Targeting 14-3-3ζ overcoming resistance to EGFR-TKI in lung adenocarcinoma via BMP2/Smad/ID-1 signaling

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SUBJECT AREAS

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14 3 3ζ, lung adenocarcinoma, EGFR TKI resistance, BMP2/Smad/ID 1 signaling, signaling pathway
Abstract

Background: 14-3-3ζ protein which acts as a putative oncoprotein has been found to promote proliferation, metastasis and chemoresistance of cancer cells in several cancers including lung adenocarcinoma (LUAD), however, its significance in epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) resistance remains unknown.

Methods: The Cancer Genome Atlas (TCGA) database was used to determine 14-3-3ζ expression in pan-cancer and LUAD. 14-3-3ζ and ID1 expression was then examined in clinical LUAD samples by immunohistochemistry (IHC). Lentiviral transfection with 14-3-3ζ-specific shRNA was used to establish stable 14-3-3ζ knockdown gefitinib resistant PC9 (PC9/GR) and H1975 cell lines. The effect of 14-3-3ζ knockdown on reversing EGFR-TKI resistance was determined in vitro by CCK-8, wound healing, transwell assays and flow cytometry. A xenograft tumor model was established to evaluate the role of 14-3-3ζ in EGFR-TKI resistance. Microarray analysis results showed the multiple pathways regulated by 14-3-3ζ-shRNA.

Results: In the present study, we firstly demonstrated that 14-3-3ζ expression was elevated and predicted unfavourable prognosis in pan-cancer including LUAD based on TCGA. In addition, high 14-3-3ζ expression was significantly associated with advanced T stage, TNM stage, present of lymph node metastasis and, importantly, poor treatment response to EGFR-TKI in LUAD patients with EGFR-activating mutations. 14-3-3ζ shRNA significantly sensitized EGFR-TKI-resistant human LUAD cells to gefitinib and, notably, reversed epithelial-to-mesenchymal transition (EMT). BMP signaling activation was decreased in EGFR-TKI resistant cells followed by 14-3-3ζ depletion in microarray analysis, which was further validated by Western blot analysis. Furthermore, the expression of 14-3-3ζ positively correlates with ID1 expression in human EGFR-mutant LUAD patient samples. In vivo, 14-3-3ζ shRNA and gefitinib treatment resulted in a significant reduction in the tumor burden compared to that treated with gefitinib alone.

Conclusion: Our work uncovers a hitherto unappreciated role of 14-3-3ζ in EGFR-TKI resistance. This study might provide a potential therapeutic approach for treating LUAD patients harboring EGFR mutations.
Full Text
Due to technical limitations, full-text HTML conversion of this manuscript could not be completed.

However, the manuscript can be downloaded and accessed as a PDF.

Supplementary Figure Legends

**Supplementary Figure S1**
The Kaplan-Meier survival curves of OS and PPS comparing high and low 14-3-3ζ expression estimates of LUAD patient according to the database of Kaplan–Meier plotter. Notes: The desired Affymetrix ID is valid: 200641_s_at (YWHAZ).

**Supplementary Figure S2**
Representative immunohistochemistry staining images of low expression and high expression of ID-1 in LUAD tissues. Magnification, ×100

**Supplementary Figure S3**
Photographs of mice at 28 days after inoculation using H1975/Control or H1975/14-3-3ζ-shRNA cells treated with PBS or gefitinib.

