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A comprehensive exploration of twist1 to identify a biomarker for tumor immunity and prognosis in pan-cancer

Yue Wang, MD[a,b], Chunhao Li, MD[b], Tianjiao Jiang, MD[b], Yiqiang Yin, MD[b,c], Yaowen Wang, MD[a], Hui Zhao, MD[b,d], Liang Yu, MD, PhD[b,*]

Abstract

Twist1 has been identified as a critical gene in tumor, but current study of this gene remains limitative. This study aims to investigate its roles and potential mechanisms across pan-cancer. The study used various databases and computational techniques to analyze twist's RNA expression, clinical data, gene mutations, tumor stemness, tumor microenvironment, immune regulation. Furthermore, the experimental method of fluorescence staining was carried out to identify twist1 expression in various tumor masses. After analyzing the protein-protein interaction of TWIST, enrichment analysis and predictive potential drugs were performed, and molecular docking was conducted to validate. We found that twist1 expression was significantly higher in various types of cancer and associated with tumor stage, grade, and poor prognosis in multiple cancers. Differential expression of twist1 was linked to gene mutation, RNA modifications, and tumor stemness. Additionally, twist1 expression was positively associated with tumor immunoregulation and immune checkpoint. Salinomycin, klugline, isocephaelince, manassantin B, and pimonidazole are predictive potential drugs targeting TWIST1. This study revealed that twist1 plays an important role in tumor, and might be a curial marker in tumor diagnose and prognosis. The study also highlighted twist1 as a promising therapeutic target for cancer treatment and provided a foundation for future research.

Abbreviations: BLCA = bladder urothelial carcinoma, BRCA = breast invasive carcinoma, CESC = cervical squamous cell carcinoma and endocervical adenocarcinoma, CHOL = cholangiocarcinoma, COAD = colon adenocarcinoma, COADREAD = colon adenocarcinoma/rectum adenocarcinoma esophageal carcinoma, DFI = disease-free survival, DNAss = DNA methylation-based stemness scores, DSS = disease-specific survival, EMT = epithelial-mesenchymal transition, ESCA = esophageal carcinoma, FPPP = FFPE pilot phase II, GBM = glioblastoma multiforme, GBMLGG = Glioma, GO = gene ontology, HNSC = head and neck squamous cell carcinoma, KIPAN = Pankidney cohort (KICH + KIRC + KIRP), KIRC = kidney renal clear cell carcinoma, KIRP = kidney renal papillary cell carcinoma, KM = Kaplan–Meier, LAML = acute myeloid leukemia, LGG = brain lower-grade glioma, LIHC = liver hepatocellular carcinoma, LUAD = lung adenocarcinoma, LUSC = lung squamous cell carcinoma, MESO = mesothelioma, NB = neuroblastoma, OS = osteosarcoma, OV = ovarian serous cystadenocarcinoma, PAAD = pancreatic adenocarcinoma, PCPG = pheochromocytoma and paraganglioma, PFI = progression-free interval, PPI = protein-protein interaction, PRAD = prostate adenocarcinoma, READ = rectum adenocarcinoma, SARC = sarcoma, SKCM = skin cutaneous melanoma, STAD = stomach adenocarcinoma, STES = stomach and esophageal carcinoma, TARGET = therapeutically applicable research to generate effective treatment, TCGA = the cancer genome atlas, THCA = thyroid carcinoma, THYM = thymoma, TMB = tumor mutation burden, TME = tumor microenvironment, twist1 = the twist family bHLH transcription factor 1, UCEC = uterine corpus endometrial carcinoma, UCS = uterine carcinosarcoma, UVM = uveal melanoma, WT = High-risk Wilms tumor.

Keywords: immunotherapy, pan-cancer, the twist family bHLH transcription factor 1, tumor immunity, tumor microenvironment

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All data generated or analyzed during this study are included in this published article [and its supplementary information files].

The studies involving human samples were approved by Shandong Provincial ENT Hospital Ethical Committee, and followed the Declaration of Helsinki.

[a] Department of Otolaryngology–Head and Neck Surgery, The first affiliated hospital of Ningbo University, Ningbo, China, [b] Department of Otorhinolaryngology-Head and Neck Surgery, Shandong Provincial ENT Hospital, Shandong University, Jinan, China, [c] Department of Pathology, Jinan Fourth People's Hospital, Jinan, China, [d] Department of Otorhinolaryngology-Head and Neck Surgery, Linyi People's Hospital, Linyi, China.

** Correspondence: Liang Yu, Department of Otorhinolaryngology-Head and Neck Surgery, Shandong Provincial ENT Hospital, Shandong University, Jinan 250000, China, (e-mail: entspyl@126.com).*

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1. Introduction

Tumors are diseases characterized by mutations and a complex tumor microenvironment (TME). The objective of tumor therapy is to eliminate malignant cancer cells while preserving nonmalignant cells, such as stroma cells and immune cells.[1,2] While there are various therapy methods available, including surgery, radiotherapy, and chemotherapy, early detection and gene-targeted treatment are crucial for a positive outcome in tumor patients. A crucial pan-cancer gene can play a key role in inhibiting tumor growth, stimulating the immune system's antitumoral response, and improving immunogenic therapy.[3,4] Hence, identifying pan-cancer genes is a valuable strategy for understanding tumorigenesis and improving tumor therapy.

The twist family bHLH transcription factor 1 (twist1) gene codes for a protein that influences embryonic development. Reflective of its significant involvement in early embryogenesis, twist1 demonstrates prominent expression within tissues originating from the mesodermal germ layer, thereby playing a substantive role in the differentiation and maturation of mesodermal tissues.[5] Positioned within the human genome at the chromosomal locus 7q21.2, twist1 operates as a transcription factor, principally exerting its regulatory functions within the nucleus of cells.[6] Recent investigations have elucidated twist1's primary expression in various cell types including myofibroblasts, alveolar epithelial cells, renal tubular epithelial cells, oral mucosa cells, hepatocytes, mammary epithelial cells, adipocytes, epidermal cells, among others. Twist1 is a critical transcription factor involved in the epithelial-mesenchymal transition (EMT), which plays a role in tumor metastasis.[7] Our previous study showed that twist1 can promote tumor metastasis by inducing EMT.[8,9] EMT affects cell interactions with the extracellular matrix, leading to intermediate cell states that act as tumor stem cells in the TME, causing tissue fibrosis and impacting the immunobiology of cancer.[10,11] EMT, with its properties of carcinogenesis, also enhances mobility and facilitates invasion.[12] Besides, certain investigations have suggested that twist-induced EMT may not serve as the primary determinant governing invasion and metastasis. Instead, it has been proposed that EMT modulation could impact the therapeutic efficacy in pancreatic cancer management.[13] Furthermore, numerous studies suggest that the TWIST1 protein promotes invasion and metastatic recurrence, which affects the tumor's stage and grade in diagnosis and influences patient outcomes.[13,14] Recent investigations have studied that TWIST1 proteins facilitate oncogenesis by impeding the cellular aging process and apoptosis. During the initial phases of cancer development, twist1 fosters tumor progression and confers resistance to therapeutic interventions by counteracting apoptotic and senescence mechanisms. Augmented expression of TWIST1 proteins has been documented to exert a significant influence on the initiation, advancement, and metastatic dissemination of cancer across various human solid tumors, encompassing diverse carcinoma and sarcoma subtypes, gliomas, neuroblastomas, and melanomas. Moreover, alterations in the expression patterns of these proteins have been observed in numerous hematologic malignancies such as leukemia, lymphoma, and myelodysplastic syndromes.

Novel immunotherapy combinations show promise as frontline treatments, with some encouraging anti-tumor effects. The most well-known immune checkpoint molecule is PD-1. Previous studies have shown that twist1 is upregulated by the PD-1 ligand, promoting tumorigenesis by mediating EMT.[15] There have also been advancements in twist1-related vaccines to improve immunotherapy against a broad range of twist1-expressing tumors.[16,17] These findings suggest that twist1 is a highly immunogenic shared antigen and a promising target for cancer immunotherapy. However, a comprehensive analysis of the landscape of twist1 in pan-cancer is still lacking. This study aims to fill this gap by performing a comprehensive analysis and revealing the landscape of twist1 in pan-cancer (Fig. 1), which may aid in mechanism research and clinical therapy.

2. Materials and methods

2.1. Data acquisition

RNA expression, clinical, and prognostic data were obtained from the TCGA, TARGET, and GTEx databases using the UCSC browser (https://xenabrowser.net/). The credible outcomes were integrated from TCGA and TARGET for supplementation, following the methodology described in a previous study.[18] The abbreviations of the tumors are listed in Table 1.

2.2. Expression analysis

After collecting those data, the R studio software was carried out to calculate differentially expressed twist1 in diversity cancers, as well as in various stages and grades of cancers.

