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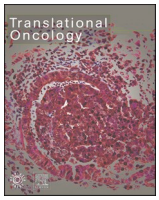

Original Research

Pan-cancer analysis of super-enhancer-induced LINC00862 and validation as a SIRT1-promoting factor in cervical cancer and gastric cancer

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ABSTRACT

Immune checkpoints inhibitors are effective but it needs more precise biomarkers for patient selection. We explored the biological significance of LINC00862 in pan-cancer by bioinformatics. And we studied its regulatory mechanisms using chromatin immunoprecipitation and RNA immunoprecipitation assays etc. TCGA and single-cell sequencing data analysis indicated that LINC00862 was overexpressed in the majority of tumor and stromal cells, which was related with poor prognosis. LINC00862 expression was related with immune cell infiltration and immune checkpoints expression, and had a high predictive value for immunotherapy efficacy. Mechanistically, LINC00862 competitively bound to miR-29c-3p to unleash SIRT1's tumor-promoting function. SIRT1 inhibitor-EX527 were screened by virtual screening and verified by in vitro and vivo assays. Notably, acetyltransferase P300-mediated super-enhancer activity stimulated LINC00862 transcription. Collectively, LINC00862 could be a diagnostic and prognostic biomarker. LINC00862 could also be a predictive biomarker for immunotherapy efficacy. Super-enhancer activity is the driver for LINC00862 overexpression in cervical cancer and gastric cancer.

Introduction

Cancer gives a great threat to human health worldwide. The incidence and mortality of cancer have been rising in recent years [1]. By assisting the immune system in recognizing cancer cells, immunotherapy has proven to be a promising anti-tumor strategy. Particularly, immune checkpoint inhibitors have benefited numerous tumor patients [2,3]. However, other than the high cost, immunotherapy also has many other deficiencies such as unique toxicity profiles [4]. Therefore, precise biomarkers for predicting the efficacy of immunotherapy are necessary.

Besides programmed cell death 1 (PD1)/ programmed cell death 1 ligand 1 (PD-L1) expression analysis, the present biomarkers for immune checkpoint inhibitors contain microsatellite instability (MSI), tumor mutational burden (TMB) and neoantigens (NEO) et.al [5–8]. Patients with high MSI, TMB and NEO status are inclined to be sensitive to immune checkpoint blockade. However, owing to the shortcomings of present biomarkers, many selected patients still fail to respond to immune checkpoint inhibitors [9]. Therefore, it is vital to select the exact patients for immunotherapy. Many new biomarkers, such as tumorous mutations, circulating tumor DNA and gut microbiota, are emerging [10,11]. Among them, non-coding RNAs, including miRNA, lncRNA and circRNA, have intrigued numerous researchers. In particular, many lncRNAs have been identified as novel biomarkers for immunotherapy [12,13]. Super-enhancer-related genes are closely related with many tumor characteristics such as immune response [14]. Recently, super-enhancer-related lncRNAs (SE-lncRNAs) are emerging as new biomarkers for predicting immunotherapy efficacy [15].

In this study, we identified a new super-enhancer-induced lncRNA-

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LINC00862. We analyzed the roles of LINC00862 in pan-cancer by bioinformatics. We found that LINC00862 could be a diagnostic and prognostic biomarker. The LINC00862 expression levels were related with a "hot" immune microenvironment, suggesting that patients with high-expressed LINC00862 may be more suitable for immune checkpoint inhibitors treatment. In cancer cells, LINC00862 competitively bound to miR-29c-3p to promote sirtuin-1 (SIRT1) expression. Targeting SIRT1 by specific inhibitor-EX527 could yield good anti-tumor effects in vitro and vivo assays. Moreover, our study identified P300-mediated super-enhancer activity as the driver for LINC00862, providing a theoretical basis for targeting LINC00862 against malignant tumors.

Materials and methods

SangerBox 3.0 database

The Sangerbox 3.0 [16] (http://vip.sangerbox.com/login.html) is an important tool for pan-cancer analysis. Firstly, the expression levels of LINC00862 were predicted by its "gene expression differential analysis" module. The prognostic value of LINC00862 was predicted by its "gene expression prognostic analysis" module. And the ESTIMATE algorithm was used to calculated the correlation between LINC00862 expression and immune score, ESTIMATE score, stromal score in pan-cancer. In order to predict the role of LINC00862 in tumor immunotherapy, we conducted correlation analysis between the expression of LINC00862 and the predictive indexes of immunotherapy that contained MSI (microsatellite Instability), TMB (tumor mutation burden), NEO (neoantigen), MATH (mutant-allele tumor heterogeneity), HRD (homologous recombination deficiency), LOH (loss of heterozygosity), purity and ploidy.

ACLBI database

The data of ACLBI database (https://www.aclbi.com/) is comprehensive and the immunoassay function is reliable. Through the "Immunity" module of ACLBI database, the correlation between expression level of LINC00862 and immune cells infiltration were calculated what used XCELL and TIMER algorithm in pan-cancer. Except immune cells infiltration, the correlation between expression levels of LINC00862 and immune checkpoint genes also was calculated by ACLBI database.

UALCAN database

The UALCAN database [17] (http://ualcan.path.uab.edu/index.html) is a comprehensive website for analyzing cancer omics data. We used "TCGA" module of UALCAN database to analyze the difference of LINC00862 expression between tumor and normal issues in 23 cancers.

GEPIA2 database

The "Expression DIY" and "Survival analysis" modules of GEPIA2 database [18] (http://gepia2.cancer-pku.cn/) were used to analyze the relationship between expression levels of LINC00862 and stages, prognosis of cancer patients.

Lnc2Cancer 3.0 database

The Lnc2Cancer 3.0 database [19] (http://bio-bigdata.hrbmu.edu.cn/lnc2cancer) is a database that provides experimental data of lncRNA or circRNA related to human cancer. The analysis of correlation between some tumor stages and LINC00862 expression was completed by this database.

Kaplan-Meier plotter database

The "Therapy" module of Kaplan-Meier Plotter [20] (http://kmplot.com/analysis/) was used to explore the survival differences using immunotherapy inhibitors. We selected the anti-PD1 treatment (Nivolumab and Pembrolizumab), anti-PD-L1 treatment (Atezolizumab and Durvalumab) and anti-CTLA4 treatment (Ipilimumab) for further analysis.

CAMOIP database

CAMOIP [21] database (https://www.camoip.net/) is used to explore the relevance between LINC00862 expression and immune cell levels in pan-cancer.

TIDE database

The expression of immune checkpoints is an important criterion to evaluate the feasibility of ICIs (immune checkpoint inhibitors) treatment scheme. The data of tumor patients who applied ICIs were collected and analyzed according to the expression of LINC00862 to predict the effect of it in tumor treatment by TIDE database [22] (http://tide.dfci.harvard.edu/). And the relationship between expression of LINC00862 and CTL level was also explored through this database.

Single-cell sequencing data analysis

We obtained some single-cell RNA-seq datasets from GEO Database (https://www.ncbi.nlm.nih.gov/), which includes CRC dataset (GSE146771), LIHC dataset (GSE166635), KIRC dataset (GSE171306) and NSCLC dataset (GSE127465). Among them, CRC dataset includes normal tissues and tumor tissues information of 10 CRC patients, LIHC dataset includes the tissues information of 2 PLC patients, KIRC dataset includes tissues information of 3 Bilateral renal cell carcinoma (RCC) patients and NSCLC dataset includes tumor tissues information of 7 NSCLC patients. We read and integrated them separately using merge method from "Seurat" R package. In order to make the results more accurate, the data were screened. The subsequent analysis excluded the genes expressed in 3 or less cells and cells with less than 200 genes. Qualified data was normalized and the top 2000 genes with highly variable characteristics were screened by ANOVA. Principal component analysis (PCA) was performed based on qualified data, and the top 40 principal components (PC) were selected for subsequent analysis. The t-SNE (t-distributed stochastic neighbor embedding) was used to perform dimensionality reduction on the top 40 PC pairs of datasets by "Seurat" R package. And FindNeighbors and FindClusters functions were used to cluster single cells into different subsets (CRC: dim = 40 and resolution = 0.1, LIHC: dim = 40 and resolution = 0.26, KIRC: dim = 40 and resolution = 0.07, NSCLC: dim = 40 and resolution = 0.06). Based on Human Primary Cell Atlas Data from "singleR" R package, CellMarker database and marker genes from collected literatures, different clusters were manual annotation. We have clustered and labeled four datasets through the above series of steps. Furthermore, we explored intercellular communication through "CellChat" R package. The communication results of all ligand-receptor interactions related to each signal pathway were calculated. And we visualized them in two directions: cells and signal pathways.

miRNA prediction and analysis

The miRNAs were predicted by the miRDB [23] database. The LINC00862 RNA and SIRT1 3′UTR sequence was analyzed by "Custom prediction" module. The expression levels of the predicted miRNAs in stomach adenocarcinoma (STAD) were determined by the "pan-cancer" module in Starbase [24] database. The expression levels of the hsa-miR-29c-3p in pan-cancer were analyzed by the "Query" module in CancerMIRNome [25] database.

