Comprehensive analysis of E47-like factors and verification of ELF4 in clear cell renal cell carcinoma

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Abstract. Clear cell renal cell carcinoma (ccRCC) is the most prominent subtype of renal cancer and E47-like factors (ELFs) are important in tumorigenesis; however, the specific role of key ELFs in ccRCC remains unclear. The present study comprehensively analyzed RNA sequencing and clinical data from multiple databases, and identified differentially expressed ELFs (ELF3-5) in ccRCC. The DNA promoter methylation, genetic variation and clinical significance of ELF3-5 in ccRCC were analyzed using the cBioPortal and UALCAN databases. The association between ELF3-5 and multiple immune cell infiltration was analyzed using Tumor Immune Estimation Resource. Subsequently, ELF4 was selected and its association with biological functions was assessed. Cell counting kit-8 (CCK-8), colony formation, Transwell, macrophage chemotaxis and polarization assays were conducted to validate the functions of ELF4. Notably, the mRNA expression levels of ELF4 were significantly upregulated in ccRCC, whereas ELF3 and ELF5 mRNA expression levels were significantly downregulated. Clinical significance analysis revealed that ELF4 showed a high clinical significance with tumor grade, clear cell type A and B subtypes, and incidence rates of amplification in genetic variation. Further analyses indicated that ELF4 may be involved in multiple immune cell differentiation. Additionally, cell experiments revealed that ELF4 inhibition downregulated 769-P and 786-O proliferation, migration and invasion. Knockdown of ELF4 in cancer cells also inhibited M2 macrophage polarization and chemotaxis towards 769-P and 786-O cells. Conclusively, the present findings indicated the clinical significance of ELF4 in ccRCC, and verified its key role in driving cell proliferation, migration and invasion, and promoting M2 macrophage polarization and chemotaxis in ccRCC.

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Introduction

Renal cell carcinoma (RCC) is a prominent tumor within the urinary system; it accounts for ~2% of global cancer diagnoses and deaths, and is projected to increase in burden worldwide (1). Clear cell RCC (ccRCC) is the predominant subtype of RCC. Despite recent advances in treating advanced and metastatic ccRCC, the 5-year survival rate of metastatic ccRCC is <10% (2). Surgical resection is currently the main treatment option for ccRCC; however, it has been reported that 30-40% of patients with local lesions experience post-surgery recurrence (3). Despite gradual improvements in immune and targeted therapies, these approaches have failed to achieve desirable progression-free survival in patients with ccRCC. Moreover, subsequent treatments for recurrent ccRCC have yielded suboptimal outcomes (4). Therefore, exploring the mechanisms underlying ccRCC development, and identifying highly sensitive and specific tumor biomarkers have emerged as current research trends.

Recent studies have highlighted the role of specific transcription factor families in the malignant progression of ccRCC (5,6). Within these families, the E-twenty-six (ETS) transcription factor family serves major roles in tumorigenesis, including that of ccRCC, with some members functioning as oncogenes and others as tumor suppressors (7). Among the ETS family, various E47-like factors (ELFs) influence the biological activity of ccRCC cells through transcriptional regulation. For example, ELF1 exhibits bidirectional suppression of the tumor suppressor TSC2 and the repair-related gene NTH1 (8). Additionally, ELF2 has been reported to promote ccRCC cell proliferation by mediating the transcription of c-Myc-induced ELF2 regulator (9). However, the molecular mechanisms underlying the carcinogenic or tumor-suppressive effects of these ELFs in ccRCC remain poorly understood.

Previous reports have highlighted the association of various ELFs with malignant progression, prognosis and infiltration in numerous types of cancer. For example, ELF1, which has been identified as a carcinogen, has been observed to regulate the cell proliferation of multiple types of cancer, including prostate and lung cancer (7,10). ELF4 has been implicated in the malignant progression of gastric cancer by regulating CDX2 (11). In tumor prognosis research, ELF4 expression has emerged as an independent predictor of poor prognosis in colorectal cancer (12). Furthermore, ELF5 expression levels

have also been linked to the survival and prognosis of patients with epithelial ovarian cancer (13). As immunotherapy gains wider application, research on the regulatory mechanisms of ELFs in the tumor immune microenvironment have gained attention. For example, a decrease in T-cell receptor (TCR) ζ chain transcription factor ELF1 and its binding to DNA may contribute to reduced or absent TCR ζ chain transcripts in tumor-infiltrating lymphocytes (14). In breast cancer, ELF5 has been identified as a key transcriptional determinant of tumor subtype and increased levels of ELF5 have been associated with enhanced leukocyte infiltration (15). Despite the significant roles played by ELFs in other cancer types, their specific functions and related mechanisms in ccRCC remain unclear.

In the present study, a comprehensive analysis of ELF1-5 in ccRCC was conducted using multiple databases and the clinical significance of ELF3-5 was confirmed in patients with ccRCC. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were also performed. Notably, the effects of ELF4 on the proliferation, migration and invasion of ccRCC cells were assessed, as were its effects on macrophage polarization and chemotaxis. The present study is expected to reveal the clinical significance, biological activity and immune infiltration of ELF4, in order to identify a potential new target for patients with ccRCC.

Materials and methods

Analysis of differentially expressed genes in multiple databases. The Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/) was used to analyze ELF1-5 expression data (16). GEPIA is a newly developed interactive web server for analyzing the RNA sequencing expression data from 9,736 tumor tissues and 8,587 normal tissues of patients obtained from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression projects (16). For Gene Expression Omnibus (GEO) analysis, raw sequencing data were obtained from the GEO database (GEO accession: GSE53757) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53757) (17). Differential expression analysis and gene expression data normalization were performed using the R package edgeR (18). The differential expression levels of ELF1-5 in ccRCC and normal tissues were also illustrated using UALCAN (http://ualcan.path.uab.edu) (19). UALCAN is a comprehensive, user-friendly web resource for analyzing cancer omics data in TCGA project. The differential expression levels of ELF1-5 in various cancer types and normal tissues were illustrated using the Oncomine database (https://www.oncomine.org) (20). Oncomine, the largest cancer gene chip database and integrated data mining platform is designed to extract valuable cancer gene information. The threshold parameters of P-value and fold-change were demarcated as 0.05 and 2, respectively.

Clinical significance analysis. The expression levels and promoter methylation levels of ELF3-5 were assessed in relation to cancer stage, subtype and tumor grade using UALCAN (19). The threshold parameters of P-value and fold-change were demarcated as 0.05 and 2, respectively. The clinical significance of ELF3-5 on the overall survival (OS) and disease-free survival (DFS) of patients with ccRCC was evaluated using GEPIA. Kaplan-Meier survival analysis and log-rank test were performed using GEPIA database, and log-rank P-values and hazard ratio (HR) values were obtained (16). A log-rank test with $P<0.05$ was considered to indicate a statistically significant difference.

cBioPortal analysis. Genetic alterations of ELF3-5 were obtained and analyzed from the cBioPortal based on TCGA project (11). As a comprehensive web resource, the cBioPortal database (http://www.cbioportal.org) is used for visualizing and analyzing multidimensional cancer genomics data.

