Silencing of long non-coding RNA TUC338 inhibits the malignant phenotype of nasopharyngeal cancer cells via modulating the miR-1226-3p/FGF2 axis

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Abstract
Long noncoding RNAs (lncRNAs) have been suggested as essential regulators in the cancer progression. LncRNA TUC338 was found to promote the malignancy of various cancers, however, the involvement of TUC338 in nasopharyngeal cancer (NPC) has not been well characterized. Here, our results found the significant overexpression of TUC338 in NPC tissues. Higher level of TUC338 was also observed in NPC cells. Interestingly, NPC patients harboring overexpressed TUC338 have worse prognosis. Functional study indicated that down-regulated TUC338 remarkably suppressed the NPC cell proliferation and cell migration. Notably, depletion of TUC338 significantly inhibited the in vivo tumor growth. Mechanistically, TUC338 acted as molecular sponge of miR-1226-3p and attenuated the negative regulation of miR-1226-3p on the expression of fibroblast growth factor 2 (FGF2). Down-regulation of TUC338 inhibited FGF2 expression in NPC cells and tumor tissues. Overexpression of FGF2 attenuated the suppressed NPC proliferation upon the depletion of TUC338. Our results demonstrated the novel function of TUC338/miR-1226-3p/FGF2 axis in NPC progression, suggesting the potential diagnosis and therapeutics significance of TUC338 in NPC.

Keywords TUC338 · miR-1226-3p · FGF2 · NPC

1 Introduction
Nasopharyngeal cancer (NPC) is a most frequently diagnosed head and neck cancer mainly occurs in Southeast Asia, especially in China [1]. Currently, the main therapeutic strategies that surgery combined with chemotherapy or radiotherapy have substantially benefited the prognosis of patients [2]. However, NPC usually develops distant metastasis and progresses into poor outcomes. Therefore, identifying key factors involved in the progression of NPC is necessary to benefit the diagnosis and treatment of NPC.

Increasing evidence has found that non-coding RNAs (ncRNAs) act importantly in the initiation and development of cancers [3–6]. MicroRNA (miRNAs) and lncRNAs are well defined two major classes of ncRNAs [7]. Specifically, lncRNAs are >200 nucleotides (nt) in length and take up more than 98 percentage of transcriptomes [8]. Dysregulation of lncRNAs is closely involved in the oncogenesis and development of cancers [9]. As a big part of ncRNAs, miRNAs, with the length of approximately 22 nt, are dysregulated and modulate the tumorigenesis [10–12]. Mechanistically, miRNAs triggered the target mRNA degradation or translation defects through binding the mRNA’s 3′-untranslated region (UTR) [13, 14].
Generally, one of the functional mechanisms of lncRNA is to physically bind and sequester miRNA to attenuate its suppressive effect on targeted mRNAs. These lncRNAs are also named as competitive endogenous RNAs (ceRNAs) [15]. The cancer-promoting effects of LncRNA TUC338 have been identified in liver cancer and tongue squamous cell carcinoma [16, 17]. Highly expressed TUC338 promoted cancer cell proliferative and invasive capacities [18–21]. Nevertheless, the reports about the function of TUC338 in NPC is not available.

Fibroblast growth factor (FGF2) is a prototypic growth factor belonging to the FGF family [22, 23]. Recent study demonstrated that FGF2 is a type of proto-oncogene that is expressed higher in many types of malignancies [24]. Overexpressed FGF2 in NSCLC promoted the progression of and was correlated with the poorer survival of NSCLC patients [25]. Regulatory mechanism by which FGF2 activity is regulated in NPC remains to be revealed.

In this study, TUC338 was up-regulated in NPC and associated with the advanced progression of NPC patients. Mechanistically, TUC338 regulated FGF2 expression by sponging miR-1226-3p and modulated the NPC pathogenesis. Our results shed light on the promising therapeutic significance of TUC338/miR-1226-3p/FGF2 axis in NPC.

