

The Role of DISP3 in Thyroid Cancer Progression: Implications for Immune Microenvironment Modulation

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Research Article

Keywords: DISP3, Thyroid cancer, Tumor immune microenvironment, Macrophage polarization, dendritic cell

Posted Date: April 21st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9394485/v1>

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Additional Declarations: No competing interests reported.

Abstract

Objective: To investigate the role of DISP3 in thyroid cancer (TC) progression and its modulation of the tumor immune microenvironment.

Methods: Bioinformatics analyzed DISP3 expression and its prognostic significance in TC. The biological impact of DISP3 on TC cells was validated through in vitro assays and in vivo BALB/c mice models. Flow cytometry and cytokine assays were performed to evaluate the regulatory mechanism of DISP3 on dendritic cell (DC) maturation and macrophage polarization.

Results: DISP3 was overexpressed in TC and significantly correlated with poorer prognosis. Bioinformatics revealed that DISP3 expression negatively correlated with DC and macrophage infiltration. Experimentally, knocking down DISP3 significantly inhibited TC cell proliferation and migration both in vitro and in vivo. Furthermore, DISP3 knockdown enhanced anti-tumor cytokine secretion, promoted DC maturation, and induced a phenotypic shift of macrophages toward the M1 (anti-tumor) phenotype.

Conclusion: DISP3 promotes TC progression by modulating the immune microenvironment, specifically by suppressing DC maturation and M1 macrophage polarization. These findings suggest DISP3 as a promising novel target for TC immunotherapy.

Introduction

Thyroid cancer (TC) is a frequent endocrine malignancy, with a steadily increasing prevalence of differentiated TC (DTC) worldwide over the past three decades(1). Although most patients achieve favorable outcomes with current treatments, about 30% experience disease progression due to recurrence and become unresponsive to current therapies(2). Frequently, TC displays local immune responses, creating a tumor microenvironment (TME) where tumor and host cells coexist(3). The immune cell composition within this microenvironment influences the tumor's clinical aggressiveness(4-6).

In cancer immune surveillance, the innate immune response initially targets and eliminates tumor cells, followed by macrophages phagocytosing the cell fragments(7, 8). Dendritic cells (DCs) process neoantigens from these cells, secrete inflammatory cytokines, and present tumor antigens to T cells, thereby facilitating an adaptive immune response(9). This activates and expands T cells and produces tumor-specific antibodies by B cells to eliminate the tumor. However, research into the TME reveals that tumors can re-educate immune cells to support tumor development(10). Reversing this trend is indispensable for tumor immunotherapy success(11).

DISP3, part of the Dispatched family, primarily regulates Hedgehog signaling, which is crucial for embryonic development, cell differentiation, and tissue homeostasis(12). Over-activation of Hedgehog signaling is linked to malignancy development, including gastric, pancreatic, basal cell, and prostate cancers(13). Nonetheless, the DISP3 function in TC remains unexplored.

Materials and Methods

Patient clinical samples

Between June and December 2024, 10 paired TC and adjacent non-cancerous tissues were collected from Jingzhou Hospital Affiliated to Yangtze University and maintained at -80°C . The study was authorized by the hospital's Ethics Committee and complied with their guidelines.

Bioinformatics analysis

Transcriptomic data (TPM-normalized) and corresponding clinical information for the Thyroid Carcinoma project (TCGA-THCA, $n=510$) and normal thyroid tissues ($n=58$) were downloaded from the TCGA database. For validation, the GEO database was utilized to ensure the reproducibility of DISP3 expression patterns.

Differential expression analysis between high- and low-DISP3 expression groups (stratified by the median) was performed using the DESeq2 package in R. Significant differentially expressed genes (DEGs) were identified using the criteria of $|\log_2 \text{FoldChange}| > 1.5$ and adjusted $P < 0.05$. To evaluate the prognostic value, univariate Cox proportional hazards regression analysis was used to calculate hazard ratios (HR) and identify prognosis-related genes ($\text{HR} > 1.2$, $P < 0.05$).

Functional enrichment was conducted using the clusterProfiler package. Overlapping genes between DISP3-correlated genes (2,197 genes), prognosis-related genes (3,214 genes), and immune-related genes from the ImmPort database (2,271 genes) were analyzed for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

To quantify the infiltration of 24 types of immune cells, the single-sample Gene Set Enrichment Analysis (ssGSEA) algorithm was employed. The correlation between DISP3 expression and immune cell abundance, including dendritic cells (DCs) and macrophages, was assessed using Spearman correlation analysis.

Cell culture

The culture of BCPAP, FTC133, K-1, and TPC-1 cell lines (Wuhan Institute of Cell Biology, China) was conducted in 1640 complete medium that contained 10% fetal bovine serum in a 5% CO₂ incubator at 37 °C.

Pathological sample processing

Tumor and adjacent non-cancerous tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned into 5µm slices. Following deparaffinization and rehydration through a graded ethanol series, heat-induced antigen retrieval was performed using microwave heating in citrate buffer (pH 6.0). To block non-specific binding, sections were incubated with 5% bovine serum albumin (BSA) and subsequently incubated with primary antibodies at 4 °C overnight. After washing, the sections were treated with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using a 3,3'-diaminobenzidine (DAB) substrate. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted.

