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Over-Expression of EFNA2 in Lung Adenocarcinoma: EFNA2 Gene Expression Correlates with Shortened Survival

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Authors' contributions

This work was carried out in collaboration among all authors. Author CL wrote the manuscript and performed bioinformatics analysis. Authors YW, HZ and XG contributed to manuscript discussion. Authors XX and GW designed the study, researched the literature, and contributed to the figures and tables. Authors YW, HZ, and XC supervised the study and contributed to data analysis. All authors read and approved the final manuscript.

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ABSTRACT

Background: The incidence of lung adenocarcinoma (LUAD) is increasing worldwide with different prognosis. Ephrin-A2（EFNA2）, a member of the Eph/ephrin family, is associated with tumor progression. However, the correlations of EFNA2 with prognosis in LUAD remain unclear. The purpose of this article is to analyze the impact of EFNA2 on the prognosis of LUAD patients through TCGA, Oncomine, and GEPIA databases, and to explore its possible mechanisms.
Methods: This article found a significant correlation between EFNA2 and shortened survival in LUAD patients through TCGA, Oncomine, and GEPIA database analysis. Therefore, we further

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invested the relationship between the expression and prognostic value of the EFNA2 gene in LUAD patients. Sequential data filtering (survival analysis, independent prognostic analysis, and clinicalcorrelation analysis) was performed. EFNA2 expression was analyzed by the Oncomine database and Tumor Immune Estimation Resource (TIMER). We evaluated the influence of EFNA2 on clinical prognosis using Kaplan-Meier plotter, the PrognoScan database and Gene Expression Profiling Interactive Analysis (GEPIA). The correlation between EFNA2 and cancer immune infiltrates was investigated by TIMER. In addition, correlations between EFNA2 expression and gene marker sets of immune infiltrates were analyzed by TIMER and GEPIA. In addition, gene enrichment analysis was performed by Metascape. Finally, a co-expression analysis was performed by the Oncomine database.

Results: A cohort of LUAD patients showed that high EFNA2 expression was associated with poorer overall survival (OS), disease-free survival (DFS) by TCGA, and EFNA2 was significantly associated with stage in LUAD. In addition, EFNA2 expression was positively correlated with infiltrating levels of B cells and CD8+ T cells. Moreover, the differential expression of EFNA2 was significantly higher in lung adenocarcinoma compared with that in normal controls. Specifically, EFNA2 was positively associated with ADAMTSL5, REEP6, PCSK4, C19orf25, and ANAPC2.

Conclusions: Our data indicate that EFNA2 is a potential diagnostic and prognostic biomarker and a promising molecular therapeutic target to attenuate LUAD progression.

Keywords: EFNA2; LUAD; prognosis; TCGA; ephrinA2.

1. INTRODUCTION

“Lung cancer is the leading cause of cancer-related deaths with an increasing incidence of lung adenocarcinoma (LUAD) subtype worldwide” [1]. Prognosis may vary in patients with the same stage tumor because cancer is characterized by genetic, epigenetic, and phenotypic changes that result in a tremendous variability in clinical behavior. Therefore, the development of additional molecular markers for survival prediction of LUAD is required. Lung cancer is a malignant tumor caused by abnormal growth of bronchial cells, and primary lung cancer is prone to metastasis. The most common pathological types include squamous cell carcinoma, adenocarcinoma, small cell lung cancer, and so on. Among them, LUAD accounts for 40%-55% of the total number of lung cancers, most of which originate from the bronchial mucosal epithelium, and more than 3/4 of the patients' lesions occur in the periphery. The disease progresses slowly, and the initial symptoms are generally not obvious, but it is easy to metastasize.

In recent years, molecular progress has changed the treatment of LUAD, and genetic testing has become a standard diagnosis and prognostic indicator and could determine the treatment target. The progress of bioinformatics and high-throughput sequencing was whether we could identify many tumor biomarkers, which could help improve the accuracy of predicting the prognosis of LUAD and find increasingly effective treatments. EFNA2 was a member of the Ephrins family. Ephrins, ligands for the Eph receptors, its physiological role not only involved cell-to-cell communication, cell adhesion, cell migration, and invasion, but also involved the regulation of blood vessel development and angiogenesis. They were promiscuous in a very complex web of relationships (Fig. 1). At present, the targeted therapy of LUAD has made outstanding progress, but there were still about 10% of patients with negative genetic testing, so we needed more genetic testing sites to improve the prognosis. And better targeted drugs can be found based on this gene. Currently, there are few articles studying the impact of EFNA2 on lung cancer. Only one article reports that EFNA2 can predict the prognosis of early lung adenocarcinoma in Asians and Caucasians [2], and only one article reports that the prepared EFNA2 targeted drugs can increase the therapeutic effect of lung cancer [3]. At present, research on the impact of EFNA2 on lung adenocarcinoma is not sufficient. In thisarticle, we analyzed EFNA2 and lungadenocarcinoma through multiple databases and analysis tools and found that EFNA2 is a good biomarker for predicting the prognosis of lung adenocarcinoma patients and is a good target for targeted therapy of lung adenocarcinoma.

