LHX2 facilitates the progression of nasopharyngeal carcinoma via activating the autocrine secretion of FGF1

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Research

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Abstract

Background: Distant metastasis and recurrence remain the obstacle for nasopharyngeal carcinoma (NPC) treatment in clinical, unfortunately, the molecular events underlying NPC growth and metastasis are poorly understood.

Methods: The expression level and prognostic value of LHX2 in pan-cancer were analyzed in TCGA database. The LHX2 expression both in protein and RNA levels in NPC tissues and normal tissues was determined by western blot, qRT-PCR and immunohistochemistry. The roles of LHX2 in oncogenesis were determined using CCK-8, colony formation assays, EdU assay, wound healing assays, transwell assay as well as in vivo mouse model. Bioinformatics analysis, the Gene Set Enrichment Analysis, Luciferase Reporter Assays and Chromatin Immunoprecipitation Assays were applied to identify the downstream effector of LHX2. The activation of indicated signal pathways were measured by western blot.

Results: The LIM-homeodomain transcription factor 2 (LHX2) expression was increased in many solid tumors and was associated with poor progression based on the data from TCGA database. LHX2 levels was also significantly upregulated in NPC tissues and cell lines. Ectopic expression of LHX2 dramatically promoted the cell viability, colony formation efficiency, migratory and invasive ability of NPC cells both in vitro and in vivo. In mechanism, the Fibroblast Growth Factor 1 (FGF1) was confirmed transcriptionally regulated by LHX2 and mediated LHX2-induced tumor-promoting effects. The elevated expression of FGF1 by LHX2 activated the phosphorylation of STAT3, ERK1/2 and AKT/ser9-GSK3 β / β -catenin signal pathway, leading to the increased proliferation ability and epithelial-to-mesenchymal transition (EMT). Treating NPC cells with FGF1-conditioned medium or human recombinant FGF1 accelerated tumor growth and metastasis, which could be blocked by tyrosine kinase inhibitor, AZD4547.

Conclusions: Our study provides mechanistic insight into how LHX2/FGF1 acts as a sufficient regulator pathway in NPC proliferation and metastasis, and the FGF1/FGFR1/2 trapping may contribute to the development of a novel target for NPC therapy.

Introduction

Nasopharyngeal carcinoma (NPC), a malignant tumor raising from the nasopharynx epithelium, has the highest incidence rate in Southeast Asia, especially in Southern China¹. Most patients diagnosed with NPC are in advanced stages, and approximately 30% of them eventually develop distant metastasis². Although, advances in intensity-modulated radiotherapy and broader application of chemotherapy have improved the local and regional control of NPC, the prognosis of NPC patients with recurrence and metastasis is still not satisfying^{3,4}. Therefore, better understanding the molecular mechanisms that underlying the initiation and progression of NPC is of crucial importance to develop efficient novel therapeutic strategies.

The LIM-homeodomain transcription factor 2 (Lhx2) belongs to the LIM homeobox gene family, which consists of two zinc-finger domains. The domains can interact with various transcriptional cofactors and serve its role in modulating the biological functions of LIM-homeodomain proteins⁵. The LIM-homeobox family proteins seems to be associated with many biological processes including asymmetric cell division, cell proliferation, cell differentiation and tissue specification^{6,7}. Lhx2 was first identified in pre-B and T-lymphoid cell lines, the expression pattern of LHX2 differed from the stages of differentiation, and highest expression of LHX2 was found in embryonic and adult tissues⁸. Lhx2 has important roles in development of the eye, bone, forebrain and olfactory sensory neurons^{9–12}. Recent studies demonstrate LHX2 play crucial roles in the occur and development progress of malignant tumors. Highly expressed LHX2 has been found in a variety of human cancer types, including pilocytic astrocytoma, chronic myelogenous leukaemia, pancreatic cancer, non-small cell lung cancer and kidney cancer^{13–15}, however, the role and mechanism of LHX2 in NPC has not been fully understood.

