# High Levels of EFNA2 in Lung Adenocarcinoma Predicts Poor Prognosis

**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.

**Methods**: In this study, we investigated the relationship between the expression and prognostic value of the EFNA2 gene in LUAD patients in The Cancer Genome Atlas (TCGA) database. Sequential data filtering (survival analysis, independent prognostic analysis, and clinical correlation 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

## 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 (FIGURE 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. using CGGA and TCGA data, Liu[2] speculate on genes that may affect the survival time of patients with low-grade gliomas. In this study, data filtering (survival analysis, independent prognostic analysis, and clinical correlation analysis) from TCGA data was used to screen the EFNA2. bioinformatics analysis of EFNA2 was performed. In addition, the relationship between EFNA2 and clinical characteristics was also studied. Furthermore, gene enrichment analysis was performed. Finally, a co-expression analysis was performed.

# MATERIALS AND METHODS

## 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.

## 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/).

## 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 $P < 0.05$.

## 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 $P < 0.05$.

## 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).

## 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

92 samples from the TCGA and GTEx databases, provides customizable functions such as tumor/normal
93 differential expression analysis, patient survival analysis, and correlation analysis.
94
95 ## Gene Enrichment Analysis
96 In this study, Metscape was used to generate an ordered list of all genes associated with the expression of
97 EFNA2. Then, Metscape was used to identify survival differences between the high and low EFNA2 groups.
98
99 ## Analysis of Immune Infiltration
100 Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) was used to
101 comprehensively study the molecular characteristics of tumor-immune interactions(18). The abundances of
102 six immune infiltrates, including B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic
103 cells were evaluated. We analyzed the relationship between the expression level of EFNA2 and the level of
104 immune infiltration in LUAD using the TIMER “gene” module. The Kaplan-Meier method was used to plot
105 the effect of EFNA2 expression and immune cell infiltration on the prognosis of patients with LUAD, and
106 clinical factors were included to construct a multivariate Cox proportional risk model. Finally, the
107 relationship between copy number variations (CNVs) of EFNA2 in different somatic cells and the level of
108 infiltration in LUAD was analyzed using the “SCNA” module.
109
110 ## Co-expression Analysis
111 Oncomine (https://www.oncomine.org) was used to screen genes that were co-expressed with EFNA2. In
112 addition, the pheatmap (https://github.com/taiyun/corrplot) package was used to plot the first 20 genes
113 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.

# RESULTS

## 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 (**Figure 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 **Figure 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 (**Figure 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 (**Figure 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 (**Figure 3C**).

## 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[3] (**Figure 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 (**Figure 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 (**Figure 4F**). The EFNA2 expression of Caucasian was significant different to African-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( $P < 0.05$).

## Relationship Between EFNA2 Expression and Prognosis of LUAD Patients

GEPIA is a public database established for expression profiling analysis of cancer and normal genes.[4] Prognostic analysis revealed that high expression of EFNA2 would lead to a short overall survival in patients with LUAD based on GEPIA database (**Figure 5A**, $P < 0.05$). In addition, EFNA2 was significantly associated with stage in LUAD analyzed by GEPIA database(**Figure 5B**, $P < 0.05$).

## 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 (**Figure 6)**. Univariate cox survival analysis showed that EFNA2, B cell, and dendritic cells were associated with the survival of patients with LUAD (**Figure 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 (**Figure 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.

## 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 **Figure 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.

## Co-expression Analysis of EFNA2

A heatmap (**Figure 10A**) of the top 20 genes positively and negatively associated with EFNA2 was plotted. In addition, a circular plot (**Figure 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 and KIAA1377.

## 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. There was still a lot of research space for targeted therapy. 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. No previous studies have reported a link between EFNA2 and lung cancer. However, 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 [5, 6] [7, 8] [9-12] [13-23]. The relative studies showed that the expression levels of EFNA2 were negative and correlated with the prognosis of prostate cancer[24], CD133 high neuroblastoma[25, 26]， breast cancer[11]， hepatocellular carcinoma[10], gastric cancer[27] and colorectal cancer[28]. 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[10]. 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[12]. The transcript for EFNA2 is also highly and shows a relative increase in more aggressive prostate cancer compared to its parent cell line, implying that EFNA2 may also play an important role in promoting prostate cancer invasion. 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[29]. Kuo et al. showed that EFNA2 might be a prognostic predictor of multiple DNA methylation biomarkers for early-stage LUAD in Asian and Caucasian populations.[30] 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[13-15, 23, 31, 32]. 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 [13-23]. In addition, research and clinical trials have confirmed the proteolytic shedding of membrane-bound Ephrin-As, which releases soluble fragments at the cellular level[33-35]. For example, membrane-bound EFNA2 has been identified as the substrate of ADAM10 and released soluble EFNA2 fragments into the cell medium [33, 34]. These data suggested that secreted EFNA2 may be useful serum markers for the diagnosis and prognosis of many tumors [36].

