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Original Article

Immunotherapeutic value of NUSAP1 associated with bladder cancer through a comprehensive analysis of 33 human cancer cases

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Abstract: To investigate the correlation between nucleolar spindle-associated protein 1 (NUSAP1) and cancer immunotherapy across 33 different types of human cancers. We conducted an analysis of The Cancer Genome Atlas (TCGA) database to retrieve gene expression data and clinical characteristics for 33 different cancer types. The immunotherapy cohorts encompassed GSE67501, GSE78220, and IMvigor210. Relevant information was extracted from the gene expression repository. We assessed the prognostic significance of NUSAP1 by examining various clinical parameters. The single-sample gene-set enrichment analysis (ssGSEA) method was utilized to gauge NUSAP1 activity and to contrast NUSAP1 transcriptome and protein levels. We delved into the correlation between NUSAP1 and various immune processes and components to gain insights into NUSAP1's role. We also discussed coherent pathways associated with NUSAP1 signal transduction and its impact on immunotherapy biomarkers. To authenticate and validate the differential expression patterns of NUSAP1 in bladder tumor tissues versus normal bladder counterparts, we utilized Western blotting (WB), real-time quantitative polymerase chain reaction (RT-qPCR), and immunohistochemistry (IHC) techniques. NUSAP1 exhibits overexpression across a spectrum of malignancies, and its expression levels correlate with overall survival (OS), disease-specific survival, and tumor stage in specific cancer types. Furthermore, NUSAP1 expression is linked to mutations, methylation patterns, and immunotherapy responses in human cancers. Meanwhile, our experiments, involving WB, RT-qPCR, and IHC, consistently demonstrated significantly higher NUSAP1 expression in bladder tumor tissues compared to normal controls. Our study underscores the potential of NUSAP1 as a promising prognostic indicator and immunotherapeutic target for a range of malignant tumors.

Keywords: Nucleolar spindle-associated protein 1, pan-cancer analysis, bladder cancer, immunotherapy, biomarker

Introduction

Bladder cancer ranks as the 10th most prevalent cancer globally and stands as the 9th leading cause of cancer-related fatalities. In the year 2020, an estimated 573,000 new cases emerged, resulting in approximately 213,000 deaths [1]. This form of cancer can be categorized into two primary types based on whether the tumor invades the muscle layer of the bladder, known as muscle-invasive bladder cancer (MIBC), and non-muscle-invasive bladder cancer (NMIBC) [2]. Bladder cancer is characterized by a high recurrence rate and a propensity for metastasis, owing to the substantial heterogeneity and genomic instability inherent in this disease [3, 4]. While clinical management of bladder cancer includes radical surgical interventions, postoperative therapies such as Bacillus Calmette-Guerin (BCG) infusion, chemotherapy, and immunotherapy, there remains a lack of universally accepted and dependable

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standard predictors for early-stage bladder cancer diagnosis and prognosis. Exploring the abnormal expression of genes within bladder cancer tissues holds the potential to uncover new molecular biomarkers that could enhance the accuracy of bladder cancer diagnosis and prognosis assessment. Nucleolar spindle-associated protein 1 (NUSAP1) has recently been recognized as a cell cycle regulatory protein that binds microtubules and dominates mitotic progression, spindle formation and stability [5, 6]. The integrity of the spindle structure ensures uniform chromosome division, which is a prerequisite for cell division. Abnormal spindle structure can lead to chromosome missegregation, which is also known as chromosome instability, resulting in tumorigenesis [7].

Numerous investigations have underscored the pivotal regulatory role of NUSAP1 in various tumor types. These studies, drawing from datasets such as The Cancer Genome Atlas (TCGA), Gene Expression Omnibus Analysis (GEO), and others, have explored its significance in diverse cancers, including astrocytoma [8], colorectal cancer [9], breast cancer [10], lung cancer [11, 12], and cervical cancer [13]. Furthermore, NUSAP1 has emerged as a critical contributor to tumorigenesis, disease progression, and metastasis. For instance, in cervical cancer, elevated NUSAP1 expression has been linked to enhanced cancer stem cell properties and the facilitation of the epithelial-mesenchymal transition process, ultimately promoting cancer metastasis [14]. Roy et al. identified NUSAP1 as a target of miRNA 193a-5p, revealing that reduced miRNA 193a-5p levels lead to increased NUSAP1 expression in hepatocellular carcinoma (HCC), with higher NUSAP1 expression associated with shorter patient survival [15]. Zhu et al. confirmed a close association between elevated NUSAP1 expression and poor prognosis in HCC patients, speculating that NUSAP1 primarily regulates HCC progression by promoting the transition from G1 to S phase [16]. Zhang et al. reported that NUSAP1 deficiency hampers cell proliferation, migration, and invasion in invasive breast cancer cells through the regulation of CDK1 and DLGAP5 expression [17]. Additionally, various signaling pathways have been implicated in NUSAP1's functions, including PI3K/Akt, Wnt/β-catenin, YAP1, and Hedgehog, all of which play pivotal roles in tumorigenesis [18]. Guo et al. discovered that NUSAP1 exerts an oncogenic role in gastric carcinogenesis by interacting with YAP1 and promoting its nuclear translocation. NUSAP1's expression positively correlates with poor clinical outcomes and YAP1 protein levels in gastric cancer [19].

Our primary objective was to investigate the potential connection between NUSAP1 and immunotherapy across 33 different human cancers. We began by collecting gene expression data and clinical information from TCGA for these 33 distinct cancer types. Additionally, we obtained immunotherapy-related datasets, including GSE67501, GSE78220, and IMvigor-210, from the GEO database. These datasets provided valuable patient details such as age, sex, survival outcomes, and tumor stage, which allowed us to evaluate the prognostic significance of NUSAP1. We then delved into the generation of NUSAP1 activity and associated potential pathways. To achieve this, we employed single-sample gene set enrichment analysis (GSEA) and conducted a thorough examination of the differences between NUSAP1 transcriptome and protein expression levels. To gain a deeper understanding of NUSAP1's role in cancer immunotherapy, we further explored its correlation with the tumor microenvironment. We examined its relationship with various immune processes and elements, including immune cell infiltration, immunosuppressive factors, immune stimulants, and the major histocompatibility complex. Additionally, we investigated the correlation between alterations in NUSAP1 and key factors such as tumor mutation burden (TMB) levels and microsatellite instability (MSI) events, which are important considerations in cancer genetics. To assess the link between NUSAP1 and immunotherapeutic response, we performed Wilcoxon tests on three independent immunotherapy cohorts. In summary, our study presents compelling evidence that sheds light on the immunotherapeutic role of NUSAP1 across a wide spectrum of cancers. These findings lay the groundwork for future functional experiments in this area. Additionally, we conducted experiments specifically focusing on NUSAP1 in bladder tumor tissues and normal controls, utilizing WB, RT-qPCR and IHC techniques.

Materials and methods

Data collection

The TCGA database (https://portal.gdc.cancer.gov/) encompasses various types of information, including gene expression, copy number variations, and single nucleotide polymorphisms. We acquired and prepared raw mRNA expression and SNP data of 33 pan-cancer cases for subsequent analysis. Gene expression data were retrieved from the Genotype-Tissue Expression (GTEx) database (https://commonfund.nih.gov/GTEx). These GTEx data were integrated with the TCGA dataset and harmonized to assess gene expression variations across diverse cancer types. We also accessed data from tumor cell lines from the Cancer Cell Line Encyclopedia (CCLE) database (https://portals.broadinstitute.org/ccle/) and examined gene expression levels in relation to tissue origin. For analysis, we applied a log transformation to RNA-seq data and employed VarScan2 for the analysis of somatic mutations. MSI data were sourced from Bonneville's study supplemental file. To analyze copy number variations (CNV), we utilized the GISTIC algorithm. Methylation data were obtained from the LinkedOmics data portal. Additionally, we investigated the relationship between NUSAP1 expression and tumor stage.

Examining NUSAP1 expression in relation to various cancers

We conducted an analysis using the limma package within the R Studio software to investigate whether there were disparities in NUSAP1 expression levels between tumor and normal groups. Furthermore, we explored the associations between NUSAP1 expression and several clinical characteristics, encompassing age, gender, and tumor stage. Additionally, we delved into the time-dependent prognostic significance of NUSAP1 across 33 cancer types. To accomplish this, we employed univariate Cox regression analysis with the survival package in R. This analysis encompassed multiple survival endpoints, including overall survival (OS), disease-free survival (DFS), disease-specific survival (DSS), and progression-free survival (PFS). When the hazard ratio (HR) exceeded 1 (HR>1), it signified that NUSAP1 expression played a role in promoting positive outcomes, including survival. We regarded variations with a *p*-value of less than 0.05 as statistically significant.

NUSAP1 and RNA modification gene correlation analysis

From the standardized pan-cancer dataset, we specifically extracted the expression data for NUSAP1 and three distinct classes of RNA modification-related genes: m1A(10), m5C(13), and m6A(21) in every sample. These datasets were subjected to correlation analysis. To ensure data consistency, we applied a log2(x+0.001) transformation to each expression value. Subsequently, we computed the Pearson correlation coefficients to examine the relationships between NUSAP1 and the marker genes associated with five different immune pathways.

Correlation analysis of NUSAP1 with gene mutations

We conducted an in-depth analysis of NUSAP1 across various cancers utilizing TCGA data through the cBioportal database. Additionally, we obtained mutation data specifically for bladder cancer from TCGA. To further investigate mutation frequencies, we categorized patients into two groups: those with NUSAP1 expression in the lowest 25% and those with NUSAP1 expression in the highest 25%. We accomplished this categorization using the R package "maftools".

Assessment of NUSAP1's connection with methylation and associated genes

We investigated the methylation status within the promoter region of NUSAP1 across various cancers via the ULCAN platform. Following this, we utilized GEPIA2 to identify the top 100 genes that displayed the strongest associations with NUSAP1. We extracted the ten most prominent genes. Subsequently, these ten genes were utilized to construct a pan-cancer correlation heat map using TIMER2. In addition, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomics (KEGG) enrichment analysis was performed. Finally, Linkdomics was used to obtain 50 positively related genes and 50 negatively related genes based on generating NUSAP1-related genes in BLCA and generating heatmaps.

Construction of protein interactions network and ncRNA network construction

We used the online website String database to analyze the interworking proteins of NUSAP1, imported the interworking network into Cytoscape, scored the size of each interworking protein according to the degree of interworking, and extracted the key core interworking network map. In addition, we found 2 key interworking proteins, performed molecular docking using HDOCK SERVER, and analyzed binding sites and hydrogen bonds in PyMol. Finally, we used ENCORI to predict NUSAP1 target miRNA fetch intersections and construct NcRNA network maps.

Assessing the immunological traits of the tumor microenvironment across multiple cancers

We embarked on an investigation into the connection between NUSAP1 and the proportions of immune cell subgroups, and we harnessed the power of CIBERSORT. Additionally, we gauged the extent of immune cell inflammation within the tumor microenvironment by applying the single-sample gene set enrichment analysis algorithm. In addition, we analyzed the correlation of NUSAP1 with Immune Checkpoint Pathways (ICP), MSI, TMB and tumor purity. Finally, the results of the ESTIMATE analysis were generated using an online tool in Sangerbox.

Analysis of drug sensitivity

Our investigation delved into drug sensitivity analysis utilizing the Cellminer database, which is centered on 60 distinct cancer cell lines cataloged through the National Cancer Institute's Center for Cancer Research (NCI). In the course of this study, we obtained both NCI-60 drug sensitivity data and RNA-seq gene expression data. Our primary focus was to unveil potential associations between genes and the responsiveness to commonly used anticancer medications, accomplished through correlation analysis. We regarded variations with a *p*-value less than 0.05 as statistically significant.

Analysis of tumor mutational burden (TMB)

Tumor mutational burden (TMB) was characterized as the cumulative count of somatic gene coding anomalies, encompassing base substitutions, insertions, or deletions, identified per million bases. In our investigation, TMB was determined by evaluating the occurrence of genetic variations and the count of variants relative to the exon length for each individual tumor sample. This calculation was conducted by dividing the entire length of the protein-coding region by the number of nonsynonymous mutation sites. To complement our analysis, we obtained the microsatellite instability values for each TCGA patient from a previously published study [20].

Collection of clinical samples

We also collected 40 bladder cancer patients and 40 normal bladder tissue specimens diagnosed at the University of Hong Kong Shenzhen Hospital from 2022.01 to 2023.8. The human tissues or specimens used in this study were obtained from previous medical records and data, not specifically collected for this study, and were exempted from informed consent by national medical ethical standards. The tissues or specimens were used only for this study and not for any other purposes, and the excess tissues or specimens would be returned at the end of the study, and the results of the study did not disclose the personal information of the sources of the tissues or specimens. Approval for this study was granted by the Ethics Committee of the University of Hong Kong Shenzhen Hospital.