2.3. Survival analysis

The prognostic values were measured using overall survival (OS), progression-free interval (PFI), disease-free survival (DFI), and disease-specific survival (DSS). Kaplan–Meier (KM) survival analysis was conducted to compare the survival outcomes between high and low twist1 expression. A Cox proportional hazards regression model was created to assess the relationship between twist1 expression and the prognosis of cancers using the coxph function of the survival R package. The KM survival analysis was calculated using the survival R package in combination with the maxstat R package.

2.4. Analysis of gene mutation and heterogeneity

Level 4 simple nucleotide variation data was downloaded from the TCGA database via GDC (https://portal.gdc.cancer.gov/). After integration of the mutation information, we got the structure domain of proteins and the results of the tumor mutation burden (TMB) using the R package of maftools.

2.5. Analysis of RNA modifications and cancer stemness

Data from multiple databases was analyzed to obtain 3 classes of RNA modification genes (m1A, m5C, and m6A) after filtering the collected samples. Referring to the previous study,[19] the DNA methylation-based stemness scores (DNAss) were obtained. Pearson's correlation was used to analyze the correlation between 5 immune pathways and DNAss across cancers.

2.6. TME and immune regulation

Based on the collected data, the expressive data of twist1 and 5 types of immune pathway were obtained. Then, ESTIMATE R package was conducted to obtain the ESTIMATESore, ImmuneScore, and StromalScore. IOBR R package was performed to estimate the immune cells infiltration across cancers.[20,21] Pearson correlation of twist1 with the score of immune infiltration and immune genes was computed using the corr.test function of psych R package.

2.7. Immunofluorescence staining

The tumor masses were embedded in paraffin and sliced into 10 μm thick sections. The studies involving human samples were approved by Shandong Provincial ENT Hospital Ethical

Committee (20220404). The sections were then blocked with PBT-1 for 60 minutes at room temperature, and incubated overnight at 4°C with primary antibody (anti-TWIST1, 1:100, AF4009; Affinity, Jiangsu, China). The following morning, the sections were incubated with an associated secondary antibody conjugated with Alexa Fluor 647 (1:1000; Invitrogen, Carlsbad, CA) and DAPI (1:1000, D9542; Sigma-Aldrich, St. Louis, MO) for 1 hour in the dark at room temperature. The fluorescently labeled sections were observed using a laser scanning confocal microscope (Leica SP8; Leica, Wetzlar, Germany).

2.8. Protein-protein interaction (PPI) analysis and enrichment analysis

To analyze the interacting proteins of TWIST1, candidate targets were obtained from the STRING database (https://string-db.org/). The PPI information was calculated with a limitation of "medium confidence" and "Homo sapiens" in the species option.

Subsequently, to further uncover the potential information of TWIST1 and its interacting targets, a Gene Ontology (GO) analysis and KEGG enrichment were performed using the STRING database. The downloaded data was plotted using Bioinformatics (http://www.bioinformatics.com.cn/) (last accessed on January 07, 2023). The results were filtered based on the adjusted *P* value corrected by the FDR algorithm.

2.9. Drug prediction

In order to excavate potential drugs targeting twist1, the DGIdb database (https://www.dgidb.org), a drug-gene website, was searched using the target gene TWIST1 and its interacting data.

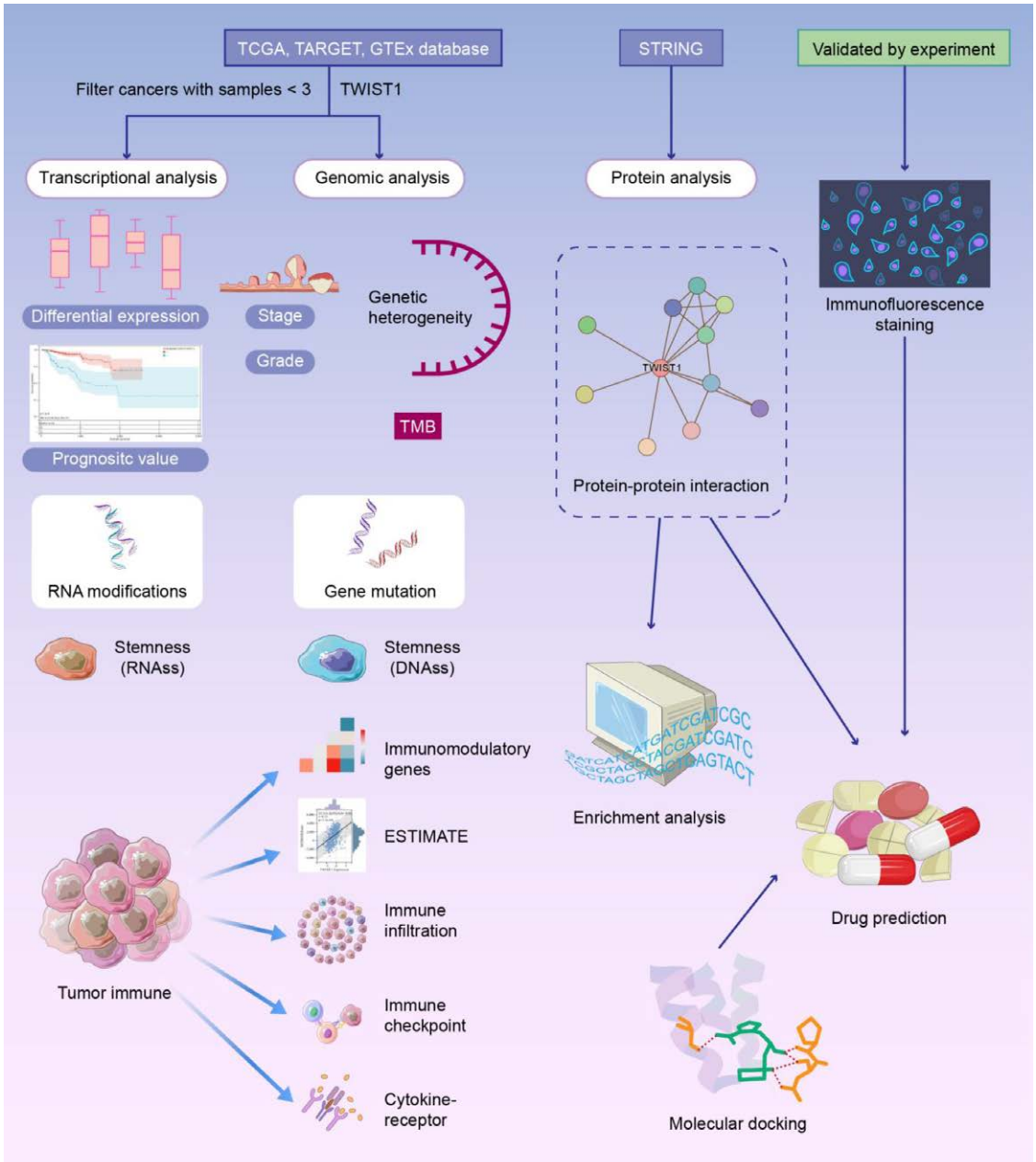

Figure 1. Graphical abstract. A flowchart.

2.10. Molecular docking

To theoretically simulate the potential effects of predictive drugs on the target sites of twist1, molecular docking was performed using AutoDock TOOLs (version 1.5.7). First, the 3D protein structure of twist1 was obtained from the PDB database (https://www.rcsb.org) and the molecular structures of the predictive drugs were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The PDBQT formats of the structures were obtained by removing water and adding hydrogen through PyMol and AutoDock Tools. Next, a 3D grid box for molecular docking that completely covered the protein chains was designed using AutoDock Tools. Finally, the docking models with the lowest binding affinity were visualized using PyMol.

2.11. Statistical analysis

The log2 transformation was performed on all collected data. The Student *t* test was utilized to compare two groups, and the one-way ANOVA was used to compare multiple groups. A Logrank test was performed to analyze prognostic values, and *P* value < .05 was considered significant.

3. Results

3.1. The expression of twist1 across pan-cancer

To determine if twist1 is differentially expressed in tumor and normal tissues, the level of inconsistent twist1 mRNA expression was integrated from multiple databases in 34 cancers. The results showed that twist1 was upregulated in 17 types of cancers compared to normal tissues in datasets of glioblastoma multiforme (GBM), glioma (GBMLGG), brain lower-grade glioma (LGG), lung adenocarcinoma (LUAD), colon adenocarcinoma/rectum adenocarcinoma esophageal carcinoma (COADREAD), prostate adenocarcinoma (PRAD), stomach adenocarcinoma (STAD), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), lung squamous cell carcinoma (LUSC), rectum adenocarcinoma (READ), pancreatic adenocarcinoma (PAAD), testicular germ cell tumors, uterine carcinosarcoma (UCS), acute myeloid leukemia (LAML), adrenocortical carcinoma, and cholangiocarcinoma (CHOL) (Fig. 2A). Meanwhile, downregulated expression of twist1 was observed in 12 types of cancer compared to normal tissues in uterine corpus endometrial carcinoma (UCEC), breast invasive carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), esophageal carcinoma (ESCA), stomach and esophageal carcinoma (STES), kidney renal papillary cell carcinoma (KIRP), skin cutaneous melanoma (SKCM), bladder urothelial carcinoma (BLCA), thyroid carcinoma (THCA), ovarian serous cystadenocarcinoma (OV), pheochromocytoma and paraganglioma (PCPG), and kidney chromophobe (Fig. 2A).