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.[37, 38]

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[39]. Regulatory B cell play an immunosuppressive role in carcinogenesis and become a therapeutic target in solid tumors[40].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 [41, 42].

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. [43]. 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 [44]. 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 Figure 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[45]. 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 I clinical tria l[46].These results indicated that EFNA2 may be a useful molecular therapeutic target to attenuate 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.

## CONCLUSION

In conclusion, this study investigated the relationship between EFNA2 and LUAD prognosis. First, sequential data filtering was used to screen the key gene EFNA2. Then, EFNA2 was analyzed for correlation with prognosis and clinical characteristics. The results show that high expression of EFNA2 was associated with worse prognosis, and EFNA2 was a high-risk factor and could be used as an independent prognostic indicator for patients with LUAD. Furthermore, Metascape gene enrichment analysis showed that EFNA2

268 could regulate the proliferation and metastasis of LUAD and that upregulation of EFNA2 in LUAD indicates
269 poor prognosis. EFNA2 mediates cell-to--to-cell interactions both in tumor cells and in the tumor
270 microenvironment, namely, the tumor stroma and vasculature. Thus, EFNA2 has been considered as
271 attractive targets for drug design, as targeting these molecules could simultaneously inhibit several aspects
272 of tumor progression. However, few studies have shown the association between EFNA2 and LUAD or
273 other cancers. Future studies will be needed to confirm the role of these genes as therapeutic targets and
274 biomarkers in LUAD. which suggests that this study may provide a scaffold for future development of
275 therapeutic strategies for LUAD.
276

277 DATA AVAILABILITY STATEMENT

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

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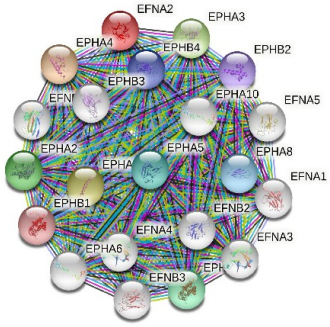
Figure legends

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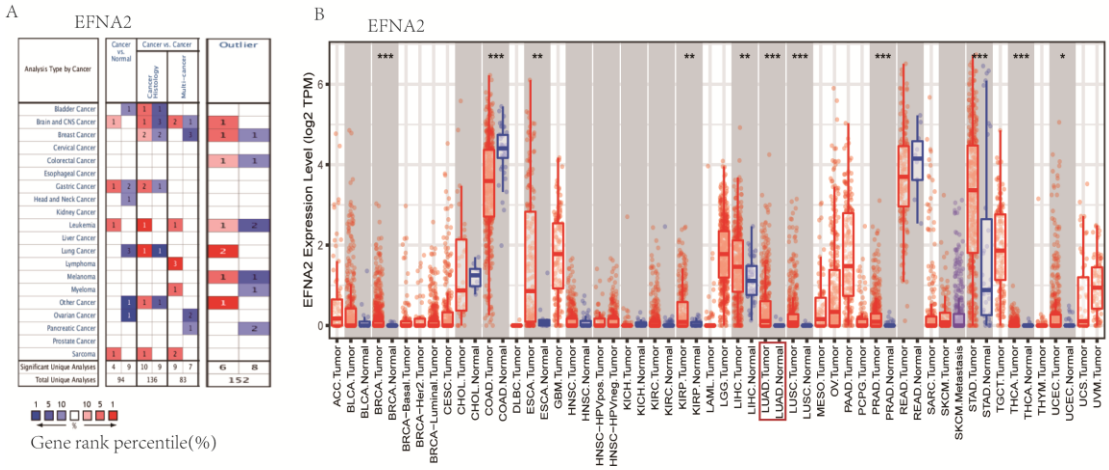
416 FIGURE 1 |Establishment of the PPI network.