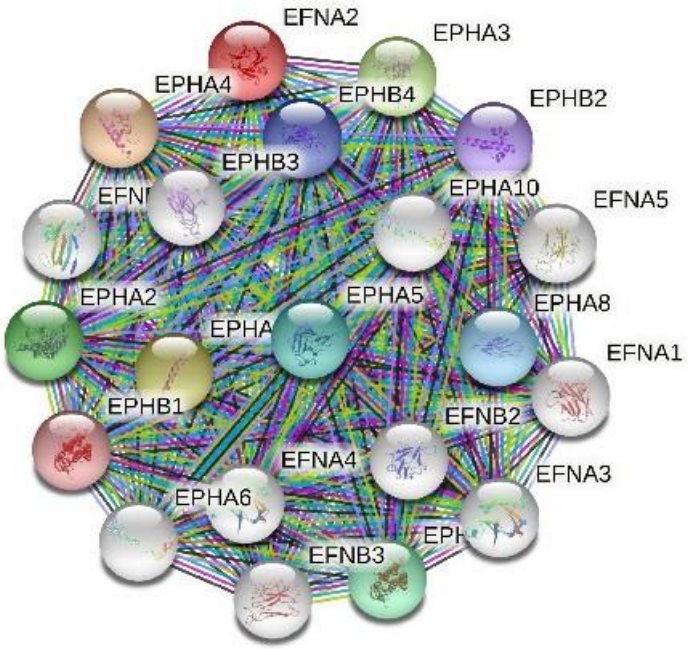

Fig. 1. Establishment of the PPI network

2. MATERIALS AND METHODS

2.1 Oncomine Database Analysis

We analyzed the EFNA2 mRNA levels in different tumors and normal tissues of multiple cancer types using the Oncomine database. (https://www.oncomine.org/resource/login.html). The threshold was determined according to the following values: P-value of 0.001, fold change of 1.5, and gene ranking of all.

2.2 Data Download and Preprocessing

Gene expression data and corresponding clinical data from LUAD patients were downloaded from TCGA (http://www.cgga.org.cn/). This dataset that contained 594 samples (DataSet ID: mRNAseq_594, Data Type: RNA sequencing) were downloaded. The gene expression data from LUAD samples were corrected in batches and integrated by loading them into the limma (14) and sva (15) packages in R software (R version 3.6.1: https://www.rproject.org/).

2.3 Survival Analysis Filtering

Survival and survminer packages were loaded in R software, and Kaplan–Meier (K-M) (16) and univariate Cox analyses were used to filter gene expression data and survival data at a significance level of $P < 0.05$.

2.4 Independent Prognostic Analysis Filtering

The gene expression data obtained from the survival analysis and integrated clinical information were analyzed using multivariate Cox analysis with R software, at a significance level of $P < 0.05$.

2.5 Analysis of the Correlation Between EFNA2 Expression and Clinical Characteristics

The correlation between EFNA2 expression and various clinical characteristics was plotted using UALCAN(http://ualcan.path.uab.edu/analysis.html).

2.6 Gepia Database Analysis

“The examination of EFNA2 expression in homogeneous subsets of LUAD was performed in GEPIA. GEPIA, an interactive web server containing RNA sequencing data based on 9,736 tumor samples and 8,587 normal samples from the TCGA and GTEx databases, provides customizable functions such as tumor/normal differential expression analysis, patient survival analysis, and correlation analysis” [4].

2.7 Gene Enrichment Analysis

In this study, Metscape was used to generate an ordered list of all genes associated with the expression of EFNA2. Then, Metscape was used to identify survival differences between the high and low EFNA2 groups.

2.8 Analysis of Immune Infiltration

"Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) was used to comprehensively study the molecular characteristics of tumor-immune interactions" (18). "The abundances of six immune infiltrates, including B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells were evaluated. We analyzed the relationship between the expression level of EFNA2 and the level of immune infiltration in LUAD using the TIMER "gene" module. The Kaplan-Meier method was used to plot the effect of EFNA2 expression and immune cell infiltration on the prognosis of patients with LUAD, and clinical factors were included to construct a multivariate Cox proportional risk model. Finally, the relationship between copy number variations (CNVs) of EFNA2 in different somatic cells and the level of infiltration in LUAD was analyzed using the "SCNA" module" [4].

2.9 Co-expression Analysis

Oncomine (https://www.oncomine.org) was used to screen genes that were co-expressed with EFNA2. In addition, the pheatmap (https://github.com/taiyun/corrplot) package was used to plot the first 20 genes positively and negatively associated with EFNA2. The Corrplot (https://github.com/taiyun/corrplot) and Circlize packages in R were used to generate a circular plot of the top five genes positively and negatively associated with EFNA2.

3. RESULTS

3.1 The Expression Levels of EFNA2 in Different Types of Human Cancers

The expression level of the EFNA2 gene in various types of cancers was identified in the Oncomine database. This analysis revealed that the EFNA2 expression was higher in lung cancer compared to normal tissues (Fig. 2A). To further evaluate EFNA2 expression in human cancers, we examined EFNA2 expression using the RNA-seq data of multiple malignancies in TCGA determined by TIMER. The differential expression between the tumor and adjacent normal tissues for EFNA2 across all TCGA tumors is shown in Fig. 2B. EFNA2 expression was significantly lower in LUAD (lung adenocarcinoma) compared with adjacent normal tissues.

