

ARHGAP36 serves as a diagnostic and therapeutic marker that mediates immune escape and promotes thyroid cancer metastasis

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Abstract. Thyroid cancer (THCA) is a prevalent malignancy of the head and neck region, yet the mechanisms underlying its tumorigenesis and metastasis remain poorly understood. Given that Rho GTPase activating protein 36 (ARHGAP36) has been implicated in various cellular processes related to cancer progression, including cell migration and invasion, it represents a promising candidate for further investigation in THCA. To investigate the gene expression differences in ARHGAP36 between tumor and normal tissues, the GEPIA and UALCAN databases were utilized. Various factors were also evaluated, including sample types, cancer stages, demographics, histological characteristics and nodal status. The LinkedOmics database was used to construct a co-expression network for ARHGAP36 in THCA. Gene Ontology (GO) process enrichment analysis for ARHGAP36-associated genes was conducted via Metascape. The TIMER and TISCH databases were employed to explore the relationships between ARHGAP36 and immune markers as well as cell clusters. Functional *in vitro* assays were performed to assess cellular behaviors such as proliferation, migration and apoptosis. The results indicated that ARHGAP36 expression was significantly elevated in THCA tissues compared with normal tissues. Co-expression analysis revealed significant links between ARHGAP36 and key genes, including IGSF1, DPYSL3, ZCCHC12, CD97, LOXL4, CST2 and WSCD. The enriched GO processes involved T-cell immunity, particularly highlighting the association between ARHGAP36 and CD4⁺ T cell infiltration. Notably, the downregulation of ARHGAP36 reduced tumor cell proliferation, migration and invasion

while enhancing apoptosis. In conclusion, the findings of the present study indicate that ARHGAP36 plays a crucial role in facilitating immune evasion and promoting THCA progression. This underscores its potential as a diagnostic marker and therapeutic target in THCA.

Introduction

Thyroid cancer (THCA) is one of the most common head and neck malignant tumors, and its incidence is increasing in China (19.42/100,000). According to the assessment report released by the International Agency for Research on Cancer of the World Health Organization, there were >820 000 new cases of thyroid cancer worldwide in 2022, ranking the seventh in the incidence of cancer (1). THCA is a common malignancy in women, characterized by high rates of recurrence, poor prognosis and mortality due to metastasis and invasion (2). However, the molecular mechanisms driving THCA progression remain poorly understood (3), highlighting the need for more comprehensive research. Understanding these processes is crucial for improving diagnostic and therapeutic strategies.

Rho GTPase activating protein 36 (ARHGAP36) has a significant role in cell migration, cytoskeletal remodeling and tumor progression (4). Rho GTPase family members such as ARHGAP4 and ARHGAP9 have been shown to promote tumor growth and metastasis via pathways such as mTOR and hypoxia-inducible factor-1 α (5,6). Evidence has indicated that ARHGAP36 plays a crucial role in tumorigenesis and progression in medulloblastoma and pheochromocytoma (7). Although ARHGAP36 contributes to tumorigenesis and progression, its role in THCA is still unknown. Therefore, the present study aimed to explore the role of ARHGAP36 in tumorigenicity in THCA.

In the present study, public databases [such as GEPIA, The Cancer Genome Atlas (TCGA), TIMER and Metascape] were utilized to explore the biological functions of ARHGAP36. Functional *in vitro* assays were also conducted to evaluate cell proliferation, migration and apoptosis. The findings of the present study position ARHGAP36 as a potential therapeutic target by mediating immune escape and promoting metastasis. Additionally, the present study underscores the importance of further investigation into the role of ARHGAP36 in THCA,

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potentially leading to novel diagnostic markers and therapeutic strategies.

Materials and methods

Public database analyses. The differential expression of ARHGAP36 mRNA between thyroid carcinoma and adjacent normal tissues was analyzed using TCGA-THCA tumor data (8) and GTEx normal tissue data (9) (accession no. phs000424.v8.p2) through the GSEA website [www.gsea-msigdb.org (10)]. The limma R package was used for statistical analysis and the Benjamini-Hochberg false discovery rate (FDR) correction (significance threshold: $\log_2(\text{fold change}) > 1$ and FDR-adjusted $P < 0.05$) was used as the significance cut-off. The UALCAN database [http://ualcan.path.uab.edu (11)] was used to evaluate the expression of ARHGAP36 in TCGA-THCA cohort across various factors, including sample types, cancer stages, patient demographics, histological characteristics and nodal metastasis status, applying one-way ANOVA with Tukey's post-hoc test for multi-group comparisons and unpaired Student's t-test for binary classifications. The LinkedOmics database [www.linkedomics.org (12)] was used to identify genes correlated with ARHGAP36 using Pearson's correlation coefficient, with $|r| > 0.3$ and FDR < 0.5 defined as significant; the top 50 positively/negatively correlated genes were visualized in heatmaps. The Metascape database [https://metascape.org (13)] was used to perform Gene Ontology (GO) process enrichment analysis for ARHGAP36 and its associated genes, highlighting key biological processes, via hypergeometric testing with Benjamini-Hochberg correction, requiring a minimum enrichment factor of 1.5, $P < 0.01$ and FDR < 0.05 . The TIMER database [https://cistrome.shinyapps.io/timer/ (14)] was used to investigate the relationships between ARHGAP36 and tumor immune components, including purity, B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils and dendritic cells based on TCGA-THCA RNA-seq data, employing a deconvoluted gene expression algorithm with tumor purity-adjusted partial correlation. Protein-protein interaction (PPI) analyses were performed by the Metascape analysis tool. The TISCH database (http://tisch.comp-genomics.org/), a publicly available resource that provides integrated single-cell RNA sequencing data from multiple tumor types, including THCA. This platform allows for cell-type-specific gene expression analysis and immune landscape profiling.

Cell culture. BHT101 and BCPAP cells (obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) were cultured in DMEM [Ubi Biotech (Shanghai) Co., Ltd.] supplemented with 10% fetal bovine serum [FBS; cat. no. U11-020A; Ubi Biotech (Shanghai) Co., Ltd.]. The culture medium was further enriched with 1% penicillin-streptomycin solution and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B to prevent contamination. Cells were maintained at 37°C in a humidified incubator with 5% CO₂. When the cell density reached 80-90% confluency, cells were detached using 0.25% trypsin-EDTA solution and centrifuged at 111 x g at 37°C for 5 min to collect the cell pellet.

Small interfering (si)RNA transfection. The specific siRNA targeting ARHGAP36 were designed and synthesized

(Shanghai Jima Pharmaceutical Technology Co., Ltd.) to optimize knockdown efficiency. Prior to transfection, cells were seeded into 6-well plates at a density of 5×10^5 cells/ml with 1.5 ml of culture medium per well and incubated at 37°C with 5% CO₂ overnight to achieve 70-80% confluency. A total of 16 h after seeding, cells were divided into two groups: A negative control (NC) group and a si-ARHGAP36 group. Each siRNA was diluted in Opti-MEM (Thermo Fisher Scientific, Inc) to a final concentration of 100 nM in a 250 μl volume and incubated at room temperature for 5 min. In a separate tube, 5 μl of Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added to 250 μl of Opti-MEM and incubated at room temperature for 5 min. The diluted siRNA solution was then gently combined with the Lipofectamine 2000 mixture and incubated at room temperature for 20 min to allow for siRNA-lipid complex formation. Next, 500 μl of this siRNA-lipid complex was carefully added to each well containing the cells, followed by gentle rocking of the plate to ensure even distribution. A total of 6 h post-transfection, the medium was aspirated and 1.5 ml of fresh complete medium was added to each well. The cells were collected 48 h after transfection for western blot analysis to assess the ARHGAP36 protein expression levels and evaluate knockdown efficiency. All procedures were conducted under sterile conditions to prevent contamination, freshly prepared reagents were used to ensure optimal transfection efficiency and strict adherence to timing at each step was maintained to ensure reproducibility. The siRNA sequences were as follows: ARHGAP36 siRNA, sense 5'-GCGGGUCAGCUCCGAGAA A-3' and antisense 5'-UUUUCGGUCAGGGGCCGC-3'; NC siRNA, sense 5'-UUCUCCGAACGUGUCACGU-3' and antisense, 5'-ACGUGACACGUUCGGAGAA-3'.

