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Comprehensive pan-cancer analysis reveals ENC1 as a promising prognostic biomarker for tumor microenvironment and therapeutic responses

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Accumulating research showed that ENC1 plays a critical role in maintaining the physiological functions. However, little is known about its role in predicting prognosis and immunotherapy response across cancers. In our results, compared to normal tissues, most cancer tissues exhibit increased ENC1 expression. We found that the most common type of genetic variation was gene mutation. In addition, a positive correlation was found between CNV and ENC1 expression. Moreover, the overexpression of ENC1 was positively correlated with poor clinical outcomes. The GSEA results showed that ENC1 is closely correlated with tumor-promoting biological functions in most cancers. ENC1 is also closely negatively associated with the infiltration levels of T cells, activated NK cells, and B cells. Most immunomodulators are positively associated with ENC1. Further, we verified that inhibition of ENC1 expression suppressed the proliferation and migration of breast cancer, pancreatic cancer and glioma cells. In conclusion, our study demonstrated that ENC1 plays a protumorigenic role in most cancers. Additionally, ENC1 is closely correlated with tumor microenvironment features and immune checkpoint inhibitors expression. Overall, ENC1 could serve as a promising potential prognostic biomarker in various tumors.

Keywords ENC1, Immunotherapy, Pan-cancer, Immunomodulators, Tumor immune infiltration, Prognostic biomarker

In recent years, cancer has gained prominence as a leading cause of death[1,2]. Often, cancer patients with advanced stages of the disease have a better quality of life and an increased chance of survival because new diagnostic and treatment methods have emerged over the past few decades[3,4]. In particular, immune checkpoint inhibitors (ICIs) has advanced significantly in treating malignant tumors[5,6]. However, a significant part of cancer patients still poorly response to ICI therapy. Considering the potential mechanisms of immunotherapy, the tumor immune microenvironment (TIME) is significant for the therapeutic response to ICIs. For example, in "hot" tumors, the massive T cell infiltration leads to the effectiveness of ICIs therapy, while the "cold" tumors do the opposite[7]. Within the TME, all immune components are collectively defined as TIME due to their unique internal interactions and essential roles in tumor biology, which contains innate immune cells, adaptive immune cells, extracellular immune factors, and cell surface molecules[8]. What's more, the composition of immune cells in TIME also affects the prognosis of malignant tumors. For example, triple-negative breast cancer (TNBC) with less T cell infiltration generally has worse prognosis than those with more T cell inflamed[9]. But, there is still a lack of suitable prognostic and therapeutic markers, so appropriate therapeutic modalities strategies for predicting clinical outcomes are urgently needed, and this can be accomplished by identifying suitable biomarkers[10]. Pan-cancer analysis is based on the mining of public databases to analyze differential gene expression, gene characteristics and immunological associations in most types of tumors to provide valuable diagnostic, prognostic and immunotherapeutic information[11].

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Ectodermal neural cortex 1 (ENC1), a member of the Kelch-related family of actin-binding proteins[12], has been demonstrated to be overexpressed in several cancers, such as medulloblastoma[13], endometrial cancer[14], lung cancer[15], glioblastomas, ovarian cancer[16], and colorectal carcinomas[17]. Thus, it may play an oncogenic role in those cancers. Moreover, previous research has proven that ENC1 is significantly upregulated in breast cancer, and its expression is related to sensitivity to radiation therapy[18]. Additionally, ENC1 was recently emphasized as a metastasis-related biomarker, suitable prognostic marker, and attractive therapeutic target in breast cancer[19]. A recent report suggested that upregulation of ENC1 may not only contribute to colorectal cancer progression and metastasis but also to the immune system through activation of the JAK-STAT pathway[20].

In human cancers, the molecular properties of ENC1 have not yet been fully described. Therefore, in our project, we explored the differential expression of ENC1 in various types of cancer according to the TCGA database and further explored its prognostic roles in 33 types of cancer. Additionally, various potential biological functions and genetic characteristics of ENC1 among cancers were analyzed via some bioinformatics websites.

Materials and methods

Data collection and processing

RNA-seq data from the TCGA and GTEx databases were collected for the analysis. Abbreviations and corresponding full names of the tumors involved in the study can be found in the Table 1. An analysis of the Gene Expression Profiling Interactive Analysis (GEPIA) database[21] was applied to explore the association between tumor stage and ENC1 expression using the function "Stage plot".

Analysis of copy number variation (CNV) and methylation

The cBioPortal platform was utilized to assess the CNV and mutation frequency of ENC1 across the TCGA database. A correlation analysis was conducted using the Gene Set Cancer Analysis (GSCA) database[22] to analyze ENC1 expression levels in relation to its mutation and CNV statuses. Additionally, the relationships between ENC1 gene alterations and prognosis across cancers were analyzed by GSCA. Comparison of the promoter methylation levels of ENC1 between different cancers and corresponding normal tissues was evaluated in the TCGA pancancer dataset by using the UALCAN website[23].

Diagnostic and prognostic analysis

A survival analysis was performed using the R packages "survival" and "survminer" and the Kaplan–Meier method (univariate Cox regression and Kaplan–Meier curve) to determine the prognosis for ENC1 across cancers based on the TCGA database. The clinical prognostic value of ENC1 was assessed based on overall survival (OS), progression-free interval (PFI), and disease-specific survival (DSS). The diagnostic performance of ENC1 across cancers was evaluated by analyzing the receiver operating characteristic (ROC) curve using R software (version 4.0.3). The ROC curves were visualized using the R packages "ggplot2" and "pROC".

Functional analysis of ENC1 across cancers

The potential biological pathways in which ENC1 might be involved were examined by using gene set enrichment analysis (GSEA). The CancerSEA database[24], which provides information on 14 functional states in 25 types of cancer, was employed to assess the correlation between ENC1 expression at the single-cell level and functional states among different types of cancers.

Analyses of immune infiltration

The relative scores of 24 immune cells in pancancer tissues were determined by using CIBERSORT (https://cibersort.stanford.edu/), a metagene analysis tool that can predict immunocyte phenotypes. Moreover, the relationship between the expression of ENC1 and the level of each immune cell infiltration was evaluated by the R packages "ggplot2" and "ggpubr". xCell (https://xcell.ucsf.edu/)[25] was used to calculate the immune infiltration scores of all types of cancers from TCGA via the R package "Immunedeconv", and an analysis of the relationship between ENC1 expression and immune infiltration scores was conducted by using Spearman's correlation test. The ESTIMATE scores for different cancers in the pancancer analysis were calculated by the R package "ESTIMATE." Moreover, the correlation between TMB or MSI and ENC1 expression was assessed by Spearman correlation analysis. The R package "ggplot2 (version 3.3.3)" was applied to visualize the results. In addition, the association between ENC1 expression and the expression of immune checkpoints and immunomodulators (including genes that activate immunity, suppress immunity, bind chemokine receptors, and modulate MHC gene expression) was analyzed by using the R package "ggplot2 (version 3.3.3)".

Drug sensitivity analysis of ENC1

The immunotherapy value of ENC1 was examined via the TISMO websites[26]. Moreover, the OPEN TARGET platform was selected to determine the role of ENC1 in diseases and to help systematically identify drug targets and priorities. In addition, GSCALite, an integrated platform that contains gene expression profiles for 33 cancer types from the TCGA, drug sensitivity information, and immunogenomic gene set data based on GDSC and CTRP, was applied to evaluate the association between ENC1 expression and drug sensitivity by Spearman analysis.

Cell lines and culture

BT-549 (HTB-122), LN229 (CRL-2611) and PANC-1 (CRL-1469) cell lines, which were purchased from the ATCC, were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) adding with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin in 5% CO_2 at 37 °C.

