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Original Article

Lactoferrin inhibits anoikis-resistance and metastasis of nasopharyngeal carcinoma cells via the AKT signaling pathway

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Abstract: Objective: To investigate the influence of lactoferrin (LTF) on the anoikis-resistance of nasopharyngeal carcinoma (NPC) cells and explore its relationship with the protein kinase B (AKT) signaling pathway. Methods: Anoikis-resistant HNE-1 and HONE-1 NPC cell lines were established. The proliferation and survival of cells were detected by Cell Counting Kit-8 (CCK8), while cell cycle and apoptosis were measured by flow cytometry. The transwell assay was used to evaluate invasion and metastasis abilities. The expression of matrix metallopeptidase-9 (MMP-9), vascular endothelial growth factor-A (VEGF-A), E-cadherin and Vimentin were assessed by Western blot. LTF plasmids and LTF shRNA plasmids were transfected into HNE-1 and HONE-1 cells, respectively, and the expression of E-cadherin, Vimentin, AKT and tropomyosin receptor kinase B (TrkB) proteins was detected by Western blot to clarify the role of LTF in anoikis-resistant NPC cells. Results: Anoikis-resistant HNE-1 and HONE-1 NPC cell lines were successfully established. Compared to parental cells, these anoikis-resistant cells exhibited enhanced survival, reduced apoptosis, and significantly increased invasive ability. They also demonstrated elevated expression of VEGF-A, MMP-9, and Vimentin, alongside decreased E-cadherin, indicating epithelial-mesenchymal transition (EMT). Furthermore, the expression of AKT and TrkB was significantly upregulated in anoikis-resistant cells. Critically, LTF overexpression reversed this aggressive phenotype: it suppressed cell survival and invasion, induced G0/G1 cell cycle arrest, promoted apoptosis, and downregulated the expression of AKT and TrkB. Conversely, LTF knockdown produced opposing effects. Conclusion: Our study revealed that LTF inhibits anoikis-resistance and metastasis of NPC cells via the AKT signaling pathway.

Keywords: Lactoferrin, nasopharyngeal carcinoma, anoikis-resistant, tropomyosin receptor kinase B/protein kinase B (TrkB/AKT)

Introduction

Nasopharyngeal carcinoma (NPC) is a tumor derived from epithelial cells, and its incidence ranks among the highest in otolaryngological malignancies [1, 2]. Unlike other types of head and neck cancer, NPC is closely related to Epstein-Barr virus infection in addition to genetic susceptibility and environmental factors [3]. Although improvements in diagnostic imaging techniques, the widespread use of intensity-modulated radiotherapy (IMRT) [4, 5], and optimization of chemotherapy regimens have significantly improved the survival of NPC patients, more than 70% of patients are diagnosed at stage III or IV due to the hidden anatomical location of the nasopharynx and atypical early symptoms [6]. Approximately 20-30% of patients with advanced NPC still experience treatment failure, mainly due to recurrence and/or metastasis (R/M) [7].

Anoikis is the process by which cells undergo cell death when detached from their surround-

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ing matrix and neighboring cells, which is distinct from apoptosis [8]. Under physiological conditions, anoikis is crucial for maintaining environment stability and structural integrity within tissues, participating in organism development, renewal and degradation, ensuring the programmed death of exfoliated cells and preventing their migration and growth elsewhere [9].

However, tumor cells may have defects in apoptotic pathways, allowing detached cells to evade apoptosis, and continue proliferating. Metastatic cancer cells exhibit various abnormal biological characteristics, among which resistance to apoptosis is particularly important. These cells survive after losing attachment to the extracellular matrix and then continue to grow and proliferate in other parts of the body via the lymphatic and blood circulation systems, leading to tumor invasion and metastasis [9, 10].

Lactoferrin (LTF) is an iron-binding protein, belonging to the transferrin family [11]. The complete amino acid sequence of human LTF consists of 792 amino acid residues, with two reversible iron-binding sites [12]. As a first line of defense the non-specific immune system, LTF has a broad range of biological functions, including antibacterial, antiviral, antioxidant, anticancer and immune system regulation [13, 14]. The LTF gene is located in the CER1 region of 3p21.3 (gene ID: 405), a region frequently deleted in the chromosomes of tumor patients [15], suggesting that loss of LTF expression may be associated with tumor development.

High LTF expression has been found in paranasopharyngeal carcinoma and nasopharyngeal chronic inflammatory tissues, and LTF is secreted in normal tissues. However, LTF is significantly downregulated in NPC tissues, and negatively correlated with tumor size, infiltration range, lymph node metastasis and recurrence after radiotherapy [16]. The effect of LTF on NPC is related to the protein kinase B (AKT) signaling pathway [17].

Anoikis resistance is a key step in tumor progression and metastasis [18]. NPC cells are highly invasive and metastatic due to their anti-apoptotic characteristics. Meanwhile, LTF has been shown to be negatively correlated with tumor progression, metastasis and invasion, leading to the hypothesis that LTF could inhibit the anti-apoptotic characteristics of tumor cells. Our study aimed to explore the effect of LTF on anoikis-resistant NPC cells and verify whether LTF is related to the AKT signaling pathway, so as to clarify the molecular mechanism by which LTF inhibits the invasion and metastasis of NPC and provide relevant theoretical basis for improving the survival rate and prognosis of patients.

Materials and methods

Suspension culture plates preparation

Suspension culture Plate Preparation poly-(2-hydroxyethyl methacrylate) HEMA powder (25249-16-5; Sigma, St. Louis, MO, USA) was dissolved in 100 mL of 95% alcohol (100 mg/mL), and vibrated until completely dissolved, the solution was diluted fourfold with 95% alcohol to a final concentration of 25 mg/mL. For coating, 1 mL per well was used for 6-well plates, 0.5 mL/well for 24-well plates, and 100 μL/well for 96-well plates. The coated plates were dried for 24 hours in a biosafety cabinet under ultraviolet radiation and used within a week.