Western blot analysis. Cells were lysed using RIPA buffer (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitors to ensure complete protein extraction. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For SDS-PAGE, 100 μg of total protein from each sample was separated on a 10% polyacrylamide gel at a constant voltage of 120 V for 2 h. Proteins were then transferred onto PVDF membranes (MilliporeSigma) using a semi-dry transfer system at 25 V for 1 h. The membranes were then blocked with 5% non-fat milk in 1X TBST (0.1% Tween) for 2 h at room temperature to reduce non-specific binding. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against ARHGAP36 (1:2,000; cat. no. PA5-31619; Thermo Fisher Scientific, Inc.) and GAPDH (loading control; 1:2,000; cat. no. ab8245; Abcam). After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:5,000; cat. no. 58802; Cell Signaling Technology, Inc.) and anti-rabbit (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) secondary antibodies for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Inc.) and imaged with a Tanon 5200 system. Densitometric analysis of the blots was performed using ImageJ software (v1.53; National Institutes of Health) to semi-quantify the protein expression levels.

Plate colony formation assay. For the plate colony formation assay, BHT101 and BCPAP cells (1×10^3 per well) were seeded into 6-well plates and allowed to adhere overnight. The next day, the medium was replaced with DMEM containing 10% FBS and 1% penicillin-streptomycin. Cells were cultured for 1 month at 37°C in a humidified 5% CO₂ incubator, with media changes every 3 days. At the end of the culture period, colonies were fixed with ice-cold 100% methanol for 10 min, stained with 0.5% crystal violet solution for 20 min at 37°C and then washed with distilled water. The plates were air-dried and colonies consisting of >50 cells in each well were manually counted.

Wound healing assay. To assess the migratory capacity of BHT101 and BCPAP cells, a scratch wound healing assay was performed. Cells were seeded at a density of 1×10^6 cells per well in 6-well plates and cultured to confluency in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. A uniform scratch was created using a sterile 200 μ l pipette tip and debris was removed by washing with PBS. The medium was then replaced with DMEM containing 1% FBS to minimize proliferation. Images of the scratch wounds were captured immediately (0 h) and again at 24 h post-scratch using an inverted light microscope. Wound closure was quantified by measuring the distance between the edges of the scratch at multiple points, and image analysis software (version 2.3; Olympus Corporation) was used to calculate the average wound width and percentage of closure.

Migration and invasion assays. To evaluate cell migration and invasion, BHT101 and BCPAP cells (2×10^5 in 200 μ l serum-free DMEM) were seeded into the top chamber of 24-well Transwell inserts with 8 μ m pores. The lower chamber contained 600 μ l of DMEM with 10% FBS as a chemoattractant. For invasion assays, the top chamber was pre-coated with Matrigel at 4°C overnight. After a 24-h incubation at 37°C in 5% CO₂, the cells that migrated or invaded through the membrane were fixed with 4% paraformaldehyde at room temperature for 15 min. After fixation, the cells were stained with 0.5% crystal violet at 37°C for 20 min. Images were captured under an Olympus BX50 light microscope (Olympus Corporation). Migrated or invaded cells were quantified by counting five random fields per membrane.

Statistical analysis. Data were analyzed using Pearson's or Spearman's tests for correlation analysis, and group differences were assessed using an unpaired Student's t-test (GraphPad Prism v6.0; Dotmatics). Results are presented as the mean $\pm$ SD, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ARHGAP36 expression levels are elevated in THCA samples compared with adjacent normal tissues. ARHGAP36 expression was analyzed across multiple databases to assess its potential role in THCA progression. The GEPIA results showed higher ARHGAP36 mRNA levels in TCGA tumor tissues versus normal tissues (Fig. 1A and B; $P < 0.01$). The UALCAN results also confirmed elevated ARHGAP36

expression in TCGA-THCA samples (Fig. 1C; $P < 0.01$). GEPIA further revealed similar findings in the THCA dataset (Fig. 1D; $P < 0.05$). These consistent results across multiple datasets strongly indicate that ARHGAP36 levels are markedly elevated in THCA samples. This suggests a potential role for ARHGAP36 in the progression and pathogenesis of THCA, positioning it as a candidate biomarker for further investigation.

Higher ARHGAP36 mRNA expression is associated with the TNM stage in patients with THCA. UALCAN data revealed a comprehensive association between ARHGAP36 expression and various clinical parameters in THCA. THCA tumor tissues exhibited significantly higher ARHGAP36 levels compared with normal tissues (Fig. 2A; $P < 0.001$). ARHGAP36 expression progressively increased from stage 1 to 4 disease (Fig. 2B; $P < 0.001$), indicating a potential role in disease progression. Elevated ARHGAP36 expression was also observed across different demographic and histological subtypes. Specifically, significant increases were noted in various ethnic groups (Fig. 2C; $P < 0.001$), female patients (Fig. 2D; $P < 0.001$), younger patients (Fig. 2E; $P < 0.001$) and distinct THCA subtypes (Fig. 2F; $P < 0.001$). Additionally, lymph node metastasis was associated with higher ARHGAP36 expression (Fig. 2G; $P < 0.001$), suggesting its involvement in aggressive tumor behavior. These findings highlight the broad relevance of ARHGAP36 in THCA, spanning multiple clinical and demographic factors. The consistent elevation of ARHGAP36 across these diverse parameters underscores its potential as a biomarker for disease progression.

ARHGAP36 downregulation reduces proliferation and induces apoptosis in THCA cells. To investigate the functional role of ARHGAP36 in THCA, ARHGAP36 expression was knocked down in BCPAP and BHT101 cells using siRNA (designated as BCPAP/si-ARHGAP36 and BHT101/si-ARHGAP36). Western blot assays showed the effectiveness of the siRNA transfection and confirmed the significant decrease in ARHGAP36 protein levels compared with the control (Fig. 3A and B; $P < 0.01$). Plate colony formation assays revealed significantly reduced proliferation in these cells compared with controls (Fig. 3C and F; $P < 0.01$). Apoptosis assays also showed increased cell death upon ARHGAP36 knockdown (Fig. 3G-J; $P < 0.01$). These findings highlight the critical role of ARHGAP36 in promoting THCA cell survival and suggest that its downregulation can significantly inhibit tumor cell proliferation while enhancing apoptosis.

