

Identification of EFNA3 as candidate prognosis marker and potential therapeutic target for adrenocortical carcinoma

YANGHAO TAI¹, XINZHE LIU¹, YIFAN ZHOU¹ and JIWEN SHANG^{1,2}

¹Department of Urology, Third Hospital of Shanxi Medical University, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Taiyuan, Shanxi 030032, P.R. China; ²Department of Urology, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, Taiyuan, Shanxi 030032, P.R. China

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Abstract. Adrenocortical carcinoma (ACC) is a rare, but highly aggressive endocrine malignancy with poor prognosis and limited treatment options. Identifying novel biomarkers and therapeutic targets is essential for improving patient outcomes. The present study aimed to systematically characterize ephrin-A3 (EFNA3) expression patterns, its prognostic and diagnostic value, and its functional role in ACC progression through multi-omics bioinformatics and *in vitro* validation. Transcriptomic, epigenetic and pharmacogenomic data were obtained from The Cancer Genome Atlas, Genotype-Tissue Expression, Genomics of Drug Sensitivity in Cancer, Cancer Therapeutics Response Portal and MethSurv databases. Expression, survival, immune infiltration, methylation and drug sensitivity analyses were conducted using the R software and online tools (GEPIA2, CIBERSORT and cBioPortal). competitive endogenous RNA (ceRNA) networks were constructed based on microRNA (miRNA)/long non-coding RNA (lncRNA) predictions. Functional assays, including CCK-8, flow cytometry, Transwell assays were performed on the ACC cell lines, SW-13 and NCI-H295R, to validate EFNA3 function. EFNA3 was significantly upregulated in numerous types of cancer and associated with poor prognosis. In ACC, upregulated EFNA3 was associated with a poor prognosis [Overall survival (OS), hazard ratio (HR)=3.14, 95% CI, 1.49-7.81; disease-specific survival, HR=4.27, 95% CI, 1.70-10.72; progression-free interval, HR=6.24, 95% CI, 2.94-13.23; P<0.05] and diagnostic efficiency (area under the curve=0.829, 95% CI, 0.760-0.897). EFNA3-mutated cases

had significantly worse OS in ACC specifically (OS, HR=2.97, 95% CI, 1.12-7.90, P=0.029; disease-free survival, HR=8.65, 95% CI, 2.14-34.93, P=0.002). β -catenin (CTNNB1) was among most frequently co-mutated genes ACC with EFNA3 (P=4.6x10⁻⁴). Genetic amplification and DNA methylation alterations were observed in the ACC cohort. EFNA3 expression negatively correlated with immune infiltration and positively correlated with several m6A/m5C regulators. ceRNA network analysis demonstrated key lncRNA-miRNA-EFNA3 axes. Drug sensitivity profiling indicated that EFNA3 expression was associated with statin and proteasome inhibitor responses. The co-expression of positively correlated gene enrichment results suggested that Wnt signaling pathway and β -catenin/T-cell factor complex may be involved in the progression of ACC mediated by EFNA3. Functionally, EFNA3 promoted ACC cell proliferation and migration *in vitro*. The present study demonstrated that EFNA3 acts as an oncogene in ACC and may contribute to tumor aggressiveness via β -catenin activation and glycolytic reprogramming, and thus may serve as a potential biomarker for prognosis, immunotherapy sensitivity and drug repurposing, particularly involving statins.

Introduction

Adrenocortical carcinoma (ACC) is a rare and highly aggressive malignancy arising from steroidogenic cells of the adrenal cortex, characterized by a 5-year overall survival rate of >35% (1). Radical surgical resection remains the only curative approach for localized ACC; however, postoperative recurrence rates are high, ranging between 70-80% (2). For advanced or metastatic ACC, therapeutic options are limited. Mitotane, the only Food and Drug Administration (FDA)-approved agent, demonstrates suboptimal efficacy and substantial toxicity (3). Combination chemotherapy regimens such as mitotane with etoposide, doxorubicin and cisplatin yields modest clinical benefit, with an objective response rate of ~30% and a median progression-free survival of 5.6 months (4). These limitations underscore an urgent need to elucidate the molecular mechanisms underlying ACC progression and to identify robust biomarkers for early diagnosis, risk stratification and development of targeted therapeutics.

Reprogramming of cancer cell metabolism is a hallmark of malignancy, with aerobic glycolysis ('Warburg effect')

Correspondence to: Professor Jiwen Shang, Department of Urology, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Third Hospital of Shanxi Medical University, 99 Longcheng Street, Xiaodian, Taiyuan, Shanxi 030032, P.R. China

E-mail: shangjiwen@sxjqh.com.cn

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facilitating increased glucose uptake and lactate production, which acidifies the tumor microenvironment (TME) and supports migration and immune evasion (5-7). Dysregulated expression and activity of glycolytic enzymes are central to this phenotype (8). Ephrin-A3 (EFNA3), a transmembrane ligand of the Eph receptor tyrosine kinase family, has been implicated in metabolic regulation and tumor progression (9,10). EFNA3 participates in bidirectional cell-cell communication through interactions with Eph receptors, modulating processes such as angiogenesis, cellular motility and tissue remodeling (11). Notably, EFNA3 functions as a glycolysis-related gene. Previous studies indicate that EFNA3 upregulation promotes glycolytic flux and proliferation in lung adenocarcinoma, correlating with unfavorable prognosis (12-14), suggesting potential roles as both a metabolic regulator and oncogenic driver.

Members of the EFNA gene family, including EFNA1 and EFNA2, exhibit distinct expression and functional profiles across tumor types. EFNA1 is frequently upregulated in various types of cancer, such as gastric cancer and melanomas, and is linked to angiogenesis, immune modulation and metastatic potential via interactions with EphA2 and hypoxia-inducible signaling (15-18). Conversely, EFNA2 expression is reduced in certain types of cancer such as gastric carcinoma, with inverse associations to CD8⁺ T-cell and dendritic cell infiltration, implicating it in immune surveillance escape (19-21). High EFNA3 expression levels are predictive of poorer survival in gastric cancer and correlate negatively with infiltration of B cells, T cells and dendritic cells, as well as with immune checkpoint activity, which indicates a role in immune evasion (22,23). Beyond its prognostic role, EFNA3 expression has been correlated with immune cell infiltration and chemotherapeutic response, indicating potential relevance to tumor immunology and therapeutic resistance (24-26).

The present study aimed to perform an integrative pan-cancer analysis of EFNA3 to evaluate its transcriptional deregulation, genetic and epigenetic alterations, prognostic relevance, associations with tumor immune infiltration and drug sensitivity, and to construct an EFNA3 ceRNA regulatory network. Furthermore, the present study aimed to investigate the effects of EFNA3 on the proliferative, migratory and anti-apoptotic capacities of ACC cells via *in vitro* experiments.

Materials and methods

Pan-cancer expression profiling of EFNA3. Transcriptome data from 15,776 samples were retrieved via the UCSC Xena Browser (<https://xenabrowser.net>; The Regents of the University of California), integrating The Cancer Genome Atlas (TCGA) (<https://portal.gdc.cancer.gov/>) and Genotype-Tissue Expression databases (<https://www.gtexportal.org/home/>). Raw RNA-Seq data (TPM+1) were log-transformed and normalized using the *rms* package in R (version 4.2.1; Posit Software, PBC). Batch-corrected data were visualized using *ggplot2* (version 3.4.0; Posit Software, PBC) as boxplots to depict EFNA3 expression across tumor and normal tissues. Differential expression was analyzed with *DESeq2* (version 1.38.3; Bioconductor), using thresholds of $|\log_2FC| \geq 1$ and $FDR \leq 0.05$ (Benjamini-Hochberg correction). Tumor stage association was analyzed using the ‘Stage

Plot’ module in GEPIA2 (<http://gepia2.cancer-pku.cn/>). The flowchart of the present study is shown in Fig. S1.

Prognostic and diagnostic evaluation of EFNA3. TCGA clinical and expression datasets were used to assess prognostic and diagnostic relevance of EFNA3. Univariate Cox proportional hazards models were constructed using the *survival* package (version 3.5-5; Posit Software, PBC), with calculated hazard ratios (HR) and 95% confidence intervals (CI). $P < 0.05$ was considered to indicate a statistically significant difference. Samples lacking complete survival data were excluded. Kaplan-Meier survival curves for OS, disease-free survival (DFS) and progression-free interval (PFI) were plotted using *survminer* (version 0.4.9; Posit Software, PBC) and *ggplot2* (version 3.4.0; Posit Software, PBC). Diagnostic value was evaluated using ROC curves generated by the *pROC* package (version 1.18.0; Posit Software, PBC).

Clinicopathological correlation in ACC. Based on median the expression level of EFNA3, patients with ACC were stratified into high- and low-EFNA3 expression groups ($n=40$ and $n=39$, respectively). Clinical parameters were compared using appropriate tests using the *stats* (version 4.2.1) and *car* (version 3.1.0) R packages (Posit Software, PBC). Visualization was performed with *ggplot2* (version 3.4.0; Posit Software, PBC). The diagnostic performance of EFNA3 in ACC was evaluated via ROC analysis (*pROC*; version 1.18.0; Posit Software, PBC) using TCGA and UCSC-derived datasets.

Somatic mutation and copy number analysis. Mutation data were obtained from cBioPortal (<http://www.cbioportal.org>) and TCGA. EFNA3 alterations [mutation type, copy number alterations (CNAs) and frequency] were analyzed using 2,683 samples from 2,565 patients. Additional ACC-specific data ($n=76$) were retrieved from the UCSC Xena (<https://xenabrowser.net/>) and the International Cancer Genome Consortium (<https://dcc.icgc.org/>) databases. Kaplan-Meier survival analyses were used to assess survival outcomes based on EFNA3 mutation status. Differential mutation profiles in EFNA3-high vs. -low expression groups were also analyzed.

Epigenetic and mRNA modification analysis. DNA methylation profiles for EFNA3 in ACC were obtained from the MethSurv (<https://biit.cs.ut.ee/methsurv/>) database. mRNA modification regulator correlations including n1-methyladenosine (m1A), 5-methylcytosine (m5C) and n6-methyladenosine (m6A), were analyzed across various types of cancer from TCGA database using the SangerBox software (version 3.0; <http://vip.sangerbox.com>). Pearson correlation coefficients and significance levels were reported.

Immune cell infiltration analysis. TME scores (stromal, immune and ESTIMATE scores) were computed using the *ESTIMATE* R package (version 1.0.13; Posit Software, PBC). Immune infiltration profiling was performed using markers from 22 immune cell types provided by the CIBERSORTx website (<https://cibersortx.stanford.edu/>) (27). Data were visualized as heatmaps using *ggplot2* (version 3.4.0; Posit Software, PBC). Spearman's correlation coefficients were used to assess statistical associations.

Competitive endogenous RNA (ceRNA) regulatory network construction. Candidate EFNA3-targeting microRNAs (miRNAs) were predicted using PITA (version 1.0; https://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), miRanda (version 3.3; <http://www.microrna.org/>) and TargetScan (version 8.0; <http://www.targetscan.org/>) software. miRNAs with a negative correlation to EFNA3 were prioritized using the StarBase (version 2.0; <http://starbase.sysu.edu.cn/>). lncRNA-miRNA interactions were derived from miRNet (version 2.0; <https://www.mirnet.ca/>) and StarBase (version 2.0; <http://starbase.sysu.edu.cn/>) under the criteria: Species=human; CLIP-Data=yes; and min stringency=5. Venn diagrams were used for intersecting target prediction using *ggplot2* (version 3.4.0; Posit Software, PBC), and *VennDiagram* (version 1.7.3; Posit Software, PBC). Final lncRNA-miRNA-EFNA3 networks were visualized using mulberry plots using *ggplot2* (version 3.4.0; Posit Software, PBC) and *ggalluvial* (version 0.12.3; Posit Software, PBC).

Drug sensitivity and interaction network analysis. Drug sensitivity data were obtained from the GSCALite (<http://bioinfo.life.hust.edu.cn/web/GSCALite/>) database, which integrates TCGA, Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Therapeutics Response Portal (CTRP) datasets. EFNA3-associated drug responses were identified based on Pearson correlations analysis with mRNA expression. FDA-approved agents were selected via DrugBank (<https://go.drugbank.com/>) annotations. Network graphs were generated using *graph* (version 1.4.1; Posit Software, PBC) and *graph* (version 2.1.0; Posit Software, PBC) packages.

Functional enrichment of co-expressed genes. Co-expressed genes were identified using LinkedOmics (<http://www.linkedomics.org>). Functional enrichment was performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms via the *clusterProfiler* (version 4.6.2; Posit Software, PBC) software. Protein-protein interaction (PPI) networks were generated using the STRING database (<https://cn.string-db.org/>) and visualized using default Benjamini-Hochberg correction for P-values.

Cell lines and culture conditions. The human ACC cell lines SW-13 (hormonally inactive) and NCI-H295R (hormonally active) were obtained from Procell Life Science & Technology Co., Ltd. Cell line authentication was confirmed via short tandem repeat profiling. Cells were maintained in DMEM/F12 medium (Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; cat. no. G24-70500; Genial Biologicals, Inc.) and 1% penicillin-streptomycin (Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd.). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

EFNA3 overexpression and knockdown via lentiviral transfection. The EFNA3 overexpression plasmid was synthesized by Shanghai Sangong Pharmaceutical Co., Ltd. The short hairpin RNA (shRNA) targeting EFNA3 and the non-targeting negative control (NC) were synthesized by Shanghai Gema Gene Biotechnology Co., Ltd. Sequences used were as follows: shNC sense (S), 5'-TTCTCCGAACGT

GTCACGT-3' and anti-sense (AS), 5'-ACGTGACACGTT CGGAGAA-3'; shEFNA3 S, 5'-GGCATGCGGTGTACT GGAACA-3' and AS, 5'-TGTTCCAGTACACCGCATGCC-3'. The EFNA3 overexpression plasmid was designed and synthesized by Shanghai Sangong Pharmaceutical Co., Ltd., and constructed by cloning the EFNA3 coding sequence into the pcDNA3.1 plasmid backbone (Thermo Fisher Scientific, Inc.). For transfection, 2.5 µg of plasmid DNA was complexed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and then added to the cell culture. Transfection was performed at 37°C for 48 h. Upon reaching 30-40% confluency in 6-well plates, cells were infected with lentivirus in medium containing Polybrene (Thermo Fisher Scientific, Inc.). Puromycin (4 µg/ml; Thermo Fisher Scientific, Inc.) was added 48 h post-infection for initial screening. Stable-transfected clones were maintained in a 2 µg/ml-puromycin environment.

Real-time quantitative PCR (RT-qPCR). According to the manufacturer's protocol, total RNA was isolated from NCI-H295R and SW-13 cells using TRIzol reagent (cat. no. R0016; Biocytogen). cDNA was synthesized from mRNA using a cDNA reverse transcription kit (cat. no. 4368814; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR amplification was performed using the SYBR Green fluorescent quantitative PCR kit (cat. no. A46012, Thermo Fisher Scientific, Inc.). The primer sequences are as follows: EFNA3 forward (F), 5'-ATGAAGGTGTTTCGTC TGCT-3' and reverse (R), 5'-CTCAAAGTCTTCCAGCAC G-3'; GAPDH F, 5'-TCAAGATCATCAGCAATGCC-3' and R, 5'-CGATACCAAAGTTGTCATGGA-3'; GAPDH was used as the internal reference gene. The relative expression level of EFNA3 mRNA was calculated using the 2^{-ΔΔC_q} method (28). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 15 sec and combined annealing/extension at 60°C for 60 sec.

Transwell migration assay. Cell migration was assessed using 24-well Transwell chambers with 8-µm pore inserts (Corning, Inc.). After serum starvation for 24 h, cells were harvested and resuspended in serum-free DMEM at 1×10⁵ cells/ml. Then, 200 µl of cell suspension was seeded into the upper chamber. The lower chamber contained 600 µl of DMEM supplemented with 10% FBS as chemoattractant. After 48 h of incubation at 37°C with 5% CO₂, non-migrated cells were removed. Migrated cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 20 min, both at room temperature. Cell images were captured using an inverted fluorescence microscope (Olympus IX73; Olympus Corporation; magnification, x400). Representative scale bars (200 µm) are shown. Each experiment was performed in triplicate and repeated three times independently.

Cell proliferation assay. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology). Cells were seeded in 96-well plates (2,000 cells/well) in 100 µl of complete medium. At 12, 24, 48 and 72 h post-seeding, the medium was replaced with 90 µl of serum-free medium with 10 µl of CCK-8 solution,

followed by 1 h incubation at 37°C. Absorbance was measured at 450 nm using a microplate reader. Each cell experiment was independently repeated three times.

Apoptosis assay. Apoptosis was quantified using Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (BD Biosciences). Transfected cells were seeded into 6-well plates and cultured to ~80% confluence. Cells were harvested, washed twice with PBS and stained in binding buffer with Annexin V-FITC and PI for 10 min at room temperature in the dark. Samples were analyzed within 1 h using flow cytometry (BD FACSCalibur™; BD Biosciences) and apoptotic populations were quantified. The total apoptosis rate was defined as the sum of early and late apoptotic populations. Data were analyzed using FlowJo software (version 10.8.1; BD Biosciences). Each cell experiment was independently repeated three times.