# 2 Materials and methods

## 2.1 Tissues

A cohort of clinical samples including 50 pairs of NPC tissues and their paired adjacent non-cancerous tissues were collected from NPC patients at the Cangzhou Central Hospital between January 2010 and August 2011. Participants were not undergone any treatments prior to the tissue collection. Tissues were stored in liquid nitrogen. The experimental procedures were approved by the Ethics Committee of Cangzhou Central Hospital (Approval Number: ECCCH20100892). Patients provided written informed consents.

## 2.2 Cell culture

CNE-1, HONE-1, SUNE-1, 5-8F cells, human nasopharyngeal epithelial NP69 cells were all purchased from ATCC (Manassas, VA, USA). The culture condition of the cells was RPMI-1640 medium plus 10% fetal bovine serum (FBS, Invitrogen, Shanghai, China) and 1% streptomycin/penicillin (Invitrogen, South Logan, UT, USA) at 37 °C with 5% CO₂. NP69 cells were maintained in keratinocyte/serum-free medium (Invitrogen, Shanghai, China) with bovine pituitary extract (Absin Bioscience Co., Ltd., Shanghai, China). All cells were incubated in a humidified CO₂ (5%) incubator at 37 °C.

## 2.3 quantitative PCR

Total RNA from cells or tissues was generated into cDNA via reverse transcription with PrimeScript Reverse Transcriptase (RT) kit (Thermo Fisher Scientific, Inc.). qPCR was performed to quantify the levels of TUC338 and miR-1226-3p with the SYBR Master Mix (Bio-Rad, USA). The levels of TUC338 or miR-1226-3p was normalized to that of GAPDH or U6 RNA, respectively. Primers used were TUC338 forward, 5'-GCA GCG ACA GTG CGA GCT, reverse, 5'-TCC GAG TGA GTT AGG AAG; GAPDH forward, 5'-GGT CTC CTC TGA CTT CAA CA, reverse, 5'-GTG AGG GTC TCT CTC TTC CT; miR-1226-3p forward, 5'-GGGCTCACAGGCCACAAAGAGCACAA; FGF2 forward, 5'-ACTGGGCTTCTAATGTGTTACG, reverse, 5'-TTG GATCCAGTTTGACTGCC.

## 2.4 In vivo xenograft mice model

4–5 weeks of BALB/c mice (female) were obtained for Charles River Laboratories and housed at SPF conditions. 200 μl of NPC cells (1 × 10⁶) with lentivirus expressed siRNA-TUC338 or siRNA-control were subcutaneously administrated into the flanks of mice. Tumors size was measured with the caliper at the interval of 5 days. After the tumor implantation of 30 days, cervical dislocation was applied to euthanize the mice and tumors were collected. The tumor length (a) and width (b) was measure. The tumor volume (V/mm³) was calculated with the formula $V = ab^2/2$. This experiment was approved in accordance with the regulations of Committee the Cangzhou Central Hospital Experimental Animal Use and Care. The maximal tumour size/burden permitted by the ethics was no more than 2000 mm³. The maximal tumour size was not exceeded 2000 mm³ in this study.
2.5 Western blot

Equal amount of protein extracted form NPC cells was separated through running SDS-PAGE and semi-dry transferred onto the 0.22 μm nitrocellulose membrane (Real-Times Biotechnology Co., Ltd., Beijing, China). The membrane was firstly pre-sealed with 5% skim milk followed by incubating with specific primary antibody against FGF2 (1:1500 dilution; #ab92337, Abcam, USA) or GAPDH (1:2000 dilution; #ab181602; Abcam, USA) overnight at 4 °C. The signals were further developed using IRdye 800-conjugated anti-IgG second antibody (1:3000 dilution; Invitrogen, USA) for 1 h at RT and detected with the ECL Western Blotting Substrate (Pierce, Thermo Fisher Scientific, Inc).