Fibroblast growth factor (FGF) signaling is fundamental for a variety of biological processes, including cell growth, tissue repair, tumor growth and metastasis. FGF1 is a member of Fibroblast growth factor family and stimulates downstream signaling cascades via binding and phosphorylating the FGFR1/2, a transmembrane tyrosine kinase that act as docking sites for interacting proteins. Accumulating evidences indicate that FGF1 promotes tumor development by facilitating cell proliferation, migration, invasion and angiogenesis^{16–18}. Aberrant expression of FGF1 has been found in a variety of human cancers, such as breast cancer, bladder cancer, hepatocellular carcinoma, ovarian cancer, pancreatic cancer, and proved to stimulate the development of these tumors^{19–23}. However, its underlying role in NPC progression remains elusive.

In the present study, based on public databases, we find that the LHX2 is upregulated in the NPC tumor tissues and cell lines. Overexpression of LHX2 promotes the cell growth and metastasis both in vitro and in vivo. In mechanism, LHX2 could activate the STAT3, ERK and AKT/ser9-GSK3 β / β -catenin via promoting the FGF1 transcription and its autocrine manner. Our findings suggest a novel therapeutic efficacy for NPC therapy by targeting LHX2/FGF1 axis.

Materials And Methods

Cell Culture and Clinical Specimens

Eight Human NPC cell lines (SUNE1, CNE1, CNE2, SUNE1, HNE1, 5-8F, 6-10B and HONE1,) were cultured in RPMI-1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). The normal nasopharyngeal epithelial cell lines NP69 was cultured in keratinocyte/serum-free medium (Invitrogen) supplemented with bovine pituitary extract (BD Bioscience, San Diego, CA, USA). Freshly frozen NPC biopsy samples and normal nasopharyngeal epithelia tissues, the paraffin-embedded (FFPE) NPC tissues were collected from Affiliated Cancer Hospital and Institute of Guangzhou Medical University. None of the patients had received anti-tumor treatment before biopsy collection. The study was approved by the

Institutional Ethical Review Board of Affiliated Cancer Hospital and Institute of Guangzhou Medical University, and informed consents have been obtained from all patients.

Immunohistochemistry assay.

Immunohistochemistry assay was performed on paraffin-embedded sections of clinical NPC tissues or xenograft mice tissues. In brief, the tissues were deparaffinized with xylene and rehydrated in a graded ethanol series; the endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide; and the slides were next operated for antigen retrieval under the citrate-mediated high-temperature. The samples were washed once with PBS, and incubated with the primary antibodies at 4 °C overnight. The sections were washed with PBS, incubated with a peroxidase-conjugated secondary antibody, and stained with diaminobenzidine (DAB). The images were captured and scored by the two experienced pathologists. The staining intensity was scored as following: 0, no staining; 1, weak, light yellow; 2, moderate, yellow brown; and 3, strong, brown. Percentage of positive cells was evaluated as: 0, negative; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. The final staining score was calculated by multiplying the intensity (0, 1, 2) and percentage scores.

Conditioned medium prepared

293T cells were transfected with FGF1 overexpression plasmid and the corresponding empty vector. After transfection for 48 h, the medium was replaced and the cells were cultured for another 48 h. The supernatant was collected and centrifuged to remove the cell debris. The conditioned medium was used for further functional studies include CCK-8 assays, colony formation assays, wound healing assays and Transwell migration and invasion assays. All experiments were conducted in triplicate.

Immunofluorescence

Transfected cells in 24-well plates were fixed and incubated with the E-cadherin (1:200; Proteintech, 20874-1-AP, Chicago, USA), Vimentin (1:200; Abcam, ab8978, USA) or β -catenin (1:200, Proteintech, 17565-1-AP, Chicago, USA) primary antibodies overnight at 4 °C. After washing with PBS, fluorescence-conjugated secondary antibodies were added for another incubation at room temperature for 1 h (1:200; Proteintech, SA00013-2 and SA00013-4, USA). Images were captured after staining with DAPI solution.