3.2. The staging and grading of tumors in relation to TWIST1 expression

To investigate the relationship between twist1 and stages and grades of tumors, the expression of twist1 was analyzed in different stages of tumors across various types of cancers. Our analysis of stages I, II, III, and IV showed that twist1 was closely related to tumor stage in 12 cancers, including colon adenocarcinoma (COAD), COADREAD, ESCA, STES, KIRP, pankidney cohort (KIPAN), STAD, KIRC, LUSC, thymoma (THYM), PAAD, and BLCA (Fig. 2B). The closest relationship was observed in Stage II for COAD, COADREAD, ESCA, STES, THYM, and PAAD, while Stage I was closely related to 4 other cancers. Moreover, significant differential expression of twist1 was found in tumor grade, with grade-associated differences identified in 7 cancers, including GBMLGG, LGG, CESC, KIPAN, HNSC, KIRC, and PAAD (Fig. 2C).

3.3. Prognostic value analysis of twist1 in pan-caner

The prognostic impact of twist1 on tumors was evaluated using multiple methods across various cancers. Our analysis included the assessment of OS, DSS, DFI, and PFI. The Cox regression model analysis of multiple datasets indicated that twist1 expression was significantly associated with OS in 14 cancers, with high levels of twist1 expression linked to poor prognosis in patients with GBMLGG, LGG, KIRP, KIPAN, GBM, KIRC, SKCM-P, BLCA, THCA, mesothelioma (MESO), uveal melanoma (UVM), and PAAD (Fig. 3A). The top 4 cancers with the most significant correlation between twist1 expression and poor outcomes were visualized using Kaplan–Meier curves (Figs. 3B-E). Our analysis also revealed a correlation between twist1 expression and DSS, with high levels of twist1 expression linked to poor prognosis in GBMLGG,

Table 1

List of abbreviations.

Abbreviations	Cancer
TCGA-ACC	Adrenocortical carcinoma
TCGA-BLCA	Bladder urothelial carcinoma
TCGA-BRCA	Breast invasive carcinoma
TCGA-CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
TCGA-CHOL	Cholangiocarcinoma
TCGA-COAD	Colon adenocarcinoma
TCGA-COADREAD	Colon adenocarcinoma/rectum adenocarcinoma esophageal carcinoma
TCGA-DLBC	Lymphoid neoplasm diffuse large b-cell lymphoma
TCGA-ESCA	Esophageal carcinoma
TCGA-FPPP	FFPE pilot phase II
TCGA-GBM	Glioblastoma multiforme
TCGA-GBMLGG	Glioma
TCGA-HNSC	Head and neck squamous cell carcinoma
TCGA-KICH	Kidney chromophobe
TCGA-KIPAN	Pankidney cohort (KICH + KIRC + KIRP)
TCGA-KIRC	Kidney renal clear cell carcinoma
TCGA-KIRP	Kidney renal papillary cell carcinoma
TCGA-LAML	Acute myeloid leukemia
TCGA-LGG	Brain lower-grade glioma
TCGA-LIHC	Liver hepatocellular carcinoma
TCGA-LUAD	Lung adenocarcinoma
TCGA-LUSC	Lung squamous cell carcinoma
TCGA-MESO	Mesothelioma
TCGA-OV	Ovarian serous cystadenocarcinoma
TCGA-PAAD	Pancreatic adenocarcinoma
TCGA-PCPG	Pheochromocytoma and paraganglioma
TCGA-PRAD	Prostate adenocarcinoma
TCGA-READ	Rectum adenocarcinoma
TCGA-SARC	Sarcoma
TCGA-STAD	Stomach adenocarcinoma
TCGA-SKCM	Skin cutaneous melanoma
TCGA-STES	Stomach and esophageal carcinoma
TCGA-TGCT	Testicular germ cell tumors
TCGA-THCA	Thyroid carcinoma
TCGA-THYM	Thymoma
TCGA-UCEC	Uterine corpus endometrial carcinoma
TCGA-UCS	Uterine carcinosarcoma
TCGA-UVM	Uveal melanoma
TARGET-OS	Osteosarcoma
TARGET-ALL	Acute lymphoblastic leukemia
TARGET-NB	Neuroblastoma
TARGET-WT	High-risk Wilms tumor

TCGA is for the cancer genome atlas; TARGET is for therapeutically applicable research to generate effective treatment.

LGG, KIPAN, KIRP, UVM, KIRC, MESO, GBM, SKCM-P, and PAAD (Fig. 3F). The Cox regression model analysis of DFI data showed that upregulated twist1 expression with poor prognosis was associated with KIRP, PRAD, PAAD, and MESO (Fig. 3G). Furthermore, the Cox regression model analysis showed that upregulated twist1 expression with poor prognosis impacted PFI in GBMLGG, LGG, CESC, KIRP, KIPAN, KIRC, UVM, and PAAD (Fig. 3H).

3.4. Relationship of twist1 expression with gene mutation and heterogeneity

To assess the relationship between twist1 expression and gene mutations, differentially expressed genes of cancers were calculated in various kinds of clinical stages. Our results showed that twist1 mutations were associated with 4 cancers, namely COAD, COADREAD, STES, and STAD (Fig. 4A). The vertical lollipop graph depicted the specific locations of the mutations, mainly including missense and nonsense mutations (Fig. 4B). Furthermore, to measure the number of mutations and the immune response of the tumor cells, we calculated TMB using integrated data. Our analysis revealed a positive correlation between twist1 expression and TMB in LUAD, and a negative correlation between twist1 expression and TMB in KIRP (Fig. 4C).

3.5. Relationship between twist1 expression and RNA modifications

In order to investigate the epigenetics influence on twist1, RNA modification was carried out. The RNA regulators were divided into 3 categories: m1A, m5C, and m6A, and also into 3 types: writers, erasers, and readers, the writer for the methyltransferase complexes, the eraser for demethylases, and the reader for

