proteins into 21 signaling pathways using Ingenuity Pathway Analysis.

tive data into three major sample groups (i.e. healthy subject, before resistance and after resistance), while in each major group the two biological replicates shared high degree of similarity in protein levels (**Figure 1**).

Out of 428 proteins there were 397 proteins, of which the expression met the criteria for further quantitative analysis. We compared the expression of these proteins between healthy subject, before TKI resistance, and after TKI resistance (**Figure 2**). The analysis identified 47 serum proteins that were differentially expressed between groups. Thirty-three out of 47 proteins were found up-regulated in patients before drug resistance was acquired, while there were 14 proteins being elevated after the resistance was developed (**Table 1**). We then selected ceruloplasmin (CP), apolipoprotein C-1 (APOC1), and alpha-1-acid glycoprotein 1 (ORM1) for further validation using ELISA.

Enrichment analysis

In order to understand the functions and signaling networks of 47 newly identified drug resistance-associated proteins, we performed enrichment analysis through IPA approach. Our analysis revealed the resistance-associated proteins were enriched in 21 signaling pathways including acute phase response signaling, LXR/RXR activation, FXR/RXR activation, role of tissue factor in cancer, clathrin-mediated endocytosis signaling, IL-12 signaling and production in macrophages, B cell receptor signaling, ERK/MAPK signaling, etc (**Figure 3**). We also performed functional enrichment analysis. The analysis indicated the TKI resistance-associated proteins exhibited various functions including humoral immune response, inflammatory response, organismal injury and abnormalities, cell-to-cell signaling and interaction, cell death and survival, cellular assembly and organization, cellular development and function and maintenance, protein degradation and synthesis, immune cell trafficking, etc (**Figure 4**). In addition, our IPA illustrated that the TKI resistance-associated proteins were enriched into 3 interaction networks (**Figure 5**). The proteins were also implicated in tissue damages and disorders in immune, cardiovascular and respiratory systems.

ELISA of CP, APOC1, and ORM1

We validated the serum levels of CP, APOC1 and ORM1 in healthy subjects, and patients with advanced lung ADC before and after TKI resistance was acquired using ELISA (**Table 2**).

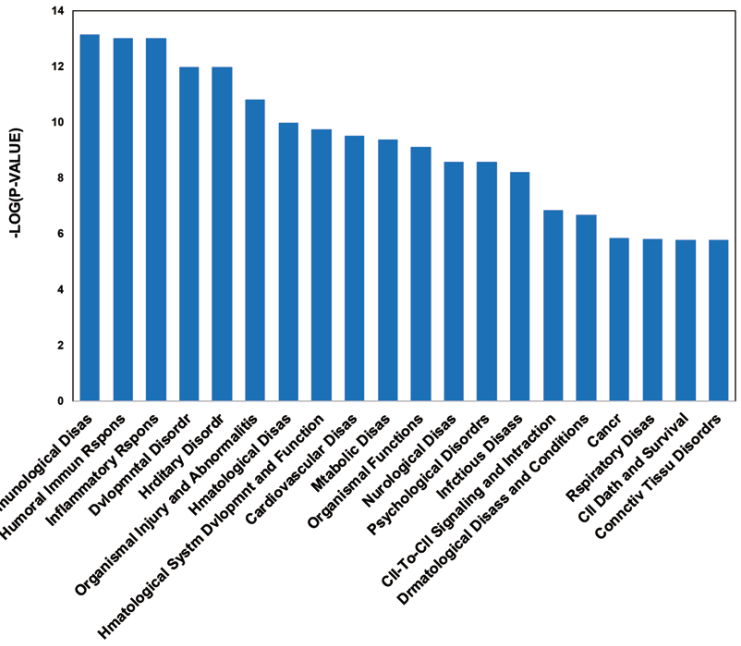

Figure 4. Enrichment of acquired TKI resistance-associated proteins into different functions using Ingenuity Pathway Analysis.

For CP and ORM1, the individual level before TKI resistance was acquired was 988.72 ± 175.08 μg/mL and 1.84 ± 0.084 μg/mL, respectively. The levels were significantly higher than those in healthy subjects (CP, 665.09 ± 103.17 μg/mL; ORM1, 1.72 ± 0.084 μg/mL ± 0.074 μg/mL) and those after TKI resistance was acquired (CP, 749.37 ± 318.42 μg/mL; ORM1, 1.77 ± 0.85 μg/mL). For APOC1, the level before TKI resistance (6.3 ± 2.88 μg/mL) was significantly lower than those in healthy subjects (11.35 ± 7.26 μg/mL) and after TKI resistance (11.15 ± 9.19 μg/mL). The comparisons were statistically significant.