2.6 Dual luciferase reporter assay

The TUC338 fragment carrying the predicted miR-1226-3p seeding sites were PCR-amplified and constructed into the pmirGLO plasmid. pmirGLO-TUC338 and miR-1226-3p or control miRNA were co-transfected into the NPC cells. The luciferase activity was examined after 48 h of transfection using the Dual-Luciferase Assay Kit (Promega, Madison, WI, USA).

2.7 Cell proliferation

Control-siRNA or siRNA-TUC338 were transfected into NPC cells and cell proliferation was determined by the Cell Counting Kit-8 (CCK-8) assay (Quanxinquanyi Biotech, Shanghai, China) following the protocol of the manufacturer. Specifically, NPC cells were seeded into the 96-well plate (1000 cells/well). After incubating with 10 μl of CCK-8 for 3 h, the cell proliferation was measured using the microplate reader at the absorbance of 450 nm.

Fig. 1 TUC338 was overexpressed in NPC. A Total RNA was extracted from the NPC tissues and adjacent tissues. The TUC338 expression was determined by RT-qPCR. B The compassion for the levels of TUC338 in NPC cell line CNE-1, SUNE-1, 5-8F and HONE-1, and the human immortalized nasopharyngeal epithelial cell lines NP69. C The correlation between the expression of TUC338 and the 5-year OS of NPC patients.
2.8 Cell cycle analysis

NPC cells were cultured in a 6-well plate and transfected with control-siRNA or TUC338-siRNA. After 36 h of the transfection, the cell cycle profile was examined by staining with propidium iodide (PI; Solarbio, Beijing, China). Briefly, the fixation with 75% ethanol was performed overnight at 4 °C. Cells were then subjected to RNase digestion for 15 min at RT and stained by PI for 20 min avoiding light. After filtering with 200-mesh membrane, the cell cycle of NPC cells was analyzed with the flow cytometry (Beckman Coulter, Epics XL).

2.9 Ago2-immunoprecipitation (IP) and pull-down assay

The cell lysates were incubated with Argonaute 2 (Ago 2) antibody at 4 °C overnight. Protein G beads were added to couple the antibody. The IP complex was treated with proteases K followed by RNA extraction. The enrichment of TUC338 was detected by RT-qPCR. For the pull-down assay, the antisense oligonucleotides that recognizing TUC338 or LacZ were conjugated to biotin and incubated with the CNE-1 or S-8F cell lysates. The biotinylated components were further captured by streptavidin beads (Invitrogen, Shanghai, China). RNA was extracted from the pull-down components and detected by RT-qPCR analysis using the primers against miR-1226-3p.

2.10 Statistical analysis

SPSS version 19.0 was used for the statistical analysis. Data was presented as the mean ± standard deviation. Difference between two groups was analyzed with Student’s t-test. Multi-sample comparison was performed with One-way ANOVA. Statistical significance was defined when \( p < 0.05 \). \(* p > 0.05\), \(** p > 0.01\), \(* * * p > 0.001\).

3 Results

3.1 TUC338 was overexpressed in NPC

To investigate whether TUC338 was involved in NPC progression, TUC338 expression in NPC tissues and matched non-cancer tissues was detected. As confirmed by the data of RT-qPCR, TUC338 was significantly overexpressed in NPC using the normal tissues as control (Fig. 1A). Meanwhile, TUC338 expression in NPC cell lines and normal cells was also compared. The data indicated the relative higher level of TUC338 in NPC cells (Fig. 1B). To deeply evaluate the clinical value of TUC338, those 50 patients were divided into TUC338-low and high expression groups based on the mean expression value of TUC338. Correlation analysis showed that patients with poorer survival had higher level of TUC338 (Fig. 1C). These findings suggested the potential clinical significance of TUC338 for the prognosis of NPC patients.