GO and KEGG enrichment analysis. The LinkedOmics (https://www.linkedomics.org/login.php) database was used to search for ELF3-5-related co-expressed genes in ccRCC (21). LinkedOmics is a publicly accessible portal integrating multi-omics data from all 32 TCGA cancer types and 10 Clinical Proteomics Tumor Analysis Consortium cancer cohorts. It is a valuable platform for biologists and clinicians to access, analyze and compare multi-omics data across various tumor types. Co-expression analysis was performed using the Pearson correlation coefficient as a statistical measure. GO analysis and KEGG pathway enrichment analysis were conducted on the ELF3-5-related co-expressed genes using the LinkInterpreter module of LinkedOmics to obtain descriptive information. The Gene Set Enrichment Analysis tool (https://www.linkedomics.org/lo_batchfile/qindex_gsea.php?fn=122773) was employed to explore the functional network of co-expressed genes, including GO (biological process, cellular component, molecular function) and KEGG pathway analyses. The rank criterion for significance was set at a false-discovery rate <0.05 and 1,000 simulations were performed.

Tumor immune estimation resource (TIMER) analysis. The TIMER web server (https://cistrome.shinyapps.io/timer/) is a comprehensive resource for analyzing immune infiltrates in various cancer types (22). The gene module of TIMER allows users to select any gene of interest and visualize the correlation of its expression with immune infiltration level in diverse cancer types. The partial Spearman's correlation analysis was performed to determine the relationship between the RNA-sequencing expression profiles of ELF3-5 in ccRCC and immune cells.

Cell culture and transfection. The 786-O and 769-P ccRCC cell lines, and the HK-2 normal human renal tubular epithelial cell line were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. 769-P and 786-O cells were cultured in RPMI-1640 medium (cat. no. C11875500BT; Gibco; Thermo Fisher Scientific, Inc.) containing 10% heat-inactivated fetal bovine serum (FBS; cat. no. 16140089; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (cat. no. P4333; MilliporeSigma). HK-2 cells were cultured in minimum Eagle's medium (cat. no. SH30244.01; Hyclone; Cytiva) containing 10% FBS. The THP-1 human monocytic leukemia cell line was also purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and were cultured in

RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin. THP-1 cells were differentiated into macrophages by treating them with 10 ng/ml phorbol-12-myristate-13-acetate (PMA; cat. no. P8139; MilliporeSigma) for 24 h at 37˚C. All cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2.

The small interfering RNA (siRNA) constructs targeting ELF4 (si-ELF4) and the corresponding negative control (si-NC) were purchased from Guangzhou RiboBio Co., Ltd. 769-P and 786-O cells were seeded into 6-well plates at $1x10^6$ cells/well. According to the manufacturer's instructions, the transfection of the aforementioned siRNAs into 769-P and 786-O cells was performed using Lipofectamine® 2000 transfection reagent (cat. no. 11668030; Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. Approximately 48 h post-transfection, cells were collected for further studies. The siRNAs were used at a concentration of 100 nM and the sequences were as follows: si-ELF4 sense, 5'-GCUGGACGACGUUCACAAUTT-3' and antisense, 5'-AUUGUGAACGUCGUCCAGCTT-3'; si-NC sense, 5'-AUCAACGAUAUCCGGUUGG-3' TT and antisense, 5'-CCAACCGGAUAUCGUUGAUTT-3'.

Macrophage polarization assay. The macrophage polarization experiment was performed as previously described (23). Si-NC and si-ELF4 groups of 769-P and 786-O cells were seeded at $1x10^6$ cells/ml in 6-well plates (3 ml/well). The supernatant of ccRCC cells was collected. PMA-induced THP-1 cells (macrophages) were seeded at $1x10^6$ cells/ml in 6-well plates (3 ml/well) in RPMI-1640 medium containing ccRCC cell supernatant and were incubated for 48 h. The mRNA expression levels of the M1 macrophage markers (IL-6, CXCL10 and CD80) and M2 macrophage markers (CD206, fibronectin and CCL22) were determined to study the effects of ccRCC cell supernatant on polarization of macrophages.

Macrophage chemotaxis assay. A chemotaxis assay was performed as previously described (24,25). Briefly, PMA-induced THP-1 cells (macrophages) were incubated with IL-4 and IL-13 (20 ng/ml IL-4 and IL-13; cat. nos. 6507IL and 213ILB; R&D Systems, Inc.) for 48 h at 37˚C to obtain M2 macrophages. Similarly, PMA-induced THP-1 cells (macrophages) were incubated with lipopolysaccharide (100 ng/ml; cat. no. L2880; MilliporeSigma) and IFN-γ (20 ng/ml; cat. no. 285-IF; R&D Systems, Inc.) for 48 h at 37˚C to obtain M1 macrophages. The supernatant of 769-P and 786-O cells (400 µl) was added to the lower compartment of 6 Transwell inserts (pore size, 3 µm; cat. no. 3414; Corning, Inc.). M1 or M2 macrophages ($4x10^4$ cells/well) were then overlaid onto the upper chamber. After 16 h at 37˚C, the migrated cells were counted using a hemocytometer (cat. no. Z359629; MilliporeSigma).

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells using TRIzol® (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA purity (OD_{260}/OD_{280} nm, 1.8-2.2) was assessed using NanoDrop 2000 (NanoDrop; Thermo Fisher Scientific, Inc.). RT was performed with 1 µg total RNA as the template using the PrimeScript™ RT reagent Kit (cat. no. RR037Q; Takara Bio, Inc.) according to the manufacturer's instructions. The relative mRNA expression levels were determined using RT2 SYBR® Green qPCR Mastermixes (cat. no. 330509; Qiagen GmbH) on the LightCycler 480 system (Roche Diagnostics). The reaction conditions included an initial single cycle at 95˚C for 10 min, followed by 45 cycles at 95˚C for 15 sec and 95˚C for 1 min. The following primer sets were used for qPCR: ELF4 forward (F), 5'-CATCATAACAGACGGGAC CTTG-3', reverse (R), 5'-GCTGGGAGACTCCATATTGAG TA-3'; GAPDH F, 5'-GAATGGGCAGCCGTTAGGAA-3', R, 5'-AAAAGCATCACCCGGAGGAG-3'; IL-6 F, 5'-CCT GAACCTTCCAAAGATGGC-3', R, 5'-CACCAGGCAAGT CTCCTCATT-3'; CXCL10 F, 5'-TGAATCCAGAATCGA AGGCCA-3', R, 5'-TGCATCGATTTTGCTCCCCT-3'; CD80 F, 5'-ACGCCCTGTATAACAGTGTCC-3', R, 5'-GAGGAA GTTCCCAGAAGAGGTC-3'; CD206 F, 5'-GCTAAACCT ACTCATGAATT-3', R, 5'-GGCAAGGCCAGCACCCGT TA-3'; fibronectin F, 5'-CCATCGCAAACCGCTGCCAT-3', R, 5'-AACACTTCTCAGCTATGGGCTT-3'; CCL22 F, 5'-GAGATCTGTGCCGATCCCAG-3', R, 5'-AGGGAATGC AGAGAGTTGGC-3'; RPS9 F, 5'-CTGGATGAGGGCAAG ATGAAG-3', R, 5'-GTCTGCAGGCGTCTCTCTAAGAA-3'. The relative mRNA expression levels were normalized to the average Cq values of GAPDH plus RPS9, and were quantified using the $2^{-\Delta\Delta Cq}$ cycle threshold method (26).

Cell counting Kit-8 (CCK-8) assay. Cell proliferation was assessed using the CCK-8 assay. Cancer cells were seeded in 96-well plates at $5x10^3$ cells/well density. According to the manufacturer's instructions, the cells were assessed at 0, 24, 48, 72 and 96 h using the CCK-8 Kit (10 µl/well; cat. no. ab228554; Abcam). The plates were incubated in the dark for 1 h at 37˚C. Cell proliferation was measured using a microplate reader (cat. no. 168-1130; Bio-Rad Laboratories, Inc.) at 450 nm.

