EFNA3 Can Serve as Potential Prognostic Biomarker for Luad Patients: A Comprehensive Analysis of the Eph/Ephrin Family

Gang Hou (hougangcmu@163.com)
The First Hospital of China Medical University https://orcid.org/0000-0003-3438-1764

Mingming Deng
China-Japan Friendship Hospital

Run Tong
China-Japan Friendship Hospital

Zhe Zhang
Shenjing Hospital of China Medical University

Tao Wang
Shenyang Kingmed Center

Chaonan Liang
The First Hospital of China Medical University: The First Affiliated Hospital of China Medical University

Xiaoming Zhou
Fourth Affiliated Hospital of China Medical University

Research

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Abstract

Background: Ephrin receptors (Eph) and their ligands called ephrins, function in various disease processes. However, the specific mechanism of Eph/ephrins in lung adenocarcinoma (LUAD) are still unclear.

Methods: Oncomine and GEPIA databases were used to explore the differential expression of Eph/ephrins in LUAD. The Kaplan–Meier plotter was selected to explore the prognostic value of Eph/ephrins. The cBioPortal database was used to analyze the genetic variation of the EFNA3 gene. Clinical LUAD tissue was analyzed by immunohistochemistry identifying the clinical value of identifying the ephrin-A3 protein. Weighted Co-expression Network Analysis (WGCNA) and Gene set enrichment analysis (GSEA) identified the potential regulatory mechanism of EFNA3.

Results: EPHA10, EFNA3/4/5 and EPHB1/2 mRNA expression levels were significantly increased in LUAD. EFNB1/2 and EPHB6 expression levels were significantly decreased. Prognostic analysis showed that EFNA3, EFNB1/2, and EPHB2 expression were significant correlate with both overall survival (OS) and progression-free survival (PFS) in LUAD patients. Next, the expression of the EFNA3 protein was increased in LUAD tissues and was designated as an independent risk prognosis factor. Mechanistically, EFNA3 may be involved in nuclear division, synaptic function, and ion channel activity-related pathways. Additionally, higher expression of EFNA3 was significantly correlated with OS in pan-cancer patients.

Conclusions: This study revealed the abnormal expression and prognostic value of Eph/Ephrin family members in LUAD. In addition, it is emphasized that EFNA3 may be a novel biomarker for the diagnosis and prognosis of LUAD patients.

Introduction

Lung cancer is the most commonly diagnosed cancer accounting for nearly 20% of cancer deaths in 2018[1]. Lung adenocarcinoma (LUAD) is a predominant pathologic subtype of lung cancer[2]. Despite progress in comprehensive therapies including surgery, radiotherapy, and targeted therapy over the past 20 years, the OS of LUAD patients remains poor[3, 4]. Therefore, continued exploration of the prognostic biomarkers and therapeutic targets for LUAD in patients is necessary thereby providing more individualized therapies for better prognosis.

Ephrin receptors (Eph) and ephrins, their membrane-anchored ligands, are essential for the development and organization of multicellular organisms. Eph/ephrins have been shown to function in various disease-related processes[5–8]. Ephs are activated by binding to ephrin ligands. Ephs and ephrins are divided into two subfamilies. First, EphA receptors (EphA1 to EphA8 and EphA10) primarily bind to GPI-anchored ephrin-A ligands (EFNA1 to EFNA5). The second are the EphB receptors (EphB1 to EphB4 and EphB6) that preferentially engage transmembrane ephrin-B ligands (EFNB1 to EFNB3)[9, 10]. Aberrant expression of Eph/ephrins has been identified in different types of human cancers. The mode of action has been implicated to affect malignant tumors through two-way signal transduction as well as
interaction with other signaling systems[11]. Recent studies have shown that EphA2, Ephrin-A1, and Ephrin-B2 are closely implicated in the prognosis of patients with LUAD[12, 13]. However, the specific mechanism of Eph/ephrins in this process is still unclear.

In this study, the expression levels and prognostic value of the Eph/ephrins family in LUAD was analyzed using bioinformatics analysis. EFNA3 was further analyzed to verify expression levels and prognostic value in LUAD through clinical samples. Finally, the biological function and regulation mechanism of EFNA3 was explored. Taken together, these findings indicate that EFNA3 plays an important role in LUAD and may act as a potential prognostic biomarker.