Total RNA was isolated from tissues or cells using TRIzol reagent according to the manufacturer's protocol. The concentration and purity of the RNA were determined spectrophotometrically. Complementary DNA (cDNA) was synthesized using a reverse transcription kit, and quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix. The primer sequences were as follows: DISP3 Forward: 5'-CCAAAGAATGACAGGAACACTGAG-3', Reverse: 5'-CTCATCTACATACCAGTAGAACTCG-3'; GAPDH Forward: 5'-CTGCTCCTCCTGTTTCGACAGT-3', Reverse: 5'-CCGTTGACTCCGACCTTCAC-3'. The PCR thermal cycling conditions consisted of an initial denaturation at 95 degrees Celsius for 5 minutes, followed by 40 cycles of 95 degrees Celsius for 15 seconds and 60 degrees Celsius for 30 seconds. Target gene expression levels were normalized to GAPDH and calculated using the 2-Delta Delta Ct method

CCK8 assay

Cells from each group were digested and resuspended in a complete medium. Cell growth was evaluated at 24, 48, 72, and 96 h by CCK-8 assay, measuring optical density at 450 nm via an enzyme marker.

Clone formation assay

Two thousand cells were seeded into six-well plates, with half of the medium refreshed daily. After 10 days, cells were collected, blotted to remove the excess medium, fixed in 4% paraformaldehyde for 10 min, and stained using crystal violet.

Transwell assay

Initially, 10,000 cells were placed in the Transwell system upper chamber (Corning, USA), incubated for 24 h at 37 °C, and stained using crystal violet for 10 min, followed by washing to remove excess stain. The migrated cells were then counted and photographed.

Scratch test

FTC133, K-1, and TPC-1 TC cells were grown in IBIDI two-well inserts placed in 24-well plates for 24 h. The inserts were removed using forceps, and 1 mL of low serum medium was introduced to each well. The healing process of the scratches was monitored and photographed at the start and 24 h after the inserts were removed.

Tumor formation in BALB/c mice

Twelve 6-week-old female BALB/c mice (average weight 24 g) were randomly assigned into two groups. Each mouse received an injection of approximately 10 million tumor cells into the left subcutaneous fat pad. Xenograft size was assessed every other day using the formula $V = 0.5 \times L \times W^2$, where L, tumor length; W, tumor width. Twenty days post-experiment, each BALB/c mouse was euthanized for tumor excision and weighing. The Jingzhou Central Hospital Animal Ethics Committee authorized the study.

Enzyme-linked immunosorbent assay

An ELISA assay was utilized to determine specific cytokine levels in the sub-cavitary culture fluid from TC and macrophage co-cultures. Herein, we used enzyme markers to determine the absorbance at 450 nm and, calculated the concentration from the calculation curve provided by the reagent vendor and expressed the results in pg/mL.

Immune cell ratio analysis

Mouse tumors were mechanically minced and incubated with collagenase IV and DNA enzyme at 37 degrees Celsius for 30 minutes with shaking. The resulting cell suspension was filtered through a 70 micrometer mesh. To prevent non-specific binding, cells were incubated with an anti-mouse CD16/32 antibody (Fc Block, BioLegend, Cat# 101302). Subsequently, the cells were stained with specific fluorophore-conjugated antibodies at 4 degrees Celsius for 20 minutes in the dark.

For macrophage polarization analysis, the following antibodies were used: APC anti-mouse F4/80 (Clone BM8, BioLegend, Cat# 123115), PE anti-mouse CD86 (Clone GL-1, BioLegend, Cat# 105007), and FITC anti-mouse CD206 (Clone C068C2, BioLegend, Cat# 141707). For mature dendritic cell (DC) identification, the antibodies included: BV421 anti-mouse CD80 (Clone 16-10A1, BioLegend, Cat# 104725) and PE anti-mouse CD86 (Clone GL-1, BioLegend, Cat# 105007). The stained cells were analyzed using LSRT Fortessa flow cytometry (BD Biosciences) and FlowJo software.

Statistical analysis

Data analyses were conducted through one-way ANOVA with Bonferroni's post hoc test or an unpaired two-sided Student's t-test using GraphPad Prism 5.0, reporting results as mean $\pm$ standard deviation. $P < 0.05$ indicated statistical significance: no significant (N.S), $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Results

DISP3 overexpression and its clinical significance

Analysis of TCGA-THCA data revealed DISP3 overexpression in TC samples, both unpaired and paired (**Figures 1A-B**), and in ten other malignancies: BLCA, BRCA, CHOL, HNSC, KIRC, LIHC, LUAD, PCPG, PRAD, and UCEC (**Figure 1C**). Clinical analysis results indicated that DISP3 overexpression is not associated with the T or N stage in TC patients but is higher in M1 compared to M0 patients (**Figures 1D-F**). Patients with DISP3 overexpression had a significantly worse prognosis (**HR = 8.94, $p = 0.004$, Figure 1G**). The ROC curve area of 0.724 indicated DISP3's potential as a diagnostic biomarker for TC (**Figure 1H**).