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418 FIGURE 2 |EFNA2 expression levels in different types of human cancers. (A) Increased or decreased EFNA2 in data sets of different cancers

419 compared with normal tissues and different pathology of cancer in the Oncomine database. (B) Human EFNA2 expression levels in different tumor

420 types from TCGA database were determined by TIMER. (*P<0.05,**P<0.01,***P<0.001).

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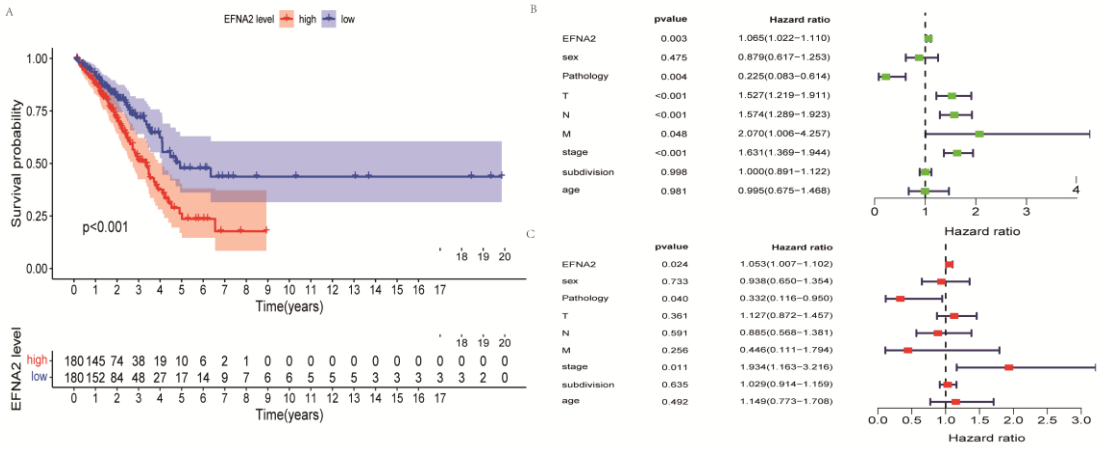