Cells and reagents

The cancer cells-Hela and HGC27 were obtained from the cell bank of the Chinese Academy of Sciences. These cells were incubated in RPMI1640 medium complemented with 10 % fetal bovine serum at 37 °C with 5 % CO2. The cells were tested for mycoplasma contamination every two months. Cell culture dishes/plates and centrifuge tubes were purchased from NEST Biotechnology Co. Ltd. The reagents used in this study are listed below: I-BET-762 (S7189, Selleck), JQ1 (S7110, Selleck), Lipofectamine™ RNAiMAX (Invitrogen, 13,778,150), X-treme GENE™ 9 DNA Transfection Reagent (Roche, 6,365,779,001), Ultra-SYBR Mixture (CW0957M, cwbiotech), EX527 (Sigma Aldrich, E7034).

siRNA transfection

1×10^5 cancer cells were seeded in six-well plates and were transfected with the specific siRNA using a transfection reagent. The siRNAs were purchased from Gene Pharma (Shanghai, China). The siRNA sequences are listed below:

LINC00862-siRNA 1# S: GUGGAUUGAAUCUCUUUAUTT, AS: AUAAAGAGAUUCAAUCCACTT; LINC00862-siRNA 2# S: GGCCUGAUCAUUUGGUUUATT, AS: UAAACCAAAUGAUCAGGCCTT; P300-siRNA 1# S: CCGGUGAACUCUCCUAUAATT, AS: UUAUAGGAGAGUUCACCGGTT; P300-siRNA 2# S: GCCUCAAACUACAAUAAAUTT, AS: AUUUAUUGUAGUUUGAGGCTT; hsa-miR-29c-3p mimics S: UAGCACCAUUUGAAAUCGGUUA, AS: ACCGAUUUCAAAUGGUGCUAUU.

Quantitative RT–PCR (qPCR)

The qPCR assays were conducted as reported previously [26]. a-Tubulin was used as the internal control. The primer sequences are listed below: a-Tubulin-F: GAAGCAGCAACCATGCGTGA, a-Tubulin-R: AAGGAATCATCTCCTCCCCCA; LINC00862-F: CCGGATGGCTACAA AGCAAC, LINC00862-R: CAGGTGCAGCACTGAAAACA; SIRT1-F: TTGG CACAGATCCTCGAACAA, SIRT1-R: ATGAAACAGACACCCCAGCTC; hsa-miR-29c-3p: TAGCACCATTTGAAATCGGTTA.

CCK-8 assays

Three thousand cells were seeded in 96-well plates. After three days, the CCK8 working reagent (C0038-500, Beyotime) was added into every well and incubated for 30 min at 37 °C. Then the optical density was detected.

Dual luciferase reporter assays

Dual luciferase reporter assays were conducted as previously reported [26]. pMIR-REPORT-LINC00862 (WT or MT) and pMIR-REPORT-SIRT1 (WT or MT) plasmids were used.

Western blotting

Western blotting was conducted as previously reported [26]. The antibodies were used in these assays: β-actin (4970 L, CST) (1:5000), SIRT1 (ab189494, Abcam) (1:1000).

Chromatin immunoprecipitation (ChIP)

The ChIP assays were conducted as reported previously [26]. The antibodies were used in these assays: Histone H3 (acetyl K27) (Abcam, ab177178), P300 (ab275378, Abcam), BRD4 (A301-985A100, Bethyl Laboratories). The primer sequences are listed below:

LINC00862-enhancer1-F: ATGGCTCTGCCCCTGAAAAA, LINC00862-enhancer1-R: CAGATCCCCATGGAACCACC; LINC00862-enhancer2-F: ACCCACCGGCAATACTTACG, LINC00862-enhancer2-R: GTGGAGAGGACGGCGTTATT; LINC00862-enhancer3-F: TTGCAAAG GAGGCAGTGTGA, LINC00862-enhancer3-R: GCATTGTGGCACTTT GAGCA.

RNA immunoprecipitation (RIP)

The RIP assays were conducted as reported previously [26]. The mRNA and miRNA were isolated for qPCR analysis. The primer sequences were the same as above.

Tissue samples collection

This research was approved by The Institute of Research Medical Ethics Committee of the Affiliated Hospital of Shandong Second Medical University. Gastric cancer tissues and paired normal tissues from fifteen patients were collected from the Affiliated Hospital of Shandong Second Medical University. All patients signed the informed consent forms.

Xenograft model

The animal experiment was approved by the Institutional Animal Care and Use Committee of Shandong Second Medical University. Ten 4–5-week-old BABL/c nude mice were used in this study. 5×10^6 Hela cells were subcutaneously injected into these mice. After 7 days, these mice were intraperitoneally injected with control reagent or EX527 (40 mg/kg), once every two days. Tumor volumes and body weights were measured every three days. All mice were sacrificed at day 20 and tumors were weighed.

Statistics

The two-sided Student's *t*-test was administered to assess statistical significance using GraphPad Prism 7. The data was presented as means ± standard deviation. $P < 0.05$ was indicated as statistically significant.

Results

LINC00862 could act as a diagnostic biomarker

First, we elaborated on the expression levels of LINC00862 in pan-cancer using The Cancer Genome Atlas (TCGA) database. Fig. S1A exhibits the expression levels of LINC00862 in the normal tissues and tumor tissues using UALCAN database with TCGA data. In order to exclude the data discrepancy in different databases, the expression levels of LINC00862 in the normal tissues and tumor tissues were also explored using Sangerbox 3.0 databases with TCGA + GTEx data (Fig. S1B). We observed the majority of human tumors with high-expressed LINC00862 spanning 17 tumor types. We obtained the intersection of these two databases. The data indicated that LINC00862 expression was consistently upregulated in 9 tumor types including bladder urothelial carcinoma (BLCA), cholangiocarcinoma (CHOL), cervical squamous cell carcinoma (CESC) and endocervical adenocarcinomahead and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), lung adenocarcinoma (LUAD), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD) and uterine corpus endometrial carcinoma (UCEC). In contrast, LINC00862 was low-expressed in kidney chromophobe (KICH). Additionally, LINC00862 expression was significantly related with tumor stages (Fig. S2). In sum, LINC00862 overexpression occurred in the majority of human tumors and LINC00862 could act as a novel diagnostic biomarker.

LINC00862 could act as a prognostic biomarker

The LINC00862 expression is markedly elevated in human tumors. Thus, we tested whether it could influence the prognosis. Indeed, TCGA data indicated that LINC00862 expression level was related with overall survival (OS), disease free interval (DFI), progression free interval (PFI)

and disease specific survival (DSS) in multiple tumors (Figs. S3–S6). Notably, LINC00862 expression was closely relevant with all of these 4 prognostic indicators in 6 tumors including breast invasive carcinoma (BRCA), KIRC, liver hepatocellular carcinoma (LIHC), LUAD, STAD, stomach and esophageal carcinoma (STES). Specifically, combining the above LINC00862 expression data, we found that LINC00862 showed a trend of shorter survival in LINC00862-high expressed tumors such as KIRC, LUAD, LIHC and STAD. On the contrary, the LINC00862 expression level was related with good prognosis in LINC00862-low expressed tumors such as BRCA. Taken together, LINC00862 could act as a prognostic biomarker.