Cell cycle analysis. Cell cycle analysis was performed using PI staining to quantify DNA content and analyzed via flow cytometry. Cells from different groups were collected and washed with ice-cold PBS. After centrifugation at 500 x g for 5 min and aspiration of the supernatant, the cell pellets were fixed in 1 ml of ice-cold 70% ethanol overnight at 4°C. Following PBS washes three times for 5 min each at room temperature with centrifugation at 500 x g for 5 min per wash, cells were incubated in PI/RNase Staining Buffer (BD Biosciences) for 30 min at 37°C in the dark. Finally, the stained cells were analyzed using a flow cytometer (BD FACSCalibur™; BD Biosciences). Data were interpreted using the CellQuest Pro software (version 6.0; BD Biosciences). Each cell experiment was independently repeated three times.

Wound healing assay. Cell migration was assessed using a wound healing assay in two human ACC cell lines, SW-13 and NCI-H295R. Cells were harvested during the logarithmic growth phase, trypsinized and seeded uniformly into 6-well plates. The cells were cultured until >90% confluence was reached. Prior to wounding, cells were serum-starved in serum-free DMEM for 24 h. A straight wound was introduced in the center area using a sterile pipette tip. The detached cells were removed by washing three times with PBS. Subsequently, serum-free cell culture medium was added. Wound images were captured at 0 and 48 h under an inverted fluorescence microscope (Olympus IX73; Olympus Corporation; magnification, x40). The same magnification and fields of view were used for each time point. Representative scale bars (500 μ m) are shown on respective images. The measurement of wound width was performed by annotating the wound edges on the images. Migration rates were quantified using ImageJ (version 1.8.0; National Institutes of Health) and calculated as follows: Cell migration rate=(scratch width at 0 h-scratch width at 48 h)/scratch width at 0 h x100). Each cell experiment was independently repeated three times.

Statistical analysis. All statistical analyses were performed using R (version 4.2.1; Posit Software, PBC). Quantitative data are expressed as mean $\pm$ standard deviation. For the comparisons in Fig. 1, the Wilcoxon rank-sum test (Mann-Whitney U test) was used for unpaired samples, while the Wilcoxon

signed-rank test was applied for paired samples. For *in vitro* comparisons, unpaired Student's t-test was used for two-group analyses. For comparisons involving ≥ 3 groups, data were assessed for normality and homogeneity of variances. The normality of data distribution was verified using the Shapiro-Wilk test, and the homogeneity of variances was assessed using Levene's test. If these assumptions were met, one-way ANOVA was performed, followed by Tukey's post hoc test for pairwise comparisons. If the assumptions were violated, the Kruskal-Wallis test was used, followed by Dunn's post hoc test. The categorical variables were compared using the χ^2 test. When the applicable conditions of the χ^2 test were violated (>20% of the expected frequency is <5) the Fisher exact test was used instead. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EFNA3 expression patterns and prognostic relevance across various types of human cancer. The differential expression of EFNA3 was assessed in a pan-cancer analysis by comparing normal tissues from the GTEx database against tumor tissues from the TCGA dataset. EFNA3 expression levels were significantly downregulated in glioblastoma multiforme (GBM), kidney chromophobe (KICH), acute myeloid leukemia-like (LAML) and skin cutaneous melanoma (SKCM) compared with that of normal tissues (Fig. 1A). Pan-cancer analysis using the TCGA data of paired tumor and paracancerous tissues from the same patients demonstrated that EFNA3 expression levels were significantly upregulated in the majority of tumor types compared with that of paired paracancerous tissues; however, significant downregulation was observed in GBM, KICH, LAML and SKCM (Fig. 1B). The expression levels of EFNA3 in cancer and normal tissues in various types of cancer are shown in Tables SI-III. Forest plots generated from Cox proportional hazard models demonstrated that EFNA3 expression was significantly associated with OS (Fig. 2A), disease-specific survival (DSS; Fig. 2B) and PFI (Fig. 2C) across various cancer types.

Prognostic and pathological correlations of EFNA3 in pan-cancer analysis. Survival data from TCGA were used to assess the prognostic role of EFNA3. The association of EFNA3 expression levels with survival events and time in various cancer types are shown in Table SIV-VI. Due to incomplete survival annotations, certain end points, specifically DSS and PFI, were unavailable for LAML. Univariate Cox regression showed that increased EFNA3 expression was associated with poor OS, DSS and PFI in bladder cancer (BLCA), kidney clear cell carcinoma (KIRC), ACC and mesothelioma (HR>1; $P < 0.05$). By contrast, increased EFNA3 expression conferred a protective role in lower-grade glioma (LGG; HR<1; $P < 0.05$; Fig. 2). EFNA3 was an adverse marker for PFI in breast cancer (BC), esophageal carcinoma (ESCA), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ) and cervical endocervical squamous carcinoma (CESC), and for DSS in liver hepatocellular carcinoma (LIHC), SKCM and CESC (Figs. 3 and S2). Specifically, the Kaplan-Meier survival analysis and log-rank testing demonstrated that, compared to those in the EFNA3-low expression group, high EFNA3 expression

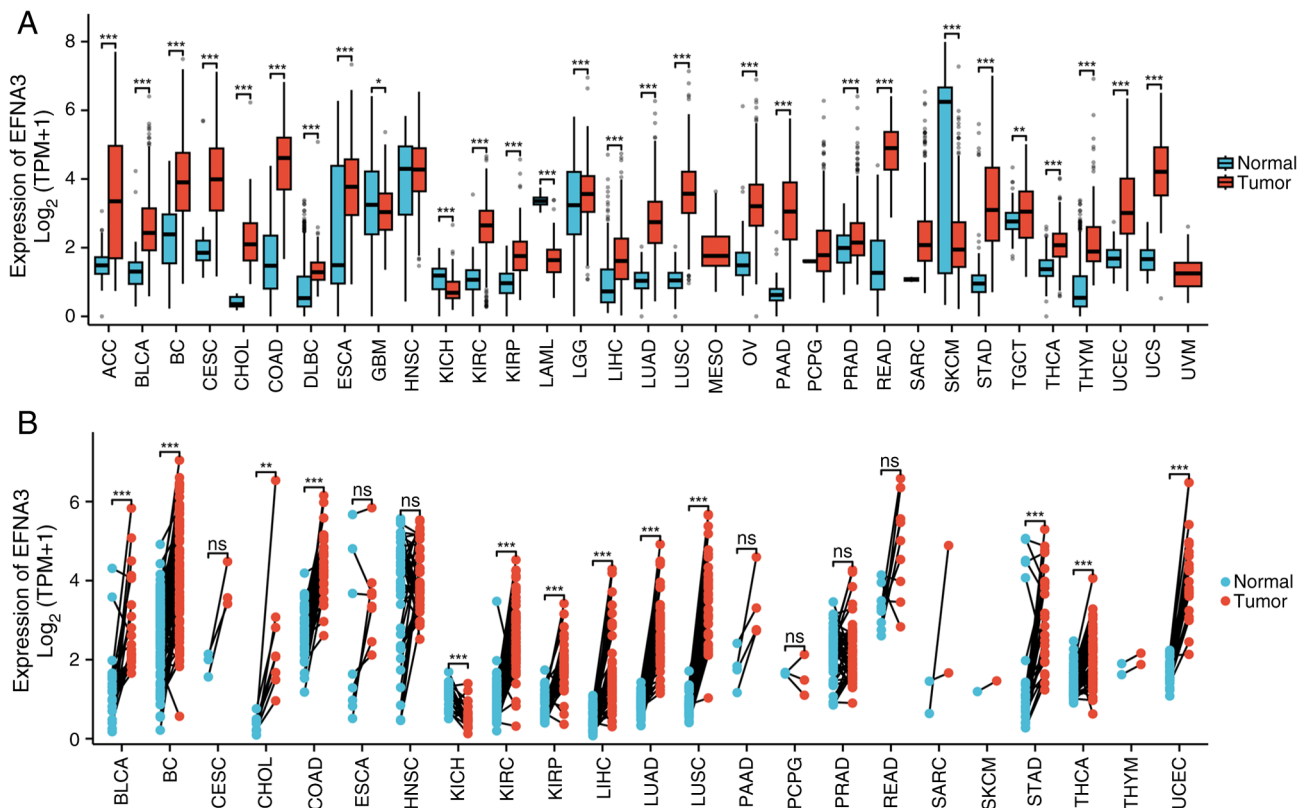


Figure 1. EFNA3 expression levels and prognosis in various types of cancer. (A) Differences in EFNA3 expression between various types of cancer and normal tissues in TCGA + GTEx. The error bars indicate the standard deviation. The Wilcoxon rank-sum test was used for analysis of unpaired samples. (B) Pairwise difference analysis of EFNA3 expression between tumors and adjacent normal tissues. The Wilcoxon signed-rank test was used for paired samples. *P<0.05, **P<0.01 and ***P<0.001. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; ns, no significance.

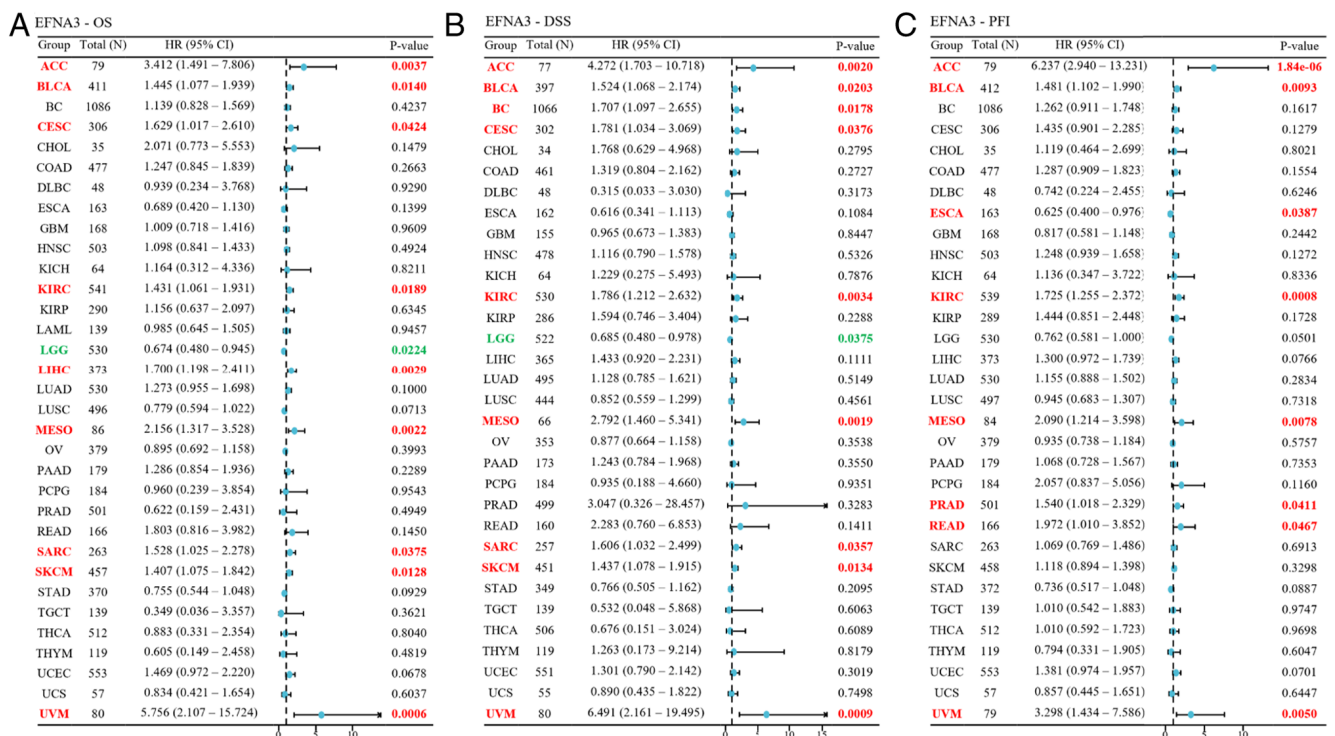


Figure 2. Forest plots demonstrate the prognostic value of EFNA3 in 33 types of cancer. (A) Forest plot of EFNA3 expression levels versus OS in patients with cancer. (B) Forest plot of EFNA3 expression levels versus DSS in patients with cancer. (C) Forest plot of EFNA3 expression level versus PFI in patients with cancer. Red text represents high expression levels associated with poor prognosis, while green text represents high expression levels associated with good prognosis. Conditional assumptions applied: Observations were independent and the risk ratio does not change over time (proportional risk assumption). The univariate Cox regression test was employed to calculate the HR with 95% CI and to determine the P-value. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval; HR, hazard ratio.

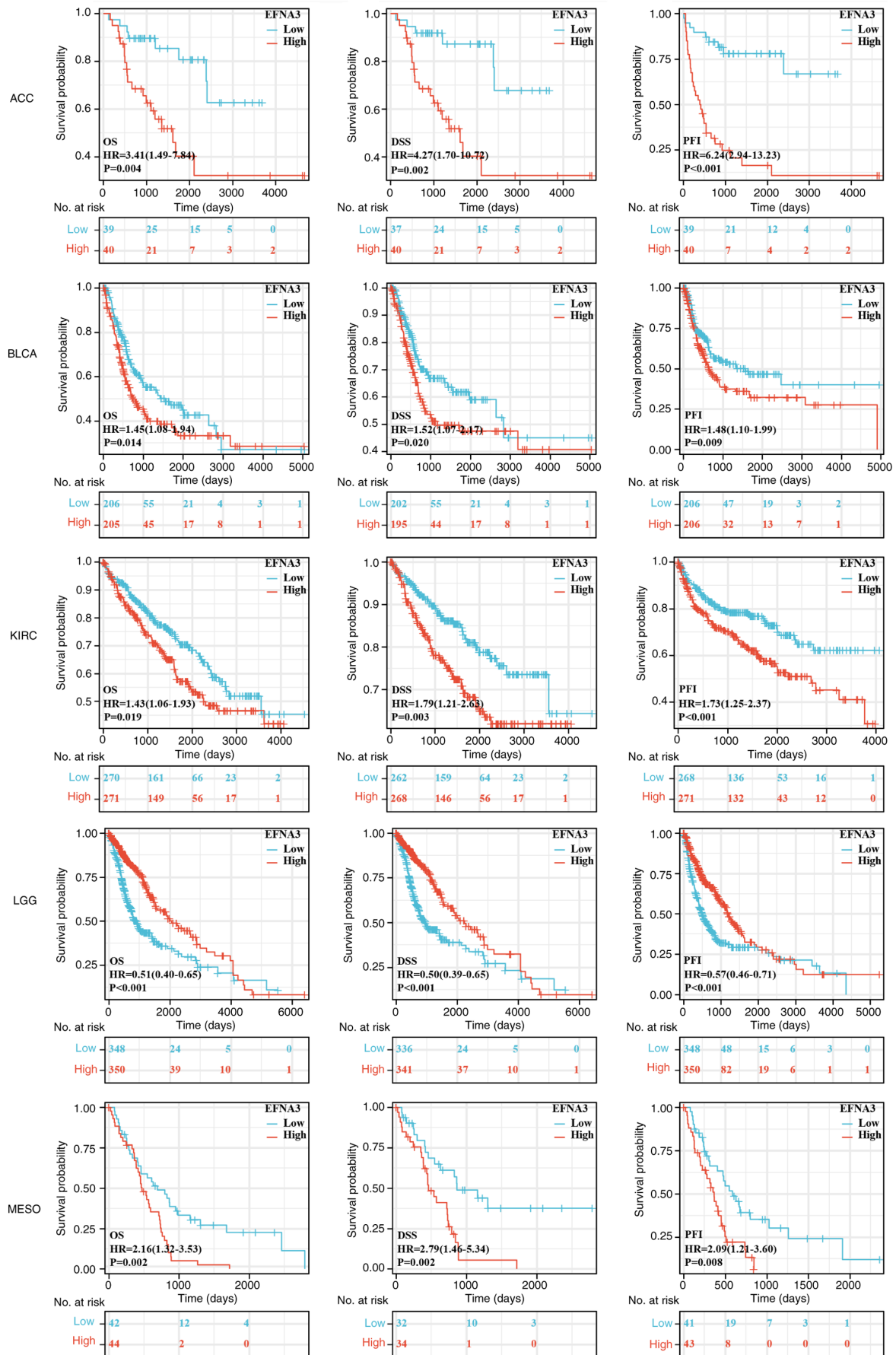


Figure 3. Prognostic value analysis. Survival curves for ACC, BLCA, KIRC, LGG and MESO in EFNA3-high and -low expression groups. The log-rank test was employed to compare the survival curves and determine the P-value. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; BLCA, bladder cancer; KIRC, kidney clear cell carcinoma; LGG, lower-grade glioma; MESO, mesothelioma; OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval; HR, hazard ratio.

was significantly associated with poorer PFI in PRAD, READ, BC, ESCA and CESC, and poorer DSS in LIHC, SKCM and CESC (Fig. 3). Significant correlations between EFNA3 and pathological stage were present in certain tumors, including ACC, cholangiocarcinoma (CHOL), ESCA, KIRP, LIHC, testicular germ cell tumors, thyroid carcinoma and SKCM (Fig. S3).