Cell culture

Two human NPC cell lines (HNE1 and HONE1), supplied by the Central South University Advanced Research Center, were used. After thawing, cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, China) supplemented with 10% fetal bovine serum (Gibco, South America, USA), 100 U/mL penicillin streptomycin (Gibco, USA), in an incubator (Thermo Fisher Scientific, USA) at 37°C with 5% CO_2.

Anoikis-resistant cells

Cells were cultured at a density of 5×10^6 cells/well in six-well poly-HEMA culture plates with medium containing 2% fetal bovine serum. Cell proliferation was detected using the Cell Counting Kit-8 (CCK8) assay (CK04, DOJINDO, Japan). Cell suspensions were prepared from logarithmically growing cells and cultured in 96-well plates at a density of 3×10^4 cells/well, 200 μL per well (10 duplicate wells per group). At the indicated time points (0 h, 24 h, 48 h, 72 h), the cells were washed, and 200 μL medium and 10 μL CCK8 solutions were added to each well, followed by incubation at 37°C with 5%

CO_2 for 4 hours. The absorbance (OD) at 450 nm was measured using a Bio-Tek microplate reader, and the growth curve was plotted using the mean values. The cell survival percentage was defined as the ratio of the average absorbance value at each time point to that at 0 h. When the absorbance value stabilized, it indicated the induction time for anoikis-resistant cells. Then, we initially screened anoikis-resistant cells.

Cell transfection

NPC cells and anoikis-resistant NPC cells were cultured in 6-well plates. When cells reached 80-90% confluency, LTF overexpression plasmid (Forward primer: 5'-cgcctccagaccgcagacatgaaactt-3', Reverse primer: 5'-ctgggccatcttcttcggttttacttc-3') and LTF-RNAi plasmid (sense strand: 5'-GGACACTTCGTCCATTCTTGA-3', antisense strand: 5'-TCAAGAATGGACGAAGTGTCC-3') along with their negative controls were transfected into each group of cells using Lipofectamine 2000. All plasmids were from Genechem, Shanghai, China, Lipofectamine 2000 transfection reagent was obtained from Thermo Fisher Scientific, USA, Catalog number: 11668500.

Western blot analysis

Collected cells at the logarithmic growth stage, Cells were washed with ice-cold PBS and lysed with RIPA lysis buffer for 15 to 30 minutes (until no visible cell deposits), centrifuged (13,188 × g) for 15 minutes at 4°C. According to BCA protein quantitative kit instruction manual testing, the absorbance was measured at a wavelength of 562 nm, a standard curve was generated to calculate the protein concentration. Western blotting was performed according to standard procedures. Gelpro 4.0 optical density analysis software was used to measure the gray value. The antibodies and dilution ratios that we used in our experiments: anti-E-cadherin (YT1454, ImmunoWay, USA, 1:1000), anti-vimentin (YT4880, ImmunoWay, USA, 1:1000), anti-VEGFA (vascular endothelial growth factor A, YT5108, ImmunoWay, USA, 1:1000), anti-MMP-9 (matrix metallopeptidase-9, YT1892, ImmunoWay, USA, 1:1000), anti-Lactoferrin (GR148596-1, Abcam, USA, 1:4000), anti-AKT (AM2059, Abzoom Biolabs, USA, 1:1000), anti-TrkB (tyrosine kinase receptor B, 13129-1-AP, Proteintech, USA, 1:800), anti-β-actin (20536-1-AP, Proteintech, USA, 1:5000). The primary antibody used in the experiment was rabbit-derived, and the secondary antibody was goat anti-rabbit. The protein loading amount was 20-30 μg per lane.

Transwell experiment

To evaluate cell invasion, Transwell chambers (CLS3412, Corning, USA) with 8-μm pores were pre-coated with Matrigel (E1270, BD Biosciences, USA) at 37°C for 30 minutes to form a basement membrane-like barrier. The lower chamber was filled with 500 μL of complete medium containing 10% fetal bovine serum as a chemoattractant. Cells were resuspended in serum-free medium at 1×10^6 cells/mL, and 100 μL of cell suspension was added to the upper chamber. After incubation at 37°C with 5% CO_2 for 48 hours, non-invading cells on the upper surface of the membrane were gently removed with a cotton swab. Invading cells on the lower surface were fixed with methanol-acetone (1:1), stained with 0.1% crystal violet, and rinsed with PBS. After decolorization, absorbance at 550 nm was measured using a microplate reader. Each experiment was performed in triplicate.

Flow cytometry

For cell cycle analysis, collected cells were washed with PBS and fixed in 1 mL ice-cold 70% ethanol at 4°C overnight. Fixed cells were washed with PBS to remove ethanol, treated with RNase A (R6148, 100 μg/mL; Sigma-Aldrich, USA) at 37°C for 30 minutes to remove RNA, and stained with propidium iodide (537-059, PI, 50 μg/mL; Sigma-Aldrich, USA) for 30 minutes at room temperature in the dark. DNA content was analyzed using a flow cytometer (NovoCyte, San Diego, CA, USA).

For apoptosis detection, an Annexin V-FITC/PI Apoptosis Detection Kit (556547, BD Biosciences, USA) was used according to the manufacturer's instructions. Briefly, cells were collected, washed twice with cold PBS, and resuspended in 1 × Binding Buffer at 1×10^6 cells/mL. Then, 100 μL cell suspension was transferred to a flow cytometry tube and stained with 5 μL Annexin V-FITC and 5 μL PI for 15 minutes at room temperature in the dark. Subsequently, 400 μL of 1 × Binding Buffer was added to each tube, and samples were analyzed by flow cytometry (NovoCyte, San Diego, CA, USA) within 1 hour. Early apoptotic cells were Annexin V-FITC positive and PI negative, while late apoptotic or

necrotic cells were both Annexin V-FITC and PI positive.

All measurements were performed in triplicate. Data were analyzed using NovoExpress 1.1.0 software (NovoCyte, San Diego, CA, USA).

