Clinicopathological and prognostic significance of Ephrin A3 in bladder urothelial carcinoma

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Abstract. Ephrin A3 (EFNA3) is a member of the Eph/ephrin tyrosine kinase family, which is associated with multiple signaling pathways involved in cell growth and tumor cell metastasis. Aberrant regulation of EFNA3 is associated with the occurrence and development of various types of cancer. However, despite the high incidence of EFNA3 upregulation in cancer, studies concerning EFNA3 in urothelial carcinoma have not, to the best of our knowledge, been conducted. In the present study, bioinformatics analyses using data from multiple online databases were performed to confirm the upregulation of EFNA3 in bladder cancer. The co-expression gene set of EFNA3 and enriched signaling pathways were also analyzed. In addition, immunohistochemistry was conducted to detect EFNA3 expression in 491 clinically confirmed bladder urothelial carcinoma samples and 80 non-cancerous bladder tissues. Kaplan-Meier survival analysis, binary logistic regression analysis, and Cox regression analysis were conducted to confirm the validity of EFNA3 in predicting patient prognosis and its significance in clinical pathology. Statistical analysis demonstrated a significant association between EFNA3 expression levels with tumor size, lymph node metastasis, distant metastasis, and pathological grade. In conclusion, high EFNA3 expression may be a potential biomarker that indicates bladder tumor occurrence and patient prognosis.

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Introduction

Bladder urothelial carcinoma (BLCA) occurs in the bladder mucosa, is the most common malignancy of the urinary tract, and its incidence increases with patient age (1). There were estimated to be >81,400 new cases and 17,000 BLCA-associated deaths in the United States in 2020 (2). Although patients with BLCA undergo aggressive treatment, including surgery, chemotherapy, radiotherapy, and immunotherapy, the 5-year overall survival (OS) rate remains unsatisfactory and recurrence and progression rates following BLCA treatment remain high, which places a considerable financial burden on the healthcare system and impacts the quality of life of patients (3,4). Patient prognosis is difficult to predict as there are no clinical biomarkers or parameters that can reliably determine disease progression (5-7). Therefore, the identification of new biomarkers is crucial for the early diagnosis, prognostic assessment, and treatment of bladder cancer.

Ephrins are a class of cell surface ligands that mediate the migration, rejection, and adhesion of neuronal, vascular, and epithelial cells by binding to members of the Eph tyrosine kinase receptor family (8). A previous study has shown that Eph receptors and ephrins serve a key role in cancer cell proliferation, invasion, metastasis, and angiogenesis as signaling molecules involved in axon guidance (9). As a member of the Ephrin family, Ephrin A3 (EFNA3) potentially plays a role in the pathology of several cancer types (10). High expression of EFNA3 in gastric cancer cells is correlated with poorer patient prognosis and is an effective prognostic indicator for the responsiveness to immunotherapy in gastric cancer (11). Meanwhile, the upregulation of EFNA3 is regulated by microRNA-210, which promotes the proliferation and invasion of oral cancer cells (12).

Collectively, the results of the aforementioned studies suggest an association between the EFNA3 gene and an unfavorable cancer prognosis. However, the precise expression levels and prognostic significance of the EFNA3 gene in the context of bladder cancer remain unclear. Thus, the aim of the present study was to investigate the expression of EFNA3 in BLCA, assess its correlation with clinicopathological characteristics, and evaluate its impact on patient prognosis. To

achieve these objectives, a publicly accessible database was initially utilized to examine EFNA3 expression in BLCA. Subsequently, immunohistochemistry (IHC) was conducted on tissues from 491 patients with BLCA to confirm the association of EFNA3 with this disease. The results of the present study indicated that EFNA3 may serve as a promising biomarker for determining both prognosis and treatment of BLCA.

Materials and methods

UALCAN database and TCGA. UALCAN (http://ualcan.path.uab.edu) is a database that uses The Cancer Genome Atlas (TCGA) database to collect clinical data from 31 cancer types. EFNA3 gene expression information and basic clinical features were obtained from TCGA (https://portal.gdc.cancer.gov) (13). UALCAN can analyze the relative expression of a gene across tumor and normal samples, as well as across various tumor subgroups that are based on the cancer stage, tumor grade, race, body weight, or other clinicopathological features. This resource serves as a platform for *in silico* validation of target genes and for identifying tumor subgroup-specific candidate biomarkers (14). Data in the UALCAN database was examined and filtered for the EFNA3 gene in BLCA using the following criteria: i) 'gene symbol: EFNA3' and ii) 'TCGA dataset: Bladder urothelial carcinoma'. Then the following conditions were selected: 'Expression'; 'Survival'; 'Correlation'; and 'Pan-cancer view'.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. LinkedOmics (http://www.linkedomics.org/login.php) uses the data of 32 cancer types from TCGA. These data can be used to analyze the relationship between mRNA gene expression and features such as methylation and mutation sites. In the present study, LinkedOmics was used to obtain information on EFNA3 expression in BLCA by setting the following filter conditions: i) 'Gene: EFNA3'; ii) 'Analysis Type: Cancer vs. Normal analysis'; iii) 'Data Type: mRNA'; iv) 'Cancer Type: Bladder urothelial carcinoma'; v) 'Gene Summary: P-value<0.05, fold change=all, gene rank=top 10%'; and vi) 'Statistical method: Pearson correlation test'. Following this, 'Gene Set Enrichment Analysis (GSEA)' tools were selected. Then the following conditions were selected: 'GO analysis (biological process)'; 'GO analysis (cellular component)'; 'GO analysis (molecular function)'; and 'KEGG pathway'.

GSEA. GSEA is a computational method used to determine whether a predefined gene set exhibits statistically significant differences between two biological states. In the present study, GSEA was used to identify potential pathways associated with EFNA3 expression and prognosis in BLCA. For this, data associated with BLCA were downloaded from TCGA (https://portal.gdc.cancer.gov/), and GSEA software (version 4.1.0; Broad Institute and the University of California) was used for analysis. The gene expression profiles of patients with BLCA were divided into high and low-expression groups based on the median value of EFNA3 expression. Gene sets were considered significantly enriched when the false discovery rate (FDR) <0.25 and P. adjust <0.05.

Protein-protein interaction networks (PPIs). The interacting proteins associated with EFNA3, and the interaction network was analyzed using the STRING search tool (http://string-db.org/). The necessary data were obtained by searching for protein names, species and other necessary information.