Kaplan-Meier survival analysis of the TCGA dataset showed that low EFNA2 expression was associated with better prognosis in patients with lung adenocarcinoma (Fig. 3A). Univariate Cox analysis showed that EFNA2 (HR = 1.065; 95% CI = 1.022-1.110; $P < 0.05$), T stage, N stage, M stage and TNM stage were high-risk factors, and pathology were low risk factors (Fig. 3B). Multivariate Cox analysis showed that EFNA2 (HR = 1.053; 95% CI = 1.007–1.102; $P< 0.05$) was independently associated with overall survival, which suggested that EFNA2 could be an independent prognostic indicator for lung adenocarcinoma. In addition, pathology and TNM stage may also be independent prognostic factors (Fig. 3C).

3.2 Analysis of the Relationship Between EFNA2 Expression and Clinical Characteristics

Analysis of 573 samples from the TCGA database showed that the differential expression of EFNA2 was significantly higher in lung adenocarcinoma compared with that in normal control by using UALCAN [5] (Fig. 4A**,** $P < 0.001$). We also can see the significant different between normal tissues and 41-88years, different stages, gender, N0-N3 nodal metastasis, different races, NOS, mixed and mucinous histological subtypes, different smoking habits, different TP53 mutations (Fig. 4B-I). In addition, analysis of 573 samples from the TCGA database showed that the differential expression of EFNA2 was significantly associated with patients race and histological subtypes (Fig. 4F). The EFNA2 expression of Caucasian wassignificant different to African and American ($P < 0.001$) or Asian ($P < 0.05$). And the EFNA2 expression of mixed subtype was significant different to lung bronchioloalveolar carcinoma, non-mucinous subtype ($P < 0.05$) or lung solid pattern predominant adenocarcinoma subtype ($P < 0.05$). The EFNA2 expression of lung adenocarcinoma not otherwise specified (NOS) subtype was significant different to the lung solid pattern predominant adenocarcinoma subtype ($P < 0.05$).

3.3 Relationship between EFNA2 Expression and Prognosis of LUAD Patients

GEPIA is a public database established for expression profiling analysis of cancer and normal genes [6]. Prognostic analysis revealed that high expression of EFNA2 would lead to a short overall survival in patients with LUAD based on GEPIA database (Fig. 5A, $P < 0.05$). In addition, EFNA2 was significantly associated with stage in LUAD analyzed by GEPIA database (Fig. 5B, $P < 0.05$).

3.4 Analysis of the Correlation between EFNA2 Expression and Clinical Characteristics in TCGA Database

Analysis using TIMER showed that EFNA2 was negatively associated with B cells and CD8+ T cells (Fig. 6). Univariate cox survival analysis showed that EFNA2, B cell, and dendritic cells were associated with the survival of patients with LUAD (Fig. 7). Furthermore, only arm level decreases in copy number variations (CNVs) of EFNA2 were associated with the extent of immune infiltration in LUAD immune cells (Fig. 8). These results showed that EFNA2 was associated with immune infiltration in LUAD using external data analysis. These findings indicated that EFNA2 might be a prognostic biomarker of LUAD and may be a target for immunotherapy.

3.5 Gene Enrichment Analysis of EFNA2

"Gene enrichment analysis is a computational method used to determine whether a group of genes is differentially expressed in two biological states. Metascape gene enrichment analysis was used to identify GO (Gene Ontology) and KEGG signaling pathways that were differentially expressed in LUAD between the low and high EFNA2 expression groups. The results showed significant differences in enrichment using Metascape" (http://metascape.org/). The most significantly enriched GO and signaling pathways were selected based on p value. As shown in Fig. 9 A regulated exocytosis, blood vessel development, cell cycle phase transition, gene ontology terms, extracellular matrix organization, cell cycle, mitotic, hemostasis, collagen formation, PID integrin1 pathway, nervous system development signaling pathway were enriched in the EFNA2 high expression phenotype.

3.6 Co-expression Analysis of EFNA2

A heatmap (Fig. 10A) of the top 20 genes positively and negatively associated with EFNA2 was plotted. In addition, a circular plot (Fig. 10B) of the top five genes positively and negatively associated with EFNA2 was generated. The results showed that EFNA2 was positively associated with ADAMTSL5, REEP6, PCSK4, C19orf25 and ANAPC2, and was negatively associated with MSLNL, SLC35F2, RAB39B, BIRC3 andKIAA1377.