Statistical analysis

All statistical analyses were performed using SPSS Statistics 26.0 software (SPSS Inc., Chicago, USA) and GraphPad Prism 9.0 software (GraphPad Software, La Jolla, CA, USA). Data from at least three independent experiments are presented as the mean ± standard deviation (SD). The normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was verified via Levene's test.

Comparisons between two independent groups (e.g., parental adherent cells vs. anoikis-resistant suspended cells of the same cell line): Unpaired (independent) Student's t-test was used.

Comparisons between two related groups (e.g., the same cell line before vs. after LTF transfection): Paired Student's t-test was used.

Comparisons among more than two independent groups (e.g., parental cells, anoikis-resistant cells, LTF-overexpressing anoikis-resistant cells, and negative control cells): One-way analysis of variance (one-way ANOVA) was applied, followed by Tukey's honest significant difference (HSD) post hoc test for pairwise comparisons (suitable for equal sample sizes and homogeneous variances, ensuring control of type I error across all comparisons).

Comparisons involving multiple time points (repeated measurements on the same sample) (e.g., CCK8 assay detecting cell survival at 0 h, 24 h, 48 h, 72 h): Repeated-measures ANOVA was used to account for within-subject correlations (avoiding overestimation of significance due to repeated sampling of the same cells), followed by Bonferroni post hoc test for pairwise comparisons between time points (conservative adjustment for multiple comparisons, ideal for small-to-moderate time point numbers).

If data did not meet the assumptions of normality or homogeneity of variances, non-parametric alternatives were adopted: Kruskal-Wallis H test (for multiple independent groups) or Friedman test (for repeated-measures data), followed by Dunn's post hoc test. A two-tailed *P*-value < 0.05 was considered statistically significant.

Results

Anoikis-resistant cell establishment

HNE1 and HONE1 cells, originally adherent, were suspended in medium containing 2% fetal bovine serum (**Figure 1A**). Cell viability was detected by CCK8 assay. The survival rate decreased significantly after 24 to 48 hours but stabilized between 48 to 72 hours (**Figure 1B**). Flow cytometry analysis of HNE1 and HONE1 cells after 48 hours of suspension culture showed that the proportion of cells in G0/G1 phase was 56.48% and 57.65%, respectively, and in S phase was 33.25% and 34.74%, respectively. Compared to adherent cells (HNE1: G0/G1 71.3%, S 22.43%; HONE1: G0/G1 75.99%, S 16.54%), suspended HNE1 and HONE1 cells had significantly decreased G0/G1 phase cells and increased S phase cells ($P < 0.001$ for both) (**Figure 1C**, **1E**). Meanwhile, the apoptosis rates of suspended HNE1 and HONE1 cells (2.07% and 2.19%, respectively) were lower than those of adherent cells (2.87% for HNE1 and 3.19% for HONE1) (**Figure 1D**, **1F**), indicating enhanced proliferation and anti-apoptotic abilities. Thus, cells suspension-cultured for 48 hours were identified as anoikis-resistant NPC cells.

Biological characteristics of anoikis-resistant NPC cells

Under microscopy, suspended cells began to aggregate after 24 hours with unclear boundaries; over time, more cells clustered together (**Figure 1A**). Given the morphological changes, we investigated whether suspended cells exhibited epithelial-mesenchymal transition (EMT) characteristics. We found that Vimentin expression was increased in anoikis-resistant NPC cells, while E-cadherin expression was decreased (**Figure 2C**), indicating EMT changes. The Transwell assay showed that the number of anoikis-resistant HNE1 and HONE1 cells passing through the Matrigel was significantly higher than that of parental adherent cells (**Figure 2A**, **2B**), suggesting enhanced invasion ability. Western blot results showed that VEGF-A and