Patient samples. The present study was a retrospective study in which bladder cancer pathological specimens were obtained. A retrospective analysis of tissue sections stored in the pathological database was performed. A total of 491 samples of BLCA tissues were collected from patients undergoing surgical resection at The Zhejiang Provincial People's Hospital (Hangzhou, China) between January 1998 and December 2011. The patient cohort consisted of 432 males and 59 females, aged 35-79 years old (median, 63.2 years old). No patients had undergone radiotherapy or chemotherapy prior to surgical resection. Moreover, 80 control samples were collected from adjacent tissues located >5 cm from the tumor edge, which is usually defined clinically as normal tissue >5 cm from the tumor margin (samples were collected between January 1998 and December 2011) (Table I). The samples were subsequently used to prepare tissue microarrays (TMAs), which were constructed by Shanghai Xinchao Biological Technology Co., Ltd. The study was a retrospective analysis, approved by the Ethics Committee of Zhejiang Provincial People's Hospital and an informed consent waiver was obtained from the Ethics Committee of Zhejiang Provincial People's Hospital (approval no. QT2022423). The study was performed in accordance with the Declaration of Helsinki (15) and all patient details were removed to protect patient privacy throughout the process.

IHC and evaluation of EFNA3 protein expression. The changes in EFNA3 protein expression were studied using the TMA consisting of the 491 human BLCA samples and 80 non-cancerous human bladder tissue samples. Briefly, slides were incubated at 68˚C for 2 h, followed by deparaffinization, dehydration in xylene, and rehydration. Subsequently, the sections were immersed in antigen retrieval buffer and boiled at 120˚C in a pressure cooker for 3 min. The sections were then treated with 3% H_2O_2 for 15 min to quench endogenous peroxidase activity and 1% bovine serum albumin to prevent non-specific binding. Then, the sections were incubated with rabbit anti-EFNA3 antibody (1:500; cat. no. PA5-86397; Thermo Fisher Scientific, Inc.) at 4˚C overnight, washed three times with PBS, incubated with biotin-labeled secondary antibody for 20 min at room temperature and then horseradish peroxidase-conjugated antibody for another 20 min. Finally, tissue sections were stained with 3,3-diaminobenzidine, counterstained with hematoxylin for 8 min at room temperature, dehydrated, washed, and mounted.

Manual IHC staining quantification. Immunostaining was assessed and scored according to the staining intensity by two independent observers blinded to the clinical and pathological data. EFNA3-positive expression was categorized into 4 classes based on staining intensity which were assigned as follows: 0, no staining; 1, light yellow (weak staining); 2, yellowish-brown (moderate staining); and 3, brown (strong staining). The proportion of stained cells was scored as follows: 0, <5% cells stained; 1, 6-25% cells stained; 2, 26-50% cells stained; and 3, ≥50% cells stained. The staining index was calculated by multiplying the intensity and proportion scores. A staining

Table I. Expression of Ephrin A3 mRNA in BLCA and non-carcinomatous bladder tissues.

	Ephrin A3 expression			
Sample	Number	Negative	Positive	P-value
BLCA	491	209	282	<0.01
Non-carcinomatous bladder tissues	80	55	25	

BLCA, bladder urothelial carcinoma.

Table II. Relationship between Ephrin A3 expression and the pathological parameters of bladder urothelial carcinoma.

	EFNA3 expression			
Clinical parameters	Low	High	χ^2	P-value
Sex			0.280	0.597
Male	182	250		
Female	27	32		
Age, years			1.935	0.164
<55	87	100		
≥55	122	182		
Size of tumor			37.221	<0.0001[c]
<3 cm	136	105		
≥3 cm	73	177		
Invasion depth			26.182	<0.0001[c]
Ta-T1	60	30		
T2-T4	149	252		
Lymph node metastasis			8.658	0.003[a]
No	171	198		
Yes	38	84		
Distant metastasis			10.264	0.001[b]
No	198	242		
Yes	11	40		
Vascular invasion			19.687	<0.0001[c]
Negative	181	196		
Positive	28	86		
Histological grade			11.436	0.01[a]
Low grade (I-II)	104	132		
High grade (III-IV)	105	150		

[a]P≤0.01, [b]P≤0.001, [c]P≤0.0001.

index ≥4 was considered to reflect high EFNA3 expression and an index of <4 was considered low EFNA3 expression.

Statistical analysis. SPSS (version 26.0; IBM Corp.) was used to perform all statistical analyses. Categorical data were assessed for statistical significance of differences using a χ^2 test or Fisher's exact test to compare the groups. Logistic regression analyses were performed to determine the effects of the EFNA3 gene and clinical factors on patient prognosis. Survival analysis was performed using the Kaplan-Meier (KM) method combined with log-rank test. KM analysis was the basis for plotting overall survival (OS) curves. Patients were grouped according to EFNA3 expression and assessed for survival status and OS time. KM survival curves were plotted using GraphPad Prism version 10 (GraphPad Software, Inc.). In addition, univariate and multivariate Cox regression analyses were used to determine the relationship between EFNA3 gene expression and clinicopathological characteristics of 491 patients with BLCA. All tests were two-tailed statistical tests. P<0.05 was considered to indicate a statistically significant difference.

Results

BLCA samples have significantly higher EFNA3 expression levels than normal tissues. EFNA3 expression level differences in various tumor and normal tissue samples were analyzed using TCGA data from UALCAN. Upregulated EFNA3 expression was observed in a number of cancer types, particularly in cervical squamous cell carcinoma and esophageal cancer, while significant differences were observed between the EFNA3 expression levels in bladder cancer and normal tissues (Fig. 1A). After analyzing 19 normal bladder and 408 BLCA tissues in TCGA, the results demonstrated that EFNA3 was expressed at high levels in patients with advanced tumor-node-metastasis (TNM) stages and non-papillary carcinomas (Fig. 1B-F).

IHC was then used to assess the EFNA3 protein expression levels in BLCA and normal bladder tissues from patients. The results demonstrated that non-tumor tissues had little EFNA3 expression and BLCA tissues had markedly high expression (Fig. 2A-I). EFNA3 protein expression was detected in 282 out of 491 (57.4%) BLCA samples and 25 out of 80 (31.3%) normal bladder tissues, indicating a significant upregulation of expression in BLCA tissues. In addition, a positive association between high EFNA3 expression and tumor size, invasion depth, lymph node metastasis, distant metastasis, vascular invasion, and histological grade was found, while there was no significant association with age and sex (Table II).

