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Global proteomic profiling identifies etoposide chemoresistance markers in non-small cell lung carcinoma

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ABSTRACT

Chemoresistance is one of the leading health concerns in cancer treatment. Understanding the mechanism of chemoresistance is the best way to improve the survival of the patient. Etoposide and its analogues are widely used as antitumor drugs in lung cancer but many etoposide resistant lung cancer cases has been identified in recent years. The present study aims to explore the cellular response of lung cancer cell lines to etoposide and finding the potential chemoresistant marker proteins. Multiple proteomic platforms like 2-DE, DIGE and iTRAQ have been used to study the global proteome profile of NCI-H460 and etoposide resistant NCI-H460R cell lines. Our study revealed that etoposide treatment leads to alteration of 83 proteins in NCI-H460R cell lines. The functional analysis highlighted the role of the differential expressed proteins in cellular signaling, apoptosis, and cytoskeleton reorganization. Our study has identified several new proteins like RHOC, DLG5, UGDH, TMOD3 in addition to known chemoresistance associated proteins. *In silico* prediction of the important selected candidates are further validated at protein and mRNA level. Further, functional studies of newly identified candidate genes RHOC and DLG5 revealed that chemotherapeutic resistance is associated with their elevated level and may serve as novel targets for therapeutic intervention.

Biological significance: Etoposide and its analogues have been used for lung cancer treatment for a while and it was reported that many non small cell lung carcinoma patients are resistant to etoposide. Although etoposide show drug resistance, the exact mechanism was not well understood. The present study focused on the global proteome analysis of NCI-H460 and NCI-H460R cell lines using multiple proteomic platforms to understand the potential chemoresistant markers for etoposide. Our multi-proteomic analysis has showed differential expression of 83 proteins involved in oxidative phosphorylation, metabolic, protein folding, cytoskeleton associated protein along with apoptotic pathway has been identified. In addition, quite a few interesting proteins such as RHOC, DLG5, HSP90, citrate synthase, UDP-glucose-6-dehydrogenase, Tropomodulin-3 are involved in chemoresistance has been observed. Overall, this is the first comprehensive proteomic study on etoposide resistant cell line NCI-H460 to explore the mechanism of chemoresistance in lung cancer.

1. Introduction

Lung cancer is one of the leading mortality oriented cancer in both men and women worldwide [1]. Various risk factors such as tobacco, alcohol, lifestyle and environmental pollution lead to lung cancer. Broadly, lung cancer is categorized into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC having low occurrence risks (15% cases) and can be treated using radiotherapy and chemotherapy whereas NSCLC has high risk of occurrence (80–85% cases) and shows chemoresistance to most of the anticancer drugs [2]. In NSCLC, the cells develop resistance through drug efflux pumps, changing the target molecule or gene mutation [3]. Moreover, the chemoresistant proteins are one of the key factors for recurrence of tumor. Besides, the diagnosis of lung cancer at the early stage is also a limitation to treat the disease. Therefore, identification of novel targets is the necessary step for treating the lung cancer effectively.

Etoposide is derived from natural product named podophylotoxin extracted from *Podophyllum peltatum*. The semi-synthetic form of etoposide is used to treat cancers such as lung cancer, sarcoma, testicular tumor, glioblastoma, blood cancer etc. [4]. Etoposide belongs to topoisomerase inhibitor family and it forms complex with DNA and topoisomerase II and prevent the ligation process [5]. Though etoposide is effective in lung cancer treatment, recently the chemoresistance has been observed against etoposide. Subsequently, many groups started exploring the mechanism of chemoresistance and reported that cancerous cells showed resistance either by altering the topoisomerase II or expressing the transport proteins viz. MDR1 (p170) and MRP to export

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the drug out [6,7]. Though few reports available in literature, the conclusive mechanism of etoposide-induced chemoresistance is still obscure.

Global proteome analysis profiles of lung cancer under drug resistance would be the right choice to explore the mechanism of chemoresistance. Gel-based and gel-free quantitative proteomics approaches have been evolved as key players in modern biology [8]. In the present study, we screened the cytotoxicity of etoposide against six selected NCI (National Cancer Institute) NSCLC cell lines and found that NCI-H460 is the most sensitive to etoposide treatment. We generated the etoposide resistant NCI-H460R cell line from the parent cell line NCI-H460. NCI-H460 and NCI-H460R cell lines were used to decipher the proteome alterations using multiple proteomic platforms such as two dimensional electrophoresis (2-DE), differential in gel electrophoresis (2D-DIGE) and isobaric tags for relative and absolute quantification (iTRAQ) in combination with LC-MALDI-TOF/TOF to understand the mechanism of chemoresistance. The applications of two modern high throughput proteomic approaches showed the global proteome profile with good coverage. Further, *in silico* analysis using DAVID, PANTHER and WebGEstalt highlighted that most of the differential expressed proteins involved in oxidative phosphorylation, apoptosis, protein folding, metabolism, and molecular chaperones. In addition, the gene expression analysis, immunoblotting and MRM analysis of selected targets have shown a good correlation with proteomic data. Additionally, gene knockdown experiment has showed that RHOC and DLG5 has role in chemoresistance. To the best of our knowledge, this is the first comprehensive global proteome analysis of etoposide resistance in lung cancer (NCI-H460 cell line) and has provided potential marker proteins involved in chemoresistance.