422

423 FIGURE 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.

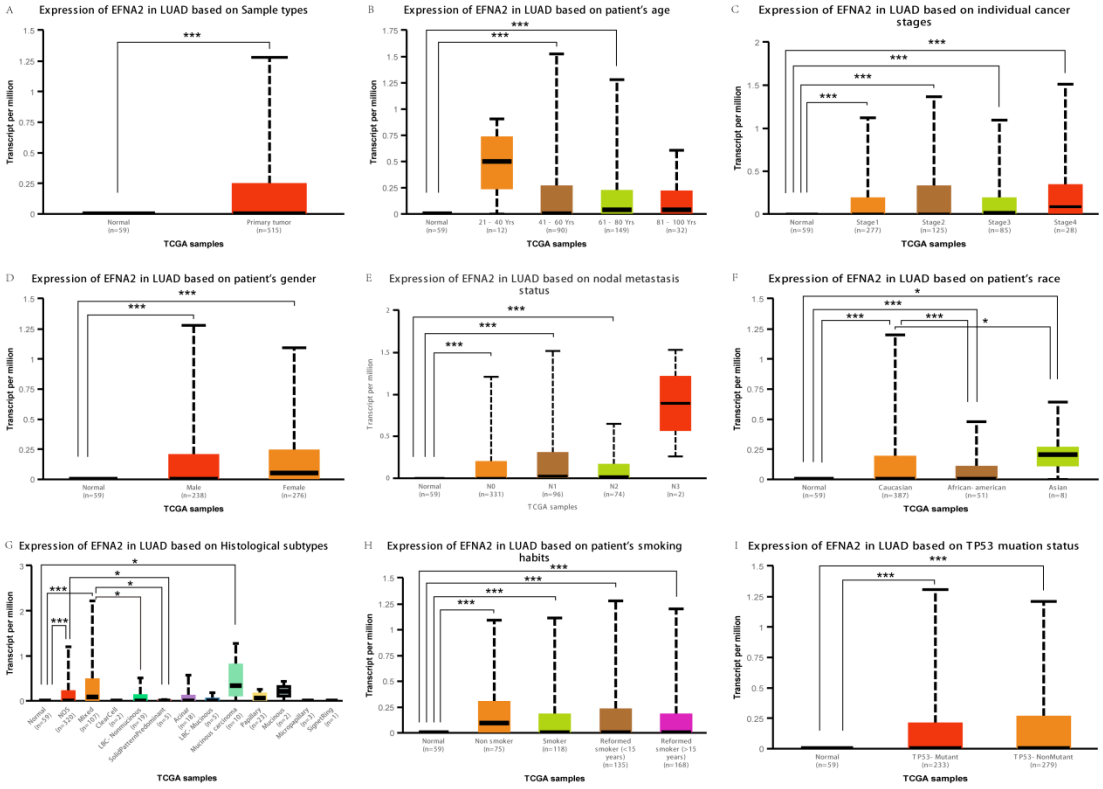



FIGURE 4| Relationship Between EFNA2 Expression based on different factors of lung adenocarcinoma Patients. (A) EFNA2 Expression based on TCGA sample types. (B) EFNA2 Expression based on patient's age. (C) EFNA2 Expression based on cancer stage. (D) EFNA2 Expression based on patient's gender. (E) EFNA2 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).

