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Original Article

Lactoferrin mediates epithelial-mesenchymal transformation by regulating the PI3K/AKT/mTOR pathway to inhibit nasopharyngeal carcinoma metastasis

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Abstract

Nasopharyngeal carcinoma (NPC) is a common malignant tumor of the head and neck. Epithelial-mesenchymal transition (EMT) is a major player in regulating NPC transfer. There is increasing evidence that lactotransferrin (LTF) is an important regulator of EMT conversion. However, the potential role and mechanisms of LTF in regulating NPC cell EMT remain unclear. In this study, quantitative real-time PCR (qRT–PCR) and Western blotting were applied to measure the expression of LTF in NPC cells. Subsequently, the influences of LTF on the proliferation, migration and invasion of NPC cells were verified by functional acquisition experiments. Finally, Western blotting was used to analyze the effects of EMT-related proteins and phosphoinositol 3-kinase (PI3K)/Akt/mammalian rapamycin target (mTOR) signaling pathways. The data of this study indicate that LTF was underexpressed in human NPC cells, and upregulation of LTF could restrain NPC cell proliferation, invasion, migration and EMT transformation. Moreover, the effects of LTF on NPC cell metastasis and EMT are partly determined by the PI3K/AKT/mTOR pathway. This study suggests that LTF is a potential biomarker of NPC and that LTF-mediated EMT progression plays a tumor-suppressive role in the progression of NPC metastasis.

Keywords: NPC, Lactoferrin, PI3K/AKT/mTOR, EMT, Metastasis.

1. Introduction

NPC is a highly aggressive squamous cell carcinoma of the head and neck originating from the nasopharyngeal mucosa epithelium. It is the most prevalent malignant tumor of the head and neck in South China and Southeast Asia, with unequal ethnic and geographical distributions [1, 2]. Current studies have demonstrated that the pathogenesis of NPC is related to Epstein–Barr virus (EBV) latent infection, regional environmental factors, genetic susceptibility and genomic instability. Statistically, 133,354 newly diagnosed cases of NPC and 80,008 deaths were reported in 2020, indicating that its morbidity and mortality rates remain high [3]. Although with the progress of radiotherapy and chemotherapy, the susceptibility and local control effect of radiotherapy are enhanced, the effectiveness of these treatments still needs to be further improved, especially the distant metastasis rate and high local recurrence rate [4, 5]. Due to the limitations of NPC and the concealment of symptoms and their aggressiveness, most NPC patients are diagnosed with advanced local-regional disease with distant metastases [6]. Locally extended tumor invasiveness and distant metastasis are associated with poor prognosis and death of NPC and are still the main reasons for patient recurrence and metastasis [5]. Therefore, a comprehensive awareness of the nosogenesis of NPC progression and metastasis may help design more effective and targeted treatment strategies.

EMT is an important cell biological phenomenon that refers to the transformation process of epithelial cells into mesenchymal cells [7]. In oncology, EMT is one of the important mechanisms by which tumor cells metastasize from site to distance. In this process, tumor cells gain stronger motor ability and better adaptability through EMT, thus escaping the attack of immune recognition and chemotherapy drugs and eventually reaching distant tissues and organs to form distant metastases [7]. Increasing evidence shows that the transformation of EMT is adjusted by PI3K/AKT/mTOR signaling [8, 9]. Studies have shown that continuous stimulation of the PI3K/AKT/mTOR pathway can lead to a metastatic phenotype of cancer [10]. Recent clinical studies have indicated that nasopharyngeal carcinoma patients with mutations in the PI3K/Akt/mTOR signaling pathway have poor prognosis [11]. In addition, preclinical and animal studies have also shown that the

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malignant progression of NPC can be weakened by targeted regulation of biomarkers related to the PI3K/AKT/mTOR pathway [12, 13]. Consequently, it is imperative to seek tumor biomarkers that simultaneously target EMT and PI3K/AKT/mTOR signaling to control the metastatic progression of NPC.

LTF is an 80 kDa iron-bound glycoprotein found primarily in external secretions, such as breast milk. LTF has many biological properties, including antiviral, anti-inflammatory, antibacterial and antitumor activities [14]. Among them, the tumor suppressive function of LTF has been reported in several tumors, including glioblastoma [15], NPC [16], and prostate cancer [17]. Studies have shown that LTF reduces the growth and metastasis of colorectal cancer [18] and breast cancer [19]. In addition, LTF overexpression has been proven to inhibit the migration ability of renal clear cell cancer cells with high metastasis potential and is negatively correlated with the progression of EMT [20]. It is noteworthy that in clinical studies, LTF expression is downregulated in NPC clinical specimens and negatively relevant to tumor progression and prognosis in NPC patients [21]. In addition, LTF can inhibit NPC cell proliferation and induce apoptosis and is associated with the inhibition of the MAPK and AKT signaling pathways [22, 23].