Figures
Figure 1

Elevated 14-3-3ζ expression positively correlates with poor prognosis of LUAD patients. (a) Relative mRNA levels of 14-3-3ζ in different tumors were analyzed by GEPIA. (b) Representative immunostaining images for 14-3-3ζ expression in 128 human LUAD tissues with EGFR mutant (Tumor) and 31 normal LUAD tissues (Nontumor). Original magnification, ×400. Histogram showing percentages of 14-3-3ζ expression for tumor and non-tumor tissues. (c) Western blot analysis of 14-3-3ζ expression in lysates originating from 10 human LUAD samples with EGFR mutant (T) and matched adjacent tissues (N). Right panel: quantification of relative expression levels of 14-3-3ζ. β-actin was used as a loading control. (d) Kaplan-Meier analysis of overall and disease-free survival for high and low 14-3-3ζ expression levels in pan-cancer determined from the GEPIA. (e) Kaplan-Meier analysis of overall and disease-free survival for high and low 14-3-3ζ expression levels in LUAD determined from the GEPIA. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)
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The clinicopathological signature of 14-3-3ζ in LUAD with EGFR-activating mutations. (a) Representative immunohistochemistry staining images of low expression and high expression of 14-3-3ζ in LUAD tissues. Magnification, ×100. (b) The protein expression level of 14-3-3ζ was classified as low or high based on the intensity and proportion of positively stained cells in these specimens. The percentages of patients with different T stage were assigned according to the expression level of 14-3-3ζ. (c) The percentages of patients with different TNM stage were assigned according to the expression level of 14-3-3ζ. (d) The percentages of patients with and without lymph node metastasis were assigned according to the expression level of 14-3-3ζ. (e) Representative immunostaining profiles of 14-3-3ζ in
drug-sensitive (PFS≥6 months, n = 21) and drug-insensitive (PFS<6 months, n = 20) LUAD tissues. Magnification, ×100 and ×400(left panel). The percentages of patients with high expression (black bar) and low expression of 14-3-3ζ (grey bar) were assigned according to different responses to EGFR-TKI (right panel). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

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in NCI-H1975 and PC9/GR cells and in cells transfected with Scrambled shRNA (Control) or 14-3-3ζ-shRNA (shRNA). (c) H1975/14-3-3ζ-shRNA (left panel), PC9/GR/14-3-3ζ-shRNA (right panel) and corresponding vector control cells were treated with the indicated doses of gefitinib for 48 h, and cell viability was analyzed by a CCK-8 assay. (d) Flow cytometric analysis of apoptosis in the indicated cells treated with gefitinib as assessed by Annexin V and 7-AAD staining. A representative flow profile is shown (left panel), and a summary of the percentage of Annexin V-positive cells is shown (right panel). (e) Transwell assays were conducted to assess TKI-resistant cell migration and invasion after 14-3-3ζ knockdown in cells cultured in the presence of gefitinib compared with those of corresponding vector control cells (i.e., crystal violet staining of migratory and invasive cells). Original magnification, ×100. (f) A wound healing assay was performed in the indicated cells as described in e. Original magnification, ×100. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)
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value of Fold Change ≥1.5 and FDR <0.05. The gray dots represent other genes that have no significant difference. (c) The bar graph shows the significant enrichment of differentially expressed genes in the classical signaling pathways. According to IPA’s internal algorithms and standards, Z-score ≥ 2 means that the pathway is significantly activated, and Z-score ≤-2 means that the pathway is significantly inhibited. (d) Heatmap showing the differential expression of BMP2/Smad5/ID-1 pathway gene signatures in NCI-H1975 cells infected with lentivirus expressing either scrambled-shRNA (blue) or 14-3-3ζ-shRNA (red). Genes and samples are listed in the rows and columns, respectively. A color key for the normalized expression data is shown at the top of the microarray heatmap (green represents downregulated genes; red represents upregulated genes). (e) NCI-H1975 and PC9/GR cells were transfected with 14-3-3ζ-shRNA (shRNA), scrambled shRNA(control), or left untreated (Untreated). Expression levels of BMP2, BMPR2, p-smad1/5, Smad1, Smad5 and Id1 were determined using Western blot. (f) The correlation between 14-3-3ζ and BMPR2, Smad5 mRNA expression was identified by the TCGA database. (g) Representative images of immunohistochemical staining for 14-3-3ζ and ID1 in serial sections of LUAD samples from patients. Case 1 is representative of a patient with non-14-3-3ζ-overexpressing lung cancer, whereas Case 2 is representative of a patient with 14-3-3ζ-overexpressing LUAD (left panel). A statistically significant correlation between 14-3-3ζ-high expression and ID1-high expression in 41 cases of LUAD tissues. The expression levels of 14-3-3ζ and ID1 were determined by immunostaining (right panel). Magnification, ×100 and ×400. (*p<0.05)
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