Materials And Methods

Bioinformatics Analysis

The Oncomine database (http://www.oncomine.org) is a tumor microarray database, that has collected 715 microarray data sets as well as 86,733 cancer and normal tissue sample data sets[14]. In this study, the Oncomine database was used to analyze the expression levels of the Eph/Ephrin family in different types of cancers.

The GEPIA database[15] (http://gepia.cancer-pku.cn/) was also used to analyze Eph/Ephrin expression in the TCGA-LUAD database.

Likewise, the Kaplan–Meier plotter (http://kmplot.com) is a tool for evaluating prognostic markers for breast cancer, ovarian cancer, lung cancer, and gastric cancer[16]. In this study, the Kaplan-Meier plotter was used to analyze the prognostic value of the Eph/Ephrin family in lung adenocarcinoma. The cBioPortal database (http://www.cbiointerportal.org) is an open-source database of DNA copy number, DNA methylation, and mutations based on the TCGA database[17]. Here, cBioPortal was used to analyze genetic variation of the EFNA3 gene.

Gene set enrichment analysis (GSEA) was performed as previously described[18]. The EFNA3-high/low groups were divided following the median expression of EFNA3 based on the TCGA database. The MSigDB KEGG gene set was used as a reference.

Patients And Tissue Samples

There were 74 primary LUAD tissue samples and paired normal lung tissue samples collected from Shengjing Hospital, China Medical University. Clinicopathological data were obtained from medical records and pathological reports. The study was approved by the Human Ethics Review Committee of Shengjing Hospital, China Medical University.

Immunohistochemistry (IHC) Analysis
IHC staining was performed and IHC scores were measured as described previously[18]. An EFNA3 (Catalog# ab89472) antibody was obtained from Abcam Corp, Ltd (Cambridge, UK), and use at a dilution of 1:100.

**Weighted Co-expression Network Analysis (wgcna)**

To explore the potential function of EFNA3, weight co-expression network analysis (WGCNA) was constructed. Approximately 4096 genes (according to variance) were extracted to construct WGCNA using a "WGCNA" package. The adjacency matrix was converted into the topological overlap matrix (TOM) when the power of $\beta$ was equal to 3 ($R^2 = 0.868$). Similar modules were merged following a height cutoff of 0.25. The module of highest correlation with EFNA3 expression was selected to explore its biological function through GO and KEGG analyses.

**Statistical Analysis**

R (4.0) software was used for statistical analyses. Statistical comparisons were calculated using Student's two-tailed t tests, p-values < 0.05 were considered statistically significant.

**Results**

**mRNA Expression level of Eph/Ephrins in LUAD based on different databases**

First, the Oncomine database was used to analyze mRNA expression levels of Eph/Ephrins in LUAD (Fig. 1A). The following threshold were used to analyze the data: 2-fold change, P value < 0.0001, and a gene grade of 10%. In most datasets, EPHA1, EPHA10, EFNA3, EFNA4, EPHB1, EPHB2, EPHB3, and EFNB3 were up-regulated in LUAD tissue compared with normal lung tissue. EPHA2, EPHB4, EPHB6, and EFNB1 were down-regulated in LUAD tissue. In order to further evaluate Eph/Ephrins expression, The Cancer Genome Atlas (TCGA) was used.

Since there were fewer normal samples in the TCGA dataset, the GTEx dataset based on GEPIA website was included for further analysis of the differential expression of Eph/Ephrins between normal and LUAD tissue (Fig. 1B). As showed in Fig. 1C, the results were consistent with data from the Oncomine database and TCGA database. The data is as follows: EPHA10, EFNA3, EFNA4, EFNA5, EPHB1, and EPHB2 expression levels were significantly upregulated in LUAD. In addition, EFNB1, EFNB2, and EPHB6 expression levels were significantly downregulated in LUAD. Based on these results, EPHA10, EFNA3, EFNA4, EFNA5, EPHB1, EPHB2, EFNB1, EFNB2, EPHB6 were used for the next analysis.

**EFNA3 acts as the most valuable prognostic biomarker in LUAD patients**
To determine the prognostic value of the selected Eph/Ephrins in LUAD patients, the Kaplan–Meier plotter database was used to analyze the relationship between expression levels and OS or PFS (Fig. 2 and Fig. 3). First, the relationship between OS was analyzed. In Figure 2A-I, upregulation of *EFNA3*, *EFNB2*, *EFNB1*, and *EPHB6* expression showed significant correlation with poor OS in LUAD patients. In contrast, *EFNA5* and *EFNB2* upregulation signified a better prognosis. Conversely, *EPHA10* and *EFNA4* expression did not show significant correlation with OS, so were excluded from this study.