Abbreviations	Full name
ACC	Adrenocortical carcinoma
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AST	Astrocytome
BLCA	Bladder urothelial carcinoma
BRCA	Breast invasive carcinoma
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	Cholangio carcinoma
COAD	Colon adenocarcinoma
COADREAD	Colon adenocarcinoma/rectum adenocarcinoma esophageal carcinoma
DLBC	Lymphoid neoplasm diffuse large B-cell lymphoma
ESCA	Esophageal carcinoma
GBM	Glioblastoma multiforme
GBMLGG	Glioma
HNSC	Head and neck squamous cell carcinoma
HGG	High grade glioma
KICH	Kidney chromophobe
KIRC	Kidney renal clear cell carcinoma
KIRP	Kidney renal papillary cell carcinoma
LAML	Acute myeloid leukemia
LGG	Brain lower grade glioma
LIHC	Liver hepatocellular carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MEL	Melanoma
MESO	Mesothelioma
NB	Neuroblastoma
NSCLC	Non-small cell lung cancer
ODG	Oligodendroglioma
OSCC	Oral squamous cell carcinoma
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PC	Pheochromocytoma
PCPG	Pheochromocytoma and paraganglioma
PRAD	Prostate adenocarcinoma
RB	Retinoblastoma
RCC	Renal cell carcinomas
READ	Rectum adenocarcinoma
SARC	Sarcoma
SKCM	Skin cutaneous melanoma
STAD	Stomach adenocarcinoma
STES	Stomach and esophageal carcinoma
TGCT	Testicular germ cell tumors
THCA	Thyroid carcinoma
THYM	Thymoma
UCEC	Uterine corpus endometrial carcinoma
UCS	Uterine carcinosarcoma
UVM	Uveal melanoma
WT	Wilms tumor

Table 1. Abbreviations and corresponding full names of the tumors involved in the study.

Cell transfection

BT-549, LN229 and PANC-1 cells were seeded in six-well plates at 1×10^5 cells/well. They were cultivated at 37 °C for 24 h in complete DMEM, and then the cells were transfected Lipofectamine 2000 (Invitrogen) with ENC1 siRNA or siRNA NC, designed and synthesized by Sangon Biotech (Shanghai, China). The sequences were as follows: control sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAG

AATT-3'; siRNA1: ENC1 sense: 5'-GUGAAGAGCUGGAGACAGATT-3', antisense: 5'-UCUGUCUCCAGCU CUUCACTT-3'; siRNA2: ENC1 sense: 5'- CAGAGAAAGAGUAAGGAAATT-3, antisense: 5'- UUUCCUUAC UCUUUCUCUGTT-3'), siRNA3: ENC1 sense: 5'- GCGAUUGGCUGCAAAGUGUTT-3', antisense: 5'- ACA CUUUGCAGCCAAUCGCTT-3'. These steps were performed on the basis of the manufacturer's instructions.

Proliferation assays

CCK-8 (Beyotime, China) assays were performed to assess viability at the indicated time points following seeding into plates and incubation (5×10^3 cells/well). Optical density was calculated at 450 nm after incubating for 2 h at 37 °C. BT-549, LN229 and PANC-1 cells were separately seeded into 96-well plates (10,000 cells/well). DNA-replicating cells were examined by an EdU detection kit (RiboBio, Guangzhou, China), which provides information on the fraction of proliferating cells. Accordingly, EdU incorporation was assessed according to the proportion of cells incorporating EdU relative to cells stained with Hoechst 33342.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from BT-549, LN229 and PANC-1 cells using SteadyPure Mag Tissue & Cells RNA extraction kit (Accurate Biology, AG21023, China). Then the Evo M-MLV Mix Kit with gDNA Clean for qPCR (Accurate Biology, AG11728, China) was applied to synthesize cDNA on the basis of the manufacturer's prompt. RT-PCR reactions were performed by SYBR Green Premix Pro Taq HS qPCR kit (Accurate Biology, AG11701, China) on the basis of the manufacturer's prompt. Using the comparative $2^{-\triangle\triangle CT}$ method, the objective mRNAs expression was calibrated to the expression of GAPDH.

Western blotting

Cell lysates were prepared using RIPA buffer supplemented with protease inhibitors. Protein concentrations were measured using a BCA assay kit (Protein concentrations were measured using a BCA assay kit (Abiowell, Changsha China). The total protein (40 μg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoridemembranes. Primary antibodies against ENC1 (15007-1-AP, 1:1000) and β-actin (66009-1-Ig, 1:50000) used for a western blot study. Densitometric analysis was conducted using ImageJ software.

Transwell assay

BT-549, LN229 and PANC-1 cells were separately assigned into 8 μm 24-well chambers (Corning, USA). 20,000 cells/well with DMEM free FBS was placed on the top chambers, and the DMEM with 10% FBS (700 μL) was filled with the bottom chambers. Cells were incubated for 24 h at 37 °C with 5% CO_2, fixed with 4% paraformaldehyde for 30 min, stained with 0.1% crystal violet for 30 min, and then counted under a microscope.

Statistical analysis

The survival analysis based on ENC1 expression was performed via a log-rank test. The relationship between ENC1 expression and TNM stage was analyzed by using Kruskal–Wallis's test. Moreover, the association between ENC1 expression and DNA methylation, immune cell infiltration, immune checkpoint expression, ESTIMATE score, immunomodulators, TMB, and MSI was evaluated by Spearman's rank correlation coefficient. One-way analysis of variance (ANOVA) was conducted to compare multiple groups in the experimental verification section. There was a statistically significant difference at $p < 0.05$.

Results

Result 1 expression of ENC1 in tumor and normal tissues

First, to detect ENC1 expression across cancers, we obtained RNA-seq data from the TCGA and GTEx databases. Our findings revealed that ENC1 expression was upregulated in most cancer types compared to normal tissues; the exceptions were KICH and KIRP, which exhibited decreased expression levels. However, no significant difference was observed in KIPAN and LIHC (Fig. 1A). According to TCGA database analysis with paired samples, the expression of ENC1 was increased in BRCA, CHOL, COAD, ESCA, HNSC, LUAD, PRAD, READ, STAD, THCA and UCEC tissues compared with normal tissues. The expression of ENC1 was decreased in KICH (Fig. 1B). Furthermore, we found that the expression of ENC1 was positively correlated with the pathological stages in ACC, BLCA, SKCM, LIHC, KICH, and PAAD via the GEPIA2.0 database (Fig. 1C–H). Our study demonstrated the overexpression of ENC1 in multiple types of tumors.

Result 2 genetic alteration analysis of ENC1

Since genetic alterations can affect gene expression[27], we explored the genetic alterations of ENC1 in human pan-cancer samples via the cBioPortal tool based on TCGA datasets. As shown in Fig. 2A, the highest alteration frequency of ENC1 (5.56% of 35 cases) was in CHOL, and the most common type of genetic variation was gene mutation. Additionally, the highest mutation frequency of ENC1 was found in UCEC (4.16%). Then, our results from the GSCA database suggested that ENC1 expression has a positive correlation with CNV in patients with LUAD, LUSC, HNSC, STAD, UCS, READ, OV, SKCM, BRCA, COAD, CESC, BLCA, KIRC, PRAD, and LGG (Fig. 2B), which suggests that CNV is one of the reasons for aberrant ENC1 expression, and the mechanisms need to be further explored. We then investigated the impact of CNVs in ENC1 on prognosis across cancers. As shown in Fig. S1B, in KIRP, UCEC, KICH, KIRC, LUAD, OV, LIHC, ACC, LAML, THCA, and MESO, the CNV of ENC1 was associated with OS. Research has shown that DNA methylation is a common epigenetic modification that can regulate gene expression (methylation and transcription are usually inversely correlated), which also has an important impact on the development of cancers[28]. Also, our results suggested that DNA methylation is one of the important factors affecting gene expression in various tumors. As shown in Fig. 2C