Colony formation assay. Approximately 48 h post-transfection, ccRCC cells were cultured in 6-well plates at $2x10^3$ cells/well and the medium was changed every 3 days. The medium was aspirated once cell colonies became visible to the naked eye. The cells were then washed twice with 1xPBS and fixed with 4% paraformaldehyde (cat. no. 158127; MilliporeSigma) for 15 min at room temperature. Following the removal of paraformaldehyde, cells were stained with 0.25% crystal violet (cat. no. C6158; MilliporeSigma) at room temperature for 25 min. Finally, the cells were washed with sterile water, dried and images were captured under a light microscope. The numbers of colonies with >50 cells were counted manually under a light microscope.

Transwell assay. The Transwell assay was performed as previously described (27). Briefly, cells were suspended in FBS-free medium and 200 µl cell suspension ($1x10^5$ cells/well) was inoculated into the upper layer of 24 Transwell inserts (pore size, 8 µm; cat. no. 3422; Corning, Inc.). The lower layer was filled with 600 µl complete medium containing 10% FBS. For the invasion assay, Matrigel (cat. no. 356234; Corning, Inc.) was diluted to a concentration of 1 mg/ml using FBS-free medium and was then added to the upper chamber of the Transwell inserts and incubated at 37˚C for 1 h. After 36 h of incubation

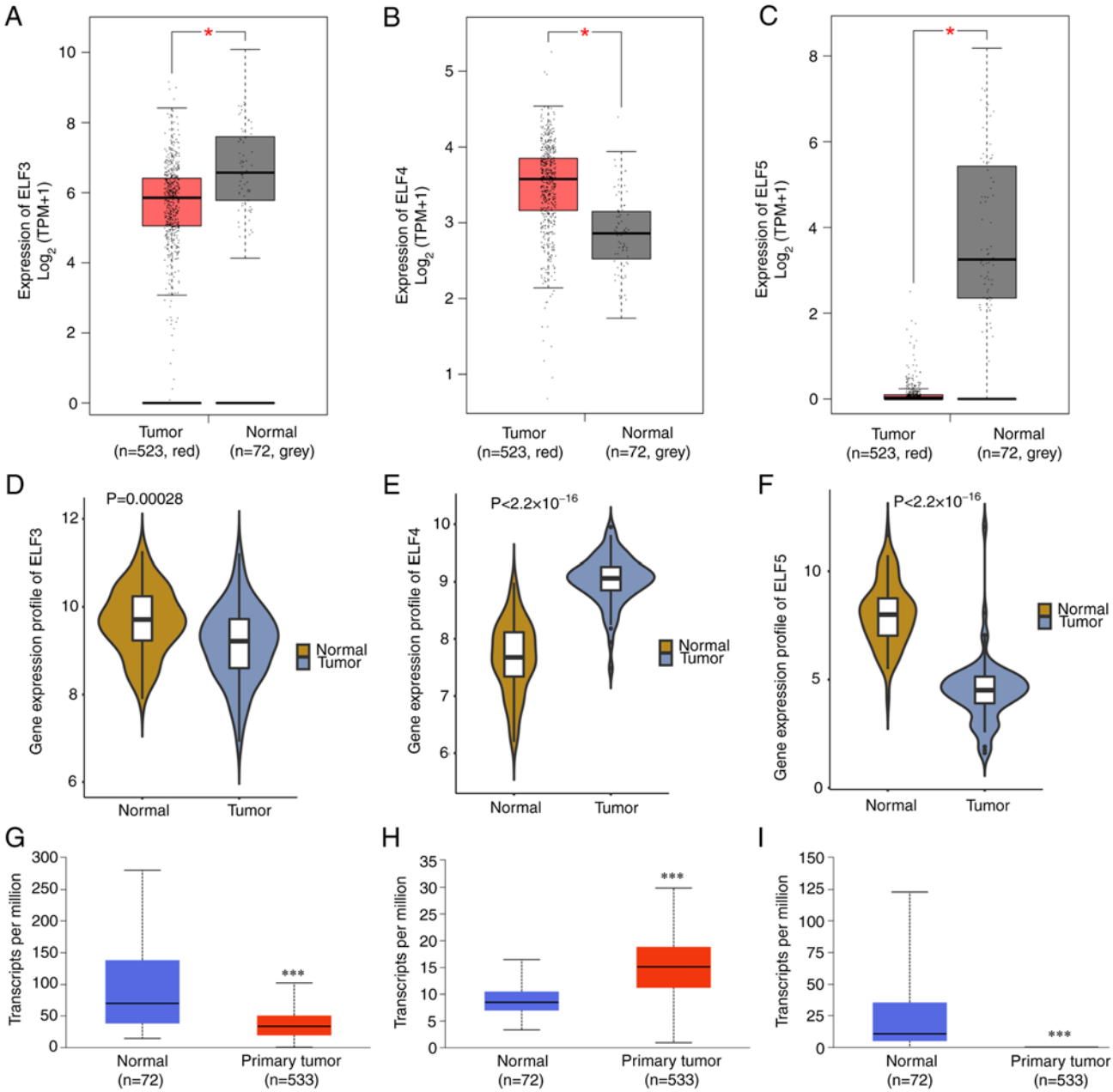

Figure 1. Differential expression analysis of ELF3-5 between ccRCC and normal tissues in multiple databases. mRNA expression levels of (A) ELF3, (B) ELF4 and (C) ELF5 in ccRCC and normal tissues in the Gene Expression Profiling Interactive Analysis database. mRNA expression levels of (D) ELF3, (E) ELF4 and (F) ELF5 in ccRCC and normal tissues in the Gene Expression Omnibus database. mRNA expression levels of (G) ELF3, (H) ELF4 and (I) ELF5 in ccRCC and normal tissues in UALCAN database. *P<0.05, ***P<0.001. ELF, E47-like factor; ccRCC, clear cell renal cell carcinoma.

at 37˚C, non-penetrating cells on the membrane were removed using cotton swabs. The cells that passed through the membrane were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min at room temperature. Subsequently, cell counting was performed under a light microscope (Olympus Corporation) at a magnification of x100.

Statistical analysis. The experimental data are presented as the mean ± standard deviation. Unpaired Student's t-test was used for two-group comparisons. Statistical analyses involving multiple group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. Data analyses were conducted using GraphPad Prism 8 (Dotmatics). The normality of data distribution was assessed using the Shapiro-Wilk or Kolmogorov-Smirnov normality test. Macrophage polarization, macrophage chemotaxis, RT-qPCR, CCK-8, colony formation and Transwell assays were repeated three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of ELF3-5 between tumor and normal tissues. The present study investigated the function of five key ELFs from the ETS family in ccRCC development. Differential analysis was performed using GEPIA, GEO and UALCAN databases. All three databases showed significantly higher expression levels of ELF4 in ccRCC tissues compared with those in normal tissues (Fig. 1B, E and H). Conversely,