3.2 Depletion of TUC338 inhibited the malignant behaviors of NPC cells

As TUC338 was aberrantly expressed in NPC, to reveal the role of TUC338 in NPC, both CNE-1 and S-8F cells were transfected with siRNA-TUC338 or siRNA-control, and the knock down efficiency of TUC338 was confirmed as indicated in Fig. 2A. The data of CCK-8 assay demonstrated that depletion of TUC338 significantly slowed the NPC cell proliferation (Fig. 2B and C). The effects of TUC338 on the growth of NPC cells were also validated by analyzing the cell cycle progression with the down-regulation of TUC338. Depletion of TUC338 significantly induced the accumulation of cells in G1 phase and reduction in S phase (Fig. 2D), suggesting G1 cell cycle arrest with knockdown of TUC338. Consistently, down-regulation of TUC338 obviously increased the apoptotic percentage of both CNE-1 and S-8F cells (Fig. 2E). Additionally, the effects of TUC338 on NPC cell migration was also determined via the transwell assay, which showed that depletion of TUC338 significantly inhibited the migration of NPC cells (Fig. 2F). Collectively, all these results demonstrated that TUC338 was required for the malignant phenotype of NPC cells.
Fig. 2 Depleted TUC338 slowed down NPC cell growth. A The depletion of TUC338 in CNE-1 and 5-8F cells was validated by RT-qPCR. B, C CCK-8 assay was done to investigate the proliferation of CNE-1 and 5-8F cells following transfection of control-siRNA or siRNA-TUC338. D Down-regulation of TUC338 in CNE-1 and 5-8F cells induced cell cycle arrest in G1 phase. E Depletion of TUC338 increased the apoptosis of both CNE-1 and 5-8F cells. F Knockdown of TUC338 inhibited NPC cell migration. Scale bar, 50 μm
3.3 Down-regulation of TUC338 inhibited in vivo NPC tumor growth

To confirm the function of TUC338 in tumorigenicity in vivo, xenograft mice model was established by subcutaneously injecting both CNE-1 and 5-8F cells, with stably expressed siRNA-TUC338 or siRNA-control. After 30 days, mice were sacrificed to harvest the tumors and the tumor weight was measured. The knockdown efficiency of TUC338 in tumors was confirmed by RT-qPCR (Fig. 3A). Significantly reduced tumor volume and weight were observed with TUC338 knocked down (Fig. 3B–D). These results demonstrated the repressed in vivo tumor formation with down-regulation of TUC338.

3.4 TUC338 acted as a competing endogenous RNA (ceRNA) to sponge miR-1226-3p

Based on the theory of ceRNA, to further understand the molecular mechanism of TUC338 in NPC, the binding between TUC338 and miRNAs were predicted with the miRDB database. According to the bioinformatics analysis, TUC338 might be a molecular sponge of miR-1226-3p among all the candidates. The putative binding sequence between TUC338 and miR-1226-3p was presented as Fig. 4A. To confirm this, the data of luciferase assay suggested that the luciferase intensity of NPC cells expressing WT-TUC338 was significantly decreased with miR-1226-3p overexpression (Fig. 4B and C). However, transfection of miR-1226-3p did not affect the luciferase activity of cells expressing mutated TUC338 that disrupted the binding with miR-1226-3p (Fig. 4B and C). Moreover, the Ago2-IP assay showed the significant enrichment of TUC338 in Ago2-IP compared to control IgG-IP (Fig. 4D). RNA pull-down assay revealed that TUC338 was specifically enriched in pull-down components using TUC338 antisense oligonucleotides but not with LacZ (Fig. 4E). miR-1226-3p was selectively enriched in TUC338 pull down assay (Fig. 4E). These findings indicated the specific physical interaction of TUC338 with miR-1226-3p. To detect whether the interaction of TUC338 affected miR-1226-3p expression, the levels of miR-1226-3p in both 5-8F and CNE-1 cells were detected. The RT-qPCR analysis showed that TUC338 overexpression
significantly decreased the expression of miR-1226-3p (Fig. 4F). Consistently, down-regulated TUC338 increased miR-1226-3p expression in NPC cells (Fig. 4G). As TUC338 was overexpressed in NPC tissues, the abundance of miR-1226-3p was also detected. Compared with the non-cancer samples, miR-1226-3p was remarkably down-regulated in NPC tissues.
**Fig. 5** MiR-1226-3p targeted FGF2 in NPC cells. 