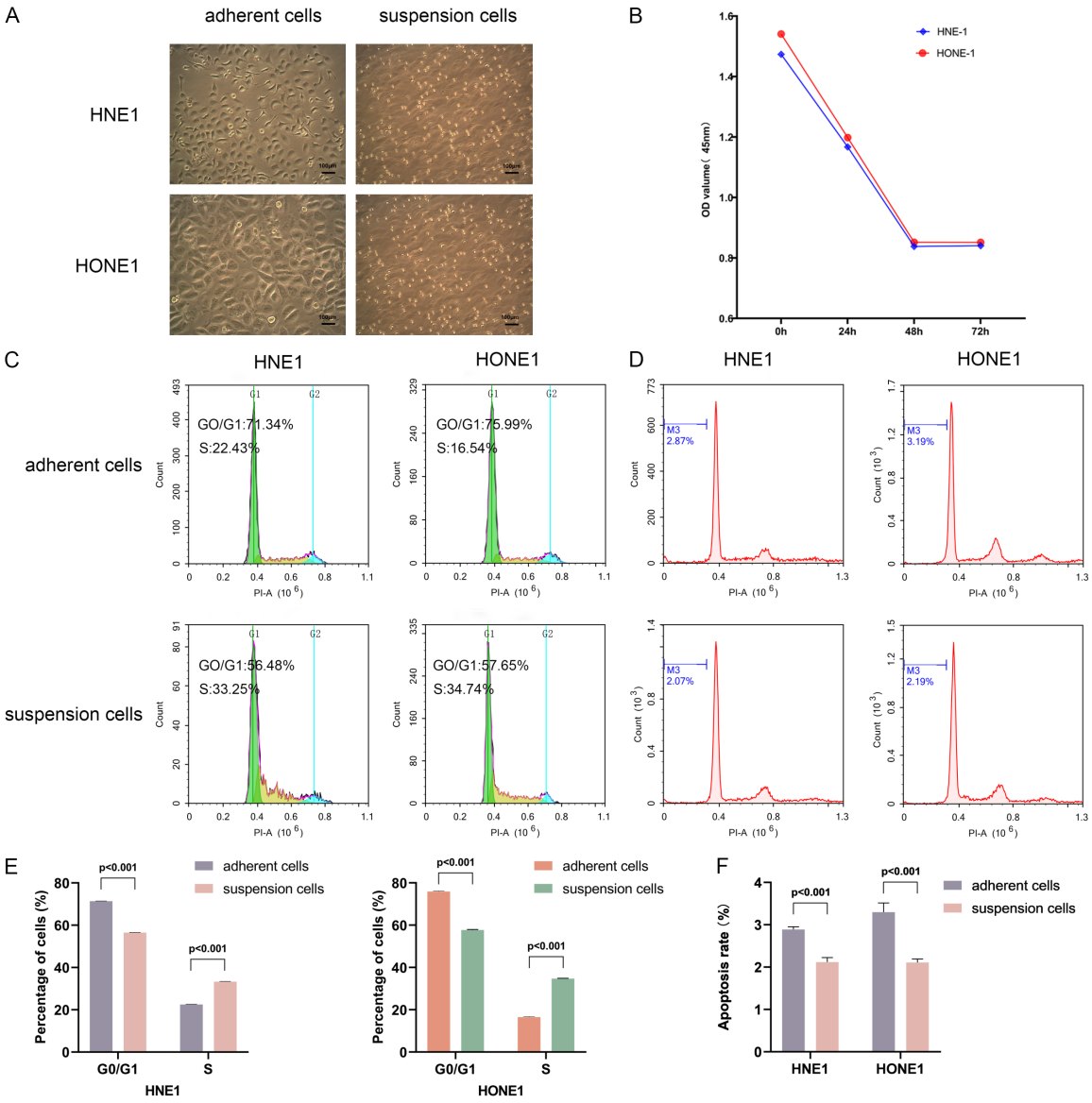


Figure 1. Establishment of anoikis-resistant NPC cells. A. HNE1 and HONE1 cell morphology in two different cultured conditions - adhesion and suspension for 48 hours (100×). B. Suspension culture for Nasopharyngeal carcinoma (NPC) HNE1 and HONE1 cells during 72 hours of Cell Counting Kit-8 (CCK8) tests and the number of cells. C. Cell cycle flow cytometry analysis. The x-axes show PI-A staining, and the Y-axes show cell counts. D. Apoptotic cell comparison of HNE1 and HONE1 cells, and that of two different cultured condition cells. E. G0/G1 and S cell comparison of HNE1 cells and HONE1 cells in different cultured conditions. In suspension culture, HNE1 and HONE1 G0/G1 cells were reduced, and S cells were increased. F. The apoptosis rates of suspended HNE1 and HONE1 cells were lower than those of adherent cells.

MMP-9 expression levels were increased in anoikis-resistant NPC cells compared to parental adherent cells (**Figure 2C**), indicating enhanced invasion and metastasis abilities. Together, the enhanced survival, reduced apoptosis (**Figure 1**) and acquired invasive/metastatic traits (**Figure 2**) demonstrated successful establishment of anoikis-resistant NPC cell models with aggressive phenotypes.

LTF inhibits the proliferation of anoikis-resistant NPC cells

LTF plasmids were transfected into HNE1 cells (low LTF expression), and LTF-RNAi plasmids were transfected into HONE1 cells (relatively high LTF expression) to verify transfection efficiency and assess biological changes. CCK8 results showed that the survival rate of LTF-