Single-cell sequencing analysis of LINC00862 expression

Then, we investigated the LINC00862 expression levels in the tumor microenvironment by single-cell sequencing data. We analyzed the LINC00862-high expressed tumors data collected in TISCH database, including colorectal cancer (CRC) (GSE146771), non-small cell lung cancer (NSCLC) (GSE127465), LIHC (GSE166635) and KIRC (GSE171306). Interestingly, comparing single-cell sequencing data revealed that in addition to the malignant cells, considerable LINC00862 also existed in multiple immune cells (Fig. 1 and Figs. S7–S9). Super-enhancer-mediated genes, especially non-coding RNA, play critical roles in determining tumor immune response [27]. Therefore, we wondered the correlation between LINC00862 expression and tumor microenvironment.

The LINC00862 expression level was related with a hot immune microenvironment

Tumor-infiltrating immune cells are the key part of the tumor microenvironment and mediate the cancer phenotype by the complicated interplay with cancer cells [28]. A prerequisite for using immune checkpoint inhibitors is the abundance of immune cell infiltration, especially the $CD8^+$ *T* cell. To address the immune relevance of LINC00862 with the tumor microenvironment, we calculated the correlation between LINC00862 expression and immune scores. We found that LINC00862 expression was positively related with immune scores, stromal scores and estimate scores in multiple tumors (Figs. S10,11). At the same time, LINC00862 expression level was positively related with immune cell infiltration levels especially in the LINC00862-high expressed tumors (Fig. 2). Collectively, all of these data indicated LINC00862 expression level was related with a hot immune microenvironment.

LINC00862 could serve as a predictive biomarker for immunotherapy efficacy

Next, we tested whether LINC00862 could predict the efficacy of immunotherapy. TCGA data indicated that LINC00862 expression level was positively related with immune checkpoints gene expression such as PD1/PD-L1 and CTLA4 in most of the tumors (Fig. 3). Furthermore, the LINC00862 expression level was related with MSI, TMB, NEO and mutant-allele tumor heterogeneity (MATH), the clinical biomarkers for immunotherapy (Figs. S12–13). Notably, the LINC00862 expression level was mainly positively related with MSI, TMB and NEO in LINC00862-high expressed tumors. Therefore, we assumed that LINC00862-high expressed patients might react better to immune checkpoint inhibitors. We downloaded TCGA-melanoma data and analyzed LINC00862 expression in relation with MSI and tumor immune dysfunction and exclusion (TIDE) scores using TIDE database. The results showed that LINC00862 expression level was positively related with MSI scores and negatively related with TIDE scores (Fig. 4A), implying that LINC00862 high-expressed melanoma patients may react better when receiving immune checkpoint inhibitors. Indeed, the Kaplan-Meier Plotter data showed that both PD1 and CTLA4 antibody-treated patients exhibited markedly better prognosis in LINC00862-high expressed melanoma (Fig. 4B). Besides, we compared the predictive power of different biomarkers for immunotherapy response by a series of human immunotherapy cohorts. LINC00862 had a good predictive value in line with other clinical biomarkers (Fig. S14). Unexpectedly, the CAMOIP data indicated that the LINC00862 expression level was negatively related with CD8+ *T* cell content in HNSC, but was positively related with CD8+ *T* cell content in KIRC (Fig. 4C). However, TIDE data showed that high-expressed LINC00862 inhibited cytotoxic T cell (CTL)-mediated anti-tumor effect in both HNSC and KIRC (Fig. 4D). This result suggested that LINC00862 could influence CD8+ *T* cell's function regardless of its level, implying that LINC00862 could also serve as a new target for immunotherapy in some tumors.

LINC00862 promoted SIRT1 expression by competitively binding to miR-29c-3p

The above data demonstrated the LINC00862 was high-expressed in multiple tumors. TCGA data showed the high expression levels of LINC00862 in CESC and STAD (Fig. S15A). And our own clinical data verified the high-expression of LINC00862 in gastric cancer patients (Fig. S15B). Single-cell sequencing data showed that LINC00862 was also high-expressed in malignant cells. Therefore, we selected CESC cell line-Hela and STAD cell line-HGC27 to explore the underlying regulatory mechanism. As one of the class III histone deacetylases (HDACs), Sirtuin-1 (SIRT1) is either as an oncogene or a tumor suppressor in tumorigenesis depending on different stages [29]. Once the tumor has come into being, SIRT1 is helpful for tumor progression. Accidentally, we discovered LINC00862 expression was positively related with SIRT1 expression in STAD (Fig. S15C). And the western blotting and qPCR assays validated the suppressive effect on SIRT1 expression after LINC00862 knockdown in cervical cancer and gastric cancer cells (Fig. 5A and Fig. S15D). Knockdown of LINC00862 could inhibit the cell viability, which was partially rescued by overexpression by SIRT1 (Fig. S15E-F), validating the key role of LINC00862/SIRT1 axis in cancer cells. To gain further insights into LINC00862-induced SIRT1 activation, we analyzed the subcellular distribution of LINC00862 by bioinformatic prediction. The results indicated LINC00862 was mainly localized in the cytoplasm (Fig. 5B). The principal regulatory mode for cytoplasm lncRNA is a ceRNA mechanism [30]. To pinpoint the specific miRNAs involved in LINC00862-induced SIRT1 activation, we searched miRDB database. Venn diagrams showed the intersection of miRNAs complementary to LINC00862 RNA and SIRT1 3′UTR (untranslated region) simultaneously (Fig. 5C). Of note, among them, only miR-29c-3p was low-expressed in STAD by analyzing Starbase data. Similarly, we validated that miR-29c-3p was low-expressed in many tumors including CESC and STAD by CancerMIRNome data (Fig. 5D–E and Fig. S15G). Additionally, miR-29c-3p expression levels were negatively related with LINC00862 and SIRT1 expression levels in STAD (Fig. S15H). Dual luciferase assays suggested that miR-29c-3p was directly bound to LINC00862 RNA and the 3′UTR of SIRT1 mRNA, and repressed their activity (Fig. 5F–H). Moreover, overexpression of miR-29c-3p abolished the SIRT1 protein expression (Fig. 5I), indicating a key role of miR-29c-3p in LINC00862-mediated SIRT1 activation. Finally, the RIP assay indicated LINC00862 and miR-29c-3p were both in the RNA-induced silencing complex (Fig. 5J–K). Overall, these data demonstrated that LINC00862 promoted SIRT1 expression by competitively binding to miR-29c-3p in CESC and STAD.

Screening sensitive drugs for SIRT1 protein

Histone deacetylases (HDACs) are closely related with tumorigenesis [31]. Histone deacetylase inhibitors (HDACis) are promising anticancer drugs. However, traditional HDACis are limited in the clinical implication owing to a range of factors. Therefore, we planned to predict some sensitive drugs to target the SIRT1 protein. Firstly, we searched the