- **A** The overexpression of miR-1226-3p after transfection was examined by RT-qPCR. 
- **B, C** Overexpression of miR-1226-3p inhibited CNE-1 and 5-8F cell proliferation. 
- **D** The possible binding of miR-1226-3p in the 3′-UTR of FGF2 as predicted by the bioinformatics analysis. 
- **E, F** Overexpression of miR-1226-3p decreased the luciferase activity of cells expressing WT instead of mutant 3′-UTR of FGF2. 
- **G, H** Transfection of miR-1226-3p decreased FGF2 in NPC cells. 
- **I** FGF2 expression in NPC was significantly increased compared with adjacent normal tissues. 
- **J, K** The correlation between expressions of TUC338, miR-1226-3p with FGF2 was analyzed with Spearman correlation test.
Collectively, TUC338 sponged miR-1226-3p in NPC.

(Fig. 4H). Meanwhile, negative correlation for the levels of TUC338 and miR-1226-3p was also found in NPC (Fig. 4I). Collectively, TUC338 sponged miR-1226-3p in NPC.

Fig. 6 Recovered FGF2 attenuated the inhibitory effects of TUC338 depletion on NPC cell proliferation. A, B Cells were transfected with control-siRNA or siRNA-TUC338. The mRNA and protein levels of FGF2 were detected. C, D The mRNA and protein levels of FGF2 in tumor tissues were examined. E, F FGF2 was recovered by transfecting pcDNA-FGF2 into NPC cells. G, H Overexpression of FGF2 attenuated the suppressed proliferation of NPC cells induced by TUC338 depletion. I Restoration of FGF2 significantly reversed TUC338 knockdown-induced apoptosis in both CNE-1 and 5-8F cells.
3.5 MiR-1226-3p targeted FGF2 in NPC cells

Previous studies demonstrated the tumor inhibitory role of miR-1226-3p in the progression of cancers, while the regulatory function of miR-1226-3p in NPC remains unclear. To investigate the involvement of miR-1226-3p in modulating the growth of NPC cells, NC-miRNA or miR-1226-3p mimics were transfected into the cells (Fig. 5A). miR-1226-3p overexpression significantly inhibited NPC cell proliferation cells (Fig. 5B and C), indicating the suppressive function of miR-1226-3p in NPC.

To deeply understand the molecular mechanism by which miR-1226-3p regulated NPC cell growth, the potential miR-1226-3p targets were searched via bioinformatics analysis. The results showed that FGF2 containing presumed binding sites of miR-1226-3p (Fig. 5D). As indicated by the luciferase assay, miR-1226-3p overexpression notably decreased the luciferase activity of NPC cells expressing WT but not mutant 3′-UTR of FGF2 (Fig. 5E and F). To evaluate the influence of miR-1226-3p on the expression of FGF2, RT-qPCR and western blot assays were carried out after the transfection of NC-miRNA or miR-1226-3p. miR-1226-3p overexpression reduced both the mRNA and protein levels of FGF2 in CNE-1 and S-8F cells (Fig. 5G and H). These findings indicated that FGF2 served as a target of miR-1226-3p in NPC. Additionally, FGF2 expression was also detected by RT-qPCR, which exerted a significant enrichment in NPC tissues compared with non-cancerous tissues (Fig. 5I). FGF2 abundance was negatively correlated with miR-1226-3p, but positively correlated with TUC338 in NPC (Fig. 5J and K).

3.6 Overexpression of FGF2 attenuated the suppressed NPC cell proliferation due to depletion of TUC338

To determine whether FGF2 mediated the role of TUC338 in NPC, we first detected the expression of FGF2 with depletion of TUC338. The data showed that transfection of siRNA-TUC338 markedly decreased the mRNA level of FGF2 (Fig. 6A). Meanwhile, the protein expression of FGF2 was also down-regulated with the depletion of TUC338 (Fig. 6B). Moreover, the FGF2 level in xenograft mouse tumor tissues was also detected. As shown in Fig. 6C and D, both the mRNA and protein abundance of FGF2 in tumors harboring depleted TUC338 was significantly decreased with tumors expressing siRNA-control as the control. These findings demonstrated that knockdown of TUC338 inhibited the expression of FGF2 both in vivo and in vitro.