Next, *EFNA3*, *EFNB2*, *EFNB1*, *EPHB6*, *EFNA5*, and *EFNB2* were selected for correlation of the relationship between expression levels and PFS. As shown in Fig. 3A-H, upregulation of *EFNA3*, *EFNB1*, and *EPHB2* expression showed significant correlation with poor PFS in LUAD patients. In contrast, *EFNB2* upregulation signified a better prognosis. Similarly, *EPHA10*, *EFNA5*, and *EPHB6* expression did not show a significant correlation with PFS. *EFNA3* was selected next since it showed the higher HR value both in prognostic analysis for OS and PFS in LUAD patients. Patients with different stage cancers require different therapeutic strategies, and have different prognoses, therefore subgroup analysis was applied[19]. As shown in Fig. 3A-C, a higher *EFNA3* expression was associated with significantly worse OS, regardless if the patient was diagnosed at stage 1 (Fig.3A), stage 2 (Fig.3B) or stage 3 (Fig.3C). Smoking was also an important risk factor for LUAD. *EFNA3* expression showed prognostic value in smoking LUAD patients (Fig. 3D), but not in patients with no smoking history (Fig. 3E).

**Genetic variations of ***EFNA3*

To determine if upregulation of *EFNA3* in LUAD tissues were caused by genetic variations, genetic variations of *EFNA3* were assessed using the cBioPortal database. This database contains information of 1272 samples from five studies (Broad, Cell 2012; MSKCC, Science 2015; OncoSG, Nat Genet 2020; TCGA, Firehose Legacy; TSP, Nature 2008). Genetic variations of *EFNA3* showed incidence rates of 12.98% in TCGA, 7.65% in Broad, and 7.28% in OncoSG (Fig. 5). Amplification was the most common type (67/67 in TCGA; 1/14 in Broad; and 7/7 in OncoSG). Based on these results, amplification might be one of the main mechanisms by which *EFNA3* is highly expressed in LUAD.

**The expression of the EFNA3 protein was increased in LUAD tissues and related to prognosis of LUAD patients**

To explore the clinical significance of the EFNA3 protein in LUAD patients, IHC to investigate the expression of EFNA3 in a tissue microarray (TMA) containing 74 LUAD tissues and adjacent normal lung tissues. Compared with normal lung tissue, the expression of the EFNA3 protein was significantly increased in LUAD tissue samples (Fig. 6A). Further analysis revealed that the IHC score for EFNA3 was significantly upregulated in cases of larger tumor sizes (Fig. 6B), lymph node metastasis (Fig. 6C), and advanced TNM stage (Fig. 6D).
LUAD patients were divided into two groups: EFNA3-high group and EFNA3-low group according to IHC scores. Chi-square test showed that EFNA3 protein expression were significantly correlated with larger tumor size (P = 0.004), lymph node metastasis (P = 0.035), and a higher TNM stage (P = 0.002) (Table 1). Moreover, EFNA3-negative patients presented with a shorter overall survival time than EFNA3-positive patients (P = 0.039) (Fig. 6E). Finally, cox regression analysis of overall survival showed that higher EFNA3 expression is the independent risk prognosis factor (HR = 3.108; 95% CI = 1.077–8.963; P = 0.036) (Table 2). Based on these results, EFNA3 could represent a new prognostic biomarker for LUAD.

Table 1
Correlation between the expression of EFNA3 and clinical characteristics in LUAD patients (n = 74).

| Clinical Pathological Parameters | Number | EFNA3 expression | P value |
|---------------------------------|--------|------------------|---------|
|                                 |        | High(n = 38)     | Low(n = 36) |
| Age                             |        |                  | 0.367   |
| < 60                            |        | 29               | 13       | 16       |
| ≥ 60                            |        | 45               | 25       | 20       |
| Sex                             |        |                  | 0.902   |
| Male                            |        | 48               | 27       | 39       |
| Female                          |        | 26               | 11       | 15       |
| Tumor size                      |        |                  | 0.004*  |
| T1                              |        | 15               | 5        | 10       |
| T2                              |        | 43               | 19       | 24       |
| T3 + T4                         |        | 16               | 14       | 2        |
| LN metastasis                   |        |                  | 0.035*  |
| No                              |        | 51               | 22       | 29       |
| Yes                             |        | 23               | 16       | 7        |
| TNM stage                       |        |                  | 0.002*  |
| I                            |        | 31               | 10       | 21       |
| II                             |        | 35               | 20       | 15       |
| III+IV                         |        | 8                | 8        | 0        |