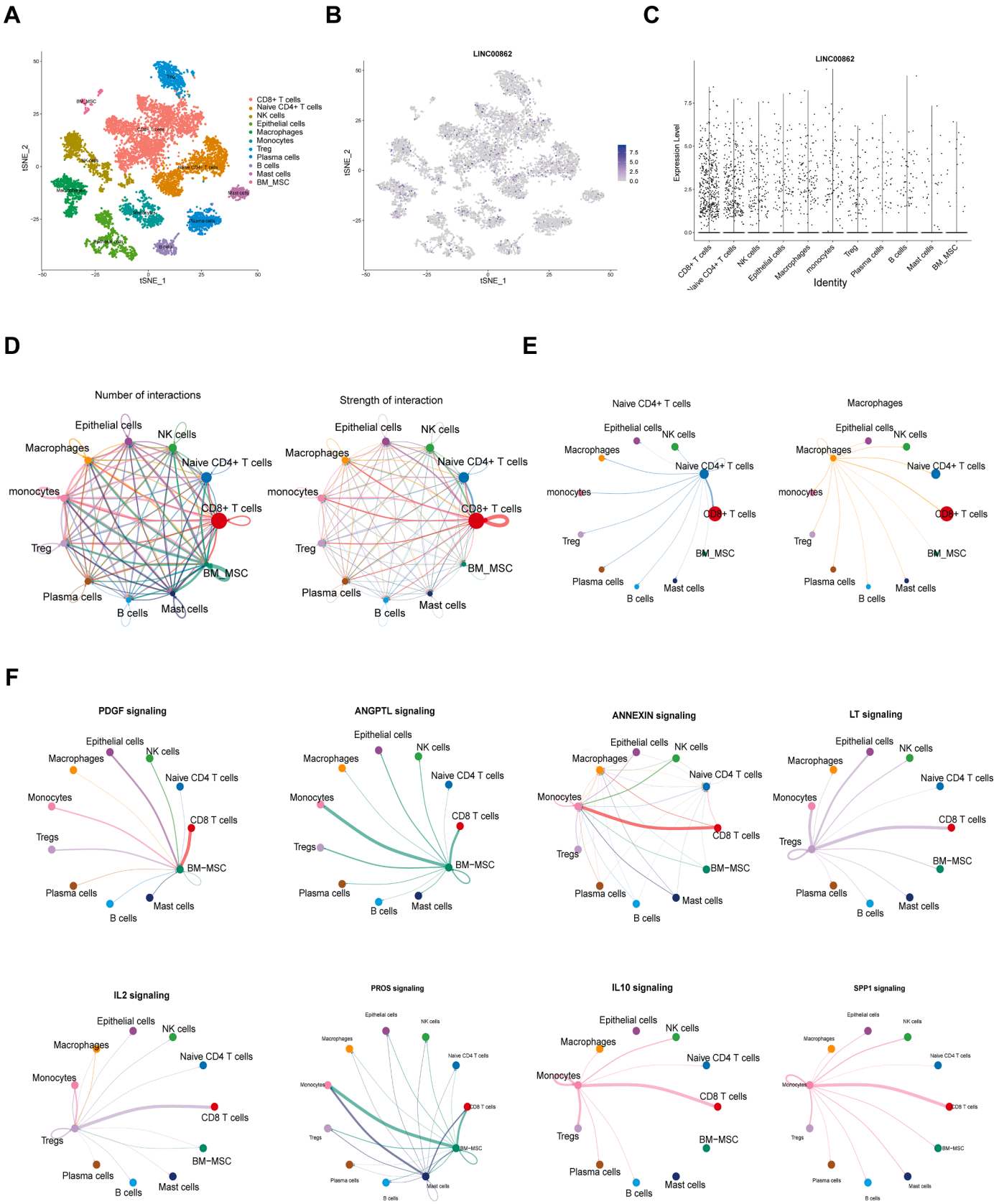

Fig. 1. Analysis of LINC00862 in colorectal cancer single-cell sequencing data (GSE146771). (A) tSNE plots of different cell types. (B) Expression distribution of LINC00862. (C) Expression levels of LINC00862 in various cell types. (D) Analysis of the number of interactions and interaction strength among different cell types. (E) Analysis of the interaction strength between Naïve CD4+ *T* cells, macrophages and other cell types. (F) Circle diagrams of PDGF, ANGPTL, ANNEXIN, LT, IL2, PROS, IL10 and SPP1 signal pathways in Naïve CD4+ *T* cells and macrophages. The width of the line represents the intensity of preference.

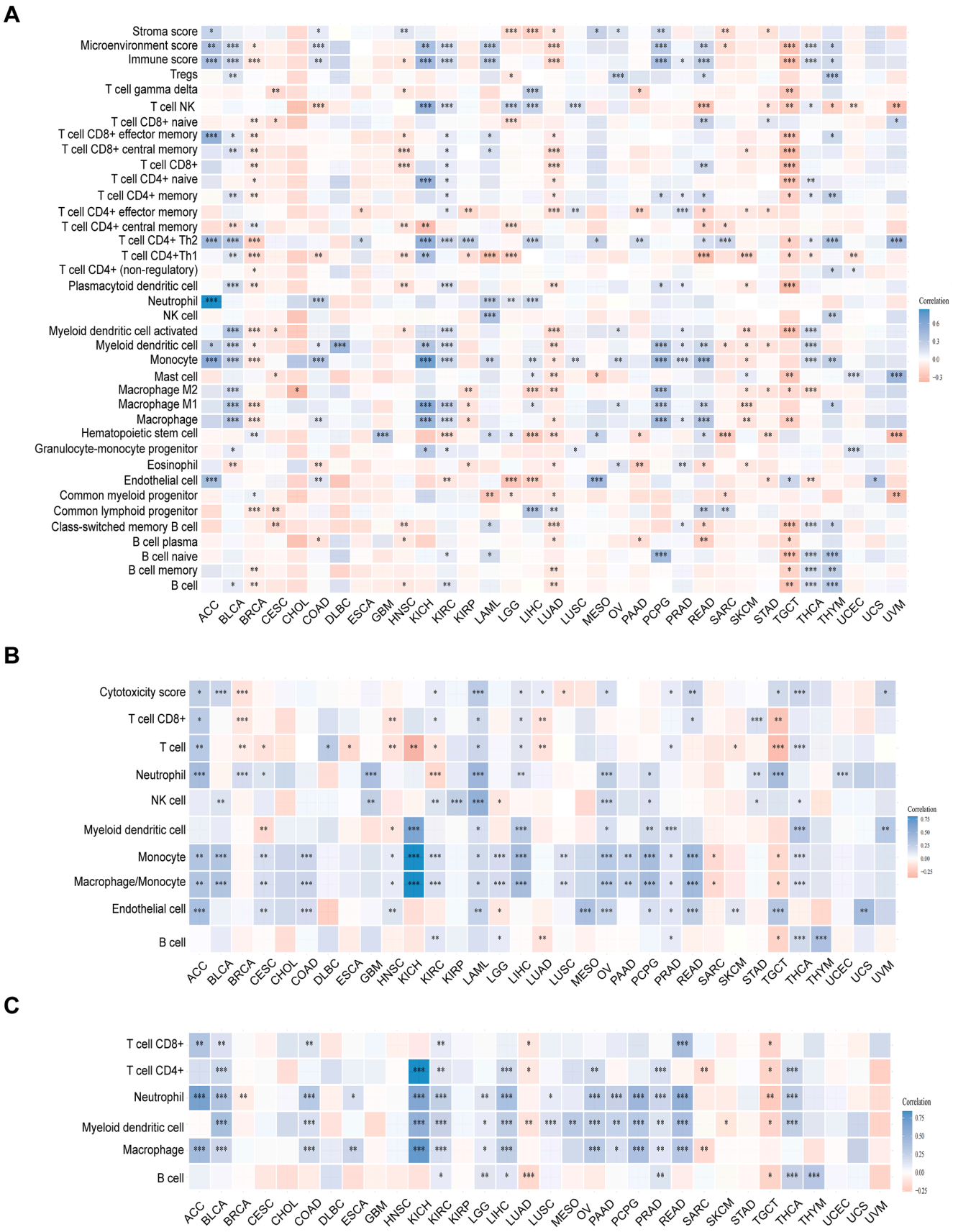

Fig. 2. The correlation between LINC00862 expression and immune cell infiltration in pan-cancer. The interrelation heatmap between LINC00862 expression and immune cell infiltration using the ACLBI database. (A: XCELL algorithm, B: MCPCOUNTER, C: TIMER algorithm) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

