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Research paper

Identification of *NMU* as a potential gene conferring alectinib resistance in non-small cell lung cancer based on bioinformatics analyses

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ABSTRACT

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, and adjuvant targeted therapy has shown great benefits for the NSCLC patients with specific genomic mutations. Alectinib, a selective anaplastic lymphoma kinase (ALK) inhibitor, has been clinically used for the NSCLC patients with ALK-rearrangement, however, irreversible therapeutic resistance for the patients receiving alectinib treatment frequently occurs. Here we show that neuromedin U (*NMU*) may confer the alectinib resistance in NSCLC via multiple mechanisms based on the integrative bioinformatics analyses. Through employing the bioinformatics analyses of three microarray datasets, *NMU*, overexpressed in both NSCLC tissues and alectinib-resistant NSCLC cells, was initially identified as potential candidate for causing alectinib resistance in NSCLC. The resistance function of *NMU* in NSCLC was validated by performing protein/gene interactions and biological process annotation analyses, and further validated by analyzing the transcription factors targeting *NMU* mRNA. Collectively, these results indicated that *NMU* may confer alectinib resistance in NSCLC.

1. Introduction

Lung cancer is the most frequently diagnosed malignancies, and also the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, which accounts for > 80% of all cases (Torre et al., 2015). Currently, surgical resection is the preferred treatment option for NSCLC patients in early-stage (Herbst et al., 2018). However, adjuvant targeted therapy has also shown dramatical benefits for the patients with specific genomic alterations, especially for the NSCLC patients with anaplastic lymphoma kinase (ALK) mutations (Naylor et al., 2016). Although these molecular targeted drugs have shown great antitumor effects, irreversible therapeutic resistance for the patients receiving the treatments is still present (Lu et al., 2017).

Alectinib is a selective ALK inhibitor with a 94% objective response ratio in NSCLC patients with ALK-rearrangement (Reckamp, 2014), therefore, it has been a mainstay treatment option for ALK-positive NSCLC patients (Ziogas et al., 2018). However, the patients receiving alectinib may subsequently acquire drug resistance, even tumor relapse (Isozaki et al., 2016). Thus, a more holistic elaboration of the mechanisms leading to acquired alectinib resistance can facilitate to develop more effective therapeutics (Katayama et al., 2014; Kodama et al., 2014). Recently, the high-throughput platforms, such as microarrays, have been increasingly valued as promising tools to obtain comprehensively gene alterations for multiple diseases (Goldstein et al., 2008; Kulasingam et al., 2010). It has been widely used in the clinic, such as cancer diagnosis and the prognostic response of targeted drugs to the tumors. These analyses also provide novel research strategies for studying the molecular mechanisms of drug resistance for tumors (Zou et al., 2015).

Therefore, here we retrieved the datasets from the Gene Expression Omnibus (GEO) and conducted a systematically bioinformatics analyses to identify the potential genes promoting alectinib resistance in NSCLC.

Abbreviations: NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase; GEO, gene expression omnibus; DEGs, differentially expressed genes; DAVID, database for annotation, visualization and integrated discovery; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; KM plotter, Kaplan-Meier plotter; NMU, Neuromedin U; HR, hazard ratio; SLC2A5, solute carrier family 2 member 5; DEPDC 7, DEP domain containing 7; SULT1C2, sulfotransferase family 1C member 2; TM4SF4, transmembrane 4 L six family member 4; CHEK1, checkpoint kinase 1; IL1RN, interleukin 1 receptor antagonist; MYCN, MYCN proto-oncogene, bHLH transcription factor; PIM1, pim-1 proto-oncogene; CDK4/6, cyclin-dependent kinases 4/6; PI3K, phosphoinositide 3-kinase; BP, biological process; CC, cellular component; MF, molecular function

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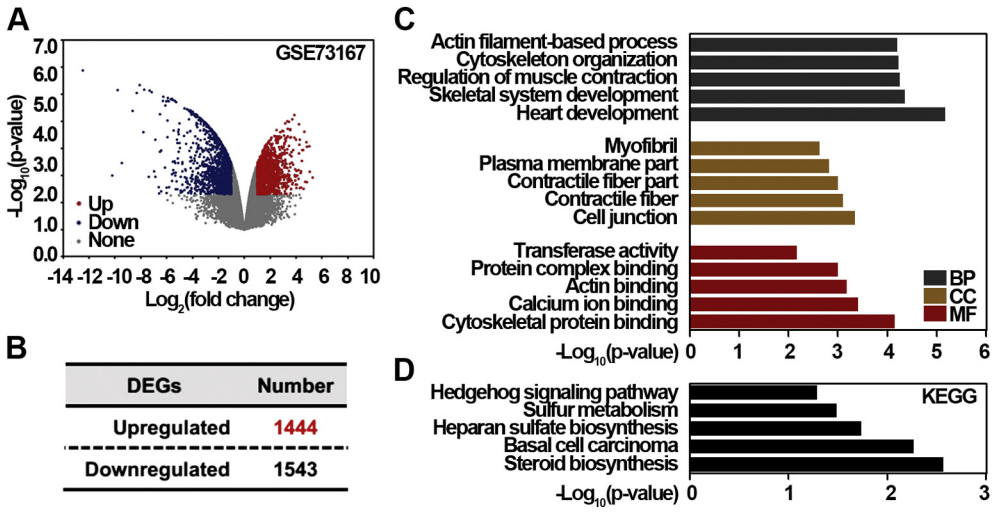