Discussion

EGFR activates oncogenic pathways to stimulate cancer growth and to promote survival through its downstream tyrosine kinase. EGFR-TKIs have been emerged as efficient molecular-targeted therapies to suppress tumor growth, and to inhibit invasion and metastasis of cancer [7]. In addition, EGFR-TKIs were demonstrated to improve the overall and disease-free survivals of patients with NSCLC [8-10]. Strikingly, the response rate of cancer patients harboring EGFR mutations could be as high as 75% [11]. Unfortunately, despite the usefulness of EGFR-TKIs in cancer therapy, their therapeutic actions and clinical benefits were shown limited by drug resistance in a considerable amount of patients receiving TKI treatment [12-17]. Different mechanisms of drug resistance have been proposed, for example, EGFR T790M mutation, MET amplification, and PIK3CA mutations [18-20]. The underlying mechanism of acquired TKI resistance however remains to be fully understood. Biomarkers useful for surveillance of drug resistance are urgently needed. In this context, the present work studied serum proteins associated with drug resistance using iTRAQ.

The genetic profiles of NSCLC patients with acquired TKI resistance provided valuable information on how the treatment regimen would be modified and improved [21]. However, in most clinical scenarios, biopsies from patients with advanced NSCLC are challenging. Non-invasive circulating biomarkers are therefore more feasible, and indeed there was study illustrating the usefulness of such biomarkers to monitor the clinical responses of patients to drug treatment [22]. In line with this finding, circulating proteins secreted from cancer cells in NSCLC patients treated with TKI were believed to be valuable biomarkers for surveillance of drug resistance (ref). In recent years iTRAQ combined with LC-MS/MS have emerged as an accurate and high-throughput quantitative method for the measurement of dynamic changes in protein levels [23, 24]. The uses of iTRAQ in cancer biomarker discovery for malignant diseases including lung adenocarcinoma have been reported [25, 26], but biomarkers for the acquired drug resistance remains to be explored.

In this study we employed iTRAQ combined with LC-MS/MS to identify serum proteins associated with acquired resistance of TKI in patients with advanced NSCLC. We examined serum samples from healthy subjects, and the samples from patients with NSCLC before and after TKI resistance was acquired. There was a total