Analysis by qRT-PCR

We initiated the analysis with the extraction of total RNA from both bladder tumors and normal bladder tissues, employing TRIzol (Invitrogen). Subsequently, we executed the reverse transcription process, converting the RNA into cDNA, following the protocol provided by the mRNA Reverse Transcription Kit (Roche). For quantitative real-time PCR (qRT-PCR), we utilized the SYBR Green RNA Kit from Applied Biosystems, headquartered in Foster City, USA. The qRT-PCR procedure was conducted in accordance with the manufacturer's guidelines. To quantify mRNA expression levels relatively, we employed the 2-ΔΔCq technique. As a reference, GAPDH was employed. The following primer sequences were used in our experiments: GAPDH Forward: CCACTCCTCCACCTTTGACG, and Reverse: CTGGTGGTCCAGGGGTCTTA; NUSAP1 Forward: 5'-CTGACCAAGACTCCAGCCAGAA-3', and Reverse: 5'-GAGTCTGCGTTGCCTCAGTTGT-3'.

Western blot analysis

After treating the cells according to the procedures, each group of cells was collected and rinsed twice with PBS, and then RIPA lysis buffer containing phosphatase inhibitors was added. The lysate was lysed in an ice water bath for 30 minutes. During the lysis process, the supernatant was lysed by an ultrasonic cell crusher and centrifuged to determine the concentration by the BCA method. For protein analysis, denatured proteins were introduced into a pre-prepared SDS-PAGE gel, with a loading of 60 μg per well. Electrophoresis was conducted to separate the proteins within the gel, followed by their transfer onto a PVDF membrane. The PVDF membrane was then immersed in a solution containing 5% skimmed milk powder for a duration of 2 hours. Subsequently, a primary antibody was applied and allowed to incubate at 4°C overnight. Afterward, the membrane underwent three washing cycles with 1× PBST (Phosphate-Buffered Saline with Tween) and was subsequently subjected to a secondary antibody (Beyotime) for 2 hours at room temperature. The membrane was rinsed three more times with 1× PBST, and the color development process was initiated using an enhanced chemiluminescence kit, specifically the one from Beyotime in Shanghai, China. Protein bands were subsequently analyzed using the ImageJ software. NUSAP1 antibody was purchased from Proteintech Biological Company, Beijing, China.

Human protein atlas database and IHC validation

We investigated NUSAP1 protein expression levels in a variety of tumor samples in the human protein atlas (HPA) online database (https://www.proteinatlas.org/) to generate HPA by integrative histology techniques.

Immunohistochemistry

Tissue specimens from various grades of bladder cancer and normal bladder tissue were initially fixed using formalin, embedded in paraffin, and subsequently sectioned into 4-micron slices. These sections underwent deparaffinization and dehydration, followed by antigen retrieval pretreatment in citrate buffer. To mitigate endogenous peroxidase activity, they were quenched using a 3% hydrogen peroxide solution. To minimize non-specific binding, any remaining antigenic sites were blocked using 10% normal goat serum. The sections were then subjected to overnight incubation at 4°C with two distinct antibodies (1:100, Abcam and 1:500, CST). Subsequently, a secondary antibody (goat anti-rabbit IgG, 1:5000, Proteintech) was applied, and the staining was developed using diaminobenzidine hydrochloride (DAB) and hematoxylin. IHC images were captured, and the protein expression levels were quantified using Image J, an image processing software, to determine the fraction of protein expression.

Statistical analysis

All statistical analyses were executed using the R language, specifically version 4.0. Univariate survival analysis was employed to compute hazard ratios (HRs) along with their corresponding 95% confidence intervals. Patient survival was assessed by Kaplan-Meier analysis, classifying individuals based on high or low gene expression levels. It's important to note that all statistical tests were two-sided, and significance was attributed to variations with a *p*-value less than 0.05.

Results

NUSAP1 expression in 33 cancers

To provide a comprehensive overview of our findings, **Figure 1** offers a summarized presentation of our analysis. **Figure 2A** illustrates that NUSAP1 exhibited differential expression in 13 out of the 33 cancer types, specifically in Bladder Urothelial Carcinoma (BLCA), Breast invasive carcinoma (BRCA), Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), Cholangiocarcinoma (CHOL), Colon adenocarcinoma (COAD), Esophageal carcinoma (ESCA), Glioblastoma multiforme (GBM), Kidney Chromophobe (KICH), Kidney renal clear cell carcinoma (KIRC), Liver hepatocellular carcinoma (LIHC), Lung adenocarcinoma (LUAD), Lung squamous cell carcinoma (LUSC), Pheochromocytoma and Paraganglioma (PCPG), Prostate adenocarcinoma (PRAD), Rectum adenocarcinoma (READ), Sarcoma (SARC), Stomach adenocarcinoma (STAD), Thyroid carcinoma (THCA), and Uterine Corpus Endometrial Carcinoma (UCEC). In BLCA, BRCA, CESC, and GBM, NUSAP1 was highly expressed

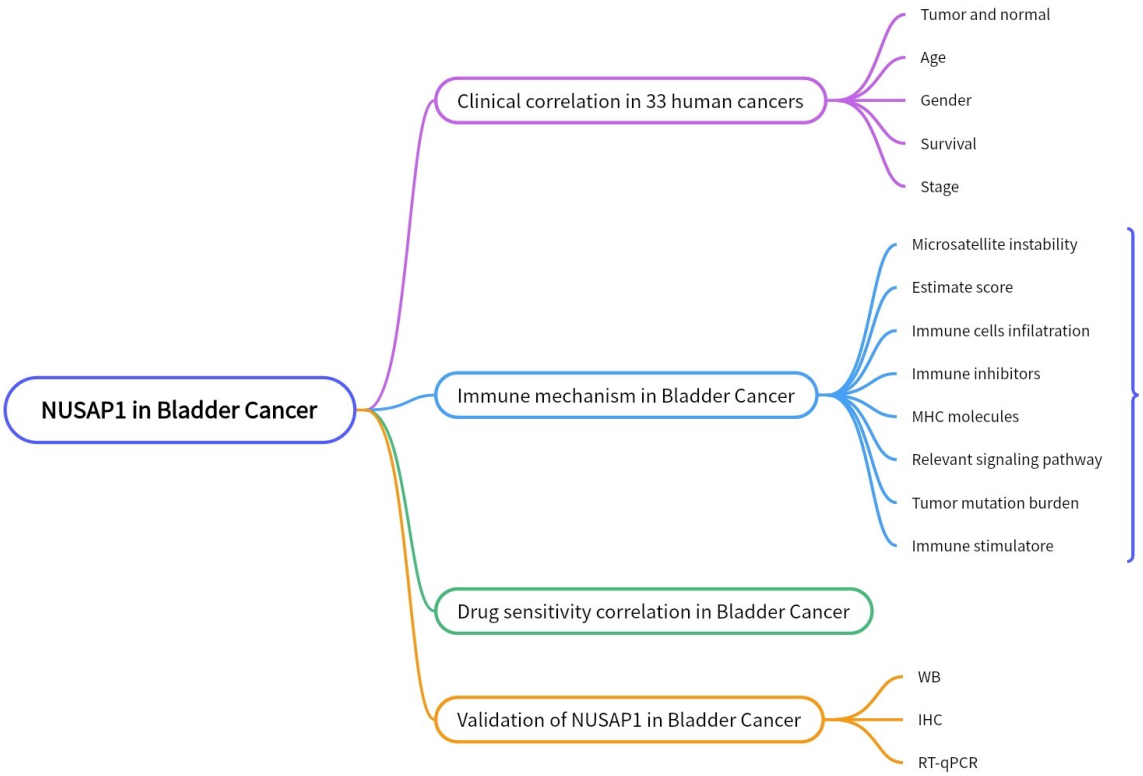

Figure 1. The whole workflow of this study. WB, Western blotting; RT-qPCR, Real-time quantitative polymerase chain reaction; IHC, Immunohistochemistry.

among tumor patients, while in KICH, KIRC, and Thymoma (THYM) tumor tissues, its expression was comparatively low (**Figure 2A**). Compared to normal bladder tissues, NUSAP1 was notably upregulated in bladder tumor tissues (**Figure 2B**), with its expression correlating with tumor grade (**Figure 2C**). Further exploration of the correlation between NUSAP1 expression, tumor stage, and grading revealed significant associations in several cancers, including Adrenocortical carcinoma (ACC), BRCA, KICH, KIRC, Kidney renal papillary cell carcinoma (KIRP), LIHC, LUAD, Skin Cutaneous Melanoma (SKCM), and THCA (**Figure 2D**). Additionally, we investigated the relationship between NUSAP1 expression and patient prognosis, examining survival indicators such as OS and PFI. Our analysis revealed strong associations between NUSAP1 expression and OS in 13 cancers, including GBM, Brain Lower Grade Glioma (LGG), KIRP, KIRC, KICH, Pancreatic adenocarcinoma (PAAD), Acute Myeloid Leukemia (LAML), ACC, Mesothelioma (MESO), LUAD, LAML, LIHC, and Acute lymphoblastic leukemia (ALL). Furthermore, KM-plot survival analysis demonstrated that high NUSAP1 expression was linked to poorer OS in nine cancers, encompassing ACC, KIRP, KIRC, LGG, LIHC, LUAD, MESO, PAAD, and PRAD (**Figure 3**). High NUSAP1 expression was also linked to worse DFS in ACC, KIRP, LGG, LIHC, LUAD, PAAD, PRAD, SARC, and Uveal Melanoma (UVM) (Supplementary Figures 1, 2). Finally, we observed significant correlations between NUSAP1 expression and PFS and DSS in most tumors (Supplementary Figure 3A). Moreover, high NUSAP1 expression was associated with unfavorable prognoses in bladder, gastric, hepatocellular, and ovarian cancers (Supplementary Figure 3B, 3C). Collectively, these results suggest a potential linkage between NUSAP1 expression and the development and progression of bladder tumors.

Correlation between NUSAP1 and gene mutation and methylation

To investigate the genetic alterations related to NUSAP1, we conducted a pan-cancer analysis using TCGA data accessed through the cBioportal database (**Figure 4**). Subsequently, we segregated the mutation data, obtained from TCGA, into two groups: NUSAP1 low 25% and

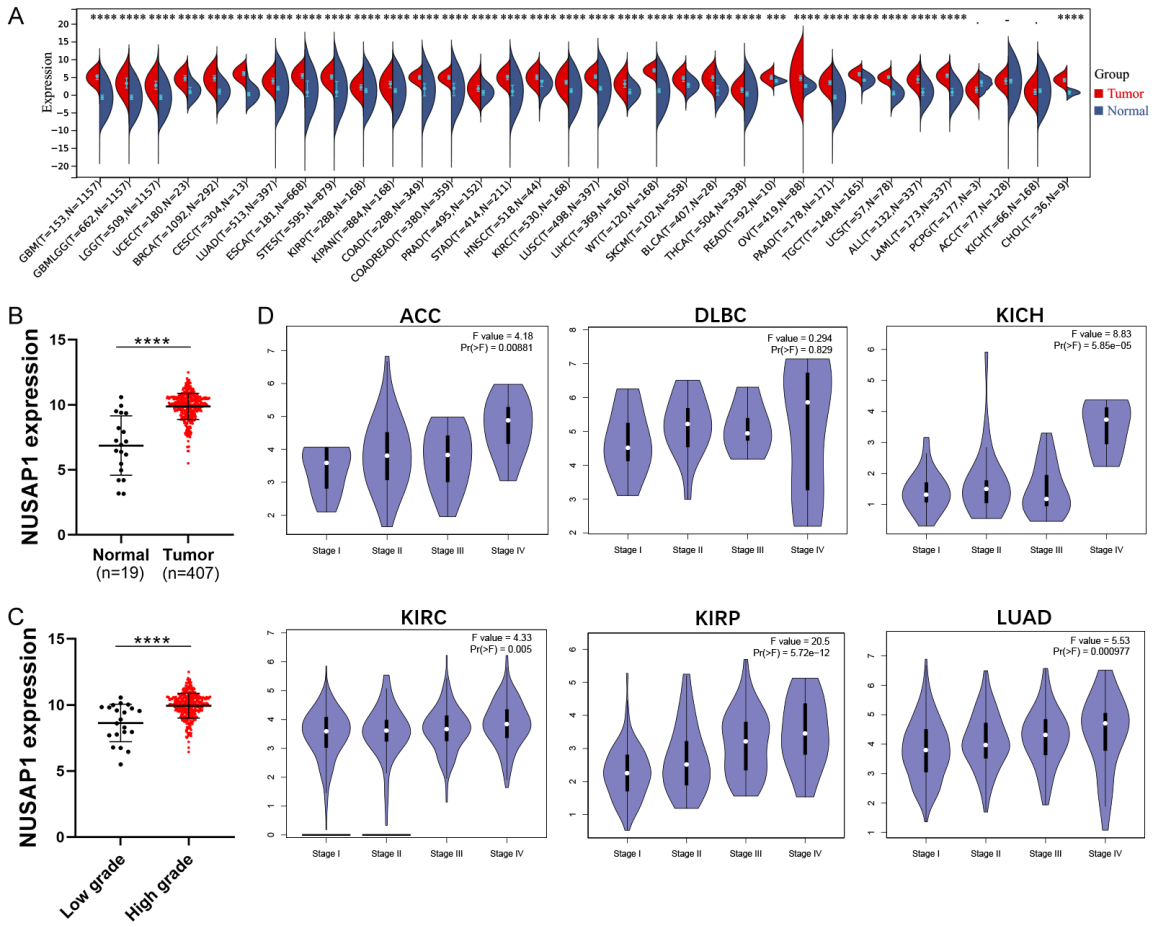

Figure 2. The NUSAP1 (Nucleolar spindle-associated protein 1) expression in normal and cancers tissues. A. The expression of NUSAP1, red labeled tumors represent upregulation, blue represent downregulation, and black represent no significant difference between tumor tissues and normal tissues. B, C. Correlation between NUSAP1 expression and WHO classification (low grade, high grade) of bladder cancer. D. Correlation between NUSAP1 expression and WHO classification (stage I-IV) of various tumors (ACC, DLBC, KICH, KIRC, KIRP and LUAD). ACC, Adrenocortical carcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LUAD, Lung adenocarcinoma.