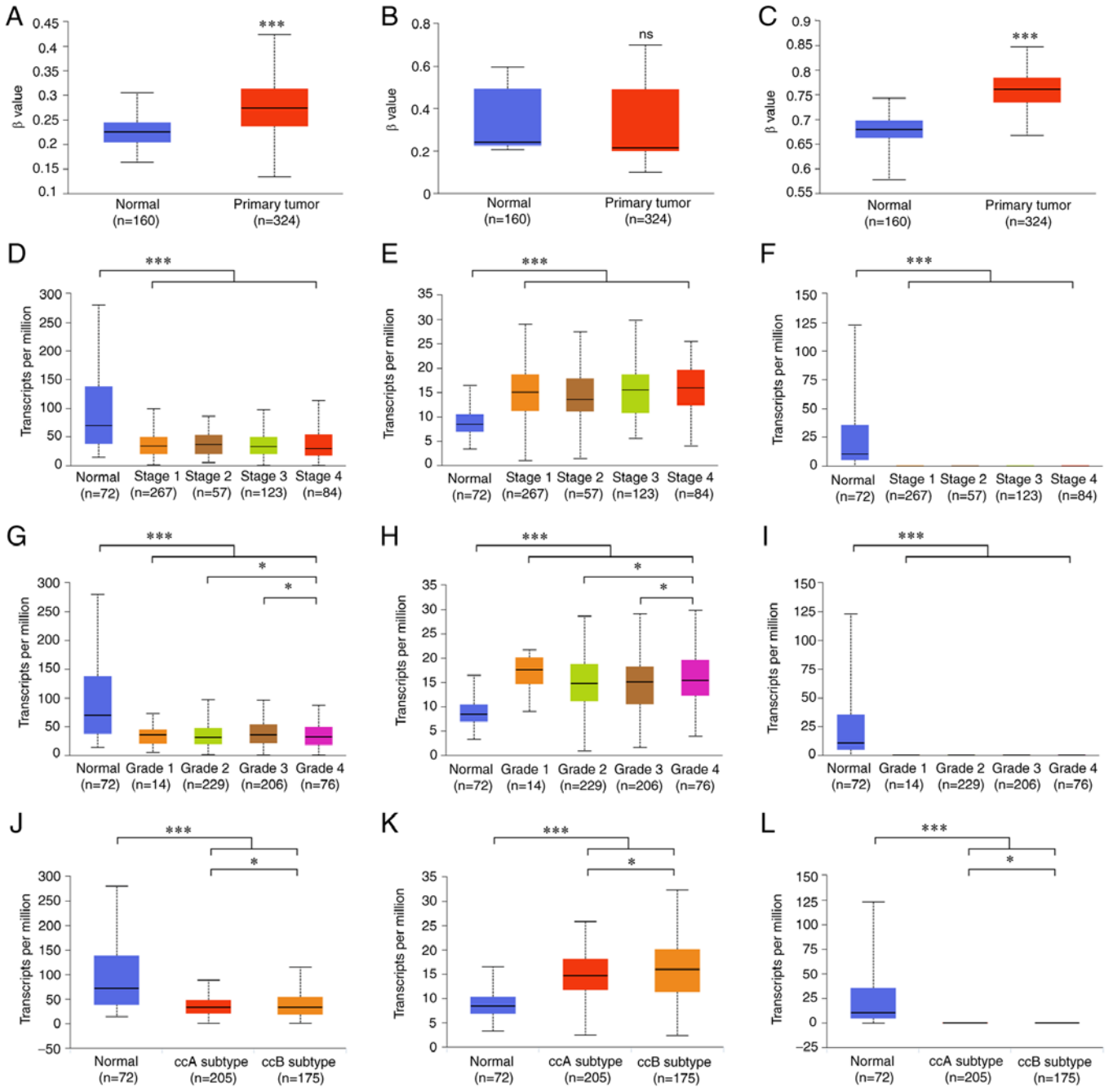

Figure 2. Clinical significance of ELF3-5 in ccRCC. Promoter methylation levels of (A) ELF3, (B) ELF4 and (C) ELF5 in normal tissues and primary ccRCC tissues in the UALCAN database. Expression levels of (D) ELF3, (E) ELF4 and (F) ELF5 in ccRCC cancer tissues of various tumor stages. Expression levels of (G) ELF3, (H) ELF4 and (I) ELF5 in ccRCC cancer tissues of various tumor grades. Expression levels of (J) ELF3, (K) ELF4 and (L) ELF5 in ccRCC cancer tissues of ccA and ccB subtypes. ns, no significance; *P<0.05, ***P<0.001. ELF, E47-like factor; ccRCC, clear cell renal cell carcinoma; ccA, clear cell type A; ccB, clear cell type B.

the expression levels of ELF3 and ELF5 showed an opposite pattern (Fig. 1A, C, D, F, G and I). Furthermore, GEPIA and UALCAN databases indicated no significant difference in the expression levels of ELF1 and 2 between ccRCC cancer tissues and normal tissues (Fig. S1A, B, E and F). Additionally, the expression profiles of ELF1-5 were analyzed in various cancer types using Oncomine, revealing differential expression across multiple types of cancer, including breast cancer, cholangiocarcinoma, chromophobe RCC, thyroid cancer and endometrial cancer (Fig. S2). Specifically, ELF3 and ELF5 exhibited lower expression levels in ccRCC compared with those in normal tissues (Fig. S2C and E). ELF4 exhibited higher expression levels in ccRCC compared with in normal tissues (Fig. S2D). Consequently, ELF3-5 were identified as key genes in the present study and further investigated for their functional relevance in subsequent investigations.

Clinical significance of ELF3-5 in ccRCC. DNA promoter methylation levels of ELF3 and ELF5 were significantly higher in ccRCC tissues compared with those in normal tissues (Fig. 2A and C). DNA promoter methylation level of ELF4 did not show a significant difference (Fig. 2B). There was no