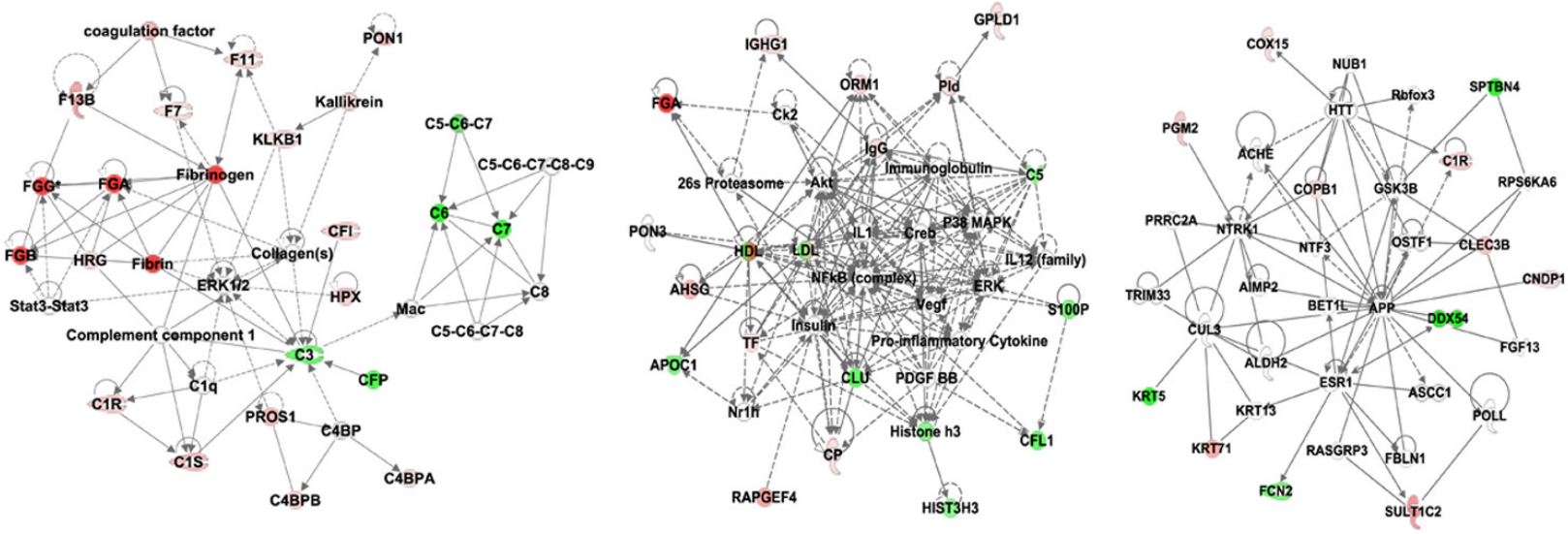

Figure 5. Building of 3 interaction networks from acquired TKI resistance-associated proteins (bold & colored blue) using Ingenuity Pathway Analysis. Up-regulated proteins are marked with red arrow, while the down-regulated are highlighted with green arrow.

Table 2. Serum levels of CP, ORM1, and APOC1 of healthy subjects and patients with lung ADC before and after TKI resistance was acquired (unit, μg/mL)

Group	t-test	ORM1	CP	APOC1
Healthy subjects (n=21)		1.72 ± 0.074	665.09 ± 103.17	11.35 ± 7.26
Before TKI resistance (n=21)		1.84 ± 0.084	988.72 ± 175.08	6.30 ± 2.88
After TKI resistance (n=21)		1.77 ± 0.85	749.37 ± 318.42	11.15 ± 9.19
Before TKI resistance vs Healthy subjects	*t*	-4.85	-7.30	2.96
	P	0.00	0.00	0.01
After TKI resistance vs Before TKI resistance	*t*	2.50	3.02	-2.31
	P	0.02	0.00	0.03

47 serum proteins found to be associated with TKI resistance. Thirty-three and 14 serum proteins were up-regulated before and after TKI resistance was acquired, respectively. We could enrich these serum proteins into some oncogenic signaling pathways. More importantly we successfully validated CP, APOC1, and ORM1 as potential biomarkers for acquired TKI resistance in patients with NSCLC using ELISA. CP, APOC1 and ORM1 are all synthesized in liver. Accumulated studies suggest that all of them should hold promise as biomarkers for malignant diseases. CP is a copper-carrying protein that serves as an anti-oxidant and a player in cell growth and blood vessel formation. In addition to Wilson disease, CP was shown elevated in malignancy and in infectious diseases [27, 28]. ORM1 is an alkaline protein that can hardly be detected in sera of healthy subjects. Serum ORM1 was found elevated in acute-phase response, inflammation and cancerous tumors [29, 30]. APOC1 is a protein component of high-density lipoprotein. It is responsible for the activation of esterified lecithin cholesterol with a key role in the exchange of esterified cholesterol between lipoproteins. APOC1 has long been utilized as a biomarker for cardiovascular diseases. In a phase I clinical study APOC1 was elevated in advanced lung adenocarcinoma comparing to the early one, indicating APOC1 as a possible target for lung cancer treatment [31].

Next-generation TKIs like afatinib and ceritinib have developed as new molecular-targeted therapies that bring hopes to patients with NSCLC. The third-generation EGFR inhibitors were also shown capable of overcoming acquired TKI resistance, achieving significant clinical benefits in patients harboring EGFR T790M [32-34]. However, despite these encouraging findings, lung cancer is still one of the leading causes of cancer-related mortality, and it remains urgent to discover new druggable targets of NSCLC. We here identified serum proteins associated with TKI resistance in clinical patients. Our subsequent analysis suggested the proteins would be new targets of NSCLC, and also potential biomarkers for drug resistance and personalized medicine. Nevertheless, the clinical utility of our identified biomarkers requires further validation in multiple cohorts. The potential adverse effect of metabolites in blood of patients will also be examined.

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Disclosure of conflict of interest

None.

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