Genetic alterations of EFNA3 and their prognostic significance. Analysis 2,683 samples from a pan-cancer database of 2,565 patients demonstrated that 15% of the cohort harbored EFNA3 alterations (Fig. 4A), with the highest frequency observed in BC (>40%; Fig. 4B). In ACC, the mutation frequency was 8%. CNAs of the 'mRNA high' and 'amplification' subtypes occurred most frequently in ACC. EFNA3 CNAs positively correlated with mRNA expression (Fig. 4C). Differential expression of genes between EFNA3 altered and unaltered groups in ACC are shown in Table SVII. Survival analyses demonstrated that EFNA3-mutated cases had significantly lower OS in the pan-cancer analysis (HR=2.52, 95% CI, 1.45-4.37; $P<0.001$) and in ACC specifically (OS, HR=2.97, 95% CI, 1.12-7.90, $P=0.029$; DFS HR=8.65, 95% CI, 2.14-34.93, $P=0.002$; Fig. 4D-F). In ACC, β -catenin (CTNNB1) was among the most frequently co-mutated genes with EFNA3 ($P=4.6\times 10^{-4}$; Fig. 4G).

DNA methylation and RNA modifications in the epigenetic regulation of EFNA3. The MethSurv software was used to identify 29 CpG methylation sites for EFNA3 in ACC (Fig. 5A and Table SVIII). The expression levels of EFNA3 positively correlated with multiple mRNA methylation regulators including m1A-, m5C- and m6A-related enzymes (Fig. 5B). The correlation and P-values between the expression level of EFNA3 and mRNA methylation regulatory factors are shown in Table SIX and SX. Top regulators included HNRNPC, ALKBH5, NSUN6, HNRNPA2B1, ELAVL1, METTL3, YTHDF2, LRPPRC and ALYREF.

Correlation between EFNA3 expression levels and the immune microenvironment. In ACC, EFNA3 expression was inversely correlated with stromal, immune and ESTIMATE scores (Fig. 6A). Using the CIBERSORT algorithm, EFNA3 expression was significantly associated with the infiltration levels of multiple immune cell subtypes (Fig. 6B), which indicated potential immunomodulatory roles. For instance, in ACC, EFNA3 expression showed the strongest positive correlation with the infiltration levels of activated dendritic cells and the strongest negative correlation with M1 macrophages, both of which were statistically significant ($P<0.05$).

EFNA3 expression and drug sensitivity prediction. were screened CTRP analysis demonstrated a positive correlation between EFNA3 expression and sensitivity to lovastatin and fluvastatin, and a negative correlation with austocystin D, ibrutinib and lapatinib (Fig. 7A). In the GDSC dataset, EFNA3 expression levels correlated positively with bortezomib, dimethyloxalylglycine and dasatinib sensitivity but negatively with CP-724714, WZ3105 and KIN001-102. Additionally, using a cut-off of $FDR<0.05$, 24 (CTRP) and 14 (GDSC) FDA-approved antitumor drugs were significantly

associated with EFNA3 expression levels (Fig. 7B and C; Tables SXI and SXII).

Association of EFNA3 with clinicopathological features in ACC. EFNA3 expression levels were significantly associated with primary treatment outcome ($P=0.015$), Weiss necrosis ($P=0.039$), tumor status ($P<0.001$), diffuse architecture ($P=0.026$), pathological stage ($P=0.034$), N-stage ($P=0.037$) and sex ($P=0.030$; Table I and Fig. 8A-F). Diagnostic ROC analysis demonstrated a high discriminatory power of EFNA3 [area under the curve (AUC)=0.829; Fig. 8G]. The ROC curve analysis demonstrated that EFNA3 exhibited diagnostic accuracy for ACC across multiple time points. The AUC was 0.764 for 1-year survival, 0.756 for 3-year survival and 0.812 for 5-year survival. Furthermore, on the 1-year ROC curve, a cut-off value of 5.526 was identified for EFNA3 expression (Fig. 8H). The time-dependent ROC analysis assessed the trend of EFNA3 diagnostic accuracy over time. The AUC remained at a high level (≥ 0.7) across all time points (Fig. 8I).

EFNA3 related ceRNA network construction in ACC. The PITA, miRanda and TargetScan databases were used to analyze and predict 85, 30 and 20 EFNA3 target miRNAs, respectively. A total of 12 target miRNAs were found to be in common from the three database predictions, including hsa-miR-30d-5p, hsa-miR-224-5p, hsa-miR-30c-5p, hsa-miR-30a-5p, hsa-miR-30b-5p, hsa-miR-30e-5p, hsa-miR-330-5p, hsa-miR-326, hsa-miR-145-5p, hsa-miR-491-5p, hsa-miR-153-3p and hsa-miR-210-3p (Fig. 9A). In addition, correlation analysis between target miRNAs and EFNA3 expression was performed to identify candidates for further investigation of ceRNA interactions. Correlation analysis demonstrated the expression levels of 6 target miRNAs negatively correlated with EFNA3 expression levels, namely hsa-miR-145-5p ($r=-0.365$, $P=9.26\times 10^{-4}$), hsa-miR-30b-5p ($r=-0.327$, $P=3.23\times 10^{-3}$), hsa-miR-30a-3p ($r=-0.44$, $P=5.06\times 10^{-5}$), hsa-miR-30c-5p ($r=-0.343$, $P=1.95\times 10^{-3}$), hsa-miR-224-5p ($r=-0.281$, $P=1.20\times 10^{-2}$) and hsa-miR-30d-5p ($r=-0.456$, $P=2.45\times 10^{-5}$; Fig. 9B). TargetScan was used to predict the potential binding sites of EFNA3 to the target miRNAs identified (Fig. 9C).

The miRNet and starBase online databases were used to further predict lncRNAs that may bind to the six target miRNAs (hsa-miR-145-5p, hsa-miR-30b-5p, hsa-miR-30a-5p, hsa-miR-30c-5p, hsa-miR-224-5p and hsa-miR-30d-5p; Fig. 10A). A negative correlation between specific lncRNAs and miRNA was observed; the ceRNA network hypothesis suggests that the lncRNA may act as a molecular sponge, sequestering the miRNA and reducing its regulatory activity, consistent with miRNA-mediated ceRNA crosstalk (29). Therefore, the starBase database was used to analyze the correlation between target lncRNA expression and miRNA in ACC. Correlation analysis proved that there are 5 target lncRNAs expression levels that are negatively correlated with hsa-miR-30d-5p, namely AC239868.1, EPB41L4A-AS1, AL049840.4, OIP5-AS1 and SNHG16 (Fig. 10B). However, only the expression level of SNHG16 was negatively correlated with hsa-miR-30c-5p (Fig. 10C). Furthermore, the expression of OIP5-AS1 and SNHG16 were negatively correlated with hsa-miR-30b-5p (Fig. 10D). The expression of MAGI2-AS3 and

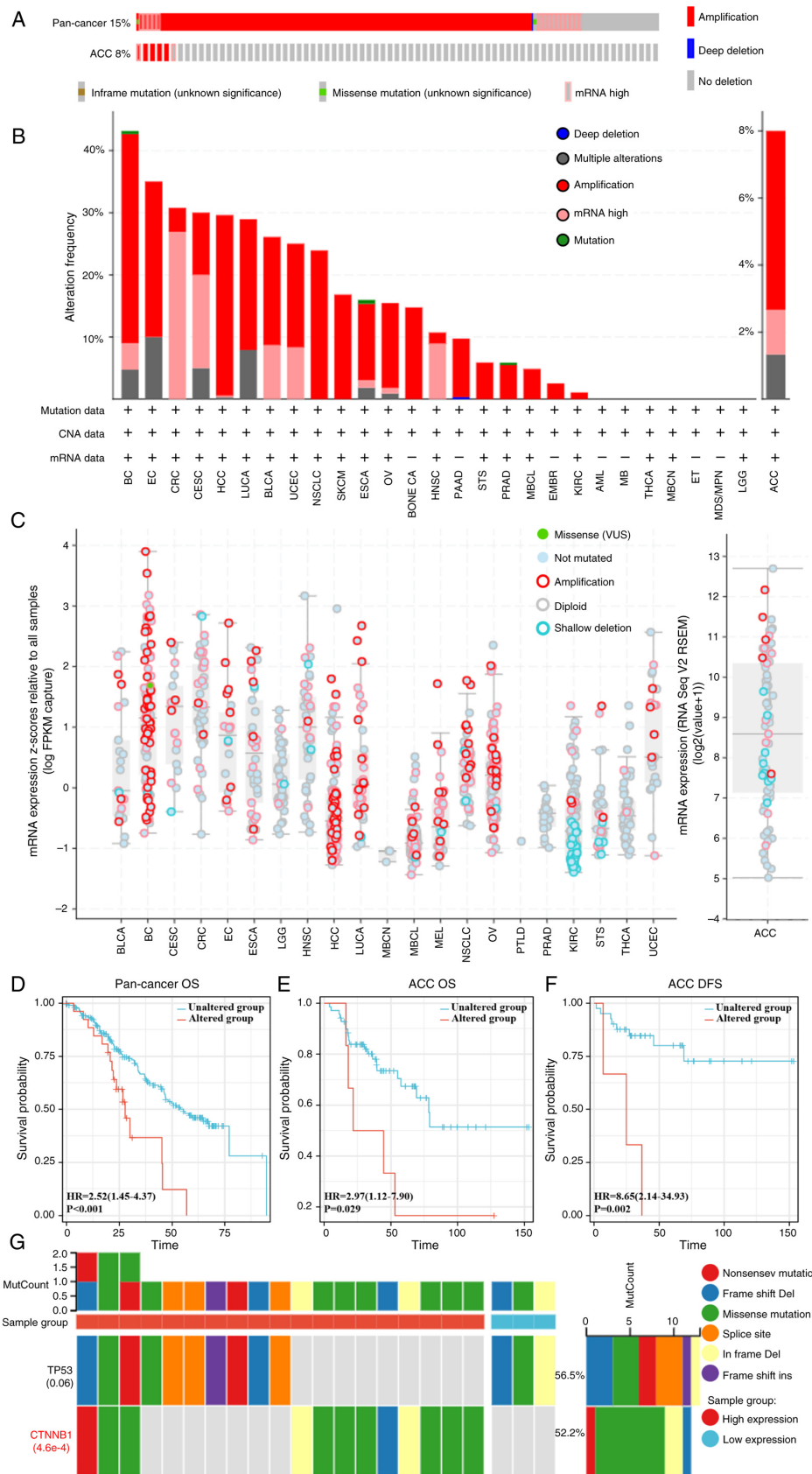


Figure 4. Analysis of genetic alterations. (A) Genetic alterations of EFNA3 in a pan-cancer database and ACC, accounting for 15% (alteration/analysis=384/2,565) and 8% (6/67) of the alterations, respectively. (B) Frequency of altered EFNA3 mutation types in different types of cancer. (C) mRNA expression of EFNA3 putative CNAs in pan-cancer tissues and ACC. Proportional risk hypothesis testing was performed using the survminer package and fitted survival regressions, and the results were visualized using the survminer package as well as the ggplot2 package. (D) Kaplan-Meier curves of EFNA3 mutation status versus OS in pan-cancer analysis. (E) Kaplan-Meier curves of EFNA3 mutation status and OS in ACC. (F) Kaplan-Meier curves of EFNA3 mutations in ACC versus DFS. (G) The top 2 genes with the highest mutation frequency in the EFNA3-high and -low expression group in ACC. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CNA, copy number alterations; Del, deletion; MutCount, mutation count; CTNNB1, β -catenin; VUS, variants of uncertain significance.

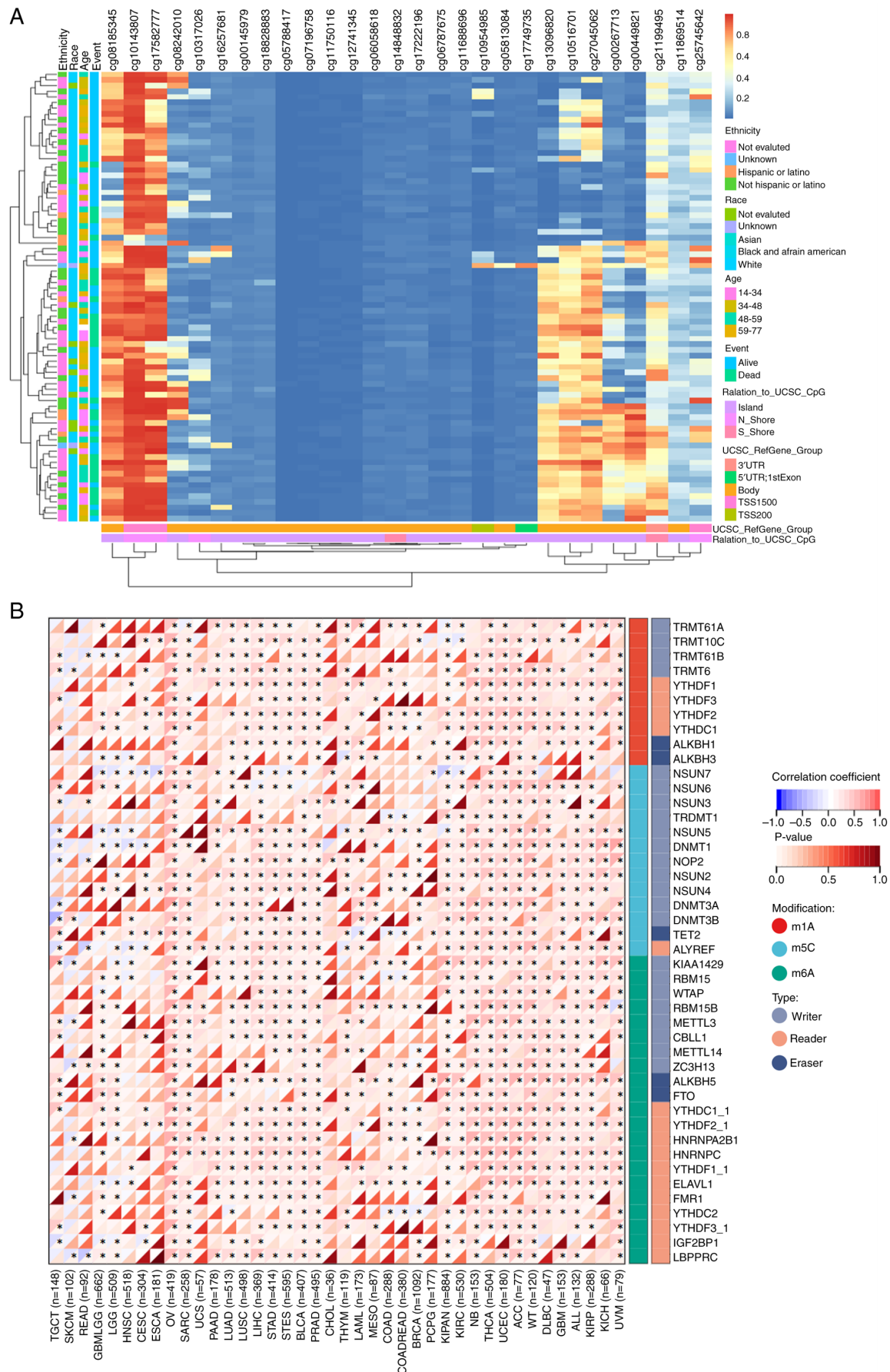


Figure 5. Analysis of EFNA3 DNA methylation in adrenocortical carcinoma and pan-cancer expression correlation with mRNA methylation regulators. (A) Heatmap of EFNA3 DNA methylation in ACC from the MethSurv database. (B) Correlation analysis of EFNA3 expression with mRNA modification methylation regulators. Correlation assessed using Pearson's ρ value and statistical significance. * $P < 0.05$. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; m1A, N1-methyladenosine; m5C, 5-methylcytosine; m6A, N6-methyladenosine

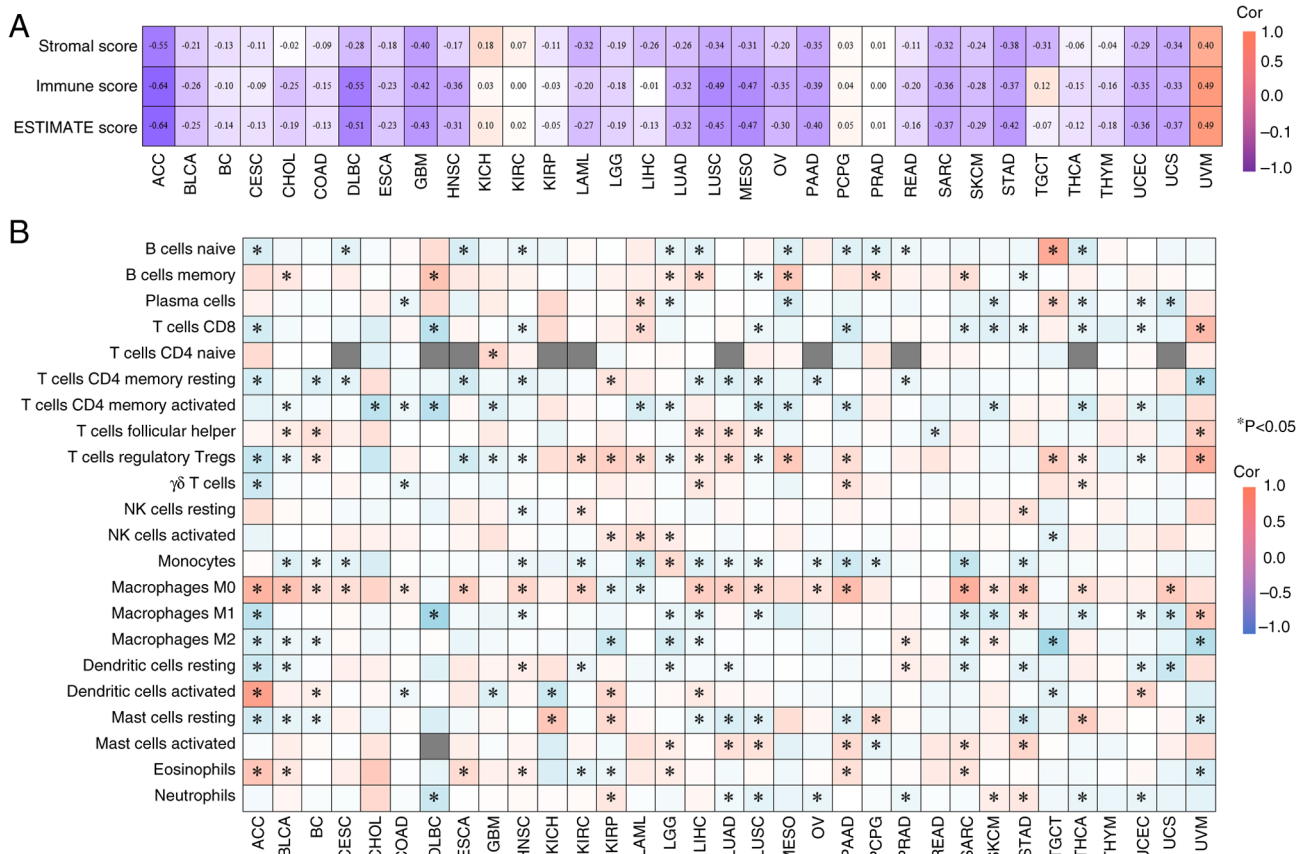


Figure 6. Correlation analysis of EFNA3 expression and immune infiltration. (A) EFNA3 expression level and immune cell infiltration were evaluated based on the ESTIMATE algorithm. (B) EFNA3 expression level and immune cell infiltration were evaluated based on the CIBERSORT algorithm. Correlation assessed using Spearman's ρ value and statistical significance. * $P < 0.05$. EFNA3, ephrin-A3; Cor, correlation; ns, no significance.

