doi: 10.4149/gpb_2025001

LncRNA ROR promotes proliferation, immune escape, and polarization of M2 macrophages in thyroid cancer by activating the PI3K/AKT pathway

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Abstract. Thyroid cancer is the most prominent type of endocrine cancer. LncRNA ROR (Linc-ROR) exerts tumor regulator function in cancers. This study elucidates the action of Linc-ROR on thyroid cancer. The Linc-ROR level were investigated in 70 thyroid cancer patients. Cell viability was detected utilizing CCK-8 method. The xenograft tumor was constructed to evaluate tumor growth. The proportion of $CD8^+$ T cells was assessed using flow cytometry. IL-10, IFN-γ, and TNF-β levels were detected utilizing ELISA assays. The Linc-ROR level was elevated in thyroid cancer patients ($n = 70$). The up-regulated Linc-ROR was associated with poor overall survival ($p = 0.0315$), lymph node metastasis ($p = 0.027$), and TNM stage ($p = 0.016$). Linc-ROR silencing restrained cell proliferation and tumor growth ($p < 0.01$). Additionally, silenced Linc-ROR suppressed the immune escape of thyroid cancer cells and polarization of M2 macrophages ($p < 0.01$). Moreover, the PI3K/AKT signaling mediated the action of silenced Linc-ROR on thyroid cancer proliferation, immune escape, and M2 macrophage polarization ($p < 0.05$). Linc-ROR may be a valuable target for thyroid cancer management. The limitations of this research are that the action of Linc-ROR on the tumor microenvironment has not been investigated in the animal model.

Key words: Thyroid cancer — Linc-ROR — Immune escape — M2 macrophages polarization — PI3K/AKT pathway

Introduction

Thyroid cancer is the most prominent type of endocrine cancer and is prevalent in the head and neck (Yan et al. 2020; Cherella et al. 2021; Zheng and Zhang 2023). The morbidity of thyroid cancer is the ninth among cancers globally and continues to rise (Bray et al. 2018; Kim et al. 2020). Thyroid cancer mainly includes papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), poorly differentiated thyroid cancer (PDTC), and anaplastic thyroid cancer (ATC), of which PTC is the most popular type (Guo H et al. 2023; Han et al. 2023). Currently, 90% of thyroid cancers present an excellent prognosis by conventional therapies (Hernando et al. 2020; Ma et al. 2023). Nevertheless, some cases may develop advanced diseases such as DTC and PDTC that are not amenable to conventional treatment, resulting in high morbidity and mortality (Hernando et al. 2020; Ma et al. 2023). Therefore, the research focusing on elucidating the mechanism of thyroid cancer progression is crucial for thyroid cancer therapy.

The tumor microenvironment is the environment that surrounds the tumor, including various cell types such as immune cells, cancer-associated fibroblasts, and pericytes (Wang et al. 2022; de Visser and Joyce 2023). Tumor cells promote tumor growth by regulating this environment

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(Wang et al. 2022). Macrophages are the primary type of tumor-infiltrating immune cells and exert vital function in tumor progression by promoting immune escape (Liu et al. 2020). Generally, macrophages can polarize into M1 and M2 macrophages based on the stimulus conditions (Zheng et al. 2022). It is accepted that tumor-associated macrophages are primarily M2-polarized macrophages that possess immunosuppressive functions and facilitate tumor development and metastasis (Quail and Joyce 2013; Zheng et al. 2022). Immune escape is an immunological mechanism underlying cancer progression (Zhang et al. 2009). The acquisition of the immune escape ability of tumor cells is closely related to the tumor microenvironment (Guo et al. 2022). Immune escape of tumor cells is mainly manifested by the inhibition of $CD8^+$ T cell activation (Yang et al. 2020). Usually, $CD8^+$ T cells significantly kill tumor cells by recognizing tumor antigens during the antitumor immune response. Nevertheless, the cytokines in the tumor microenvironment affected the activation of cytotoxic T lymphocytes and the sensitivity of tumor cells to them (Zhang et al. 2009; Yang et al. 2020). Tumor immune escape is a considerable obstacle to clinical therapy of cancers (Eichmüller et al. 2017; Aktar et al. 2022). Therefore, modulation the tumor microenvironment to prevent immune escape is a promising cancer therapy strategy.

LincRNA ROR (Linc-ROR) is a newly discovered cytoplasmic long non-coding RNA (lncRNA) in induced pluripotent stem cells (iPSCs) (Loewer et al. 2010). Accumulating studies discovered that Linc-ROR exerted vital functions in cancer development (Zhan et al. 2016; Gao et al. 2021; Tian et al. 2021; Zhang et al. 2021). For instance, Zhan et al. reported that Linc-ROR accelerated pancreatic cancer metastasis and growth *via* activating the ZEB1 pathway (Zhan et al. 2016). Gao et al. found that Linc-ROR promoted epithelial-mesenchymal transition (EMT) in retinoblastoma (Gao et al. 2021). Zhang et al. revealed that Linc-ROR enhanced hepatocellular carcinoma resistance to doxorubicin (Zhang et al. 2021)

Interestingly, the influence of Linc-ROR on thyroid cancer progression was discovered recently (Fan et al. 2022). Fan et al. demonstrated that silenced Linc-ROR restrained cell proliferation, migration, and invasion and accelerated cell apoptosis in PTC (Fan et al. 2022). Furthermore, Linc-ROR participated in regulating tumor microenvironments (Hardin et al. 2018; Sun et al. 2021). Sun et al. found that exosomal Linc-ROR mediated crosstalk between cancer cells and adipocytes to promote tumor growth in pancreatic cancer (Sun et al. 2021). Hardin et al. discovered that the exosomes from thyroid cancer stem-like cells could communicate with and regulate adjacent and distant tumor microenvironments by transferring Linc-ROR to EMT (Hardin et al. 2018). However, the further action of Linc-ROR on thyroid cancer development, especially in the tumor microenvironment, such as immune escape and the polarization of M2 macrophages, remains unclear.

Thus, this research aims to elaborate on the effect of Linc-ROR on the tumor microenvironment of thyroid cancer by determining cell proliferation, tumor growth, immune escape, and M2 macrophage polarization and explore the potential mechanism.