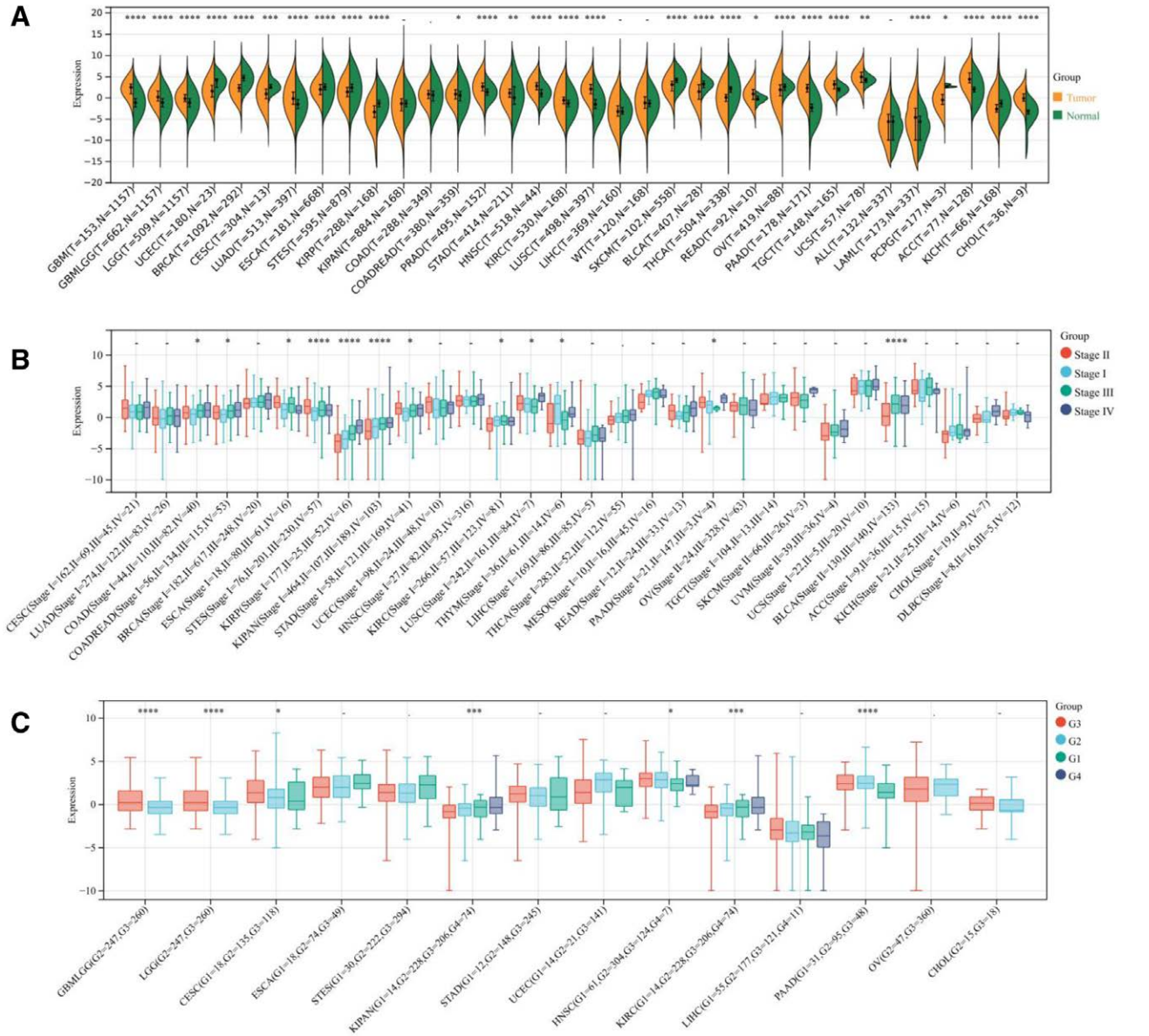

Figure 2. The expression of twist1 in pan-cancers. (A) The mRNA expressions of twist1 in pan-cancers (yellow) and normal tissues (green) was integrated from the TCGA, TARGET, and GTEx databases. (B) The expression of twist1 in different stages of pan-cancers. Stage I, blue, stage II, red, stage III, green, and stage IV, purple. (C) The expression of TWIST1 in different grades of pan-cancers. Grade 1, green, grade 2, blue, grade 3, red, and grade 4, purple. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

the binding proteins. The results showed that twist1 expression was positively correlated with RNA regulators across various cancers (Fig. 4D). Particularly, twist1 expression was markedly positively associated with most RNA modifications in PAAD, OV, liver hepatocellular carcinoma (LIHC), and UCEC. Twist1expression was also significantly positively associated with most cancers in the RNA regulators YTHDF3, DNMT1, ALKBH5, and YTHDF3.

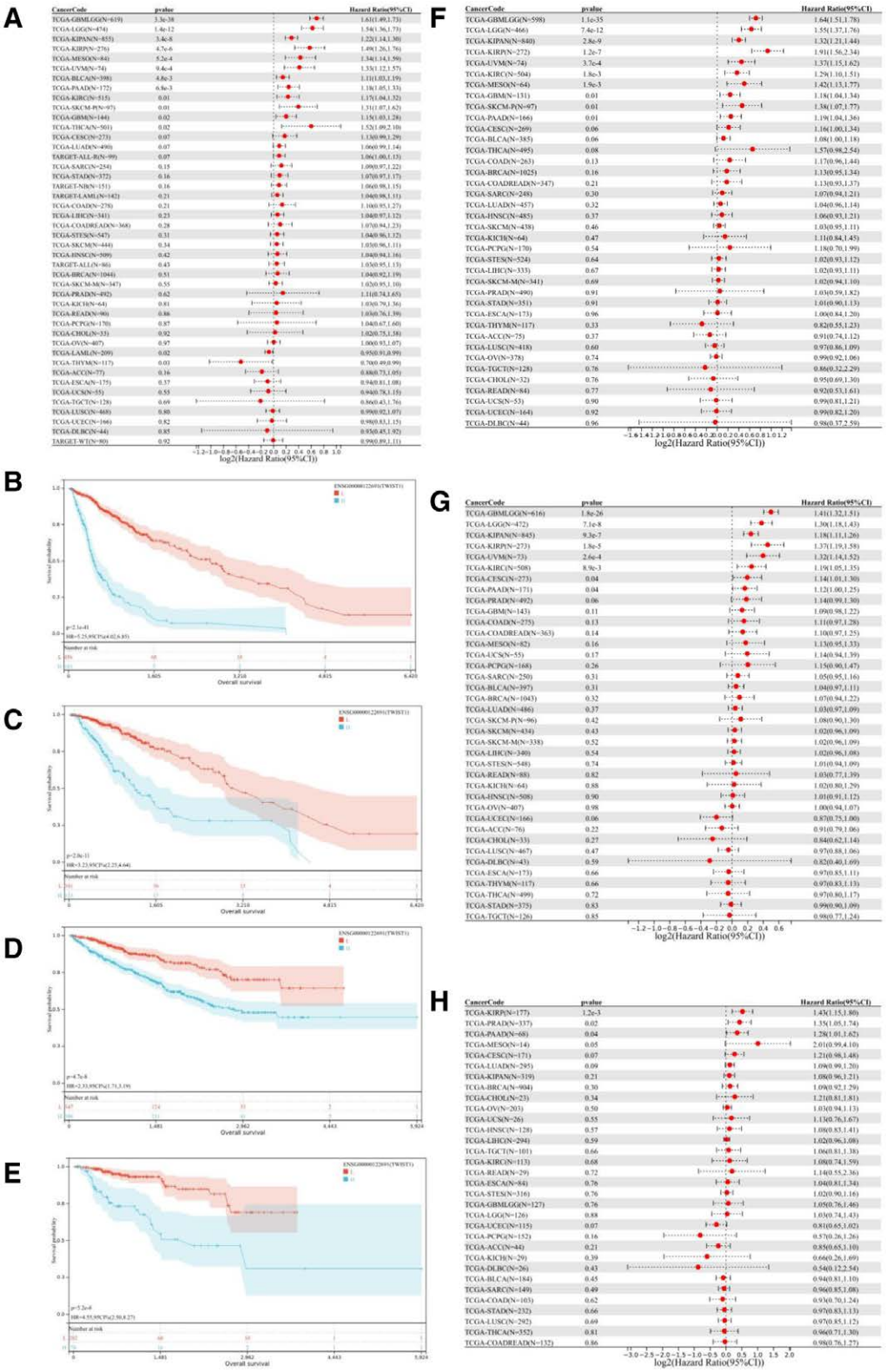

Figure 3. The prognostic significance of twist1 expression. (A) The relationship between twist1 and overall survival (OS). (B–E) Kaplan–Meier analysis of the relationship between twist1 expression and OS in GBMLGG, LGG, KIPAN, and KIRP. (F) The relationship between twist1 and disease-specific survival (DSS). (G) Association between twist1 and disease-free interval (DFI). (H) Association between twist1 and progression-free interval (PFI).

3.6. Pan-cancer analysis of twist1 expression and caner stemness

Besides the factors mentioned above that contribute to tumorigenesis, we conducted an evaluation of cancer stemness to reflect the potential of a tumor's ability to self-renew. This correlational study showed that the expression of twist1 in 21 cancers was associated with cancer stemness (Fig. 4E). The expression of twist1 was positively correlated with cancer stemness in 9 types of cancer, including GBMLGG, LGG, SARC, KIRP, KIPAN, KIRC, THYM, THCA, and UCS. On the other hand, a negative correlation between twist1 and cancer stemness was found in 12 types of cancer, including COAD, COADREAD, BRCA, ESCA, STES, STAD, PRAD, HNSC, LIHC, PCPG, BLCA, and lymphoid neoplasm diffuse large b-cell lymphoma.

3.7. Relationship of twist1 expression and tumor purity

In order to understand the components that make up the tumor microenvironment (TME), the ESTIMATE algorithm was carried out to evaluate the scores of immune and stromal cells across various cancers. The results demonstrated that a majority of cancers were significantly positively correlated with the three immune-scores. The heatmap visualizes the *P* value of the correlation between twist1 expression and the three scores (Fig. 5A). Additionally, Figure 5B–J displays the top 3 cancers with the strongest correlations between twist1 expression and each score.

3.8. TME and immune checkpoint associated with twist1 in pan-caner analysis

To analyze the immune phenotypes further, the significance of twist1 and infiltrating immune cells was investigated across all types of cancer. The results revealed that twist1 expression was significantly correlated with immune infiltration in 44 types of cancer, as shown in Figure 6A. Interestingly, twist1 expression was strongly correlated with almost all infiltrating cells in KIPAN and GBMLGG. In nearly all types of cancer, astrocytes and fibroblasts were also found to be correlated with twist1 expression.

Also, to investigate whether twist1 affected immunomodulators, the genes contributing to immunoregulation associated with expression twist1, were explored across cancers. As can be seen in Figure 6B, most of the cancers were positive related to immunomodulators, including chemokine, receptor, MHC, immunoinhibitory and immunostimulatory. Surprisingly, twist1 expression was strongly positive associated with all the of regulators surveyed in PAAD, UVM, THCA, BRCA, and OV. And an opposite result was found in neuroblastoma (NB) and LAML with a negative association. Similarly, the expression of twist1 in most of cancers were positively associated with these immunomodulators, including CCL3, CCL4, CCL11, CCL26, CCL8, CXCL8, CD276, CXCR4, and TNFSF18.