Correlation and enrichment analyses

To ascertain DISP3's impact on TC prognosis, we performed a single-gene correlation analysis to identify co-expressed genes. The top 20 correlated genes were visualized in a heatmap, identifying the intersection of DISP3 co-expressed genes with TC prognosis-related genes (**Figures 2A-B**). The GO/KEGG enrichment analysis suggested that DISP3 may function through cell projection membranes and glycosyltransferase activity (**Figure 2C**). For further exploration of DISP3's role in tumor immunity, an enrichment analysis was conducted by intersecting DISP3-related immunity genes with TC prognosis-related genes (**Figure 2D**). The results suggested that DISP3 might promote TC development by affecting cytokine activity and cytokine-cytokine receptor interactions (**Figures 2E-F**).

DISP3 and its association with immune cell infiltration (ICI)

Building on the enrichment analysis, which highlighted DISP3's role in cytokine signaling within the tumor immune microenvironment (IME), the relation between DISP3 expression and ICI was examined. The DISP3 overexpression was correlated with increased pDC and NK CD56bright cell infiltration, whereas DC, Th17, aDC, and macrophage infiltration decreased (**figure 3A**). Specifically, in the DISP3 high-expression group, overall DC infiltration was reduced, with an escalation in pDC content and a reduction in aDC infiltration (**figure 3B-E**).

***Ex vivo* and *in vitro* experiments confirm DISP3 overexpression and its role in TC progression**

Herein, we conducted *ex vivo* experiments to assess DISP3's impact on TC, revealing significantly overexpressed DISP3 in TC cell lines, unlike the normal thyroid cell line Nthy-ori3-1 (**Figure 4A**). Immunohistochemical analysis of paired samples from 10 TC patients showed higher DISP3 levels in cancerous tissues than in adjacent normal tissues (**Figures 4B-C**).

Due to the aggressive nature of follicular TC and the prevalence of papillary thyroid carcinoma (PTC), we utilized FTC133, K-1, and TPC-1 cell lines for *in vitro* studies. Moreover, we selected appropriate siRNAs to achieve DISP3 knockdown in these cell lines, achieving satisfactory efficiencies (**Figure 4D**). Clone formation assay results revealed that DISP3 knockdown significantly inhibited TC cell proliferation (**Figure 4E**). Additionally, scratch and Transwell assay outcomes showcased DISP3 knockdown reduced cell migration and invasion (**Figures 4F-G**). These experiments were repeated three times and analyzed statistically (**Figures 4H-J**). The CCK-8 and BALB/c mice tumor formation assay results manifested a significant inhibition in TC growth following DISP3 knockdown (**Figures 4K-O**).

DISP3 modulates TC-IME

To explore DISP3's impact on the TC-IME, we developed a model co-culturing TC cells with M0 macrophages (**Figure 5A**). In this setup, tumor cells in the Transwell's upper compartment secrete cytokines that migrate to the lower compartment, influencing macrophages mimicking the TME's interaction between cancer and immune cells. Then, we assessed cytokine levels in the lower chamber cultures of both groups using ELISA. The study revealed that DISP3 knockdown in tumor cells resulted in elevated production of pro-inflammatory cytokines, including IL-1 β /4 and TNF- α , while reducing those of anti-inflammatory cytokines like IL-6/10 (**Figure 5B**). Additionally, flow cytometry analysis of immune cells in tumors from mice demonstrated that DISP3 knockdown increased M1 macrophage infiltration, decreased M2 macrophages, and significantly elevated mature DCs (**Figures 5C-F**).

Discussion

On a global scale, TC represents a common endocrine malignancy with rapidly increasing incidence. While most patients with DTC have a favorable prognosis, some may develop aggressive forms that resist standard treatments such as surgery, radioactive iodine, and thyroid hormone suppression. Recently, immunotherapy has become a promising approach for managing TC through the immune system to target and eliminate cancer cells. Pembrolizumab(14) and nivolumab(15), targeting PD-1 and CTLA-4 proteins, have shown

efficacy in certain patients with advanced, treatment-resistant conditions by boosting immune responses against tumors. Nonetheless, their limited success indicates significant potential for advancing immunotherapy in TC.

The TME encompasses both the internal and external conditions where tumor cells develop, proliferate, and metastasize and includes cancer cells, immune cells, cytokines, chemokines, and extracellular matrix. The tumor IME, a subset of the TME, consists of innate and adaptive immune cells, extracellular immune factors, and cell surface molecules, playing a pivotal role in tumor initiation and progression through complex interactions. Immune cells in the TME have been reported to be related to TC aggressiveness. Tumor-associated macrophages (TAMs) are particularly connected with clinical outcomes(16). Anaplastic TC, known for its poor prognosis, exhibits a high TAM density. In TC, TAMs are predominantly M2 macrophages, which promote a microenvironment conducive to tumor growth, survival, and angiogenesis(17, 18).