NUSAP1 high 25%, and analyzed them separately to generate a waterfall plot illustrating the mutation frequency. Furthermore, we delved into exploring the potential link between NUSAP1 and methylation. Our analysis encompassed the evaluation of the methylation status within the promoter region of NUSAP1 across various tumor types, facilitated by the ULCAN platform (**Figure 5**).

Analysis and functional enrichment of NUSAP1

We first obtained 100 NUSAP1-related genes, and the top 10 genes were put in TIMER2 to generate a pan-cancer correlation heat map (**Figure 6A-D**). Then, the correlation scatter plots of NUSAP1 and these 10 genes were generated separately. In order to delve deeper into the functional implications of the correlation between NUSAP1 and these genes, we conducted a Gene Ontology enrichment analysis. Additionally, we performed a KEGG analysis, which unveiled significant enrichment in several pathways. These pathways encompassed the cell cycle, DNA replication, p53 signaling pathway, FoxO signaling pathway, Human T-cell leukemia virus 1 infection, and microRNA in cancer. This comprehensive analysis provides valuable insights into the potential functional associations between NUSAP1 and these genes in various cellular processes and pathways. These results suggest that NUSAP1 may image tumorigenesis and progression through these interactions.

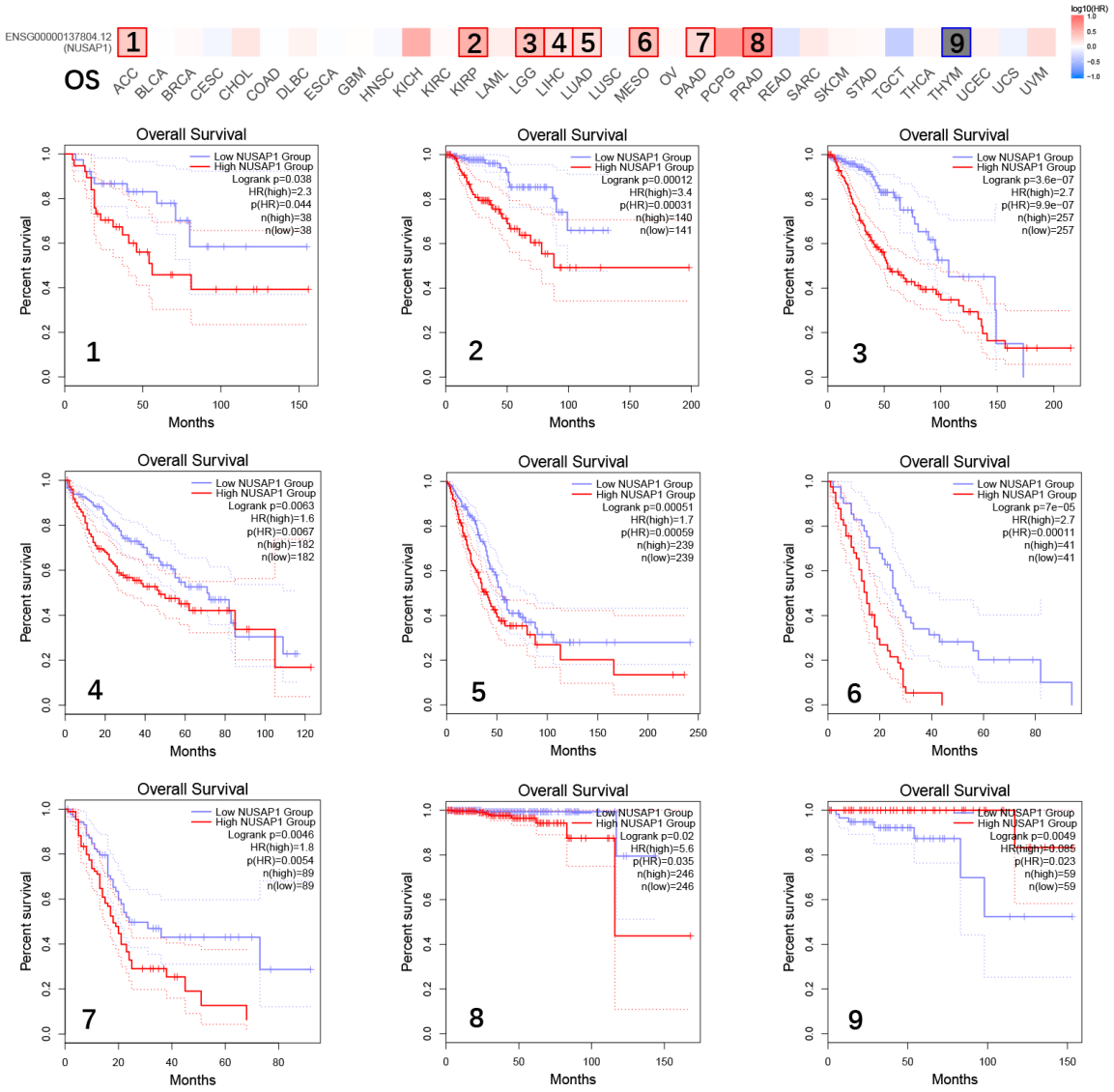

Figure 3. OS (Overall survival) for different NUSAP1 (Nucleolar spindle-associated protein 1) expression in multiple tumor.

Construction of protein interaction network map

We used the online tool STRING PPI to analyze the interworking proteins of NUSAP1, we imported the interworking network into Cytoscape, scored the size of each interworking protein according to the degree of interworking, and constructed the protein interworking network graph (**Figure 7A**). In addition, we extracted the key core interworking networks (**Figure 7B**). Finally, we found 2 key interworking proteins, performed molecular docking using HDOCK SERVER, and analyzed the binding sites and hydrogen bonds in PyMol (**Figure 7C**). To further explore the regulatory relationship of NcRNAs upstream of NUSAP1, we predicted NUSAP1 target miRNAs by combining five online tools, including miRDB, miRWalk, miRabel, TargetScan and ENCORI, and took the intersections to obtain five miRNAs (**Figure 8A**). Then, we selected hsa-miR-22-3p by miRDB binding score (**Figure 8B**) and used ENCORI to analyze the upstream target lncRNAs and circRNAs of hsa-miR-22-3p and generated network diagrams in Cytoscape (**Figure 8C**, **8D**).

NUSAP1 is closely related to m6A modification

We analyzed the relationship between NUSAP1 and m6A modification in pan-cancer using the online tool, and our results suggested that

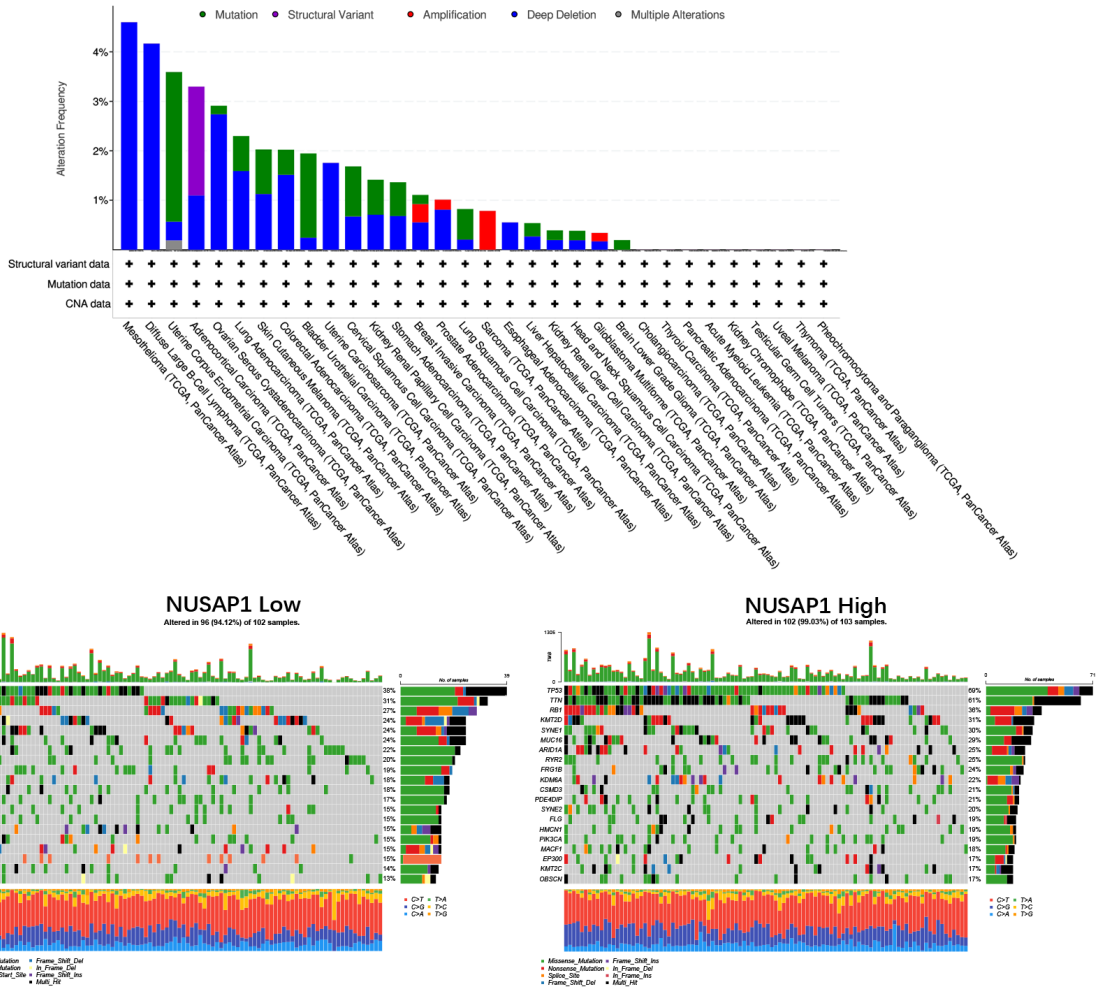

Figure 4. Comprehensive analysis of different genomic features associated with NUSAP1 (Nucleolar spindle-associated protein 1). The gene alteration features of NUSAP1 in 32 different tumors. Red labels represent tumors with the top 3 amplification levels. Green represent tumors with the highest mutation levels and blue represent tumors with the lowest mutation levels. Only the top 20 genes with the highest mutation rates were shown.

most of the m6A-associated genes were closely associated with a variety of tumors (**Figure 9A**). Then, we also predicted the m6A modification sites of NUSAP1 transcripts in SRAMP and took the six sites with the highest confidence to visualize (**Figure 9B**).

Results of the expression and immune correlation analysis of NUSAP1

Our analysis unveiled close correlations between NUSAP1 and alterations in immune cell infiltration within the tumor microenvironment. Furthermore, we observed that NUSAP1 expression exhibited significant associations with Immune Checkpoint Pathways (ICP), MSI, TMB, and Neoantigen load (Supplementary Figure 5). Finally, we explored the correlation between NUSAP1 and the ESTIMATE algorithm, which assesses the level of immune cell infiltration in tumors (**Figure 10**). The analysis revealed strong associations between NUSAP and immune scores in various tumor types, including bladder and kidney cancers (Supplementary Figure 4). These findings underscore the close links between NUSAP1, immune cell infiltration, and immunotherapeutic responses in a diverse range of tumor microenvironments.