Fig. 1. Identification and functional characterization of DEGs from GSE73167 dataset. (A) Volcano plot of DEGs between alectinib-resistant NSCLC cell lines and the parental cell line. Red dots, significantly upregulated DEGs in alectinib-resistant cells; blue dots, significantly downregulated DEGs in alectinib-resistant cells; gray dots, no significant difference. $P < 0.05$ and fold-change > 2 were considered as significant. (B) The distribution of significant DEGs in alectinib-resistant cells. Top 5 GO terms (C) and KEGG enriched pathways (D) of significantly upregulated DEGs in alectinib-resistant cells were indicated. BP, biological process; CC, cellular component; MF, molecular function. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Microarray data

In our current study, the gene expression profiles of GSE73167, GSE19804, and GSE10072 were obtained from the GEO (https://www.ncbi.nlm.nih.gov/geo) as we previously introduced (Huang et al., 2018). GSE73167 dataset is consisted of 3 alectinib-resistant H2228 replicates and 1 parental H2228 cells, which is the NSCLC adenocarcinoma cell line (Isozaki et al., 2016); GSE19804 dataset is composed of 60 pairs of human NSCLC and adjacent normal tissues, which most of the tumors are adenocarcinomas (93%) (Lu et al., 2010); GSE10072 contains 58 human lung adenocarcinoma tissues and 49 normal lung tissues (Landi et al., 2008).

2.2. Processing of microarray data

The raw microarray data files of these 3 downloaded datasets were analyzed by using the GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/). This online tool can be used to compare two or more groups of samples in the same experimental settings (Barrett et al., 2013). *P* value adjusted to 0.05 and $|\log FC| > 1$ were set as the cut-off criteria.

2.3. Functional and pathway enrichment analyses

To investigate functional annotations of the differentially expressed genes (DEGs), we then employed the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.abcc.ncifcrf.gov/) to conduct the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. $P < 0.05$ was set as the statistically significant threshold.

2.4. Survival analysis

Kaplan-Meier plotter (KM plotter, https://www.kmplot.com) is an online survival analysis tool, which can be used to assess the effect of 54,675 genes on overall survival via using 10,461 cancer samples including 5143 breast, 1816 ovarian, 2437 lung, and 1065 gastric cancer patients (Lanczky et al., 2016). To evaluate the association between neuromedin U (*NMU*) level and its clinical outcomes, KM plotter was then employed to compute the survival curves. *NMU* was entered as the gene symbol, and the analysis was performed. Patients with lung cancer were respectively separated into high- and low-expression groups based on the level of *NMU*, and the overall survival was next analyzed. The hazard ratio (HR) with 95% confidence intervals and log rank *p* value were calculated.

2.5. Analysis of NMU by GeneMANIA and Coremine

To predict potential functions of *NMU*, we subsequently employed the GeneMANIA (https://www.genemania.org/), a widely used web interface for functional prediction of specific genes, as previously reported (Warde-Farley et al., 2010). In addition, the annotation of biological processes involving *NMU*, NSCLC, and drug resistance was further conducted via consulting the Coremine Medical online database (https://www.coremine.com/medical/).

2.6. Identification of transcription factors targeting NMU

To obtain the transcription factors regulating *NMU*, FunRich (https://www.funrich.org), an interaction network analysis tool that can identify the enriched transcription factors for genes, was then employed (Benito-Martin and Peinado, 2015).

2.7. Statistical analysis

GraphPad Prism 5 software (GraphPad Software, Inc., USA) was employed to analyze the data. A significant difference was indicated as $p < 0.05$.

3. Results

3.1. Identification and functional characterization of upregulated DEGs in alectinib-resistant NSCLC cells

To obtain the potential genes conferring alectinib resistance in NSCLC, we first retrieved the GSE73167 dataset that contains alectinib-resistant and its parental cells, and further identified 2987 DEGs by using 2-fold change and *p* value < 0.05 as the threshold cutoff (Fig. 1A). Among these DEGs, in detail, 1444 were significantly upregulated, while 1543 were significantly downregulated in alectinib-resistant cells (Fig. 1B). To characterize the functions of these significantly upregulated DEGs, GO and KEGG analyses were then performed as previously introduced (Xing et al., 2016). Top 5 GO terms and enrichment pathways were exhibited, and these significantly upregulated DEGs were highly associated with heart development, cytoskeletal protein binding, and cell junction (Fig. 1C). Interestingly, as shown in Fig. 1D, these significantly upregulated DEGs were also enriched in steroid, heparan biosynthesis, and also basal cell carcinoma (Fig. 1D).

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