Finally, the role of twist1 in immunotherapy was studied by analyzing immune checkpoint expression. The results showed that a positive correlation was found between twist1 expression and immune checkpoints in most cancers (Fig. 6C). Moreover, twist1 expression was significantly

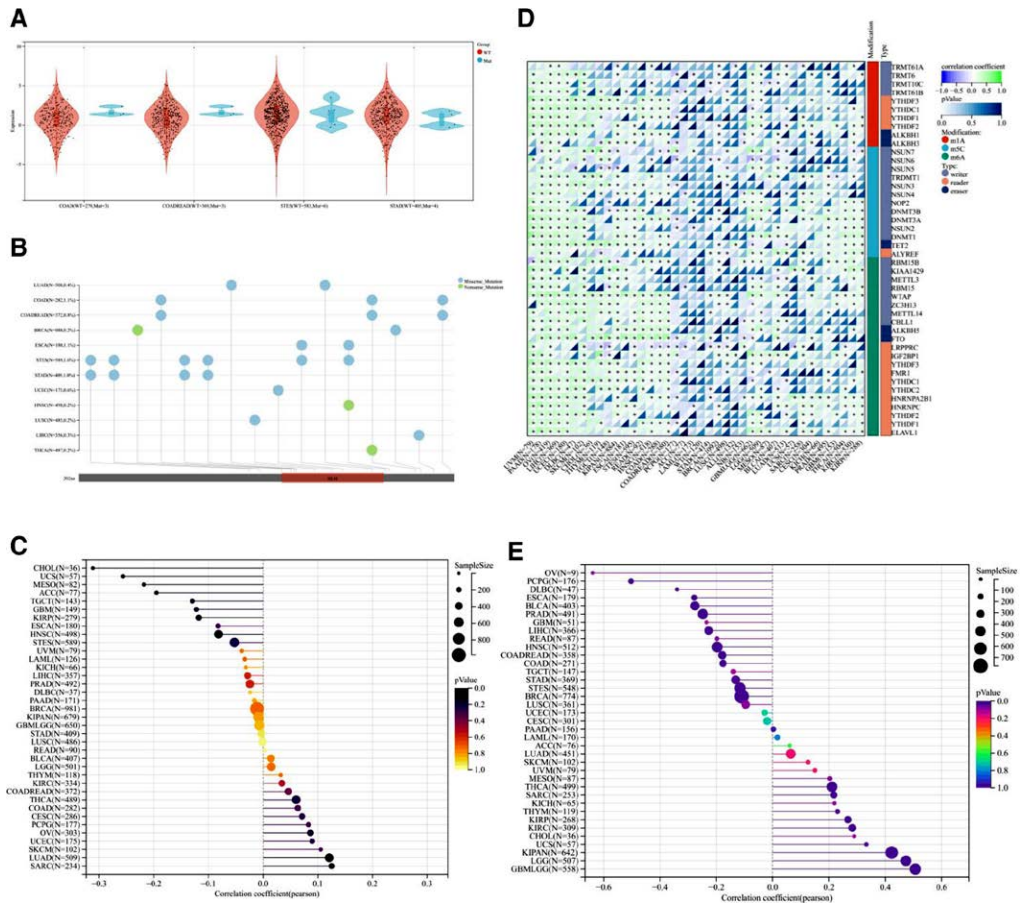

Figure 4. The expression of twist1 was correlated with DNA, RNA, and stemness instability in cancers. (A) The association between twist1 expression and gene mutation. (B) The landscape of twist1 single nucleotide variants in pan-cancers, including missense and nonsense mutations. (C) The correlation between twist1 expression and tumor mutational burden (TMB). (D) A heatmap depicts the relationship between RNA modifications and twist1 expression. (E) The correlation between twist1 expression and tumor stemness.

positively related to PAAD, READ, COAD, COADREAD, and OV in all of the targeted immune checkpoints. Only in NB was there a clear negative relationship between twist1 and these immune checkpoints. Among the other immune checkpoints, CD276 was definitely positively related to twist1 across all types of cancer.

3.9. PPI network construction and enrichment analysis

To deeply excavate the function of TWIST1, a PPI network was obtained from the STRING database. The network consisted of a central seed, represented by TWIST1, and 10 connected seeds, which represent the proteins interacting with TWIST1 (Fig. 7A). These interacting proteins included TCF3, TCF12, SOX2, ZEB1, G3BP2, STAT3, TP53, HIF1A, RUNX2, and PPARGC1A.

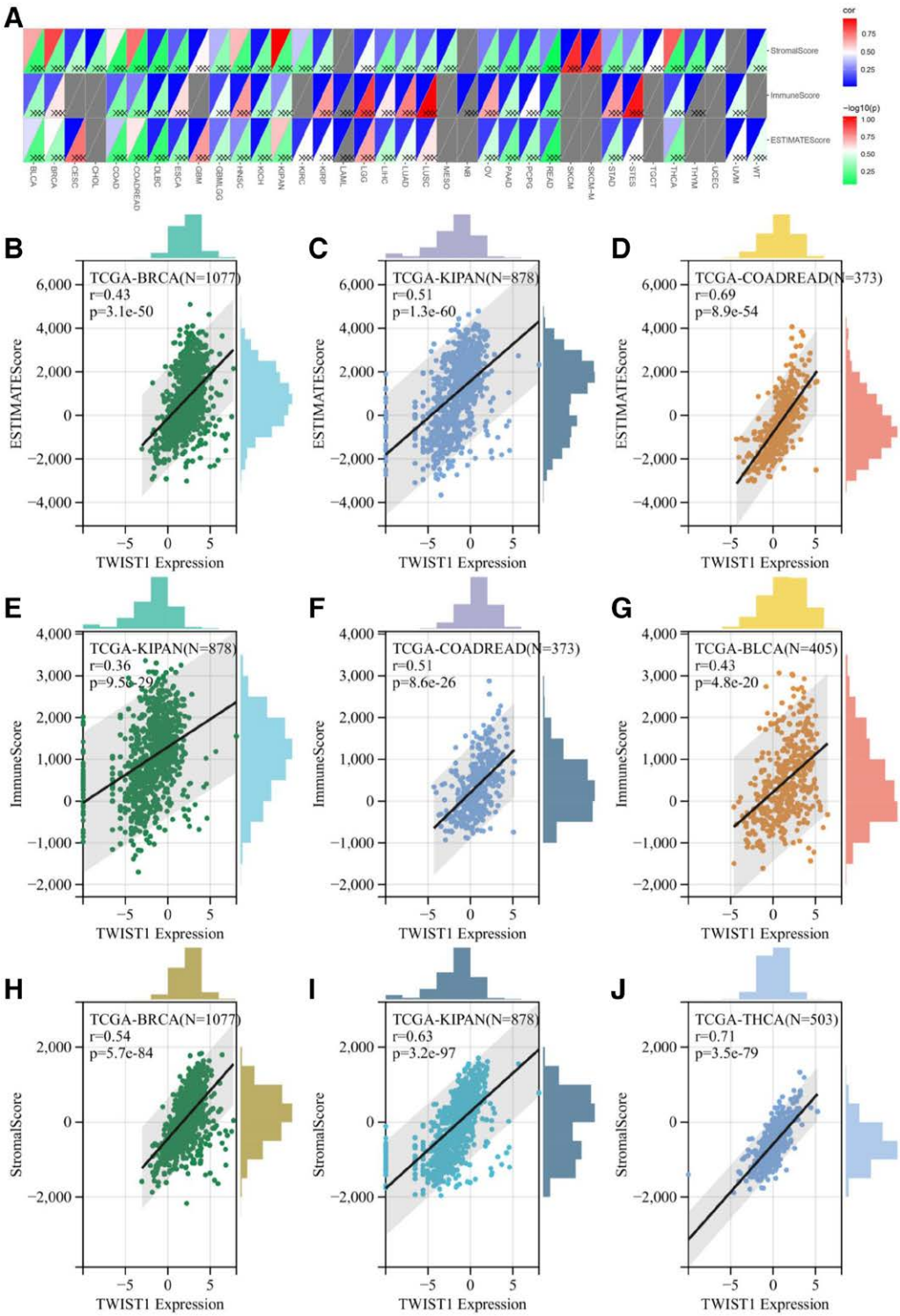

Figure 5. The expression of twist1 was correlated with tumor purity. (A) A heatmap shows the correlation between twist1 expression and ESTIMATEScore, ImmuneScore, and StromalScore. (B–D) Pearson correlation analysis of the relationship between TWIST1 expression and the top 3 tumors with ESTIMATEScore. (E–G) Pearson correlation analysis of the relationship between twist1 expression and the top 3 tumors with ImmuneScore. (H–J) Pearson correlation analysis of the relationship between twist1 expression and the top 3 tumors with StromalScore. *P < .05, **P < .01, ***P < .001, and ****P < .0001.