ARHGAP36 downregulation inhibits migration and invasion. Wound healing assays demonstrated a reduced migration in ARHGAP36 knockdown cells (Fig. 4A-D; $P < 0.05$). Transwell migration assays also showed significantly fewer si-ARHGAP36 cells traversing through Transwell inserts (Fig. 4E-H; $P < 0.05$). Invasion assays revealed similar results, with significantly decreased invasive behavior (Fig. 4E-H; $P < 0.05$). These findings collectively indicate that the downregulation of ARHGAP36 expression impairs THCA cell migration and invasion, highlighting its critical role in facilitating these processes. This evidence supports the potential of ARHGAP36 as a therapeutic target to inhibit tumor metastasis in THCA.

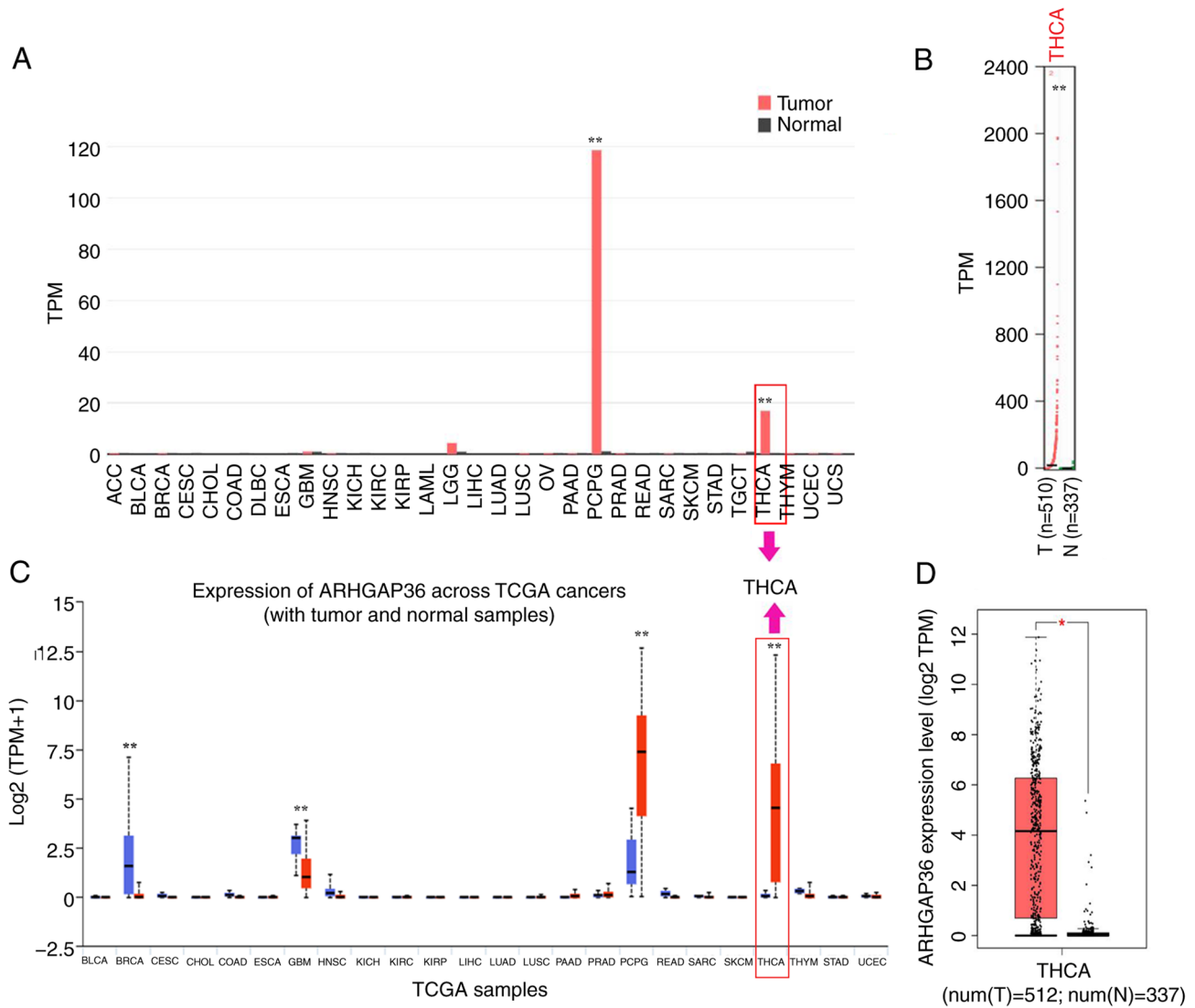


Figure 1. ARHGAP36 expression is higher in THCA samples than in normal tissues. GEPIA database analyses of ARHGAP36 mRNA expression in (A) TCGA and (B) TCGA-THCA tumor tissues compared with adjacent normal tissues. (C) ARHGAP36 expression in TCGA tumor and normal tissues. (D) Differential ARHGAP36 mRNA expression in the THCA dataset (TCGA). * $P < 0.05$, ** $P < 0.01$. ARHGAP36, Rho GTPase activating protein 36; THCA, thyroid cancer; TCGA, The Cancer Genome Atlas; TPM, transcripts per million.

ARHGAP36 gene co-expression network in THCA. To explore the genes co-expressed with ARHGAP36, the LinkedOmics database was utilized. A total of 2,436 genes were found to be positively correlated and 1,764 genes were negatively correlated with ARHGAP36 (Fig. 5A). Heatmaps identified the top 50 genes most strongly correlated with ARHGAP36 expression (Fig. 5B and C). Key positively correlated genes included immunoglobulin superfamily member 1 (IGSF1; Fig. 5D; $\rho = 0.853$, $P = 4.5 \times 10^{-145}$), dihydropyrimidinase like 3 (DPYSL3; Fig. 5E; $\rho = 0.604$, $P = 6.63 \times 10^{-52}$) and zinc finger CCHC-type containing 12 (ZCCHC12; Fig. 5F; $\rho = 0.55$, $P = 1.44 \times 10^{-41}$), indicating strong associations between these genes and ARHGAP36. Negatively correlated genes included SET domain containing 3 actin N3(tau)-histidine methyltransferase, COX15 and ring finger protein 157 (Fig. 5G-I), highlighting potential antagonistic relationships in THCA pathogenesis. These findings provide insights into the molecular network surrounding ARHGAP36, suggesting its involvement in complex regulatory pathways that influence

THCA progression. This co-expression analysis supports the hypothesis that ARHGAP36 plays a role in THCA by interacting with key regulatory genes.