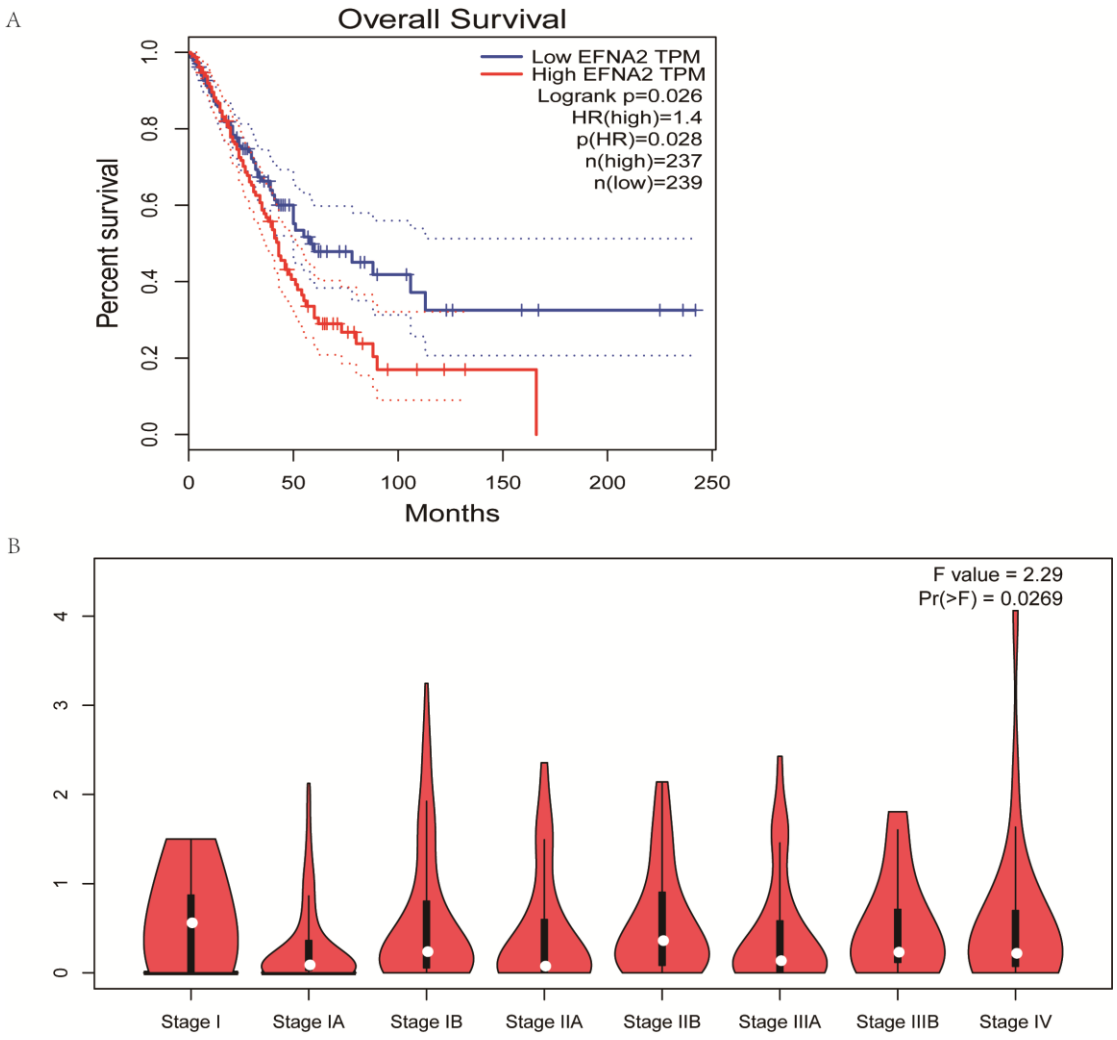



FIGURE 5 | Relationship between EFNA2 expression and prognosis of LUAD patients based on GEPIA database. (A) The relationship between EFNA2 expression levels and overall survival in LUAD was analyzed by GEPIA database.P < 0.05. (B) EFNA2 was significantly associated with stage in LUAD analyzed by GEPIA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

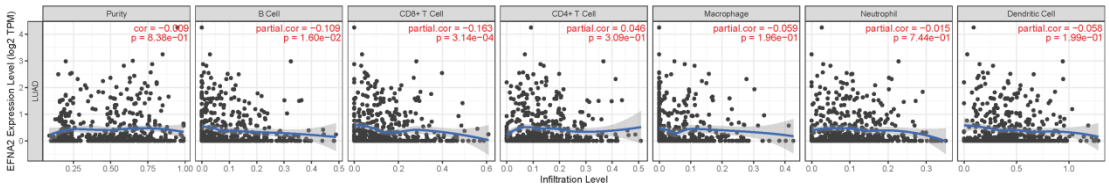
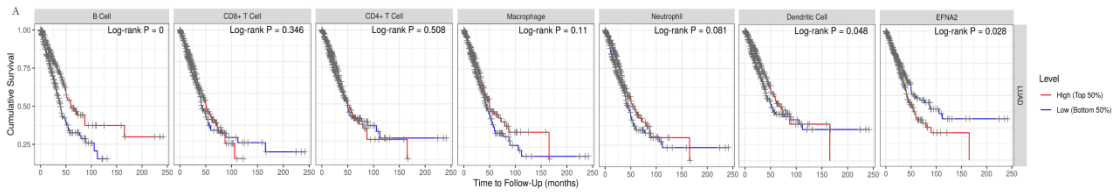



FIGURE 6 | Correlation between the expression of EFNA2 and immune infiltration of LUAD cells.



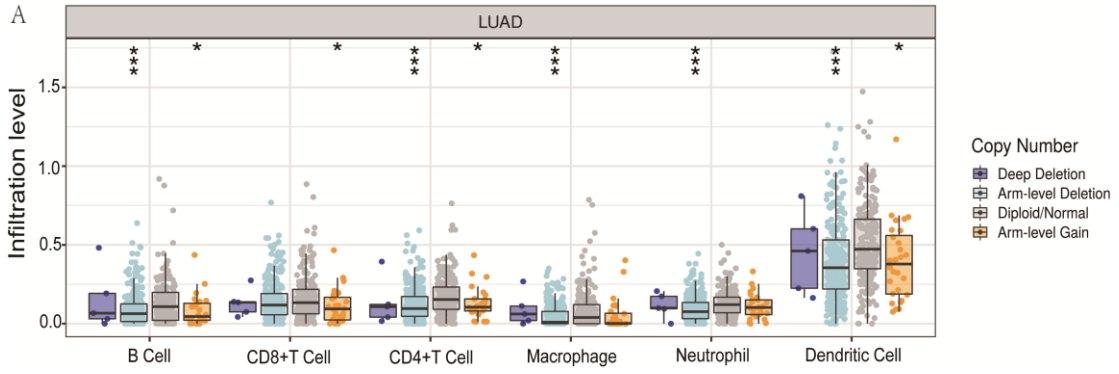

442

443 FIGURE 7 | Survival curve for immune cell infiltration. Kaplan–Meier survival curves based on top and bottom sample partitions with 50%

444 immune penetration. Red indicates a high degree of infiltration and blue indicates a low degree of infiltration. P < 0.05 was considered significant

445 and P < 0.0001 was reported as 0.