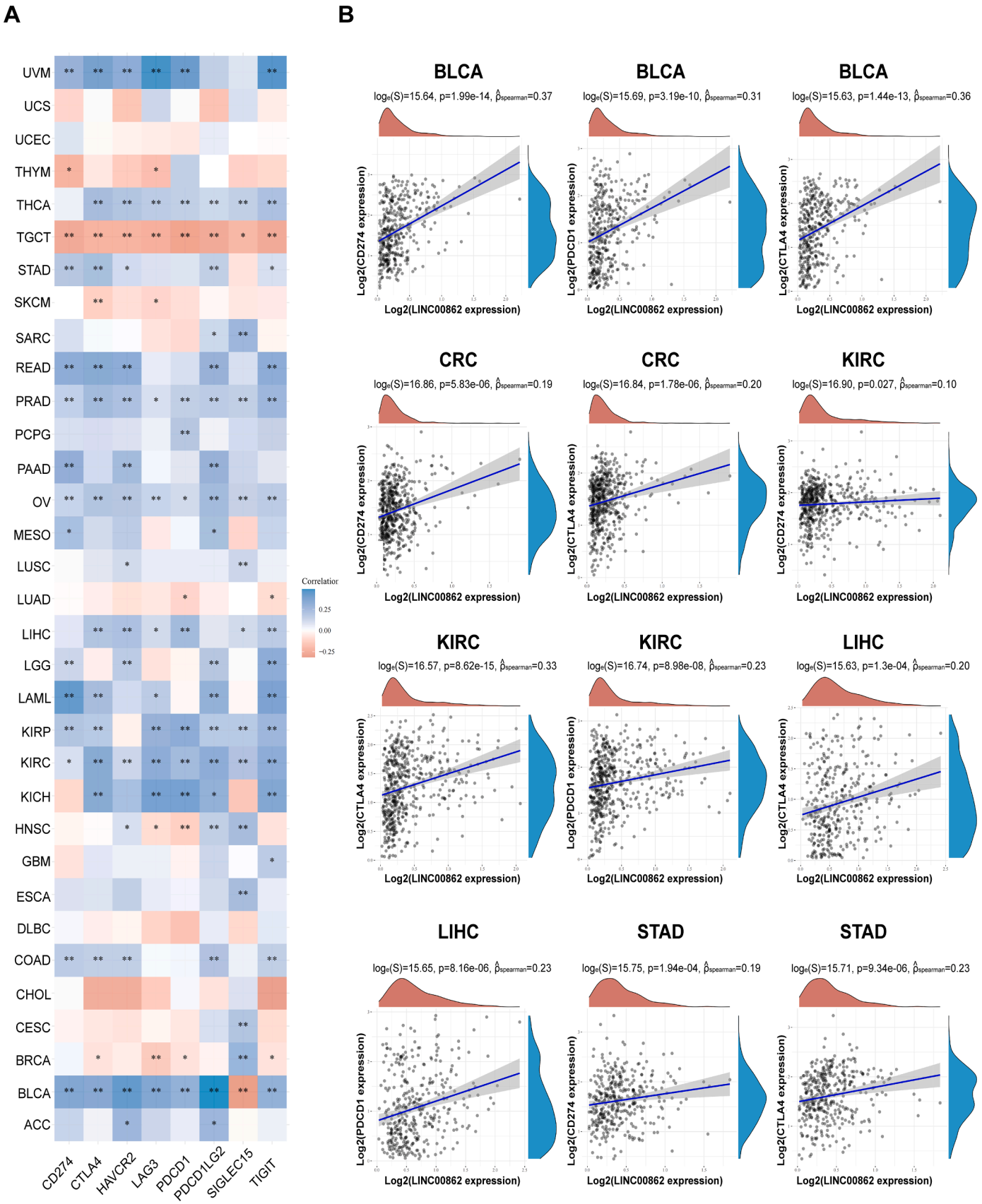

Fig. 3. The correlation between LINC00862 and immune checkpoint genes expression. (A) The correlation mapping of LINC00862 expression with immune checkpoint genes in pan-cancer using the ACLBI database. (B) Scatter plots of the correlation between LINC00862 and PD1 (PDCD1), PD-L1 (CD274) or PD-L2 (PDCD1LG2) expression using the ACLBI database. (*$p < 0.05$, **$p < 0.01$).

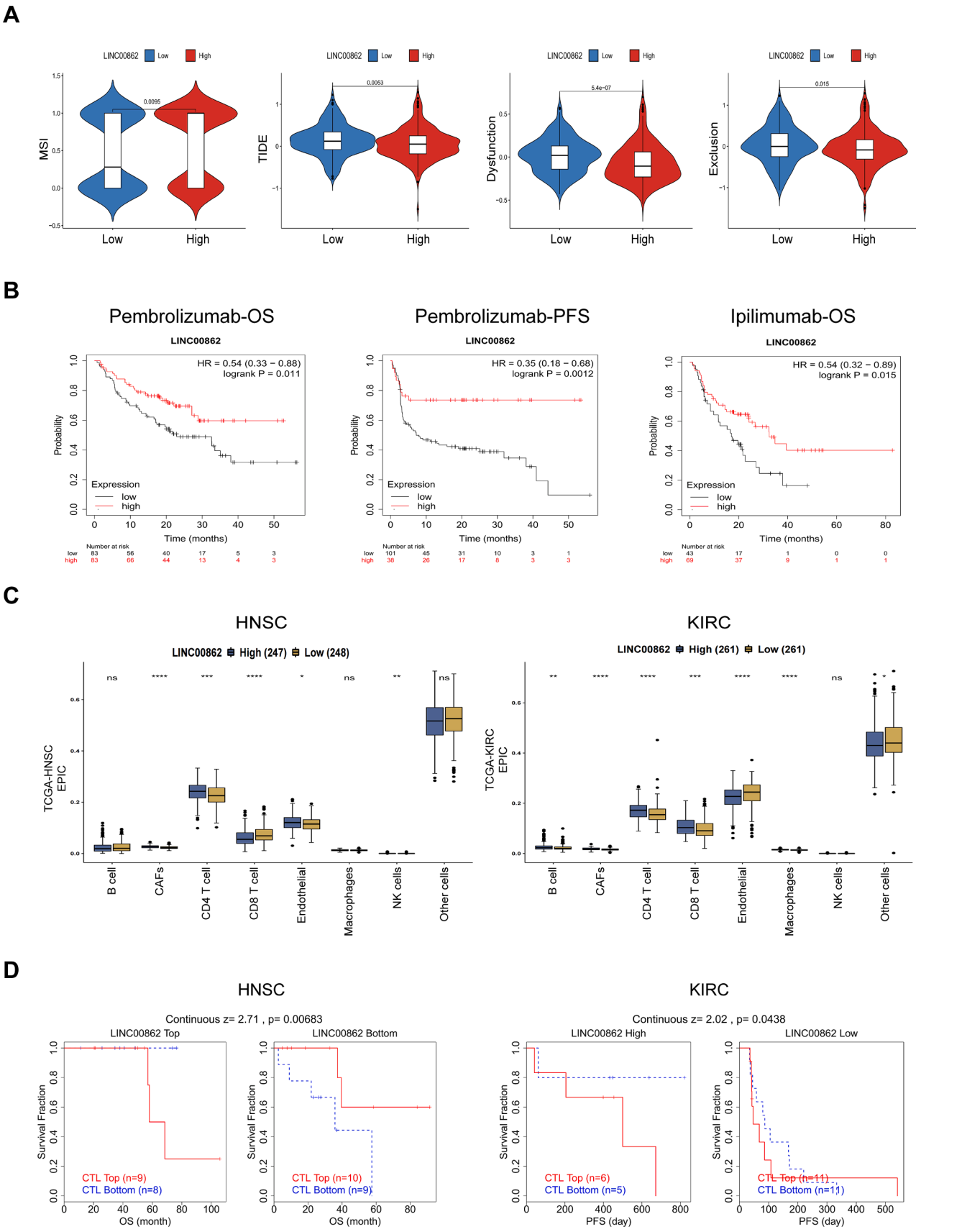

Fig. 4. LINC00862 predicted immunotherapy efficacy. (A) Relationship between LINC00862 expression and MSI, TIDE scores in melanoma. (B) The OS and PFS analysis of LINC00862 high or low expressed melanoma patients receiving PD1 or CTLA4 antibody. (C) The relevance between LINC00862 expression and immune cell levels in HNSC and KIRC. (D) The OS and PFS analysis of HNSC and KIRC patients with different LINC00862 expression and CTL levels. (CTL, cytotoxic T cell; OS, overall survival; PFS, progression free survival).