Materials and Methods

Clinical samples

Seventy primary thyroid cancer cases who received thyroidectomy surgery in The First Hospital of Changsha were enrolled in the research. No cases underwent chemotherapy or radiotherapy preoperatively. Tumor and adjacent normal tissues of all cases were obtained and preserved. Peripheral blood samples were obtained from all cases 1 month before surgery. Among the 70 cases, 38 were defined as high-risk differentiated thyroid cancer and 32 as low-risk differentiated thyroid cancer. The criteria for defining high-risk differentiated thyroid cancer were: differentiated thyroid cancer consistent with a tumor larger than 4 cm or age greater than 55 years, lymph node metastasis for cervical clearance surgery, or extra glandular invasion, follicular carcinoma, and with distant metastases. All cases signed informed consent. The Ethics Committee of The First Hospital of Changsha authorized this research (Approval number 2023006).

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA samples in thyroid cancer tissues and cells (BCPAP, IHH4, KTC, TPC-1, and Nthy-Ori 3-1) were isolated utilizing TRIzol (Beyotime, Shanghai, China), and then cDNA was generated by the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). After that, the cDNA was subjected to the qPCR assay by applying the SYBR Premix EX Taq kit (Takara, Shiga, Japan) following the supplier's protocols. The primers used were: Linc-ROR: F, 5'-CTCAGTGGGGAAGACTCCAG-3', R, 5'-AGGAAGCCTGAGAGTTGGC-3' (Fan et al. 2022); CD206: F, 5'-GCAGAAGGAGTAACCCACCC-3', R, 5'-TGGCAAATGAAGGCGTTTGG-3' (Fan et al. 2019); CD168: F, 5'-AGAACCAACTCAAGCAACAGG-3', R, 5'-AGGAGACGCCACTTGTTAATTTC-3' (Guo K et al. 2023). The thermal cycling program was: 95°C (30 s), 40 cycles of 95°C (5 s), 60°C (30 s). The gene expressions were detected utilizing the $2^{-\Delta\Delta Ct}$ method *via* normalizing to β-actin.

Cell culture

Human thyroid cancer cells BCPAP, IHH4, KTC, and TPC-1, human thyroid follicular epithelial cells Nthy-Ori 3-1, and human monocytic leukemia cells THP-1 were obtained from

the BeNa Culture Collection (Beijing, China). Cell lines were authenticated by STR profiling, and mycoplasma testing was performed. Cells were maintained in RPMI-1640 medium plus 10% FBS (Gibco, Waltham, MA, USA) at 37°C with 5% CO_2. Besides, the medium of THP-1 cells was added to 0.05 mM β-mercaptoethanol. The peripheral blood mononuclear cells (PBMCs) were obtained from human peripheral blood and co-cultured with adherent TPC-1 or IHH4 cells at a ratio of 3:1 in terms of cell numbers for 48 h. In indicated experiments, TPC-1 cells were incubated with 10 μM 740Y-P for 48 h (Selleck Chemicals, Shanghai, China) (Yin and Huang 2021), and THP-1 cells were incubated with 50 nM phorbol-12-myristate-13 acetate (PMA) for 24 h to induce differentiation (Bahrami and Haji Molla Hoseini 2021). In other experiments, PMA-induced THP-1 cells were co-cultured with the supernatants of TPC-1 and IHH4 cells for 24 h. To obtain the cell supernatants of TPC-1 and IHH4, the culture medium of these cells after 24 h incubation with 0.5% FBS in RPMI 1640 were harvested, centrifuged for 5 min at 1000 rpm, and filtered by 0.22 μm filter.

Cell transfection

The shRNA target Linc-ROR (sh-Linc-ROR) (sh-Linc-ROR#1: GCCTCTGCACTCTTATGGAAGGAGGAAAT; sh-Linc-ROR#2: GGAGAGGAAGCCTGAGAGT) was packaged into the lentiviral vector pLKO.1 (Addgene, Cambridge, MA, USA) and shRNA negative control (sh-NC) (sh-NC: TTCTCCGAACGTGTCACGT) was used as the negative control. The modified pLKO.1, psPAX2, and pMD2G were then transfected into 293T cells using Lipofectamine 2000 (Invitrogen, MA, USA) following the supplier's protocols. After 48 h, viral supernatants were obtained, filtered, and applied to infect TPC-1 and IHH4 cells to obtain cells stably expressed sh-Linc-ROR.

CCK-8 assay

TPC-1 and IHH4 cells were plated into 96-well plates (10^4 cells/well). After 24, 48, and 72 h, CCK-8 (10 μl) was mixed to cells for 4 h, respectively. The absorbance at 450 nm was determined using the microplate reader (Multiskan FC, Thermo Fisher Scientific, MA, USA). This assay was repeated three times.

Xenograft tumor experiment

Ten 6-week-old BALB/c nude mice were obtained from Charles River Laboratories (Beijing, China). All mice were grouped into the sh-NC and sh-Linc-ROR groups ($n = 5$ in each group). To construct the xenograft tumor, the TPC-1 cells (5×10^6) stably transfected into sh-NC or sh-Linc-ROR, respectively, were injected into the mice *via* subcutaneous. After 7 days of injection, tumor indexes were measured every 7 days until 35 days, and tumor volume was detected with the formula $V = (\text{Length} \times \text{Width}^2)/2$. At 35 days post-injection, mice were euthanized by CO_2 inhalation. Tumors were excised to conduct the subsequent analysis. The animal research was performed in accordance with the national and international regulations and policies. The Ethics Committee of The First Hospital of Changsha authorized the research (Approval number 2023006).

Immunohistochemistry and hematoxylin-eosin (HE) staining

After fixation and paraffin embedding, the tumor tissues were made into 5 μm slices. The slices were deparaffinized, rehydrated, heated to 95°C for 30 min in 10 mmol/l sodium citrate (pH 6.0) for antigen retrieval, and treated with 3% hydrogen peroxide for 1 h to block the endogenous peroxidase activity. After blocked nonspecific binding, the slices were incubated with the anti-Ki-67 antibody (1:150, ab156956, Abcam) for 12 h. After that, the slices were treated with goat anti-rabbit IgG H&L (HRP) (1:1000) (Abcam, Cambridge, UK) for 1 h and dyed with DAB (Beyotime, Shanghai, China). For HE staining, the sections were dyed with hematoxylin and eosin. The results were analyzed utilizing the microscope (LSM700, Zeiss, Gottingen, Germany).