Clinical significance of EFNA3 expression in BLCA prognosis. KM survival data demonstrated that the overall survival time was shorter in the high EFNA3 expression group compared with the low expression group (Fig. 3A and B). An analysis of the clinical sample data demonstrated that the mean survival time of patients with high EFNA3 expression was 38±1.03 months, which was significantly shorter than that of the low expression group (45±1.21 months) (Fig. 3C). ln addition, a univariate analysis of factors affecting survival was conducted and demonstrated that survival time was associated with tumor size (P=0.026), lymph node metastasis (P<0.01), vascular invasion (P<0.01), depth of invasion (P<0.01), distant metastasis (P=0.009), histological grade (P<0.01), and EFNA3 expression (P<0.01) (Table III). After entering these factors into a Cox proportional risk regression model, the results demonstrated that vascular invasion, histological grade, and depth of invasion were independent factors affecting the prognosis of patients

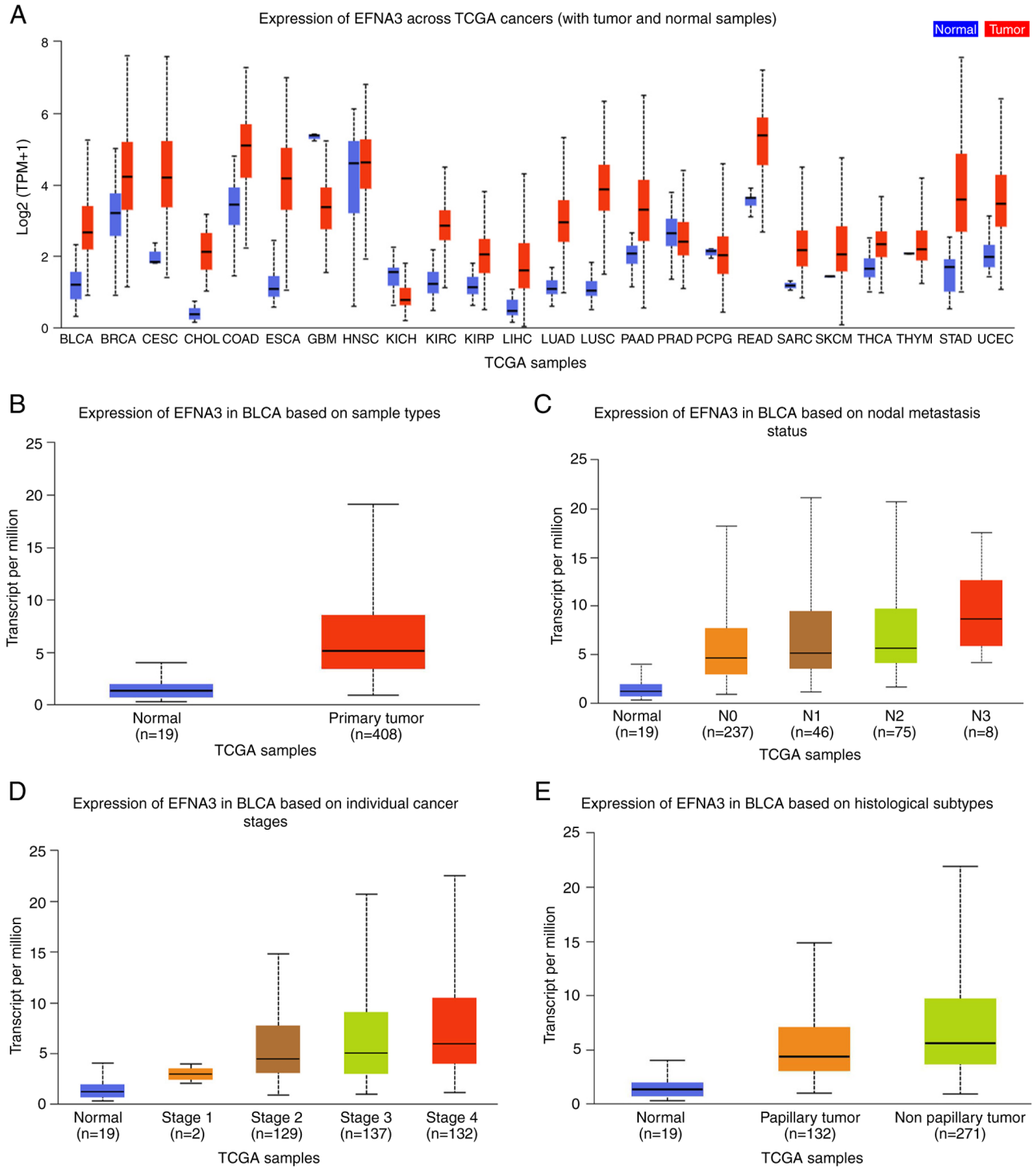

Figure 1. EFNA3 mRNA expression in BLCA and normal tissues. (A) EFNA3 mRNA expression in various cancer types based on data from The Cancer Genome Atlas database (using UALCAN). (B-E) EFNA3 transcription in subgroups of patients with BLCA stratified by grade, stage, and other criteria (using UALCAN). (B) Boxplot showing relative EFNA3 expression in BLCA and normal tissue samples. (C-E) Association between EFNA3 expression and BLCA histological grade, (C) BLCA nodal metastasis status, (D) BLCA TNM stage, and (E) BLCA histological subtypes. BLCA, bladder urothelial carcinoma; EFNA3, ephrin A3; TNM, tumor-node-metastasis; TPM, transcripts per million.

with BLCA (Table III). Although EFNA3 expression was not an independent factor, it contributed to the development of BLCA, which may be one of the adverse factors affecting patient prognosis. The EFNA3 gene was also subjected to binary logistic regression analysis and the results revealed that EFNA3 (P=0.036) had some therapeutic value in the early diagnosis of bladder cancer.

Genes co-expressed with EFNA3 in BLCA (GO and KEGG pathway analyses). Using the LinkedOmics web tool, co-expression of the EFNA3 gene in 408 BLCA samples from TCGA database was investigated. Genes positively correlated with EFNA3 (red spots; FDR=0.05) and genes negatively correlated with EFNA3 (green spots; FDR=0.05) are shown in the volcano plot in Fig. 4A. The top 50 genes