T cell-mediated immunity is a key GO biological process linked to ARHGAP36. To gain deeper insights into the biological processes associated with ARHGAP36, a Metascape analysis was conducted. This analysis revealed that ARHGAP36-associated genes are significantly enriched in processes such as 'Pancreatic cancer subtypes,' 'Prolactin signaling pathway' and 'T cell mediated immunity' (Fig. 6A). PPI networks further supported these findings, illustrating the interconnectivity between ARHGAP36 and its associated genes (Fig. 6B and C). These networks highlight key pathways and interactions that may underline the functional role of ARHGAP36 in THCA. The analysis revealed that ARHGAP36 may be intricately involved in multiple signaling pathways critical for THCA development and progression. Notably, it appears to interact with components of the MAPK

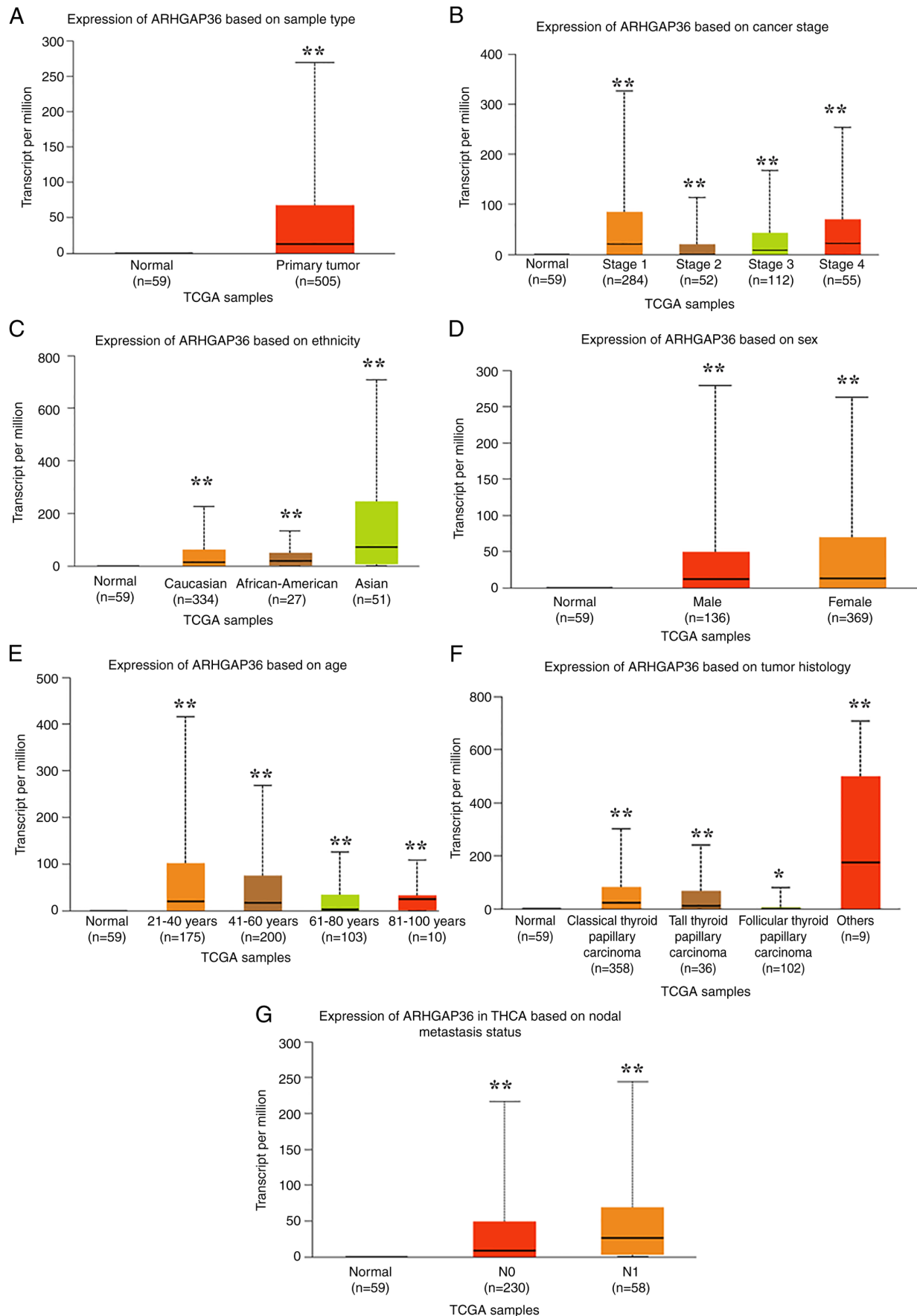


Figure 2. Higher ARHGAP36 mRNA expression is associated with TNM stage in patients with THCA. (A) ARHGAP36 expression in tumor tissues is significantly higher than in normal tissues. (B) ARHGAP36 expression increases across tumor stages 1-4. Elevated ARHGAP36 expression by (C) ethnicity, (D) sex, (E) age and (F) THCA subtype. (G) Higher ARHGAP36 expression in patients with lymph node metastasis. * $P < 0.05$, ** $P < 0.01$. The UALCAN data, which was used for all analyses, revealed a comprehensive association between ARHGAP36 expression and various clinical parameters in THCA. ARHGAP36, Rho GTPase activating protein 36; THCA, thyroid cancer; TCGA, The Cancer Genome Atlas.