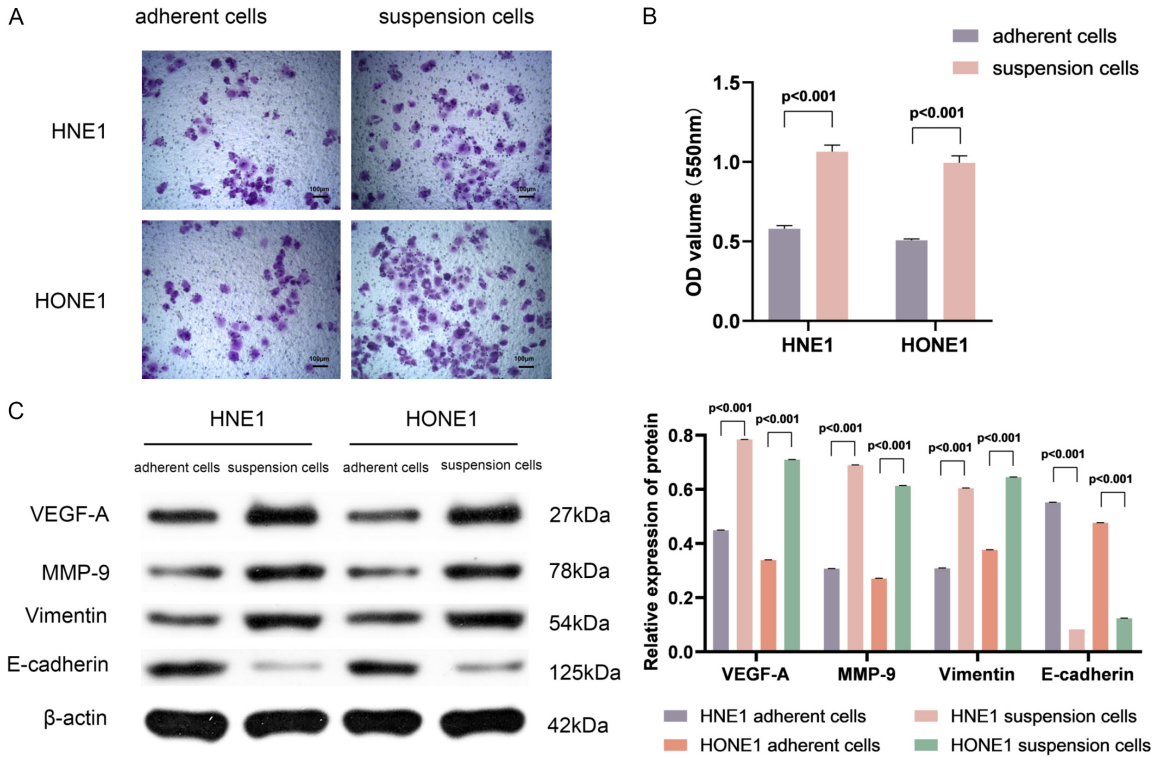
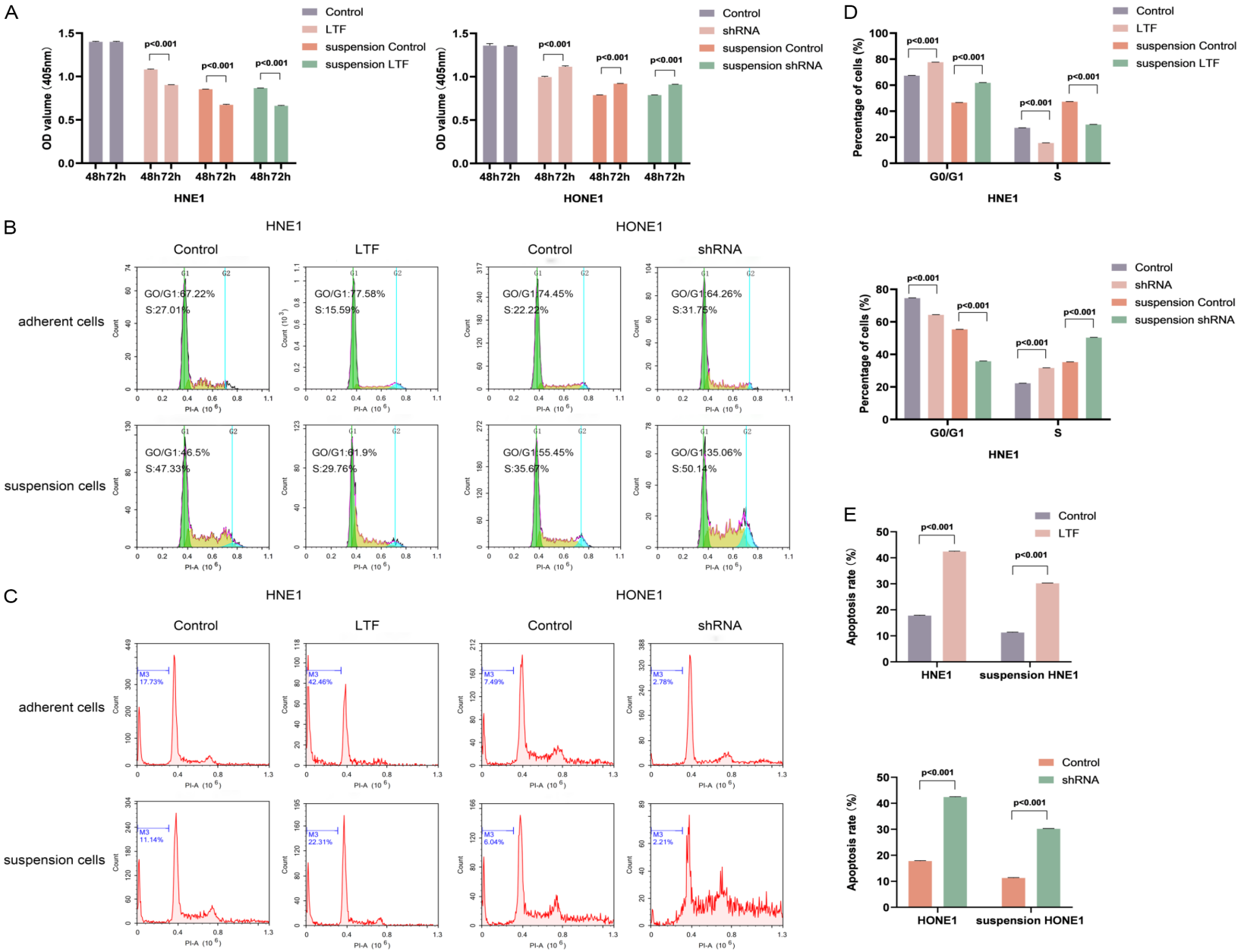


Figure 2. Biological characteristics of anoikis-resistant NPC cells. A. Transwell assay analysis the invasion ability of nasopharyngeal carcinoma cells under different culture conditions (100×). B. The number of anoikis-resistant HNE1 and HONE1 cells through the basal gel was more than that of their parent adherent cells. C. epithelial-mesenchymal transition (EMT) changes of nasopharyngeal carcinoma cells in different culture states. In Western blot analysis, the expression levels of vascular endothelial growth factor-A (VEGF-A), matrix metallopeptidase-9 (MMP-9) and Vimentin of the HNE1 and HONE1 anoikis-resistant NPC cells were increased, while the expression levels of e-cadherin were decreased.

overexpressing HNE1 cells was lower than that of the control group at the 48-hour and 72-hour time points, while the survival rate of LTF-knockdown HONE1 cells was higher than that of the control group, with the difference becoming statistically significant at the 72-hour time point (**Figure 3A**). Furthermore, as consistently observed across all time points, the survival rate of anoikis-resistant NPC cells was significantly higher than that of adherent cells (**Figure 3A**). Flow cytometry results showed that compared to control cells, LTF-overexpressing HNE1 parental and anoikis-resistant HNE1 cells had increased G0/G1 phase cells (77.58% and 61.9%, respectively), decreased S phase cells (15.59% and 29.76%, respectively) (**Figure 3B**, **3D**), and significantly increased apoptosis rates (42.46% and 30.09%, respectively) (**Figure 3C**, **3E**). In contrast, HONE1 parental and anoikis-resistant cells with LTF knockdown had decreased G0/G1 phase cells (64.26% and 35.06%, respectively), increased S phase cells (31.75% and 50.14%, respectively) (**Figure 3B**, **3D**), and significantly decreased apoptosis rates (2.78% and 2.21%, respectively) (**Figure 3C**, **3E**). These results indicate that LTF inhibits DNA synthesis, reduces cell proliferation, and promotes apoptosis in NPC.