LINC00205 were negatively correlated with hsa-miR-224-5p (Fig. 10E). There were 4 target lncRNAs expression levels that are negatively correlated with hsa-miR-30a-5p, respectively EPB41L4A-AS1, PVT1, HELLPAR and DLEU2 (Fig. 10F). Based on the ceRNA hypothesis, regarding the inverse relationship between miRNA and lncRNA or mRNA (29), 14 pairs of ceRNA networks (EPB41L4A-AS1-hsa-miR-30a-5p-EFNA3, PVT1-hsa-miR-30a-5p-EFNA3, HELLPAR-hsa-miR-30a-5p-EFNA3, DLEU2-hsa-miR-30a-5p-EFNA3, OIP5-AS1-hsa-miR-30b-5p-EFNA3, SNHG16-hsa-miR-30b-5p-EFNA3, SNHG16-hsa-miR-30c-5p-EFNA3, AC239868.1-hsa-miR-30d-5p-EFNA3, EPB41L4A-AS1-hsa-miR-30d-5p-EFNA3, AL049840.4-hsa-miR-30d-5p-EFNA3, OIP5-AS1-hsa-miR-30d-5p-EFNA3, SNHG16-hsa-miR-30d-5p-EFNA3, MAGI2-AS3-hsa-miR-224-5p-EFNA3 and LINC00205-hsa-miR-224-5p-EFNA3) were constructed based on the correlation analysis results (Fig. 10G).

Analysis of genes and functions co-expressed with EFNA3 in ACC. The LinkedOmics database was used to analyze EFNA3 co-expression in ACC; under the condition of $FDR < 0.05$, 2,000 genes were significantly positively correlated with EFNA3 expression levels (Fig. 11A), while 2,967 genes were significantly negatively correlated with EFNA3 expression levels. The genes that were positively and negatively correlated with EFNA3 expression levels in ACC are provided in Tables SXIII.

The top 50 genes most significantly positively (Fig. 11B) and negatively (Fig. 11C) correlated with EFNA3 expression levels are displayed in the heat map. Tables SXIV-XV summarizes the GO and KEGG enrichment analyses of genes positively and negatively correlated with EFNA3 expression. As shown in the functional enrichment analysis, genes positively correlated with EFNA3 expression were significantly enriched in pathways and biological terms including 'Cushing syndrome', 'Wnt signaling pathway', 'C2H2 zinc finger domain binding', 'forebrain development', and the 'β-catenin-TCF complex' (Fig. 11D). Genes negatively correlated with EFNA3 expression were significantly associated with immune-related processes and molecular functions, such as 'T-cell-mediated immunity', 'N-glycan processing', 'immunological synapse formation' and 'cytokine receptor activity' (Fig. 11E).

The STRING database was used to investigate the PPI network of EFNA3; EFNA3 was associated with ephrin type-A receptor 4 (EPHA4), ephrin type-A receptor 2 (EPHA2), ephrin type-A receptor 3 (EPHA3), ephrin type-A receptor 7 (EPHA7), ephrin type-A receptor 5 (EPHA5), 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ-1, ephrin type-A receptor, ephrin type-B receptor 1 and ephrin type-B receptor 3 (0.999, 0.948, 0.936, 0.929, 0.924, 0.914, 0.904, 0.895, 0.88 and 0.869 respectively; Fig. 11F). The confidence scores represent the calculated probability that these associated proteins have a functional interaction with EFNA3. EPHA4, EPHA2 and EPHA3 had the highest

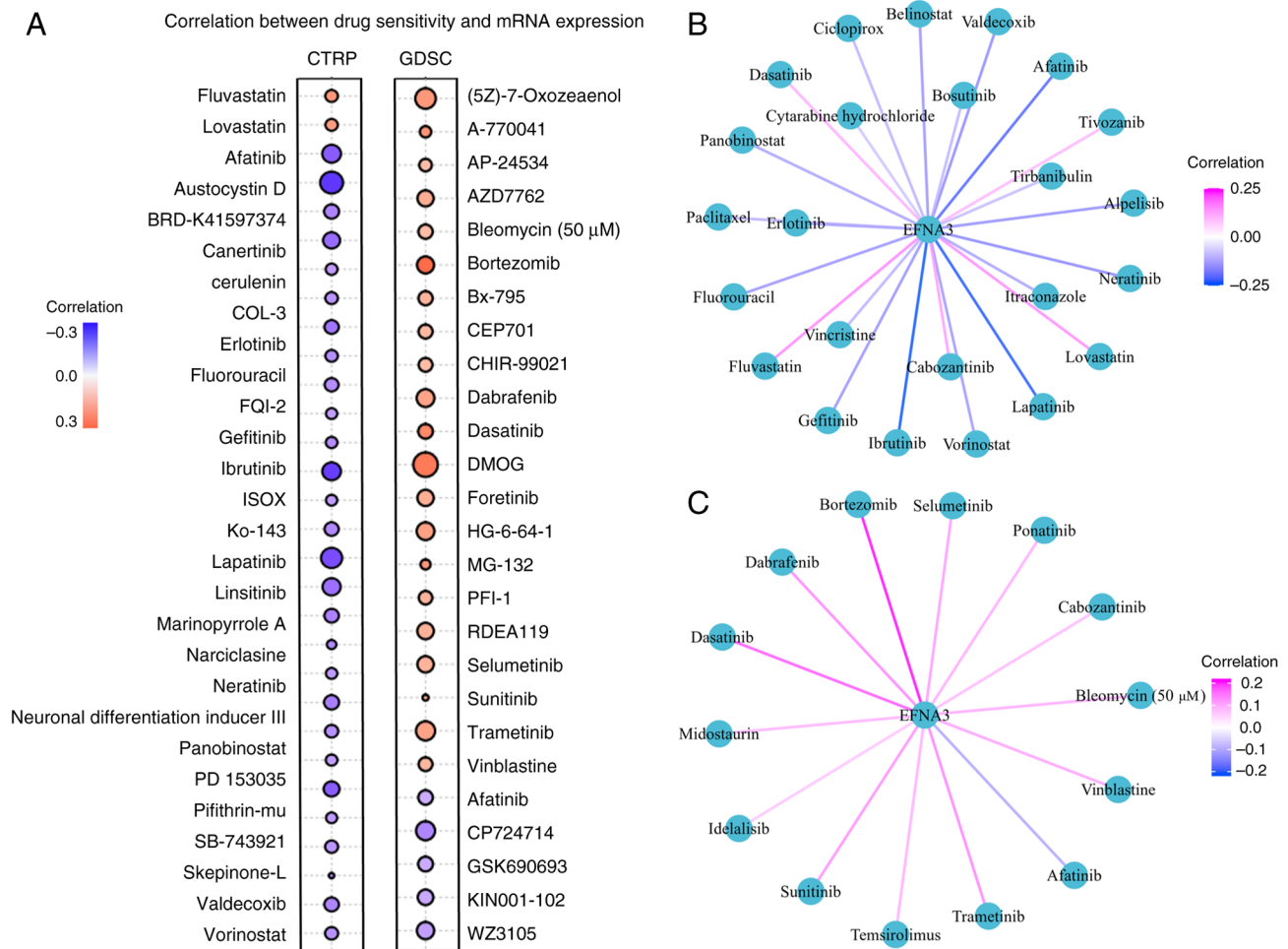


Figure 7. Pan-cancer analysis of drug sensitivity of EFNA3-related drugs. (A) The relationship between GDSC and CTRP drug sensitivity and EFNA3 mRNA expression. (B) FDA-approved EFNA3-related chemotherapeutic agents from CTRP drug sensitivity analysis. (C) FDA-approved EFNA3-related anticancer drugs from GDSC drug sensitivity analysis. EFNA3, ephrin-A3; GDSC, Genomics of Drug Sensitivity in Cancer; CTRP, Cancer Therapeutics Response Portal; FDA, Food and Drug Administration.

correlation with EFNA3, suggesting that these genes may serve a promoting role in certain types of tumors. These results suggested that EFNA3 may be closely related to the occurrence and development of ACC.

Validation of ACC cell transfection efficiency. ACC cell transfection efficiency was demonstrated using RT-qPCR. In NCI-H295R and SW-13 cell lines, the mRNA expression level in EFNA3-OE group was significantly increased compared with that of the vector group, and the EFNA3 mRNA expression in sh-EFNA3 group was significantly decreased compared with that of the shNC group ($P < 0.05$; Fig. 12).

EFNA3 enhances the viability and invasiveness of ACC cells in vitro. CCK-8 assays demonstrated that EFNA3 overexpression significantly promoted cell viability compared with that of the control group, whereas EFNA3 knockdown had the opposite effect. Flow cytometric analysis demonstrated that EFNA3 knockdown induced apoptosis and G_1/S phase cell cycle arrest. Conversely, EFNA3 overexpression significantly reduced apoptosis and facilitated S-phase progression (Fig. 13A-F). In the Transwell and wound healing assays, EFNA3 overexpression significantly enhanced cell migration

capabilities, respectively. By contrast, EFNA3 knockdown significantly impaired these abilities (Fig. 14A-D).

Discussion

EFNA3, a glycolysis-associated gene, has been implicated in the progression of several malignancies, including BC, hepatocellular carcinoma (HCC), oral squamous cell carcinoma, pancreatic adenocarcinoma, lung adenocarcinoma (LUAD), pheochromocytoma and stomach adenocarcinoma, and has been proposed as a diagnostic and prognostic biomarker in these contexts (14,30-33). A recent multi-omics study demonstrated epigenetic regulation of EFNA3 in metastatic pheochromocytomas and paragangliomas, identifying a differentially methylated probe (cg12741345) located within the gene body of EFNA3 (34). The present study also found the cg12741345 probe among the 28 CpG sites identified in ACC, which suggested potential epigenetic dysregulation of EFNA3 in ACC pathogenesis.

To the best of our knowledge, no comprehensive pan-cancer analysis of EFNA3 incorporating multi-dimensional data has been reported. The present results demonstrated that EFNA3 is significantly upregulated in tumor tissues when compared

Table I. Correlation between EFNA3 expression levels and clinicopathological characteristics.

Characteristic	Low expression of EFNA3, n (%)	High expression of EFNA3, n (%)	P-value
Total patients	39 (49.4)	40 (50.6)	
Pathological N stage			0.037
0	37 (46.8)	31 (39.2)	
1	1 (1.3)	8 (10.1)	
Unknown	1 (1.3)	1 (1.3)	
Pathological stage			0.034
I	6 (7.6)	3 (3.8)	
II	23 (29.1)	14 (17.7)	
III	5 (6.3)	11 (13.9)	
IV	4 (5.1)	11 (13.9)	
Unknown	1 (1.3)	1 (1.3)	
Tumor status			<0.001
Tumor free	29 (36.7)	10 (12.7)	
With tumor	8 (10.1)	30 (38.0)	
Unknown	2 (2.5)	0 (0.0)	
Primary therapy outcome			0.015
Progressive disease	4 (5.1)	14 (17.7)	
Stable disease	1 (1.3)	1 (1.3)	
Partial response	1 (1.3)	0 (0.0)	
Complete response	30 (38)	16 (20.3)	
Unknown	3 (3.8)	9 (11.4)	
Sex			0.030
Female	19 (24.1)	29 (36.7)	
Male	20 (25.3)	11 (13.9)	
Weiss-Necrosis			0.039
Absent	12 (15.2)	5 (6.3)	
Present	24 (30.4)	33 (41.8)	
Unknown	3 (3.8)	2 (2.5)	
Weiss-Diffuse architecture			0.026
Absent	5 (6.3)	14 (17.7)	
Present	24 (30.4)	18 (22.8)	
Unknown	10 (12.7)	8 (10.1)	

The percentages presented are derived from the proportion of each variable category relative to the total sample size (n=79). Cases were categorized as unknown when source of the sample was not defined by clinical characteristics. EFNA3, ephrin-A3; N, node.

with that of adjacent normal tissues across multiple cancer types, including BLCA, CHOL and colon adenocarcinoma. However, in GBM, KICH, LAML and SKCM, EFNA3 expression levels were significantly reduced. These cancer types have not been well explored regarding the biological role of EFNA3, and the clinical significance of this downregulation remains currently unclear.

EFNA3 is involved in multiple cellular functions, including tumor malignancy, angiogenesis, energy metabolism and intratumoral hypoxia. In HCC, EFNA3 upregulation correlates with more aggressive tumor behavior, promotion of self-renewal, proliferation, migration and tumor stemness (35). Mechanistically, under hypoxic conditions, hypoxia-inducible factor 1 α (HIF-1 α) increases EFNA3 expression in HCC by increasing copy number (12). Deng *et al* (14) demonstrated that

knocking down EFNA3 significantly inhibits the proliferation and glycolytic capacity of LUAD cells. Yiminniyaze *et al* (36) further demonstrated that EFNA3 induces epithelial-mesenchymal transition by enhancing ERK and AKT phosphorylation levels, while upregulating MMP2 and MMP9 expression. In choroidal melanoma, EFNA3 promotes cell proliferation and migration by activating the STAT3/AKT signaling pathway (37). In prostate cancer, EFNA3 knockout suppresses disease progression by reducing Ras/Braf/MEK/Erk1/2 phosphorylation levels (38). In pancreatic ductal adenocarcinoma cells, EFNA3 enhances tumor angiogenesis and cell permeability through the Wnt/ β -catenin pathway (39). This divergent expression pattern may reflect cancer-type-specific regulatory mechanisms or TME influences. Further experimental studies are warranted to elucidate the role of EFNA3