*Significant correlation.
Table 2
Cox regression analysis of overall survival in LUAD patients.

| Variables          | Univariate analysis |          |          | Multivariate analysis |          |          |
|--------------------|---------------------|----------|----------|-----------------------|----------|----------|
|                    | HR                  | 95% CI   | P value  | HR                    | 95% CI   | P value  |
| Age (years)        |                     |          |          |                       |          |          |
| (≤ 60 vs > 60)     | 1.180               | 0.529–2.629 | 0.686   |                       |          |          |
| Gender             | 0.949               | 0.419–2.150 | 0.900   |                       |          |          |
| (male vs female)   |                     |          |          |                       |          |          |
| pT stage           | 1.489               | 0.898–2.469 | 0.123   |                       |          |          |
| pN stage           | 3.162               | 1.435–6.965 | 0.004*  | 1.336                 | 0.479–3.725 | 0.580   |
| pTNM stage         | 2.583               | 1.505–4.432 | <0.001* | 1.530                 | 0.738–3.173 | 0.253   |
| EFNA3 expression   | 4.272               | 1.597–11.423 | 0.004*  | 3.108                 | 1.077–8.963 | 0.036*  |

Factors for which P < 0.05 in univariate analysis were subsequently used for multivariate analysis.

Wgcna And Gsea Analysis

To identify the potential regulatory mechanism of EFNA3, TCGA dataset were used to construct the co-expression network through WGCNA analysis. Clinical features, including OS time, OS status, pathological parameters and EFNA3 expression were obtained from the TCGA dataset (Fig. 7A). The parameters were established by setting the soft-threshold power to 3 (scale free R² = 0.868) and the height was set to 0.25. In this study, 12 modules were identified (Fig. 7B-D). The association between the modules and clinical features was measured by the correlation between module eigengene (ME) values and clinical features. Data were visualized by heatmap profiles. The results showed that the purple module was the most closely corrected with EFNA3 expression (Pearson co-efficient = 0.23, P = 1E-05; Fig. 7E). A scatter plot of purple module eigengenes is shown in Fig. 7F. In the purple module, 168 genes were selected as hub genes to use for GO and KEGG analysis.

In the purple module genes, nuclear division, synaptic function, and ion channel activity-related pathways were the most frequently noted pathways in the GO analysis. The most enriched GO terms in the Biological Process (BP) category was “meiotic cell cycle.” In the Cellular Component (CC) category, “chromatoid body” was most enriched and in the Molecular Function (MF) category “ion gated channel activity” was most abundant. Furthermore, KEGG pathway enrichment analysis results contained “neuroactive ligand-receptor interaction,” and “calcium signaling pathway” enrichments (Fig. 8B). Finally, GSEA was used to identify the mechanism and functional differences between the EFNA3-high expression group and the EFNA3-low expression group. As showed in Fig. 8C, “cholesterol homeostasis,”
"DNA repair," "glycolysis," and "oxidative phosphorylation" was enriched in the EFNA3-high expression group, whereas, "IL2-STAT5 signaling" was enriched in the EFNA3-low expression group.

**Generalization value of EFNA3 in pan-cancer**

To investigate whether EFNA3 has broad value, a series of studies were performed on EFNA3 across all cancers. EFNA3 expression was significantly upregulated in more than half (17/33) of the cancer types analyzed (Fig. 9A). K-M survival analysis showed that high EFNA3 expression had a significant association with short OS in adrenocortical carcinoma (ACC), kidney renal clear cell carcinoma (KIRC), brain Lower Grade Glioma (LGG), liver hepatocellular carcinoma (LIHC), mesothelioma (MESO), skin cutaneous melanoma (SKCM) and uveal melanoma (UVM) (Fig. 9B–9J). Finally, EFNA3 is an extremely significant gene in the survival statistics of large number of pan-cancer samples (N = 9544, HR = 1.3, p = 1.1e-10) based on the GEPIA website (Fig. 9K).