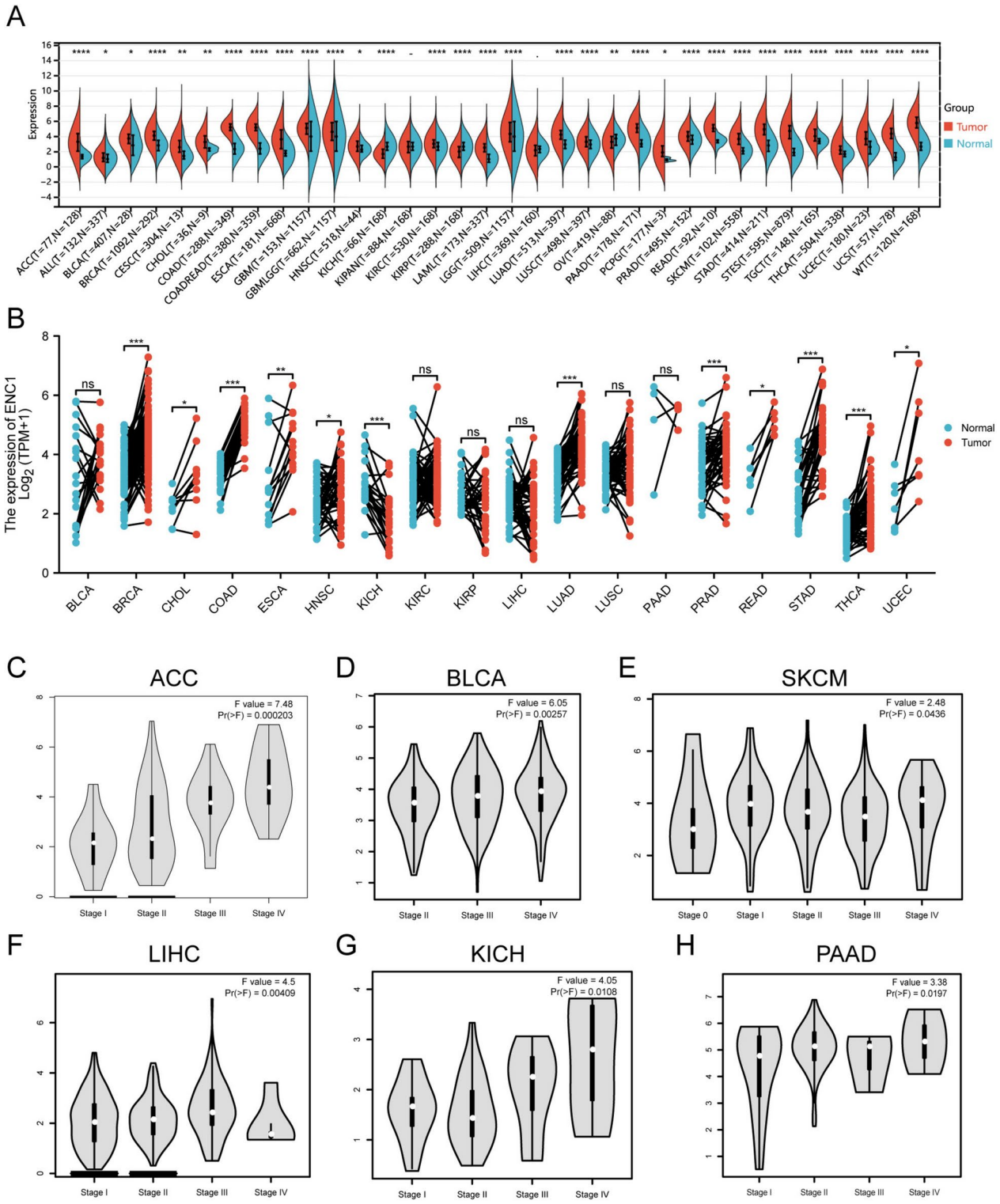

Fig. 1. The expression of ENC1 in normal tissues and cancer tissues. (**A**) ENC1 expression across cancers based on the TCGA and GTEx databases. (**B**) The expression of ENC1 in paired samples from the TCGA datasets. (**C**) The correlation between ENC1 expression and pathological stages in ACC, BLCA, SKCM, LIHC, KICH, and PAAD. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

(left), DNA methylation levels were different in LUSC, KIRP, THCA, KIRC, PRAD, PAAD, BLCA, BRCA, LUAD, UCEC, COAD, and LIHC. Moreover, the DNA methylation levels had a negative correlation with ENC1 mRNA expression in PCPG, LUSC, ESCA, UVM, SKCM, MESO, SARC, TGCT, ACC, KIRP, UCEC, CESC, THYM, LUAD, LIHC, BRCA, HNSC, PRAD, KIRC, and THCA (Fig. 2C (Right), Fig. S1). Then, we further investigated

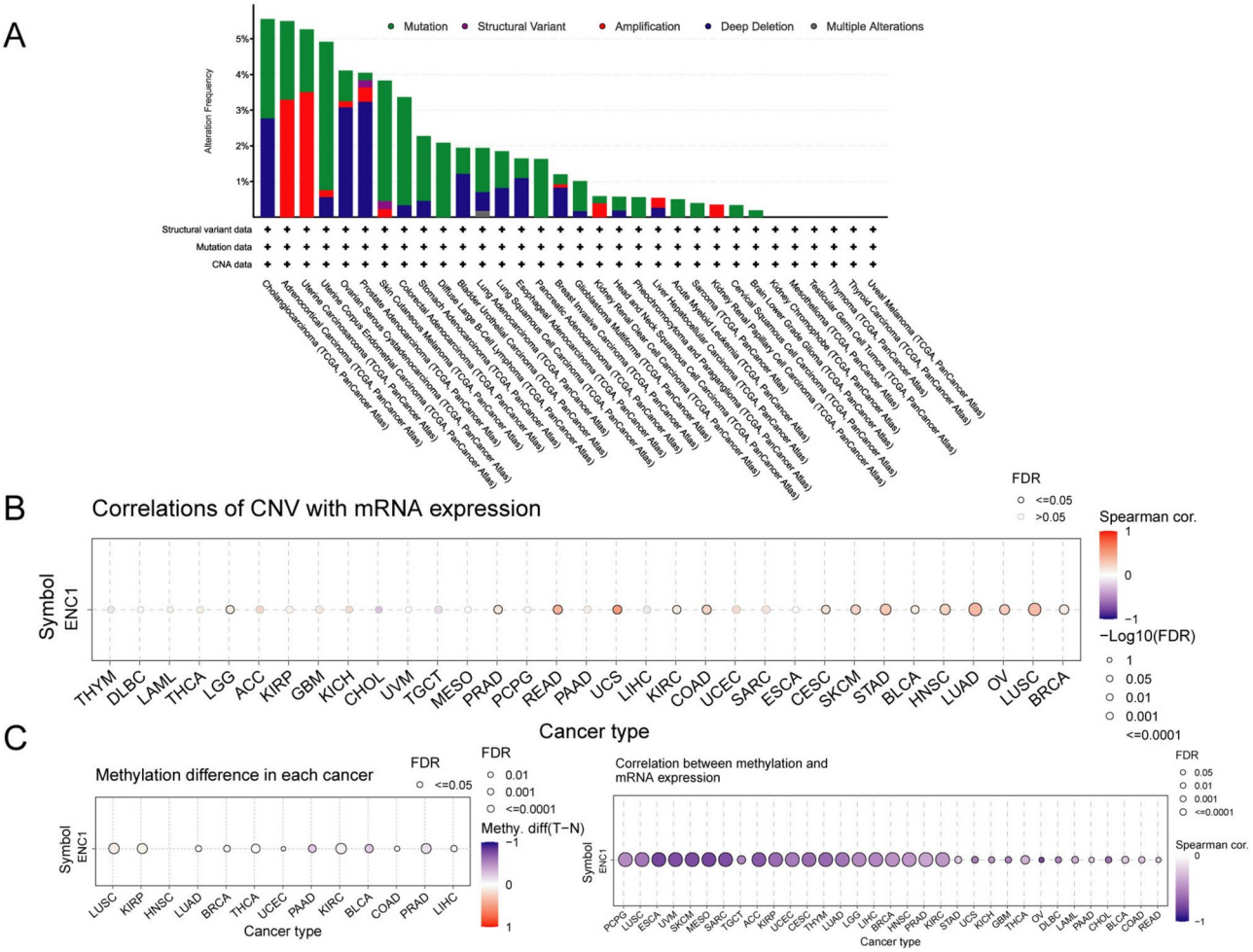

Fig. 2. Genetic alteration analysis of ENC1. (**A**) The alteration frequency of ENC1. (**B**) The correlation of CNV with ENC1 expression in 33 types of cancer. (**C**) The methylation difference in a subset of cancers (left). The correlation of methylation with ENC1 expression in 33 types of cancer.

the relationship between ENC1 expression and four methyltransferase genes, including DNMT1, TRDMT1, DNMT3A, and DNMT3B. Our results revealed a close association between them and ENC1 expression in PRAD, READ, SKCM, KIRC, KIRP, BRCA, MESO, LIHC, and ACC (Fig. 2D).