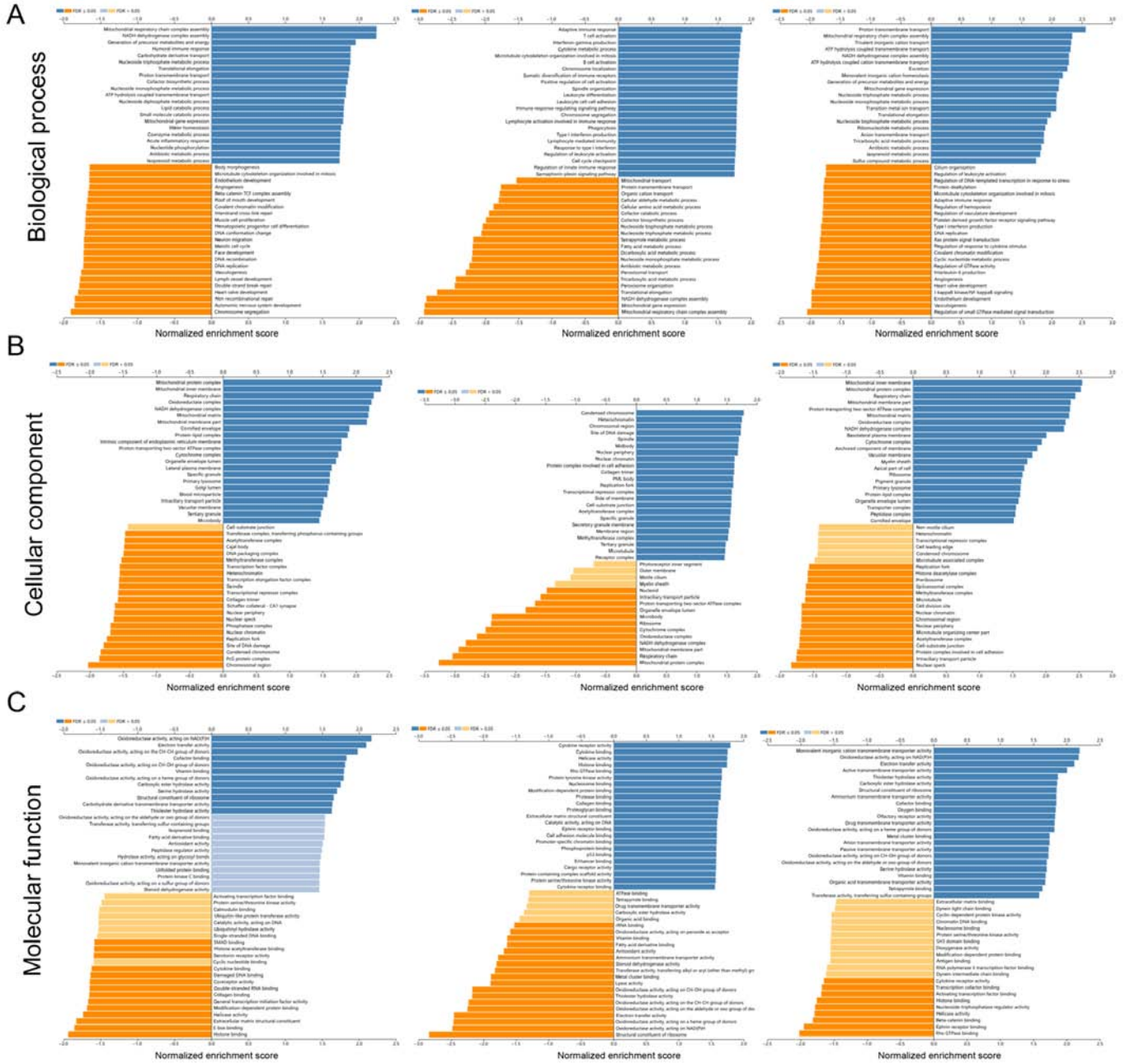

Figure 3. Gene Ontology functional enrichment analysis of ELF3-5. (A) Biological process, (B) cellular component and (C) molecular function enrichment analysis of ELF3-5. Blue represents normalized enrichment score <0. Orange represents normalized enrichment score >0. Dark blue and dark orange represent FDR ≤0.05. Light blue and light orange represent FDR >0.05. ELF, E47-like factor; FDR, false-discovery rate.

significant difference in the expression of ELF3-5 in patient tissues at different cancer stages (Fig. 2D-F). By contrast, ELF3 and ELF4 expression exhibited differences depending on tumor grade (Fig. 2G and H). There was no significant difference in the expression of ELF5 in patient tissues at different tumor grades (Fig. 2I). Additionally, ELF3-5 exhibited different expression levels in clear cell type A (ccA) and B (ccB) subtypes (Fig. 2J-L). Moreover, genetic variations of ELF3-5 were analyzed using the cBioPortal database, revealing mutations, amplifications and deep deletions in these three genes in some types of cancer (Fig. S3A-C). Amplification variation existed in all three genes; however, only ELF4 showed a high amplification variation in ccRCC, exhibiting 1.27 and 0.02% incidence rates of amplification and mutation, respectively (Fig. S3B). Furthermore, the prognostic significance of ELF3-5 was investigated in patients with ccRCC by assessing OS and DFS. The results indicated no significant association between ELF3-5 expression and patient survival (Fig. S4).

Enrichment analysis of ELF3-5 in ccRCC. GO and KEGG enrichment analyses were performed to investigate the potential functions and pathways associated with the differential expression of ELF3-5. Among the enrichment functions showing the strongest association with genes co-expressed with ELF3, 'mitochondrial respiratory chain complex', 'chromosome segregation', 'oxidoreductase activity, acting on NAD(P)H' and 'histone binding' were associated with tumorigenesis and tumor progression (Fig. 3A-C). The ELF4-related

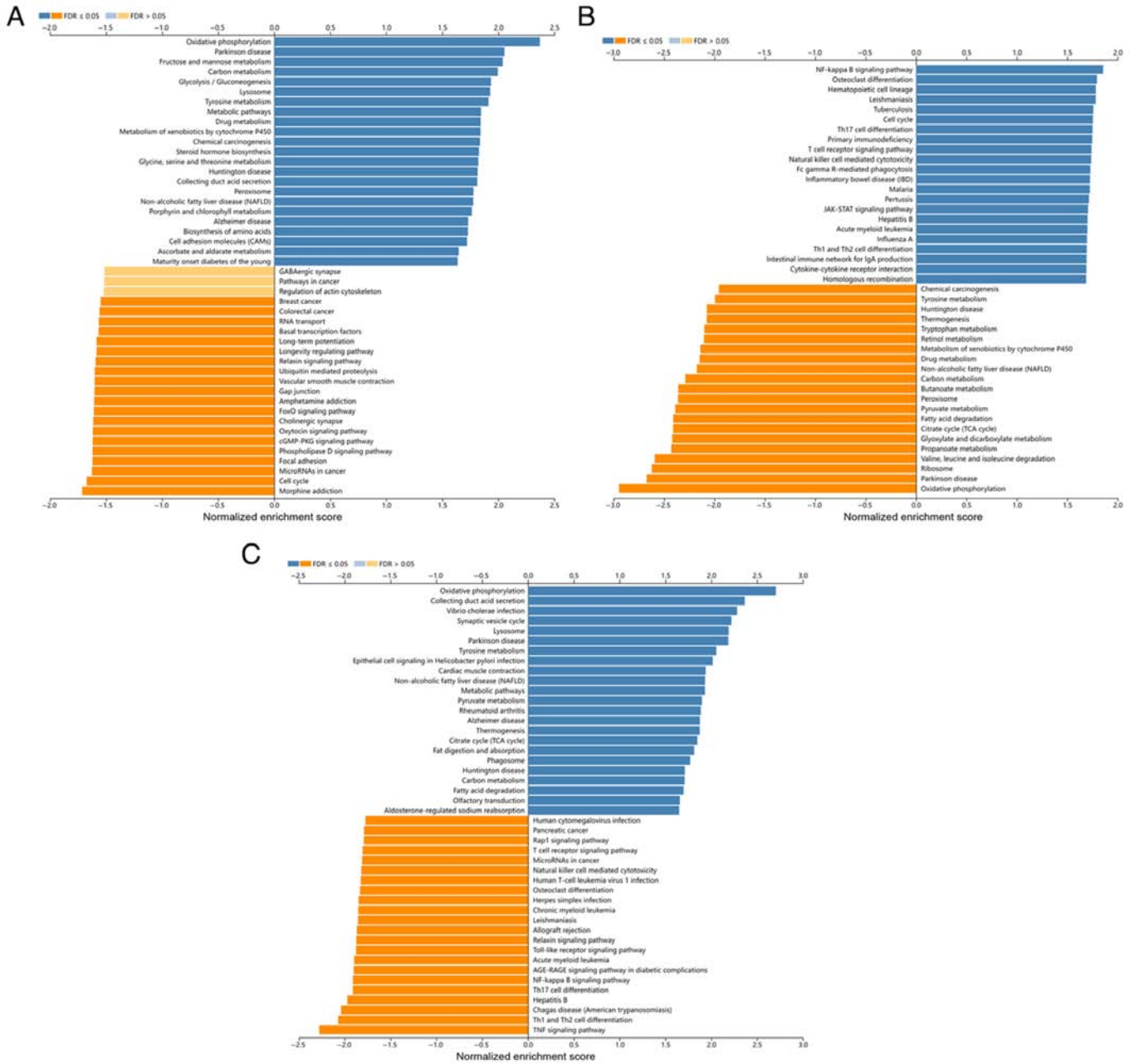

Figure 4. KEGG enrichment analysis of ELF3-5. KEGG enrichment analysis of (A) ELF3, (B) ELF4 and (C) ELF5. Blue represents normalized enrichment score <0. Orange represents normalized enrichment score >0. Dark blue and dark orange represent FDR ≤0.05. Light blue and light orange represent FDR >0.05. ELF, E47-like factor; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false-discovery rate.