**Discussion**

In this study, we first focused on 22 Eph/Ephrin family members and investigated their expression and prognostic value in LUAD tissue. First, EPHA10, EFNA3, EFNA4, EFNA5, EPHB1, EPHB2, EFNB1, EFNB2, and EPHB6 were differentially expressed in LUAD tissue. Furthermore, EFNA3, EFNB1, EFNB2, and EPHB2 showed a significant correlation with OS and PFS in LUAD patients. Previous studies confirmed that EPHB2 was overexpressed in LUAD tissue, and is an independent prognostic biomarker[20]. This is consistent with the results presented here. In addition, decreased expression levels of EPHB6 are associated with an increased risk of metastasis development in LUAD. Consistently, our study suggested that EPHB6 was downregulated in LUAD tissue and serves as a prognostic protective factor[21]. These findings indicate that Eph/Ephrin family members play an important role in the occurrence and development of LUAD, but the function and molecular mechanism still needs to be clarified by experiments.

The present study further focused on EFNA3, which had both significant differential expression and the most significant prognostic value. Studies had found that the expression levels of EFNA3 was inhibited in the process of skeletal muscle satellite cell formation[22]. Another study showed that EFNA3 promotes the proliferation and invasion of peripheral nerve sheath tumor cells, and is regulated by miR-210[23]. Importantly, several studies showed that EFNA3 was involved in tumor angiogenesis[23, 24]. However, the functions of EFNA3 in pathogenesis and progression of LUAD is still unclear. Results from the cBioPortal database suggested amplification may be one of the main mechanisms by which EFNA3 is over-expressed in LUAD. In this study, the expression level and prognostic value were further verified in LUAD through clinical samples. The protein expression of EFNA3 was up-regulated in LUAD tissue compared with normal lung tissue. In addition, the expression of the EFNA3 protein was significantly related to clinicopathological characteristics. Thus, EFNA3 gene expression is an independent prognostic risk biomarker.
According to previous studies, overexpression of ephrin-A3 could reduce glutamate transporter levels in astrocytes, and EphA4/ephrin-A3 signaling could regulate synaptic function and plasticity[25]. In addition, it has been confirmed that EFNA3 could regulate the EMT process by the PI3K/AKT signaling pathway in oral cancer[26]. In order to further explore the potential mechanism of EFNA3 expression in lung adenocarcinoma, WGCNA analysis was conducted. In this study, purple modules related to EFNA3 were screened out. Further enrichment analysis showed that EFNA3 is closely related to nuclear division, synaptic function, and ion channel activity. In additional, GSEA analysis showed "cholesterol homeostasis," "glycolysis," and "oxidative phosphorylation" were enriched in the EFNA3-high expression group, suggesting that EFNA3 may be closely related to the metabolic ability of lung adenocarcinoma cells. In short, EFNA3 may promote the malignant progression of LUAD through these potential pathways. The specific mechanism still needs further exploration.

Another important finding from this study is that EFNA3 also has universal prognostic value in pan-cancer. EFNA3 expression is significantly upregulated in more than half (17/33) of the cancer types investigated. Despite EFNA3 were differential expressed in various tumors, they are significantly related to shorter OS in pan-cancer patients, which suggests that EFNA3 has a wide range of clinical applications. However, this study has some limitations. The molecular mechanism of EFNA3 in LUAD were analyzed through bioinformatics, and not confirmed by experiment in vivo and in virto. Experimental studies will be performed and reported in a future study.

In conclusion, this study revealed the abnormal expression and prognostic value of Eph/Ephrin family members in LUAD. In addition, it is suggested that EFNA3 may be a novel biomarker for the diagnosis and prognosis of LUAD patients.

**Declarations**

**Ethics approval and consent to participate**

All procedures performed in studies involving humans were reviewed and permitted by The Shengjing Hospital of China Medical University.

**Consent for publication**

NA

**Availability of data and materials**

All data generated or analyzed during this study are included in this article.

**Competing interests**

The authors declare no conflicts of interest.

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Authors' contributions

Mingming Deng: Investigation, Data curation, Writing – original draft. Run Tong: Investigation, Data curation. Zhe Zhang: Methodology. Tao Wang: Data curation. Chaonan Liang: Writing – review & editing. Xiaoming Zhou: Writing - review & editing. Gang Hou: Conceptualization, Writing - review & editing, Project administration,

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