446




447

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

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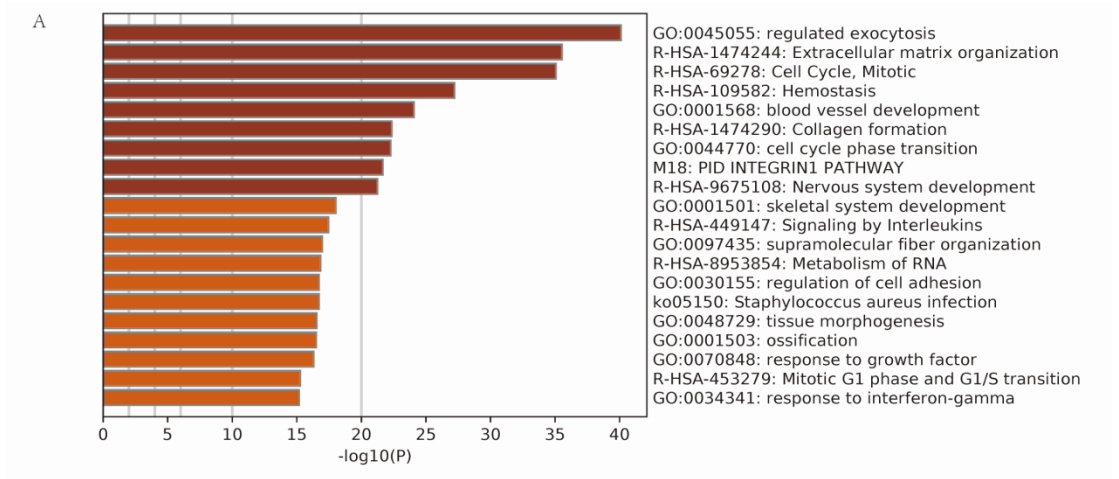


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451 FIGURE 9 | Gene Set Enrichment Analysis of EFNA2. Heatmap of enriched terms across input gene lists, colored by p-values.

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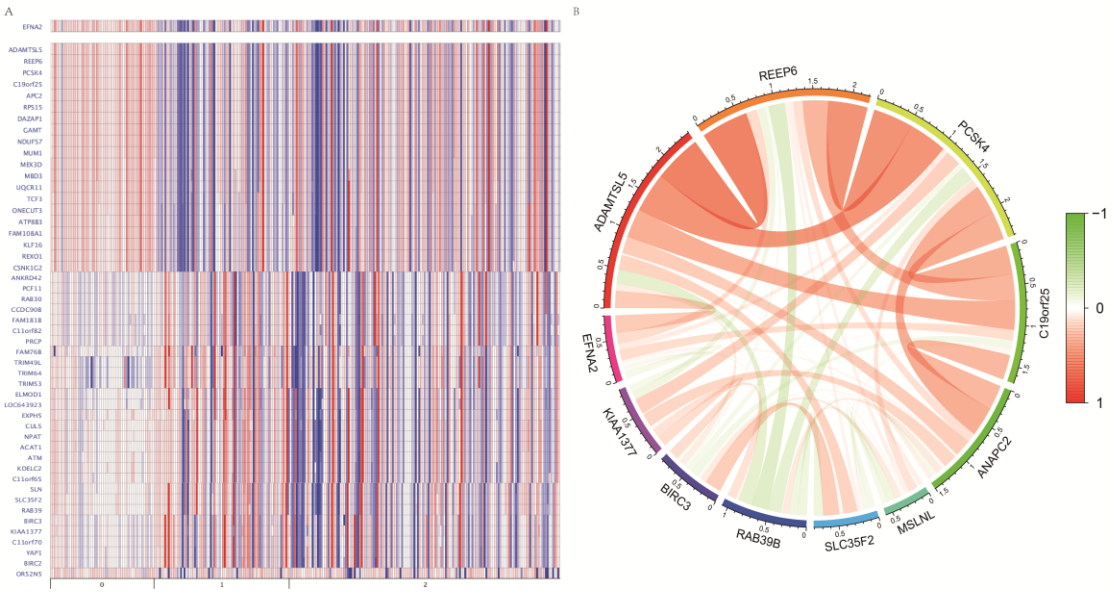


FIGURE 10 | Co-expression analysis of EFNA2 using the TCGA database. (A) Heatmap of the top 20 genes positively and negatively associated with EFNA2. (B) Circular plot of the top five genes positively and negatively related to the EFNA2 gene. Green represents negative association,and red represents positive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