To further elucidate the biological processes (BP) of twist1 and its interacting targets, a GO enrichment analysis was performed using the STRING database. The top 5 significantly enriched BPs were visualized in a bubble chart (Fig. 7B). The results showed that twist1's potential functions primarily include regulation of transcription, DNA-templated regulation, regulation of cell proliferation, cellular response to cytokine stimulation, positive regulation of transcription by RNA polymerase II, and embryonic morphogenesis. The GO enrichment analysis indicated that twist1 expression played a positive role in tumor cell invasion, metastasis, and recurrence.

Similarly, in order to study twist1's signaling pathways across all types of cancer, a KEGG enrichment analysis was

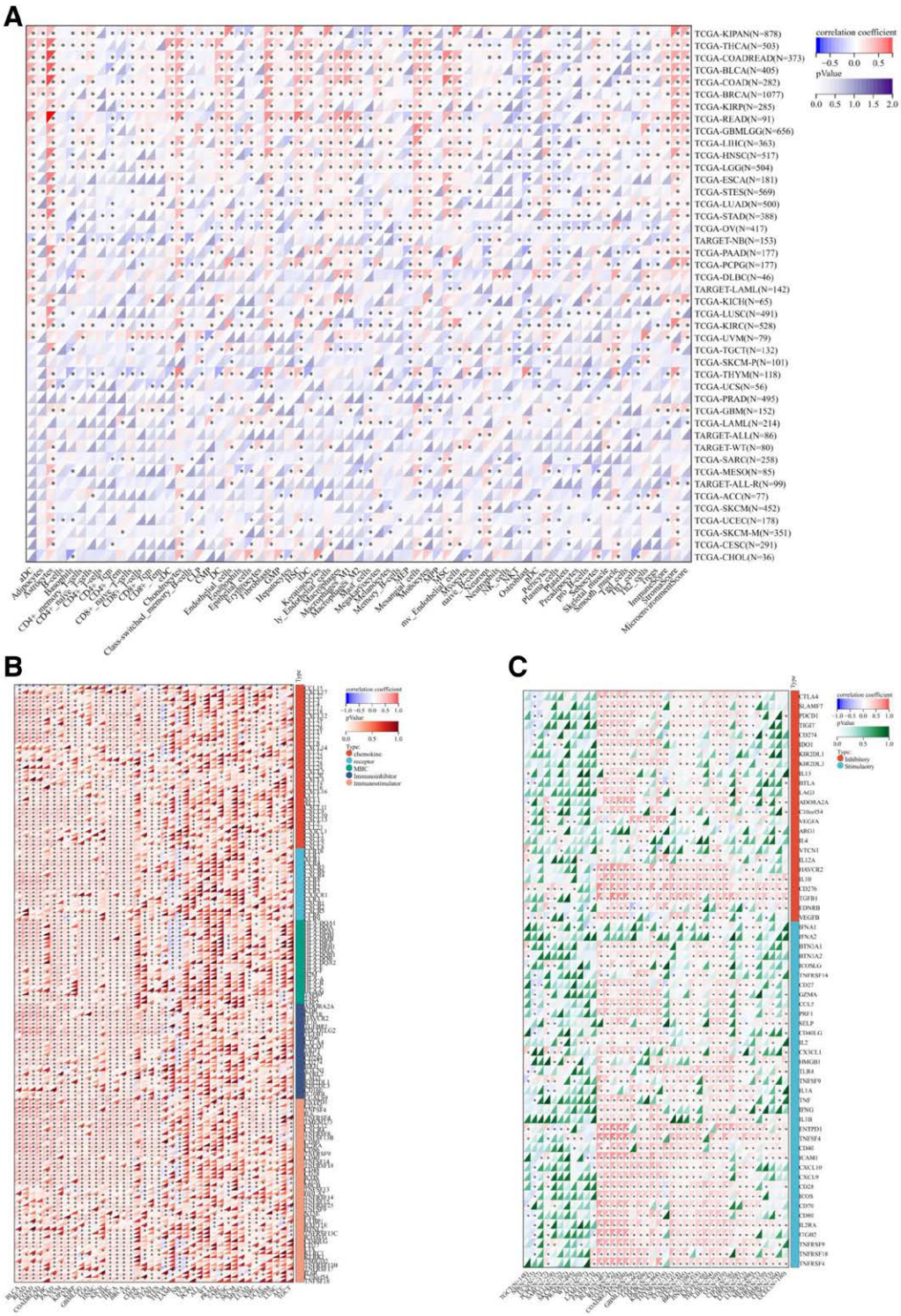

Figure 6. The expression of twist1 in tumor immunity in pan-cancer. (A) The relationship between twist1 expression and immunocyte infiltration across pan-cancer. (B) The relationship between twist1 expression and immunoregulation across pan-cancer. (C) The association between twist1 expression and immune checkpoint across pan-cancer.

performed using the STRING database. The top 5 enriched pathways were displayed in bubble diagrams, including Th17 cell differentiation, signaling pathways regulating stem cell pluripotency, the PD-L1 expression and PD-1 checkpoint pathway in cancer, transcriptional misregulation in cancer, and central carbon metabolism in cancer (Fig. 7C). The role of twist1 in these pathway enrichments was consistent with its role in the GO analysis, affecting the occurrence and development of tumors.

3.10. Immunofluorescence staining of TWIST1 expression on tumor

To experimentally validate TWIST1 expression, immunofluorescence staining was conducted on various types of tumor sections. The results showed that TWIST1 was expressed in 5 cancers, including CHOL, COADREAD, STAD, UCEC, and THCA (Fig. 8). TWIST1 expression was detected in the nuclei, and the fluorescence intensity appeared lower in tumor masses of UCEC, and THCA (Fig. 8). On the other

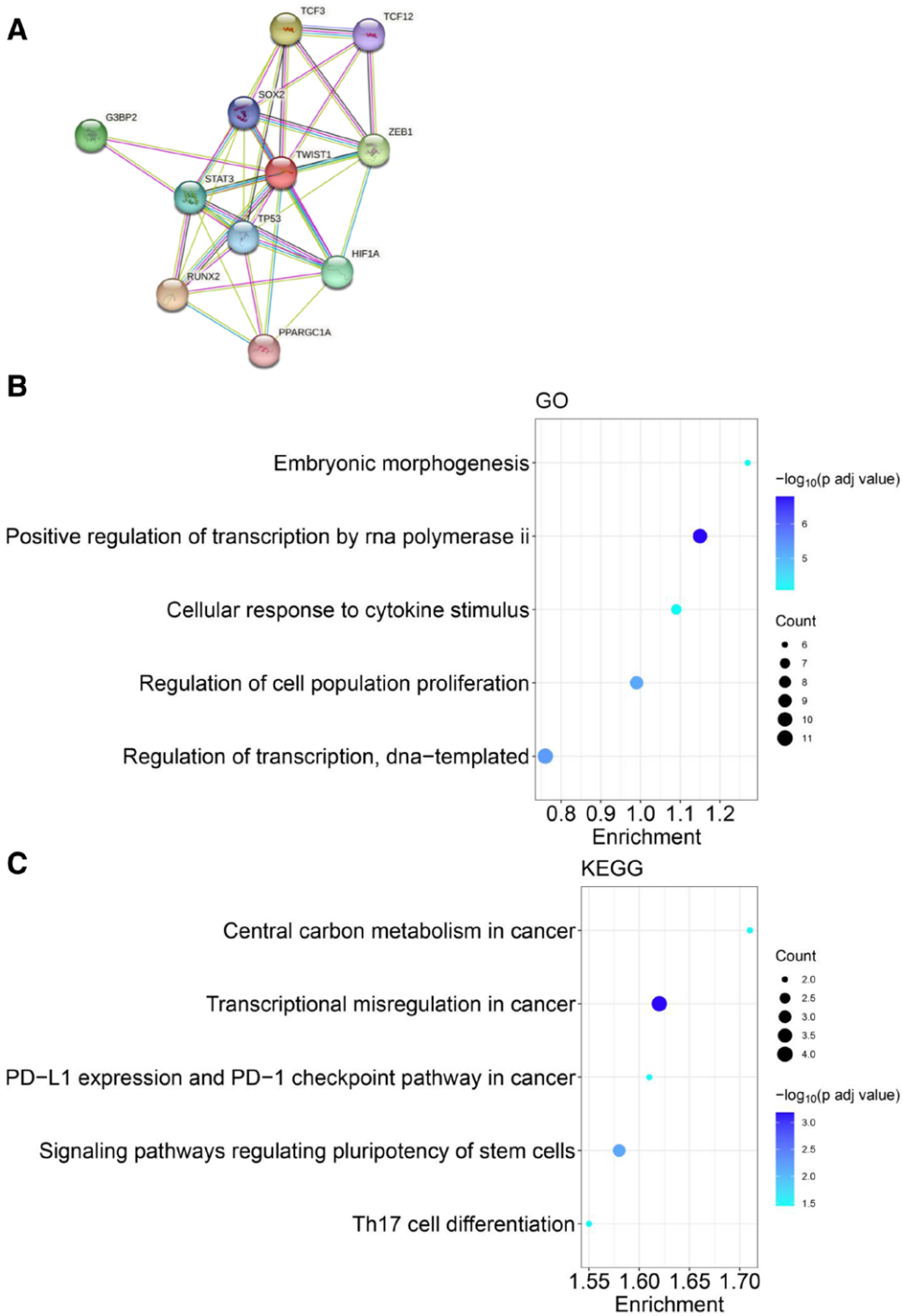

Figure 7. The enrichment analysis of TWIST1 and its interacting targets. (A) The protein-protein interaction network of TWIST1 and its top 10 interacting proteins. (B) The top 5 biological processes (BPs) of twist1 based on GO enrichment analysis. (C) The top 5 pathways of twist1 based on the KEGG enrichment analysis.

hand, the fluorescence intensity of TWIST1 seemed higher in the tumors of CHOL, COADREAD, and STAD. This result of fluorescence intensity was consistent with the differential expression found in various databases (Fig. 2A).