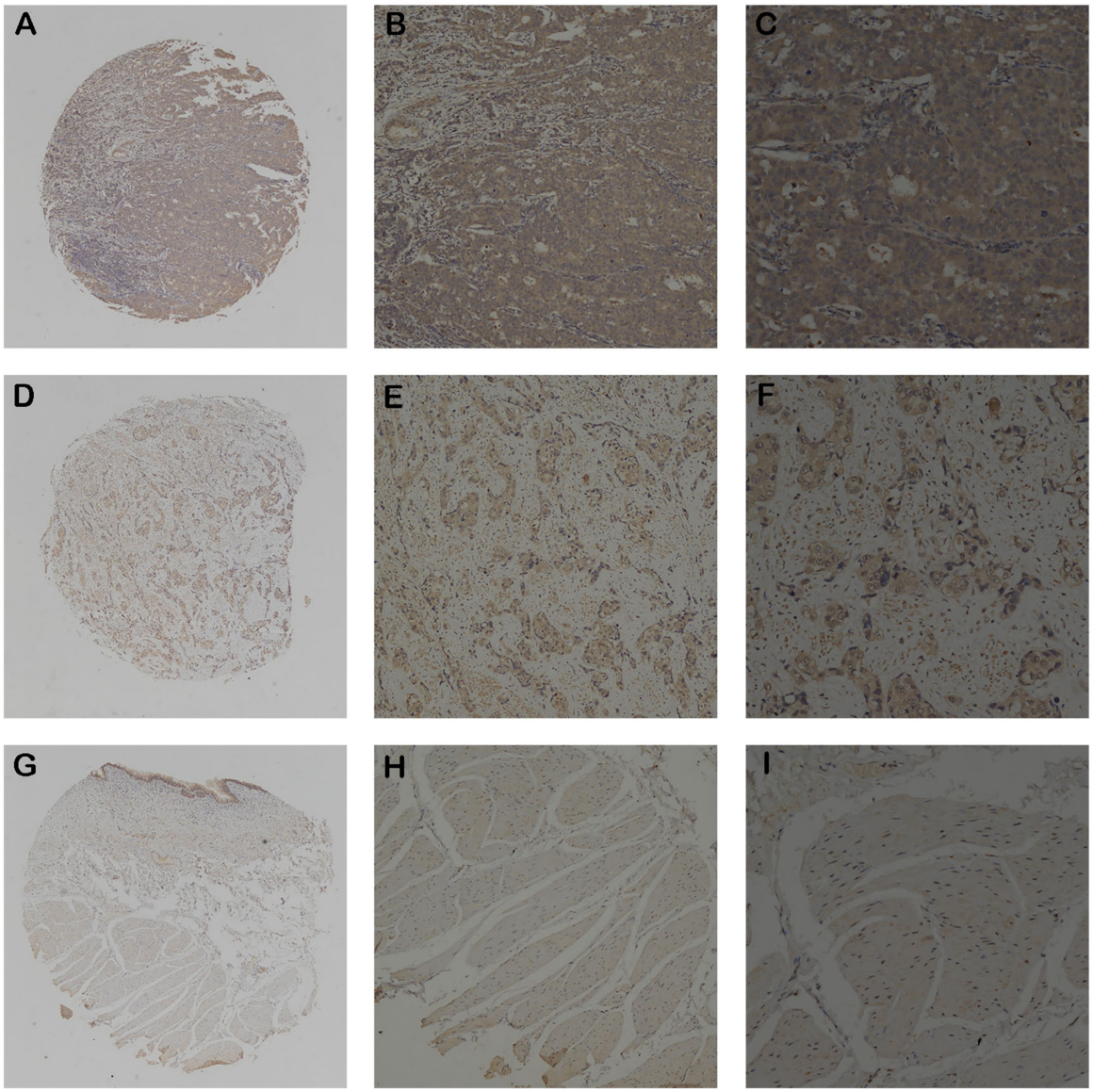

Figure 2. Representative images of EFNA3 staining. (A-C) High level of EFNA3 expression in BLCA. (D-F) Immunohistochemical staining of EFNA3 in normal bladder tissue. (G-I) Low level of EFNA3 expression in BLCA. Magnification: original magnification, x40 (left column); x200 (middle column), and x400 (right column). BLCA, bladder urothelial carcinoma; EFNA3, ephrin A3.

positively and negatively correlated with EFNA3 are shown in the heat map in Fig. 4B and C. The top three positively associated genes were ephrinA4 (EFNA4), ADAM metalloprotease domain 15 (ADAM15) and serine protease 27 (PRSS27) (Fig. 4D-F), and the top three negatively associated genes were centrosomal protein 120, DENN domain-containing 4A and KIAA1109 (Fig. 4G-I). The results of the GO enrichment analysis of the LinkedOmics data demonstrated that genes co-expressed with EFNA3 were located mostly in a mitochondrial protein complex, respiratory chain, anchored component of membrane, Sm-like protein family complex or the cytosol (Fig. 5A). The genes were involved in mitochondrial gene expression, NADH dehydrogenase complex assembly, the nucleoside monophosphate metabolic process, protein localization in the endoplasmic reticulum and the ephrin receptor signaling pathway (Fig. 5B). This co-expression of genes plays an important role in ribosomal RNA binding, ephrin receptor binding, unfolded protein binding, threonine-type peptidase activity, and oxidoreductase activity, acting on a heme group of donors (Fig. 5C). The KEGG pathway analysis revealed that the genes co-expressed with EFNA3 were primarily enriched in the ribosome, Parkinson's disease, the proteasome, carbon metabolism, fructose and mannose metabolism, the spliceosome and systemic lupus erythematosus (Fig. 5D). These findings indicated that EFNA3 had a broad transcriptome impact.

GSEA. To investigate the potential functional processes of EFNA3 in BLCA, TCGA data were used to perform a GSEA to locate KEGG pathways that were enriched in samples with high EFNA3 expression. Significantly enriched pathways were chosen based on their normalized enrichment score. The results demonstrated that 'REPRODUCTION', 'M_PHASE', 'NEURONAL_SYSTEM', 'EPIGENETIC_REGULATION_OF_GENE_EXPRESSION', 'HCMV_EARLY_EVENTS'

Table III. Univariate and multivariate analysis of the correlation between clinicopathological parameters and survival time of patients with bladder urothelial carcinoma.

A, Univariate

Covariate	Coefficient	Standard error	HR	95% CI	P-value
Age range, years: >55 vs. ≤55	0.267	0.138	1.31	0.996-1.712	0.053
Tumor size: ≥3 cm vs. <3 cm	0.297	0.133	1.35	1.037-1.748	0.026[a]
Sex: male vs. female	0.129	0.196	1.14	0.775-1.670	0.511
Lymph node metastasis: positive vs. negative	0.524	0.15	1.69	1.258-2.267	<0.0001[c]
Vascular invasion: positive vs. negative	1.082	0.145	2.95	2.219-3.922	<0.0001[c]
Distant metastasis: positive vs. negative	0.533	0.205	1.7	1.140-2.549	0.009[b]
Ephrin A3 expression: high vs. low	0.516	0.137	1.68	1.280-2.192	<0.0001[c]
Depth of invasion: Ta-T1 vs. T2-T4	0.954	0.206	2.6	1.736-3.885	<0.0001[c]
Histological grade	0.563	0.069	1.76	1.533-2.011	<0.0001[c]

B, Multivariate

Covariate	Coefficient	Standard error	HR	95% CI	P-value
Tumor size: ≥3 cm vs. <3 cm	-0.036	0.139	0.97	0.734-1.268	0.797
Lymph node metastasis: positive vs. negative	0.135	0.16	1.15	0.836-1.568	0.399
Vascular invasion: positive vs. negative	0.547	0.161	1.73	1.259-2.371	0.001[b]
Distant metastasis: positive vs. negative	0.386	0.218	1.47	0.960-2.254	0.076
Ephrin A3 expression: high vs. low	0.275	0.15	1.32	0.982-1.765	0.066
Depth of invasion: Ta-T1 vs. T2-T4	0.745	0.213	2.11	1.387-3.197	<0.0001[c]
Histological grade	0.459	0.079	1.58	1.355-1.848	<0.0001[c]

[a]P≤0.01, [b]P≤0.001, [c]P≤0.0001. HR, hazard ratio; CI, confidence interval.