Flow cytometry

PBMCs were collected, resuspended, and made into cell suspension (1×10^7 cells/ml). The cell suspension was added to the mixture of anti-CD3-FITC and anti-CD8-APC antibodies (Abcam, Cambridge, MA, UK) for 30 min treatment at 4°C without light. After that, cells were harvested by centrifugation for measured using the FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed utilizing FlowJo software (Tree Star, Inc.).

ELISA assays

IL-10, IFN-γ, and TNF-β production in PBMCs or THP-1 cells were detected utilizing the ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the supplier's protocols. This assay was repeated three times.

Western blot

Protein samples were separated using RIPA (Beyotime, Shanghai, China), quantitated concentration, run on SDS-PAGE, and transferred to the PVDF membrane. After that, the membranes were blocked with 5% skim milk powder in Tris-buffered saline containing 0.1% Tween 20 and probed to primary antibodies including PD-L1 (1:500, ab205921), CD206 (1:1000, ab64693), CD168 (1:1000, ab262941), p-PI3K (1:1000, ab138364), PI3K (1:1000, ab227204), p-AKT (1:1000, ab8933), AKT (1:1000, ab8805), GAPDH (1:2500, ab9485) and β-actin (1:5000, ab8227)

(Abcam, Cambridge, MA, UK) overnight at 4°C and treated with secondary antibody for 1 h at room temperature. The bands were detected by applying the ECL chemiluminescence system (Beyotime, Shanghai, China) and quantitated utilizing Image J (NIH, Bethesda, MD, USA).

Statistical analysis

Data collected from at least three replicates were exhibited as mean ± standard deviation (SD), and data analysis was accomplished utilizing SPSS Statistics 22.0 (SPSS, Chicago, IL, USA). The student's *t*-test (non-paired) determined the difference between the two groups. The multiple groups' differences were detected using one-way ANOVA with Tukey's *post hoc* test. The paired *t*-test analyzed the significance between thyroid cancer tumor and adjacent normal tissues. The significance between the Linc-ROR level and the clinical characteristics of thyroid cancer cases was assessed utilizing the chi-square test. The survival of thyroid cancer cases was assessed using the Kaplan-Meier analysis with the log-rank test. $p < 0.05$ was identified as statistically significant.

Results

Linc-ROR is elevated in thyroid cancer and associated with poor prognosis

To investigate the influence of Linc-ROR on thyroid cancer development, the Linc-ROR level in thyroid cancer was first evaluated. The Linc-ROR level was up-regulated in tumor tissues of 70 thyroid cancer patients ($p < 0.01$, Fig. 1A). Among these 70 patients, 38 were defined as high-risk differentiated thyroid cancer and 32 as low-risk differentiated thyroid cancer. Linc-ROR presented higher expression in low-risk differentiated patients than high-risk differentiated patients ($p < 0.01$, Fig. 1B). Besides, the Linc-ROR level in thyroid cancer cases at TNM stage III–IV was higher than that at TNM stage I–II ($p < 0.01$, Fig. 1C). Additionally, 70 cases were divided into the high Linc-ROR expression group ($n = 35$) and low Linc-ROR expression group ($n = 5$) by designating the median value of Linc-ROR expression as the cut-off. Afterward, the correlation between the Linc-ROR level and the clinical pathological features was determined. Results revealed that thyroid cancer cases with high Linc-ROR levels presented poor overall survival ($p = 0.0315$, Fig. 1D). Furthermore, the Linc-ROR level was related to lymph node metastasis and TNM stage ($p < 0.05$), while it had no association with age, gender, tumor size, and extrathyroidal extension of thyroid cancer patients (Table 1). Moreover, the Linc-ROR level in thyroid cancer cells was assessed. It was observed that the Linc-ROR expression was enhanced in TPC-1, BCPAP, KTC, and IHH4 cells compared to Nthy-Ori 3-1 cells ($p < 0.01$, Fig. 1E). TPC-1 and IHH4 cells exhibited higher Linc-ROR expression than BCPAP and KTC cells, so TPC-1 and IHH4 cells were selected for follow-up assays (Fig. 1E). Therefore, the Linc-ROR expression was elevated in thyroid cancer and associated with poor prognosis.