Plasmids, Virus Production, siRNA and Transfection

The full length of LHX2 and FGF1 were synthesis and cloned into the lentiviral plasmid pSin-EF2-puromycin (Addgene, Cambridge, MA, USA). pSin-EF2-LHX2/FGF1-puromycin or negative control pSin-EF2-puromycin vector was then co-transfected into 293T cells with the VSVG and PSPAX packaging plasmid (Addgene, Cambridge, MA, USA) using Lipofectamine 3000 reagent (Invitrogen). The supernatant was harvested and used to infect HONE1 and SUNE1 cells, stable clones were selected using 0.5 μ g/ml puromycin. The short hairpin RNA targeting LHX2 (sh-LHX2) (Supplementary Table 1) was synthesized and then cloned into pLKO.1. Small interfering RNA targeting FGF1 (si-FGF1) (Supplementary Table 1) was synthesized by RiboBio (Guangzhou, China). For LHX2 and FGF1 knockdown, HONE1 and SUNE1

were transfected with sh-LHX2 plasmids (4 µg) or siFGF1(50 nM) with Lipofectamine 3000 reagent (Invitrogen) according to the manuscript and then harvested for assays 48 h after transfection.

Quantitative Reverse Transcription (RT)-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using a Reverse Transcription Kit (Promega, Madison, WI). Quantitative PCR reactions were performed using SYBR Green Real-Time PCR Master Mix Kit (Invitrogen). The experiment was performed in triplicate and the relative expression was calculated with the $2^{-\Delta\Delta CT}$ method. GAPDH was applied as endogenous controls. Primer sequences are shown in Supplementary Table 2.

Western blot

Protein was extracted with lysis buffer (Beyotime, Shanghai, China) and then quantified by BCA Protein Quantitation Kit (Beyotime, Shanghai, China). Equal amounts of protein (30µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MD) Later on. The membrane was blocked with 5% BSA and incubated with primary antibodies overnight at 4 °C. After incubating with secondary antibodies for 1 h at room temperature, the proteins were detected by enhanced chemiluminescence reagents. primary antibodies against β-catenin(51067-2-AP), GAPDH (60004-1-Ig), E-cadherin (20874-1-AP) were purchased from Proteintech Group. Antibodies against LHX2 (ab243030), Vimentin (ab8978), FGF1 (ab9588), ZEB1(ab203829), and TWIST1(ab50581) were purchased from Abcam. Antibodies against STAT3 (#9139), Phospho-STAT3 (#9145), ERK (#4695), Phospho-ERK (#4370), AKT(#2920), Phospho-AKT (#4060), Phospho-GSK3 (#5558), Phospho-FGFR (#3471), Phospho-FRS2(#3861) were purchased from Cell Signaling Technology

CCK-8 Assay, Clone Formation Assay and Edu assay

For CCK-8 assay, 2×10^3 cells were placed into 96-well plates, incubated with CCK-8 reagent (Dojindo, Japan) and detected absorbance values at 450 nm every 24 hours. For the colony formation assay, 6×10^2 cells were seeded into 6-well plates, refreshed new medium every 3 days. After 4 weeks culture, the colonies then fixed in methanol, stained with 0.1% crystal violet, and counted. Following the standard EDU Assay protocol, tumor cells were added EDU (Invitrogen,Cat. A10044, USA) as recommended dose and photoed by Fluorescence microscopy.