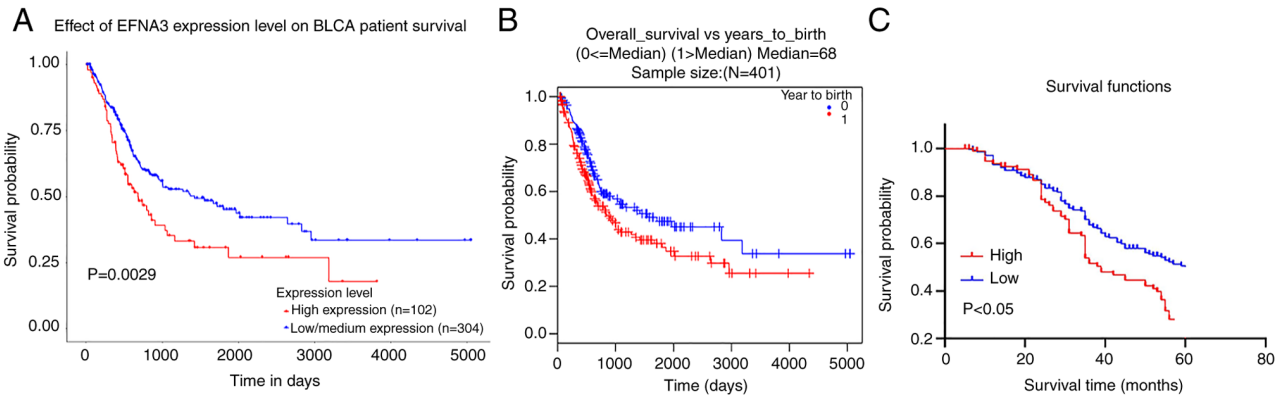

Figure 3. The prognostic value of EFNA3 expression levels in patients with BLCA. Survival curves were plotted based on the (A) UALCAN and (B) LinkedOmics databases. (C) Kaplan-Meier survival curves showed that patients with BLCA with high EFNA3 expression levels have a poorer prognosis than those with low EFNA3 expression (P<0.05). BLCA, bladder urothelial carcinoma; EFNA3, ephrin A3.

and 'CHROMOSOME_MAINTENANCE' were mostly enriched in samples with high EFNA3 expression (Table IV and Fig. 6A-F). These data implied that EFNA3 may aid in the advancement of BLCA by participating in a number of cancer-related signaling pathways.

PPI networks. The STRING database was used to generate PPI networks of EFNA3 in BLCA. The results demonstrated that EFNA3 interacted with Eph receptor A1 (16), EPHA2, EPHA3, EPHA4, EPHA5, EPHA7, EPHA10, EPHB1, EPHB3, and phospholipase C γ1 (Fig. 7A). In addition, the protein interactions were analyzed using the GeneMANIA tool and the data demonstrated that EPHA1, EPHA3, EPHA5, EFNA4, Ras P21 protein activator 1, EPHA2, EPHA10, EPHA4, EFNA5, EFNB3, EFNB2, EFNB1, and PIK3R2 interacted with EFNA3 (Fig. 7B).

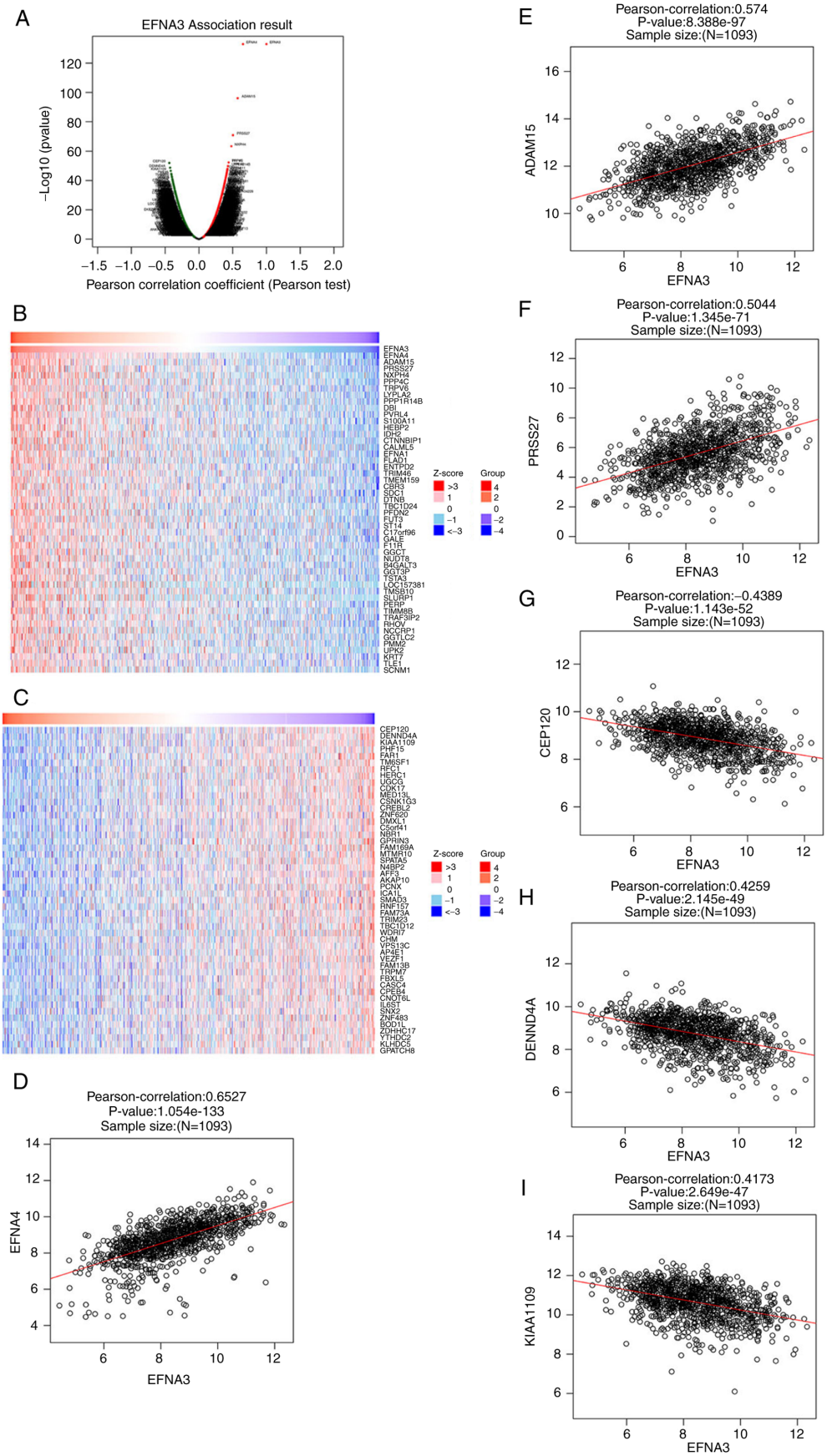

Figure 4. Genes co-expressed with EFNA3 in BLCA (using LinkedOmics). (A) Pearson's test was used to analyze correlations between EFNA3, and genes differentially expressed in BLCA. (B and C) Heat maps showing the top 50 genes positively and negatively correlated with EFNA3 in BLCA. Red indicates positively correlated genes and green indicates negatively correlated genes. (D-F) The positive correlation between EFNA3 and the top three genes (EFNA4, ADAM15, and PRSS27). (G-I) The negative correlation between EFNA3 and the top three genes (CEP120, DENND4A, and KIAA1109). ADAM15, A distintergase and metalloprotease domain 15; BLCA, bladder urothelial carcinoma; CEP120, centrosomal protein 120; DENND4A, DENN domain-containing 4A; EFNA3, ephrin A3; EFNA4, ephrinA4; PRSS27, serine protease 27.