Based on the above background, we hypothesized that LTF inhibits NPC transfer by mediating EMT transformation through the regulation of the PI3K/AKT/mTOR pathway. In this article, we aimed to investigate the effect and mechanism of LTF-mediated EMT transformation on the malignant behavior of NPC cells. The results may provide a promising perspective on EMT in NPC progression and, more importantly, may also provide a new theoretical basis for targeted therapy of NPC.

2. Materials and Methods

2.1. Cell culture and transfection

The human NPC cell line (HONE-1) and non-NPC cell line (NP-69) were obtained from the Chinese Academy of Sciences (CAS). Cell culture was performed according to the instructions provided by the CAS. All cells were validated using short tandem repeat analysis and were negative for mycoplasma contamination.

Human source PCDNA3.1-LTF overexpression and pcDNA3.1 vector were purchased from Suzhou Jima Gene Co., LTD. The cDNA of LTF1 was amplified and subcloned and inserted into the pcDNA3.1 vector, and the final construct was verified by sequencing for overexpression of LTF. An empty pcDNA3.1 vector was applied as a control. Then, the above plasmids were transfected into the cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) when the cell confluence reached 70% to 80%.

2.2. qRT–PCR

RNA extraction was carried out using TRIzol (Invitrogen, Carlsbad, CA, USA), and the concentration of RNA was determined. Reverse transcription was performed using a HiScript II first-strand cDNA synthesis kit (Vazyme, Nanjing, China). Then, real-time PCR analysis of LTF gene expression was performed on a CFX96 Touch 1855195 real-time fluorescence quantitative PCR instrument, and GAPDH was selected as the internal control. Gene expression was normalized by $2^{-\Delta\Delta}$Ct. All primers used in this study are listed in Table 1.

2.3. Western blot (WB)

Cells in each group were collected, cell lysate containing protease inhibitor (Beyotime, Shanghai, China) was added for protein extraction, and the total protein concentration was determined by the BCA method. Previously, biomarker samples or intracellular total protein extracts were electrophoretically isolated using 10% sulfate polyacrylamide gel (NCM Biotech) and then transferred to a polyvinylidene fluoride membrane.

After blocking with 5% skim milk powder, a 1:1000 dilution of primary antibody was added for incubation. After that, the membrane containing protein bands was incubated with HRP polymerized secondary antibody (bs-0296G-HRP) at room temperature for 2 hours. ECL luminescent solution (Thermo Fisher Scientific, Waltham, MA, USA) was used for chemiluminescence, pictures were taken under a JP-K6000 chemiluminescence imager, and images were obtained and analyzed by ImageJ software. All primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). These included mouse anti-LTF monoclonal antibody (abs100035), rabbit anti-N-cadherin polyclonal antibody (abs 131133), E-cadherin antibody (abs130068), mouse anti-vimentin monoclonal antibody (abs149750) (Absin, China), phospho-PI3 kinase p85 (Tyr458)/p55 (Tyr199) (E3U1H) rabbit mAb (17366), PI3 kinase p85 (19H8) rabbit mAb (4257), phospho-Akt (Ser473) (D9E) XP® rabbit mAb (4060), Akt (pan) (C67E7) rabbit mAb (4691), phospho-mTOR (Ser2448) (D9C2) XP® rabbit mAb (5536), mTOR (7C10) rabbit mAb (2983), and β-actin-loading control (bsm-33033 M).

2.4. CCK-8

The HONE1 cells were transferred to plates at a density of 2000 cells per well and incubated for 4-8 hours. After transfection, the cells were incubated for 24, 48, 72 and 96 hours. Two hours before the end of incubation, 10 μL CCK8 (Bioss, Woburn, MA, USA) reagent was added to each well. Finally, the optical density was recorded at 450 nm using a microplate reader.

2.5. Scratch test

HONE1 cells were inoculated into the plate at 1×10^6 cells per well and transfected according to the experimental groups when the cells were approximately 80% confluent. On the second day, the sterile pipette tip was pressed vertically against the line marked on the back of the board to create a scratch. After the cells were rinsed with sterile PBS, images were obtained under an inverted microscope, and cell scratch broadband (migration distance) was recorded at 0 h and 24 h.

2.6. Transwell migration and invasion assays

Cells (2×10^4) were transfected with the plasmid using an 8-μm pore filter for 48 h and then coated with or without

Table 1. Primer sequences.