LTF weakens the invasive ability of anoikis-resistant NPC cells

The invasive ability of anoikis-resistant NPC cells was significantly enhanced compared to untreated parental adherent cells. In both parental and anoikis-resistant NPC cells, the number of LTF-overexpressing HNE1 cells passing through the Matrigel decreased, while that of LTF-inhibited HONE1 cells increased (**Figure 4A**, **4B**). Western blot showed that VEGF-A and MMP-9 expression was increased in anoikis-resistant NPC cells, decreased in LTF-overexpressing HNE1 cells, and upregulated in LTF-inhibited HONE1 cells (**Figure 4C**). These results indicate that LTF inhibits the invasion and metastasis of NPC cells.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C
D
Percentage of cells (%)
G0/G1
S
E
Apoptosis rate (%)
HNE1
suspension HNE1
HONE1
suspension HONE1
p<0.001

Figure 3. Lactoferrin (LTF) inhibits the proliferation of anoikis-resistant NPC cells. A. CCK8 experimental analysis of the proliferation of NPC cells and anoikis-resistant NPC cells with LTF and ShRNA-LTF. Over-expression of LTF reduced survival, while LTF knockdown cells survival rate increased. B. Cells for cell cycle flow cytometry analysis. The x-axes show PI-A staining, and the Y-axes show cell counts. C. Cells for apoptosis flow cytometry analysis. The x-axes show PI-A staining, and the Y-axes show cell counts. D. LTF suppressed S phase cell growth and promoted the growth of G0/G1 phase cells. Meanwhile, anoikis-resistant cells with ShRNA-LTF G0/G1 phase cells decreased, and S phase cells increased. E. LTF induced apoptosis of anoikis-resistant cells. Anoikis-resistant cells of HNE1 and HONE1 treated with LTF had stronger resistance than parent adherent cells. LTF knockdown cells lowered apoptosis.

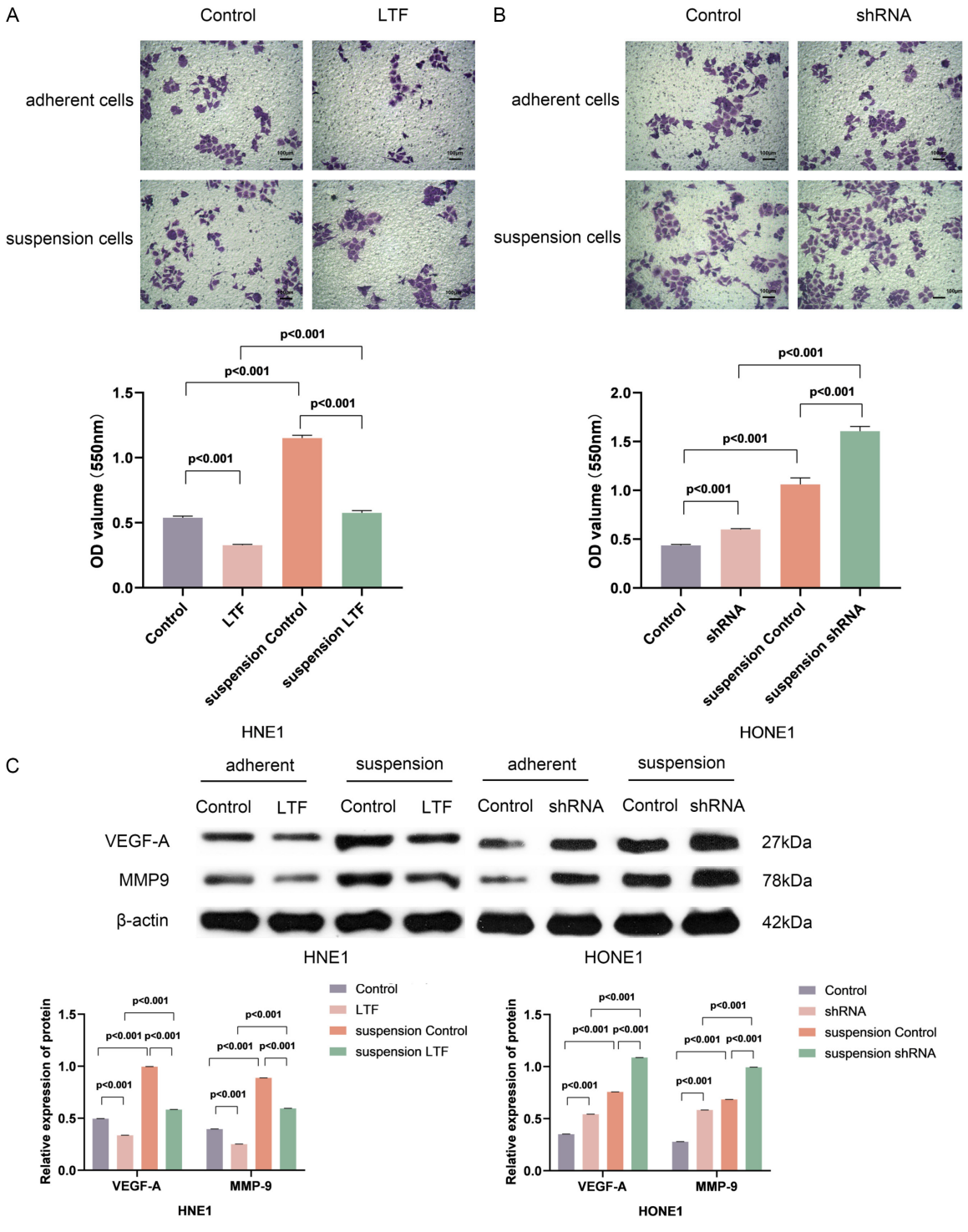

Figure 4. LTF affects the invasive ability of anoikis-resistant NPC cells. A. Transwell experimental testing results of parental cells and anoikis-resistant NPC cells (100×). The number of HNE1 cells with LTF overexpression through the basal gel decreased. B. LTF weakened the invasive ability of anoikis-resistant NPC cells. The number of HONE1 cells with LTF interference through the basal gel increased (100×). C. Western blot analysis of EMT marker proteins (Vimentin, E-cadherin) and metastasis marker protein (MMP-9, VEGF-A) expression for two different cultured conditions of HNE1 and HONE1 cells with LTF and ShRNA-LTF.