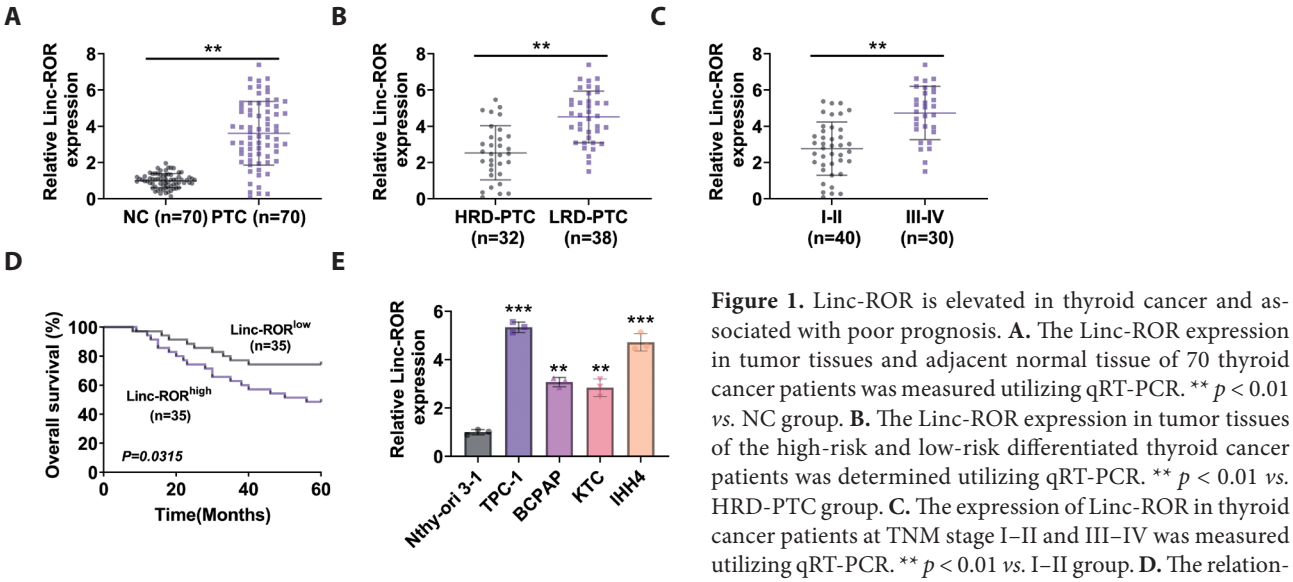

Figure 1. Linc-ROR is elevated in thyroid cancer and associated with poor prognosis. **A.** The Linc-ROR expression in tumor tissues and adjacent normal tissue of 70 thyroid cancer patients was measured utilizing qRT-PCR. ** $p < 0.01$ *vs.* NC group. **B.** The Linc-ROR expression in tumor tissues of the high-risk and low-risk differentiated thyroid cancer patients was determined utilizing qRT-PCR. ** $p < 0.01$ *vs.* HRD-PTC group. **C.** The expression of Linc-ROR in thyroid cancer patients at TNM stage I–II and III–IV was measured utilizing qRT-PCR. ** $p < 0.01$ *vs.* I–II group. **D.** The relationship between Linc-ROR expression and overall survival of thyroid cancer patients was assessed using Kaplan-Meier analysis. **E.** The Linc-ROR expression in human thyroid follicular epithelial cell line Nthy-Ori 3-1 and human thyroid cancer cell lines BCPAP, IHH4, KTC, and TPC-1 was measured utilizing qRT-PCR. ** $p < 0.01$; *** $p < 0.001$ *vs.* Nthy-Ori 3-1 group.

Linc-ROR silencing restrains thyroid cancer cell proliferation and tumor growth

To clarify the action of Linc-ROR on thyroid cancer development, the Linc-ROR expression was silenced by transfecting into shRNA target Linc-ROR. Results showed that both shRNAs target Linc-ROR knocked down the Linc-ROR level in TPC-1 and IHH4 cells ($p < 0.05$, Fig. 2A). Given that sh-Linc-ROR#1 exhibited higher efficiency than sh-Linc-ROR#2, sh-Linc-ROR#1 was chosen for follow-up assays. Results discovered that Linc-ROR silencing significantly suppressed TPC-1 and IHH4 cell viability ($p < 0.01$, Fig. 2B). Besides, Linc-ROR silencing restrained xenograft tumor growth of thyroid cancer ($p < 0.01$, Fig. 2C,D). The Linc-ROR level in xenograft tumors was decreased ($p < 0.01$, Fig. 2E). Furthermore, Linc-ROR silencing caused abnormal morphology of tumor cells (Fig. 2F). The Ki-67 level in xenograft tumor tissues was reduced after Linc-ROR silencing ($p < 0.01$, Fig. 2G). Thus, Linc-ROR silencing restrained thyroid cancer cell proliferation and tumor growth.

Linc-ROR silencing suppresses the immune escape of thyroid cancer cells

To study the action of Linc-ROR on the tumor microenvironment of thyroid cancer, the influence of Linc-ROR on the immune escape of thyroid cancer cells was determined by co-culturing TPC-1 and IHH4 cells with PBMCs. After the co-cultivation, the percentage of $CD8^+$ T cells in PBMCs was higher in the sh-Linc-ROR group than in the sh-NC group ($p < 0.01$, Fig. 3A). Besides, silenced Linc-ROR increased the level of IFN-γ in PBMCs ($p < 0.01$, Fig. 3B). Conversely, the IL-10 level in PBMCs was suppressed by silenced Linc-ROR ($p < 0.01$, Fig. 3C). In addition, Linc-ROR silencing reduced the PD-L1 level in TPC-1 and IHH4 cells ($p < 0.01$, Fig. 3D). Hence, silenced Linc-ROR suppressed the immune escape of thyroid cancer cells.

Linc-ROR silencing suppresses the polarization of M2 macrophages

To deeply investigate the function of Linc-ROR on thyroid cancer tumor microenvironment, the role of silenced Linc-ROR in macrophage polarization was explored by co-culturing PMA-induced THP-1 cells with TPC-1 and IHH4 cell supernatants. Results discovered that silenced Linc-ROR reduced the levels of CD206 and CD168 in THP-1 cells after the co-cultivation ($p < 0.01$, Fig. 4A–C). Besides, silencing of Linc-ROR decreased TNF-β and IL-10 levels in THP-1 cells after the co-cultivation ($p < 0.01$, Fig. 4D). Accordingly, Linc-ROR silencing suppressed the polarization of M2 macrophages.

Table 1. Correlation between Linc-ROR expression and the clinical pathological features of 70 thyroid cancer patients

Characteristic	All cases	Linc-ROR expression		*p*-value
		High ($n = 35$)	Low ($n = 35$)	
Age (years)				0.402
< 55	53	28	25	
≥55	17	7	10	
Gender				0.621
Male	26	14	12	
Female	44	21	23	
Tumor size (cm)				0.087
< 2	54	24	30	
≥2	16	11	5	
Lymph node metastasis				0.027*
Yes	27	18	9	
No	43	17	26	
Extrathyroidal extension				0.382
Yes	15	9	6	
No	55	26	29	
TNM stage				0.016*
I+II	40	25	15	
III+IV	30	10	20	

A chi-square test was used for comparing groups between low and high Linc-ROR expression. * $p < 0.05$.