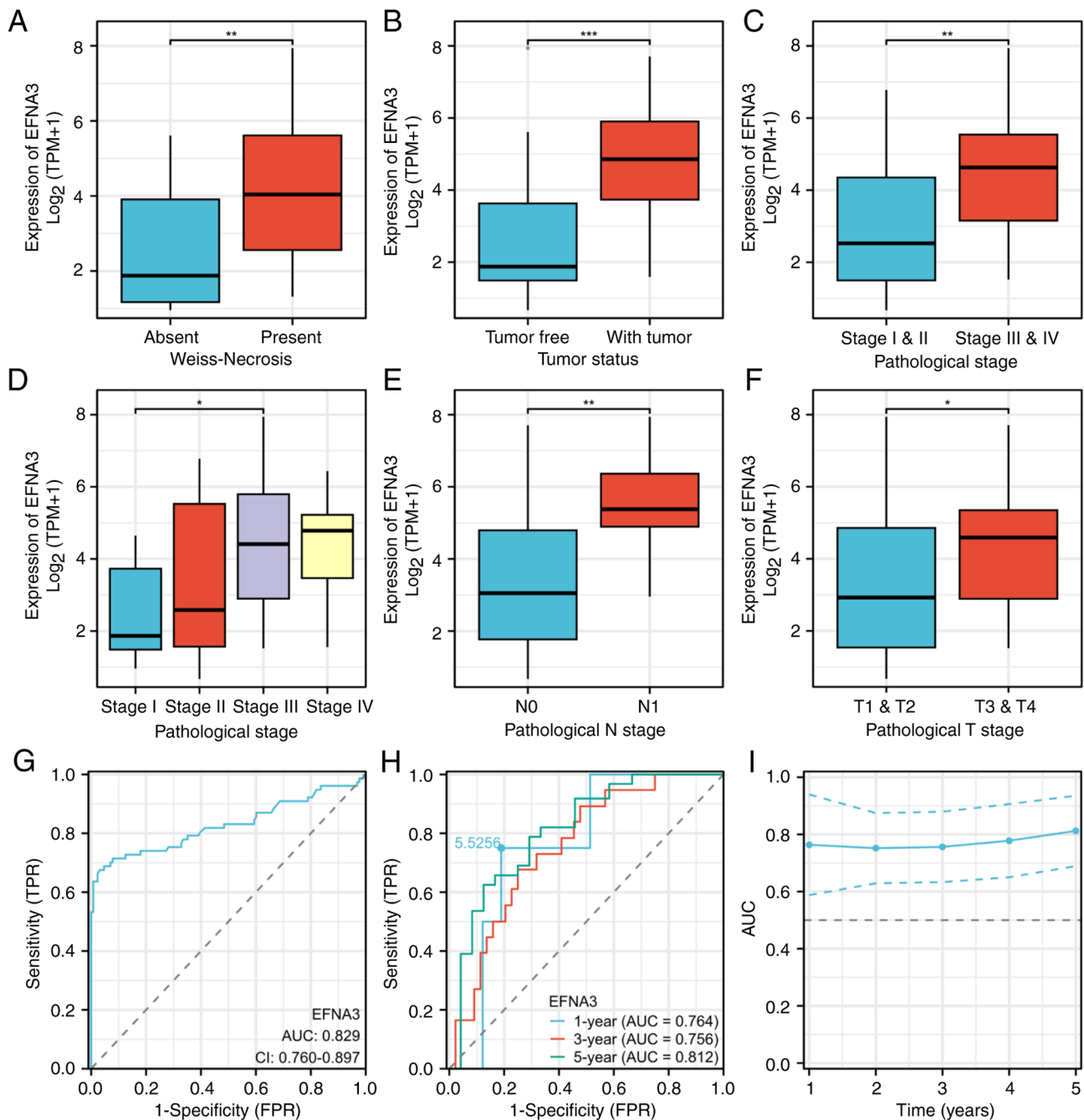


Figure 8. Association between EFNA3 expression and clinicopathological features in ACC. Association of EFNA3 expression levels with (A) Weiss-Necrosis, (B) tumor status, (C) pathological stage: I & II vs. III & IV), (D) pathological stage: I vs. II vs. III vs. IV (Kruskal-Wallis and Dunn's analysis), (E) pathological N stage and (F) pathological T stage: T1 & T2 vs. T3 & T4 (Mann-Whitney U test). (G) Diagnostic value of EFNA3 in ACC. (H) Time-dependent prognostic value of EFNA3 at 1-, 3- and 5-years. (I) CI of time-dependent diagnostic value; normal group, n=128; tumor group, n=77. The error bars indicate the standard deviation. ROC analysis was performed using the pROC package; the AUC and cumulative survival rate corresponding to each time point were calculated using the time ROC package. *P<0.05, **P<0.01 and ***P<0.001. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; AUC, area under the curve; N, node; T, tumor; TPR, true positive rate; FPR, false positive rate.

in these malignancies. In the future, *in vivo* models can be used to elucidate whether reduced EFNA3 expression confers tumor-suppressive effects or reflects compensatory biological processes, in order to expand the current understanding of EFNA3 as a context-dependent modulator in tumor biology.

Genetic alterations such as single nucleotide and CNAs are key drivers of oncogenesis and tumor progression (40). In the present pan-cancer analysis, EFNA3 exhibited a notably high mutation frequency, >40% in BC, suggesting a potential

tumor-promoting role in this context. Within the ACC cohort, the most prevalent genomic events associated with EFNA3 were elevated mRNA expression and gene amplification, both indicative of CNA-driven dysregulation. Survival analyses across pan-cancer datasets demonstrated that EFNA3 mutations were associated with poorer overall survival, supporting its relevance as a clinically significant genomic alteration. Specifically, patients harboring EFNA3 mutations in ACC showed significantly reduced OS and DFS compared with

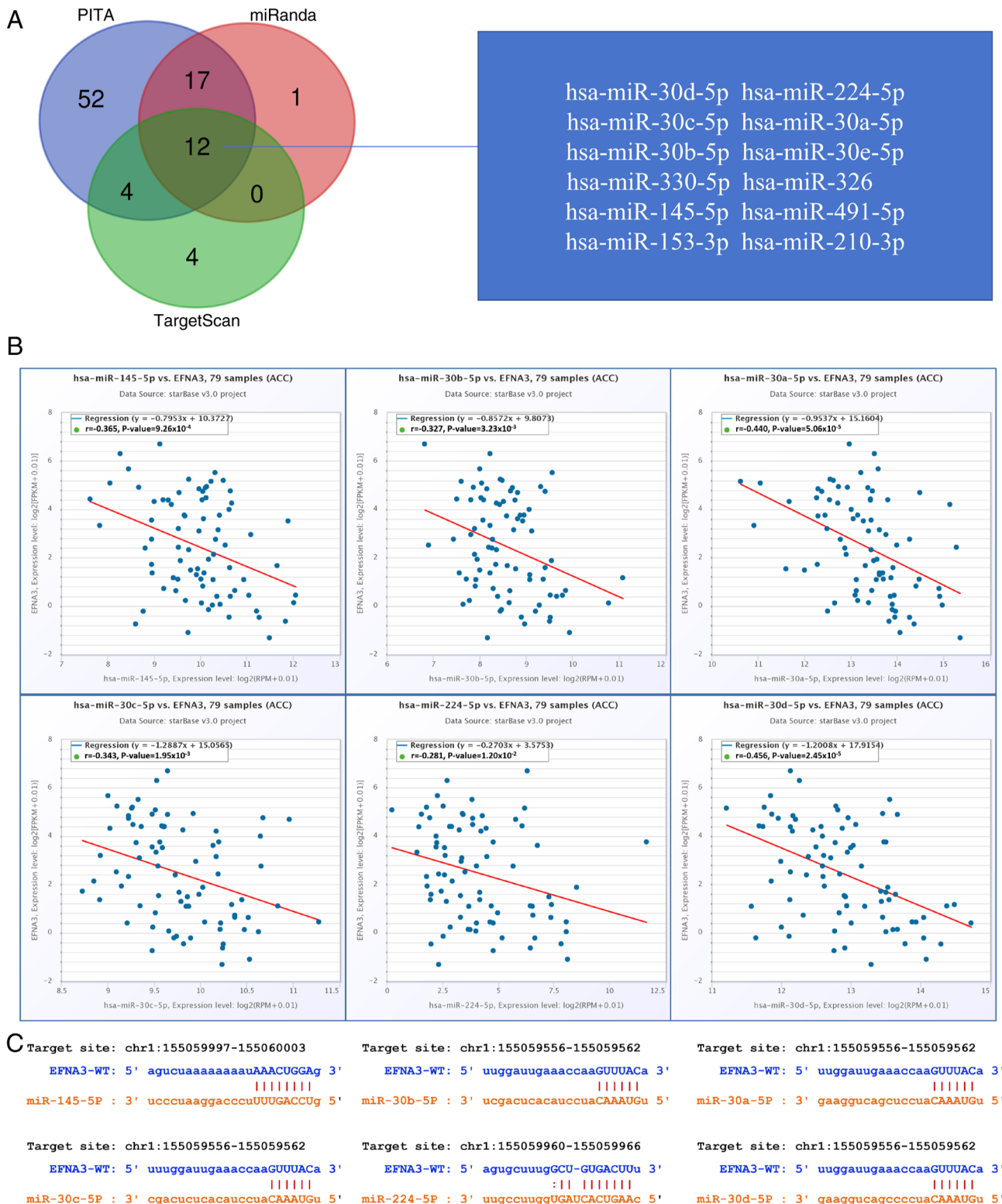


Figure 9. Prediction of miRNAs targeting EFNA3 in ACC. (A) Venn diagram showing the prediction results of EFNA3 targets using the PITA, miRanda and TargetScan software. (B) Scatter plots demonstrate the miRNA-mRNA associations with significant correlation. The starBase software was used to analyze the correlation between EFNA3 and the target miRNA. (C) The TargetScan software was used to predict the potential binding site of EFNA3 to the target miRNA. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; miRNA, microRNA; WT, wild-type; chr, chromosome.

those with wild-type EFNA3, underscoring its potential role as a negative prognostic marker.

To further elucidate the genetic landscape associated with EFNA3 expression, mutation profiles between high- and low-EFNA3 expression groups were compared. CTNNB1

emerged as a differentially mutated gene, suggesting possible co-regulatory or downstream interactions. By contrast, EFNA3 expression did not significantly differ between groups stratified by TP53 or PRKAR1A mutation status, two well-established drivers in ACC, which suggested that EFNA3 regulation may

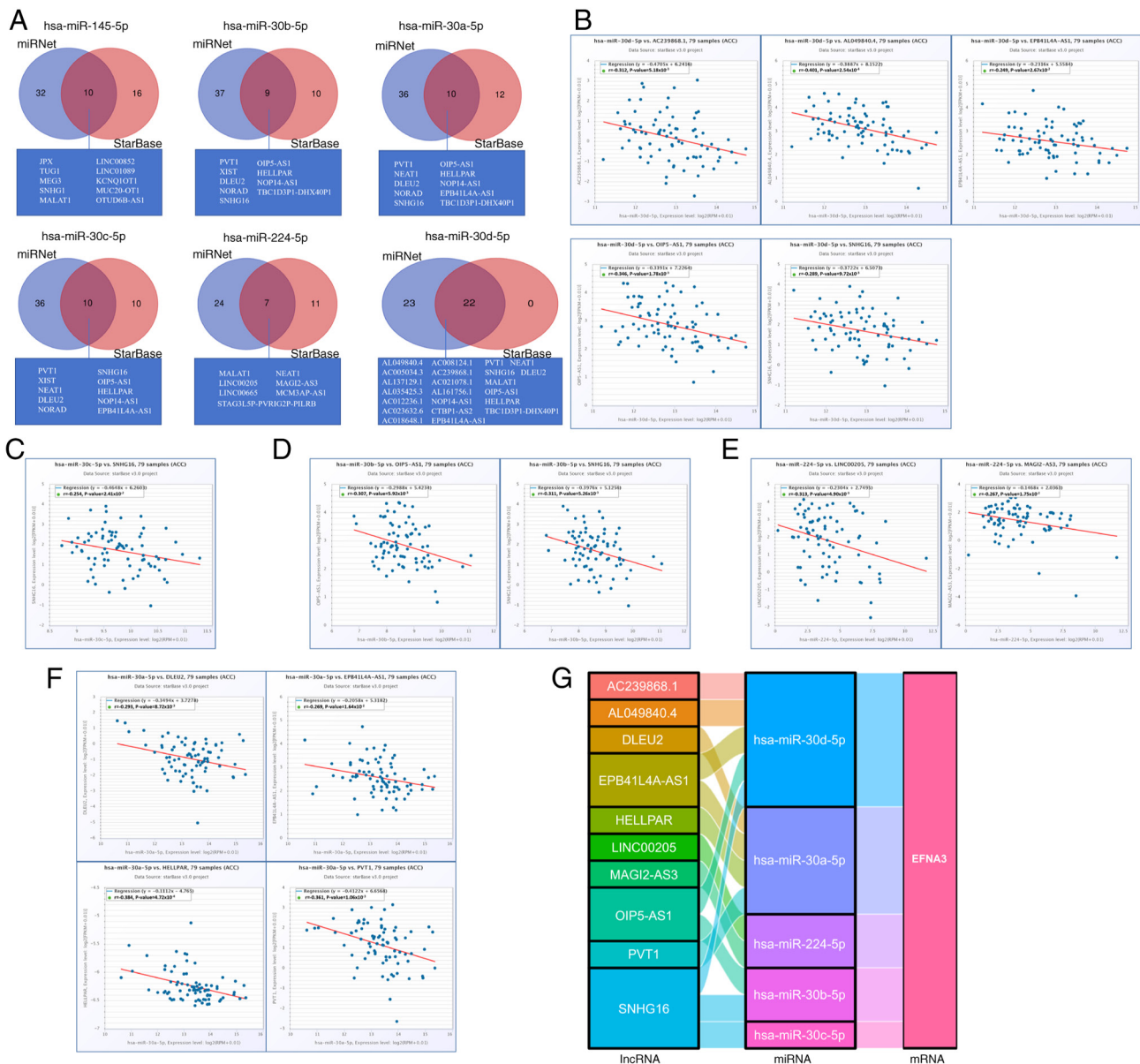


Figure 10. Prediction of lncRNA and ceRNA network construction in ACC. (A) Venn diagrams display the target lncRNAs of hsa-miR-145-5p, hsa-miR-30b-5p, hsa-miR-30a-5p, hsa-miR-30c-5p, hsa-miR-224-5p and hsa-miR-30d-5p respectively. The starBase software was used to analyze the correlations between miRNAs and the target lncRNAs. Scatter plots demonstrate the miRNA-mRNA associations with significant correlation as follow, lncRNA related to (B) hsa-miR-30d-5p, (C) hsa-miR-30c-5p, (D) hsa-miR-30b-5p, (E) hsa-miR-224-5p and (F) hsa-miR-30a-5p. (G) The Sankey diagram displays the lncRNA-miRNA-mRNA EFNA3 regulatory network in line with the competitive endogenous RNA hypothesis. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; miRNA, microRNA; lncRNA, long non-coding RNA.

operate through mechanisms independent of TP53/PRKARIA signaling.

Previous studies have shown that CTNNB1 mutations in ACC are primarily missense mutations localized to exon 3, which impair β -catenin degradation and promote Wnt pathway activation (41,42). Future research should elucidate whether specific CTNNB1 mutation types, such as exon 3 hotspots versus null alleles, modulate EFNA3 expression or function. A mechanistic dissection of EFNA3-CTNNB1 cross-talk may yield novel insights into the oncogenic circuitry of ACC.

Unlike genomic mutations, epigenetic modifications, such as DNA methylation and RNA methylation, alter gene expression without changing the DNA sequence itself (43). These modifications are increasingly recognized as pivotal regulators

of oncogenesis and tumor behavior (44). The present study identified 28 CpG methylation sites associated with EFNA3 in ACC; a CpG island methylator phenotype (CIMP) has been previously reported in ACC, with the CIMP-high subgroup associated with poorer clinical outcomes, compared with that of the CIMP-low subgroup (45,46). Aberrant DNA methylation in promoter or gene body regions can silence tumor suppressor genes or activate oncogenes, thereby influencing tumor aggressiveness and therapeutic response (47). Although no methylation-targeted therapy has been clinically approved for ACC, DNA methylation inhibitors such as 5-azacytidine and decitabine have demonstrated efficacy in other types of cancer, such as BC and ACC, and are under investigation in preclinical ACC models (48-53). Decitabine demonstrates

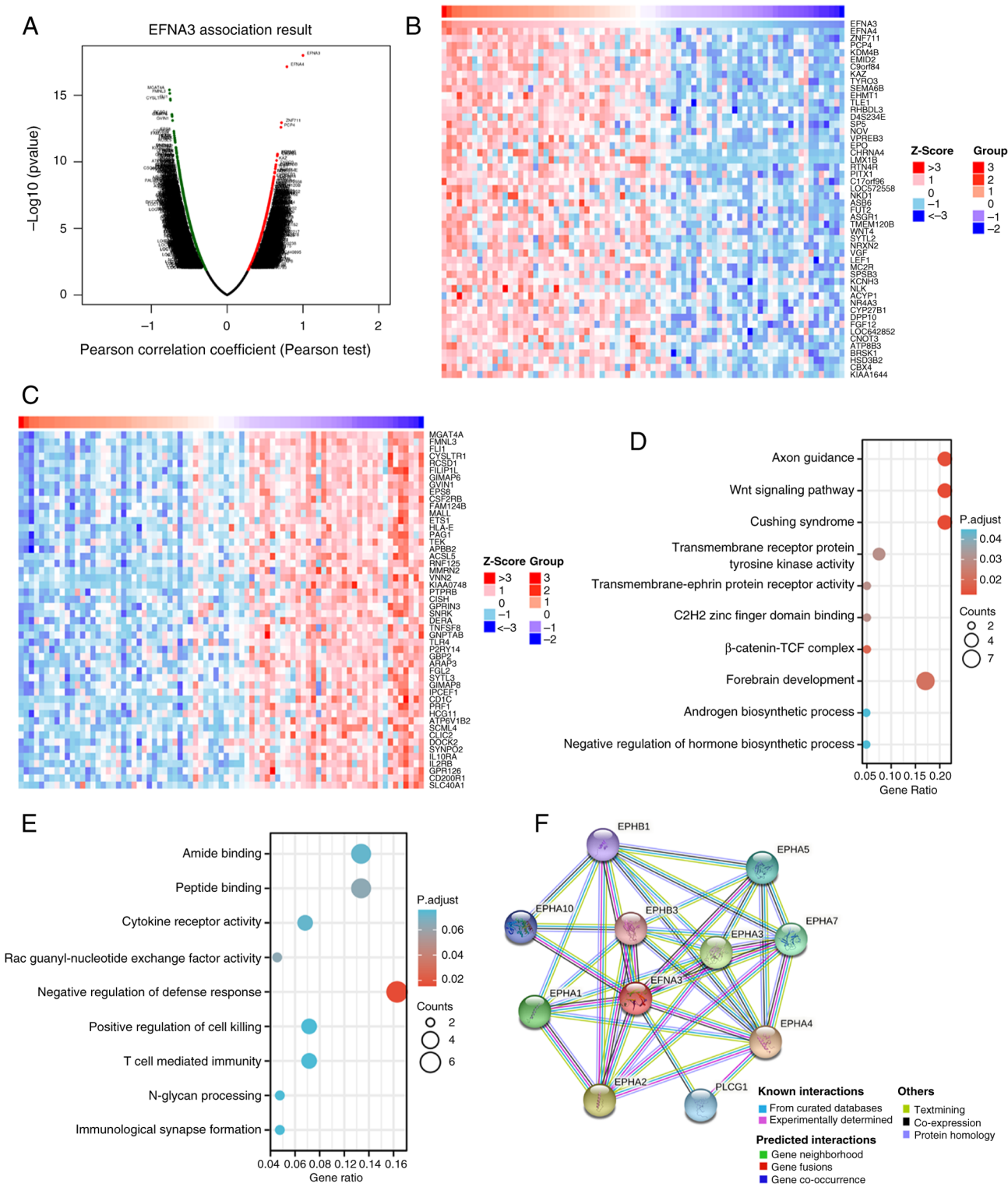


Figure 11. Enrichment analysis of EFNA3 functional networks in ACC. (A) The Pearson test was used to identify genes highly related to EFNA3 identified in ACC. (B) The heat map shows the top 50 genes positively related to EFNA3 in the ACC cohort. (C) The heat map shows the top 50 genes negatively related to EFNA3 in the ACC cohort. (D) Enrichment of GO and KEGG terms for the top 50 genes positively related to EFNA3. (E) Enrichment of GO and KEGG terms for the top 50 genes negatively related to EFNA3. (F) Protein-protein interaction network of EFNA3. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; P.adjust, adjusted P-value.

anti-tumor effects in ACC cells at clinically relevant concentrations by reactivating silenced genes in the 11q13 region, suggesting a role for epigenetic mechanisms in adrenocortical carcinogenesis and indicating its potential as an adjuvant treatment for advanced cases (49). The present data supported the

hypothesis that EFNA3 methylation patterns may contribute to ACC pathogenesis and could be leveraged as a predictive biomarker or therapeutic target.