Result 3 the prognostic value of ENC1 in cancers

To further explore the potential prognostic value of ENC1 in different types of cancer, we evaluated the prognostic significance of ENC1 in a pancancer analysis through cox proportional hazards analysis. And the results showed that overexpression of ENC1 was significantly positively associated with a poor prognosis in terms of OS in ACC, KICH, GBML, KIPAN, MESO, PAAD, LGG, BRCA, LAML, DLBC, LUSC, and KIRP (Fig. 3A). The expression of ENC1 was associated with DSS in ACC, KICH, GBMLGG, LUSC, BRCA, DLBC, LIHC, PAAD, LGG, CESC, KIPAN, and MESO (Fig. 3B). The results for DFI were basically consistent with those for OS, suggesting that ENC1 upregulation is correlated with a poor clinical prognosis in ACC, KICH, CESC, LUSC, PAAD, GBMLGG, BRCA, KIPAN, DLBA, and LIHC (Fig. 3C). Based on Kaplan–Meier survival analysis, the upregulation of ENC1 was correlated with a worse OS in ACC, BRCA, CESC, DLBC, GBMLGG, HNSC, KICH, KIRP, LGG, LUSC, MESO, SARC, UCS, and UCEC (all $p < 0.05$, Fig. 4A–P). However, in KIRC and PCPG, low ENC1 expression was related to shorter OS (Fig. 4H, 4M). Furthermore, we then analyzed the diagnostic value of ENC1 across cancers by constructing ROC curves. Our study has demonstrated that ENC1 has a high diagnostic sensitivity with AUC > 0.8 in 14 types of cancer, including ACC, BRCA, CHOL, COAD, COADREAD, ESAD, ESCA, LAML, LUAD, PAAD, READ, STAD, UCEC, and UCS (Fig. S1). In brief, our research implied that there is a significant correlation between ENC1 expression and prognosis in many types of cancer, and high levels of ENC1 expression could indicate a worse outcome.

Result 4 biochemical functions of ENC1 correlated with ENC1 expression in multiple cancers

Moreover, to explore the biological processes associated with ENC1 expression, we investigated the correlation between ENC1 expression and the tumor-related biological functional status based on the CancerSEA database.

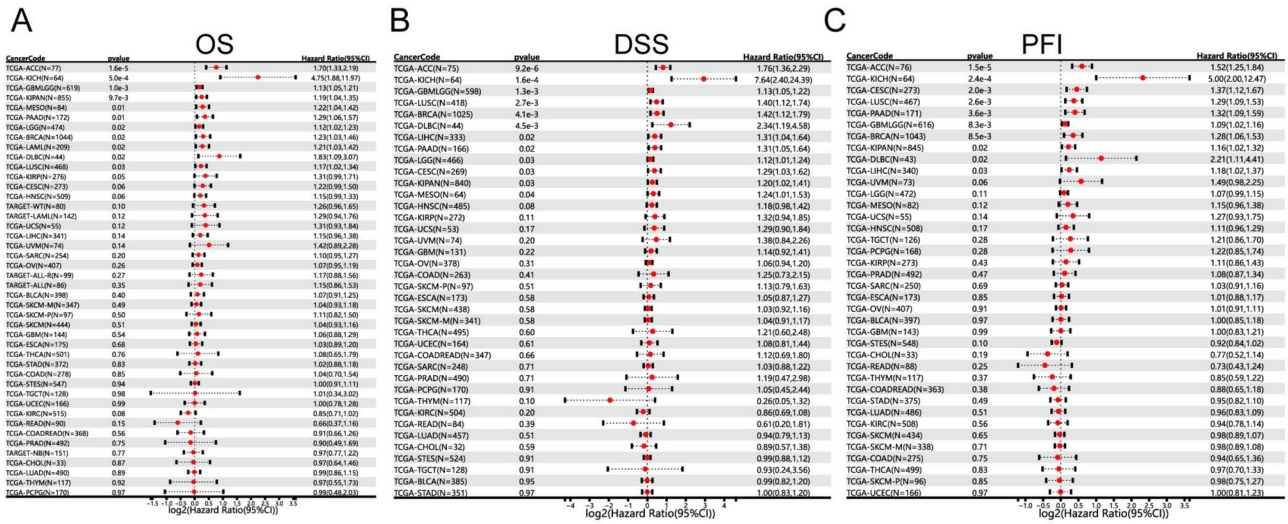

Fig. 3. The prognostic value of ENC1 across cancers. (**A**–**C**) Forest plot of the correlation of ENC1 expression with OS, DSS, and PFI in 33 types of cancer. OS (overall survival), DSS (disease-specific survival), PFI (progression-free interval).

Our results showed that ENC1 has a strong positive relationship with angiogenesis, stemness, quiescence, and differentiation (Fig. 5A). It is worth emphasizing that ENC1 expression is positively correlated with EMT, hypoxia, invasion, metastasis, proliferation, stemness, and quiescence but negatively correlated with apoptosis, inflammation and DNA repair in BRCA. Moreover, we explored signaling pathways related to ENC1 expression based on GSEA. This result indicated that ENC1 expression strongly correlated with epithelial-mesenchymal transition (EMT), KRAS signaling, TNFα signaling via NF-κB, angiogenesis, and IL6_JAK_STAT3 signaling in most types of cancers (Fig. 5B). As discussed above, our results suggest that ENC1 expression exhibit a positive correlation with numerous oncogenic signaling pathways in some cancers.

Result 5 correlation of ENC1 expression with tumor immune infiltration across cancers

The tumor immune microenvironment (TIME) is a crucial factor in the initiation and spread of cancer. Delving deeper into the study of TIME will unravel the intricacies of cancer progression and potentially uncover additional therapeutic targets for immunological interventions[29]. To further clarify the immunological features of ENC1 in the tumor microenvironment, we explored the association between ENC1 expression and infiltrated immune inflammatory cells across cancers by using CIBERSORT (Fig. 6A) and the xCell algorithm (Fig. 6B). We found that ENC1 expression was closely linked to immune cell infiltration in most tumors, as shown in the immune heatmap. High ENC1 expression was associated with decreased infiltration of T cells, activated NK cells, and B cells in BRCA, CESC, HNSC, LAML, LUAD, LUSC, TGCT, THCA, THYM, UCEC, and UCS ($P < 0.05$), while it was associated with increased infiltration of most immune cells in DLBC (Fig. 6B). Therefore, the above results indicated that the expression of ENC1 may be inversely correlated with immune infiltration in BRCA, CESC, HNSC, LAML, LUAD, LUSC, TGCT, THCA, THYM, UCEC, and UCS, implying a role in suppressing tumor immunity within these tumors.

Result 6 ENC1 expression and immune response across cancers

As previously mentioned, the therapeutic efficacy of ICIs is significantly influenced by the TIME. Next, we further investigated the relationship between ENC1 expression and immunomodulators, including immunostimulators, immunoinhibitors, chemokine ligands, chemokine receptors and MHC genes by a pancancer gene coexpression analysis, based on public databases (Fig. 7A-B). The results revealed that almost all immune-related genes were coexpressed with ENC1, and the majority of immunomodulators were positively associated with ENC1 expression ($p < 0.05$), but these results differed in ACC and MESO. Previous studies indicate that patients with high TMB are more likely to benefit from ICI therapy than those with low TMB[30], and MSI can also assist in predicting the efficacy of ICIs. Then, we explored the association between ENC1 expression and TMB or MSI across cancers by Spearman correlation analysis (Fig. 7C-D). Our study demonstrated that the expression of ENC1 was positively associated with TMB in 7 types of cancer, including GBMLGG, LAML, ESCA, STES, THYM, READ, and ACC. However, ENC1 expression was negatively associated with TMB in 7 types of cancer, including LUAD, KIRP, HNSC, LUSC, LIHC, THCA, and UVM. Additionally, we found that ENC1 expression was closely related to MSI in 6 types of cancer; the correlation was positive in BRCA, KIPAN, HNSC, THCA, and DLBC and negative only in LAML. In summery, our resluts showed that ENC1 were closely associated with common immune checkpoints, TMB and MSI in many cancers.