Table IV. Gene sets enriched in the high ephrin A3 expression phenotype of bladder urothelial carcinoma.

Gene set name	Normalized enrichment score	Nominal P-value	False discovery rate q-value
REACTOME_REPRODUCTION[a]	1.98614599	0.00175131	0.01645493
REACTOME_M_PHASE[a]	1.84556691	0.00175439	0.01645493
REACTOME_NEURONAL_SYSTEM[a]	1.61850743	0.00176056	0.01645493
REACTOME_EPIGENETIC_REGULATION_OF_GENE_EXPRESSION[a]	1.84724447	0.00176367	0.01645493
REACTOME_HCMV_EARLY_EVENTS[a]	2.14477408	0.0017762	0.01645493
REACTOME_CHROMOSOME_MAINTENANCE[a]	1.93203135	0.00177936	0.01645493

[a]Gene sets with a nominal P-value <0.05 and a false discovery rate q-value <0.05 were considered significant.

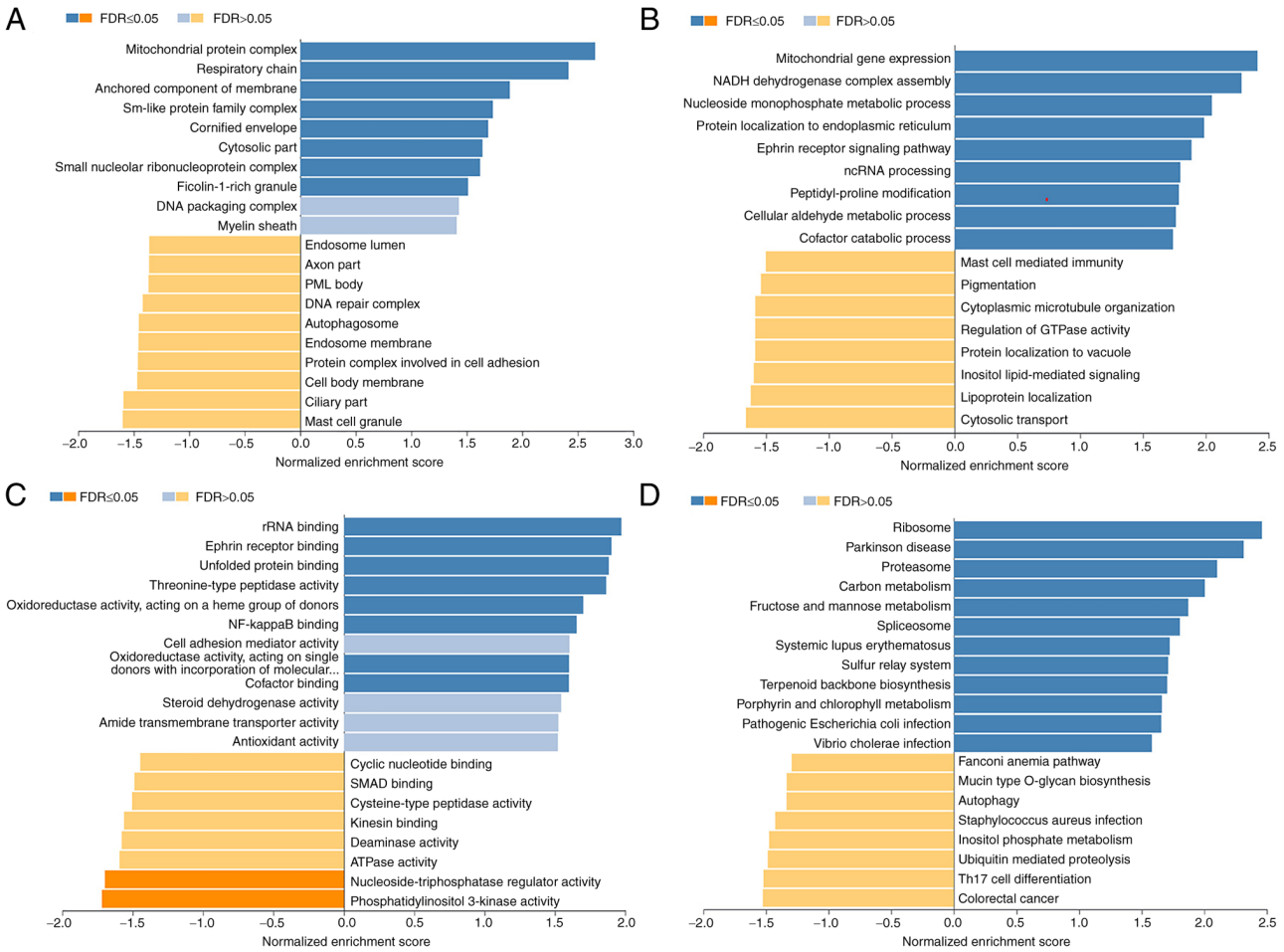

Figure 5. Significantly enriched GO annotations and KEGG pathways of EFNA3 in BLCA (using LinkedOmics). The significantly enriched GO annotations and KEGG pathways of genes co-expressed with EFNA3 in BLCA were analyzed using gene set enrichment analysis. The GO annotations were divided into (A) cellular components, (B) biological processes, and (C) molecular functions. (D) KEGG pathway analysis. The x-axis represents the NES, and the y-axis represents the GO term. BLCA, bladder urothelial carcinoma; EFNA3, ephrin A3; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; FDR, false discovery rate; p.adj, adjusted P-value.