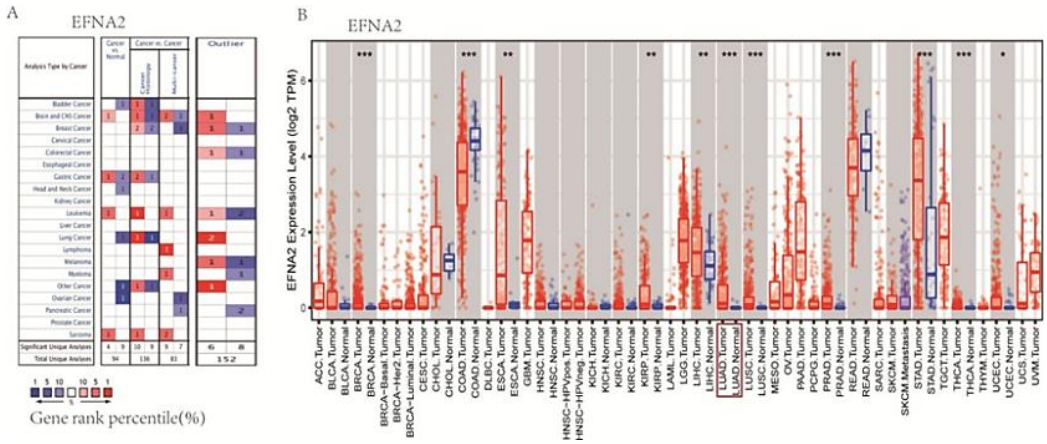

Fig. 2. EFNA2 expression levels in different types of human cancers. (A) Increased or decreased EFNA2 in data sets of different cancers compared with normal tissues and different pathology of cancer in the Oncomine database. (B) Human EFNA2 expression levels in different tumor types from TCGA database were determined by TIMER. (*P<0.05, **P<0.01, ***P<0.001)

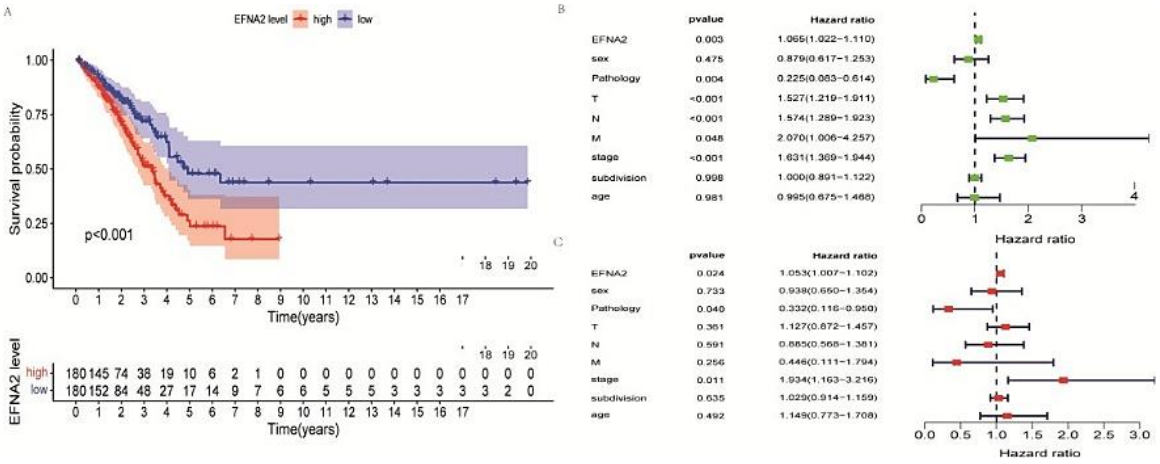

Fig. 3. Bioinformatics analysis of EFNA2 using the TCGA database (LUAD, n = 367). (A) Survival analysis of patients with lung adenocarcinoma in the high EFNA2 and low EFNA2 groups. Red indicates high expression and blue indicates low expression. P < 0.001. (B) Univariate analysis of EFNA2. (C)Multivariate analysis of EFNA2