Linc-ROR silencing suppresses the PI3K/AKT pathway activation

Given that the PI3K/AKT signaling was critical in cancer progression, the function of Linc-ROR on the PI3K/AKT signaling was clarified. It was discovered that silenced Linc-ROR greatly suppressed the phosphorylation levels of PI3K and AKT, while it did not affect the total PI3K and AKT levels in TPC-1 and IHH4 cells ($p < 0.01$, Fig. 5A). Besides, similar results of Linc-ROR on the PI3K/AKT pathway activity were observed in tissues of xenograft tumors ($p < 0.01$, Fig. 5B). In short, Linc-ROR silencing suppressed the PI3K/AKT pathway activation.

Linc-ROR silencing restrains thyroid cancer cell proliferation, immune escape, and M2 macrophage polarization by suppressing the PI3K/AKT pathway

To elucidate the mechanism of Linc-ROR on thyroid cancer development and tumor microenvironment, the activator of the PI3K/AKT pathway 740Y-P was used to treat TPC-1 cells. Results showed that 740Y-P treatment strengthened the cell viability of TPC-1 cells inhibited by silenced Linc-ROR ($p < 0.05$, Fig. 6A). Besides, silenced Linc-ROR increased the level of IFN-γ in PBMCs after the co-cultivation with TPC-1 cells, but 740Y-P treatment

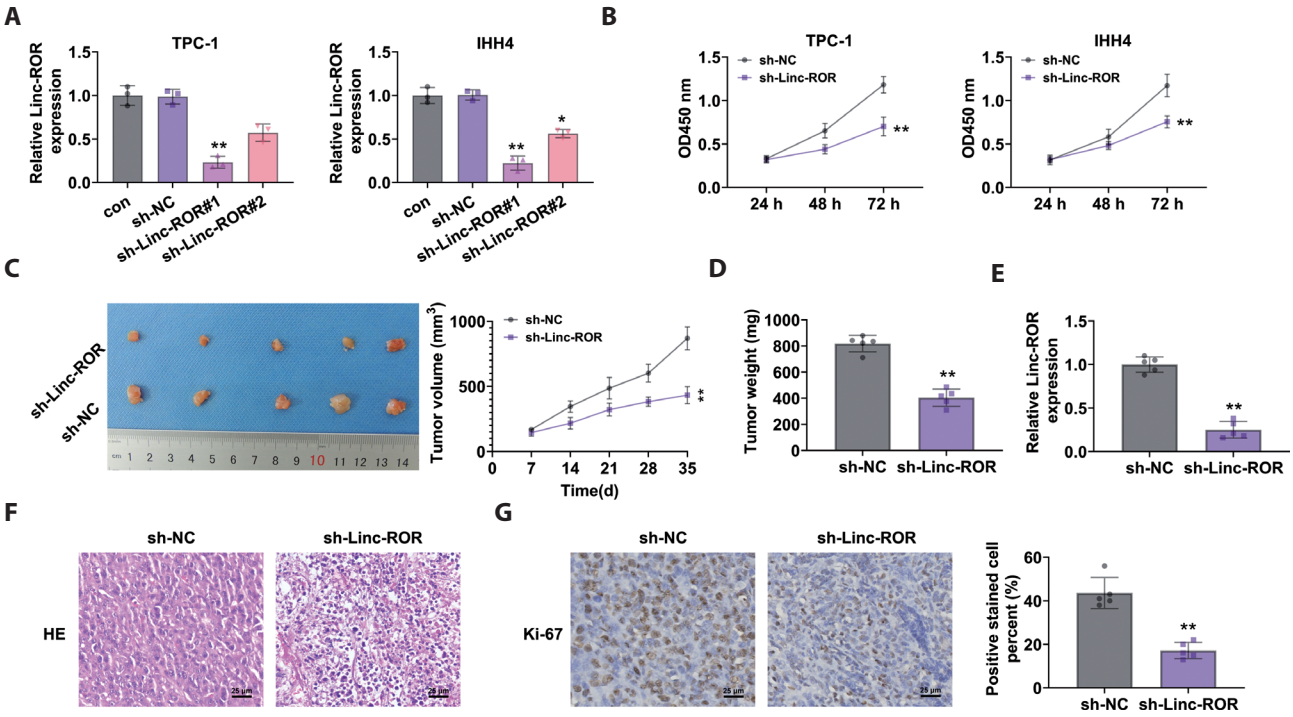

Figure 2. Linc-ROR silencing restrains thyroid cancer cell proliferation and tumor growth. **A.** Linc-ROR expression in TPC-1 and IHH4 cells was measured utilizing qRT-PCR after transfected with sh-Linc-ROR. **B.** The viability of TPC-1 and IHH4 cells was determined utilizing CCK-8 assay after transfected with sh-Linc-ROR. **C.** Images of the xenograft tumors constructed using TPC-1 cells after transfected with sh-Linc-ROR at the endpoint and the tumor volumes of mice were monitored every 7 days until 35 days. **D.** Mice weights were measured after transfected with sh-Linc-ROR. **E.** Linc-ROR expression in xenograft tumor tissues was measured utilizing qRT-PCR. **F.** Pathological evaluation of tumor tissue was performed using HE staining. **G.** The levels of Ki-67 in tumor tissues were evaluated using immunohistochemistry. ** $p < 0.01$ *vs.* sh-NC group.