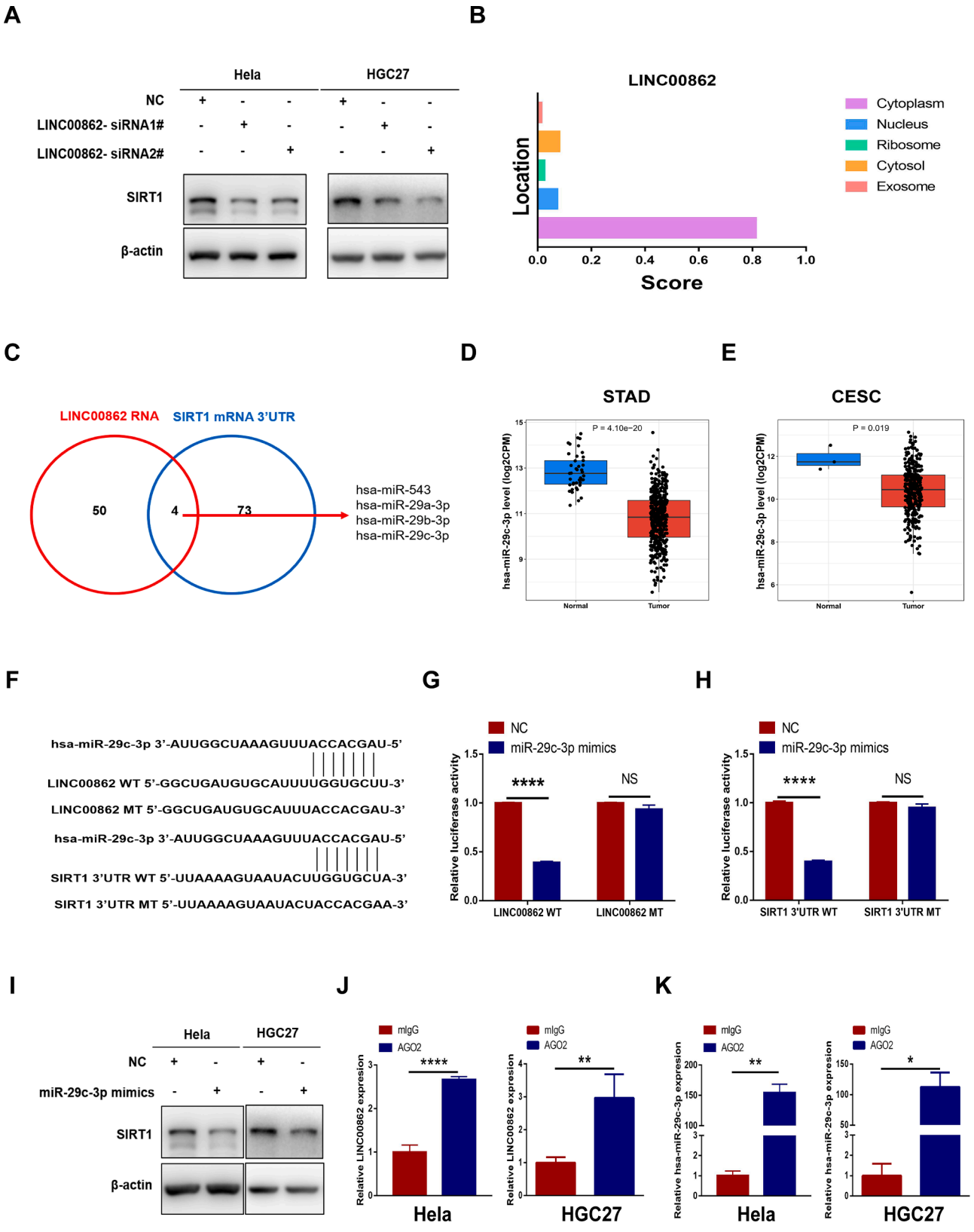

Fig. 5. LINC00862 promoted SIRT1 expression by competitively binding to miR-29c-3p. (A) Relative SIRT1 expression after LINC00862 knockdown. (B) The subcellular localization of LINC00862 predicted by lncLocator database. (C) The Venn diagrams of a series of miRNAs complementary to LINC00862 RNA and SIRT1 3′UTR region predicted by bioinformatic tools. (D-E) The relative expression of miR-29c-3p in STAD and CESC using CancerMIRNome database. (F) Schematic representation of the predicted target site for miR-29c-3p in LINC00862 RNA and SIRT1 3′UTR region. (G-H) Dual luciferase assays of LINC00862 or SIRT1 3′UTR constructions with intact or mutated seed sequences for miR-29c-3p. (I) The effect of miR-29c-3p mimics on SIRT1 protein levels. (J-K) The relative expression levels of LINC00862 and miR-29c-3p by RNA immunoprecipitation assays. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

sensitive drugs for SIRT1 protein by CTRP and GDSC database. Three compounds were discovered including EX527, Salermide and SRT-1720 in CTRP database while only EX527 was in GDSC database. We obtained the 3D structure of SIRT1 protein and drugs structure by the Alpha Fold [32] protein structure and PubChem [33] database, respectively (Fig. 6A). And the possible binding sites and boxes on SIRT1 protein surface were predicted by GHECOM algorithm [34] (Fig. 6B). Then we performed molecular docking between SIRT1 protein and these three drugs by using DOCK and PyMol software (Fig. 6C). The estimated free energy of binding was showed in Table S1. The interaction between SIRT1 protein and the drugs were analyzed by Ligplus software (Fig. 6D). Finally, we selected EX527 for further verification. In the vitro assay, EX527 could significantly inhibit cervical cancer and gastric cancer cell viability (Fig. S15I), further validating the effect of targeting SIRT1. And the inhibiting effect required dose and was incremental. In the vivo assay, EX527 also exhibited a significantly suppressive effect on tumor formation (Fig. 6E–G). Overall, we predicted several sensitive drugs for the SIRT1 protein and validated EX527's effect on cervical cancer and gastric cancer cells.

P300-mediated super-enhancer activity was responsible for LINC00862 expression

Subsequently, we mined the driving forces behind LINC00862 overexpression in CESC and STAD. In an effort to search for the potential mechanisms, we searched the UCSC database and found abundant H3K27ac signals existing LINC00862 gene loci. The ENCODE data [35] confirmed H3K27ac signals in many cancer cells (Fig. 7A). Our ChIP-qPCR assays also verified the enrichment of H3K27ac at the LINC00862 gene loci in cervical cancer and gastric cancer cells (Fig. 7B). As is generally known, H3K27ac is the best marker of super-enhancer which frequently occurs in cancers [36]. These data implied the possibility that super-enhancer was involved in activating LINC00862. To further demonstrate that LINC00862 expression was directly controlled by super-enhancer, we targeted BRD4, the known H3K27ac signal reader, to inhibit the super-enhancer activity. First, we found the enrichment of BRD4 at the LINC00862 gene loci was also significantly elevated (Fig. 7C). Then, TCGA data showed a positive correlation between LINC00862 and BRD4 expression in STAD (Fig. S15J). More importantly, BRD4 inhibition by inhibitors abolished LINC00862 expression (Fig. 7D). A recent study suggested that acetyltransferase P300 was necessary for the super-enhancer activity [37]. Whether the super-enhancer activity at LINC00862 gene loci was also subject to P300? TCGA data also showed a positive correlation between LINC00862 and P300 expression in STAD (Fig. S15J). Coincidentally, our study demonstrated that the occupancy of P300 at the LINC00862 gene loci was also increased (Fig. 7E). Again, P300 inhibition by siRNAs abolished LINC00862 expression (Fig. 7F). Collectively, LINC00862 expression in CESC and STAD depended on P300-mediated super--enhancer activity (Fig. 7G).