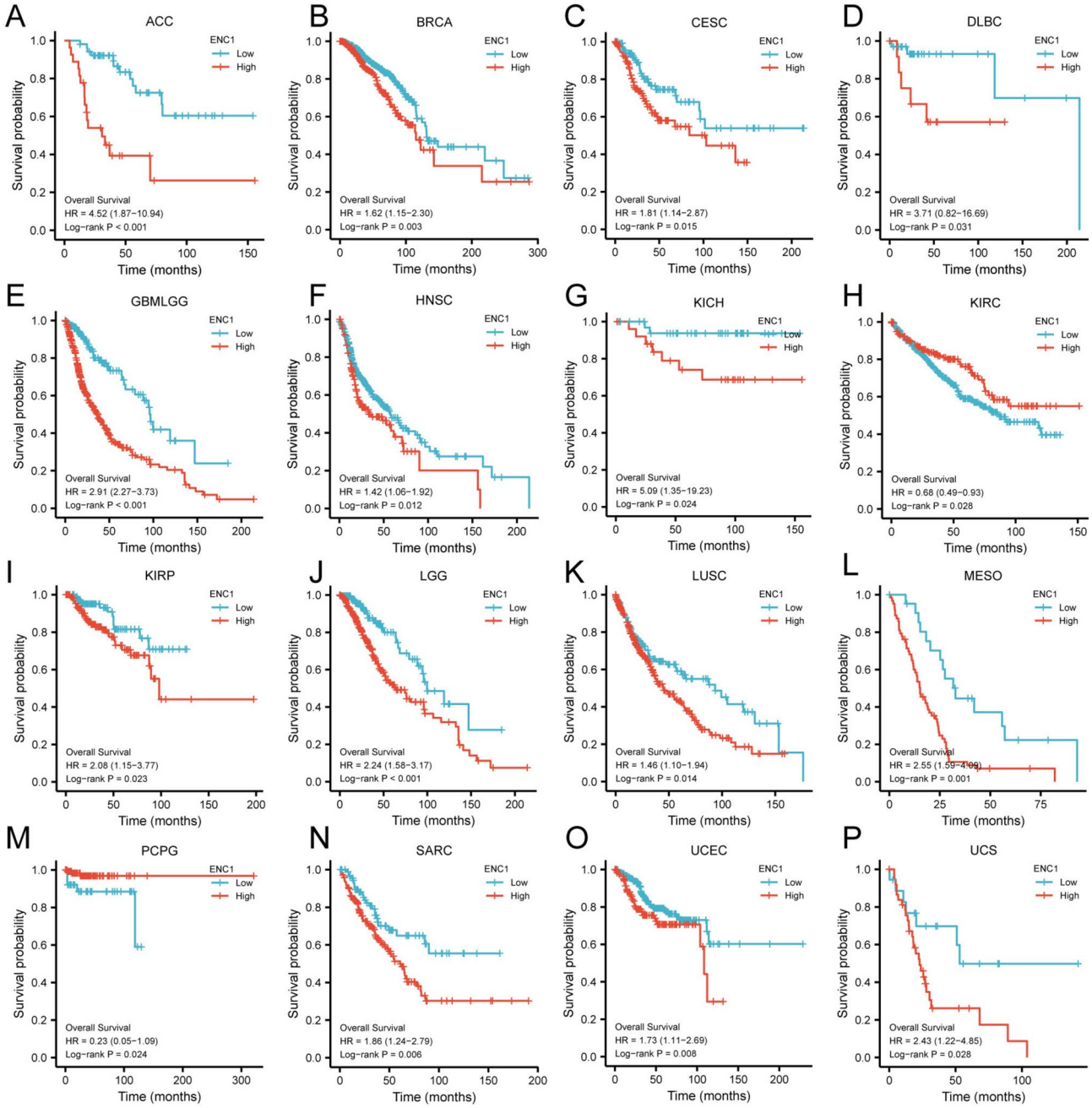

Fig. 4. Kaplan–Meier analysis of the correlation between ENC1 expression and OS. (**A**–**P**) Survival and prognosis analysis of ENC1 in different cancers.

Result 7 value of ENC1 in predicting the response to immunotherapy across cancers

In addition, according to public databases, we examined the potential value of ENC1 in predicting the immunotherapy response ENC1 across cancers. Interestingly, based on studies of tumor models in 10 murine immunotherapy cohorts (Fig. 8A) and cell lines in 16 cytokine treatment cohorts (Fig. 8B), ENC1 serves as a powerful predictor of immunotherapy response. Therefore, ENC1 can significantly predict the effectiveness of immunotherapy, highlighting its potential as a valuable tool for enhancing cancer treatment outcomes. Afterward, we analyzed public databases to predict effective drugs and small molecules according to ENC1 expression. We conducted Spearman correlation analysis to evaluate the correlation between the expression of ENC1 in the gene set and the sensitivity to small molecules/drugs (IC50). According to website prompts, a positive correlation means that high ENC1 expression indicates resistance to the small molecule/drug, while a negative correlation means that high ENC1 expression indicates sensitivity to the small molecule/drug. As shown in Fig. 8C, we found that the IC50s of many drugs were positively correlated with ENC1 expression according to the GDSC database. Methotrexate, FK866, and Vorinostat were the top three drugs. In addition,

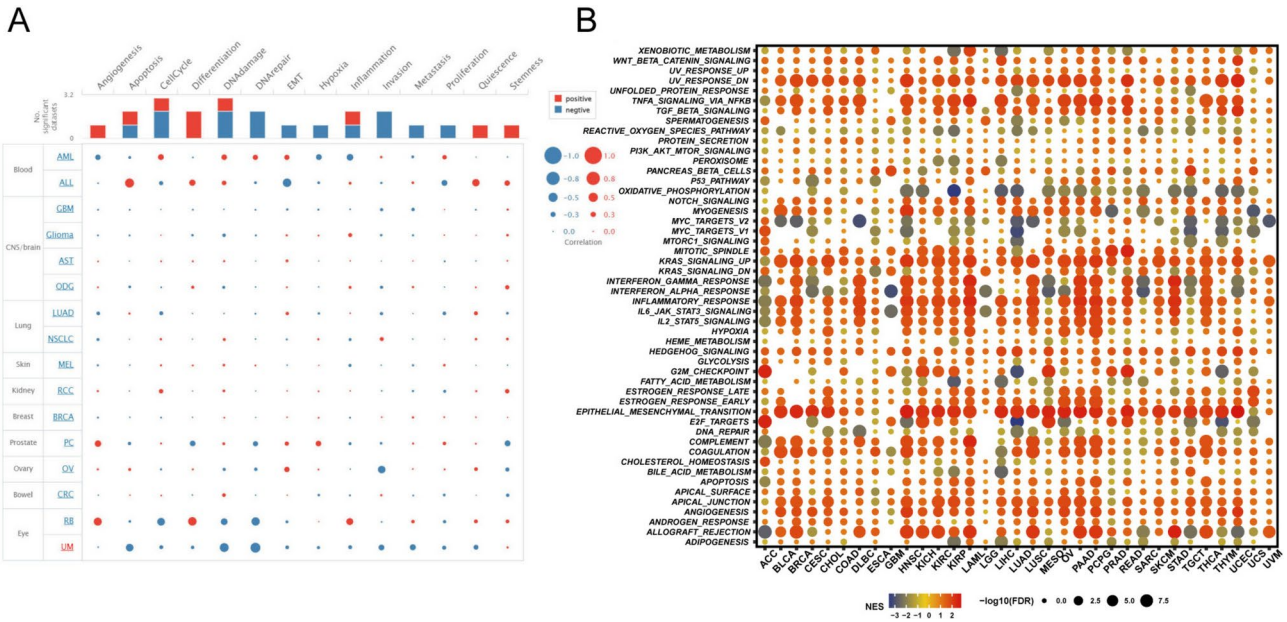

Fig. 5. The expression of ENC1 correlated with biological functional status across cancers. (**A**) The association of ENC1 expression with 14 functional states in 25 types of cancer in CancerSEA. (**B**) Correlation analysis between the 50 hallmark pathways and ENC1 expression across TCGA cancers.

many drugs had IC50s that were positively correlated with ENC1 expression according to the CTRP database. BRD-K30748066, teniposide, and GSK461364 were the top three drugs (Fig. 8D).