Furthermore, significant positive correlations between EFNA3 expression and mRNA modification regulators across

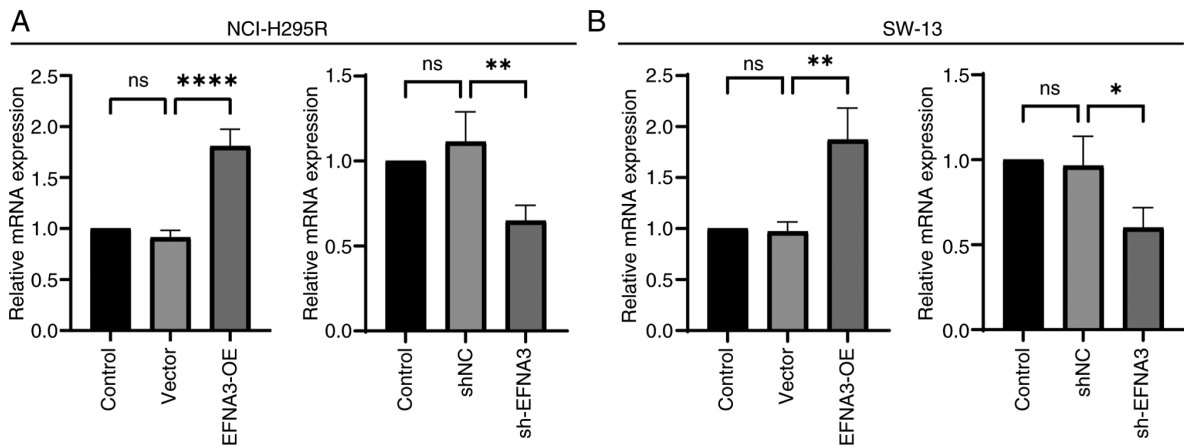


Figure 12. Validation of EFNA3 knockdown and overexpression efficiency. Verification of EFNA3 expression efficiency after knockdown and overexpression in (A) NCI-H295R cells and (B) SW-13 cells. The error bars indicate the standard deviation. * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.0001$. One-way ANOVA was used for statistical analysis. Each experiment was independently repeated three times. EFNA3, ephrin-A3; ns, not significant; OE, overexpressed; NC, negative control; sh, short hairpin.

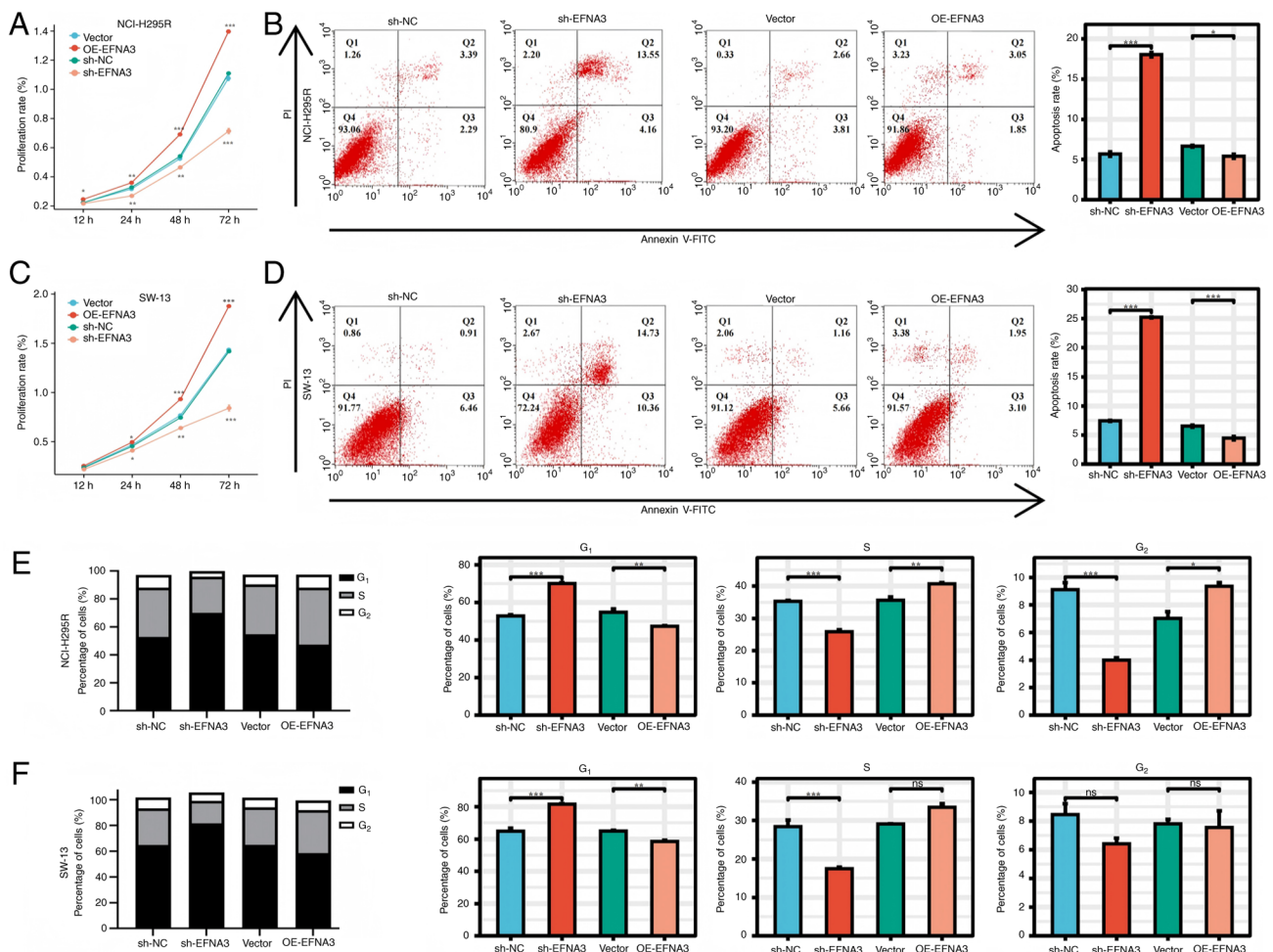


Figure 13. Effects of knockdown and overexpression of EFNA3 in viability, apoptosis and cell cycle in ACC cells. Effects on (A) cell viability and (B) apoptosis in the NCI-H295R cell line. Effects on (C) cell viability and (D) apoptosis in the SW-13 cell line. Effect on cell cycle in the (E) NCI-H295R and (F) SW-13 cell lines. The error bars indicate the standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. One-way ANOVA was used for statistical analysis. Each experiment was independently repeated three times. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; OE, overexpressed; NC, negative control; sh, short hairpin.

pan-cancer datasets were demonstrated. In ACC specifically, several m6A modulators demonstrated expression alterations with prognostic implications. For instance, HNRNPC,

a known splicing regulator, was downregulated in ACC and associated with a worse prognosis (54). Conversely, ALKBH5 and YTHDF2 were upregulated and linked to

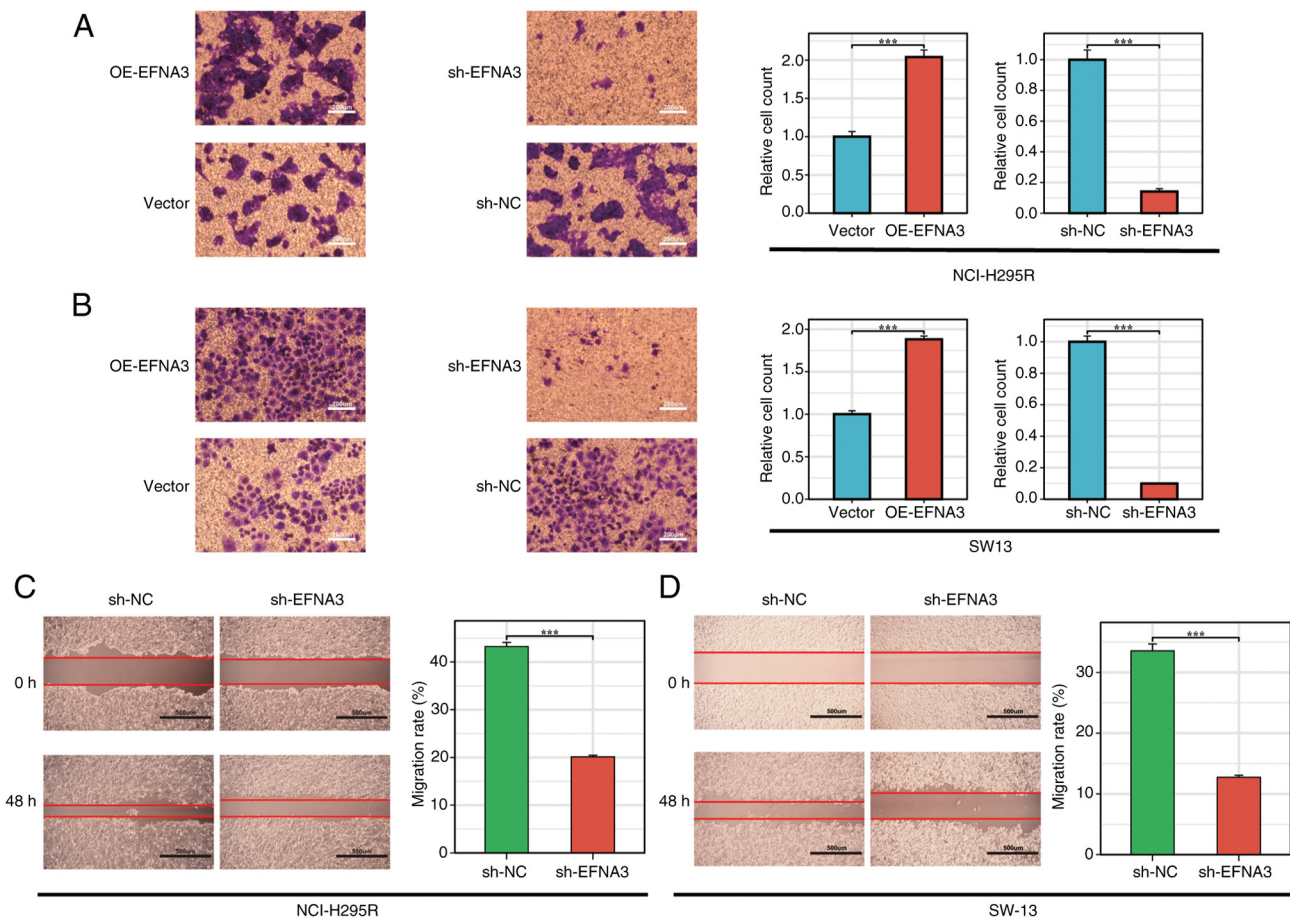


Figure 14. Effects of knockdown and overexpression of EFNA3 on the invasive and migratory capacities of ACC cells. Effects on cell migration in (A) NCI-H295R and (B) SW-13 cell lines (magnification x400; scale bar, 200 μ m). Effect on cell migration ability of (C) NCI-H295R and (D) SW-13 cell lines (magnification x40; scale bar, 500 μ m). The error bars indicate the standard deviation. *** $P < 0.001$. One-way ANOVA was used for statistical analysis. Each experiment was independently repeated three times. EFNA3, ephrin-A3; ACC, adrenocortical carcinoma; OE, overexpressed; NC, negative control; sh, short hairpin.

tumor progression (54-58). METTL3, a methyltransferase, was downregulated and associated with a favorable prognosis. These findings suggested that EFNA3 may be integrated into broader epigenetic regulatory networks, including m6A RNA methylation pathways, that modulate tumor biology in ACC. Collectively, the present results suggested a complex regulatory landscape in which EFNA3 is subject to both DNA- and RNA-level epigenetic control, offering novel insights into its diagnostic and therapeutic relevance in ACC. Future studies may explore combinations with DNA methyltransferase inhibitors (such as decitabine) or m6A modulators to reverse EFNA3-driven oncogenicity, leveraging epigenetic vulnerabilities common in types of endocrine cancer.

Immunotherapy has revolutionized the treatment paradigm for various types of cancer, offering durable responses in a subset of patients, such as patients with relapsed ovarian cancer and relapsed gastric cancer (59). However, a significant proportion of individuals fail to achieve sustained benefits, which is often attributed to the complexity and heterogeneity of the TME (60,61). Immunotherapy is primarily used in patients with advanced ACC after the failure of traditional chemotherapy (62). Among these treatments, pembrolizumab is the most studied and recommended in guidelines, although its monotherapy objective response rate remains

limited (63-65). The present study identified a significant correlation between EFNA3 expression levels and immune cell infiltration across multiple cancer types, including ACC. This suggested that EFNA3 may serve a role in modulating the immune landscape, potentially impacting tumor immune evasion and responsiveness to immunotherapeutic agents. In ACC specifically, where immune-based treatment options currently remain limited, EFNA3 may serve as a potential immunological biomarker or therapeutic target. Given its association with immune infiltration, EFNA3 might be involved in shaping the immunosuppressive or immunoactive features of the TME. Further studies are warranted to delineate its mechanistic role in regulating immune cell recruitment, antigen presentation or checkpoint molecule expression. The correlation between EFNA3 and TME features demonstrated in the present study provide a rationale for evaluating EFNA3 not only as a diagnostic or prognostic marker but also as a modulator of tumor-immune interactions, opening avenues for combination strategies involving EFNA3-targeted agents and immune checkpoint inhibitors (such as anti-programmed cell death protein 1 and programmed cell death ligand 1) in ACC.

The ceRNA hypothesis proposes that lncRNAs can regulate gene expression by sequestering miRNAs, thereby preventing them from binding to target mRNAs (66). Increasing evidence

has implicated the EFNA3-centered ceRNA network in various tumor types. For example, miR-210-3p has been shown to target EFNA3, thereby modulating the PI3K/AKT pathway and influencing tumor progression in oral squamous cell carcinoma (67). Similarly, miR-210-mediated suppression of EFNA3 has been reported to affect cell proliferation and invasiveness in peripheral nerve sheath tumors (68). Under hypoxic conditions, EFNA3 can also be regulated through HIF-induced lncRNA activation, promoting metastatic spread in BC (69).

The present study constructed a lncRNA-miRNA-EFNA3 regulatory axis in ACC, highlighting novel non-coding RNA molecules such as OIP5-AS1 and hsa-miR-30d-5p. Previous studies have shown that OIP5-AS1 can act as a ceRNA to modulate oncogenic signaling in endocrine tumors, while miR-486-3p is downregulated in adrenocortical neoplasms and may serve as a tumor suppressor (70,71). The ceRNA network involving EFNA3 identified in the present analysis demonstrated a potential mechanism of post-transcriptional regulation that could contribute to tumor progression, immune modulation and drug resistance in ACC. Furthermore, targeting the lncRNA-miRNA-EFNA3 regulatory axis may have therapeutic potential. For example, salazosulapyridine has been proposed to exert anticancer effects in ACC by interacting with the OIP5-AS1-miR-92a-3p-SLC7A11 pathway (72). The present findings underscored the potential of EFNA3-centered ceRNA regulatory networks as diagnostic tools and therapeutic targets in ACC, warranting further functional validation.