Wound Healing Assay

Cells in 6-well culture plates were cultured with serum-free medium for 12 h, a P- 200 pipette tip was used to scrape the cells straightly. The detached cells were washed with PBS gently for twice and the remaining cells were cultured in serum-free medium. After incubation for 36 h, the width of the scratch was detected and the migration rate was quantified

Migration and Invasion Assays

Cells were digested and suspended in serum-free medium. 3×10^4 cells were placed into the upper 8- μ m pore size Transwell chambers (Corning, NY, USA), which were coated without or with Matrigel (BD Biosciences, San Diego, CA, USA). Culture medium with 10% FBS was placed into the lower chamber to act as a chemoattractant. After incubation for 12 h or 24 h, The cells were fixed in paraformaldehyde and stained with 0.1% crystal violet. Cells on the undersides of the filters were observed and counted under $200 \times$ magnification.

Xenograft tumor models and Immunohistochemistry assay

5 weeks old female nude mice were purchased from Guangdong Medical Laboratory Animal enter (Guangzhou, China). Mice were divided into 4 groups at random, 2×10^6 LHX2 overexpressed or FGF1 overexpressed NPC cell lines and their control cell lines were injected into the dorsal flank of mice. One week after cell injection, the mice were treated with AZD4547 (12.5 mg/kg/d) or equal volume of vehicle once daily by oral gavage for 3 weeks. Animals were sacrificed 30 days later and the tumors were excised for further examination.

The xenograft tumors were fixed in paraformaldehyde and embedded in paraffin. Then the slices of xenograft mice tissues were deparaffinized, hydration, antigen retrieval and block, and then followed by the immunostaining analysis of LHX2 (1:500, Abcam, ab243030, UK), Ki67 (1:500, Abcam, ab16667, UK), PCNA (1:500, Abcam, ab92552, UK), E-cadherin (1:300, proteintech, 20874-1-AP,USA) and Vimentin(1:200, Abcam, ab8978, UK) according to the manufacturer's instructions.

For the tumor metastasis model, 1×10^6 LHX2 overexpressed or FGF1 overexpressed NPC cell lines and their control cell lines were injected into the tail vein of nude mice. One week after cell injection, the mice were administrated with AZD4547(12.5 mg/kg) or vehicle orally every other day. Animals were sacrificed 30 days later, lungs were removed, and H&E staining was conducted.

Luciferase Reporter Assays

pGL3 luciferase reporter plasmids containing wild type or mutant promoter region of FGF1 were constructed. Transfected cells were co-transfected with the indicated luciferase reporters and Renilla luciferase reporter for 24 h. The cells were harvested and the luciferase activity was detected using a Dual Luciferase Reporter Assay Kit (Promega).

Chromatin Immunoprecipitation Assays

The Chromatin Immunoprecipitation (ChIP) Assays were performed as previously described. In brief, LHX2 overexpression or control cells were prepared for cross-linking and sheared to 200–500 bp by sonication. The chromatin fraction was immunoprecipitated with anti-LHX2 (1:50, Abcam, UK), IgG (1:50, Abcam, UK) (negative control). Real-time PCR assays were conducted to detect the enrichment of FGF1 promoter occupancy. The sequences of the ChIP primers were shown in Supplementary table 3.

GSEA Analysis

The Gene Set Enrichment Analysis software (version 2.0.13, www.broadinstitute.org/gsea/) was performed to identify LHX2 expression related pathways in GSE12452 and GSE53819. In brief, by calculating the enrichment score for each gene set using Kolmogorov-Smirnov statistic, we identified the metastasis-related pathway and Fibroblast growth factor were associated with LHX2 expression.

Statistical analysis

Statistical analysis was performed with SPSS 22.0 software (SPSS, Chicago, USA). The Student's t-test was used to analyze the significant difference between two groups. Spearman correlation analysis was applied to analyze the relationship between LHX2 and FGF1 mRNA expression in the GEO datasets and the TCGA datasets. All tests were two-tailed; P-values ≤ 0.05 were considered statistically significant.

Results

LHX2 is highly expressed in NPC clinical specimens and cell lines.