Genes	Primer sequences (5'-3')
LTF	F:5'-TGCAGGCAAATGTGGTTTGG-3'
	R: 5'-CTGATCTCCTAACCACCGCC-3'
GAPDH	F: 5'-GGTCTCCTCTGACTTCAACA-3'
	R: 5'-GTGAGGGTCTCTCTCTTCCT-3'

Note: F, forward; R, reverse.

50 μL Matrigel (Corning, Corning, NY, USA) in serum-free medium. DMEM containing 10% FBS was added as an attractant. Migration after incubation for 12 hours and invasion after 48 hours: The cells that did not migrate or invade were gently removed, and the cells that migrated to the bottom of the membrane were fixed with 4% paraformaldehyde. Ultimately, dye with crystal violet solution for 30 minutes and observe under 100× microscope.

2.7. Statistical analysis

Data were analyzed and plotted using GraphPad Prism 9 (Version 9.5.0, La Jolla, CA, USA). Photoshop was used to organize the image. All maps are presented as the means ± SDs, and the significant difference between groups was tested by one-way test. A P value less than 0.05 was considered significant (* P<0.05, ** P<0.01, *** P<0.001).

3. Results

3.1. Reduced LTF was present in NPC cells

To clarify the expression pattern of LTF in NPC, we detected the expression of LTF in NPC cells (HONE1) and a non-NPC cell line (NP69) via qRT–PCR and WB. We found that LTF expression was lower in HONE1 cells than in NP69 cells (P<0.01) (Figure 1A/B). Our data illustrated that LTF might be a tumor suppressor in NPC.

3.2. LTF participates in the adjustment of NPC cell proliferation and metastasis

To investigate whether LTF participates in the regulation of NPC cell progression, we transfected an LTF-targeting pcDNA 3.1 overexpression vector into HONE1 cells. Figure 2A confirmed that LTF was overexpressed in HONE1 cells. Subsequently, cell viability was evaluated by the CCK-8 method. As manifested in Figure 2B, compared with the pcDNA 3.1 group, NPC cell viability in the overexpressed group was observably reduced at 72 and 96 hours (P<0.001). Subsequently, HONE1 cell migration was analyzed by scratch and Transwell tests. As shown in Figure 2C/D, HONE1 cell mobility was depressed after LTF overexpression vs. the pcDNA 3.1 group (P<0.01), and the number of migrating cells was depressed dramatically (P<0.001). Next, the invasion of HONE1 cells was analyzed by Transwell assay. As revealed in Figure 2E, compared with the pcDNA 3.1 group, the number of invasive HONE1 cells after LTF overexpression was signally reduced (P<0.01). These data suggested that upregulation of LTF hindered the viability, migration and invasion of NPC cells, confirming that LTF is an important factor regulating NPC metastasis.

3.3. The influences of LTF on NPC cell metastasis and EMT are partly determined by the PI3K/AKT/mTOR pathway

The PI3K/Akt/mTOR pathway is one of the main downstream signal transduction pathways of LTF, and LTF exerts anticancer effects in various cancers by influencing the PI3K/Akt/mTOR pathway [24, 25]. Thus, it is reasonable to speculate that the change in NPC cell phenotype caused by LTF may be due to the regulation of the PI3K/AKT/mTOR pathway. To find evidence to support this hypothesis, we incubated pcDNA 3.1-LTF-transfected NPC cells with the PI3K agonist 740Y-P and detected the expression and phosphorylation of proteins associated with the PI3K/AKT/mTOR pathway via WB. As shown in Figure 3A-G, compared with the pcDNA3.1 group, upregulation of LTF in HONE1 cells inhibited the expression of p-PI3K, p-Akt and p-mTOR (P<0.05), and 740y-P treatment reversed the inhibition of LTF overexpression, suggesting that upregulation of LTF inhibits the PI3K/AKT/mTOR pathway in NPC cells. Subsequently, we further measured the levels of pro-EMT-related markers (N-cadherin, Vimentin) and epithelial markers (E-cadhe-

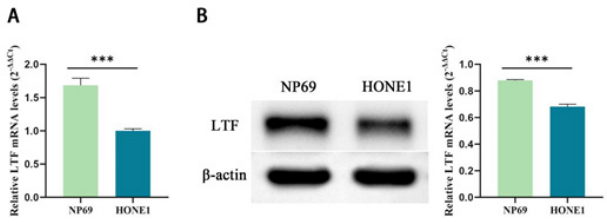

Fig. 1. Reduced LTF was present in NPC cells. (A/B) qRT–PCR and WB were used to evaluate the expression of LTF in HONE1 and NP69 cells. **P < 0.01, ***P < 0.001 vs. HONE1, N = 3.