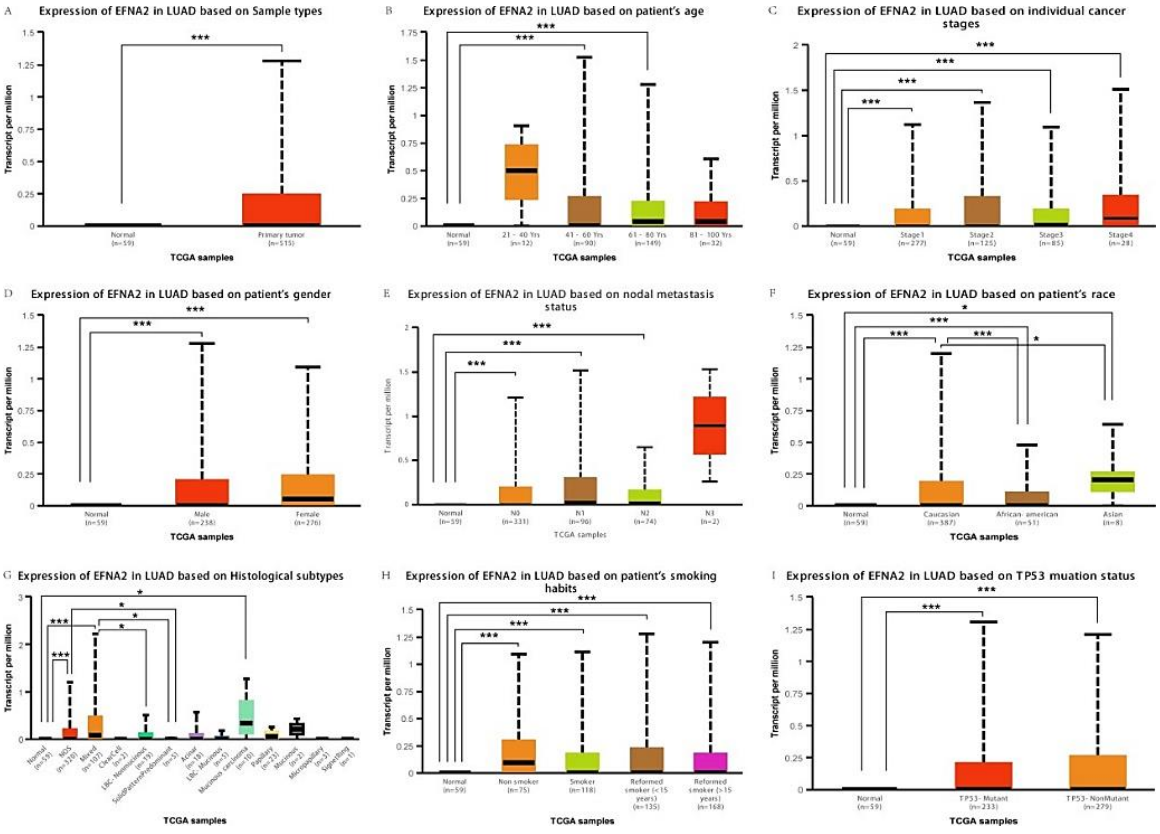

Fig. 4. Relationship between EFNA2 Expression based on different factors of lung adenocarcinoma Patients. (A) EFNA2 Expression based on TCGA sample types. (B) EFNA2 Expression based on patient's age. (C) EFNA2 Expression based on cancer stage. (D) EFNA2 Expression based on patient's gender. (E) EFNA2 Expression based on nodal metastasis. (F) EFNA2 Expression based on patient's race. (G) EFNA2 Expression based on histological subtypes. (H) EFNA2 Expression based on patient's smoking. (I) EFNA2 Expression based on TP53 mutation status. (*P<0.05, **P<0.01, ***P<0.001)

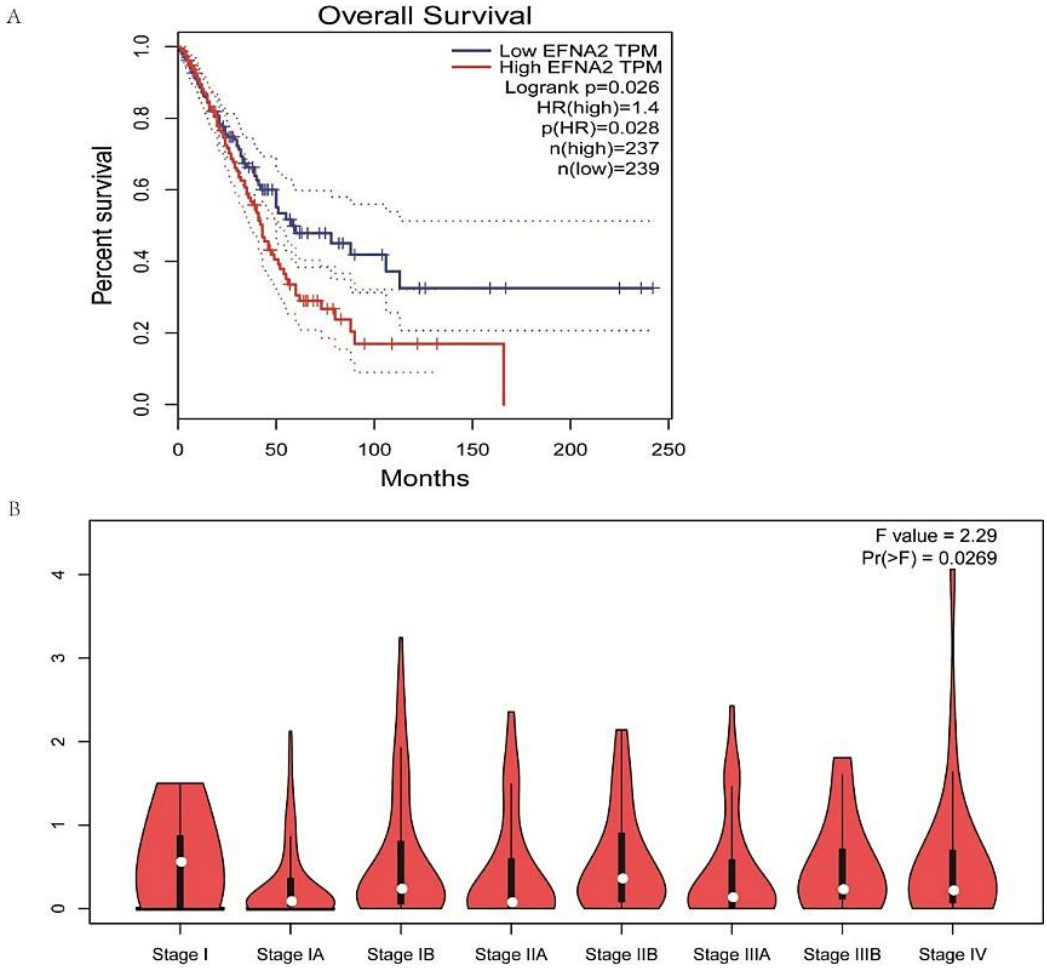

Fig. 5. Relationship between EFNA2 expression and prognosis of LUAD patients based on GEPIA database. (A) The relationship between EFNA2 expression levels and overall survival in LUAD was analyzed by GEPIA database. P < 0.05. (B) EFNA2 was significantly associated with stage in LUAD analyzed by GEPIA database. P < 0.05