DCs, essential for antigen presentation and cytokine secretion, are usually rare in normal thyroid tissue but are more prevalent in PTC(19). These DCs often display an immature phenotype, possibly due to secreting immunosuppressive cytokines such as IL-10 by the tumor cells(20). Immature DCs exhibit limited antigen-presenting capabilities and struggle to activate the cytotoxic T cells' tumor-killing function(21).

This study utilized bioinformatics to examine DISP3 expression, prognosis, and fundamental characteristics in TC. Through the integration of analyses on co-expressed, prognosis-related, and immune-related genes, we clarified the mechanism of DISP3, a potential pathogenic gene in TC, and conducted both *in vivo* and *in vitro* experiments. DISP3 is recognized as a key regulator of the Hedgehog signaling pathway, which is crucial for embryonic development and tissue homeostasis. Over-activation of Hedgehog signaling has been linked to the development of various malignancies. It is highly plausible that DISP3 exerts its effects on the tumor immune microenvironment through the Hedgehog pathway, as this signaling cascade is known to play a role in modulating cytokine networks and immune cell recruitment. Herein, DISP3 knockdown improved the immune response within the TME and decreased TC malignant behavior. Elevated pro-inflammatory cytokines, TNF- α , and diminished secretion of immune-suppressive factors, IL-10, indicated an enhanced immune response. Concurrently, the increase in mature DCs improved tumor antigen presentation. Additionally, the shift from M2 to M1 macrophages strengthened innate immunity, transforming 'cold' immune tumors and potentially enhancing T-cell response to other immunotherapies(22, 23). While the immunohistochemical analysis in our study confirmed higher DISP3 levels in cancerous tissues than in adjacent normal tissues, it is important to acknowledge the limitation of the relatively small clinical sample size (n=10) used for this validation. Although these paired samples provided statistically significant preliminary evidence, future validation in

larger, multi-center cohorts is necessary to firmly establish the diagnostic and prognostic value of DISP3 in clinical practice. Additionally, our findings that DISP3 levels are higher in M1 stage patients compared to M0 stage patients further highlight its potential as a marker for advanced disease.

The shortcoming of this study is that the tumor IME involves more interactions of immune cells, cytokines, and extracellular matrix, and more complex and realistic models of the human body are needed to simulate this process. In addition, the effectiveness of targeted DISP3 therapy in synergy with other immunotherapies needs further validation.

Abbreviations

BLCA bladder cancer

BRCA breast cancer

CHOL bile duct cancer

DCs dendritic cells

DTC differentiated thyroid cancer

HNSC head and neck cancer

IME immune microenvironment

KIRC kidney clear cell carcinoma

LIHC liver cancer

LUAD lung adenocarcinoma

PCPG Pheochromocytoma & Paraganglioma

PRAD prostate cancer

PTC papillary thyroid carcinoma

TC thyroid cancer

UCEC Endometrioid Cancer

Declarations

Ethics approval and consent to participate

The studies involving humans were approved by Ethics Committee of jingzhou central hospital (Tz20240724). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Ethics Committee of jingzhou central hospital (Tz20240819). The study was conducted in accordance with the local legislation and institutional requirements.

Consent for publication

Not applicable.

Availability of data and material

The data that support the findings of this study are available in The Cancer Genome Atlas (TCGA) database (<https://tcia.at/home>) and Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>).

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Funding

Not applicable.

Authors' contributions

LQ and YT obtained clinical specimens; YT performed the data analysis; WC performed the formal analysis; WC and YT performed immunohistochemical analysis; LQ and SD wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