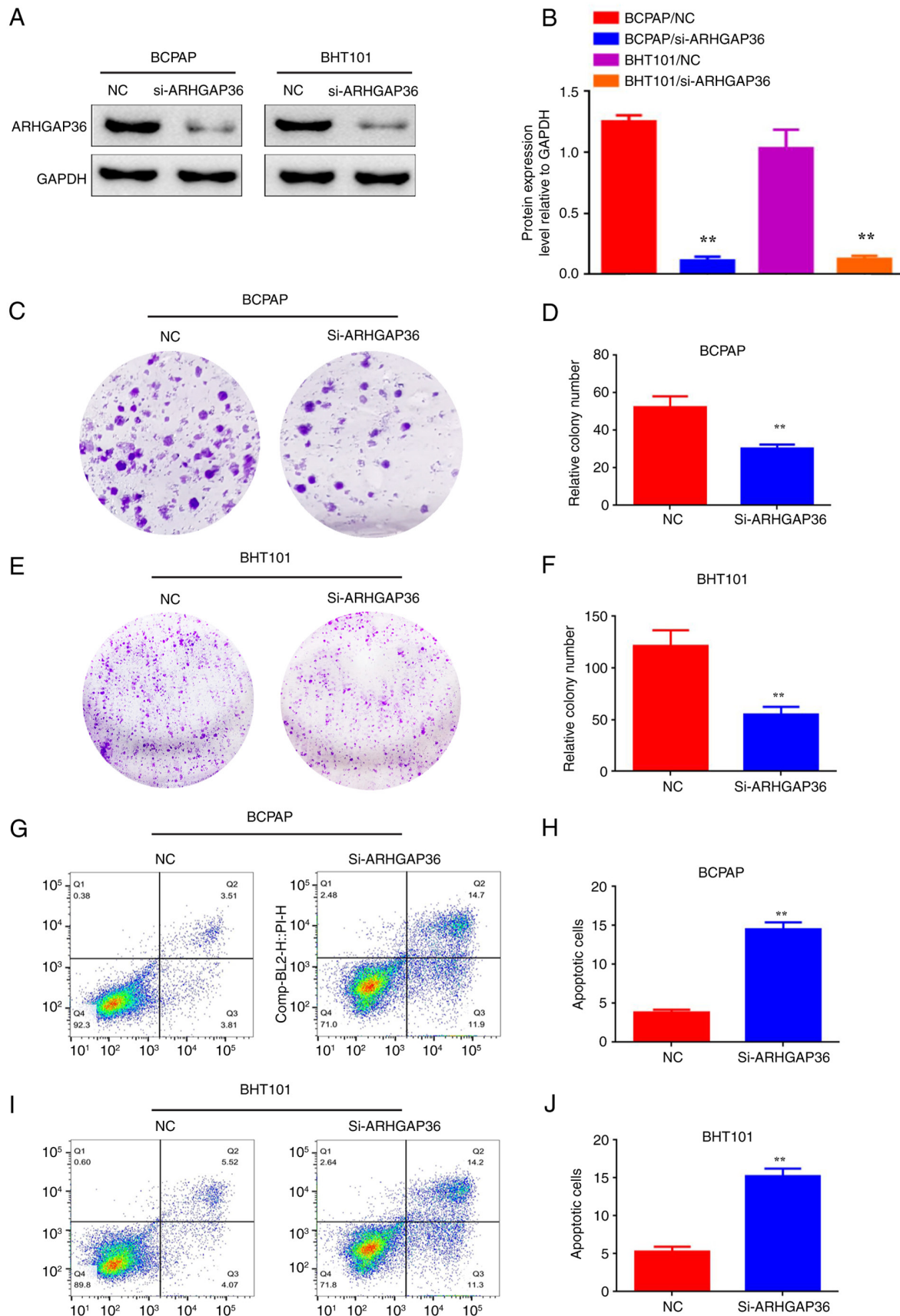


Figure 3. ARHGAP36 downregulation reduces proliferation and induces apoptosis in THCA cells. (A) Western blot assays show the effectiveness of the siRNA transfection and confirm the significant decrease in ARHGAP36 protein levels. (B) Protein ratio in cells. (C) Colony formation assays show reduced proliferation in BCPAP/si-ARHGAP36 cells. (D) Colony numbers for BCPAP/si-ARHGAP36 cells. (E) Colony formation assays show reduced proliferation in BHT101/si-ARHGAP36 cells. (F) Colony numbers for BHT101/si-ARHGAP36 cells. (G) Apoptosis assays show ARHGAP36 downregulation induces the apoptosis of BCPAP. (H) Apoptosis rates in BCPAP cells. (I) Apoptosis assays show ARHGAP36 downregulation the induces apoptosis of BHT101 cells. (J) Apoptosis rates in BHT101 cells. ** $P < 0.01$. ARHGAP36, Rho GTPase activating protein 36; THCA, thyroid cancer; NC, negative control; siRNA, small interfering RNA.

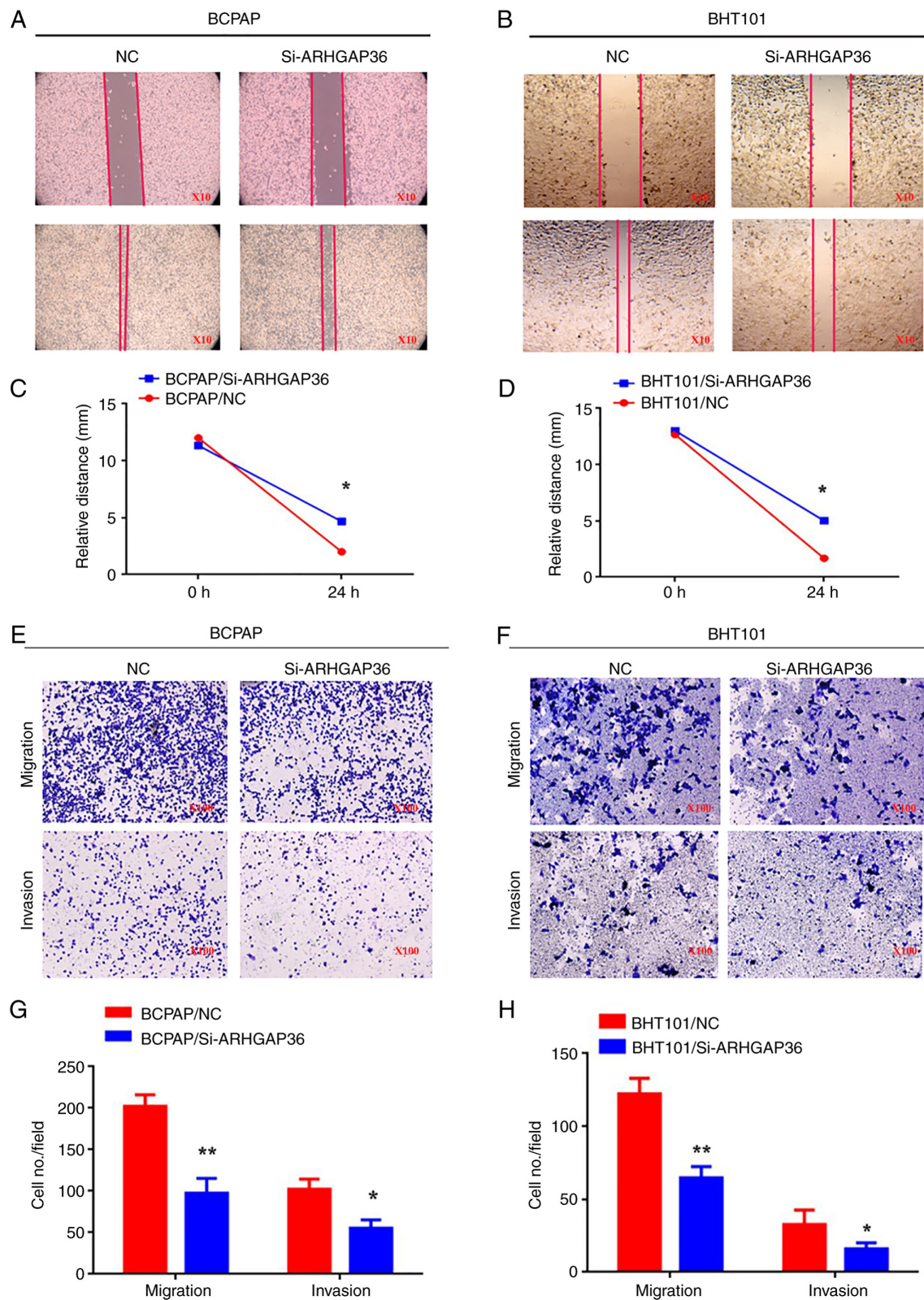


Figure 4. ARHGAP36 downregulation reduces the migration and invasion of thyroid cancer cells. Wound healing assays reveal reduced movement in ARHGAP36 knockdown (A and C) BCPAP/si-ARHGAP36 and (B and D) BHT101/si-ARHGAP36 cells. Transwell migration assays show decreased migration in (E and G) BCPAP/si-ARHGAP36 and (F and H) BHT101/si-ARHGAP36 cells. Transwell invasion assays indicate reduced invasion in (E and G) BCPAP/si-ARHGAP36 and (F and H) BHT101/si-ARHGAP36 cells. * $P < 0.05$, ** $P < 0.01$. ARHGAP36, Rho GTPase activating protein 36; si, small interfering (RNA); NC, negative control.

pathway, which is known to play a pivotal role in cell proliferation, differentiation and survival (15). These results suggest that ARHGAP36 is involved in diverse biological processes

beyond THCA, potentially influencing pathways related to other cancer types and immune responses. The enrichment in T cell-mediated immunity is particularly noteworthy,