Discussion

Bladder cancer is the second most common malignancy of the urinary tract and ranks tenth among the most prevalent types of cancer worldwide (4). Although patients are now able to receive effective treatment with advances in surgical techniques and the advent of neoadjuvant chemotherapy, bladder cancer remains highly susceptible to recurrence after treatment and places a great burden on families of patients (17). Immunotherapy, such as immune checkpoint blockade, now offers a novel avenue of treatment for BLCA, improving the survival of patients with advanced bladder

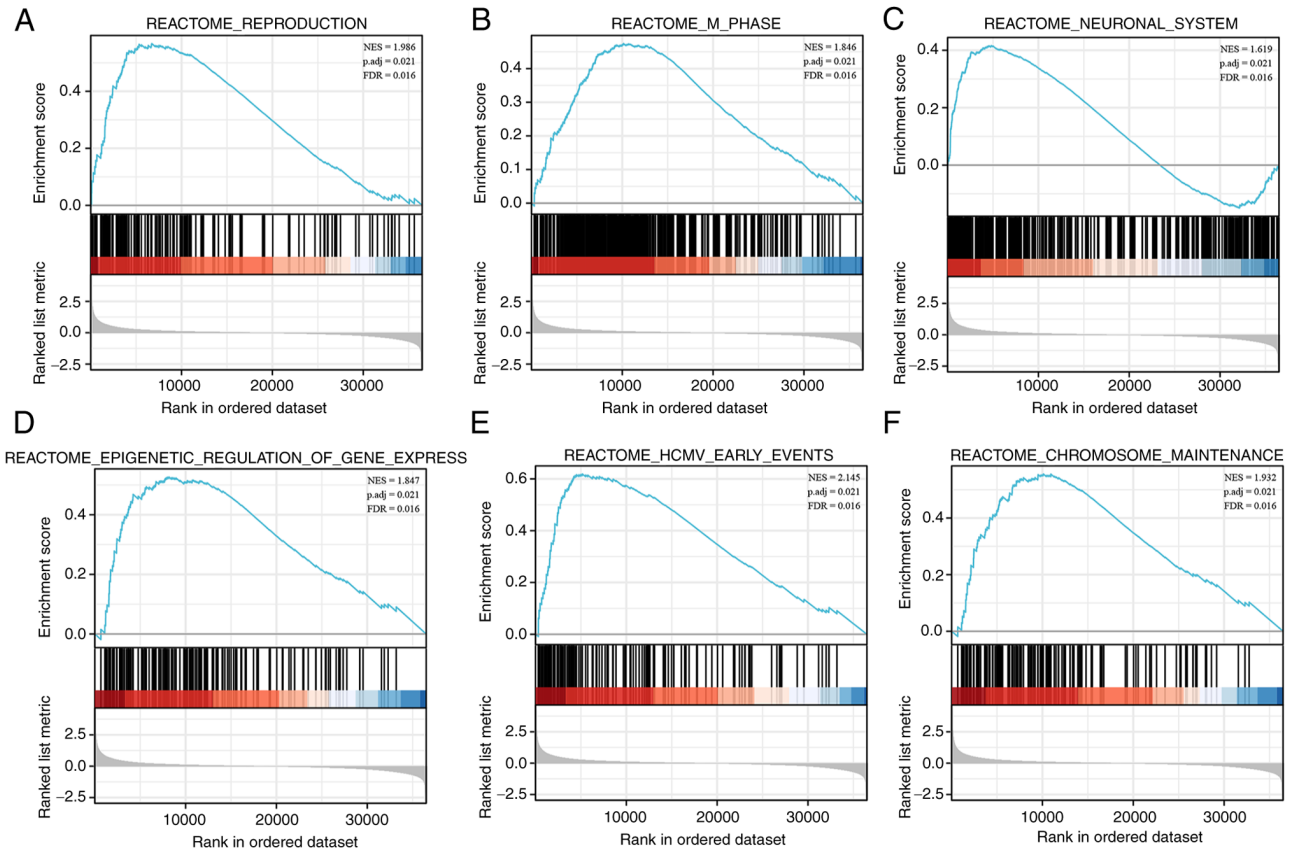

Figure 6. GSEA reveals potential signaling pathways of EFNA3. The analysis showed that (A) reproduction, (B) M-phase, (C) neuronal system, (D) epigenetic regulation of gene expression, (E) HCMV-early event, and (F) chromosome maintenance were enriched in the EFNA3 high expression group. The top panels indicate the enrichment scores for each gene, while the bottom panels show the ranking metrics of each gene. y-axis, ranking metric values; x-axis, ranks for all genes. BLCA, bladder urothelial carcinoma; EFNA3, ephrin A3; GSEA, Gene Set Enrichment Analysis; HCMV, human cytomegalovirus.

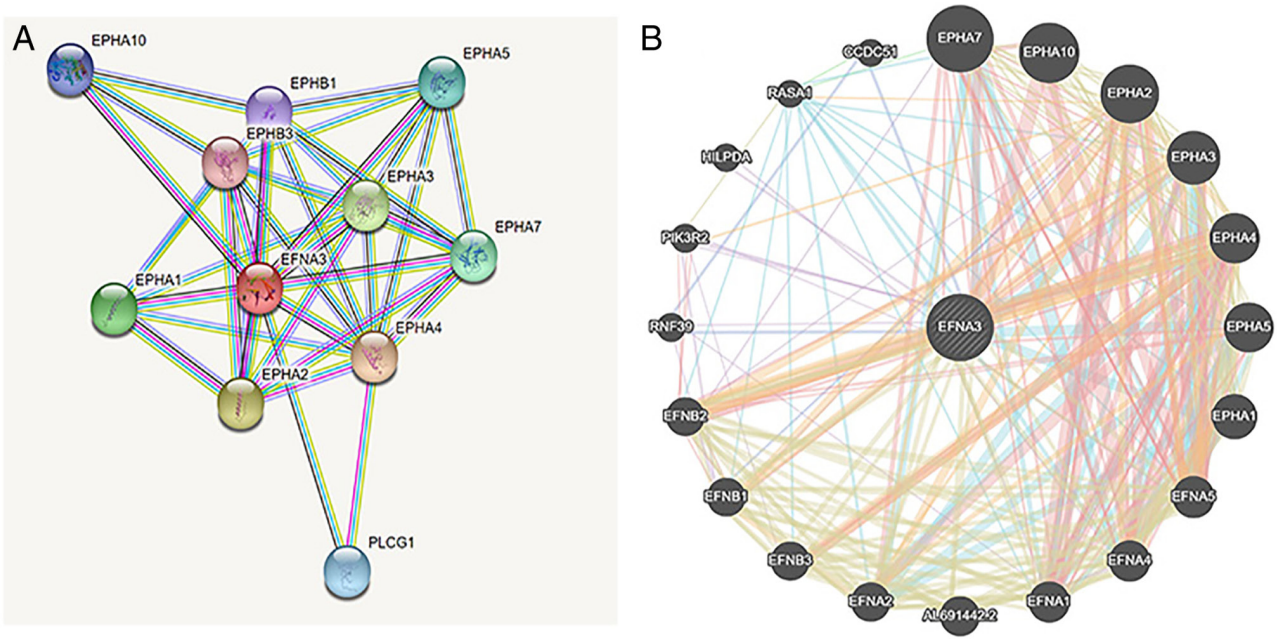

Figure 7. Protein-protein interaction network of EFNA3. Interactions between EFNA3 and other genes were obtained from the (A) STRING and (B) GeneMANIA web portal results. EFNA3, ephrin A3.

cancer; however, the results remain unsatisfactory (18,19). Therefore, there is an urgent need for new biomarkers both for early screening and for the assessment of treatment effectiveness in bladder cancer.