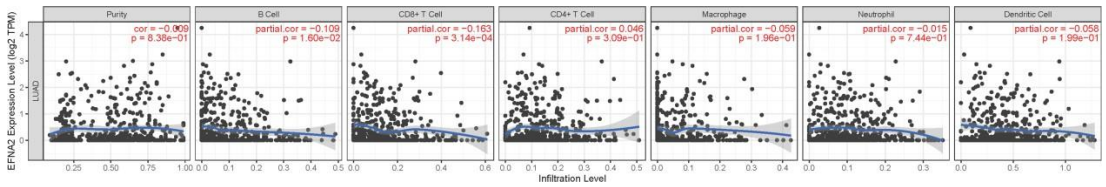

Fig. 6. Correlation between the expression of EFNA2 and immune infiltration of LUAD cells

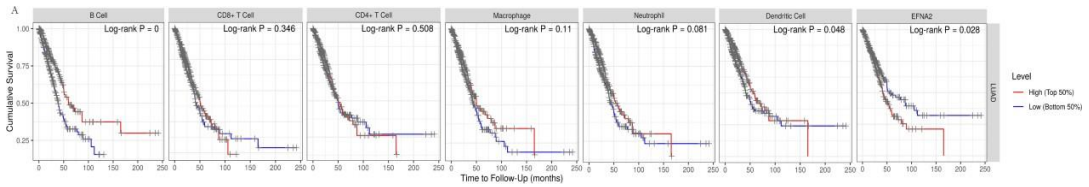

Fig. 7. Survival curve for immune cell infiltration. Kaplan–Meier survival curves based on top and bottom sample partitions with 50% immune penetration. Red indicates a high degree of infiltration and blue indicates a low degree of infiltration. P < 0.05 was considered significant and P < 0.0001 was reported as 0

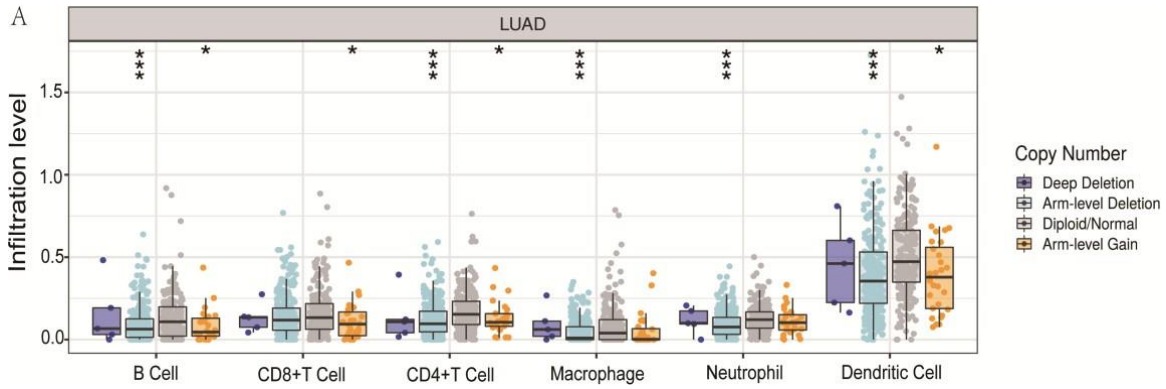

Fig. 8. Relationship between copy number variation of EFNA2 and immune infiltration level in LUAD. *P < 0.05; ***P < 0.001

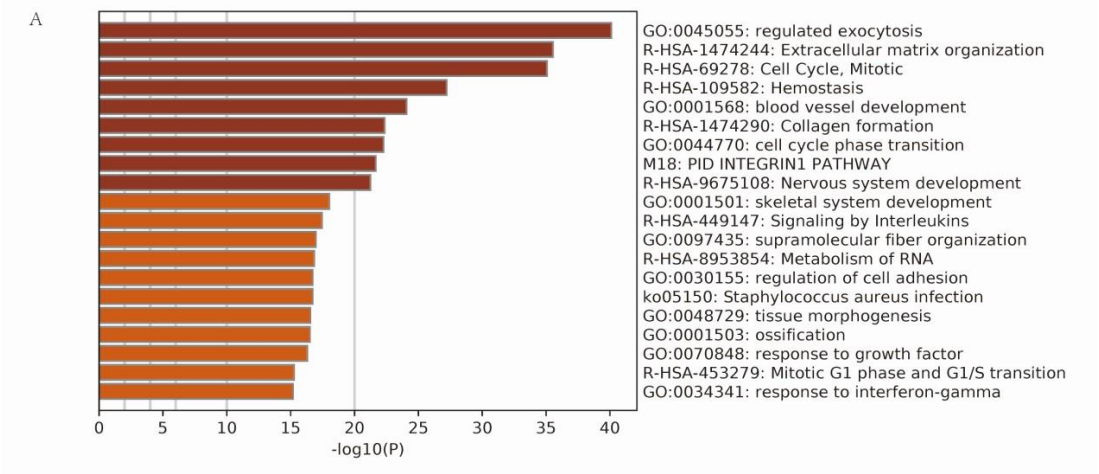

Fig. 9. Gene Set Enrichment Analysis of EFNA2. Heatmap of enriched terms across input gene lists, colored by p-values