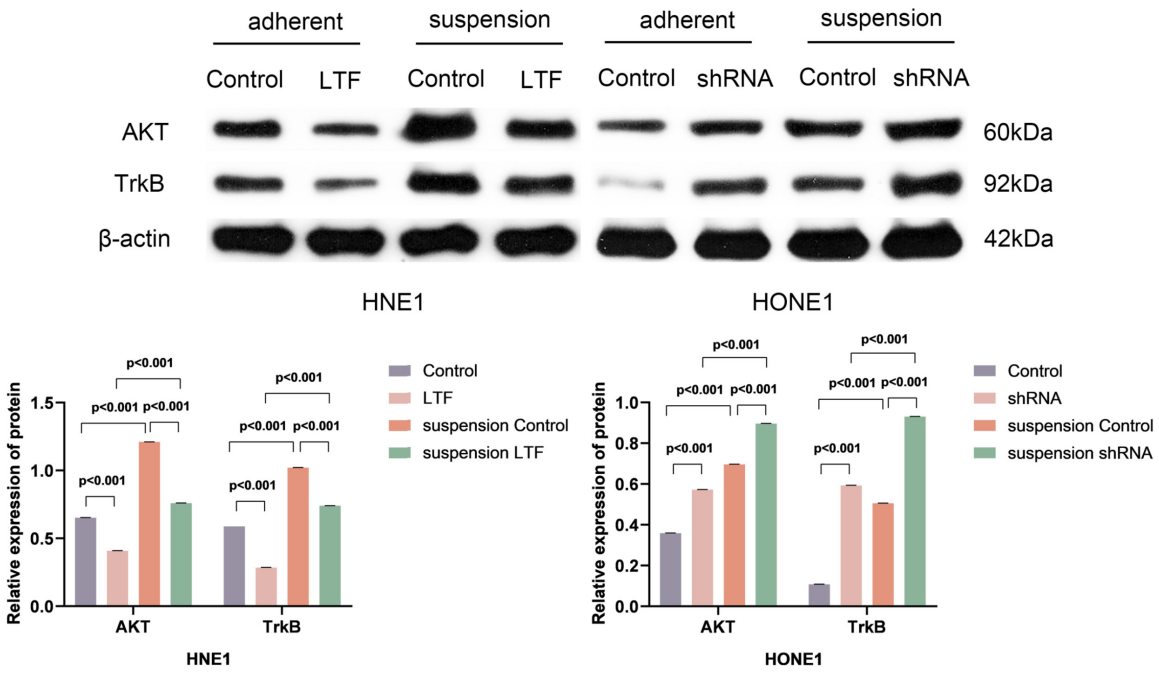

Figure 5. LTF regulates the AKT signaling pathway. Western blot analysis of AKT and TrkB expression for two different cultured conditions of HNE1 and HONE1 cells with LTF and ShRNA-LTF. LTF inhibited the expression of AKT signaling pathway protein (AKT and TrkB).

LTF regulates the AKT signaling pathway

We first confirmed LTF modulation efficiency at the protein level by Western blot. As expected, LTF expression was significantly upregulated in HNE1 cells transfected with the LTF overexpression plasmid and downregulated in HONE1 cells transfected with LTF-shRNA compared to their respective controls (data not shown). We then examined key molecules in the AKT pathway. The expression of AKT and TrkB was significantly higher in anoikis-resistant NPC cells than in parental adherent cells. After LTF overexpression in HNE1 cells, AKT and TrkB expression decreased, while LTF knockdown in HONE1 cells significantly increased their expression (**Figure 5**).

Discussion

Anoikis-resistance is a rate-limiting step for nasopharyngeal carcinoma (NPC) metastasis, enabling detached tumor cells to survive circulation and colonize distant tissues [9, 19]. Lactoferrin (LTF), a downregulated tumor suppressor in NPC [15, 20], has been shown to inhibit AKT signaling [17], but its role in regulating anoikis-resistance - a critical metastatic phenotype - remains unexplored. This study fills this gap by investigating LTF's impact on anoikis-resistant NPC cells and the underlying molecular mechanisms.

Consistent with Ng et al. [21], who established anoikis-resistant NPC cells with pro-metastatic traits, our cell model validated the reliability of in vitro anoikis-resistance induction. However, our work extends this foundation by identifying LTF as a key regulator of these aggressive phenotypes, a connection not addressed in prior studies on NPC anoikis-resistance. EMT and elevated VEGF-A/MMP-9 are conserved features of anoikis-resistant cells across tumors [22-24], but our study links these traits to LTF deficiency in NPC, providing a molecular expla-

nation for why LTF downregulation correlates with poor prognosis [16].

Our research group previously found that the basal LTF expression of HNE1 cells is relatively low, so it is suitable for LTF overexpression research. In contrast, HONE1 cells have relatively high basal LTF expression, making them suitable for LTF knockdown assays. Therefore, LTF overexpression plasmids were transfected into HNE1 cells, and LTF-RNAi plasmids were transfected into HONE1 cells for the best experimental results. For transfection experiments, cells were harvested 48 hours post-transfection for analysis. Transfection efficiency was confirmed by quantifying LTF mRNA levels via quantitative real-time PCR (qRT-PCR) and LTF protein expression via Western blot; these results are not shown in this article. Our results demonstrate that LTF modulates anoikis-resistant NPC cell behaviors, which aligns with LTF's conserved tumor-suppressive role [25, 26], but with critical differences from prior work.