Validation of NUSAP1 in bladder tumors

To further validate the NUSAP1 expression between bladder tumor tissues and normal bladder tissues, we conducted experiments using 30 pairs of bladder tumor tissues and their corresponding normal bladder tissues.

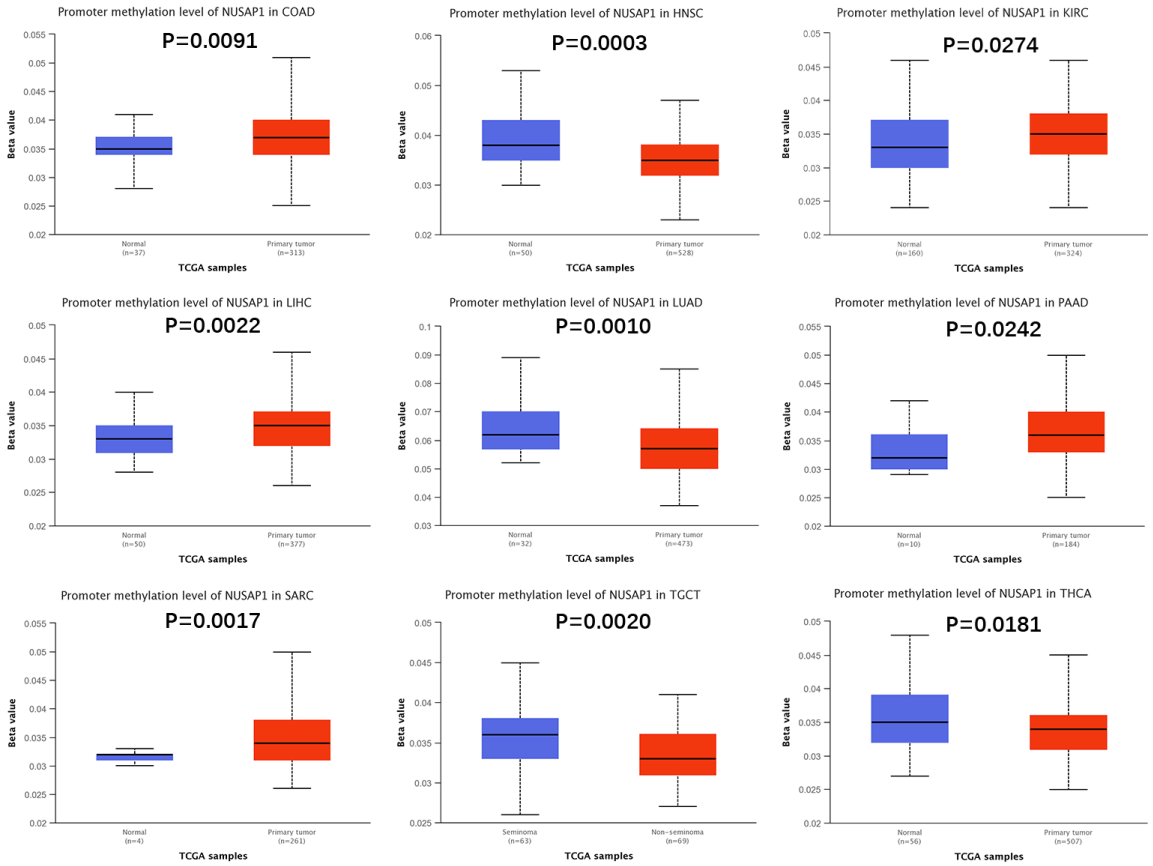

Figure 5. Promoter methylation level of NUSAP1 (Nucleolar spindle-associated protein 1) between tumor and normal tissues of multiple tumors in the TCGA (The Cancer Genome Atlas) database.

The results from RT-qPCR demonstrated a significant increase in NUSAP1 expression in bladder tumor tissues compared to normal bladder tissues (**Figure 11C**, **11D**). This finding was corroborated by Western blot validation, which also showed significantly higher NUSAP1 expression in tumor tissues (**Figure 11A**, **11B**). Moreover, we obtained IHC staining images of NUSAP1 in various tumor tissues and normal tissues from the Human Protein Atlas database. These results reinforced the observation that NUSAP1 was significantly overexpressed in a variety of tumor tissues, including bladder cancer, renal clear cell carcinoma, cervical cancer, testicular cancer, and skin cancer (**Figure 12**). These findings collectively underline the heightened expression of NUSAP1 in tumor tissues across different cancer types.

Discussion

Bladder cancer has been found as a common malignancy in urology. It is currently one of the top 10 major malignancies worldwide [20]. About 20% of patients with bladder cancer are already MIBC at the time of presentation, and patients have a poor prognosis with high bladder tumor recurrence, progression and mortality [21]. There are also limitations of radiotherapy, chemotherapy, immunotherapy and gene-targeted therapy for intermediate and advanced bladder tumors. Within the tumor microenvironment, abnormalities are likely to cause extensive heterogeneity of tumors, resulting in obvious heterogeneity in the responses of patients with bladder cancer to treatments (e.g., gene-targeted therapy and immunotherapy). The construction of biomarkers that identify predictors of bladder cancer prognosis and the development of new prognosis models could guide the clinical treatment of patients with bladder cancer.

Extensive evidence underscores the pivotal regulatory role of NUSAP1 in various tumors, with high expression noted in colorectal can-

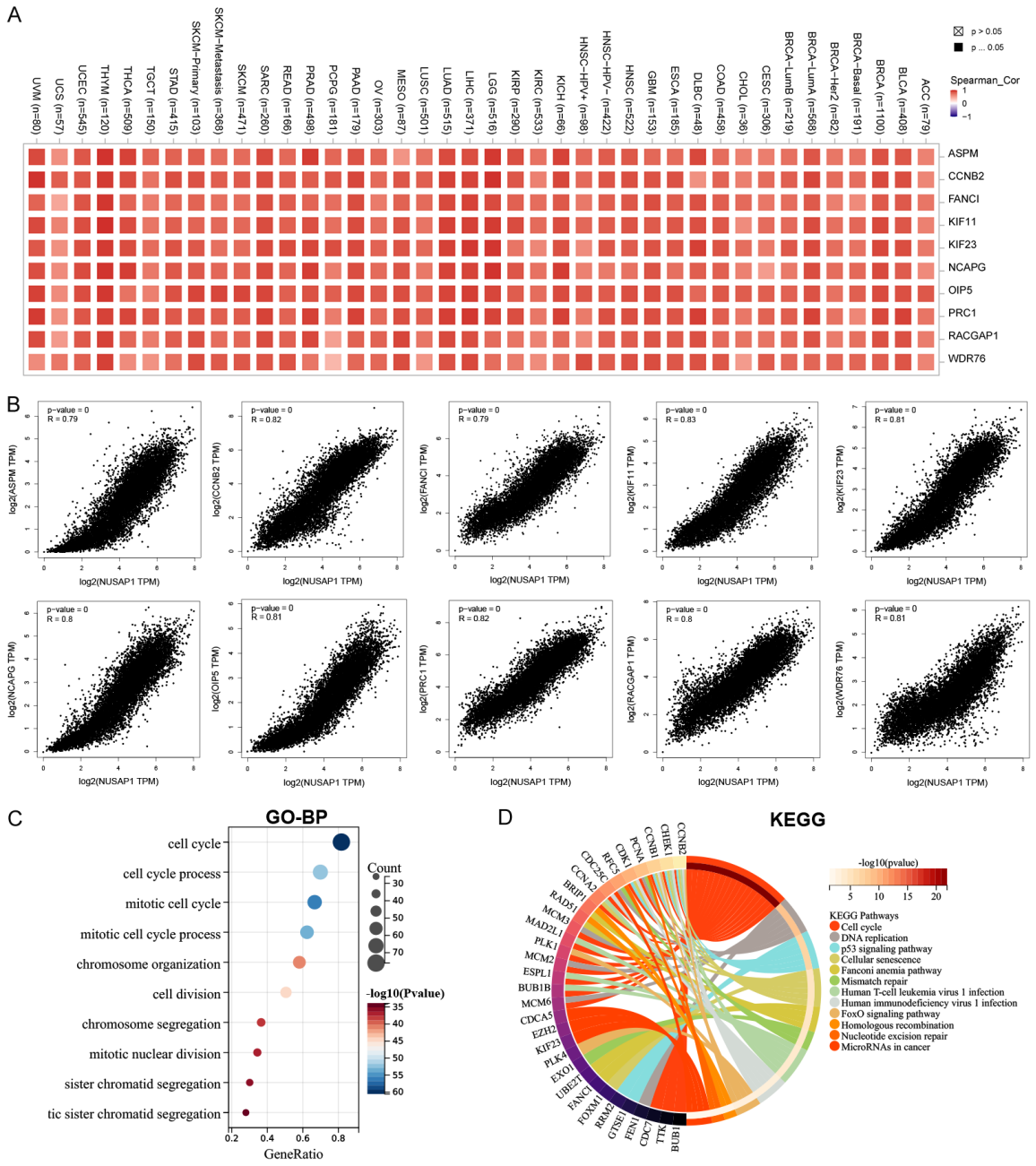

Figure 6. Enrichment analysis of NUSAP1 (Nucleolar spindle-associated protein 1)-related genes in pan-cancer. A. The top 10 NUSAP1-associated genes were displayed by GEPIA2 website. B. Corresponding heatmap data of NUSAP1 and its positively correlated genes obtained by TIMER2.0. C. GO (Gene ontology)-BP (Biological Process) analysis was applied to analyze the functional enrichment of the crossover genes. D. Alterations in different biofunctional classifications in bladder cancer with NUSAP1 expression.

cer, breast cancer, lung cancer, and cervical cancer. In our comprehensive pan-cancer analysis, we systematically explored the involvement of NUSAP1 across 33 human cancers. Our findings initially indicated that NUSAP1 exhibited elevated expression in 13 tumor tissues when compared to their non-tumor counterparts. These included BLCA, BRCA, CESC, CHOL, COAD, ESCA, GBM, LIHC, LUAD, LUSC, PCPG, PRAD, READ, SARC, STAD, THCA, and UCEC. Conversely, low levels of NUSAP1 expression were observed in KICH, KIRC, and THYM tumor tissues compared to non-tumor tissues. These results largely align with prior research,

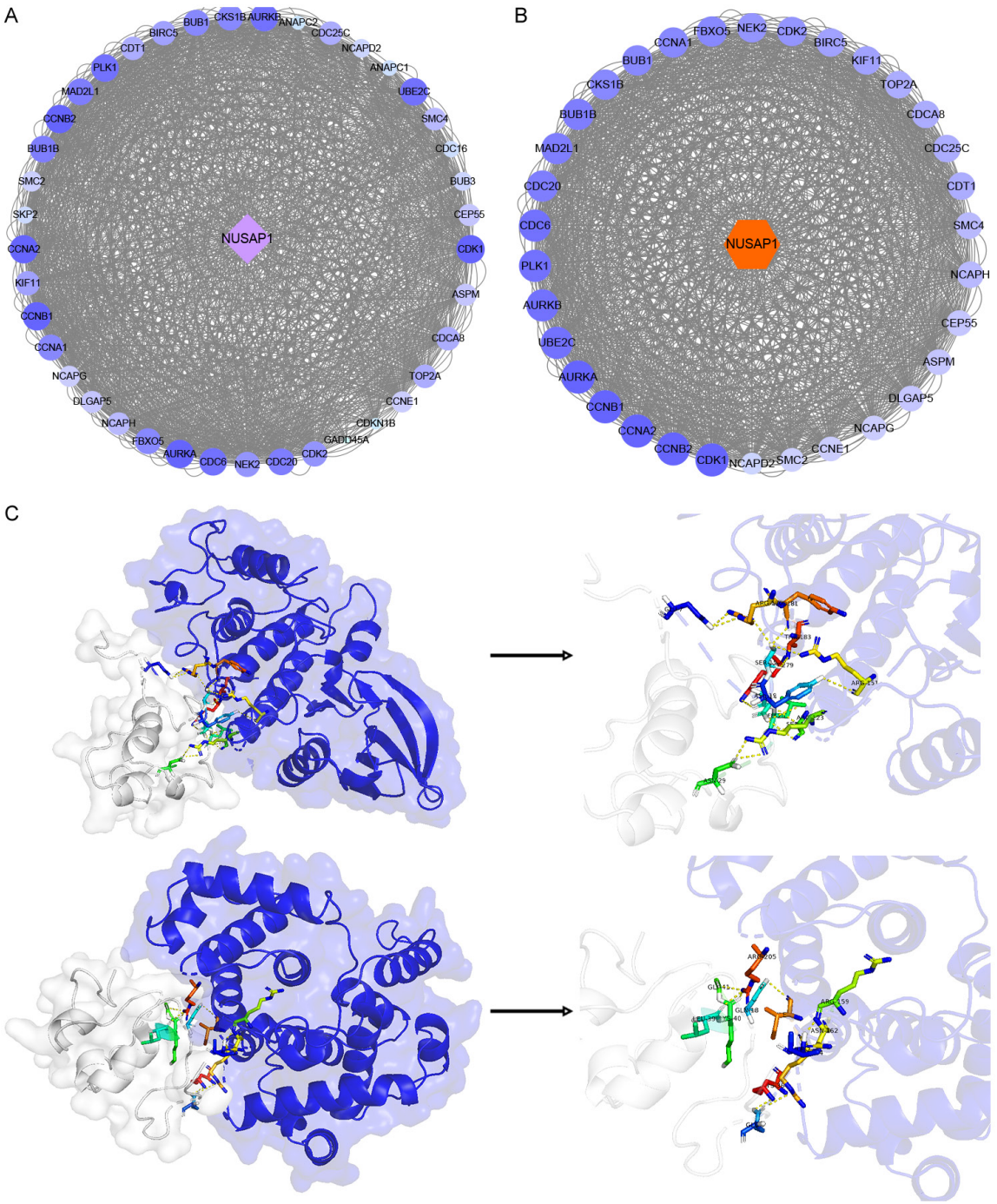

Figure 7. A. Analyzing NUSAP1 (Nucleolar spindle-associated protein 1) interacting proteins using String, importing the network into Cytoscape, and scoring the size of each interacting protein according to the degree of interactions; B. Extracting the key core interacting network; C. Finding the two key interacting proteins, molecularly docking the network using the HDOCK SERVER, and analyzing the binding sites and hydrogen bonds in PyMol.