Discussion

Immune checkpoints inhibitors have changed the landscape of tumor treatment. However, only a small portion of tumor patients have a response to these inhibitors. Considering the severe side effects and high cost, it is crucial to explore precise biomarkers for patient selection. As the standard clinical biomarkers, PD-L1 and TMB are imperfect owing to the dynamic alteration during the course of tumors. Non-coding RNA such as lncRNA or circRNA has been screened to predict immune response in many tumor types [38–40], exhibiting a strong advantage owing to their easy testability. Enhancers have emerged as an important feature for tumors [41]. Super-enhancer (SE) is composed of multiple adjacent enhancers. Enhancer RNAs (eRNAs) are transcribed from enhancer regions and play critical roles in tumorigenesis [42–44]. Super-enhancer-mediated RNA (seRNA), especially lncRNA, have drawn the scientists' attention because of their correlation with immune escape. For example, Zhao et.al validated the importance of SE-LINC00945 in immunotherapy and chemotherapeutics for glioma [45]. Li et.al found SE-TM4SF1-AS1 was negatively related with immune cell infiltration and was responsible for T cell-mediated immune response in STAD [15]. Meanwhile, our latest study demonstrated that SE-PRR7-AS1 expression was inversely connected with tumor-infiltrating immune cells and had a better predictive value for immunotherapy efficacy compared to other biomarkers [46]. All of these researches indicated some SE-lncRNAs could be related with an immunosuppressive microenvironment. Therefore, we supposed whether these SE-lncRNAs overexpression may lead to the formation of "cold tumors", facilitating immune escape? Of course, our assumption needs more clinical assays to verify.

In this study, we identified a new super-enhancer-induced lncRNA-LINC00862. At present, there is no research about the roles of LINC00862 in cancer. We comprehensively analyzed the roles of LINC00862 in pan-cancer by bioinformatics. Our results indicated that LINC00862 could be a new diagnostic and prognostic biomarker. Meanwhile, we also explored the relevance of LINC00862 with immune characteristics. We are the first study to systematically analyze the immune relevance of LINC00862 in pan-cancer. We reported that the LINC00862 expression level was related with a "hot" immune microenvironment and could be a predictive biomarker for immunotherapy. Especially, LINC00862 expression level was positively related with existing biomarkers and immune checkpoint genes in most tumors. MSI and TIDE scores are key factors for immunotherapy response prediction [47,48]. LINC00862 expression was positively related with MSI scores and negatively related with TIDE scores, implying a preference to immune checkpoints inhibitors. Indeed, we found the melanoma patients with high-expressed LINC00862 had a better prognosis when receiving PD1 and CTLA4 inhibitors. Now, not all of the selected patients who are in accordance with current testing standards are really suitable for immune checkpoint inhibitors. Even with a hot immune microenvironment and positive PD1/PDL1 expression, many patients are still resistant to immune checkpoint inhibitors. Our study discovered that LINC00862 may impair the function of cytotoxic T cell whatever their levels. Maybe the joint detection of LINC00862 expression and cytotoxic T cell function could be benefiting for selecting the exact patients for immunotherapy. Whether targeting LINC00862 could react better when combining immune checkpoint inhibitors than using immunotherapy alone? Whether targeting LINC00862 could obtain ideal effect in the patients who have failed in immunotherapy? These assumptions also need further clinical assays to verify. Overall, our study provided another clue to reveal the underlying mechanisms for immune checkpoint inhibitors resistance.

Subsequently, we explored the roles and regulatory mechanisms of LINC00862 in cervical cancer and gastric cancer. We verified that LINC00862 was indispensable for gastric cancer progression and this effect depended on SIRT1 activity. SIRT1 is one of the class III histone deacetylases (HDACs) which are dysregulated in tumor progression [49]. SIRT1 is a potential new target for gastric cancer [50,51]. Several non-coding RNAs interacting with SIRT1 have been identified [52]. For example, lncRNA HOXC-AS1 promoted esophageal squamous cell carcinoma progression by stabilizing SIRT1 mRNA [53]. And LINC01133 inhibited pancreatic adenocarcinoma pyroptosis by miR-30b-5p/SIRT1 axis [54]. Our results demonstrated that LINC00862 could exert its effect on cervical cancer and gastric cancer by stabilizing SIRT1 expression, shedding light on the underlying regulatory mechanism. And the dual luciferase and western blotting assays suggested that the LINC00862-induced effect was miR-29c-3p-specific. Latest study confirmed that miR-29c-3p repressed the malignant phenotype of gastric cancer [55]. In line with their research, our study revealed the inhibitory role of miR-29c-3p in gastric cancer by suppressing SIRT1 expression. SIRT1 is one key metabolic sensor in cellular metabolism pathways and plays vital roles in influencing immune responses [56].

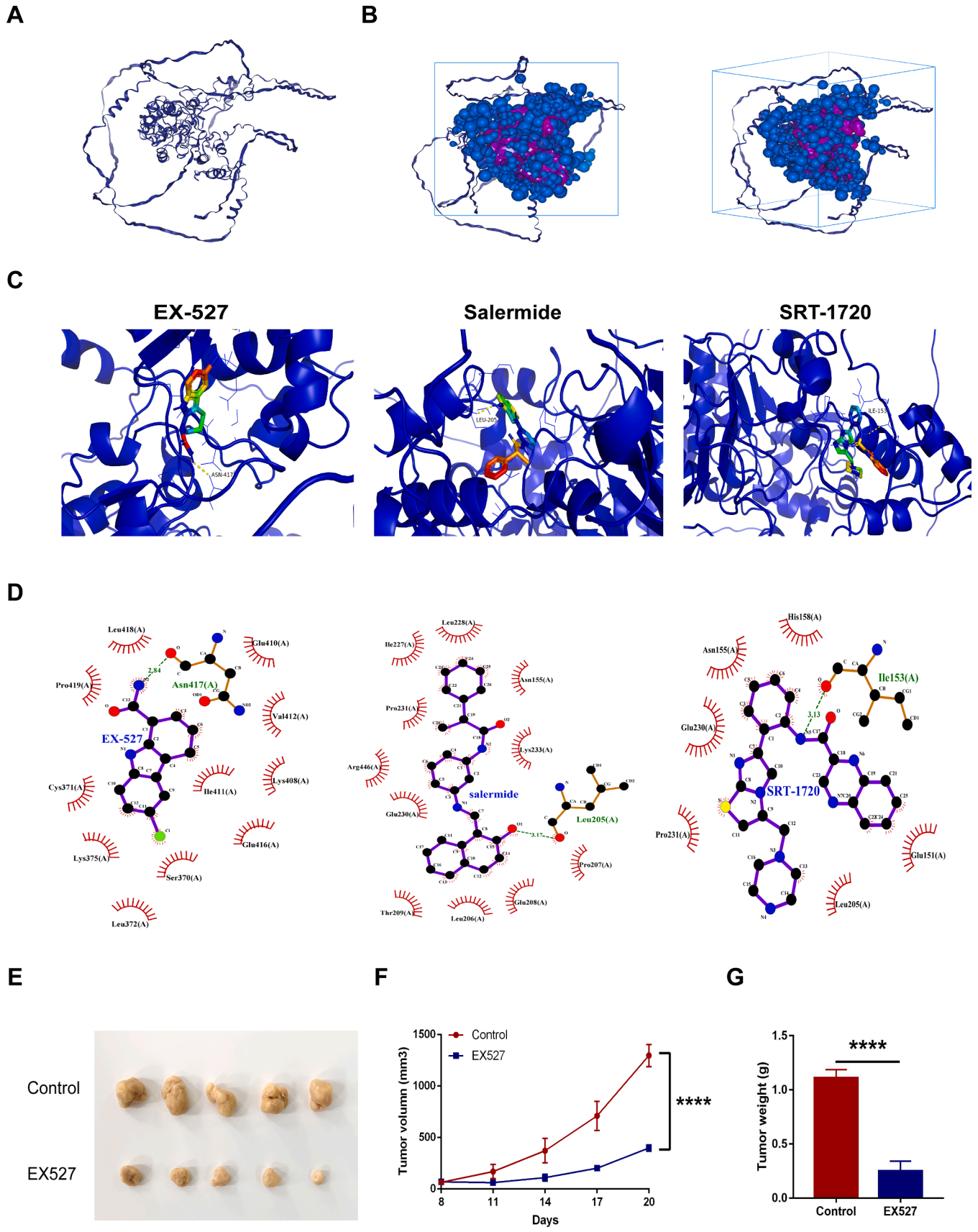

Fig. 6. Screening sensitive drugs for SIRT1 protein. (A) The spatial structure diagram of the SIRT1 protein. (B) Potential binding sites and box of SIRT1 protein. (C) The optimal docking space conformation of the drugs to SIRT1 protein. (D) The interaction force between SIRT1 protein and drugs. (E) Images of dissected tumors. (F) Tumor volumes were measured in xenograft mice model. (G) Summary of mean tumor weight measured at end point. (Data are represented as mean ± SD, ****$P < 0.0001$).