Notably, our study differs from and advances previous insights into LTF's role in NPC. Deng et al. [17] previously reported that LTF represses AKT signaling to inhibit NPC cell proliferation, but their work focused on primary tumor growth and did not address anoikis-resistance - the core biological process enabling metastasis. In contrast, we demonstrate that LTF's inhibition of AKT signaling directly targets anoikis-resistance, linking LTF to a metastatic step that accounts for ~20-30% of advanced NPC treatment failures [7]. This distinction is critical: while proliferation inhibition suppresses primary tumor size, targeting anoikis-resistance directly blocks the dissemination of tumor cells, a more unmet clinical need for preventing recurrence.

Our identification of LTF as an upstream regulator of TrkB/AKT in NPC anoikis-resistance also differs from prior work on TrkB/AKT signaling. Li et al. [27] reported that BDNF/TrkB activates AKT to promote anoikis-resistance in prostate cancer, and Zou et al. [28] noted TrkB/AKT inhibition suppresses tumor metastasis, but neither study identified LTF as a natural antagonist of this pathway. In NPC, Deng et al. [17] linked LTF to AKT inhibition but ignored TrkB-a key upstream activator of AKT in anoikis-resistance [29]. Our study bridges this gap by demonstrating that LTF downregulates both TrkB and AKT, revealing a more complete molecular cascade: LTF → TrkB/AKT suppression → anoikis-resistance reversal. This novel regulatory axis explains how LTF specifically targets metastatic phenotypes, rather than general proliferation.

While LTF's tumor-suppressive role has been reported in other cancers (e.g., breast [25], oral [26]), our findings highlight NPC-specific relevance and extend mechanistic understanding. For example, Zhang et al. [25] showed LTF induces ferroptosis in triple-negative breast cancer, and Chea et al. [26] reported LTF reverses EMT via ERK1/2 in oral squamous cell carcinoma. In contrast, our study demonstrates LTF acts via TrkB/AKT in NPC, suggesting LTF may employ tissue-specific signaling pathways to suppress tumors. This difference underscores the need for cancer-type-specific investigations of LTF, and our work provides the first mechanistic framework for LTF in NPC metastasis.

Despite these insights, our study has several limitations. First, our findings are based primarily on in vitro experiments using two NPC cell lines (HNE1, HONE1). In vivo validation using animal models (e.g., nude mouse models of NPC metastasis) is needed to confirm LTF's anti-metastatic efficacy in a physiological microenvironment, as in vitro conditions cannot fully replicate the complex tumor-stroma interactions that regulate anoikis-resistance in vivo. Second, we only explored the TrkB/AKT pathway and did not investigate downstream effectors (e.g., Bad, Caspase-9) that directly mediate apoptosis, or potential cross-talk with other signaling pathways (e.g., MAPK) involved in anoikis-resistance - elucidating these molecules would provide a more detailed mechanistic cascade of LTF's action. Third, we did not validate our findings in clinical samples beyond referencing existing literature [16, 17]; analyzing LTF expression and TrkB/AKT activation in a larger cohort of NPC patient tissues would strengthen the clinical relevance of the LTF/TrkB/AKT axis as a potential prognostic marker or therapeutic target. Fourth, we did not examine the difference in endogenous LTF expression between parental adherent cells and anoikis-resistant suspended cells. This data gap prevents us from clarifying whether the acquisition of anoikis-resistance is accompanied by downregulation of endogenous LTF - a key question that

would directly link LTF deficiency to the development of metastatic traits in NPC. Without this comparison, the physiological relevance of LTF's regulatory role in anoikis-resistance remains partially unresolved.

To address these limitations, future studies will focus on three key areas: (1) Establishing NPC metastasis models in nude mice to evaluate LTF's in vivo effect on anoikis-resistance and distant metastasis; (2) Expanding experiments to additional NPC cell lines (e.g., 5-8F, CNE2) and patient-derived organoids to improve generalizability, given NPC's inherent heterogeneity; (3) Investigating the interaction between LTF and downstream AKT effectors (e.g., FOXO3a) or other pathways (e.g., TGF-β) to clarify the full molecular network regulating anoikis-resistance; (4) Detecting endogenous LTF expression in adherent vs. anoikis-resistant suspended NPC cells via Western blot and qPCR to determine whether anoikis-resistance acquisition is associated with reduced LTF expression. This will verify if endogenous LTF downregulation is a driving factor for anoikis-resistance, strengthening the causal link between LTF deficiency and NPC metastasis. Additionally, clinical studies will explore whether LTF expression correlates with TrkB/AKT activation and patient outcomes (e.g., metastasis-free survival) to validate LTF as a prognostic biomarker.

In summary, our study demonstrates that LTF inhibits anoikis-resistance and metastasis of NPC cells by downregulating the TrkB/AKT signaling pathway. Compared to prior work, we: (1) Link LTF to NPC anoikis-resistance (a critical metastatic step ignored in previous LTF/NPC studies); (2) Identify TrkB as a key upstream target of LTF in AKT inhibition; (3) Reveal a tissue-specific regulatory axis for LTF in NPC. These findings advance the understanding of LTF's role in tumor metastasis and highlight LTF as a potential therapeutic target to overcome anoikis-resistance in NPC.

Conclusion

Our study demonstrates that LTF inhibits anoikis-resistance and metastasis of NPC cells by downregulating the TrkB/AKT signaling pathway. Key findings include: (1) LTF modulates the biological behaviors of anoikis-resistant NPC cells - overexpression reduces cell survival, restricts invasive capacity, arrests the cell cycle at G0/G1 phase, and promotes apoptosis, while LTF knockdown has the opposite effects; (2) Mechanistically, LTF targets the TrkB/AKT axis to suppress epithelial-mesenchymal transition (EMT) and reduce the expression of metastasis-related markers (VEGF-A, MMP-9), which are critical for sustaining anoikis-resistance; (3) This work fills a gap in prior research: while LTF was previously linked to AKT inhibition in NPC, we are the first to connect this pathway to anoikis-resistance - a rate-limiting step in NPC metastasis. Clinically, our results highlight LTF as a potential therapeutic target to overcome anoikis-resistance and reduce NPC metastasis, offering a novel direction to improve treatment efficacy and patient outcomes for advanced NPC.

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Disclosure of conflict of interest

None.

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