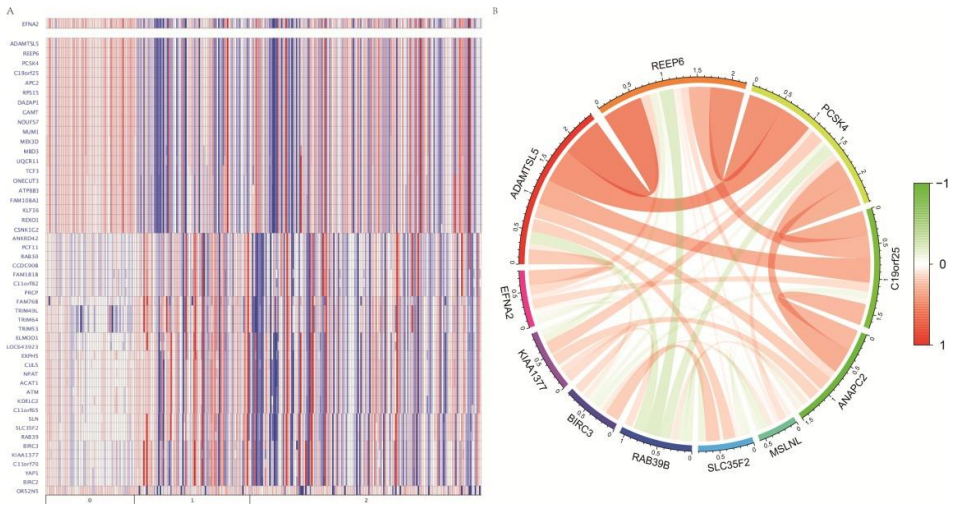

Fig. 10. Co-expression analysis of EFNA2 using the TCGA database. (A) Heatmap of the top 20 genes positively and negatively associated with EFNA2. (B) Circular plot of the top five genes positively and negatively related to the EFNA2 gene. Green represents negative association, and red represents positive association

4. DISCUSSION

The treatment methods of LUAD included surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy. At present, surgery, radiotherapy, and chemotherapy have been used for many years, and the therapeutic effect has been basically applied to the limit. This requires us to explore new treatment directions. In addition to external environmental factors [7], the most important cause of lung cancer is genetic changes [8,9]. “Therefore, we studied the related genes of LUAD, hoping to find new treatment methods or prognostic factors. We showed that EFNA2 was a high-risk factor and could be an independent prognostic indicator in patients with LUAD using comprehensive univariate and multivariate Cox analyses. Taken altogether, these results indicated that EFNA2 was upregulated in LUAD, and EFNA2 had a prognostic value in LUAD, indicating that EFNA2 had important regulatory functions in LUAD. EFNA2 is a member of the ephrin family whose genes have been reported to be frequently overexpressed in a wide variety of cancer types directly regulating critical steps of cellular adhesion, tumor growth, chemo repulsion, invasion, metastasis, angiogenesis, axon guidance, tissue border formation” [10,11,12,13, 14-17,18-29]. The relative studies showed that the expression levels of EFNA2 were negative and correlated with the prognosis of prostate cancer [30], CD133 high neuroblastoma [31,32] breast cancer [16], hepatocellular carcinoma [16], gastric cancer[33] and colorectal cancer [34]. For example, Feng et al. found that “EFNA2 is significantly upregulated in both cancerous cell lines and clinical tissue samples of hepatocellular carcinoma (HCC) and compared with the normal ones” [15]. Chakraborty's study found that EFNA2Trp112Cysmutant may be a contributing factor to the development of non-small cell lung cancer [35]. In addition, Fox et al. reported that “the expression of EFNA2 is significantly higher in CPTX cells (human local prostate tumor) compared to NPTX cells (normal human prostate epithelium), suggesting that EFNA2 may promote the transformation of the normal prostate epithelial cell into one with a malignant phenotype” [17]. Zhao found that “ectopic expression of EFNA2 can promote the invasion and metastasis of prostate cancer cells, promote blood vessel proliferation, and silence EFNA2, which can reduce the invasiveness and metastasis of prostate cancer cells” [36]. In addition, EFNA2 participates in the regulation of diverse cellular processes and gene expression through chromatin remodeling, and the expression of EFNA2 at the transcription level would lead to the activation of signaling pathways related to tumor progression. For example, Liu et al. showed that “blocking EFNA2 expression inhibits the metastasis ability of human liver cancer cell line HepG2” [37]. Kuo discovered that “EFNA2 can predict the prognosis of early lung adenocarcinoma”.[2] “Moreover, ephrins family via Ephrin receptor (Eph)–ephrin interactions regulate critical steps of angiogenesis, blood vessel formation malignant transformation, tumor metastasis, tumor differentiation, and outcome” [18-20,28], [38-41]. “For example, upregulation of EphA2 has been observed in many malignant tumors and is associated with accelerated cell proliferation, stimulating angiogenesis, and promoted cell migration and invasion, increasing cancer cell survival” [18-28]. Psilopatis reviewed Ephrin/Eph family targeted treatment of gynecological tumors, breast cancer and lung cancer [42-44] , Papadakos reviewed Ephrin/Eph family targeted treatment of colon cancer [45] , Hadjimichael reviewed Ephrin/Eph family targeted treatment of bone and chondrosarcoma [46] , and found that Ephrin/Eph family targeted treatment can not only promote the regression of tumors, but also increase the effect of radiotherapy, chemotherapy and targeted treatment [35] , which is a new hope for tumor treatment, More than ten drugs have been discovered in the treatment of lung cancer. Huang has prepared a drug targeting EFNA2, which has a significant killing effect on lung cancer cells and has entered phase I clinical research [3]. “In addition, research and clinical trials have confirmed the proteolytic shedding of membrane-bound Ephrin-As, which releases soluble fragments at the cellular level” [47-49]. “For example, membrane bound EFNA2 has been identified as the substrate of ADAM10 and released soluble EFNA2 fragments into the cell medium” [47,48]. “These data suggested that secreted EFNA2 may be useful serum markers for the diagnosis and prognosis of many tumors” [50].