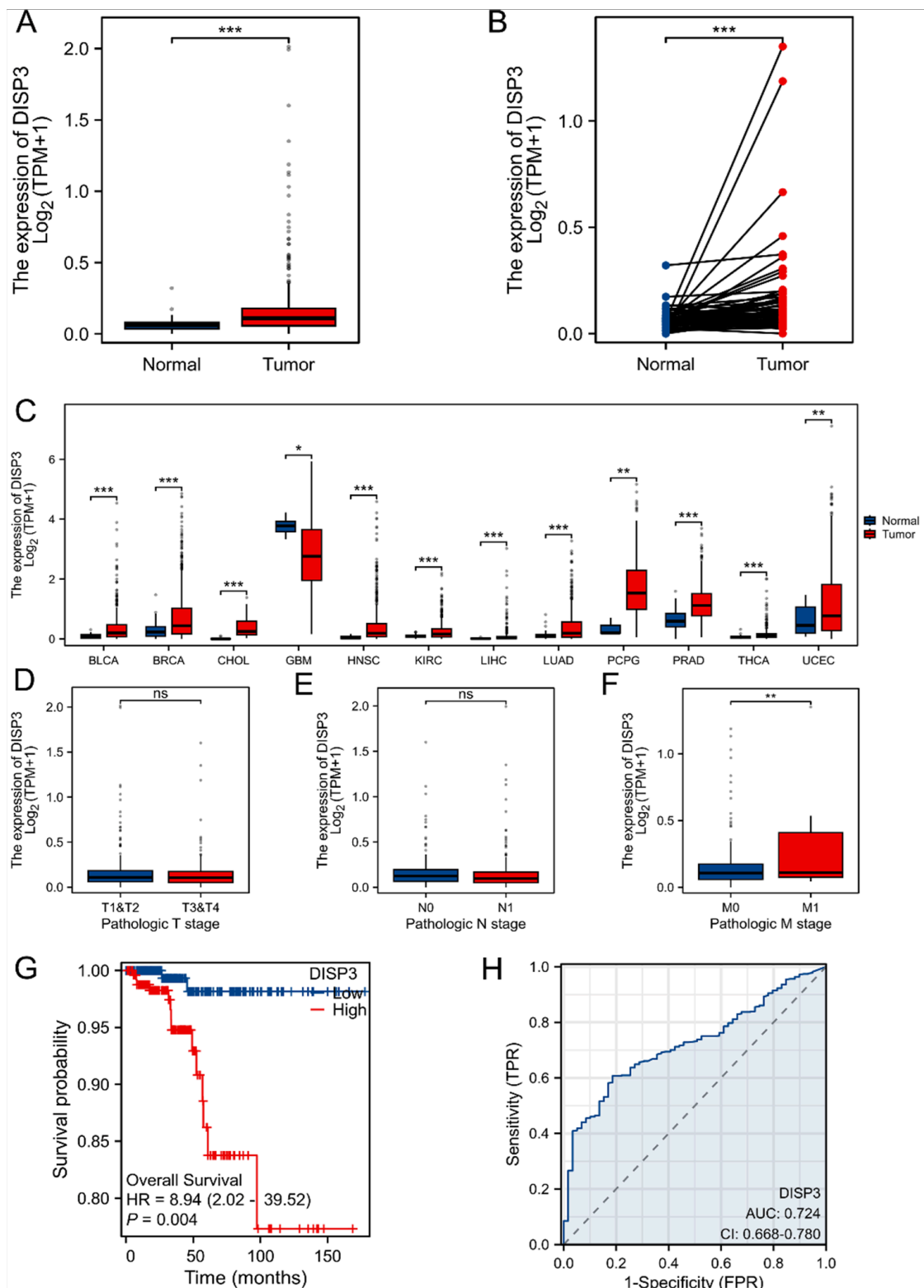


Figure 1

Expression of DISP3 in thyroid cancer (TC) and prognosis. (A-C) DISP3 expression in TC unpaired **(A)**, paired samples **(B)** and pan-cancer **(C)**. **(D-F)** Correlation of DISP3 with T- **(D)**, N- **(E)**, and M-stages **(F)** in TC patients. **(G)** Correlation of DISP3 expression with OS, a prognostic indicator in TC patients. **(H) ROC curve:** DISP3 in TC. **Data interpretation:** mean $\pm$ SD. ns no significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