LHX2 level was increased in many solid tumors based on the data from TCGA database, and most of them are epidermal-derived tumors (Fig.S1A). Besides, High expression of LHX2 predicted poor survival in several solid tumors (Fig.S1B), suggesting its oncogenic role in tumor development. However, the effect of LHX2 on NPC has not been fully determined. To verify the expression of LHX2 in NPC, we firstly compared the expression level of LHX2 between NPC and normal tissues in three microarray-based high-throughput NPC datasets (GSE12452, GSE53819 and GSE64634). As shown in Fig. 1A, LHX2 mRNA expression was significantly upregulated in NPC clinical specimens. In accordance with the published microarray data, LHX2 expression was higher in NPC biopsy samples than in normal nasopharyngeal epithelia tissues both at mRNA and protein level (Fig. 1B and 1C). In addition, we examined the LHX2 expression in the nasopharyngeal epithelial cells (NPEC) and NPC cell lines via western blotting assays. Similarly, LHX2 protein levels were significantly upregulated in the NPC cell lines (Fig. 1D). Furthermore, the LHX2 protein levels were substantially higher in the NPC tissues with regional lymph node and distant metastasis than in the tissues without metastasis (Fig. 1E and 1F). Taken together, these data suggest that LHX2 is upregulated in NPC and might influence the NPC malignancy.

LHX2 promotes NPC cells growth both *in vitro* and *in vivo*.

To evaluate the effects of LHX2 on NPC cell growth, LHX2 overexpression or control NPC cells were constructed (Fig. 2A and Fig.S2A). The CCK-8 assay demonstrated that LHX2 overexpressed HON1 and SUNE1 cells achieved significantly faster viability than the control cells (Fig. 2B). Moreover, overexpressing of LHX2 could remarkably increase the colony formation abilities of HONE1 and SUNE1 cells (Fig. 2C and Fig.S2B). Consistently, similar positive effects of LHX2 overexpression on the proliferation were validated by 5-ethynyl-2'-deoxyuridine (EdU) assays (Fig. 2D). In contrast, LHX2 knocking down cells using two different shRNAs were also constructed (Fig.S2C and 2D). As shown in Fig.S3E-3G, LHX2 silencing clearly decreased the cell proliferation as well as colony formation ability.

Next, we screened multiple survival signaling pathways via western blot and found LHX2 overexpression activated p-STAT3, p-ERK, and p-AKT (Fig. 2E).

To further explore the role of LHX2 on NPC in vivo, xenograft tumor models injected with transfected NPC cells was established. The tumors injected with LHX2 overexpression HONE1 and SUNE1 cells grew at a faster rate and possessed a bigger volume than that injected with the control cells (Fig. 2F and 2G). In parallel, the average weight of tumors with LHX2 overexpressed cells were significantly higher than control tumors (Fig. 2H). Immunohistochemistry assay of xenograft tumor-derived tissues showed an increased expression of cell proliferation marker Ki67 and PCNA in LHX2 overexpressing groups compared with the vector groups (Fig. 5D). Collectively, these findings indicate that LHX2 promotes NPC cell growth both in vitro and vivo via activating pro-survival signaling pathways.

LHX2 strengthens NPC cells migration and metastasis.

GSEA analysis based on the GSE12452 and GSE53819 databases revealed that LHX2 was associated with tumor metastasis (Fig. 3A). To investigate the role of LHX2 in NPC metastasis, HONE1 and SUNE1 cells, stably overexpressing LHX2 or the control vector, were subjected to wound healing assay and Transwell assay. Ectopic expression of LHX2 significantly strengthened the migration and invasion of NPC cells (Fig. 3B and 3C). On the contrary, LHX2 silenced NPC cells showed a decreased migratory and invasive abilities (Fig.S3). Furthermore, we explore the role of LHX2 in tumor metastasis in vivo in a lung metastasis model. LHX2 overexpressed NPC cells and the control cells were injected into the tail veins of nude mice. The results shown ectopic LHX2 overexpression significantly increased the number of metastatic nodules, as confirmed by testing H&E-stained lung sections (Fig. 3F-3H). These results demonstrated that LHX2 promotes the cell migration, invasion and metastasis in NPC cells.