Drug repurposing offers a cost-effective and time-efficient strategy to identify new therapeutic options for rare and refractory malignancies such as ACC. In the present study, drug sensitivity correlation analysis was performed based on EFNA3 expression was performed, identifying 24 EFNA3-associated compounds in the CTRP database and 14 in the GDSC database. Notably, EFNA3 expression was significantly correlated with sensitivity to HMG-CoA reductase inhibitors (statins), which have gained interest for their potential antitumor effects (73,74). Statins, primarily used to treat hypercholesterolemia, have demonstrated tumor-suppressive effects in multiple cancer types, including HCC, breast, lung and colorectal cancer (75-80). Mechanistically, statins exert anti-tumor effects through inhibition of the mevalonate pathway, leading to suppression of AKT/NF- κ B signaling, induction of apoptosis via caspase cascade activation and impairment of metastatic potential through modulation of MAPK and mTOR pathways (81-88). Simvastatin, for example, has been shown to activate AMPK, upregulate p21 and induce apoptosis in HCC cells (86). HMG-CoA reductase inhibitors reduce isoprenoid synthesis by inhibiting the mevalonate pathway, thereby affecting the tumor procession (89).

Despite this promising pharmacological profile, limited studies have examined the application of statins in endocrine malignancies, particularly in ACC. Given the dependence of ACC cells on cholesterol biosynthesis and isoprenoid metabolism (90), the mevalonate pathway represents a potential target. Furthermore, given the key role of EFNA3 in glycolysis, combining EFNA3 inhibition with glycolytic inhibitors or statins represents a metabolic 'double-hit' strategy against the Warburg-dependency of ACC. The present results highlight EFNA3 as a potential biomarker for predicting statin sensitivity

in ACC. The therapeutic implications of this finding warrant further validation through *in vitro* mechanistic assays and *in vivo* efficacy studies. Furthermore, integrating statins with EFNA3-targeted strategies may provide a synergistic approach to disrupt tumor metabolism and reduce ACC aggressiveness.

The Wnt/ β -catenin signaling cascade serves a pivotal role in ACC by regulating tumor cell proliferation, migration and metabolic reprogramming. Dysregulation of Wnt/ β -catenin pathway, commonly through activating mutations in the CTNNB1 gene, is a hallmark of ACC pathogenesis (91,92). In the present transcriptome-based co-expression analysis, EFNA3 was closely associated with genes involved in Wnt signaling, suggesting a potential regulatory interaction. Specifically, EFNA3 expression was significantly increased in CTNNB1-mutated samples, demonstrating an association between EFNA3 activity and Wnt/ β -catenin pathway dysregulation. Previous research has demonstrated that β -catenin and Transcription Factor 4 modulate the expression of EphB receptors and their ligands, including ephrin-B1, thereby orchestrating spatial organization along epithelial axes such as the crypt-villus axis in colorectal cancer (93). The present findings suggested a similar interaction may exist between EFNA3 and β -catenin in ACC, which potentially contributes to malignant transformation. Furthermore, EFNA3 is a glycolysis-related gene, and the Wnt/ β -catenin pathway is a known driver of metabolic reprogramming in cancer cells (94). This pathway enhances aerobic glycolysis by upregulating glycolytic enzymes, promoting a tumor-favorable microenvironment characterized by increased lactate production and glucose uptake (95-101). In colon and breast cancer, activation of Wnt signaling induces pyruvate dehydrogenase kinase 1 expression and modulates adipogenic enzymes, respectively, reinforcing glycolytic flux and tumor proliferation (96-98).

The present *in vitro* experiments demonstrated that EFNA3 promotes invasive behavior in ACC cells. These data suggested that EFNA3 may act as a downstream effector or modulator of Wnt/ β -catenin signaling to coordinate both metabolic and invasive phenotypes in ACC. Future mechanistic studies are warranted to dissect the precise molecular interactions between EFNA3, β -catenin and glycolysis-related signaling pathways in ACC progression. These findings present EFNA3 not only as a key mediator of Wnt-driven ACC pathogenesis but also as a potential node for combinatorial therapeutic intervention. Given the established challenges in targeting Wnt/ β -catenin signaling directly in endocrine tumors, primarily the disruption of normal somatic stem cell function critical for cellular repair and tissue homeostasis (102), EFNA3 inhibition offers a tractable approach to disrupt downstream oncogenic outputs (such as metabolic reprogramming and invasion) while potentially synergizing with Wnt pathway modulators or endocrine-disrupting agents.

Despite the comprehensive nature of the present study, several limitations should be acknowledged. First, the bioinformatics analyses were primarily based on the relatively small TCGA-ACC cohort. Future studies incorporating larger, multi-center datasets are needed to strengthen the robustness and generalizability of these findings. Although the integrative bioinformatics and *in vitro* findings suggested that EFNA3 is associated with enhanced sensitivity to HMG-CoA reductase inhibitors, clinical evidence remains lacking. Large-scale,

randomized controlled trials are needed to validate the therapeutic efficacy of these agents and safety in patients with ACC. Second, the ceRNA regulatory network involving EFNA3 was constructed through computational predictions and partially supported by molecular data. However, extensive experimental validation, particularly through gain and loss-of-function assays *in vivo*, is required to confirm biological relevance and establish causal relationships between EFNA3 and these pathways. Third, although significant epigenetic alterations associated with EFNA3 expression in ACC were observed, the potential of DNA methylation inhibitors as a therapeutic strategy remains unexplored in clinical settings. Additional preclinical studies are necessary to determine whether demethylating agents can reverse EFNA3-mediated oncogenic effects. While the present *in vitro* experiments demonstrated EFNA3 enhances ACC cell invasiveness, the absence of *in vivo* validation remains a key constraint. The precise molecular mechanism governing the interplay between EFNA3, Wnt/ β -catenin signaling and metabolic reprogramming requires further elucidation through mechanistic studies in animal models complemented by patient-derived organoids.

Future research should also explore the feasibility of targeting EFNA3 as a multi-modal biomarker for ACC prognosis, immunomodulation and drug responsiveness. Future research should focus on verifying the synergistic potential of EFNA3-targeted therapy with four major categories of drugs: Immune checkpoint inhibitors (to reverse immunosuppression), epigenetic modulators (to regulate the methylation/m6A network), metabolic disruptors (to block glycolysis and mevalonate pathways) and endocrine-specific drugs (adrenal diuretics and steroid synthesis inhibitors). This is essential to overcome ACC drug resistance, achieve synergistic effects, and are key to translating the present findings into clinically actionable strategies.

The present study identified EFNA3 as a potential oncogenic driver and prognostic biomarker in adrenocortical carcinoma. Through integrative bioinformatics analyses and *in vitro* validation, it was demonstrated that EFNA3 may modulate tumor invasiveness, potentially through its interaction with the Wnt/ β -catenin signaling pathway and glycolytic reprogramming. EFNA3 expression was also associated with immune cell infiltration, epigenetic alterations and drug sensitivity, particularly to HMG-CoA reductase inhibitors, highlighting its potential as a multifaceted therapeutic target. Furthermore, construction of a ceRNA regulatory network provided novel insights into the post-transcriptional control of EFNA3 in ACC. These findings collectively support the translational relevance of EFNA3 and warrant further mechanistic and clinical investigation.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YT, XL and JS designed and implemented the study. YT and YZ performed acquisition, analysis or interpretation of data for the work. YT, XL, YZ and JS contributed to drafting the work or revising it critically for important intellectual content. JS supervised the project, acquired funding and provided final approval of the published version. YT and XL confirm the authenticity of all the raw data. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and approved the final 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.

References

1. Else T, Kim AC, Sabolch A, Raymond VM, Kandathil A, Caoili EM, Jolly S, Miller BS, Giordano TJ and Hammer GD: Adrenocortical carcinoma. *Endocr Rev* 35: 282-326, 2014.
2. Sinclair TJ, Gillis A, Alobuia WM, Wild H and Kebebew E: Surgery for adrenocortical carcinoma: When and how? *Best Pract Res Clin En* 34: 101408, 2020.
3. Del Rivero J, Else T, Hallanger-Johnson J, Kiseljak-Vassiliades K, Raj N, Reidy-Lagunes D, Srinivas S, Gilbert J, Vaidya A, Aboujaoude E, *et al.*: A review of mitotane in the management of adrenocortical cancer. *Oncologist* 29: 747-760, 2024.
4. Fassnacht M, Dekkers O, Else T, Baudin E, Berruti A, de Krijger R, Haak H, Mihai R, Assie G and Terzolo M: European society of endocrinology clinical practice guidelines on the management of adrenocortical carcinoma in adults, in collaboration with the European network for the study of adrenal tumors. *Eur J Endocrinol* 179: G1-G46, 2018.
5. Chen L, Huang L, Gu Y, Cang W, Sun P and Xiang Y: Lactate-Lactylation hands between metabolic reprogramming and immunosuppression. *Int J Mol Sci* 23: 11943, 2022.
6. Ganapathy-Kanniappan S and Geschwind JH: Tumor glycolysis as a target for cancer therapy: Progress and prospects. *Mol Cancer* 12: 152, 2013.
7. Boedtker E and Pedersen SF: The acidic tumor microenvironment as a driver of cancer. *Annu Rev Physiol* 82: 103-126, 2020.
8. Liu S, Shen G, Zhou X, Sun L, Yu L, Cao Y, Shu X and Ran Y: Hsp90 promotes gastric cancer cell metastasis and stemness by regulating the regional distribution of glycolysis-related metabolic enzymes in the cytoplasm. *Adv Sci* 11: e2310109, 2024.
9. Nievergaal E, Lackmann M and Janes PW: Eph-dependent cell-cell adhesion and segregation in development and cancer. *Cell Mol Life Sci* 69: 1813-1842, 2012.

10. Himanen J, Saha N and Nikolov DB: Cell-cell signaling via Eph receptors and ephrins. *Curr Opin Cell Biol* 19: 534-542, 2007.
11. Kou CJ and Kandpal RP: Differential expression patterns of Eph receptors and ephrin ligands in human cancers. *Biomed Res Int* 2018: 7390104, 2018.
12. Stewen J, Kruse K, Godoi-Filip AT, Zenia, Jeong H, Adams S, Berkenfeld F, Stehling M, Red-Horse K, Adams RH and Pitulescu ME: Eph-ephrin signaling couples endothelial cell sorting and arterial specification. *Nat Commun* 15: 2539, 2024.
13. Pasquale EB: Eph receptors and ephrins in cancer progression. *Nat Rev Cancer* 24: 5-27, 2024.
14. Deng M, Tong R, Zhang Z, Wang T, Liang C, Zhou X and Hou G: EFNA3 as a predictor of clinical prognosis and immune checkpoint therapy efficacy in patients with lung adenocarcinoma. *Cancer Cell Int* 21: 535, 2021.
15. Hao Y and Li G: Role of EFNA1 in tumorigenesis and prospects for cancer therapy. *Biomed Pharmacother* 130: 110567, 2020.
16. Yamashita T, Ohneda K, Nagano M, Miyoshi C, Kaneko N, Miwa Y, Yamamoto M, Ohneda O and Fujii-Kuriyama Y: Hypoxia-inducible transcription factor-2alpha in endothelial cells regulates tumor neovascularization through activation of ephrin A1. *J Biol Chem* 283: 18926-18936, 2008.
17. Nakamura R, Kataoka H, Sato N, Kanamori M, Ihara M, Igarashi H, Ravshanov S, Wang Y, Li Z, Shimamura T, *et al*: EPHA2/EFNA1 expression in human gastric cancer. *Cancer Sci* 96: 42-47, 2005.
18. Cui Y, Chang Y, Ma X, Sun M, Huang Y, Yang F, Li S, Zhuo W, Liu W, Yang B, *et al*: Ephrin A1 stimulates CCL2 secretion to facilitate pre-metastatic niche formation and promote gastric cancer liver metastasis. *Cancer Res* 85: 263-276, 2024.
19. Wilson K, Shiuan E and Brantley-Sieders DM: Oncogenic functions and therapeutic targeting of EphA2 in cancer. *Oncogene* 40: 2483-2495, 2021.
20. Mao L, Yuan W, Cai K, Lai C, Huang C, Xu Y, Zhong S, Yang C, Wang R, Zeng P, *et al*: EphA2-YES1-ANXA2 pathway promotes gastric cancer progression and metastasis. *Oncogene* 40: 3610-3623, 2021.
21. Li Y, Peng Q and Wang L: EphA2 as a phase separation protein associated with ferroptosis and immune cell infiltration in colorectal cancer. *Aging (Albany NY)* 15: 12952-12965, 2023.
22. Pei J, Zhang C, Yusupu M, Zhang C and Dai DQ: Screening and validation of the hypoxia-related signature of evaluating tumor immune microenvironment and predicting prognosis in gastric cancer. *Front Immunol* 12: 705511, 2021.
23. Xie R, Yuan M and Jiang Y: The pan-cancer crosstalk between the EFNA family and tumor microenvironment for prognosis and immunotherapy of gastric cancer. *Front Cell Dev Biol* 10: 790947, 2022.
24. Bhatia S, Oweida A, Lennon S, Darragh LB, Milner D, Phan AV, Mueller AC, Van Court B, Raben D, Serkova NJ, *et al*: Inhibition of EphB4-Ephrin-B2 signaling reprograms the tumor immune microenvironment in head and neck cancers. *Cancer Res* 79: 2722-2735, 2019.
25. Janes PW, Vail ME, Ernst M and Scott AM: Eph receptors in the immunosuppressive tumor microenvironment. *Cancer Res* 81: 801-805, 2021.
26. Ma W, Zhu M, Wang B, Gong Z, Du X, Yang T, Shi X, Dai B, Zhan Y, Zhang D, *et al*: Vandetanib drives growth arrest and promotes sensitivity to imatinib in chronic myeloid leukemia by targeting ephrin type-B receptor 4. *Mol Oncol* 16: 2747-2765, 2022.
27. Chen B, Khodadoust MS, Liu CL, Newman AM and Alizadeh AA: Profiling tumor infiltrating immune cells with CIBERSORT. *Methods Mol Biol* 1711: 243-259, 2018.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
29. Salmena L, Poliseno L, Tay Y, Kats L and Pandolfi PP: A ceRNA hypothesis: The Rosetta Stone of a hidden RNA language? *Cell* 146: 353-358, 2011.
30. Kuang L, Pang Y and Fang Q: TMEM101 expression and its impact on immune cell infiltration and prognosis in hepatocellular carcinoma. *Sci Rep* 14: 31847, 2024.
31. Wu Q, Li P, Tao X, Lin N, Mao B and Xie X: A novel super-enhancer-related risk model for predicting prognosis and guiding personalized treatment in hepatocellular carcinoma. *BMC Cancer* 24: 1087, 2024.
32. Lin P and Yang H: EFNA3 is a prognostic biomarker for the overall survival of patients with hepatocellular carcinoma. *J Hepatol* 77: 879-880, 2022.
33. Wang L, Song Y, Wang H, Liu K, Shao Z and Shang Z: MIR-210-3p-EphrinA3-PI3K/AKT axis regulates the progression of oral cancer. *J Cell Mol Med* 24: 4011-4022, 2020.
34. Chatzikyriakou P, Brempou D, Quinn M, Fishbein L, Nobnerini R, Anastopoulos IN, Tufton N, Lim ES, Obholzer R, Hubbard JG, *et al*: A comprehensive characterisation of phaeochromocytoma and paraganglioma tumours through histone protein profiling, DNA methylation and transcriptomic analysis genome wide. *Clin Epigenetics* 15: 196, 2023.
35. Husain A, Chiu Y, Sze KM, Ho DW, Tsui Y, Suarez EMS, Zhang VX, Chan L, Lee E, Lee JM, *et al*: Ephrin-A3/EphA2 axis regulates cellular metabolic plasticity to enhance cancer stemness in hypoxic hepatocellular carcinoma. *J Hepatol* 77: 383-396, 2022.
36. Yiminniyaze R, Zhang X, Zhu N, Wang J, Li C, Wumaier G, Zhou D, Li J, Xia J, Zhang Y, *et al*: EphrinA3 is a key regulator of malignant behaviors and a potential prognostic factor in lung adenocarcinoma. *Cancer Med* 12: 1630-1642, 2023.
37. Li Y, Peng Q and Wang L: EphA2 as a phase separation protein associated with ferroptosis and immune cell infiltration in colorectal cancer. *Aging (Albany NY)* 15: 12952-12965, 2023.
38. Pei J, Zhang C, Yusupu M, Zhang C and Dai D: Screening and validation of the hypoxia-related signature of evaluating tumor immune microenvironment and predicting prognosis in gastric cancer. *Front Immunol* 12: 705511, 2021.
39. Deng M, Tong R, Zhang Z, Wang T, Liang C, Zhou X and Hou G: EFNA3 as a predictor of clinical prognosis and immune checkpoint therapy efficacy in patients with lung adenocarcinoma. *Cancer Cell Int* 21: 535, 2021.
40. Kiri S and Ryba T: Cancer, metastasis, and the epigenome. *Mol Cancer* 23: 154, 2024.
41. Zheng S, Cherniack AD, Dewal N, Moffitt RA, Danilova L, Murray BA, Lerario AM, Else T, Knijnenburg TA, Ciriello G, *et al*: Comprehensive pan-genomic characterization of adrenocortical carcinoma. *Cancer Cell* 29: 723-736, 2016.
42. Sun-Zhang A, Juhlin CC, Carling T, Scholl U, Schott M, Larsson C and Bajalica-Lagercrantz S: Comprehensive genomic analysis of adrenocortical carcinoma reveals genetic profiles associated with patient survival. *ESMO Open* 9: 103617, 2024.
43. Sun L, Zhang H and Gao P: Metabolic reprogramming and epigenetic modifications on the path to cancer. *Protein Cell* 13: 877-919, 2022.
44. Orsolic I, Carrier A and Esteller M: Genetic and epigenetic defects of the RNA modification machinery in cancer. *Trends Genet* 39: 74-88, 2023.
45. Clay MR, Pinto EM, Cline C, Tran QT, Lin T, Dyer MA, Shi L, Wu H, Pounds SB, Zambetti GP, *et al*: DNA methylation profiling reveals prognostically significant groups in pediatric adrenocortical tumors: A report from the international pediatric adrenocortical tumor registry. *JCO Precis Oncol* 3: PO.19.00163, 2019.
46. Mohan DR, Lerario AM, Else T, Mukherjee B, Almeida MQ, Vinco M, Rege J, Mariani BMP, Zerbinini MCN, Mendonca BB, *et al*: Targeted assessment of G0S2 methylation identifies a rapidly recurrent, routinely fatal molecular subtype of adrenocortical carcinoma. *Clin Cancer Res* 25: 3276-3288, 2019.
47. Li C, Tang Y, Li Q, Liu H, Ma X, He L and Shi H: The prognostic and immune significance of C15orf48 in pan-cancer and its relationship with proliferation and apoptosis of thyroid carcinoma. *Front Immunol* 14: 1131870, 2023.
48. Oliver J, Garcia-Aranda M, Chaves P, Alba E, Cobo-Dols M, Onieva JL and Barragan I: Emerging noninvasive methylation biomarkers of cancer prognosis and drug response prediction. *Semin Cancer Biol* 83: 584-595, 2022.
49. Suh I, Weng J, Fernandez-Ranvier G, Shen WT, Duh Q, Clark OH and Kebebew E: Antineoplastic effects of decitabine, an inhibitor of DNA promoter methylation, in adrenocortical carcinoma cells. *Arch Surg* 145: 226-232, 2010.
50. Endo A, Ly T, Pippa R, Bensaddek D, Nicolas A and Lamond AI: The chromatin assembly factor complex 1 (CAF1) and 5-Azacytidine (5-AzaC) affect cell motility in src-transformed human epithelial cells. *J Biol Chem* 292: 172-184, 2017.
51. Li X, Li Y, Dong L, Chang Y, Zhang X, Wang C, Chen M, Bo X, Chen H, Han W and Nie J: Decitabine priming increases anti-PD-1 antitumor efficacy by promoting CD8+ progenitor exhausted T cell expansion in tumor models. *J Clin Invest* 133: e165673, 2023.
52. Wang Y, Tong C, Dai H, Wu Z, Han X, Guo Y, Chen D, Wei J, Ti D, Liu Z, *et al*: Low-dose decitabine priming endows CAR T cells with enhanced and persistent antitumor potential via epigenetic reprogramming. *Nat Commun* 12: 409, 2021.