Result 8 effect of ENC1 on proliferation and migration

In view of the above bioinformatics analysis, we found that there was an upregulation of ENC1 in multiple types of cancer, including breast cancer, pancreatic cancer and glioma. Also, the increased ENC1 expression was poor prognostic in these cancers. We then further verified the biological function of ENC1 in breast cancer, pancreatic cancer and glioma cells. The results demonstrated that the significantly reduction of cell proliferation in BT-549, LN229 and PANC-1 after ENC1 knockdown by CCK-8 after confirmed the knockdown efficiency of ENC1 in the si-RNA-treated group (Fig. 9A–F). Also, the effects of ENC1 on cell proliferation and migration of BT-549, LN229 and PANC-1 cells were respectively analyzed by EdU and transwell assays (Fig. 9G–J). As the results showed that the ratio of proliferative and migrated cells significantly reduced after ENC1 knockdown. To sum up, suppression of ENC1 expression restrains the proliferation and migration of breast cancer, pancreatic cancer and glioma cells, which implied that ENC1 is closely correlated with the progression of breast cancer, pancreatic cancer and glioma.

Discussion

Research has reported that ENC1 is highly expressed in the neuroectodermal region of the ectoderm during gastrulation and is subsequently expressed in the nervous system[31]. Additionally, ENC1 expression is upregulated in breast carcinoma[19], ovarian cancer[16], lung cancer[15], colorectal carcinoma[32], and human brain cancers, such as glioblastomas and astrocytomas. Our results were consistent with those of previous studies, which indicated that ENC1 may play a potential procarcinogenic role if overexpressed in multiple types of cancers.

Genetic variations, including CNVs and SNVs, perform a very important function in tumorigenesis and tumor progression[33]. To date, few studies have reported the correlation between ENC1 gene variations and cancers. Thus, we explored the genetic characteristics of ENC1 across cancers by bioinformatics analysis. Our results suggested that the most common type of genetic variation was gene mutation, and ENC1 expression had a positive correlation with CNV in multiple types of cancers. Additionally, we found that ENC1 mutations were closely correlated with prognosis in multiple types of cancer. Additionally, it is well known that DNA promoter methylation causes epigenetic changes that increase cancer vulnerability and promote advancement by regulating chromatin structure, transcription, and cotranscriptional RNA processing[34–36]. Our results suggested that the level of DNA methylation is negatively associated with mRNA expression in LUAD, CESC, BLCA, COAD, READ, GBM, PCPG, LUSC, KIRC, BRCA, LIHC, PAAD, and TGCT, with only a few tumor types with positive correlations.

Moreover, we found that ENC1 has outstanding predictive and diagnostic value in most types of tumors, such as ACC, KICH, GBML, KIPAN, MESO, PAAD, LGG, BRCA, LAML, DLBC, LUSC, and KIRP. The AUC of the ROC curve confirmed the diagnostic value of ENC1 in the diagnosis of most cancers. Evidence has also been found in a previous study: high ENC1 expression could predict a worse prognosis among patients with ovarian cancer[16], and upregulated ENC1 expression is a potential diagnostic marker correlated with a poor prognosis in breast cancer[19]. Additionally, it has been proven that high expression of ENC1 predicts unfavorable

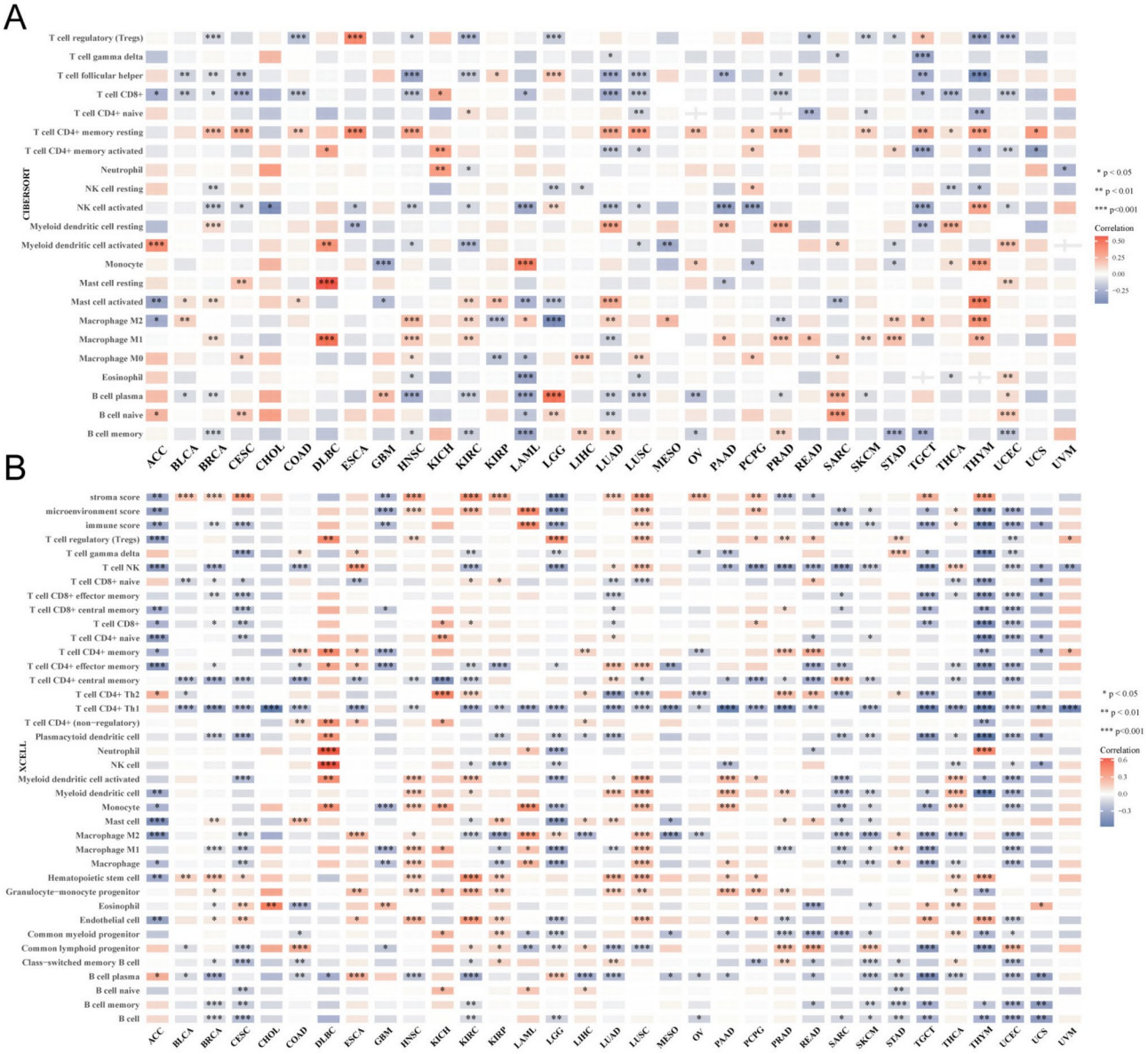

Fig. 6. Correlation of ENC1 with tumor immune infiltration across cancers. Immune cell infiltration analyzed by CIBERSORT (**A**) and xCell (**B**).

clinical outcomes in patients with READ[20]. Therefore, these results confirmed that ENC1 has the potential to be a prognostic biomarker in multiple cancers.

In addition, we investigated the biological function of ENC1 across cancers. We found that ENC1 expression was positively related to EMT, invasion, metastasis, proliferation, stemness, and quiescence. This was consistent with the results of published studies, which showed that ENC1 induced EMT and stemness via JAK2/STAT5/AKT signaling in READ[20]. Some studies have also proposed that knockdown of ENC1 suppresses the growth, colony formation, migration and invasion of breast cancer cells by regulating the β-catenin pathway[19]. Therefore, ENC1 plays a critical role in the progression of cancers, possibly by promoting EMT, invasion, and stemness and preventing apoptosis.