3.11. Drug prediction targeting twist1 and validation by molecular docking

The importance of pan-cancer research is to ensure the biological role of tumors and to find effective methods for treating them. Based on our analysis, we explored potentially effective drugs targeting twist1. The top 35 significant drugs were listed in a bubble chart (Fig. 9A). The top five predictive drugs were salinomycin, klugline, isocephaelince, manassantin B, and pimonidazole. To analyze the interactions between TWIST1 and the top 5 predictive drugs, we performed molecular docking. The results showed that all 5 drugs interacted with TWIST1 with binding affinities less than zero. Salinomycin had the strongest interaction with TWIST1, with a binding affinity of -5.36 kcal/mol. It formed 2 hydrogen bonds with LYS346 and 1 hydrogen bond with HIS341 and LYS349 (Fig. 9B). Klugline had a binding affinity of -9.63 kcal/mol and interacted with TWIST1 via 2 hydrogen bonds with ASP459 and 1 hydrogen bond with ASP337, PRO458, and MET457 (Fig. 9C). Isocephaelince had a binding affinity of -7.65 kcal/mol and interacted with TWIST1 via 1 hydrogen bond with PHE454, ASP459, and ASP337 (Fig. 9D). Manassantin B had a binding affinity of -5.22 kcal/mol and interacted with TWIST1 through 2 hydrogen bonds with LYS349 and 1 hydrogen bond with LYS346 (Fig. 9E). Pimonidazole had a binding affinity of -4.3 kcal/mol and interacted with TWIST1 through 2 hydrogen bonds with ASP337 and PRO336, and 1 hydrogen bond with ALA455 (Fig. 9F). The results indicated that TWIST1 and the predictive drugs formed tight bonds through complex interactions.

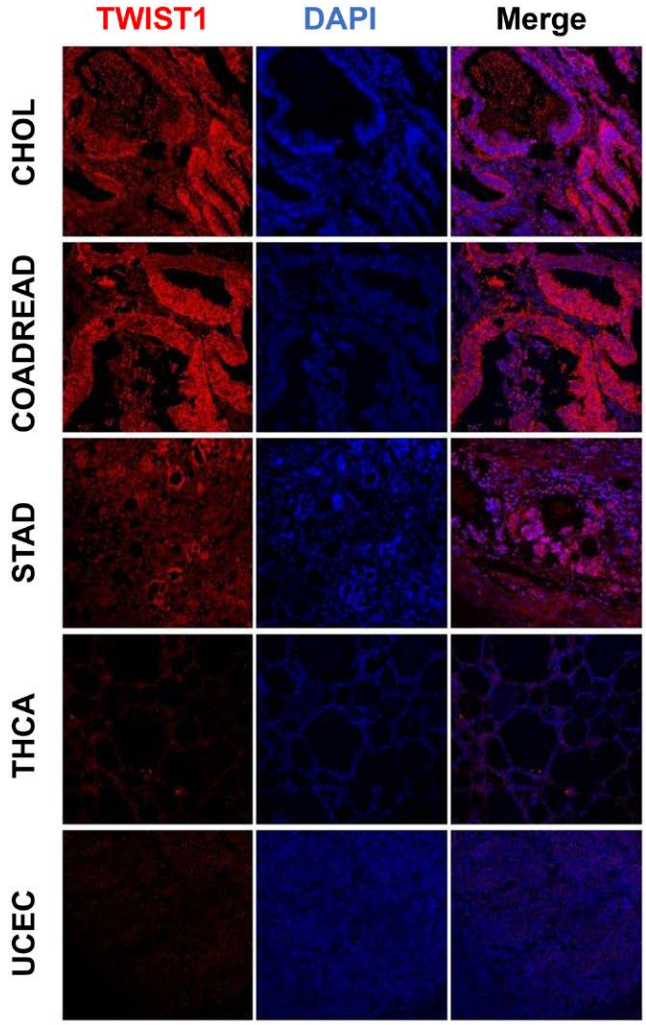

Figure 8. Immunofluorescence staining of TWIST1 in tumor tissue sections. The figure depicts the staining of TWIST1 in tumor tissue sections, with high expression of TWIST1 in CHOL, COADREAD, and STAD, and low expression in UCEC and THCA. twist1, red; cellular nuclei, blue (4′,6-diamidino-2-phenylindole) (×200 magnification).

4. Discussion

This study comprehensively investigated the role of twist1 expression in 34 cancers, incorporating complex data from the TCGA, TARGET, and GTEx. The findings suggested that twist1 expression changes were linked to tumorigenesis. Our previous research showed that upregulated twist1 expression was associated with the occurrence and metastasis of cancers in HNSC, which was consistent with this study.[8,22] Other studies have also shown that twist1 expression was upregulated in LUAD, LUSC, STAD, PAAD, and COADREAD. The current results showed that high twist1 expression in CHOL, COADREAD, and STAD and low expression in UCEC and THCA were supported by strong evidence from Immunofluorescence staining. The same relationship of twist1 with STAD can be found in study of Qi et al[23] Our results showed a significant relationship between twist1 expression and tumor stage. However, our study did not find a relationship between twist1 and the prognosis in STAD, which may be due to the contribution of other virulence genes that are coordinately expressed with twist1. Multi-database evidence suggested that overexpressed twist1 is associated with a poor prognosis in KIRP. Previous analysis by Sara Lovisa has found that twist1 plays an important role in the hybrid phenotype of EMT in kidney cancers.[24] A previous clinical observation by Arezoo Rasti showed that the level of twist1 expressed in the cytoplasm is positively associated with a poor prognosis in 252 patients with definite kidney tumors.[25] Many researchers have also shown that twist1 expression is linked to the tumor stage and grade of KIRP,[25,26] which was consistent with our multiple analyses. However, our results for tumor stage were focused on early stages, which might be due to more positive willingness and aggressive treatment of patients in the early stages compared to those in advanced stages.

Furthermore, our study found a positive association between twist1 expression and gene mutation in COAD, COADREAD, STES, and STAD. Upregulated twist1 expression was previously linked to genome instability and cellular heterogeneity in COAD.[27] According to a study by Cai M. Roberts et al, the Twist box of twist1 is a critical domain for activating downstream signaling for tumor metastasis.[28] Our results confirmed these findings and showed that twist1 expression impacted cancer stemness across pan-cancer. Notably, some studies are consistent with our findings, including the direct or indirect regulation of twist1 in tumor stemness.[29,30] For example, Katharina Proestling found a positive relationship between twist1 expression and endometrium,[31] which was in agreement with our results showing that twist1 affects stemness in UCS.

Here, we showed a strong positive association between twist1 expression and tumor purity, including ImmuneScore, StromalScore, and ESTIMATEScore across various cancers. Twist1 overexpression was found to regulate the TME. Analysis of immune infiltration showed that twist1 expression affects TME in KIPAN, in relation to tumor stemness and poor prognosis. Interestingly, a positive association between twist1 expression and tumor purity was also detected in KIPAN. Moreover, the fibroblast was one of the most important infiltrating cells, consistent with analysis of prognostic, staging and grading

of tumors. Twist1 plays a crucial role in EMT through renal fibrosis, inducing kidney diseases.[24,32,33] Our current data also confirmed a positive association between twist1 expression and GBMLGG. TWIST1 regulates EMT, inducing invasion and migration in GBMLGG.[34,35] Astrocytes are the major component of the tumor body in GBMLGG, and previous studies have reported the association of twist1 and astrocytes in cancers. Infiltrated astrocytes have a significant impact on tumor invasion, chemotherapy effects, and patient prognosis.[36–38] Therefore, the results suggest that intervention in twist1 expression to alter TME may be a promising method for tumor therapy.