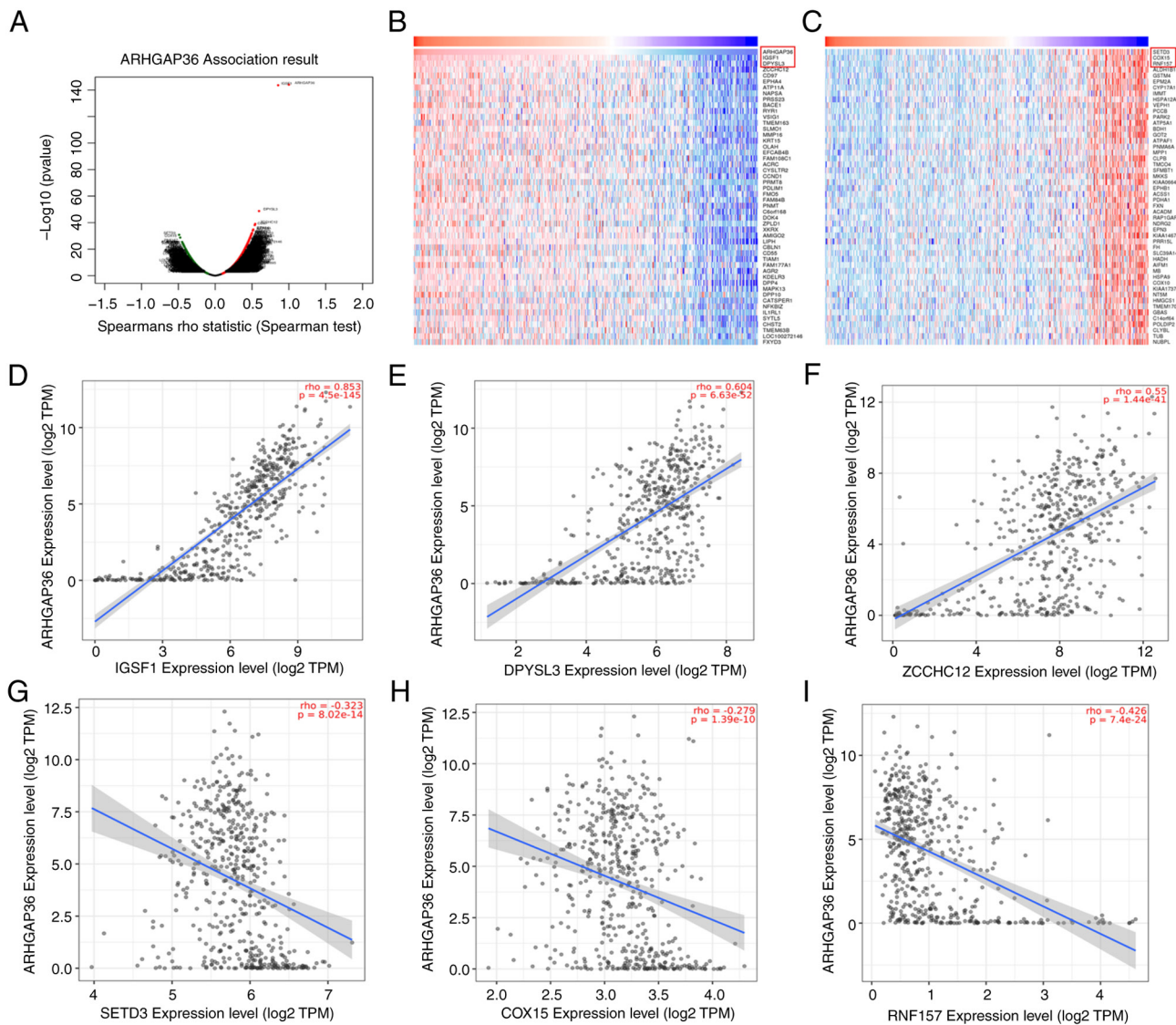


Figure 5. ARHGAP36 gene co-expression network in thyroid cancer. (A) Volcano plot of genes co-expressed with ARHGAP36 in TCGA. Heatmaps of the top 50 (B) positively and (C) negatively correlated genes. Positive correlations between ARHGAP36 and (D) IGSF1, (E) DPYSL3 and (F) ZCCHC12. Negative correlations with (G) SETD3, (H) COX15 and (I) RNF157. ARHGAP36, Rho GTPase activating protein 36; IGSF1, immunoglobulin superfamily member 1; DPYSL3, dihydropyrimidinase like 3; ZCCHC12, zinc finger CCHC-type containing 12; TPM, transcripts per million. SETD3, SET domain-containing protein 3; RNF157, ring finger protein 157.

indicating a potential role for ARHGAP36 in modulating the tumor microenvironment and immune evasion mechanisms.

ARHGAP36 is associated with CD4⁺ T cell infiltration. To investigate the relationship between ARHGAP36 expression and immune cell infiltration in THCA, an analysis using the TIMER database was conducted. TIMER analysis indicated a significant but weak correlation between ARHGAP36 expression and CD4⁺ T cell infiltration (Fig. 7D; $\text{cor} = 0.295$, $P = 3.04 \times 10^{-11}$). Additional weak correlations were observed with neutrophils (Fig. 7F) and dendritic cells (Fig. 7G), further supporting the possible role of ARHGAP36 in modulating immune cell infiltration. These findings suggest that ARHGAP36 may influence the tumor microenvironment by affecting the recruitment and activity of various immune cells. The positive correlation with CD4⁺ T cells is particularly noteworthy, as it highlights the potential involvement of ARHGAP36 in adaptive immunity and immune evasion

mechanisms in THCA. These results collectively underscore the importance of ARHGAP36 in shaping the immune landscape of THCA, positioning it as a potential target for immunotherapeutic strategies.

ARHGAP36 is linked to immune escape. To further explore the role of ARHGAP36 in the tumor microenvironment, its expression was analyzed using the TISCH database. This analysis revealed that ARHGAP36 is expressed in epithelial cells within THCA tissues and exhibits a weak positive correlation with several immune checkpoint molecules, including PDCD-1 (programmed cell death protein 1; PD-1), lymphocyte-activation gene 3 (LAG3), CD274 (programmed death-ligand 1; PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (Fig. 8A-F). These findings suggest that ARHGAP36 may facilitate immune escape in THCA by interacting with these key immune checkpoints. The observed weak correlations imply that ARHGAP36 could contribute to the