implying that NUSAP1 may function as a tumor promoter in various human cancers. Further analysis combining Cox analysis and the Kaplan-Meier method unveiled that high NUSAP1 expression was linked to decreased OS in 13 tumors, specifically GBM, LGG, KIRP, KIRC, KICH, PAAD, LAML, ACC, MESO, LUAD, LAML, LIHC, and ALL. Additionally, elevated NUSAP1 expression was associated with poorer OS in ACC, KIRC, KIRP, LGG, LIHC, LUAD,

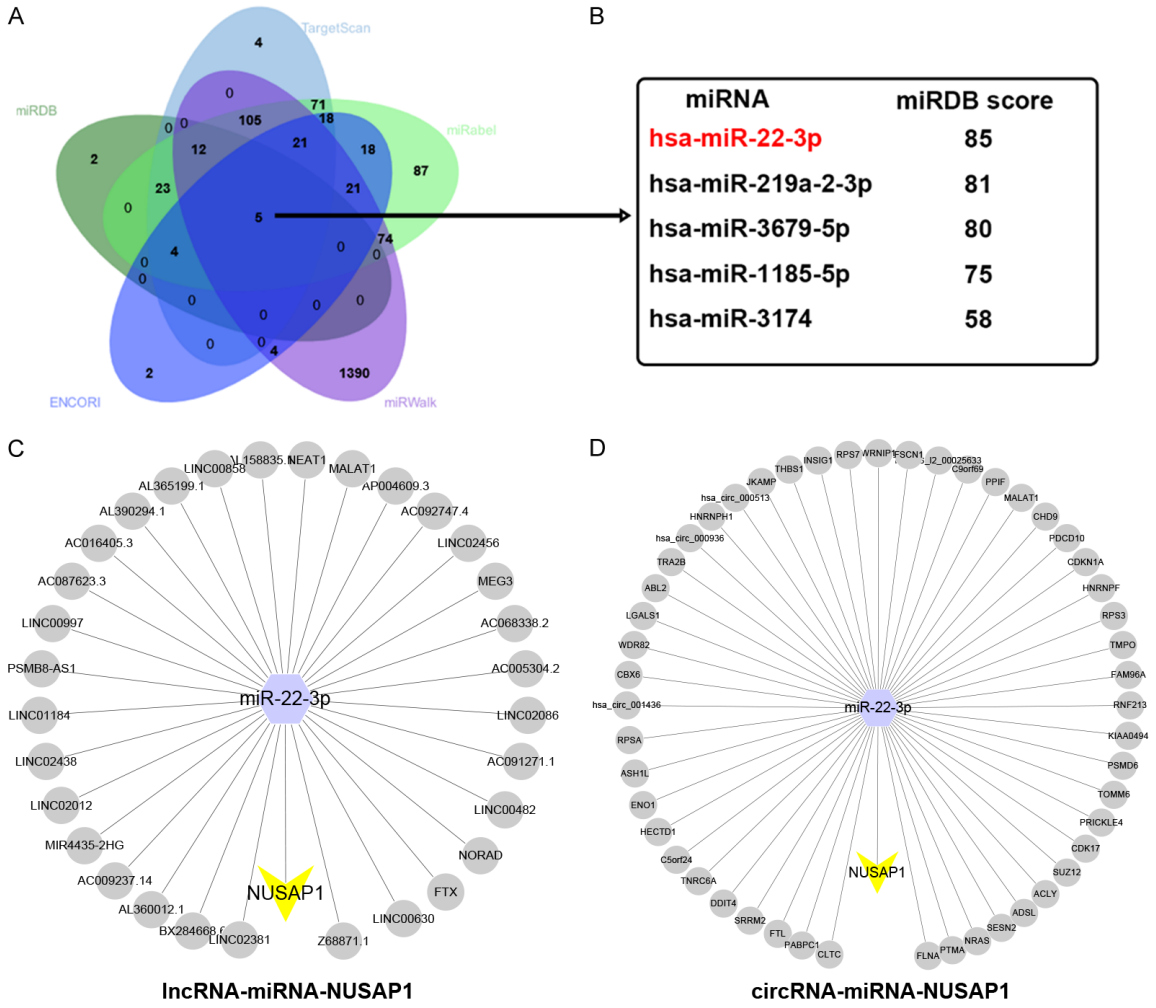

Figure 8. NcRNA network construction. A. Using miRDB, miRWalk, miRabel, TargetScan and ENCORI to predict NUSAP1 (Nucleolar spindle-associated protein 1) target miRNAs to take the intersection to get 5 miRs and do Venn diagram. B. Select hsa-miR-22-3p according to miRDB binding scores. C, D. Analyze the upstream targets of hsa-miR-22-3p for lnc and circ using ENCORI, and generate the network diagram in Cytoscape.

MESO, LHYM, and PAAD. High NUSAP1 expression also predicted inferior disease-free survival in ACC, KIRP, LIHC, LUAD, PAAD, SARC, and THCA. These results underscore the potential of NUSAP1 as a prognostic biomarker. Recent studies have also reported differential expression of NUSAP1 in various cancers. Catherine A et al. reported increased NUSAP1 mRNA and protein expression levels following RB1 deletion in prostate cancer cells, where downregulation of RB1 reduced the proliferation and invasive ability of these cells [22]. Bazzocco S et al. highlighted high NUSAP1 expression in colorectal cancer [23]. Fang et al. found elevated NUSAP1 expression in renal clear cell carcinoma tissue specimens and cell lines, with high NUSAP1 expression closely associated with shorter OS in patients [24]. Shan's findings in our study further emphasize the significance of NUSAP1 in the prognosis of these tumors, reinforcing the credibility of our analysis [25]. However, the mechanism of the role of NUSAP1 in tumorigenesis is unclear and needs further research. We speculate that the regulation of NUSAP1 activity associated with tumors may contribute to obtaining results that could help improve therapeutic techniques.

Considering the substantial impact of NUSAP1 on the intricate landscape of the tumor immune microenvironment, further comprehensive investigations are warranted to delve into the intricate interplay between immune cells, the

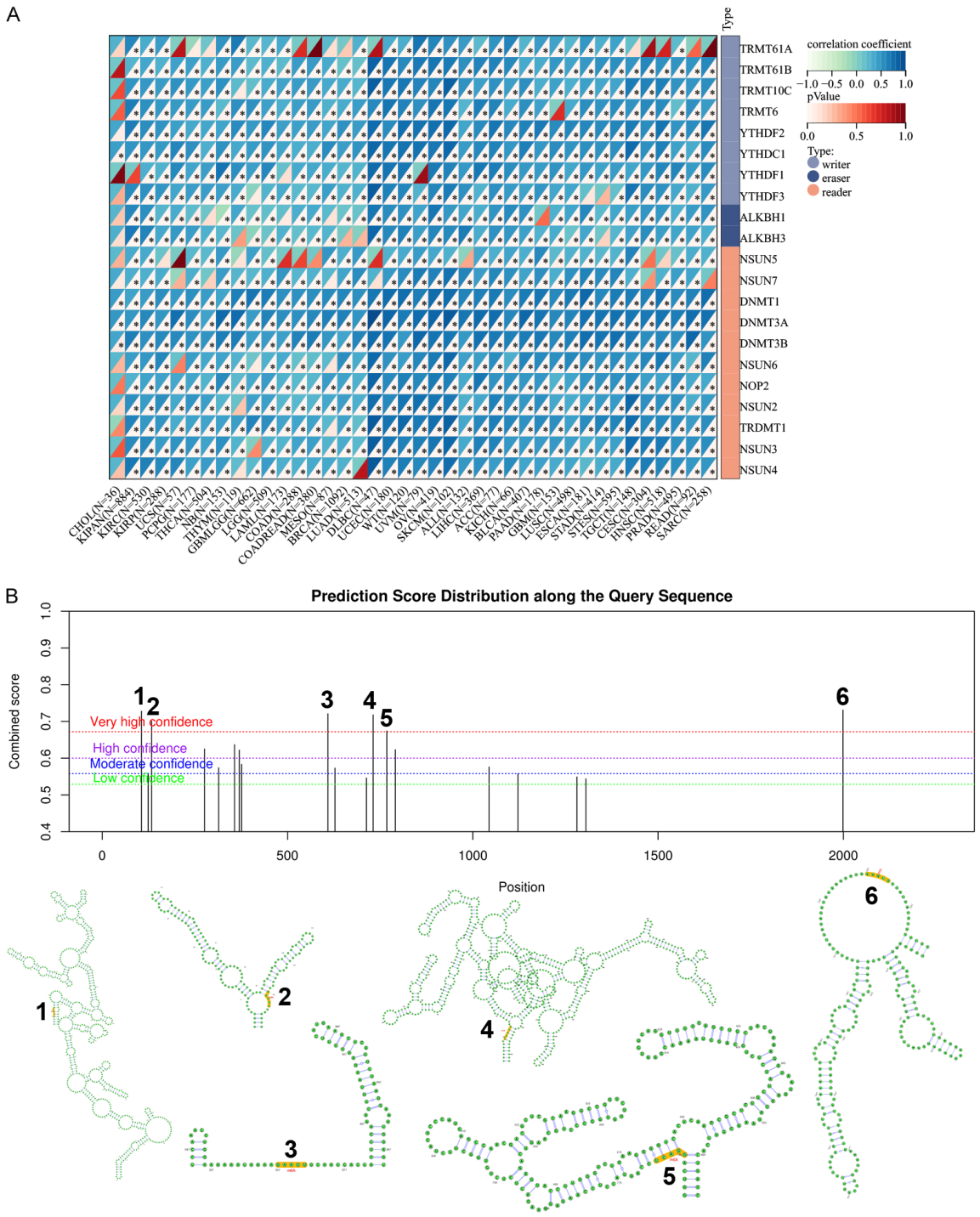

Figure 9. A. The relationship between NUSAP1 (Nucleolar spindle-associated protein 1) and m6A modification was analyzed in Sangerbox, The m6A modification sites of NUSAP1 transcripts were predicted in SRAMP; B. The six sites with the highest confidence were taken for mapping.

tumor microenvironment, immunomodulatory factors, and the intricate dynamics governing immunotherapeutic responses. Such endeavors hold significant promise in fostering a profound understanding and unveiling hidden insights that can propel our comprehension of this complex field to unprecedented depths. This study encompassed the analysis of 33 distinct types of human cancers to glean pertinent insights. The primary objective was to investi-