Furthermore, we investigated the correlation between ENC1 expression and the tumor microenvironment (TME) across cancers; the TME is the ecosystem surrounding the tumor that includes immune cells, stromal cells, the extracellular matrix, and blood vessels[37,38]. The TME plays an important role in tumor development, immunological therapy response, immune escape, and clinical outcome[39–42]. Our results suggested that ENC1 expression is negatively correlated with immune cell infiltration in most types of tumors. High expression of ENC1 was correlated with decreased levels of T cells, activated NK cells, and B cells in BRCA, CESC, HNSC, LAML, LUAD, LUSC, TGCT, THCA, THYM, UCEC, and UCS. A previous study showed that ENC1 expression was positively correlated with the levels of CD8+ T cells and neutrophils but negatively associated with those of CD4+ T cells and B cells in UCEC[14]. ENC1 expression was also found to be negatively correlated

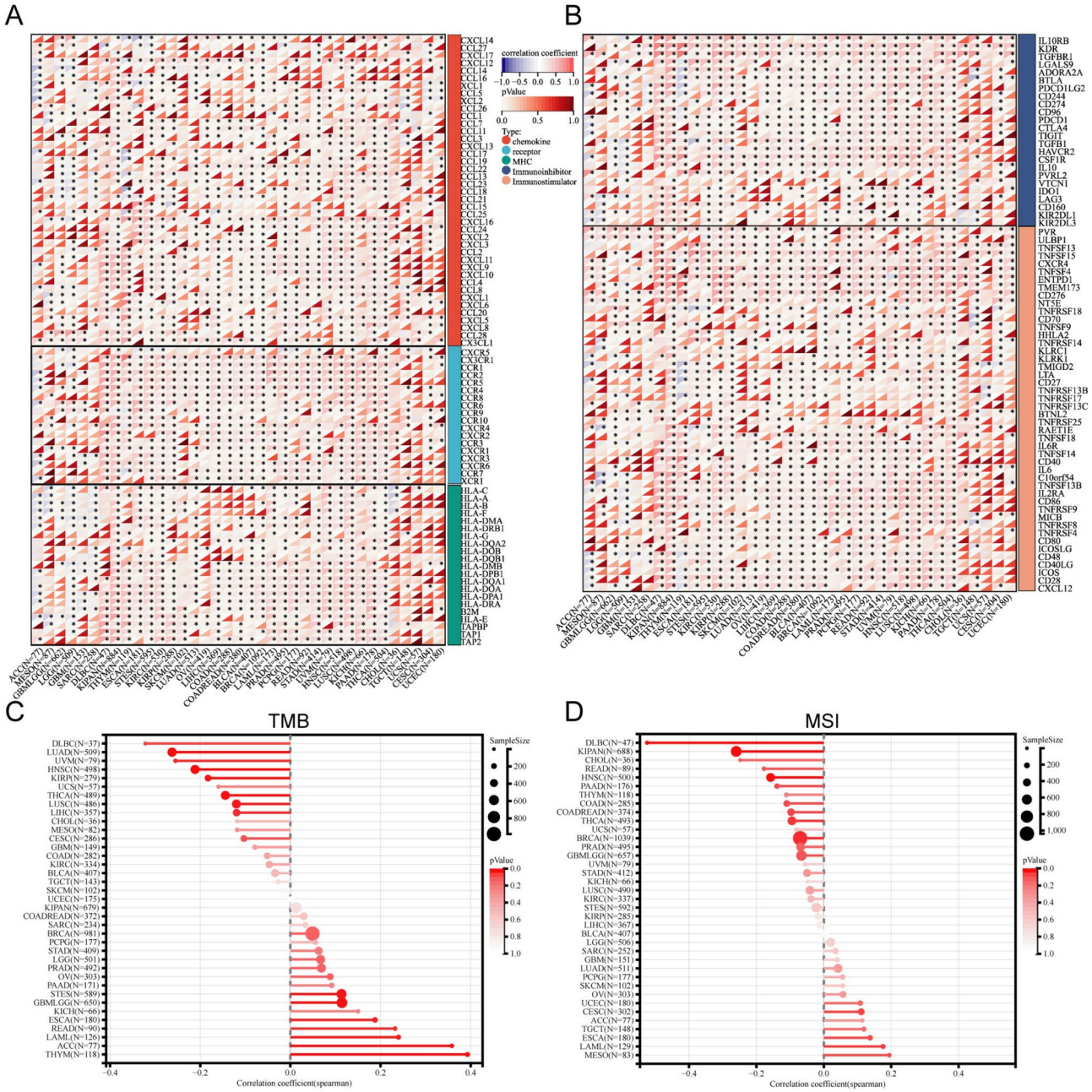

Fig. 7. Relationship between the expression of ENC1 and immune response across cancers. (**A**) Correlation between ENC1 expression and the expression of immune-related genes (chemokine genes, receptor genes, and MHC genes). (**B**) Correlation between ENC1 expression and the expression of immunoinhibitors and immunostimulators. Bar chart of the relationship between ENC1 expression and TMB (**C**) and MSI (**D**). TMB (tumor mutational burden), MSI (microsatellite instability).

with one set of immune checkpoints (CD244 and CTLA4) but positively correlated with other checkpoints (CD274 and CD276). Our studies elucidated that ENC1 expression was positively associated with the levels of the majority of immunomodulators, including immunostimulators, immunoinhibitors, chemokine ligands, chemokine receptors and MHC genes, which are involved in tumor progression and the development of targeted immunotherapies. Based on the above, we proposed that ENC1 expression may affect immune infiltration in the majority of cancer types. However, the deeper mechanism remains to be further explored.

Additionally, the specific mechanisms and pathways of tumor immunity have facilitated exciting breakthroughs in the past few decades, while there are still limitations in the application of tumor immunotherapy[43–45]. For instance, most patients are still insensitive to immunotherapy, there are few effective ways to predict the efficacy of immunotherapy, and the optimal biomarkers to assess the efficacy of immunotherapy are difficult to detect[46–49]. However, immunotherapy still has promising application prospects in cancer treatment, and

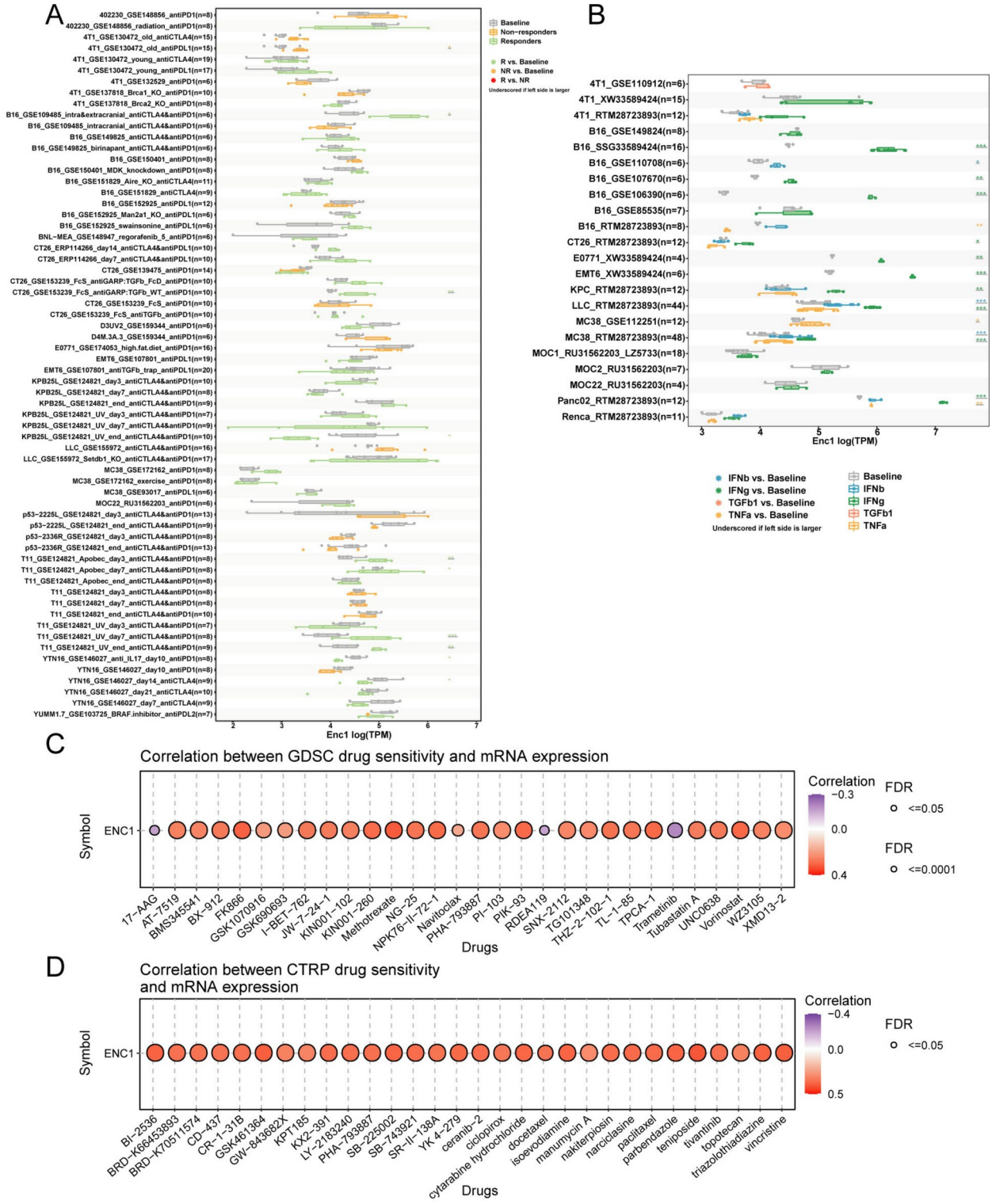

Fig. 8. Value of ENC1 in predicting the immunotherapy response across cancers. Value of ENC1 in predicting the immunotherapy response in murine tumor model immunotherapy cohorts based on in vivo studies (**A**) and in cell line cytokine treatment cohorts based on in vitro studies (**B**). Correlation analysis between ENC1 expression and drug sensitivity based on the GDSC (**C**) and CTRP (**D**) datasets.