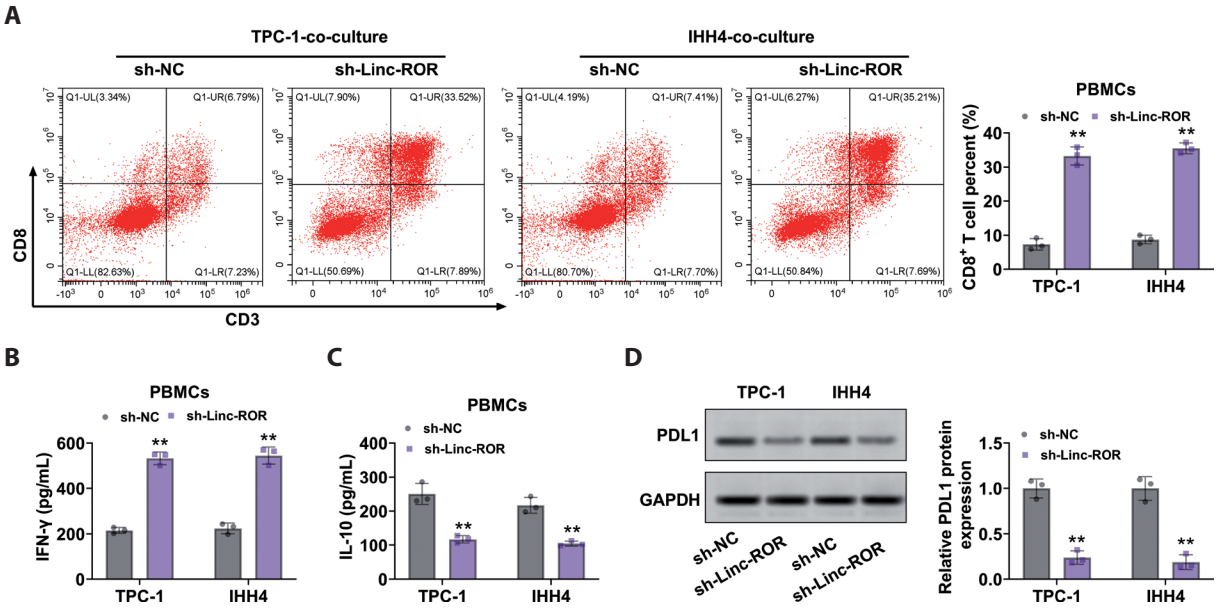

Figure 3. Linc-ROR silencing suppresses the immune escape of thyroid cancer cells. **A.** The percentage of $CD8^+$ T cells in PBMCs was evaluated by flow cytometry after co-culturing with TPC-1 or IHH4 cells with Linc-ROR silencing. **B, C.** The levels of IFN-γ and IL-10 in PBMCs were determined by ELISA after co-culturing with TPC-1 or IHH4 cells with Linc-ROR silencing. **D.** The PD-L1 level in TPC-1 and IHH4 cells was determined by Western blot after transfected with sh-Linc-ROR. ** $p < 0.01$ *vs.* sh-NC group.

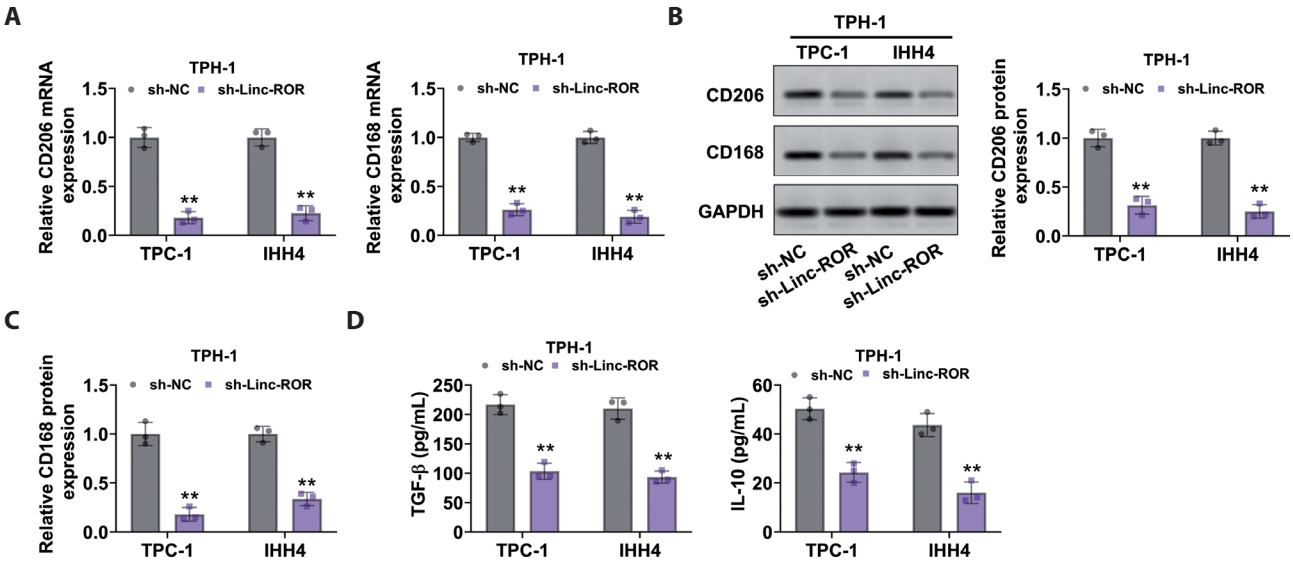

Figure 4. Linc-ROR silencing suppresses polarization of M2 macrophages. **A.** The CD206 and CD168 mRNA levels in THP-1 cells after co-culturing with TPC-1 or IHH4 cells with Linc-ROR silencing were measured utilizing qRT-PCR. **B, C.** The CD206 and CD168 protein levels in THP-1 cells after co-cultured with TPC-1 or IHH4 cells with Linc-ROR silencing were measured utilizing Western blot. **D.** The levels of TNF-β and IL-10 in THP-1 cells after co-culturing with TPC-1 or IHH4 cells with Linc-ROR silencing were measured by ELISA. ** $p < 0.01$ *vs.* sh-NC group.

reversed this phenomenon ($p < 0.05$, Fig. 6B). In addition, Linc-ROR silencing reduced the level of IL-10 in PBMCs after the co-cultivation with TPC-1 cells, which was abolished by the 740Y-P treatment ($p < 0.05$, Fig. 6C). Moreover, the CD206 and CD168 levels in THP-1 cells after the co-cultivation with TPC-1 cells were suppressed by silenced Linc-ROR but were abrogated by 740Y-P treatment ($p < 0.05$, Fig. 6D). These findings indicated that silenced Linc-