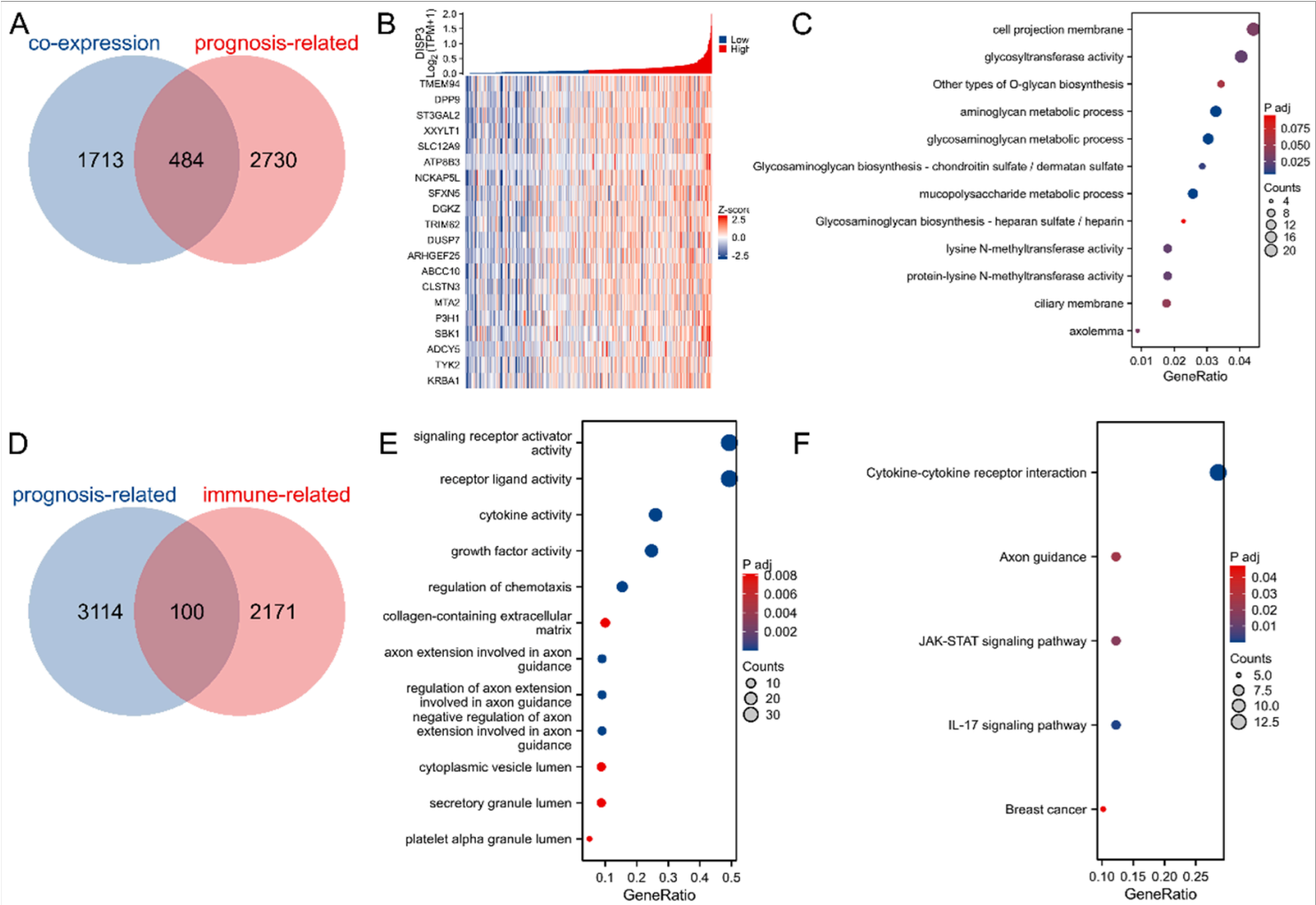


Figure 2

GO/KEGG analyses of DISP3-related genes. (A) Intersection of DISP3 co-expressed genes with thyroid cancer (TC) prognosis-related genes. (B) **Heatmap:** DISP3 co-expressed genes. (C) **GO/KEGG analysis:** Intersection of DISP3 co-expressed genes with TC prognosis-related genes. (D) Intersection of TC prognosis-related genes with immune-related genes. (E-F) Full GO (E) and KEGG analyses (F) of prognosis-immunity gene intersections.

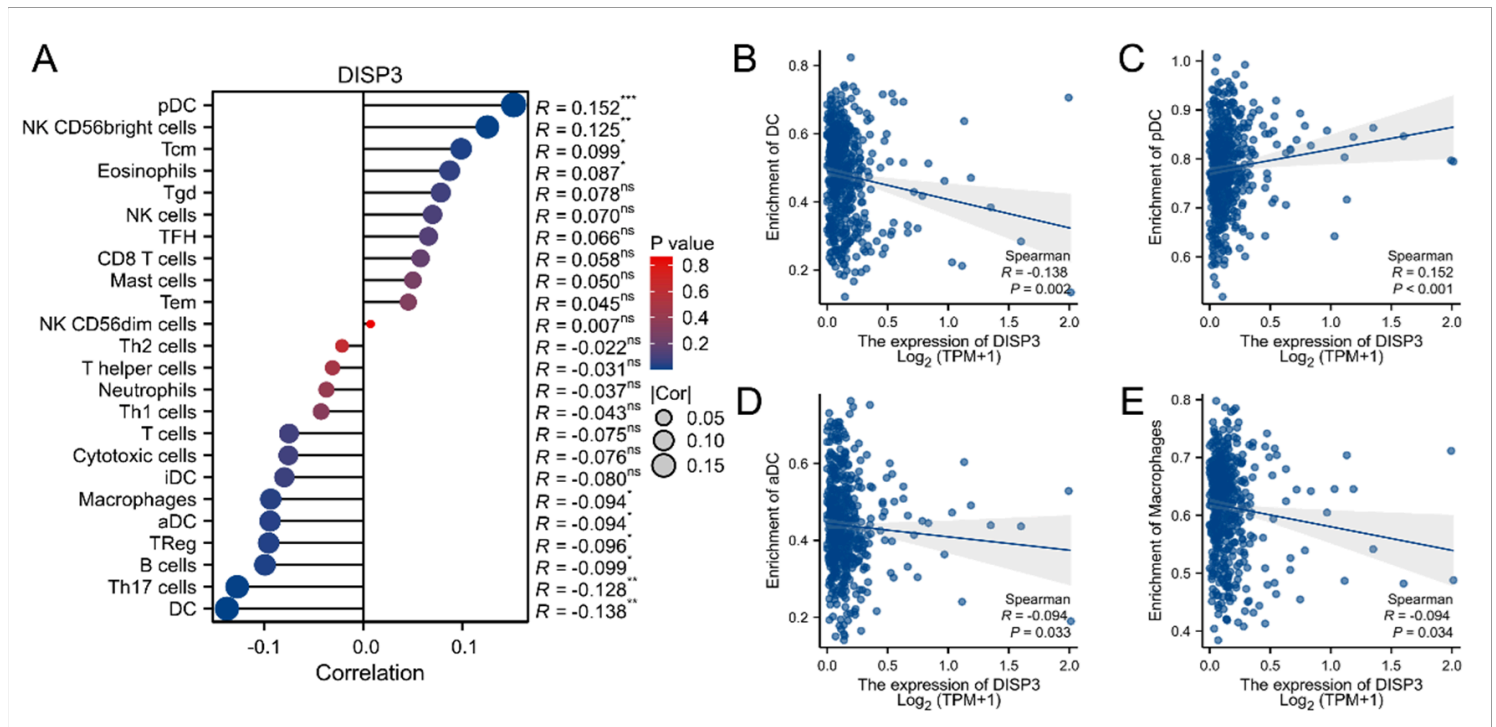


Figure 3

Relationship between DISP3 and immune infiltration (ICI) in thyroid cancer (TC)