2. Materials and methods

2.1. Cell lines and culturing conditions

NSCLC cancer cell lines viz. A549, EKVX, HOP-62, HOP-92, NCI-H226 and NCI-H460 were grown in RPMI-1650 medium as monolayer supplemented with 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2 atmosphere in a humid condition. NCI-H460R cells were generated from parental NCI-H460 cells. The resistant cell line was generated by continuous exposure of NCI-H460 cells to stepwise increasing concentration of etoposide (1-10 μM). Both the cell lines were sub-cultured at the confluency of 80%.

2.2. Determination of inhibitory concentration

The cytotoxic effect of etoposide was evaluated by MTT (3-(4, 5 dimethylthiazol-2-yl)-2–5 diphenyltetrazolium bromide) assay. 3×10^3 cells (A549, EKVX, HOP-62, HOP-92, NCI-H226 and NCI-H460 cell lines) were seeded in each well of 96 well plates for 24 h. Different concentrations of the etoposide (0–100 μM) was added to the respective wells and incubated for 48 h. MTT solution (5 mg/ml stock) was added to each well after 48 h and incubated for another 3.5 h in humid 5% CO_2 incubator. Then, MTT solution containing media was replaced with MTT solvent [iso-propanol, HCl (4 mM) and triton X-100 (0.01%)] for each well and incubated for 15 min at room temperature (RT) with gentle shaking. Finally absorbance of the solution was measured at 590 nm using Elisa plate reader (Thermo scientific MULTISKAN GO V3.2). All experiments were carried out at least in biological triplicate. The percentage of viable cells was calculated as compared with DMSO treated control.

2.3. Cell cycle analysis using fluorescence activated cell sorting

Exponentially growing NCI-H460 and NCI-H460R cells were collected and washed with ice cold PBS for three times. The cells were fixed with 95% chilled ethanol and kept at 4 °C for 24 h. The cells were washed with ice cold PBS for three times and stained with propidium iodide (50 μg/mL) having RNAse (50 μg/ml) in PBS. The intensity of propidium iodide was measured using BD FACS Calibur instrument and the data was analyzed using Cell Quest Pro software. All the experiments were carried out in triplicate.

2.4. Colony formation assay

To assess the drug resistance ability of NCI-H460R cell line, we performed colony formation assay. Cells were collected after 48 h of exposure to 1 μM etoposide for both NCI-H460, NCI-H460R and 5000 cells were seeded in 35 mm culture dishes. Cells were allowed to grow for 14 days with changing fresh media in every 3 days. Colonies were washed with PBS, fixed in 100% methanol for 10 min and stained for 30 min in shaking with crystal violet. Further, the plates were washed with 1× PBS to remove excess stain and image of the plate was captured.

2.5. Whole cell protein extraction from cell lines

Exponentially growing NCI-H460 and NCI-H460R cells were harvested and washed with PBS buffer for three times followed by resuspension in lysis buffer (8 M urea, 2% CHAPS and 30 mM Tris) having nuclease and protease inhibitor. Cells were lysed on ice for 15 min and subsequently centrifuged at 15,000 rcf for 15 min at 4 °C. To the cell lysate, six volumes of chilled acetone was added and kept at −20 °C for overnight. Protein pellet was collected after centrifugation at 8000 rcf for 15 min at 4 °C. The protein pellet was dissolved in rehydration buffer (7 M urea, 2 M thiourea and 2% CHAPS). The protein sample was passed through 3 kD molecular weight cut-off (MWCO) centrifugal filter (Millipore) to remove the salt interferences. Finally, the protein concentration was measured using 2D Quant kit (GE healthcare).

2.6. Two-dimensional gel electrophoresis and image analysis

The desalted protein (1 mg per strip) was diluted with DeStreak rehydration buffer (GE healthcare) having DTT and IPG buffer. The processed protein was subjected to rehydration on 24 cm IPG strips (linear range of pH 3–10) for 18 h at RT. The rehydrated strips were focussed using Ettan IPGphor 3 instrument (GE healthcare). The following voltage settings were used for the isoelectric focussing: 200 V for 4 h (step), 500 V for 1 h (step), 1000 V for 1 h (step), 1000–8000 V for 3 h (gradient), 8000 V for 8 h (step) and 500 V for 4 h (step). The focussed IPG strips were equilibrated with 15 mL of equilibration solution-I (8 M urea, 1.5 M Tris–HCl pH 8.8, 1% DTT and BPB) and equilibration solution-II (8 M urea, 1.5 M Tris–HCl pH 8.8, 2.5% IAA, glycerol and BPB) for 15 min each with continuous gentle shaking. The second-dimension was performed using 12.5% SDS-PAGE. Gels were stained with PhastGel CBB followed by destaining to visualize the protein spots. The scanned images of the gels were analyzed using Image Master 2D Platinum 7 (IMP7 from GE healthcare) by importing triplicate images of parent and resistant cell line gels. The spot detection and matching was done automatically and manual inspection was done to remove the artifacts. The protein spots showing the statistically significant differential expression were considered for downstream MALDI-TOF/TOF identification.

2.7. CyDye labeling, DIGE and data analysis

Proteins were quantified and labeled with Cydyes as reported by Reddy et al. [9]. In brief, the pH of the protein sample was adjusted to 8.5 using 50 mM NaOH. Protein (60 μg) from both parent and resistant cell lines were taken and labeled with 400 pmol of Cydyes. The parent sample was labeled with Cy5, resistant sample was labeled with Cy3 and internal control having 1:1 concentration of both samples was labeled with Cy2. Dye swapping was performed to get rid of dye labeling biasness. A schematic diagram of the workflow used for proteomic