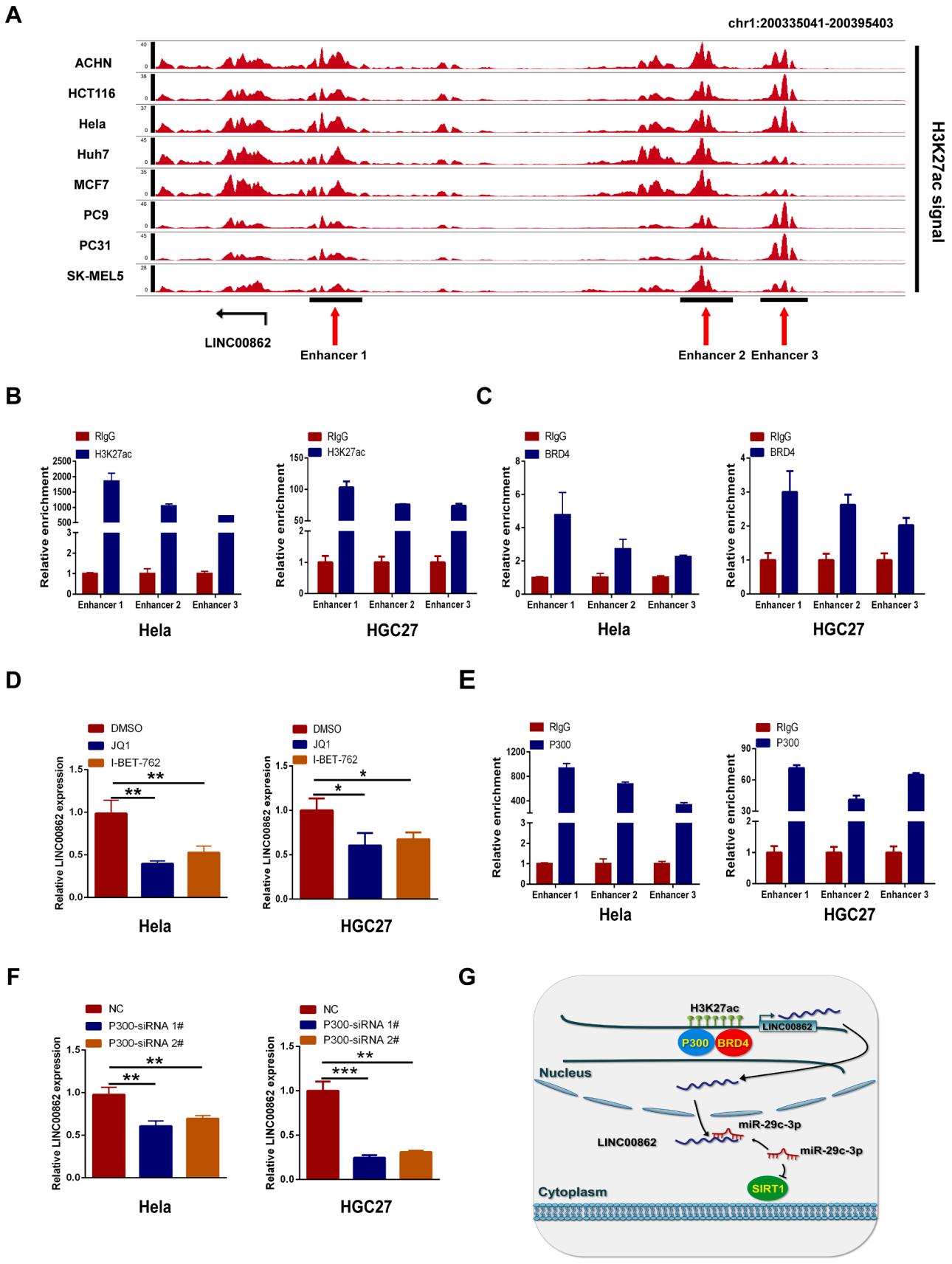

Fig. 7. P300-mediated super-enhancer activity stimulated LINC00862 transcription. (A) Gene tracks of H3K27ac ChIP-seq occupancy at LINC00862 gene loci in different cell lines. (B) The relative enrichment levels of H3K27ac to LINC00862 gene loci by ChIP-qPCR. (C) The relative enrichment levels of BRD4 to LINC00862 gene loci by ChIP-qPCR. (D) The relative LINC00862 expression after the treatment with JQ1 (1uM) or I-BET-762 (2uM) by qPCR. (E) The relative enrichment levels of P300 to LINC00862 gene loci by ChIP-qPCR. (F) The relative LINC00862 expression after P300 knockdown by qPCR. (G) Working model. (*$P < 0.05$, **$P < 0.01$).

Recent study demonstrated that SIRT1 could be delivered by extracellular vesicles to regulate immune response [57]. Therefore, we suppose whether LINC00862 regulates the immune microenvironment by either SIRT1 or other genes? Of course, more in vitro and in vivo assays are needed to validate our hypothesis. Owing to the infeasibility for targeting lncRNA in the clinical work now, we wanted to target SIRT1 to achieve the effect of inhibiting this signal axis in tumors. Recently, many new drugs targeting SIRT1 have been designed [58–61]. But the effect and toxicity of these drugs needs more in vitro and vivo assays to test. In our study, by comprehensive bioinformatic prediction and molecular docking analysis, three sensitive drugs targeting SIRT1 were screened out. More importantly, we validated the effect of SIRT1 inhibitor-EX527 on cervical cancer cell by in vivo assays for the first time. EX527 has been verified to be effective in many tumor types [62–64]. Our study was helpful to expand the indications of this drug in tumors. Certainly, more clinical trials are needed to determine the effectiveness and toxicity of this drug.

Notably, our study also revealed the driving forces of LINC00862 in cervical cancer and gastric cancer. We discovered that abundant H3K27ac signals, the best marker for super-enhancer, exist at the LINC00862 gene loci. Thus, our research highlighted LINC00862 as a new super-enhancer lncRNA. Many molecular mechanisms likely exist to ensure super-enhancer activation and eRNA transcription [65,66]. Xu et al. found transcription factor YY1 cooperated with BRD2/4 to coactivate downstream targets [67]. Extraordinarily, acetyltransferase P300 was involved in activating super-enhancer-mediated gene transcription [37]. By coincidence, our study demonstrated that the LINC00862 expression was up to P300-mediated super-enhancer activity. Interestingly, SIRT1 could regulate different histone deacetylation such as H1K26ac, H3K9ac, H3K56ac and H4K16ac [68]. Our study indicated that super-enhancer activity (H3K27ac) could enhance LINC00862 expression which was in favor of SIRT1 expression. Hence, we assume that histone acetylation and deacetylation state could regulate mutually. Maybe a dynamic equilibrium exists between them. Collectively, our research analyzed the roles of LINC00862 in pan-cancer and revealed the specific regulatory mechanisms for LINC00862 in cervical cancer and gastric cancer cells. Of course, our article also has some limitations. For example, we need more clinical samples to further validate our hypothesis. More in vitro and in vivo assays are needed for exploring how LINC00862 regulates the immune microenvironment. And where is the hub site for super-enhancer activity at LINC00862 gene loci? All of these questions will be clarified in our future studies.

Conclusion

Collectively, LINC00862 could be a diagnostic and prognostic biomarker. LINC00862 could also be a predictive biomarker for immunotherapy. Super-enhancer activity is the driver for LINC00862 overexpression in cervical cancer and gastric cancer.

Availability of data and materials

Data are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This research was approved by The Institute of Research Medical Ethics Committee of the Affiliated Hospital of Shandong Second Medical University.

CRediT authorship contribution statement

Shaojun Liu: Writing – original draft, Methodology. **Zhaohui Wang:** Resources. **Lei Hu:** Validation. **Chao Ye:** Formal analysis. **Xubin Zhang:** Investigation. **Zhiqiang Zhu:** Investigation. **Jiaqiu Li:** Writing – original draft, Software, Project administration, Methodology, Conceptualization. **Qi Shen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2024.101982.

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