LHX2 promotes the epithelial-mesenchymal transition of NPC via activating β -catenin signal pathway.

GSEA analysis based on the GSE12452 demonstrated that LHX2 was associated with the β -catenin signaling (Fig. 4A). In light of the activation of p-AKT by LHX2 (Fig. 2E), we hypothesized that LHX2 might facilitate β -catenin stability via downregulating GSK3 β activity by phosphorylation at Ser9. As determined by western blot, LHX2 overexpression caused a remarkable increase in nuclear levels of β -catenin and the total expression levels of β -catenin and p-Ser9-GSK3 β in both HONE1 and SUNE1 cells (Fig. 3SE). qPCR assay revealed that the expression level of β -catenin targeted genes was substantially increased in LHX2 overexpressed NPC cells, including ZEB1 and TWIST1 (Fig. 4C). We also evaluated the subcellular localization of β -catenin by immunofluorescence analysis and found LHX2 overexpression induced the accumulation of nuclear β -catenin (Fig. 4D). ZEB1 and TWIST1 are two key mediators in EMT, LHX2 overexpression led to an increased expression of the Vimentin, ZEB1 and TWIST1, while a decreased expression of E-cadherin was detected by LHX2 overexpression, as shown by Western blot and immunofluorescence staining results (Fig. 4E and 4F). Moreover, a decreased level of E-cadherin and an increased level of Vimentin were also determined in tumors derived from LHX2 overexpression NPC cells (Fig. 4G). Taken together, LHX2 induces the epithelial-mesenchymal transition through the activation of β -catenin signal pathway.

LHX2 promotes NPC growth and metastasis via transcriptionally activating FGF1 expression

To investigate the downstream genes and potential mechanism regulated by LHX2, we referred to the accessible online Transcription Factor ChIP-seq database (<http://dc2.cistrome.org/>) and analyzed the genes with strong correlation with LHX2 in NPC GEO datasets. We found FGF1 was a putative target of LHX2 that LHX2 motif (Fig. 4A) could bind to its promoter. In addition, mRNA expression of FGF1 was positively correlated to LHX2 expression in NPC clinical specimens (Fig. 4B). Consistently, spearman correlation analysis of TCGA datasets in other types of cancers further verified the positive correlation between FGF1 and LHX2 expression (Fig.S4). FGF1 was confirmed highly expressed in NPC clinical specimens at mRNA level (Fig. 4C). Moreover, overexpression of LHX2 increased the FGF1 expression while knocking down LHX2 could reduce its expression as detected by western blot and real-time PCR assays. (Fig. 4D-4G). What's more, high expression of FGF1 was observed in LHX1 overexpressing xenografts (Fig. 4H), and a positive correlation between FGF1 protein expression and LHX2 protein expression was confirmed on the consecutive paraffin sections of NPC tissues (Fig. 4I). Then, we performed a luciferase reporter gene assay to determine whether FGF1 is a direct target of LHX2. There were two potential sequence binding enrichment (SBE) motifs for the LHX2 transcription factor in the human FGF1 gene on the promoter region. To confirm the specific LHX2 binding sites in its promoters, deletions and selective mutations were generated into the promoter sequences. We identified binding site 2 on the FGF1 promoter was the putative LHX2 binding site (Fig. 4J). Furthermore, ChIP real-time PCR assay confirmed the affinity of LHX2 to the FGF1 promoter (Fig. 4K). These findings suggest that LHX2 binds directly to specific sites on the FGF1 promoter region and transcriptionally activates its expression.