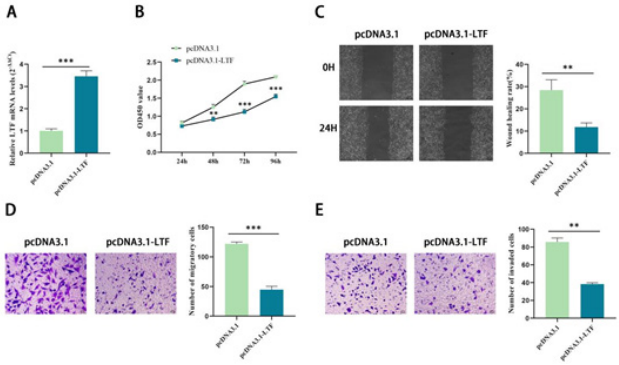

Fig. 2. LTF was involved in the regulation of NPC cell proliferation and metastasis. The LTF-targeting pcDNA 3.1 overexpression vector and control vector were transfected into HONE1 cells. (A) qRT–PCR test of the transfection efficiency of LTF in HONE1 cells. (B) CCK-8 detection of cell proliferation activity. (C/D) Scratch test of cell migration; (E) Transwell test of cell migration and invasion. **P < 0.01, ***P < 0.001 vs. pcDNA3.1, N = 3.

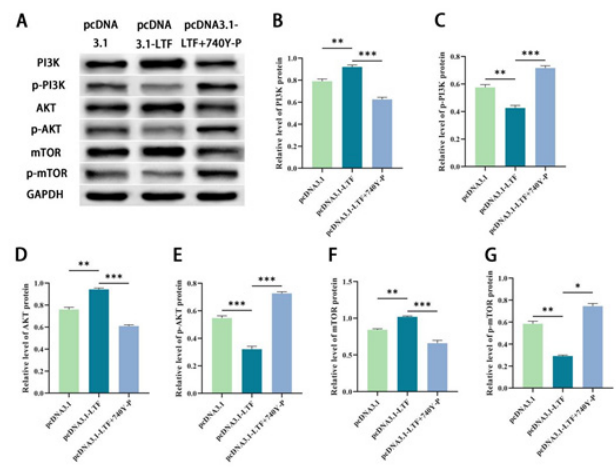

Fig. 3. LTF inactivated the PI3K/AKT/mTOR pathway in NPC cells. NPC cells transfected with pcDNA 3.1-LTF were incubated with the PI3K agonist 740Y-P. (A-G) WB analysis of PI3K/AKT/mTOR axis phosphorylation levels (p-PI3K/PI3K, p-AKT/AKT, p-mTOR/mTOR) in HONE1 cells. **P < 0.01, ***P < 0.001 vs. pcDNA3.1, N = 3. *P < 0.05, ***P < 0.001 vs. pcDNA3.1-LTF, N = 3.

rin). The results are manifested in Figure 4A-D. Compared with the pcDNA3.1 group, the levels of N-cadherin and vimentin in HONE1 cells in the overexpressed LTF group were decreased, while the level of E-cadherin was increased (P<0.01). However, 740y-P treatment dramatically reversed the inhibition of LTF overexpression on the above aspects. The above data illuminated that the influences of LTF on NPC cell metastasis and EMT are determined by the PI3K/AKT/mTOR pathway.

4. Discussion

Local recurrence and distant metastasis remained major obstacles to the clinical treatment of NPC. EMT has become a key process of cell invasion and metastasis in most epithelial tumors (including NPC) [24, 25]. Recent studies have shown that a large number of genetic or epigenomic changes and genetic instability jointly control NPC occurrence, which is a complex process involving the interaction regulation of numerous molecular networks [26, 27]. Therefore, in this study, we sought to find new tumor suppressor genes and biomarkers with a view to providing prospective strategies for the treatment of NPC metastasis and recurrence. Here, we showed for the first time that LTF, as a potentially useful target gene for NPC tumor therapy, directly affected the proliferation and metastasis of NPC cells by altering EMT. In addition, the link between LTF and downstream pathways has been clearly demonstrated.