functions included 'adaptive immune response', 'T cell activation', 'mitochondrial respiratory chain complex assembly', 'mitochondrial protein complex', and 'cytokine receptor activity', which are associated with immune response and malignant progression (Fig. 3A-C). The ELF5-related functions included 'proton transmembrane transport', 'regulation of small GTPase-mediated signal transduction', 'mitochondrial inner membrane', and 'nuclear speck' (Fig. 3A-C). KEGG pathway analysis revealed that ELF3 was mainly enriched in 'Oxidative phosphorylation', 'Cell cycle', 'Phospholipase D signaling pathway' and 'cGMP-PKG signaling pathway' (Fig. 4A). KEGG pathway analysis revealed that ELF5 was mainly enriched in 'Oxidative phosphorylation', 'Collecting duct acid secretion' and 'TNF signaling pathway' (Fig. 4C). These pathways were closely related to ccRCC development. By contrast, ELF4 was associated with more immune-related signaling pathways, including 'Th17 cell differentiation', 'Primary immunodeficiency', 'T cell receptor signaling pathway', 'Natural killer cell mediated cytotoxicity', and 'Th1 and Th2 cell differentiation' (Fig. 4B). These results indicated that the ELF4 expression network was closely related to immune response and the immune microenvironment in ccRCC.

Correlation analysis between ELF3-5 expression and immune infiltrate. The TIMER database was used to investigate the relationship between ELF3-5 expression and immune infiltrate. The expression levels of ELF3 and ELF5 showed no

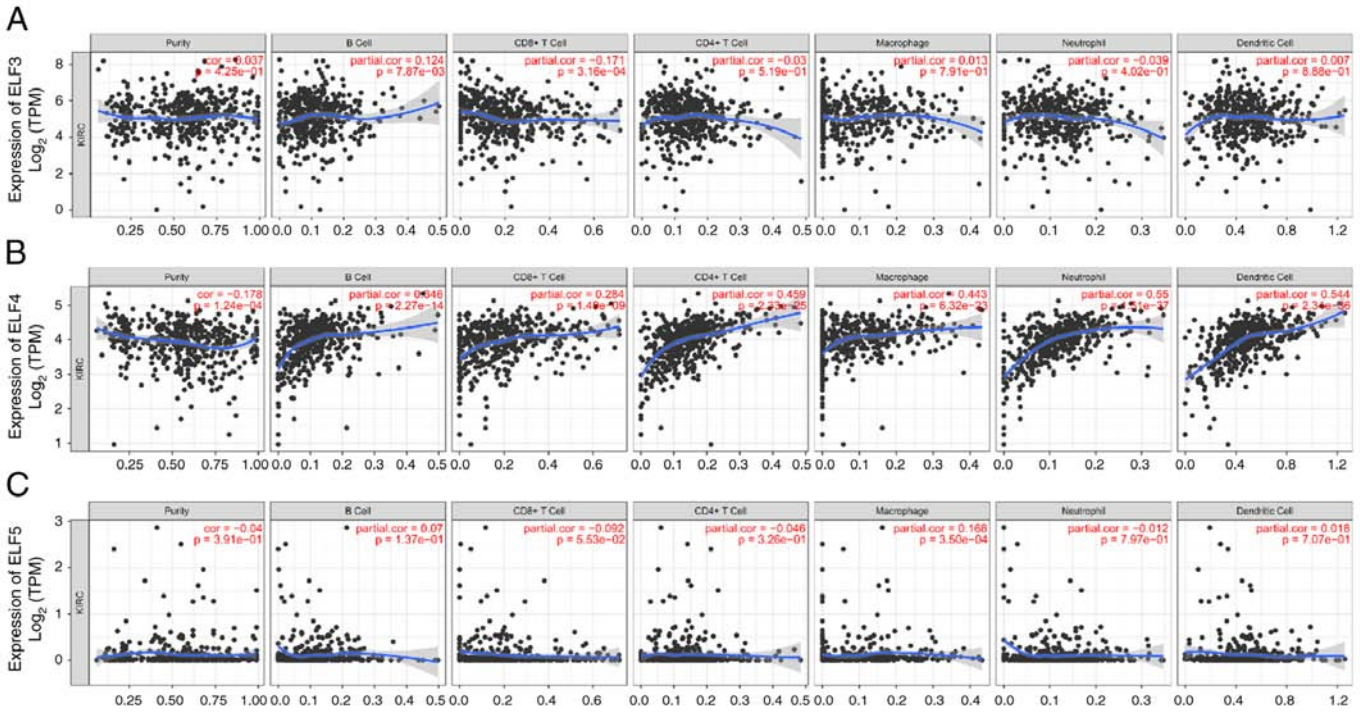

Figure 5. Correlation between ELF3-5 and immune infiltration in clear cell renal cell carcinoma. Correlation between expression levels of ELF3 (A), ELF4 (B), ELF5 (C) and tumor purity, B cells, $CD8^+$ and $CD4^+$ T cells, macrophages, neutrophils and dendritic cells. ELF, E47-like factor.

significant association with most immune cell infiltration levels (Fig. 5A and C). However, ELF4 exhibited a notable correlation with various immune cells, including B cells, $CD4^+$ T cells, macrophages, neutrophils and dendritic cells (Fig. 5B). These findings suggested a specific role for ELF4 in immune infiltration in ccRCC. Therefore, the present study further investigated the effects of ELF4 on the proliferation, migration, invasion and immune escape of ccRCC cells.

ELF4 promotes ccRCC cell proliferation, migration and invasion. ELF4 expression was detected in HK-2 and ccRCC cells to validate its potential function. ELF4 exhibited high expression levels in 769-P and 786-O cells compared with those in HK-2 cells (Fig. 6A). Subsequently, ELF4 expression was knocked down in the two ccRCC cell lines (Fig. 6B). A decrease in 769-P and 786-O cell proliferation was detected upon ELF4 knockdown compared with that in the si-NC group (Fig. 6C and D). The colony formation assay results also revealed that knockdown of ELF4 expression could reduce the colony formation of ccRCC cells compared with that in the si-NC group (Fig. 6E). Furthermore, Transwell assay results indicated a reduction in cell migration and invasion in the si-ELF4 group compared with those in the si-NC group (Fig. 7A and B). These findings suggested that activating ELF4 may promote ccRCC cell proliferation, migration and invasion.

ELIF4 regulates M2 macrophage polarization and chemotaxis of M2 macrophages to ccRCC cells. M1 and M2 macrophage marker expression levels were detected in ccRCC and macrophage co-culture experiments. In 769-P cells, higher transcription levels of the M1 macrophage marker CXCL10 were detected in the si-ELF4 group compared with that in the si-NC group (Fig. 8A). Conversely, the expression levels of the M2 macrophage markers CD206 and CCL22 were lower in the si-ELF4 group compared with those in the si-NC group (Fig. 8B). Knockdown of ELF4 expression had no impact on M1 marker expression in the 786-O cell and macrophage co-culture system (Fig. 8C); however, it did decrease the expression levels of M2 markers (Fig. 8D). Regarding macrophage chemotaxis, the present findings revealed that knockdown of ELF4 in ccRCC cells did not regulate the migration rate of M1 macrophages towards cancer cells (Fig. 8E); however, it did inhibit the migration rate of M2 macrophages towards cancer cells (Fig. 8F). These results suggested that ELF4 could promote M2 macrophage polarization and chemotaxis of M2 macrophages to ccRCC cells.