(A) Interconnection of DISP3 expression with various ICI in TC. **(B-E)** Correlation of DISP3 expression with DC **(B)**, pDC **(C)**, aDC **(D)**, and macrophage **(E)** in TC. **Data interpretation:** mean $\pm$ SD. ns no significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

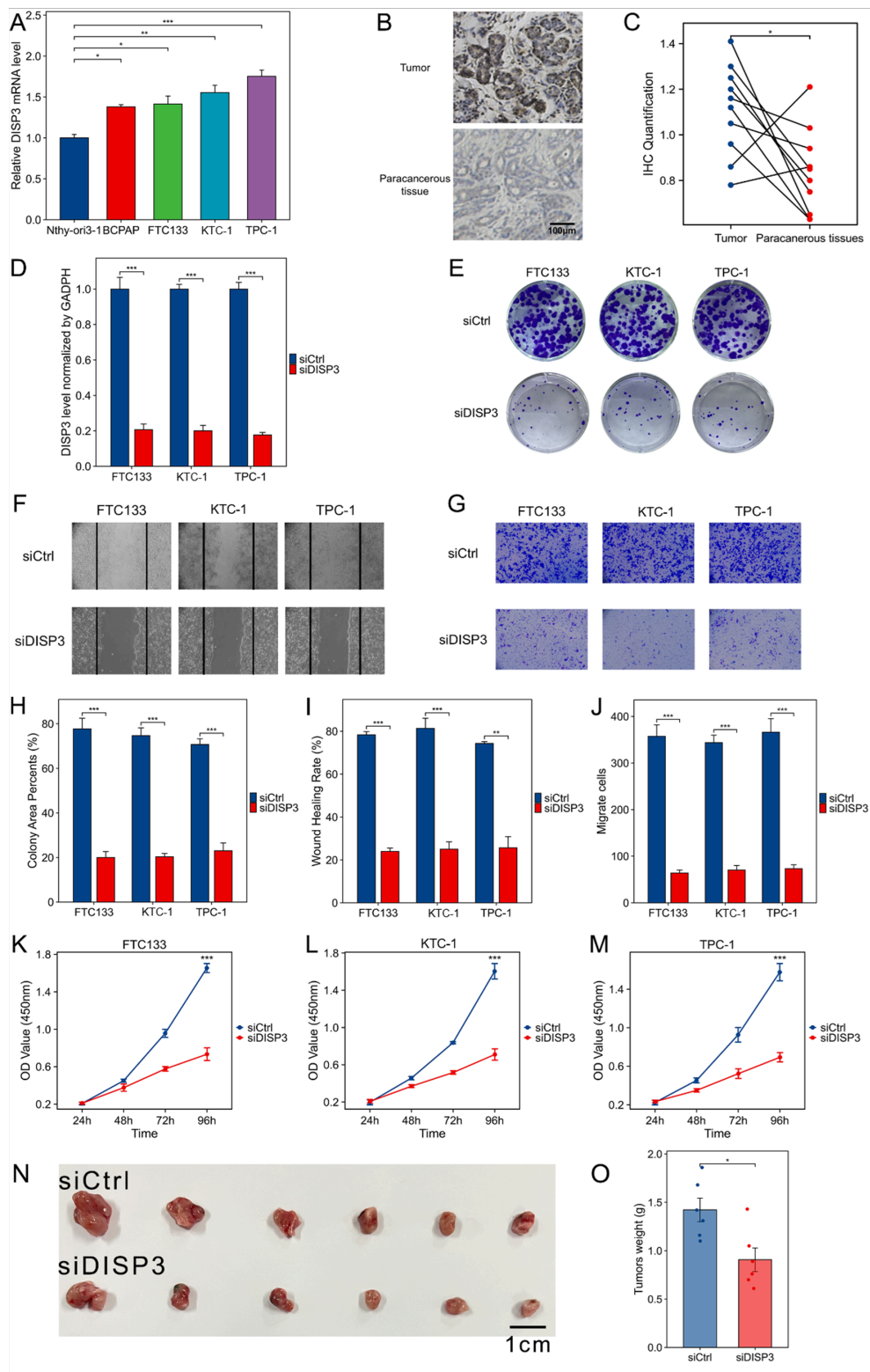


Figure 4

DISP3 knockdown inhibits thyroid cancer (TC) malignant biological behavior

(A) DISP3 expression in various cell lines of the thyroid gland. **(B-C)** Immunohistochemical typical pictures **(B)** and statistical analysis **(C)** of paired tissues from patients in our center. **(D)** qRT-PCR: Efficiency of DISP3 knockdown. **(E)** Clone formation assay:

Proliferative capacity of TC. **(F) Scratch assay:** Migration ability of TC. **(G) Transwell assay:** Invasion ability of TC. **(H) Quantitative analysis:** Clone formation assay. **(I-J) Quantitative analysis:** Scratch and **(J) Transwell** experiments. **(K-M) CCK-8 assay:** Growth potential of FTC-133, K1, and TPC-1. **(N-O) Size of tumor formation in BALB/c mice (N)** and statistical analysis **(O)**. Data interpretation: mean $\pm$ *SD*. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

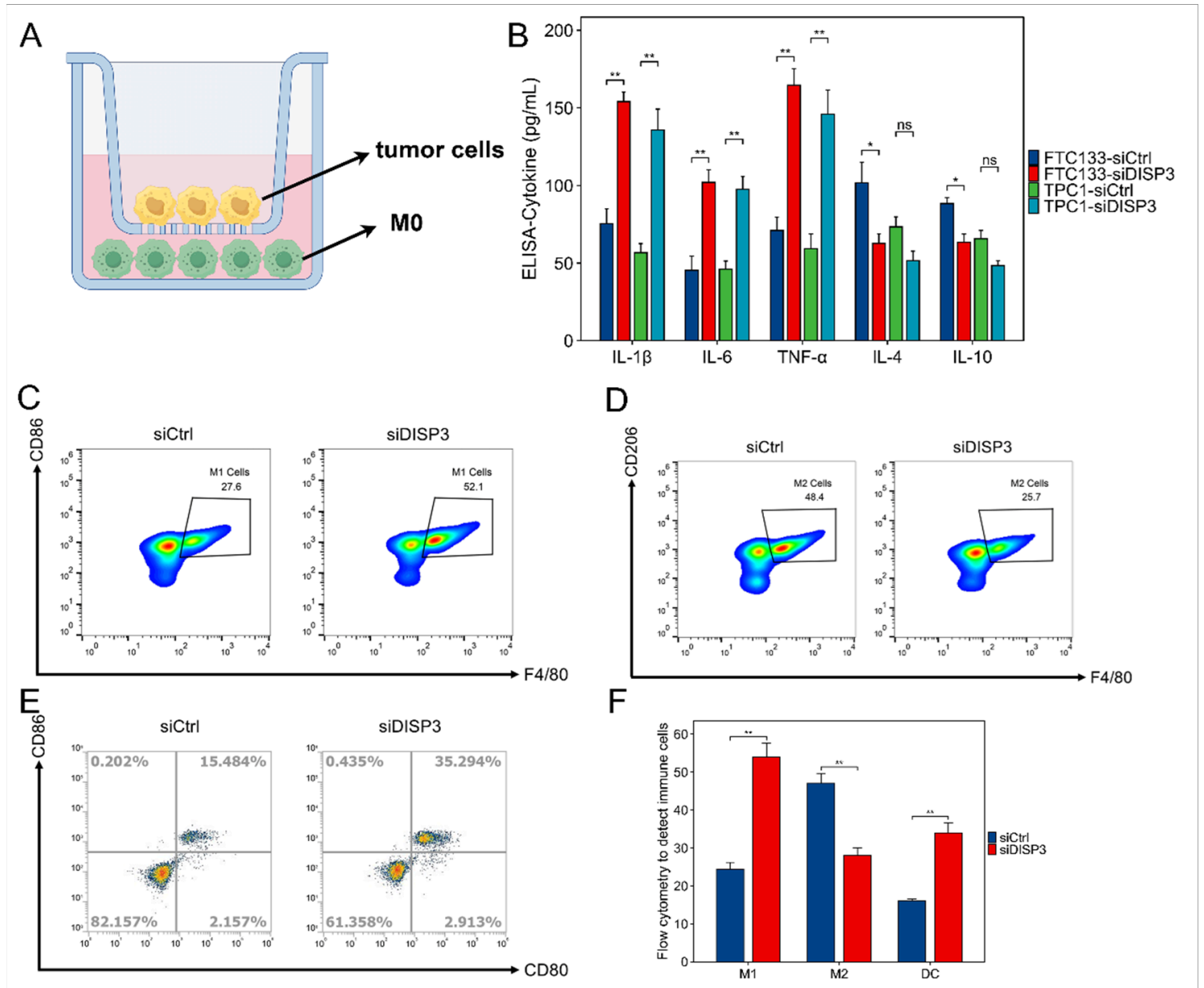


Figure 5

DISP3 reverses the pro-tumor immune microenvironment of thyroid cancer (TC). **(A)** Schematic of the co-culture model of TC with macrophages. **(B) ELISA:** Cytokines in the lower chamber of Transwell. **(C-F) Flow cytometry:** M1 macrophages **(C)**, M2 macrophages **(D)**, and mature DCs **(E)** in control and experimental tumors and

quantitative analysis **(F)**. **Data interpretation:** mean $\pm$ *SD*. ns no significant, * $p < 0.05$, ** $p < 0.01$.