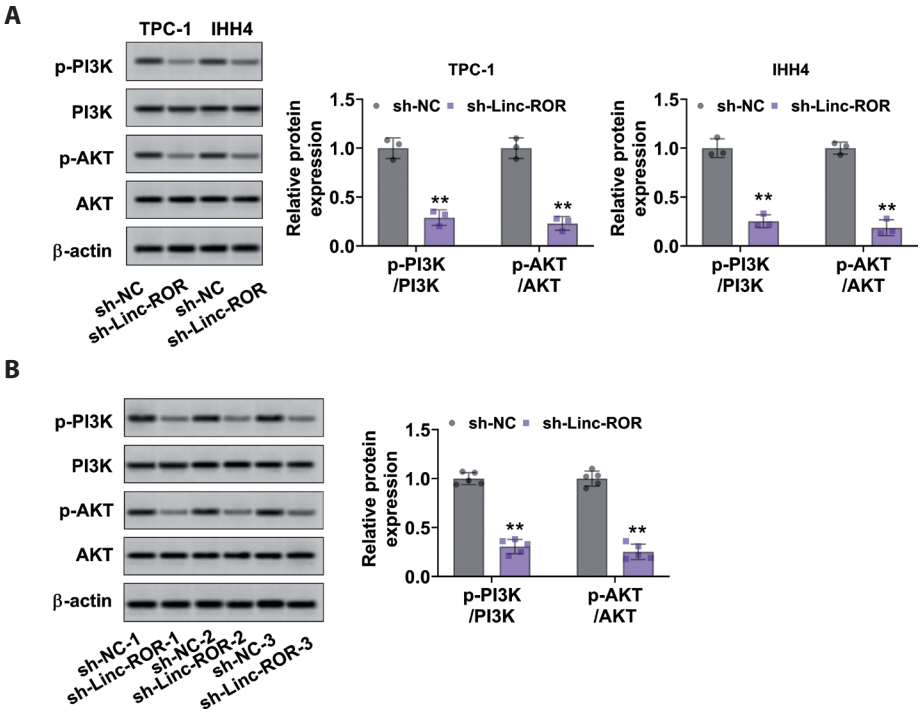

Figure 5. Linc-ROR silencing suppresses the PI3K/AKT pathway activation. **A.** The protein levels of p-PI3K, PI3K, p-AKT, and AKT in TPC-1 and IHH4 cells after transfected with sh-Linc-ROR were assessed using Western blot. **B.** The protein levels of p-PI3K, PI3K, p-AKT, and AKT in tissues of mice xenograft tumors after transfected with sh-Linc-ROR were evaluated by Western blot. ** $p < 0.01$ *vs.* sh-NC group.

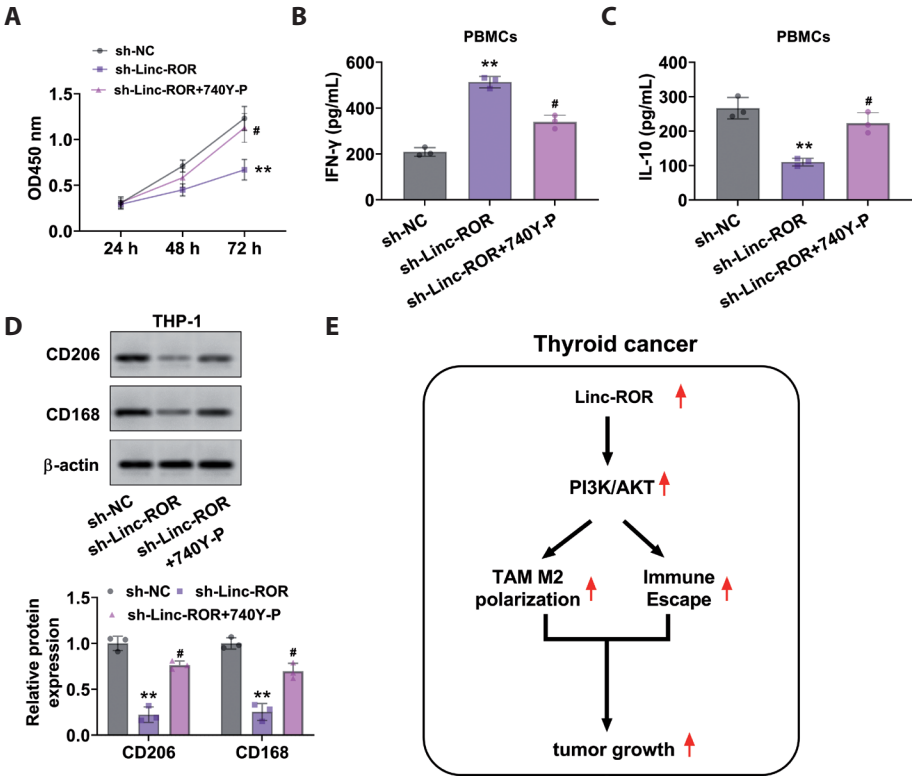

Figure 6. Linc-ROR silencing restrains thyroid cancer cell proliferation, immune escape, and M2 macrophage polarization through inhibiting the PI3K/AKT pathway. **A.** The viability of TPC-1 cells was detected using CCK-8 assay after transfected with sh-Linc-ROR and treated with 740Y-P. **B, C.** The levels of IFN-γ and IL-10 in PBMCs were determined by ELISA after co-culturing with TPC-1 with Linc-ROR silencing and treated with 740Y-P. **D.** The CD206 and CD168 protein levels in THP-1 cells were measured utilizing the Western blot after co-culturing with TPC-1 cells with Linc-ROR silencing and treated with 740Y-P. **E.** Proposed molecular mechanisms of Linc-ROR in thyroid cancer. ** $p < 0.01$ *vs.* sh-NC group; # $p < 0.05$ *vs.* sh-Linc-ROR group.

ROR restrained thyroid cancer cell proliferation, immune escape, and polarization of M2 macrophages by inhibiting the PI3K/AKT signaling. Taken together, Linc-ROR could regulate immune escape and M2 macrophage polarization to modulate tumor growth *via* the PI3K/AKT pathway in thyroid cancer (Fig. 6E).