LTF is a well-conserved monomer 80 kDa single polypeptide-chain glycoprotein containing approximately 690 amino acid residues that was originally described as an iron-binding molecule with antimicrobial properties and is now known to be a multifunctional protein [28, 29]. Emerging research suggested that the high bioavailability of LTF and its high selectivity for controlling tumor proliferation and metastasis made LTF a promising biomarker and therapeutic target in several cancers [30]. For example, the expression of LTF in breast cancer was associated with poor prognosis in patients and the clinical physiology of the disease [31] and restrained the invasive process of breast cancer [32]. Besides, LTF attenuated the growth and metastasis phenotypes of several cancers [20, 33]. This evidence suggests that LTF may well become a target for NPC. Here, we demonstrated that LTF was downregulated in NPC cells. Moreover, we observed that the upregulation of LTF restrained NPC cell proliferation, migration, and invasion. These results confirmed that LTF acts as a tumor suppressor in NPC.

It is well known that the activation of EMT is the main cause of NPC transfer [34, 35]. Researches show that, abnormal EMT activation increased mesenchymal signatures and reduced epithelial signatures, promoting cancer cell initiation, invasion, metastasis and chemotherapy resistance, thereby enabling tumor cells to spread throughout the body. Recently, many studies have found that transcription factors inhibit the EMT process at the molecular level [36, 37]. Importantly, previous studies have found that LTF reverses EMT transformation in several cancers [20, 38]. Similarly, in our research, we found that upregulation of LTF observably altered the expression of EMT markers; that is, the expression of E-cadherin increased while that of N-cadherin and Vimentin decreased. These results illuminated that LTF was an important contributor to the progression of EMT, inhibiting the migration and invasion of NPC cells by regulating EMT transformation.

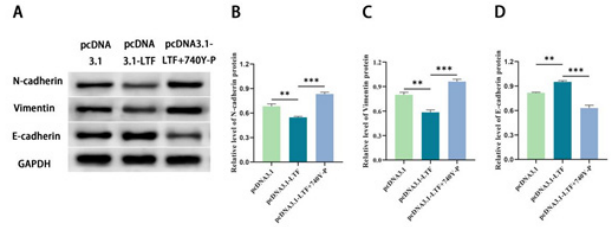

Fig. 4. The effects of LTF on NPC cell metastasis and EMT were partly determined by the PI3K/AKT/mTOR pathway. NPC cells transfected with pcDNA 3.1-LTF were incubated with the PI3K agonist 740Y-P. (A-D) WB analysis of N-cadherin-, Vimentin-, and E-cadherin-related proteins in HONE1 cells. **P < 0.01 vs. pcDNA3.1, N = 3. ***P < 0.01 vs. pcDNA3.1-LTF, N = 3.

The PI3K/AKT/mTOR pathway is known to play a crucial role in many biological processes [11, 39]. Extensive evidence suggests that activation of the PI3K/AKT/mTOR pathway is a strong EMT-inducing mechanism in NPC. For example, Liu et al. reported that APLNR hindered EMT processes in NPC via PI3K/AKT/mTOR signaling [8]. Cheng et al. reported that Tenascin-C promoted tumor cell proliferation and EMT activity by activating the PI3K/AKT/mTOR signaling pathway in NPC cells, thus playing a role as an oncogene [40]. Emerging evidence suggests that the LTF protein plays a significant role in the adjustment of the PI3K/AKT/mTOR pathway [16, 24]. However, until now, the role of LTF in EMT in NPCS has been unclear. More importantly, no studies have focused on the EMT process mediated by LTF and PI3K/AKT/mTOR signaling. In this study, WB analysis illuminated that NPC cells significantly depressed the activation of the PI3K/AKT/mTOR pathway after upregulation of LTF. Importantly, treatment with the PI3K agonist 740y-P reversed the inhibitory effect of LTF overexpression on EMT transformation. These results verified that the underlying mechanism by which LTF inhibited EMT transformation was at least partially mediated by LTF regulation of the PI3K/AKT/mTOR pathway.

However, there were some limitations to our findings. For example, the function of LTF in accelerate the NPC cell malignant phenotype has only been studied *in vitro*, and *in vivo* studies are also critical for future studies. Second, due to the variety of targets for LTF, we could not rule out the possibility that LTF was involved in the regulation of other transcription factors and signaling pathways.

5. Conclusion

In summary, our study verified that LTF is a novel oncogenic factor in NPC that influences NPC progression and EMT by regulating the PI3K/Akt/mTOR pathway. LTF inhibited NPC cell metastasis and EMT, and its mechanism had something to do with the inhibition of PI3K/Akt/mTOR axis. These findings provide a promising idea for the diagnosis and therapy of NPC.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed consent
The authors declare not used any patients in this research.

Data availability
The data are available from the corresponding author upon reasonable request.

Authors' contributions
Min Xu, Ye Fan: Conceptualization, methodology, writing original draft preparation. Guoying Zou, Qi Yang: Investigation, software, statistical analysis. Fei Xu: Reviewing and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

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