Discussion

Abnormal expression of ELFs has been identified in various malignant tumors, influencing their biological processes (28); however, the regulatory mechanisms and clinical significance of certain ELFs in ccRCC remain unclear. The present study comprehensively analyzed the clinical significance and key pathways associated with ELF3-5 in ccRCC using multiple databases. Moreover, the proliferation-promoting effects of the core gene ELF4 and its regulation of macrophages were assessed *in vitro*.

The present study demonstrated that ELF3 and ELF5 exhibited lower expression levels in ccRCC tissues compared with those in normal tissues, whereas ELF4 expression was higher. Furthermore, the clinical significance of these three key genes were explored in ccRCC. Previous research has highlighted ELF3 as a methylation-driven gene in lung adenocarcinoma (29). In addition, DNA methylation levels at the ELF5 promoter region have been identified as potential

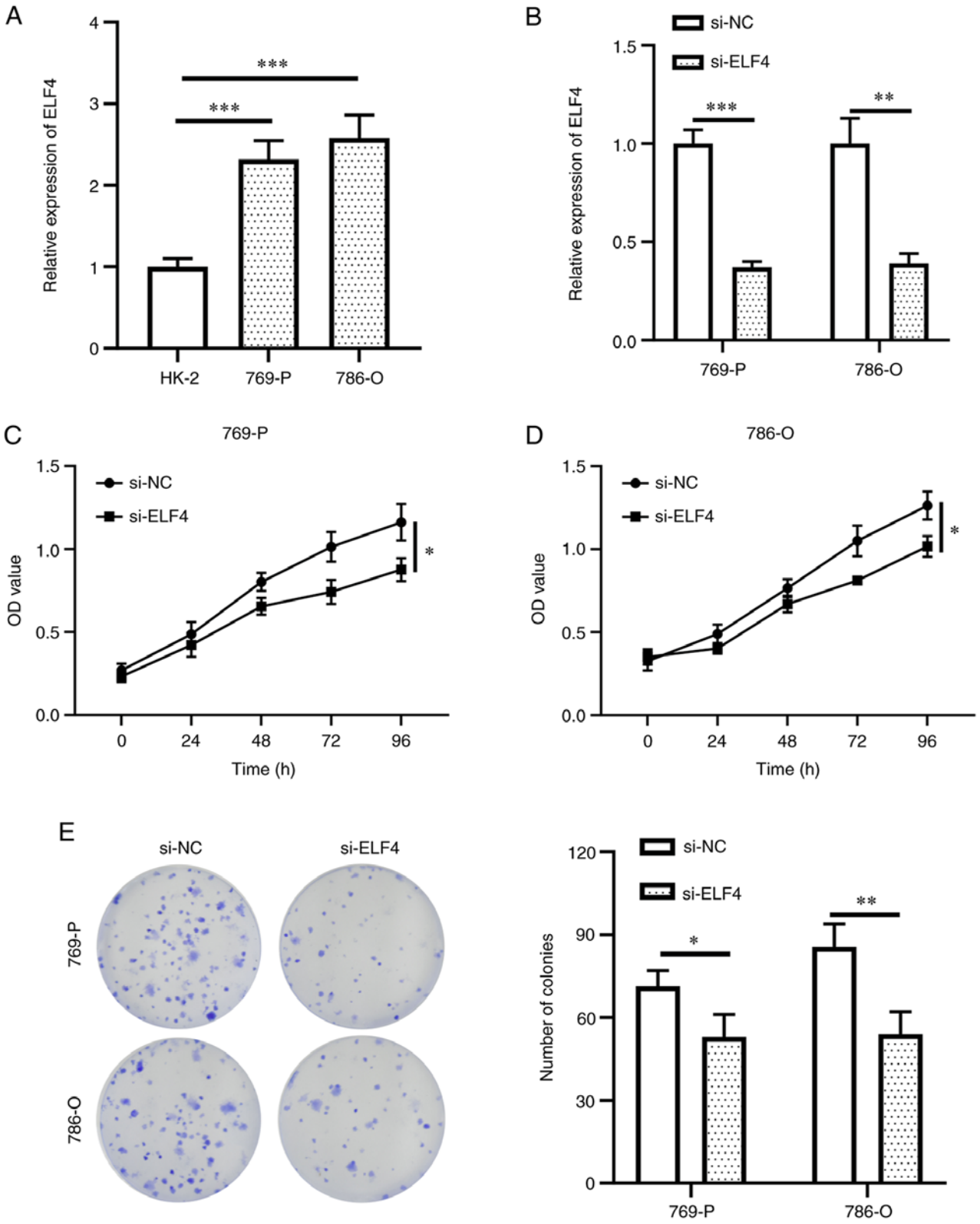

Figure 6. ELF4 knockdown inhibits ccRCC cell proliferation. (A) RT-qPCR analysis of ELF4 expression in HK-2, 769-P and 786-O cell lines. (B) RT-qPCR analysis of the impact of si-ELF4 transfection on ELF4 expression in ccRCC cells. Effect of si-ELF4 on (C) 769-P and (D) 786-O cell proliferation measured by Cell Counting Kit-8 assay. (E) Effect of si-ELF4 on 769-P and 786-O cell proliferation measured by colony formation assay. Data are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. ELF, E47-like factor; ccRCC, clear cell renal cell carcinoma; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; si, small interfering.

breast-specific biological clocks for identifying the risk of breast cancer (30). Demethylation of ELF5 has also been explored as a potential therapeutic strategy in urothelial cancer (31). The present study detected higher methylation levels of ELF3 and ELF5 in primary tumor tissues compared with those in normal tissues. Notably, ELF4 methylation has previously

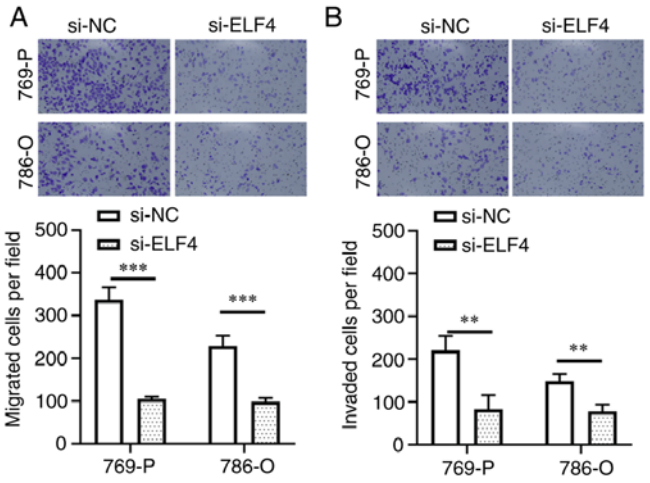

Figure 7. ELF4 knockdown inhibits ccRCC cell migration and invasion. Effect of si-ELF4 on ccRCC cell (A) migration and (B) invasion assessed via Transwell assay (magnification, x100). Data are presented as the mean ± SD. **P<0.01, ***P<0.001. ELF, E47-like factor; ccRCC, clear cell renal cell carcinoma; NC, negative control; si, small interfering.