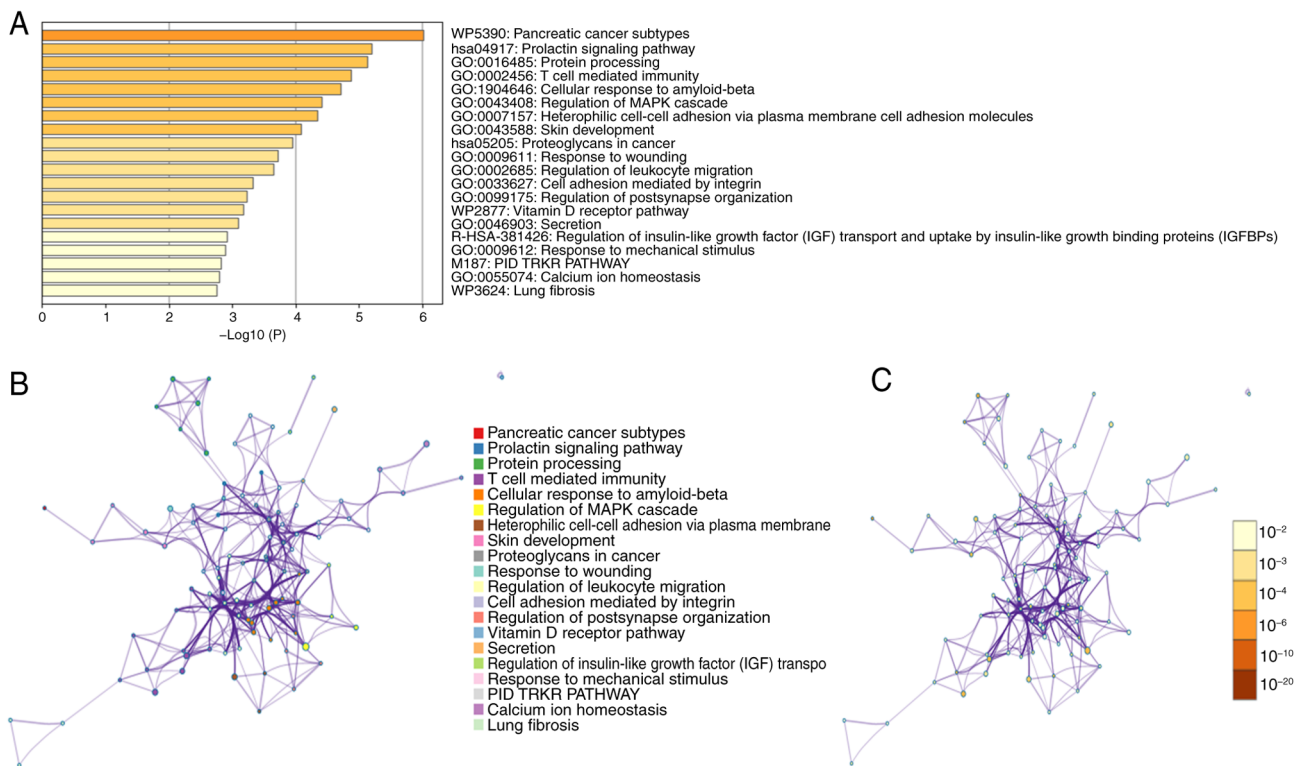


Figure 6. T cell-mediated immunity is significantly enriched among genes related to ARHGAP36. (A) Gene Ontology biological processes enriched in ARHGAP36-associated genes. (B,C) Protein-protein interaction networks with functional clusters represented by color. ARHGAP36, Rho GTPase activating protein 36.

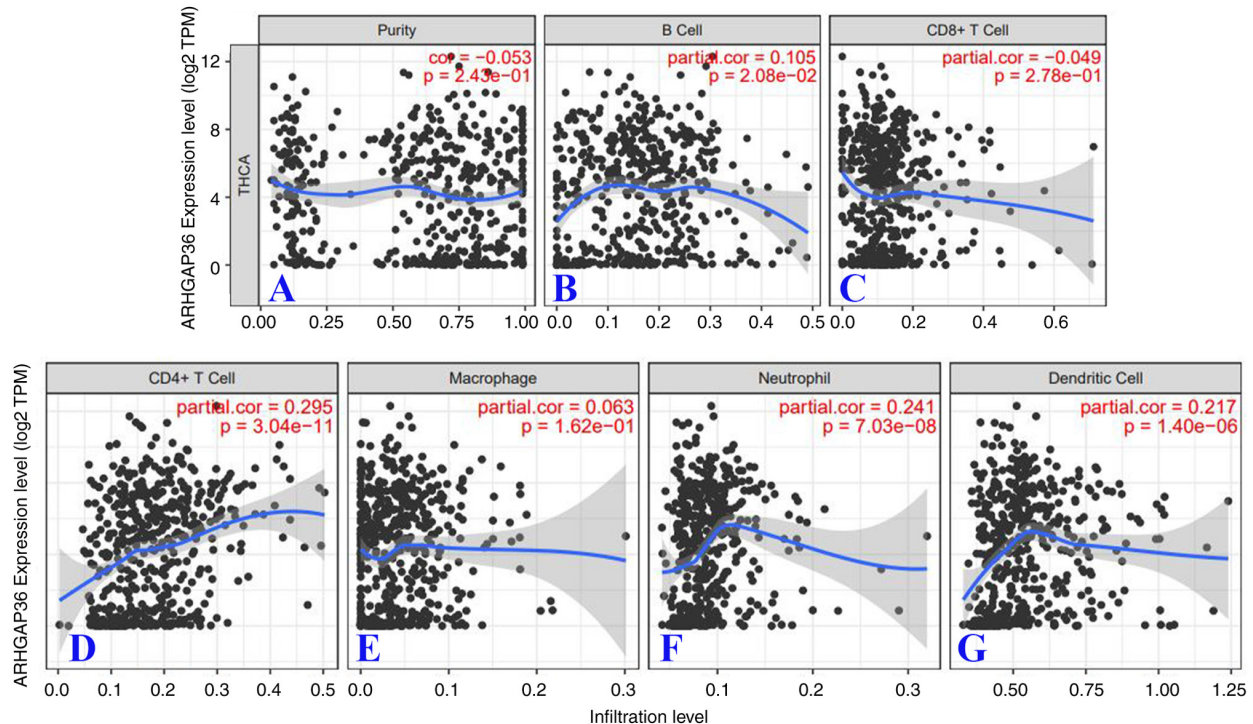


Figure 7. ARHGAP36 weakly correlates with immune CD4⁺ T cell infiltration in thyroid cancer. TIMER analysis of ARHGAP36 correlations with (A) purity, (B) B cells, (C) CD8⁺ T cells, (D) CD4⁺ T cells, (E) macrophages, (F) neutrophils and (G) dendritic cells. ARHGAP36, Rho GTPase activating protein 36; TPM, transcripts per million.

suppression of immune surveillance and the establishment of an immunosuppressive tumor microenvironment. The positive

weak correlations with PD-1, LAG3, PD-L1 and CTLA4 highlight the potential of ARHGAP36 as a mediator of immune