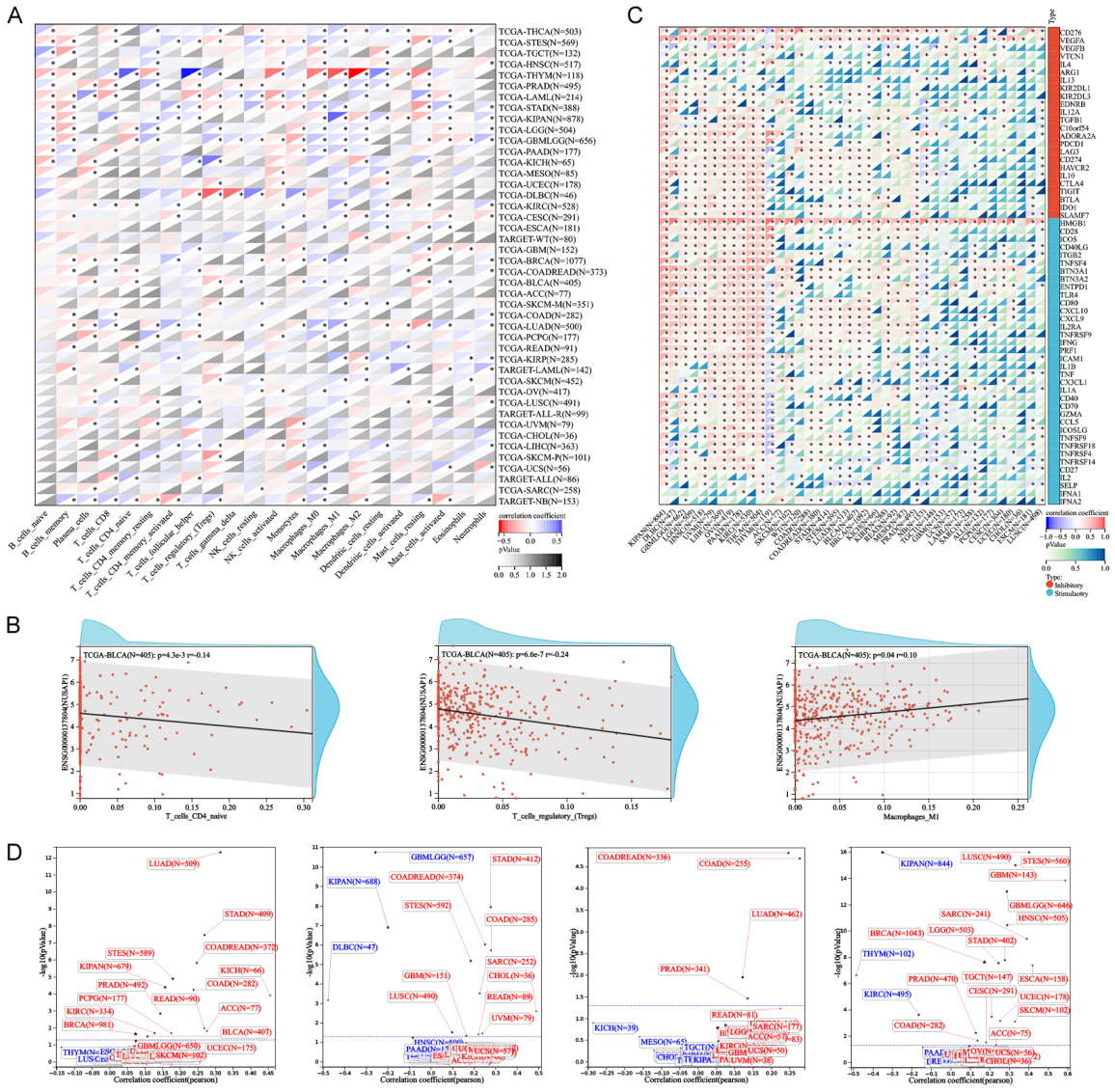

Figure 10. Correlation of NUSAP1 (Nucleolar spindle-associated protein 1) with (A) immune cell infiltration, (B) immune cells, (C) immune-related genes, (D) tumor purity in pan-cancer.

gate the immune-related mechanisms intertwined with urinary tumors. The investigation entailed an examination of NUSAP1 expression alongside clinical characteristics. Notably, COX regression analysis unearthed NUSAP1 as a prognostic factor in the context of bladder cancer. Furthermore, correction curves were constructed for bladder cancer patients at the fifth and seventh years, revealing consistent model effects.

Our study on immune-related aspects indicates that NUSAP1 assumes a distinct role in modulating the immune microenvironment within tumors. The atypical expression of NUSAP1 may have repercussions on the immune functionality within tumors. One intriguing finding is the significant correlation between NUSAP1 expression and varying degrees of immune cell infiltration in human cancers. Furthermore, our investigation revealed high NUSAP1 levels to be notably positive in certain cancers like GBM, KIRC, LGG, THCA, and LAML, while significantly negative in others such as UCEC, BRCA, CESC, LUAD, ESCA, Stomach and Esophageal carcinoma (STES), SARC, STAD, Head and Neck squamous cell carcinoma (HNSC), LIHC, SKCM, BLCA, Ovarian serous cystadenocarcinoma (OV), and Testicular Germ Cell Tumors (TGCT). This suggests that NUSAP1 may impact cancer

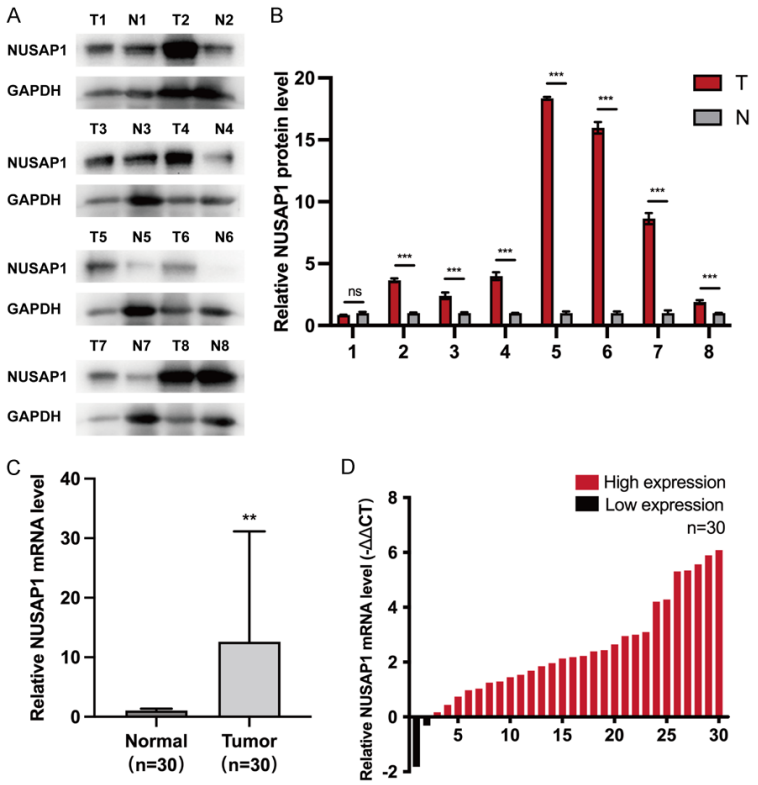

Figure 11. Tissue validation of NUSAP1 (Nucleolar spindle-associated protein 1) expression in bladder cancer and normal bladder tissues. A. WB (Western blotting) validation of NUSAP1 expression in 30 pairs of bladder cancer tissues and normal bladder tissues; B. The differences in expression were analyzed using image J; C. RT-qPCR (Real-time quantitative polymerase chain reaction) was performed to detect the altered expression; D. The statistically analyzed the differential expression alterations.

progression by influencing immune cell infiltration in malignant tumors. Immunoregulatory molecules exert a pivotal role within the tumor microenvironment, and our analysis unveiled a close association between NUSAP1 expression and immune-related factors, encompassing Major histocompatibility complex (MHC), immune activators, immunosuppressors, chemokines, and chemokine receptor proteins. Notably, the nature of these regulatory relationships varied across different cancers.

It's important to highlight that our research outcomes underscore the significant prognostic implications of this gene in these tumors, affirming the robustness and reliability of our analytical findings. Furthermore, it has been reported that there exists a correlation between NUSAP1 and the prognosis of bladder cancer. Unveiling these elusive mechanisms holds paramount importance in unraveling the molecular intricacies that drive the onset and progression of this formidable disease. We can infer that the modulation of NUSAP1 activity, particularly in the context of bladder cancer, has the potential to yield insights that may enhance therapeutic strategies and outcomes. In the context of advanced-stage bladder cancer, conventional treatment modalities such as surgical intervention, radiotherapy, and chemotherapy often fall short of achieving satisfactory outcomes. The inherent challenges posed by late-stage bladder cancer necessitate the exploration and development of innovative therapeutic approaches that can effectively address the complexity and resilience exhibited by this formidable disease. Perhaps further research should be dedicated to investigating gene targets and their correlation with immune checkpoint inhibitors across various cancer types. Such investigations have the potential to aid in predicting the outcomes of anti-tumor immunotherapy. In our pursuit to understand the association between NUSAP1 and the sensitivity to anti-tumor drugs, we examined the correlation between NUSAP1 and drug Half maximal (50%) inhibitory concentration (IC50) using the Cellminer database. Our findings indicated that high NUSAP1 expression was linked to resistance to multiple anti-tumor drugs. Nevertheless, it's important to acknowledge that factors and mechanisms influencing drug sensitivity in cancer are intricate and multifaceted. Therefore, we delved deeper into exploring the mechanisms underlying NUSAP1's impact on drug sensitivity. Additionally, our Kaplan-Meier survival analysis revealed a significant association between high NUSAP1 expression and poorer patient prognosis, as well as its effect on immunotherapy outcomes. Based on these results, we can explore cancer immunotherapy based on NUSAP1, which may provide a research basis for the development of tumor immunotherapy strategies.

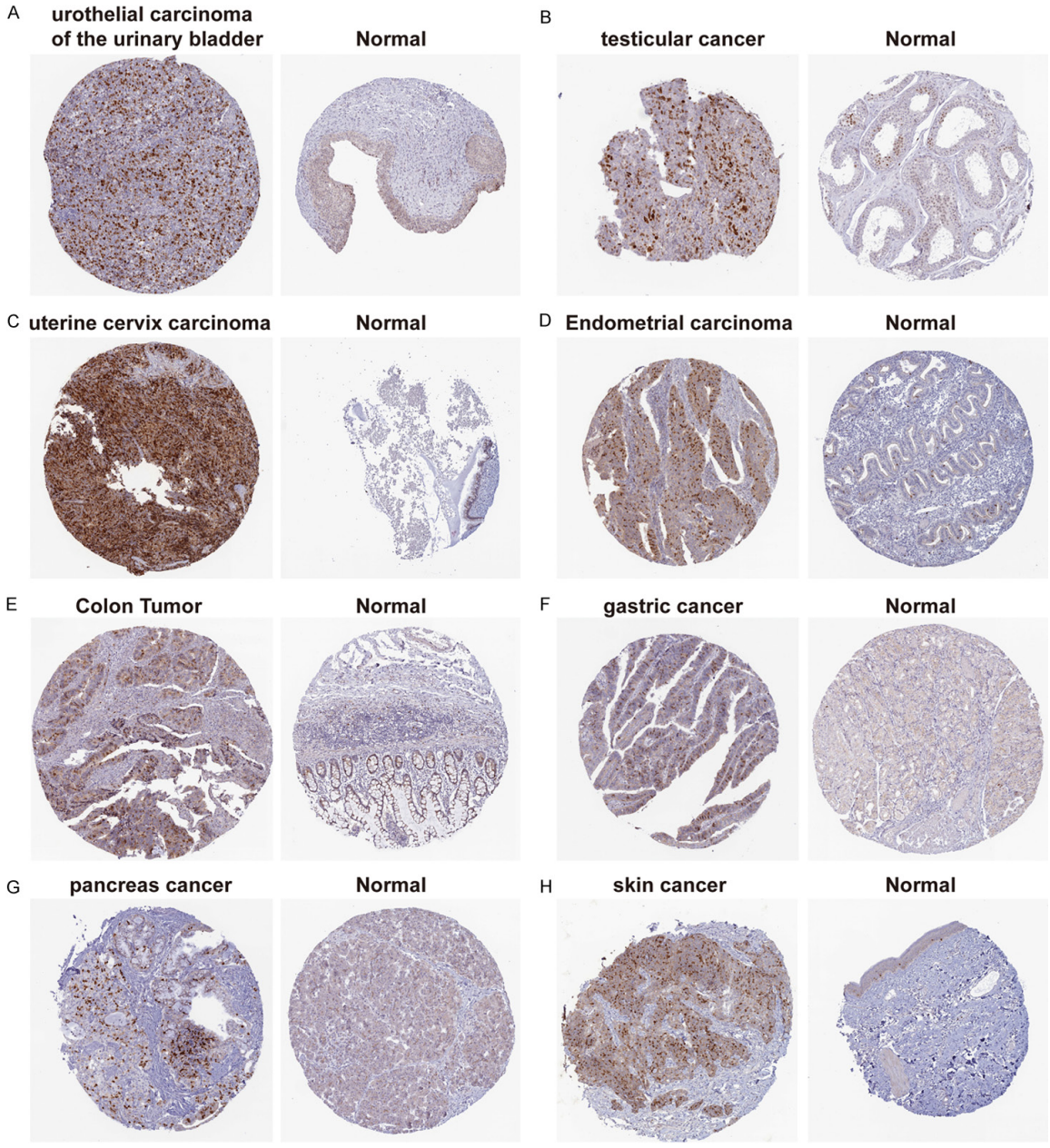

Figure 12. Changes in NUSAP1 (Nucleolar spindle-associated protein 1) expression. A. Bladder uroepithelial cancer; B. Testicular cancer; C. Cervical cancer; D. Endometrial cancer; E. Colon cancer; F. Gastric cancer; G. Pancreatic cancer; H. Skin cancer tissues and their normal tissues.