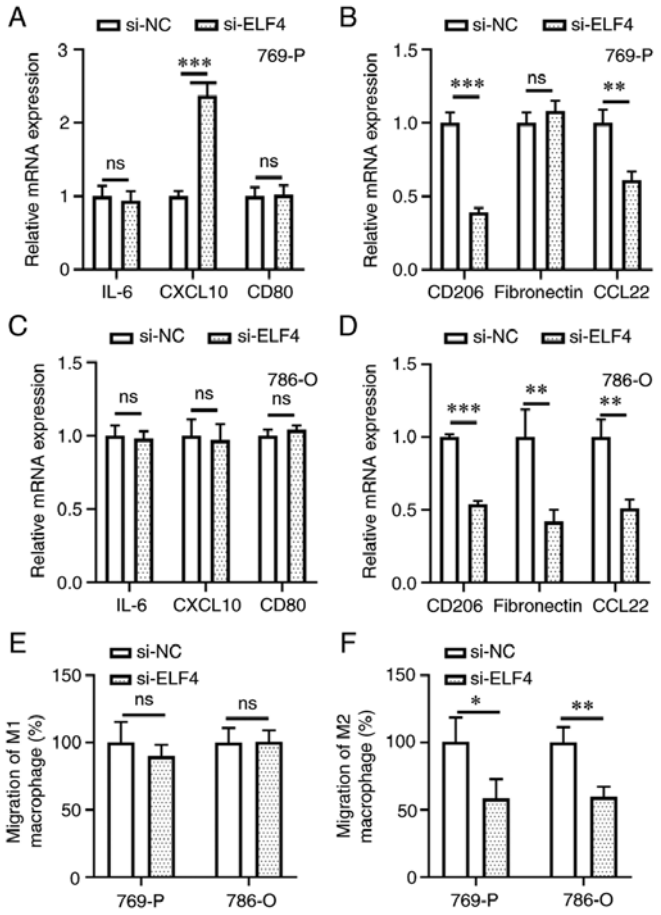

Figure 8. ELF4 knockdown inhibits M2 macrophage chemotaxis and polarization in clear cell renal cell carcinoma. Effect of ELF4 on the expression levels of (A) M1 and (B) M2 macrophage markers in macrophages, estimated through 769-P cell co-culture experiments. Effect of ELF4 on the expression levels of (C) M1 and (D) M2 macrophage markers in macrophages, estimated through 786-O cell co-culture experiments. Effect of ELF4 on the chemotaxis of (E) M1 and (F) M2 macrophages to cancer cells. ns, no significance; *P<0.05, **P<0.01, ***P<0.001. ELF, E47-like factor; NC, negative control; si, small interfering.

been reported to be significantly upregulated during liver cell carcinogenesis (32), and hypermethylation of the ELF4 promoter region in colitis preparations has been associated with disease progression to colorectal cancer (33). However, no significant difference was observed in the methylation level of ELF4 between ccRCC tissues and normal tissues in the present study. Clinical significance serves a crucial role in exploring the diagnostic value of biomarkers. ELF3 has been shown to have clinical significance in non-small cell lung cancer, where the inhibition of ELF3 mediated the synthetic lethality of PARP inhibitor (34). In epithelial ovarian cancer, the expression levels of ELF5 were related to pathological surgical stage, pathological grade and lymph node metastasis (13). Clinical analysis has also revealed associations between ELF4 expression and tumor size, pathological grade and clinical stage in squamous cell carcinoma of the cervix (35). In the present study, ELF3 and 4 exhibited different expression patterns across different grades, and ELF3-5 showed differential expression levels in ccA and ccB subtypes. Regarding genetic variations, all three genes exhibited amplification variations, but it was only ELF4 that showed a high amplification variation in ccRCC. Kafita *et al* (36) reported that high amplification variation of ELF4 in cancer was associated with worse disease outcomes and increased resistance to anticancer drugs. These reports and findings highlighted the high clinical significance of ELF3-5, particularly ELF4, in ccRCC.

ELF3-5, as members of a transcription factor family, have been implicated in regulating tumor progression through various signaling pathways. ELF3 can promote resistance in gallbladder cancer cells via the PKMYT1/CDK1 signaling pathway (37), whereas ELF5 can inhibit the p53/p21 pathway, leading to the induction of acute myeloid leukemia (38). In glioblastoma, ELF4 controls genes associated with receptor tyrosine kinase and receptor tyrosine kinase pathways (39). Therefore, identifying the key regulatory pathways of these three ELF genes was crucial for understanding the molecular mechanisms underlying the impact of ETS family genes on cancer cell development. ELF3-5 were revealed to be associated with various functions in the present study, including biological regulation, metabolic processes, membrane functions, protein binding and nucleic acid binding. Notably, ELF4 was particularly linked to immune-related signaling pathways. A previous study highlighted the critical involvement of ELF4 in the cancer immune response (40). It has also been reported to be associated with immune cell infiltration and immune-related feature genes (CD14, CD163, CD33) in cholangiocarcinoma (41). Similarly, the present study revealed that ELF4 expression in ccRCC was closely related to the infiltration levels of multiple immune cell types compared with ELF3 and ELF5. These results suggested an important role for ELF4 in regulating cancer cell activity and tumor-related immune cell infiltration.

Previous cancer studies have indicated that ELF4 functions as an oncogene. It has been shown to promote neuroblastoma proliferation and maintain an undifferentiated state (42). ELF4 has also been implicated in endometrial cancer, where it acts as an oncogene by binding to the CTNNB1 promoter in cancer cells (43). The present findings in ccRCC cell lines further support the role of ELF4 in promoting cell

proliferation, migration and invasion in 769-P and 786-O cell lines. Moreover, abnormal ELF4 expression was shown to influence the regulation of M2 polarization and the chemotaxis of macrophages to cancer cells. In lung cancer, ELF4 in macrophages has been shown to rescue immunotherapy efficacy (44). ELF4 also exhibits transcriptional activation of macrophage colony-stimulating factors in ovarian cancer (45). These findings underscore the significance of ELF4 in regulating cancer cell abilities, inducing M2 polarization of macrophages, and their chemotaxis towards ccRCC cells. This highlights the crucial role of ELF4 in the tumor microenvironment of ccRCC.

The present study has certain limitations that should be acknowledged. Firstly, the clinical significance of ELFs was primarily assessed through bioinformatics analysis using public databases; therefore, it is crucial to gather larger clinical samples of ccRCC to further validate the clinical significance of ELFs. Secondly, although the study uncovered the involvement of ELF4 in macrophage polarization and chemotaxis, the immune escape mechanism of ELF4 in ccRCC remains to be elucidated. Future investigations should explore the regulatory effects of ELF4 on other immune cell types in ccRCC. Moreover, inconsistencies in the expression results of certain ELFs across different databases necessitate additional sequencing data for further verification. Finally, the specific molecular mechanism by which ELF4 regulates ccRCC cells and M2 macrophages warrants in-depth exploration. A number of the findings from *in vitro* experiments also require future *in vivo* validation.

In conclusion, ELF members display varying degrees of abnormal expression and serve important roles in ccRCC tumorigenesis and progression. The present study comprehensively analyzed the clinical significance and tumor-immune interaction of ELF4. The results revealed that ELF4 was significantly upregulated in ccRCC tumor tissues, indicating its high clinical significance in ccRCC. The present study further elucidated the promoting effects of ELF4 on ccRCC cell proliferation, migration and invasion. Additionally, the results suggested that ELF4 could regulate macrophage polarization and chemotaxis to ccRCC cells. These findings provide novel insights into our understanding of the involvement of ELFs in ccRCC development.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the GEPIA (http://gepia.cancer-pku.cn/), GEO database (https://www.ncbi.nlm. nih.gov/geo/query/acc.cgi?acc=GSE53757), UALCAN (http://ualcan.path.uab.edu), Oncomine(https://www.oncomine.org),cBioPortal(http://www.cbioportal.org), LinkedOmics (https://www.linkedomics.org/login.php) and TIMER web server (https://cistrome.shinyapps.io/timer/) databases. All other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and WC conceived and designed the study. WC acquired funding. LMo, LMa and QZ conducted the experiments and bioinformatics analysis. JL drafted the paper and WC revised the manuscript. JL and WC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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