In addition to the effects of twist1 on immune infiltration in the TME, our findings provide insight into how twist1 impacted immunoregulation. The present evidence showed a positive correlation between twist1 and immunomodulatory genes across different cancers. Among these factors, activation of CXCR4 by TWIST1 has been reported to play a role in cancer progression in various studies.[39–41] Based on the present data and on those available in the literature, we hypothesize that twist1 expression is strongly linked to factors in immunomodulation. We also explored the correlation between twist1 and immune checkpoints. Our results showed significant positive correlations between

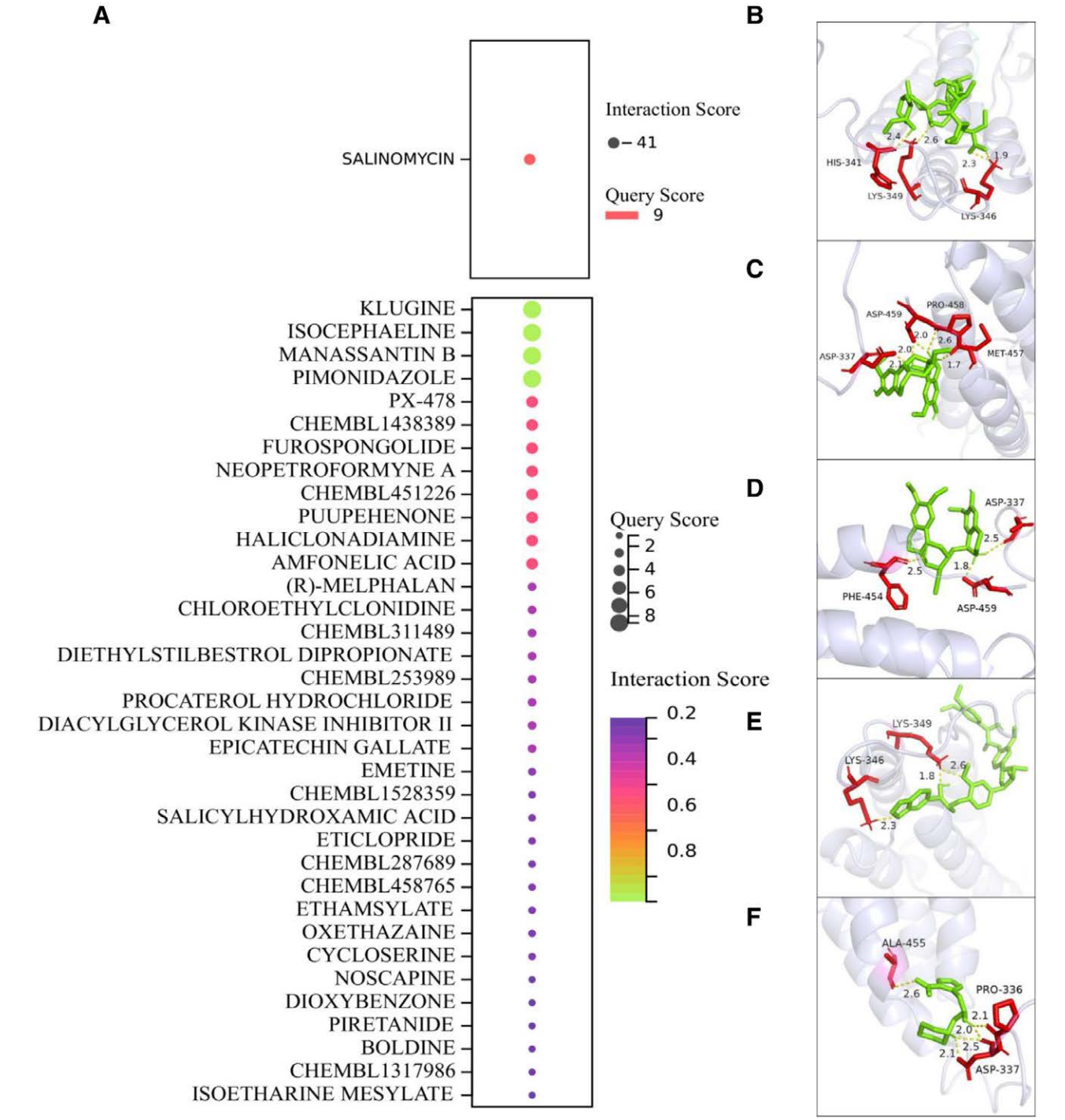

Figure 9. The predictive potential drugs for twist1. (A) A bubble chart showing the predicted drugs targeting twist1. (B) The molecular structures of the most effective drugs docked to the protein chain of TWIST1 (2 mjv).

twist1 and immune checkpoints, consistent with other bioinformatics analyses in cancers such as PAAD, READ, and COADREAD. Our expression analysis also revealed that twist1 expression was upregulated in these three types of tumors. Overexpression of twist1 has been linked to poor prognosis, RNA modifications, and resistance to immunotherapy in PAAD. Our results of immune checkpoints were strong supplements for immune cells infiltration in the complex TME. Yuki Yajima et al also found that twist1 is a promising target for immune-based cancer treatment due to its association with highly immunogenic antigens.[16] Based on these evidence, we propose that twist1 could be a critical target for understanding tumor development, progression, and potential immunotherapies.

Twist1 is well-established as a key player in inducing embryonic development and EMT in cancers. Our results of GO and KEGG pathway enrichment analysis provide further evidence for twist1's central role in these processes. Our analysis showed that twist1 is involved in cell proliferation and metabolism. Pan et al also experimentally validated that twist1 regulates cancer cell occurrence and metastasis through its effects on EMT.[42] Irrespective of the specific mechanism by which twist1 influences tumor cells, twist1 emerges as a promising therapeutic target for cancer treatment. Recent studies have further highlighted this potential, revealing that humanized mice, upon vaccination with TWIST1-peptide or specific vaccinations delivered via adeno-associated vectors, elicited activation of twist-specific T-cells in cancer contexts.[16,17] In addition to vaccinations, investigations into breast cancer have identified LY-290181, also known as 2-amino-4-[3-pyridyl]-4H-naphtho[1,2-b]pyran-3-carbonitrile, as a compound capable of reducing migration, invasion, and multicellular tumor spheroid invasion through down-regulation of twist expression.[43] Furthermore, a modified poly(amidoamine) (PAMAM) dendrimer combined with siRNA-based targeting of twist1 has shown promise in inhibiting invasion and migration, offering a potential avenue for treating patients with triple-negative breast cancer.[44] Moreover, dihydrorotenone, a drug that deactivates cancer-associated fibroblasts through experimental targeting of twist1, has demonstrated efficacy in preclinical studies.[45] Moreover, our analysis also identified potential drugs that target twist1 and its interacting targets to combat tumorigenesis. Salinomycin was identified as a potent anti-tumor agent that primarily targets tumor stem cells.[46] It has been shown to regulate tumorigenesis through various pathways. Wang et al found that salinomycin acts as an anti-tumor agent by affecting cancer cell activity.[47] Klugline and isocephaeline, which are terpenoid, have been reported to inhibit tumor cell activation, according to previous study by Zhou et al.[48] Previous studied reported that manassantin B had been shown to inhibit angiogenesis and transcription activation against tumorigenesis.[49,50] Kristina Y. Aguilera et al found that pimonidazole is associated with hypoxia levels in cancers.[51] Our molecular docking results indicated that these potential drugs could directly interact with TWIST1 in its natural state. The binding of TWIST1 with salinomycin, klugline, and isocephaeline was found to be favorable. These results provide strong evidence that these predicted drugs are effective against twist1-induced diseases.

Our study has some limitations that need to be addressed. Although we have previously studied the function of twist1 expression in HNSC, the pathways by which twist1 affects other types of cancer need to be further investigated through in vivo experiments. Clinical studies on patients are also imperative to fully understand the role of twist1 in cancers. However, our findings on twist1 provide novel insights that have not been fully explored in previous studies and can serve as valuable reference for future immunotherapy studies targeting twist1 in cancers.

To the best of our knowledge, this is the first study to analyze the effects of twist1 on pan-cancer. In summary, our findings suggest that varying levels of twist1 expression are associated with different types of cancer, with upregulated twist1 expression having a significant correlation with tumorigenesis and poor prognosis. This study also highlights the association between twist1 expression and clinical factors, such as tumor stage and grade, as well as gene mutations, RNA modifications, and cancer stemness. The strong positive correlation between twist1 expression and TME provides evidence that twist1 plays a role in influencing immunotherapy in tumors.

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Author contributions

Conceptualization: Yue Wang, Tianjiao Jiang, Yiqiang Yin, Liang Yu.
Data curation: Chunhao Li.
Formal analysis: Yaowen Wang, Hui Zhao.
Investigation: Chunhao Li, Tianjiao Jiang, Yiqiang Yin.
Methodology: Yue Wang, Liang Yu.
Supervision: Liang Yu.
Visualization: Chunhao Li, Tianjiao Jiang, Yiqiang Yin.
Writing – original draft: Yue Wang, Liang Yu.
Writing – review & editing: Liang Yu.

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