Moreover, our observations unveiled a noteworthy association between NUSAP1 and two prominent immunotherapy biomarkers (TMB and MSI), across diverse tumor types. Such correlations provide valuable insights into the potential interplay between NUSAP1 and the underlying immunogenicity within the tumor microenvironment, thereby laying the foundation for exploring novel therapeutic strategies harnessing the power of immunotherapy. We also noticed that the tumor neoantigen load can be effectively assessed by examining TMB [26]. MSI represents a robust mutant phenotype resulting from defects in DNA mismatch repair and serves as a critical predictor for responses to immunotherapy. We observed a significant correlation between NUSAP1 expression in tumor tissues and TMB in several cancers, including PCPG, UCEC, SKCM, COAD, PRAD, STAD, LIHC, LUAD, ACC, KICH, and THCA.

Moreover, high NUSAP1 expression was notably associated with MSI in tumor tissues from UCEC, STAD, PRAD, KIRC, COAD, BRCA, GBM, and Lymphoid Neoplasm Diffuse Large B-cell Lymphoma (DLBC). These findings suggest that NUSAP1 may impact TMB and MSI in tumors, which, in turn, affect patient responses to Immuno-checkpoint inhibitor (ICI) treatment. This information can be valuable for predicting the prognosis of immunotherapy in cancer. Therefore, we hypothesized that tumors with high NUSAP1 expression, high TMB, and MSI might exhibit improved responses to ICI treatment. Methyltransferases are well-established epigenetic markers in cancers, and some methyltransferases are now validated therapeutic targets [27, 28]. Additionally, it has been reported that tumors with mismatch repair protein defects may be more susceptible to immune checkpoint inhibitors [29, 30]. In our study, we explored the association of NUSAP1 with methyltransferases and mismatch repair proteins and identified significant correlations between NUSAP1 expression and these factors in several cancer types.

It is indeed worth noting that the current body of literature exploring the functional aspects of NUSAP1 in the context of bladder cancer remains limited. These findings hold significant promise in augmenting the development of targeted therapeutic approaches for bladder cancer, thereby potentially enhancing the efficacy and precision of treatment interventions in this challenging disease. Our comprehensive pan-cancer analysis has uncovered a substantial connection between NUSAP1 and the prognosis and progression of various human cancers. Furthermore, this research has unveiled a significant correlation between NUSAP1 and crucial immune markers, such as immune cell infiltration, immunomodulatory factors, and immune biomarkers. These findings shed light on the potential mechanisms linking NUSAP1 to the immune system. The identification of immune-related effects of NUSAP1 through this study could offer valuable insights for targeted therapeutic approaches in bladder cancer. Our follow-up plan is to validate our conclusions in other database, and we need to experimentally validate them at the molecular level and animal level to investigate the association with clinical tumor tissue specimen information and patient prognosis, with the expectation that they can be used clinically to guide the treatment and prognostic assessment of patients with bladder cancer.

Conclusion

This study represents one of the few pioneering investigations that specifically delve into the immunotherapeutic potential encapsulated within NUSAP1 in the context of bladder cancer. By shedding light on the intricate interplay between NUSAP1 and the immune system within the unique microenvironment of bladder cancer, our research serves as a steppingstone toward unraveling novel avenues for harnessing the immunotherapeutic value of NUSAP1 in the battle against this formidable disease. This research contributes significantly to our understanding of NUSAP1's role in the development of tumors, suggesting its potential as both a prognostic biomarker and an immunotherapeutic target.

Disclosure of conflict of interest

None.

Abbreviations

NUSAP1, Nucleolar spindle-associated protein 1; TCGA, The Cancer Genome Atlas; ssGSEA, Single-sample gene-set enrichment analysis; WB, Western blotting; RT-qPCR, Real-time quantitative polymerase chain reaction; IHC, Immunohistochemistry; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; BCG, Bacillus Calmette-Guerin; GEO, Gene Expression Omnibus; HCC, Hepatocellular carcinoma; GSEA, Single-sample gene set enrichment analysis; ICP, Immune Checkpoint Pathways; TMB, Tumor mutation burden; MSI, Microsatellite instability; GTEx, Genotype-Tissue Expression; CCLE, Cancer Cell Line Encyclopedia; CNV, Copy number variations; OS, Overall survival; DFS, Disease-free survival; DSS, Disease-specific survival; PFS, Progression-free survival; HR, Hazard ratio; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomics; NCI, National Cancer Institute's Center; HPA, The human protein atlas; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; ESCA,

Esophageal carcinoma; GBM, Glioblastoma multiforme; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; STAD, Stomach adenocarcinoma; THCA, Thyroid carcinoma; UCEC, Uterine Corpus Endometrial Carcinoma; THYM, Thymoma; ACC, Adrenocortical carcinoma; KIRP, Kidney renal papillary cell carcinoma; SKCM, Skin Cutaneous Melanoma; LGG, Brain Lower Grade Glioma; PAAD, Pancreatic adenocarcinoma; LAML, Acute Myeloid Leukemia; MESO, Mesothelioma; ALL, Acute lymphoblastic leukemia; UVM, Uveal Melanoma; STES, Stomach and Esophageal carcinoma; HNSC, Head and Neck squamous cell carcinoma; OV, Ovarian serous cystadenocarcinoma; TGCT, Testicular Germ Cell Tumors; MHC, Major histocompatibility complex; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; IC50, Half maximal (50%) inhibitory concentration; ICI, Immunocheckpoint inhibitor.

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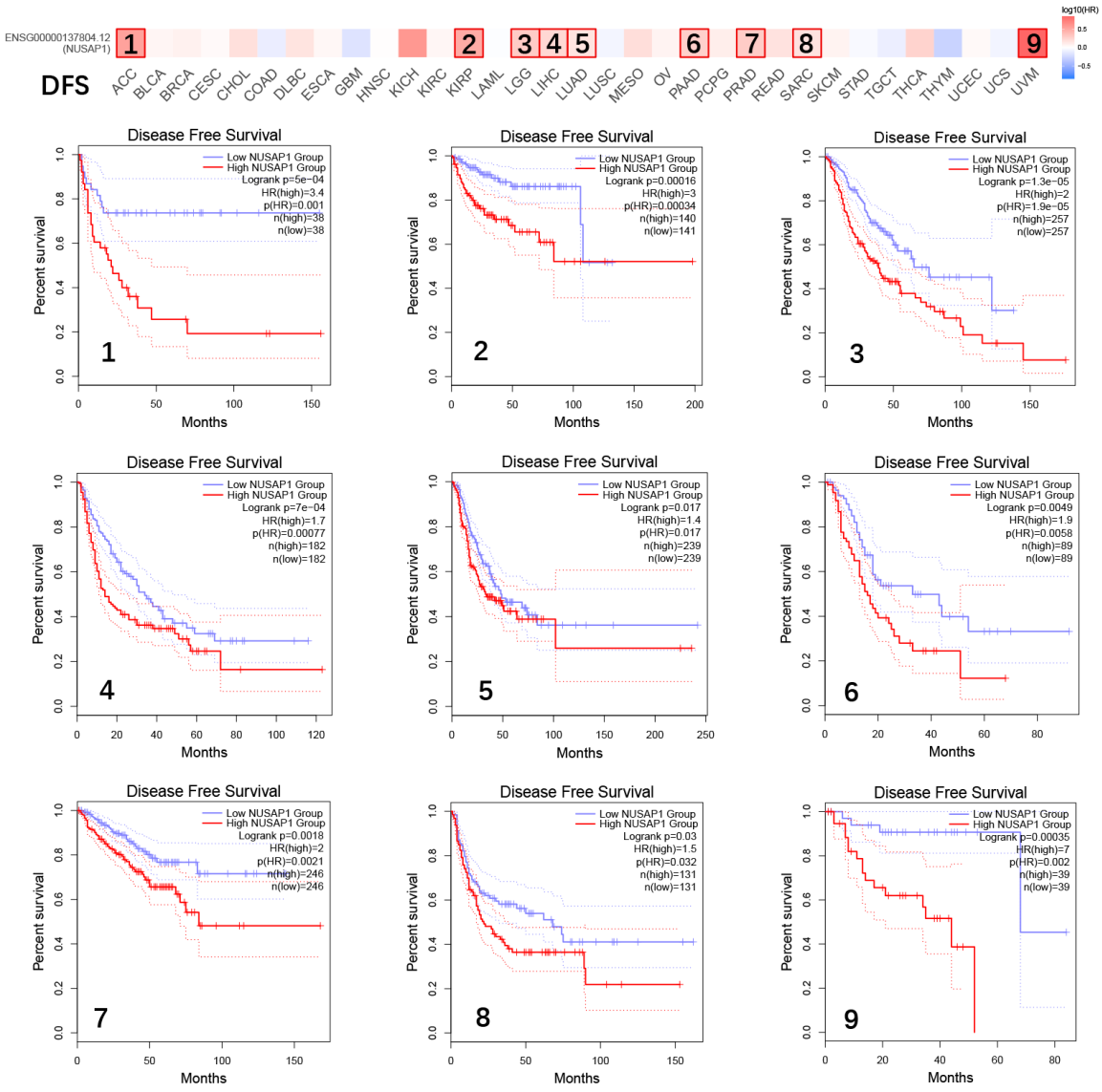
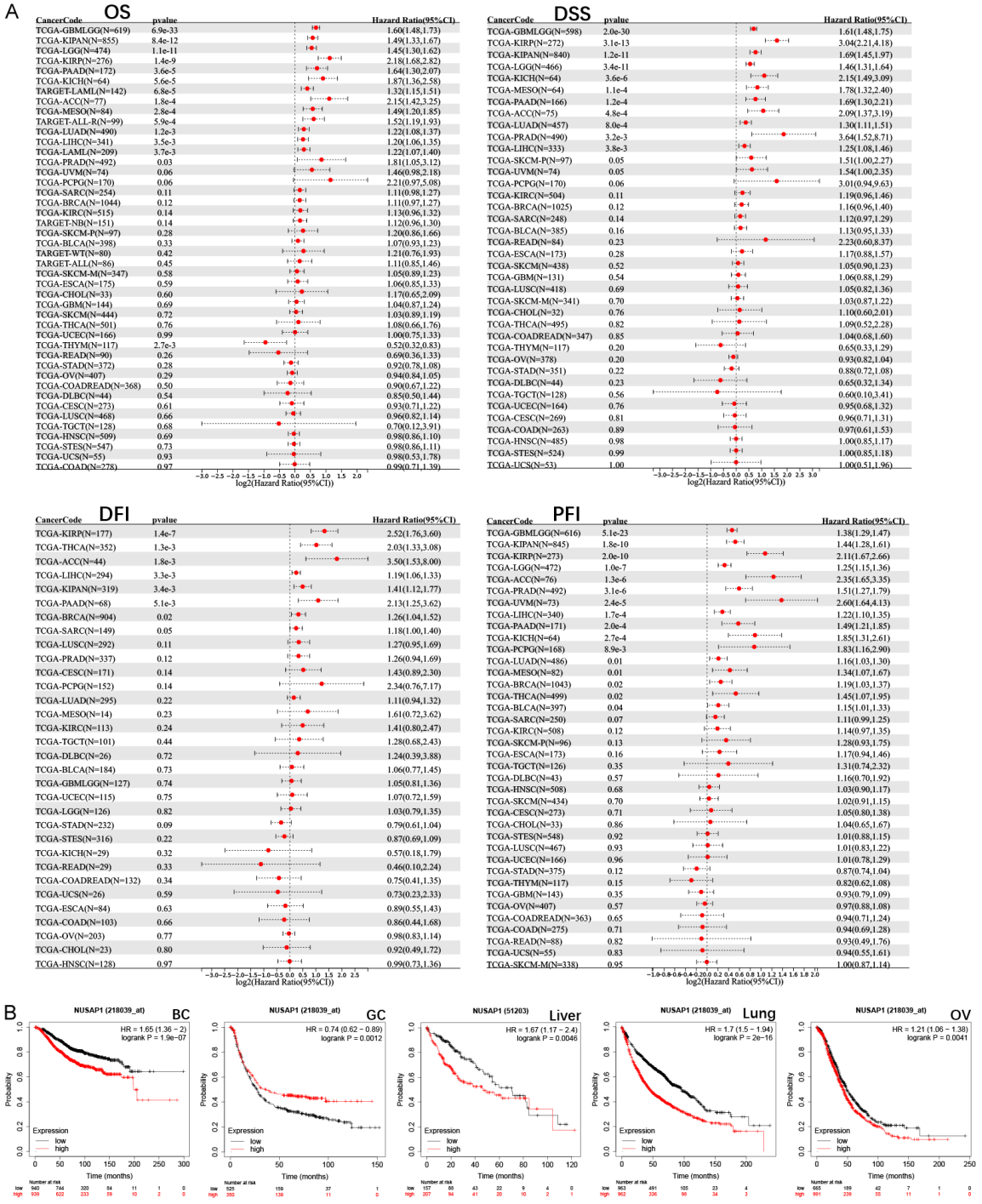
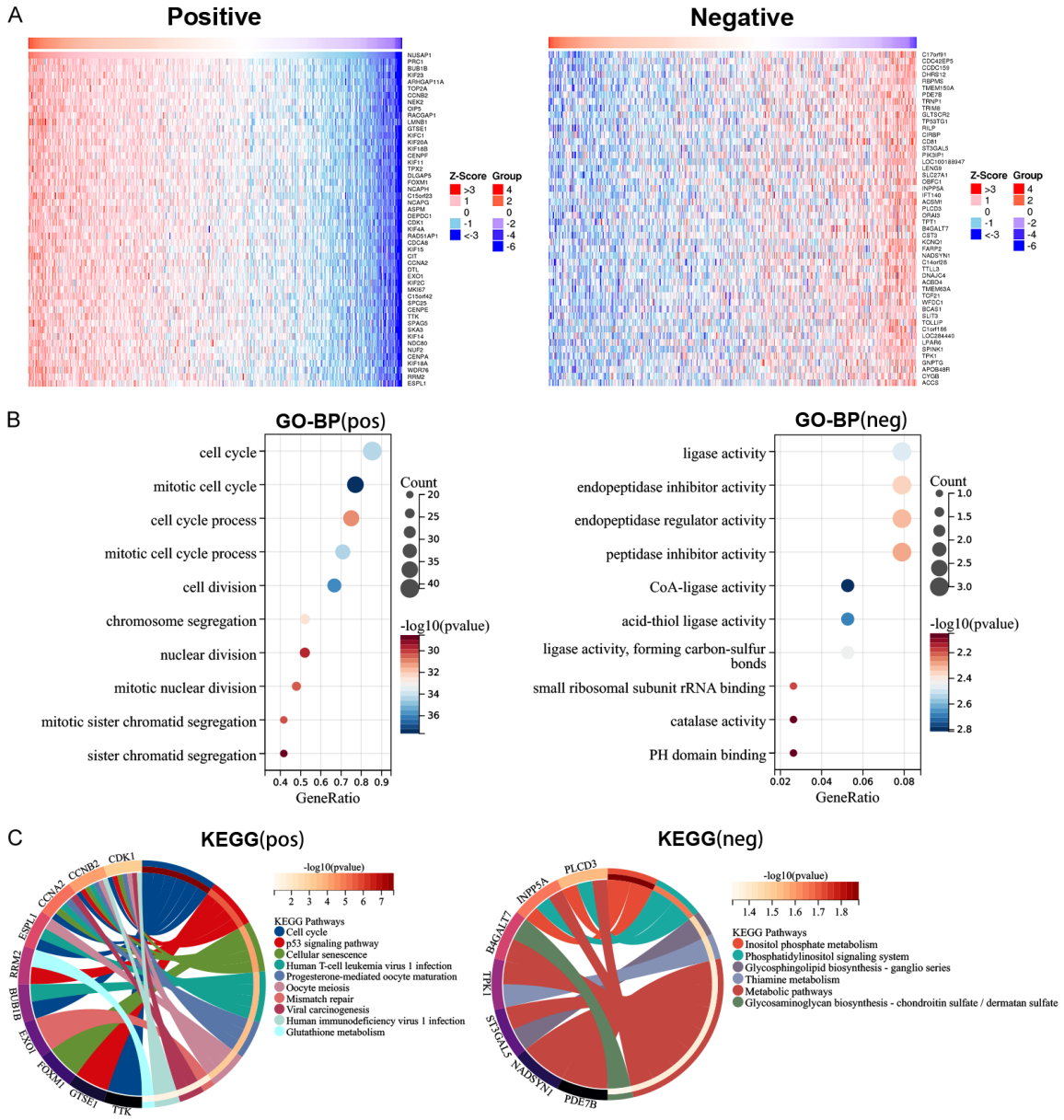