“Besides, gene enrichment analysis was performed to obtain further information about the role of EFNA2 in tumor progression. The results of TIMER showed that a regulated exocytosis, blood vessel development, cell cycle phase transition, Gene ontology terms, extracellular matrix organization, cell cycle, mitotic, hemostasis, collagen formation, PID integrin1 pathway, nervous system development signaling

pathways were enriched in the EFNA2 high expression phenotype. The extracellular matrix regulates tissue development and homeostasis, and its dysregulation contributes to neoplastic progression. The extracellular matrix serves not only as the scaffold upon which tissues are organized but provides critical biochemical and biomechanical cues that direct cell growth, survival, migration, and differentiation and modulate vascular development and immune function" [51,52].

"The gene ontology terms of EFNA2 were generally enriched in B cell related mediated immunity, humoral immune response, and innate immune response. B lymphocyte was recognized to participate in regulating the immune response to murine and human tumors" [53]. "Regulatory B cell play an immunosuppressive role in carcinogenesis and become a therapeutic target in solid tumors [54]. Recent studies indicated that the B lymphocytes exists in all stages of cancer and plays important roles in shaping tumor development in lung cancer and thus influences the prognosis of lung cancer patients" [55,56].

"Finally, co-expression analysis showed that EFNA2 was positively associated with ADAMTSL5, REEP6, PCSK4, C19orf25, and ANAPC2, and was negatively associated with MSLNL, SLC35F2, RAB39B, BIRC3 and KIAA1377. As previously reported, ADAMTSL5 was an epigenetically activated gene underlying tumorigenesis and drug resistance in hepatocellular carcinoma and pointed to a role for ADAMTSL5 in maintaining the function of key oncogenic signalling pathways, suggesting that it may act as a master regulator of tumorigenicity and drug resistance". [57]. Moreover, proliferation and metastasis of lung cancer cells lacking REEP5 and REEP6 were markedly decreased compared to the control group, and they could be novel regulators of G-protein-coupled receptor signaling [58]. These reports suggested that ADAMTSL5 and REEP6 were associated with the regulation of proliferation and metastasis of cancer. Our study showed EFNA2 to be associated with ADAMTSL5 and REEP6, which indicated that EFNA2 might be associated with the regulation of proliferation and metastasis of cancer.

Ephs and ephrins were regarded as promising candidates for drug development. However, Eph and ephrin had been considered as undruggable target molecules because the interactions between Eph and ephrin, shown in Fig. 1, were not specific and promiscuous in a very complex web of relationships. Many processes that involve fast changes in cellular motility and/or morphology depend on ephrin–Eph signaling pathway. Therefore, there are no drugs against the Eph/ephrin family for medical use included in the clinical guidelines so far. Dasatinib is the only drug for medical use that shows an inhibitory effect on EphA2 activity [59]. However, dasatinib has not been used for any anti-EphA2 therapy so far. Recently, Richard Huang et al. discovered EFNA2 targeted immunoliposomes incorporating pH-sensitive taxane prodrugs were developed for sustained delivery of active drugs to solid tumors, and this drug had entered a Phase Iclinical trial [3].These results indicated that EFNA2 may be a useful molecular therapeutic target to attenuate

4.1 LUAD progression

We speculated that EFNA2 may be used as a prognostic indicator for LUAD, and future studies will be needed to explore the protein in a multidisciplinary way in the future, hoping to find a molecular predictor with great clinical value.

5. CONCLUSION

This study looked into the connection between EFNA2 and the prognosis for LUAD. Initially, sequential. The important gene EFNA2 was screened via data filtering. After then, EFNA2 was examined for a relationship with outcome as well as clinical features. The findings indicate that elevated EFNA2 expression was linked to lower prognosis, and EFNA2 was a high-risk factor that could be utilized on its own to predict outcomes. Indicator for LUAD sufferers. Additionally, an examination of Metascape gene enrichment revealed that EFNA2 potentially control LUAD's growth and metastasis, and EFNA2's overexpression in LUAD suggests bleak prognosis. Cell-to-cell contacts within tumor cells and the tumor itself are mediated by EFNA2. Microenvironment, specifically the vascular and tumor stroma. Consequently, EFNA2 has been regarded as desirable objectives for medication design, as targeting.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

DATA AVAILABILITY STATEMENT

The data that support the findings of this work are obtainable from the corresponding author based on reasonable request.

FUNDING

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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