Discussion

Thyroid cancer is a frequent endocrine cancer with high morbidity (Bray et al. 2018; Kim et al. 2020; Yan et al. 2020; Cherella et al. 2021; Zheng and Zhang 2023). Although most thyroid cancer patients present an excellent prognosis, current therapy strategies are unsatisfactory for advanced patients (Hernando et al. 2020; Ma et al. 2023). Linc-ROR exerts critical functions in cancer development, including thyroid cancer, while its role in the tumor microenvironment of thyroid cancer was unclear. Thus, this research investigated the function of Linc-ROR on thyroid cancer development, especially tumor microenvironment. The Linc-ROR level in thyroid cancer was first studied. Linc-ROR was up-regulated in thyroid cancer and associated with poor overall survival, lymph node metastasis, and TNM stage of thyroid cancer cases. The findings aligned with the reported studies (Shi et al. 2017; Fan et al. 2022). Fan et al. reported that the Linc-ROR expression was increased in PTC tissues and cells (Fan et al. 2022). Shi et al. (2017) revealed that renal cell carcinoma patients with high Linc-ROR levels exhibited shorter survival. Furthermore, the influence of Linc-ROR on thyroid cancer development was determined. It was found that Linc-ROR silencing restrained thyroid cancer cell proliferation and tumor growth. Similarly, Fan et al. (2022) proved silenced Linc-ROR inhibited cell proliferation, migration, and invasion, suppressed tumor growth *in vivo* and accelerated cell apoptosis in PTC. Collectively, these findings indicated that the enhanced Linc-ROR was correlated with poor prognosis of thyroid cancer and regulated thyroid cancer growth.

It is accepted that cancer progression could be regulated *via* modulating immune escape (Kim et al. 2019; Zhang et al. 2023). For instance, Kim et al. revealed that exosomal PD-L1 promoted tumorigenesis via modulating immune escape in non-small cell lung cancer (Kim et al. 2019). At present, the influence of Linc-ROR on immune escape remains unclear. Thus, we studied the action of Linc-ROR silencing on immune escape in thyroid cancer and discovered that Linc-ROR silencing restrained the immune escape of thyroid cancer cells. So far, this is the first report on the regulatory effect of Linc-ROR on immune escape in thyroid cancer. Macrophages are considered the primary type of tumor-infiltrating immune cells in the component

of the tumor microenvironment and exert vital function in tumor development (Liu et al. 2020). Tumor-associated macrophages are primarily M2 macrophages, which exert immunosuppressive function and promote tumor progression (Quail and Joyce 2013; Zheng et al. 2022). Increasing evidence showed that inhibition of M2 macrophage polarization could suppress tumor development (Xu F et al. 2018; Jang et al. 2023). Interestingly, lncRNAs were also proven to regulate macrophage polarization in cancers (Zong et al. 2021; Gong et al. 2023; Ding et al. 2024). At present, it is unclear whether Linc-ROR could affect macrophage polarization. Therefore, we focused on solving this question and found that Linc-ROR silencing suppressed the polarization of M2 macrophages. These findings on immune escape and macrophage polarization further proved the action of Linc-ROR on modulating the tumor microenvironment. These findings on tumor microenvironment were consistent with the previous reports (Hardin et al. 2018; Sun et al. 2021). Sun et al. (2021) found that exosomal Linc-ROR mediated crosstalk between cancer cells and adipocytes to promote tumor growth in pancreatic cancer. Hardin et al. discovered that the exosomes from thyroid cancer stem-like cells could communicate with and regulate adjacent and distant tumor microenvironments by transferring Linc-ROR to induce EMT (Hardin et al. 2018). However, previous studies have not investigated immune escape and macrophage polarization, which are crucial in the tumor microenvironment. Therefore, this is the innovation of this research. This research provides a basis for the potential application of Linc-ROR in thyroid cancer therapy. Furthermore, PD-L1 was highly expressed in advanced thyroid cancer cases (Ahn et al. 2017), which indicated that immune escape and changes in the tumor microenvironment were presented in advanced thyroid cancer. Therefore, Linc-ROR may have great potential in treating advanced thyroid cancer. In future research, we will further validate the action and mechanism of Linc-ROR in regulating the tumor microenvironment of advanced thyroid cancer in animal models.

The PI3K/AKT signaling is prominent in modulating tumor tumorigenesis and progression (Porta et al. 2014). Furthermore, the PI3K/AKT pathway regulated the tumor microenvironment (Qiu et al. 2019; Sun et al. 2022). It has been widely accepted that inhibition of PI3K not only inhibited tumor progression but also reshaped the immunosuppressive tumor microenvironment (Sun and Meng 2020). Thus, we supposed that Linc-ROR might regulate the immune escape of thyroid cancer cells and M2 macrophages by modulating the PI3K/AKT signaling. As expected, silenced Linc-ROR restrained thyroid cancer cell proliferation, immune escape, and M2 macrophage polarization *via* repressing the PI3K/AKT signaling. Similarly, Xu XY et al. (2018) found that overexpressed Linc-ROR promoted endometrial cell proliferation by activating the PI3K/Akt signaling. Nevertheless, the previous study has not revealed that the PI3K/AKT signaling mediated the regulation of Linc ROR on the tumor microenvironment. According to this evidence, we concluded that Linc-ROR could regulate immune escape and M2 macrophage polarization to modulate tumor growth *via* the PI3K/AKT signaling in thyroid cancer. However, whether Linc-ROR modulates tumor microenvironment through the competing endogenous RNA (ceRNA) mechanism has not been explored, which will be investigated in further research.

In conclusion, Linc-ROR was up-regulated in thyroid cancer and correlated with a poor prognosis. Silenced Linc-ROR suppressed cell proliferation, immune escape, and M2 macrophage polarization by inhibiting the PI3K/AKT signaling in thyroid cancer. Linc-ROR may be a promising target for thyroid cancer management. The limitations of this research are that the action of Linc-ROR on the tumor microenvironment has not been investigated in the animal model and that it has not been investigated whether Linc-ROR modulates tumor microenvironment through the competing endogenous RNA (ceRNA) mechanism, which will be addressed in the further study.

Funding. This study was supported by the grants: General projects of Hunan Provincial Health Commission (grant number: B202302056660) and Hunan Province Science and Technology Innovation Plan Project (grant number: 2021SK53106).

Conflicts of interest. The authors declare that they have no competing interests.

Ethical approval. The Ethics Committee of The First Hospital of Changsha authorized the study (Approval number. 2023006).

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Received: June 25, 2024
Final version accepted: January 3, 2025