immune checkpoint blockade is a good technique[50]. Therefore, we explored the value of ENC1 in predicting the immunotherapy response of multiple cancers based on a public database. We found that ENC1 significantly predicted the treatment response of 10 immunotherapy-treated mouse tumor models in vivo and 16 cytokine-treated cell lines in vitro. These results confirmed that the overexpression of ENC1 could be a rational and

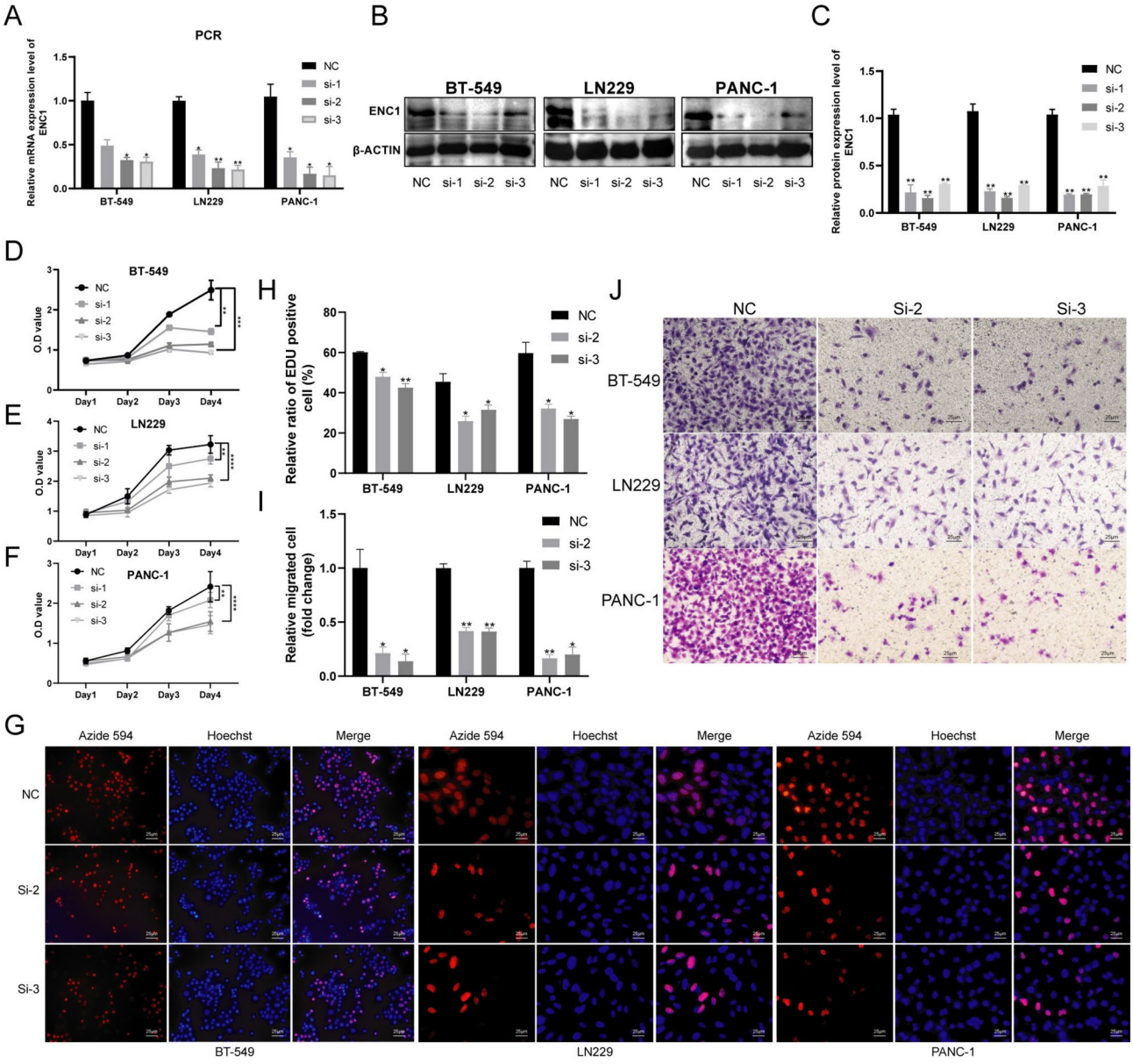

Fig. 9. The effect of ENC1 on proliferation and migration. (**A**-**C**) The knockdown efficiency of ENC1 mRNA and protein level in BT-549, LN229 and PANC-1 cell line verified by qRT-PCR and WB respectively. Quantitative comparisons for WB between samples was derived from the same experiment and that blots were processed in parallel. (**D**-**F**) The cell proliferation of BT-549, LN229 and PANC-1 about the ENC1-siRNA group compared to the control group though CCK-8 assay. (**G**-**H**) The cell proliferation was analyzed by EdU assay. (I-J) Transwell assays was performed to analysis the migration of BT-549, LN229 and PANC-1 cell transferred by si-RNA comparing the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

effective biomarker for the response to immunotherapy and that ENC1 may regulate the immunomodulatory response of cancers by regulating immune cell infiltration.

Furthermore, we investigated effective small molecules and drugs through the GDSC and CTRP databases. The results of ENC1 expression in the GDSC database showed that the top 3 most effective drugs were methotrexate, FK866 and vorinostat, and the top 3 least effective drugs were BRD-K30748066, teniposide and GSK461364. For example, methotrexate is mainly used as an antifolic acid antitumor drug through the inhibition of dihydrofolate reductase to prevent the synthesis of tumor cells and inhibit the growth and reproduction of tumor cells, especially for patients with chorionic epithelial carcinoma, malignant mole, all kinds of acute leukemia, breast cancer, lung cancer, head and neck cancer, digestive tract cancer, cervical cancer and malignant lymphoma[51–55]. Methotrexate is effective and can extend the time to tumor recurrence. Therefore, this information provides new perspectives for predicting the therapeutic scope of drugs and searching for new targeted effective drugs for these cancers.

In conclusion, our study had elucidated that the higher expression of ENC1 was found in the pan-cancer than normal tissue, also the up-regulated expression of ENC1 was predicted a poor prognosis in majority types of cancer. Additionally, we found that ENC1 was correlated with TME, tumor infiltration immune cells, and ICI biomarkers. Furthermore, we demonstrated that inhibition of ENC1 expression suppresses the proliferation and migration of breast cancer, pancreatic cancer and glioma cells.

Data availability

These data were derived from the following resources at TCGA (https://www.cancer.gov/ccg/research/genome-sequencing/tcga) and GTEx (https://www.gtexportal.org/home/index.html) databases, GSCA (http://bioinfo.life.hust.edu.cn/GSCA/#/), UALCAN (https://ualcan.path.uab.edu/analysis.html), CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA/), and xCell (https://xcell.ucsf.edu/).

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Author contributions

Liyun Zeng conceived the article. Zhenyu Cao analyzed the data and arranged all the figures. Liyun Zeng and Zhenyu Cao wrote the manuscript in consultation. Jinfeng Zhu and Zicheng Wang conducted the experiments and modified the manuscript. Yuhuai Peng collected the experimental data. All authors reviewed the manuscript and approved the submitted version.

Declarations

Conflict of interest

The authors declare no conflict of interest.

Additional information

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