Ephrins are cell surface ligands that bind to Eph receptors on adjacent cells. Ephrins act as cell signaling molecules in pathways that repel and attract each other, mediating the migration and adhesion of cancer cells (20). The present study focused on EFNA3, which the present study showed to be significantly differentially expressed in bladder cancer cells and has a high prognostic value. A previous study has identified EFNA3 as a key driver of hepatocellular carcinogenesis and progression in the hypoxic microenvironment (21). Notably, several studies have found that EFNA3 is involved in tumor angiogenesis (22,23). However, to the best of our knowledge, the impact of EFNA3 in BLCA is unknown.

In the present study, it was found that EFNA3 expression in BLCA tissues was significantly higher than that in normal bladder tissues, through the analyses of several online databases. It was also demonstrated that EFNA3 was positively associated with pathological grade and tumor stage. In addition, EFNA3 protein expression was detected by IHC, and it was found that the EFNA3 expression levels were higher in BLCA tissues than in normal tissues, a result consistent with the mRNA results obtained from bioinformatics analyses. In summary, high expression of EFNA3 may have a facilitating effect on the proliferation and invasion of tumor cells.

Next, the clinicopathological features of patients with BLCA were correlated with the EFNA3 immunohistochemical scoring in the present study. The results demonstrated that EFNA3 was not only highly expressed in BLCA but was also associated with tumor size ($P<0.05$), depth of invasion ($P<0.05$), distant metastasis ($P=0.001$), lymph node metastasis ($P=0.003$), vascular invasion ($P<0.05$), and pathological grade ($P=0.01$). However, EFNA3 expression was not associated with patient age or sex. In the present study, univariate survival analysis was conducted to assess the prognostic value of EFNA3 in BLCA and it was found that EFNA3 expression levels, tumor size, lymph node metastasis, distant metastasis, vascular invasion, TNM stage, and pathological grade may affect the prognosis of BLCA. However, multivariate survival analysis demonstrated that only vascular invasion, pathological grade, and TNM stage were independent prognostic factors of BLCA. This suggested that the mechanism of BLCA development is complex and that non-independent prognostic factors affecting its prognosis may be associated with other factors, such as tumor size and distant metastases. Moreover, survival analysis of 491 samples was assessed and it was found that patients with BLCA with high EFNA3 expression had a worse prognosis than those with low expression. This result was consistent with the results of the KM Plotter database analysis. Therefore, it can be concluded that high EFNA3 expression promotes tumor cell growth and can be used to predict tumor metastasis and progression in BLCA.

To further understand the role of EFNA3 in BLCA, GO and KEGG analyses on genes co-expressed with EFNA3 were conducted in the present study. The results demonstrated that EFNA3 played an important role in the structure and function of the mitochondria, ribosomes, DNA, and proteins. The most notable site of enrichment was the ribosome. Increased synthesis of ribosomes leads to a corresponding increase in protein synthesis, which ultimately affects the development of tumor cells and plays an important role in the development of cancer (24,25). The enrichment of EFNA3 in the ribosome suggests that its expression levels are closely related to ribosome biosynthesis in bladder cancer cells. However, this hypothesis needs to be verified by relevant experiments in subsequent studies. Nonetheless, the present study provides new insights into the mechanism of BLCA development.

In the present study, the results of the GSEA demonstrated that EFNA3 was involved in biological processes, such as the cell cycle, transcription factors, and epigenetics. Previous studies have shown that epigenetic changes are closely associated with tumor development and prognosis in bladder cancer and that alterations in DNA hypermethylation and histone acetylation affect the aberrant expression of a large number of genes (26,27). Based on these findings, the following possible regulatory network for EFNA3 is suggested: EFNA3 may influence cell proliferation and apoptosis by regulating the cell cycle and DNA replication and by regulating transcription factor activity at the transcriptional level.

The top three genes positively associated with EFNA3 in the present study were EFNA4, ADAM15, and PRSS27. It has been demonstrated that EFNA4 was expressed in 82.9% of osteosarcoma cases and its high expression was associated with poor prognosis (28). Moreover, knock down of ADAM15 mRNA expression significantly reduced the invasive ability of bladder cancer cells through vascular endothelial monolayer, suggesting that ADAM15 may be involved in the proliferation and migration of bladder cancer cells (29). In addition, PRSS27 mRNA expression was higher in resected esophageal squamous cell carcinoma (ESCC) tissue samples than in normal esophageal mucosal tissues, suggesting that high PRSS27 expression is an indicator of poor prognosis in patients with ESCC (30). In summary, EFNA3 co-expression of related genes may play an important role in tumor development. It is therefore hypothesized that EFNA3 may promote the development of BLCA.

Unfortunately, the present study inevitably has some limitations, the first of which is the small sample size. In this retrospective analysis, the use of different surgical methods and resection ranges in patients with different pathological stages has led to challenges in obtaining sufficient paired samples of non-cancerous bladder tissue are certainly limitations. For example, the surgical margin distance for non-muscle invasive bladder cancer is often <5 cm, which leads to the fact that noncancerous bladder tissue may not be taken in every bladder cancer specimen. At the same time, it is worth stating that RNA sequencing data in public databases may not be representative of the entire BLCA population. Secondly, TMAs were used instead of whole tissue sections, which may not reflect the full heterogeneity of primary BLCA. The third point is that tools such as Image J for automated semiquantitative analysis of EFNA3 staining were lacking. In the present study, gene expression levels in tissue sections were assessed based on the number of positively stained cells and the intensity of staining, rather than using histochemistry score (H-score), Allred-score, and immunoreactive scoring systems. This allows for possible visual bias and lack of accuracy in IHC scoring. Finally, IHC-based EFNA3 protein expression was assessed, which remains a semi-quantitative method. Therefore, more quantitative examinations are required.

In conclusion, the understanding of the mechanisms of the molecular oncogenic action in BLCA of EFNA3 has been

enriched by merging oncogene expression data from public databases with IHC results from clinical samples. The present study demonstrated that EFNA3 plays a significant role in BLCA, and since upregulation of EFNA3 expression in BLCA was associated with a shorter survival time and reduced survival, it may bring novel approaches to BLCA prevention and therapy. Further understanding EFNA3 expression in BLCA will aid in the identification of patients with a high metastatic potential. As a result, EFNA3 expression levels could be used to predict bladder cancer invasion and prognosis in the future.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the TCGA repository, LinkedOmics, STRING (http://string-db.org/), UALCAN, GEPIA, and GeneMANIA. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QZ and HW designed the study. HW, YFW, KL and CFZ collected the data. YFW, YNH and ZFS analyzed and interpreted the data. YNH and KL confirm the authenticity of all the data. YFW and HW wrote the manuscript. All authors edited and reviewed the manuscript.

Ethics approval and consent to participate

Ethical approval was received for the study from the Clinical Research Ethics Committee of Zhejiang Provincial People's Hospital (approval no. QT2022423; Hangzhou, China). Informed consent was waived by the Clinical Research Ethics Committee of Zhejiang Provincial People's Hospital. All methods were performed in accordance with relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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