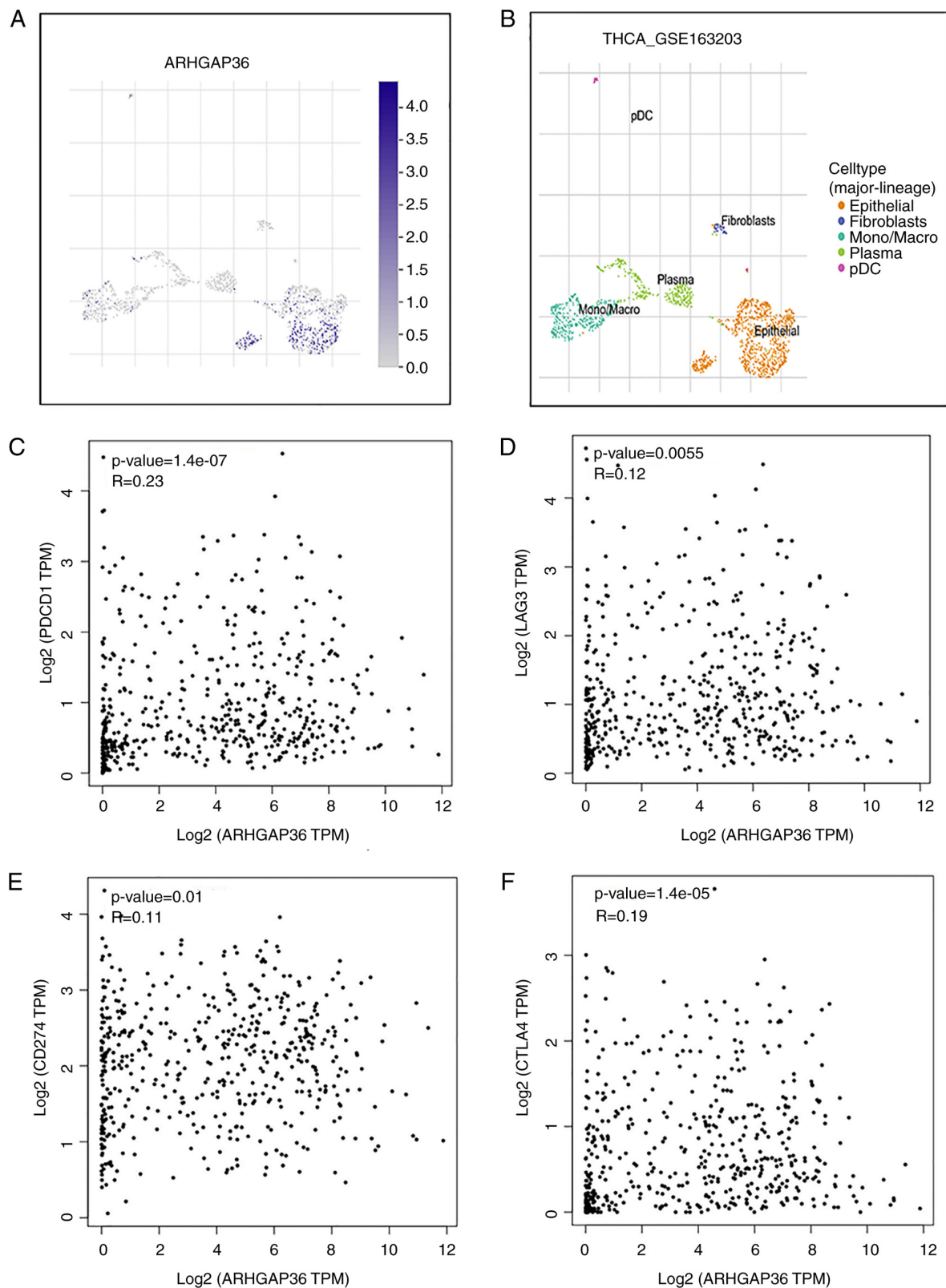


Figure 8. ARHGAP36 is associated with immune escape. (A,B) Single-cell expression matrices from the TISCH database show ARHGAP36 abundance in immune cell clusters. Positive associations between ARHGAP36 and immune checkpoints, (C) PDCD-1, (D) LAG3, (E) CD274 and (F) CTLA4. ARHGAP36, Rho GTPase activating protein 36; TPM, transcripts per million; LAG3, lymphocyte-activation gene 3; CTLA4, cytotoxic T-lymphocyte-associated protein 4.

evasion mechanisms in THCA. This evidence supports the hypothesis that targeting ARHGAP36 might enhance the efficacy of immunotherapies aimed at overcoming immune resistance in THCA.

Discussion

The metastasis of THCA is driven by a complex interplay of mechanisms involving genetic diversity,

epithelial-mesenchymal transition and the tumor microenvironment (16-18). Thyroid carcinoma, accounting for ~2.5% of all malignancies, is exhibiting an increasing incidence rate and represents the fifth most common cancer in women in the USA (19). Lymph nodal involvement in THCA is very common and lymph node micrometastases are observed in up to 90% of cases (20). Despite extensive research, the biological processes underlying THCA metastasis remain poorly understood, necessitating further exploration to provide a comprehensive understanding of its mechanisms.

ARHGAP36, an atypical member of the Rho GTPase-activating protein family, has roles in spinal cord development and tumorigenesis by suppressing protein kinase A and activating Gli transcription factors (21). ARHGAP36 features unique structural domains, but its role in THCA metastasis has not been extensively reported. Investigating ARHGAP36 in this context could have significant clinical value. The present study revealed that ARHGAP36 expression is significantly higher in THCA tissues compared with normal tissues, as demonstrated through analyses using the GEPIA and UALCAN databases. Higher ARHGAP36 levels were associated with sample types, cancer stages and patient demographics. *In vitro* experiments validated these findings, showing that knocking down ARHGAP36 expression significantly reduced THCA cell proliferation, migration and invasion, while inducing apoptosis. These results confirm that ARHGAP36 downregulation impairs the aggressive properties of THCA cells.

In the present study, 2,436 genes positively correlated and 1,764 genes negatively correlated with ARHGAP36 were identified using the LinkedOmics database. Notably, ARHGAP36 expression was strongly correlated with genes such as IGSF1, DPYSL3 and ZCCHC12. IGSF1, a plasma membrane glycoprotein associated with conditions such as hypothyroidism and delayed puberty, is linked to THCA cell growth, metastasis and apoptosis (22-24). The immune-targeting potential of ARHGAP36 complements existing cancer immunotherapies, suggesting that ARHGAP36 may act as a biomarker influencing immune infiltration in THCA.

In the present study, Metascape analysis identified key GO biological processes associated with ARHGAP36, including prolactin signaling, protein processing and T cell-mediated immunity. TIMER database analyses demonstrated weak correlations between ARHGAP36 expression and CD4⁺ T cell infiltration in THCA. TISCH data further showed ARHGAP36 expression in epithelial cells and weak correlations with immune checkpoints such as PDCD-1, LAG3, CD274 and CTLA4, suggesting a possible role in immune escape. Tumor immune escape allows cancer cells to evade immune detection, enabling survival and metastasis. Immune cells, such as macrophages and T cells, are suppressed in the tumor microenvironment by cytokines and tumor interactions, facilitating cancer progression (25,26). The positive correlation between ARHGAP36 and immune checkpoint molecules highlights its potential involvement in modulating the tumor immune microenvironment.

While the present study provides valuable insights into the functional impact and underlying mechanisms of ARHGAP36 in THCA cells, it is important to acknowledge limitations regarding long-term follow-up data. The present

research primarily focused on short-term cellular effects and did not include extensive longitudinal patient data. This lack of long-term follow-up data makes it challenging to fully assess the role of ARHGAP36 in the prognosis of patients with THCA over extended periods. Longitudinal studies are essential for understanding how changes in ARHGAP36 expression influence patient outcomes, survival rates and disease progression in the long term. Future research should aim to address this gap by conducting comprehensive longitudinal studies and aggregating multicenter data to better elucidate the prognostic significance of ARHGAP36 in THCA.

The present study primarily focused on the impact of down-regulating ARHGAP36 expression on THCA cells, providing detailed insights into its functional consequences and underlying mechanisms. The experimental data demonstrated that reduced ARHGAP36 expression significantly affected cell proliferation, migration and invasion, highlighting its potential as a therapeutic target in THCA. However, the present investigation has limitations in comparing and discussing other potential treatment methods. Therefore, reviewing relevant literature to explore whether changes in ARHGAP36 expression might influence the efficacy of radiotherapy or interact with other treatment strategies, may provide theoretical foundations for future research directions. In future studies, we aim to conduct experiments using THCA cells or tissues to explore whether the suppression of ARHGAP36 impacts the expression of immune checkpoint molecules. This will help elucidate the mechanisms by which ARHGAP36 influences immune responses.

In conclusion, the results of the present study indicate that ARHGAP36 may promote THCA metastasis by mediating immune escape, making it a potential prognostic biomarker and therapeutic target for THCA. However, further studies are needed to elucidate the precise mechanisms through which ARHGAP36 regulates immune escape and interacts with other components of the tumor microenvironment.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LY and JL performed the experiments and edited, drafted and wrote the manuscript; LY, SH, GW and YG conducted the data analysis; study design and general supervision were led by SH and GW; data interpretation was performed by LY and YG. LY and JL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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