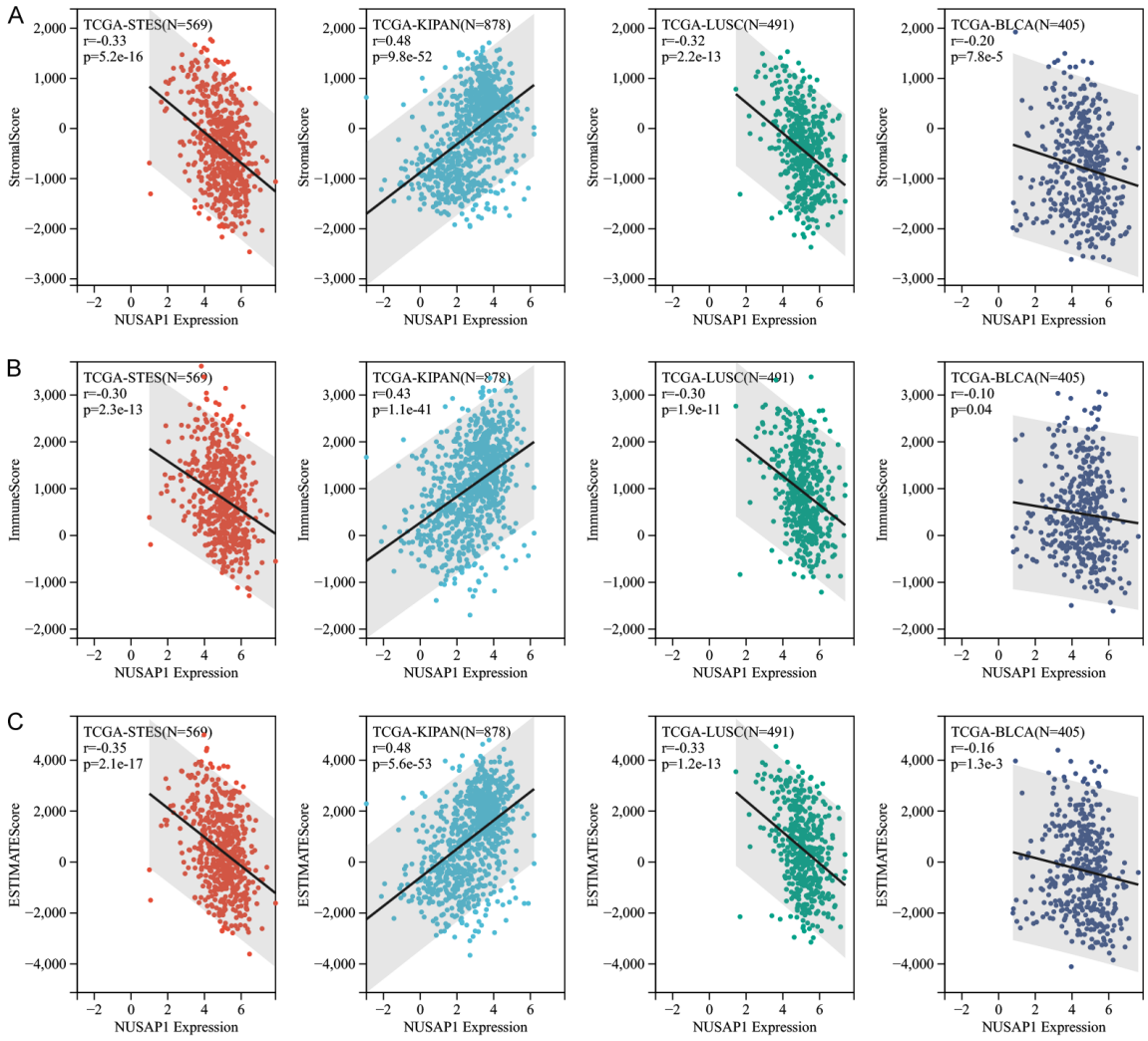
Supplementary Figure 1. DFS (Disease-free survival) of different NUSAP1 (Nucleolar spindle-associated protein 1) expression levels in multiple tumor.



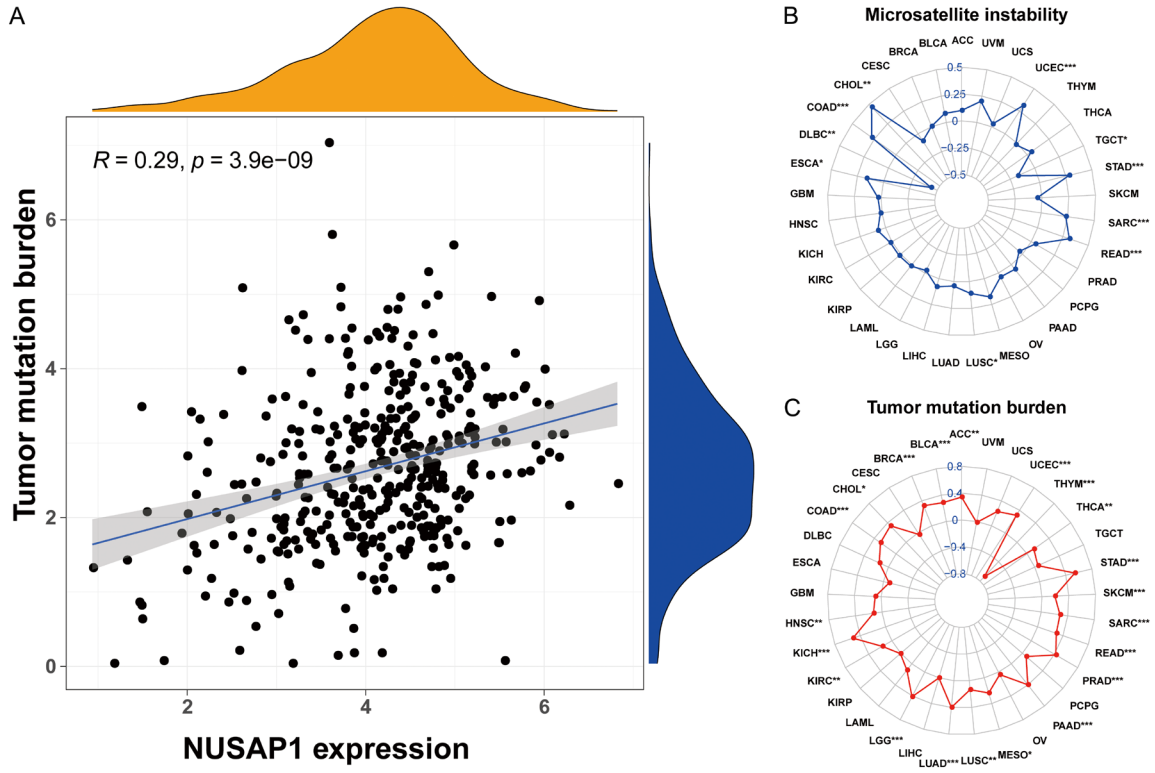
Supplementary Figure 2. A. Correlation analysis of different NUSAP1 (Nucleolar spindle-associated protein 1) expression levels with OS (Overall survival), DSS (Disease-specific survival), DFI (Disease free interval) and PFI (Progression free interval). B. Correlation analysis of high and low NUSAP1 expression with OS in bladder, gastric, liver, lung and ovarian cancers.



Supplementary Figure 3. Analysis of NUSAP1 (Nucleolar spindle-associated protein 1)-related genes in bladder cancer. A. The heat map displays the enrichment patterns of the top 100 genes correlated with NUSAP1. B. GO (Gene ontology)-BP (Biological Process) enrichment analysis was performed on the top 50 NUSAP1-positive or NUSAP1-negative correlated genes. C. KEGG (Kyoto Encyclopedia of Genes and Genomics) enrichment analysis was conducted to analyze the top 50 genes associated with NUSAP1.



Supplementary Figure 4. Correlation between NUSAP1 (Nucleolar spindle-associated protein 1) expression and Estimate score (A), Immunity score (B) and StromalScore (C) in STES, KIPA, LUSC and BLCA. STES, Stomach and Esophageal carcinoma; KIPA, NPan-kidney cohort; LUSC, Lung squamous cell carcinoma; BLCA, Bladder Urothelial Carcinoma.



Supplementary Figure 5. (A) Correlation analysis of NUSAP1 (Nucleolar spindle-associated protein 1) expression with MSI (Microsatellite instability) (B), TMB (Tumor mutation burden) (C).