53. Sarhadi VK and Armengol G: Molecular biomarkers in cancer. *Biomolecules* 12: 1021, 2022.
54. Xu F, Guan Y, Ma Y, Xue L, Zhang P, Yang X and Chong T: Bioinformatic analyses and experimental validation of the role of m6A RNA methylation regulators in progression and prognosis of adrenocortical carcinoma. *Aging (Albany NY)* 13: 11919-11941, 2021.
55. Fu Y, Sun S, Bi J, Kong C and Yin L: Expression patterns and prognostic value of m6A RNA methylation regulators in adrenocortical carcinoma. *Medicine (Baltimore)* 100: e25031, 2021.
56. Zhang S, Zhao BS, Zhou A, Lin K, Zheng S, Lu Z, Chen Y, Sulman EP, Xie K, Bogler O, *et al*: m(6)A Demethylase ALKBH5 maintains tumorigenicity of glioblastoma stem-like cells by sustaining FOXM1 expression and cell proliferation program. *Cancer Cell* 31: 591-606, 2017.
57. Xiong J, He J, Zhu J, Pan J, Liao W, Ye H, Wang H, Song Y, Du Y, Cui B, *et al*: Lactylation-driven METTL3-mediated RNA m⁶A modification promotes immunosuppression of tumor-infiltrating myeloid cells. *Mol Cell* 82: 1660-1677, 2022.
58. Wang L, Dou X, Chen S, Yu X, Huang X, Zhang L, Chen Y, Wang J, Yang K, Bugno J, *et al*: YTHDF2 inhibition potentiates radiotherapy antitumor efficacy. *Cancer Cell* 41: 1294-1308, 2023.
59. Barbari C, Fontaine T, Parajuli P, Lamichhane N, Jakubski S, Lamichhane P and Deshmukh RR: Immunotherapies and combination strategies for immuno-oncology. *Int J Mol Sci* 21: 5009, 2020.
60. Rui R, Zhou L and He S: Cancer immunotherapies: Advances and bottlenecks. *Front Immunol* 14: 1212476, 2023.
61. Wu T and Dai Y: Tumor microenvironment and therapeutic response. *Cancer Lett* 387: 61-68, 2017.
62. Raj N, Zheng Y, Kelly V, Katz SS, Chou J, Do RKG, Capanu M, Zamarin D, Saltz LB, Ariyan CE, *et al*: PD-1 blockade in advanced adrenocortical carcinoma. *J Clin Oncol* 38: 71-80, 2020.
63. Habra MA, Stephen B, Campbell M, Hess K, Tapia C, Xu M, Ahnert JR, Jimenez C, Lee JE, Perrier ND, *et al*: Phase II clinical trial of pembrolizumab efficacy and safety in advanced adrenocortical carcinoma. *J Immunother Cancer* 7: 253, 2019.
64. Fassnacht M, Puglisi S, Kimpel O and Terzolo M: Adrenocortical carcinoma: A practical guide for clinicians. *Lancet Diabetes Endo* 13: 438-452, 2025.
65. Remde H, Schmidt-Pennington L, Reuter M, Landwehr L, Jensen M, Lahner H, Kimpel O, Altieri B, Laubner K, Schreiner J, *et al*: Outcome of immunotherapy in adrenocortical carcinoma: A retrospective cohort study. *Eur J Endocrinol* 188: 485-493, 2024.
66. Khalafizadeh A, Hashemizadegan SD, Shokri F, Bakhshinejad B, Jabbari K, Motavaf M and Babashah S: Competitive endogenous RNA networks: Decoding the role of long non-coding RNAs and circular RNAs in colorectal cancer chemoresistance. *J Cell Mol Med* 28: e18197, 2024.
67. Wang L, Song Y, Wang H, Liu K, Shao Z and Shang Z: MiR-210-3p-EphrinA3-PI3K/AKT axis regulates the progression of oral cancer. *J Cell Mol Med* 24: 4011-4022, 2020.
68. Wang Z, Yin B, Wang B, Ma Z, Liu W and Lv G: MicroRNA-210 promotes proliferation and invasion of peripheral nerve sheath tumor cells targeting EFNA3. *Oncol Res* 21: 145-154, 2013.
69. Gomez-Maldonado L, Tiana M, Roche O, Prado-Cabrero A, Jensen L, Fernandez-Barral A, Guijarro-Munoz I, Favaro E, Moreno-Bueno G, Sanz L, *et al*: EFNA3 long noncoding RNAs induced by hypoxia promote metastatic dissemination. *Oncogene* 34: 2609-2620, 2015.
70. Long B, Yang X, Xu X, Li X, Xu X, Zhang X and Zhang S: Long noncoding RNA ASB16-AS1 inhibits adrenocortical carcinoma cell growth by promoting ubiquitination of RNA-binding protein HuR. *Cell Death Dis* 11: 995, 2020.
71. Li S, Monazzam A, Razmara M, Chu X, Stalberg P and Skogseid B: MiR-486-3p was downregulated at microRNA profiling of adrenals of multiple endocrine neoplasia type I mice, and inhibited human adrenocortical carcinoma cell lines. *Sci Rep* 11: 14772, 2021.
72. Subramanian C, McNamara K, Croslow SW, Tan Y, Hess D, Kiseljak-Vassiliades K, Wierman ME, Sweedler JV and Cohen MS: Novel repurposing of sulfasalazine for the treatment of adrenocortical carcinomas, probably through the SLC7A11/xCT-hsa-miR-92a-3p-OIP5-AS1 network pathway. *Surgery* 177: 108832, 2025.
73. Gouirand V, Gicquel T, Lien EC, Jaune-Pons E, Da Costa Q, Finetti P, Metay E, Duluc C, Mayers JR, Audebert S, *et al*: Ketogenic HMG-CoA lyase and its product beta-hydroxybutyrate promote pancreatic cancer progression. *EMBO J* 41: e110466, 2022.
74. Yarmolinsky J, Bull CJ, Vincent EE, Robinson J, Walther A, Smith GD, Lewis SJ, Relton CL and Martin RM: Association between genetically proxied inhibition of HMG-CoA reductase and epithelial ovarian cancer. *JAMA* 323: 646-655, 2020.
75. Jiang W, Hu J, He X, Jin W and He X: Statins: A repurposed drug to fight cancer. *J Exp Clin Cancer Res* 40: 241, 2021.
76. Dorsch M, Kowalczyk M, Planque M, Heilmann G, Urban S, Dujardin P, Forster J, Ueffing K, Nothdurft S, Oeck S, *et al*: Statins affect cancer cell plasticity with distinct consequences for tumor progression and metastasis. *Cell Rep* 37: 110056, 2021.
77. Feng J, Dai W, Mao Y, Wu L, Li J, Chen K, Yu Q, Kong R, Li S, Zhang J, *et al*: Simvastatin re-sensitizes hepatocellular carcinoma cells to sorafenib by inhibiting HIF-1alpha/PPAR-gamma/PKM2-mediated glycolysis. *J Exp Clin Cancer Res* 39: 24, 2020.
78. Yao X, Xie R, Cao Y, Tang J, Men Y, Peng H and Yang W: Simvastatin induced ferroptosis for triple-negative breast cancer therapy. *J Nanobiotechnol* 19: 311, 2021.
79. Ma W, Wei S, Li Q, Zeng J, Xiao W, Zhou C, Yoneda KY, Zeki AA and Li T: Simvastatin overcomes resistance to tyrosine kinase inhibitors in patient-derived, oncogene-driven lung adenocarcinoma models. *Mol Cancer Ther* 23: 700-710, 2024.
80. Xie W, Peng M, Liu Y, Zhang B, Yi L and Long Y: Simvastatin induces pyroptosis via ROS/caspase-1/GSDMD pathway in colon cancer. *Cell Commun Signal* 21: 329, 2023.
81. Budillon A, Leone A, Passaro E, Silvestro L, Foschini F, Iannelli F, Roca MS, Macchini M, Bruzzese F, Bermejo ML, *et al*: Randomized phase 2 study of valproic acid combined with simvastatin and gemcitabine/nab-paclitaxel-based regimens in untreated metastatic pancreatic adenocarcinoma patients: The VESPA trial study protocol. *BMC Cancer* 24: 1167, 2024.
82. Jing Z, Yuan W, Wang J, Ni R, Qin Y, Mao Z, Wei F, Song C, Zheng Y, Cai H and Liu Z: Simvastatin/hydrogel-loaded 3D-printed titanium alloy scaffolds suppress osteosarcoma via TF/NOX2-associated ferroptosis while repairing bone defects. *Bioact Mater* 33: 223-241, 2024.
83. Lee YG, Chou F, Tung S, Chou H, Ko T, Fann YC and Juan S: Tumoricidal activity of simvastatin in synergy with rhoa inactivation in antimigration of clear cell renal cell carcinoma cells. *Int J Mol Sci* 24: 9738, 2023.
84. Okubo K, Miyai K, Kato K, Asano T and Sato A: Simvastatin-romidepsin combination kills bladder cancer cells synergistically. *Transl Oncol* 14: 101154, 2021.
85. Fuentes-Fayos AC, G-Garcia ME, Perez-Gomez JM, Montero-Hidalgo AJ, Martin-Colom J, Doval-Rosa C, Blanco-Acevedo C, Torres E, Toledano-Delgado A, Sanchez-Sanchez R, *et al*: Metformin and simvastatin exert additive antitumor effects in glioblastoma via senescence-state: Clinical and translational evidence. *Ebiomedicine* 90: 104484, 2023.
86. Dong G, Huang X, Jiang S, Ni L and Chen S: Simvastatin mitigates apoptosis and transforming growth factor-beta upregulation in stretch-induced endothelial cells. *Oxid Med Cell Longev* 2019: 6026051, 2019.
87. Wang S, Ho HJ, Lin J, Shieh J and Wu C: Simvastatin-induced cell cycle arrest through inhibition of STAT3/SKP2 axis and activation of AMPK to promote p27 and p21 accumulation in hepatocellular carcinoma cells. *Cell Death Dis* 8: e2626, 2017.
88. Stine JE, Guo H, Sheng X, Han X, Schointuch MN, Gilliam TP, Gehrig PA, Zhou C and Bae-Jump VL: The HMG-CoA reductase inhibitor, simvastatin, exhibits anti-metastatic and anti-tumorigenic effects in ovarian cancer. *Oncotarget* 7: 946-960, 2016.
89. Afshordel S, Kern B, Clasohm J, Konig H, Priester M, Weissenberger J, Kogel D and Eckert GP: Lovastatin and perillyl alcohol inhibit glioma cell invasion, migration, and proliferation-impact of Ras-/Rho-prenylation. *Pharmacol Res* 91: 69-77, 2015.
90. Conde J, Fernandez-Pisonero I, Lorenzo-Martin LF, Garcia-Gomez R, Casar B, Crespo P and Bustelo XR: The mevalonate pathway contributes to breast primary tumorigenesis and lung metastasis. *Mol Oncol* 19: 56-80, 2025.
91. Tai Y and Shang J: Wnt/ β -catenin signaling pathway in the tumor progression of adrenocortical carcinoma. *Front Endocrinol (Lausanne)* 14: 1260701, 2023.
92. Rubin B, Pilon C, Pezzani R, Rebollato A and Fallo F: The effects of mitotane and 1 α ,25-dihydroxyvitamin D₃ on Wnt/ β -catenin signaling in human adrenocortical carcinoma cells. *J Endocrinol Invest* 43: 357-367, 2020.
93. Batlle E, Henderson JT, Beghtel H, van den Born MMW, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, *et al*: Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 111: 251-263, 2002.

94. Zhang C, Liu L, Li W, Li M, Zhang X, Zhang C, Yang H, Xie J, Pan W, Guo X, *et al*: Upregulation of FAM83F by c-Myc promotes cervical cancer growth and aerobic glycolysis via Wnt/beta-catenin signaling activation. *Cell Death Dis* 14: 837, 2023.
95. Yang H, Shen J, Wang Y, Liu Y, Shen D and Quan S: Tankyrase promotes aerobic glycolysis and proliferation of ovarian cancer through activation of Wnt/ β -Catenin Signaling. *Biomed Res Int* 2019: 2686340, 2019.
96. Sprowl-Tanio S, Habowski AN, Pate KT, McQuade MM, Wang K, Edwards RA, Grun F, Lyou Y and Waterman ML: Lactate/pyruvate transporter MCT-1 is a direct Wnt target that confers sensitivity to 3-bromopyruvate in colon cancer. *Cancer Metab* 4: 20, 2016.
97. Du Y, Jiang Y, Hou Y and Shi Y: Complement factor I knockdown inhibits colon cancer development by affecting Wnt/beta-catenin/c-Myc signaling pathway and glycolysis. *World J Gastrointest Oncol* 16: 2646-2662, 2024.
98. Vergara D, Stanca E, Guerra F, Priore P, Gaballo A, Franck J, Simeone P, Trerotola M, De Domenico S, Fournier I, *et al*: beta-catenin knockdown affects mitochondrial biogenesis and lipid metabolism in breast cancer cells. *Front Physiol* 8: 544, 2017.
99. Halma MTJ, Tuszynski JA and Marik PE: Cancer metabolism as a therapeutic target and review of interventions. *Nutrients* 15: 544, 2023.
100. Sun W, Jia M, Feng Y and Cheng X: Lactate is a bridge linking glycolysis and autophagy through lactylation. *Autophagy* 19: 3240-3241, 2023.
101. Williams JL, Smith C, Hall C, Khaled Z, Maharaj A, Kwong R, Pittaway J, Casas J, Parvanta L, Abdel-Aziz TE, *et al*: Elevated sphingosine-1-phosphate lyase leads to increased metabolism and reduced survival in adrenocortical carcinoma. *Eur J Endocrinol* 188: Ivac007, 2023.
102. Krishnamurthy N and Kurzrock R: Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. *Cancer Treat Rev* 62: 50-60, 2018.



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