To demonstrate whether TUC338 regulated NPC cell proliferation via FGF2, the expression of FGF2 was overexpressed by transfecting pcDNA-FGF2 into NPC cells (Fig. 6E and F). CCK-8 assay was performed after cells were transfected with siRNA-TUC338 and pcDNA-FGF2. As indicated in Fig. 6G and H, up-regulation of FGF2 significantly attenuated the reduced proliferation of NPC cells induced by TUC338 depletion. Consistently, restoration of FGF2 also markedly reversed TUC338 knockdown-induced NPC cell apoptosis (Fig. 6I). These results demonstrated that TUC338 modulated the malignancy of NPC cells at least via regulating FGF2 by sponging miR-1226-3p.

4 Discussion

Accumulating data have suggested the active participation of lncRNAs in various physiological conditions and contribute greatly to the oncogenesis of cancers [3, 5]. Abnormal expression of lncRNA has been found in NPC [26, 27]. In the current study, TUC338 was overexpressed in NPC tissues and cells. Highly expressed TUC338 was correlated with NPC patients’ poorer survival. Results of functional experiments indicated that down-regulation of TUC338 significantly inhibited the NPC cancer development and therefore serves as a potential oncogenic lncRNA in NPC.

It is well established that the biological function of lncRNAs depends on the miRNAs and proteins to which they bind [4]. Our results revealed a new miRNA target for TUC338, miR-1226-3p, which has been shown to be involved in the pathogenesis of cancers. The interacting of TUC338 decreased the level of miR-1226-3p in NPC cells. Consistent with the overexpression of TUC338, miR-1226-3p was obviously reduced in NPC samples with the non-cancer adjacent tissues as the control. Previous reports have showed the tumor inhibitory function of miR-1226-3p in the progression of cancers [28, 29]. In this study, overexpressed miR-1226-3p reduced the viability of NPC cells, suggested the negative role of miR-1226-3p in NPC.

The essential roles of FGF2 has been reported in cancer progression [30–32]. Highly expressed FGF2 was associated with the unfavorable prognosis of lung cancer patients [33]. To deeply understand the function of miR-1226-3p in NPC, the targets of miR-1226-3p were predicted and FGF2 was found as a candidate. MiR-1226-3p bound FGF2 3′-UTR and decreased its level in NPC. Interestingly, accumulating evidence demonstrated that FGF2 was targeted by different miRNAs and regulated tumorigenesis [34–38]. For example, miR-889-3p inhibited the viability and invasive capacities of cervical cancer cells through directly inhibiting FGF2 [38]. FGF2 was regulated by miR-497-5p and inhibited the proliferation of NSCLC cells [37]. In this
study, consistent with the negative regulation of miR-1226-3p by TUC338, down-regulation of TUC338 significantly decreased the level of FGF2. Restoration of FGF2 reversed the suppressive function of TUC338 in NPC cell proliferation and apoptosis. These results uncovered the functional mechanism of TUC338/miR-1226-3p/FGF2 pathway in the progression of NPC.

5 Conclusions

Our findings demonstrated the overexpression of TUC338 in NPC. Down-regulation of TUC338 inhibited NPC tumorigenesis both in vitro and in vivo. Functional analysis suggested that TUC338 exerted its potential oncogenic role partially via the miR-1226-3p/FGF2 axis. These findings indicated the potential application of TUC338 in the diagnosis and therapy of NPC.

Author contributions JW and YZ designed the study. JW performed the experiments. LPL, XJ, BW, XDH, and WWL validated and discussed the data. YZ wrote the manuscript. All authors have contributed to the manuscript. All authors declared that they have no conflict of interests. All authors read and approved the manuscript.

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Data availability The data generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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