To explore whether FGF1 contributes to LHX2-induced promotion in NPC cell proliferation, migration and invasion, we depletion FGF1 expression in NPC cells stably overexpressing LHX2. CCK-8 assay demonstrated that knocking down FGF1 significantly abolished the enhanced effect of LHX2 on NPC viability (Fig.S5A). In addition, Wound healing assay and Transwell assay revealed the increased migratory and invasive abilities in LHX2 overexpression NPC cells were partially attenuated by the FGF1 silencing (Fig.S5B and 5C). Western blot assay confirmed that co-transfected with FGF1 siRNA reversed the EMT progress inhibited by LHX2 overexpression (Fig.S5D). These results suggest that FGF1 serves as a functional target of LHX2 in NPC cells.

Autocrine secretion of FGF1 promotes the malignant behaviors of NPC cells

FGFs has been identified as secreted proteins produced by cancer cells. To explore the autocrine secretion of FGF1 by NPC cells, NPC cells transfected with FGF1 overexpression plasmid or empty vector were constructed. The conditioned medium (CM) derived from HONE1 and SUNE1 cells was centrifuged and added into the indicated NPC cells (Fig. S6A). Notably, FGF1-CM could activate the STAT3, ERK and AKT signaling as evidence by the elevated expression level of phosphorylation of STAT3, ERK1/2 and AKT, which was consistently with the effect of LHX2 overexpression (Fig.S6B, Fig. 2E). In addition, Ser9-GSK3 β / β -catenin signaling as well as EMT-related proteins was stimulated by FGF1-CM (Fig.S6C). What's more, FGF1 expression was positive associated with ZEB1 and TWIST1 expression in pan-cancer (Fig.S7

and S8). As to investigate the function effect of secreted FGF1, HONE1 and SUNE1 cells treated with FGF1-CM were subjected to CCK-8 assay, colony formation assays, wound healing assay and Transwell assays. FGF1-CM significantly accelerated NPC cell proliferation rate, as well as migratory and invasive ability (Fig.S6D-6G). We then determined the effects of FGF1 recombinant protein on the NPC proliferation and metastasis. Extracellular FGF1 caused the activation of FGFR1/2, and thus stimulated the cascade of phosphorylation of downstream proteins, including FRS2, STAT3, ERK and AKT (Fig. 6A). In addition, β -catenin signaling and EMT-related proteins was also stimulated by FGF1 (Fig. 6B). Furthermore, we treated NPC cells with 5 μ M AZD4547, a FGFR specific inhibitor, followed by FGF1 treatment. As expected, AZD4547 significantly abrogated FGF1-mediated induction of STAT3/ERK/AKT signaling and EMT (Fig. 6A-6B). Consistently, administration of FGF1 dramatically increased the cell proliferation as well as migration and invasion of NPC cells, which could be attenuated by AZD4547 (Fig. 6C-6F and Fig.S9). Next, xenograft tumor models and lung metastasis model were constructed using FGF1 stably overexpressed HONE1 cells. In keeping with the *in vitro* observations, FGF1 overexpression significantly accelerated the tumor growth and lung metastasis, which could be sufficiently blocked by AZD4547 treatment. Together, these findings revealed an oncogenic role of autocrine FGF1 secretion in NPC tumorigenesis and the FGF/FGFR trapping by AZD4547 could reverse these effects.

Discussion

This study demonstrated that LHX2 is frequently upregulated in NPC clinical specimens and cell lines. Ectopic expression of LHX2 promotes NPC cell viability, colony formation ability, migration and invasion both *in vitro* and *in vivo*. Furthermore, the role of LHX2 in NPC might depend on its transcriptional promotion of FGF1 and activated FGF1/FGFR signaling. Overall, our findings provide a new insight into potential mechanism by which LHX2 regulates FGF1 expression in NPC progression.

After searching downstream targets of LHX2, we identified that FGF1 expression was positively correlated with LHX2 expression in NPC, and the expression of FGF1 is significantly affected by LHX2. Furthermore, LHX2 could promote its expression via directly binding to the promoter region. FGF1 belongs to the human fibroblast growth factors family, which consist of a superfamily of polypeptides that play important roles in multiple biological processes, such as proliferation, migration, angiogenesis and differentiation²⁴⁻²⁶. Overexpression of FGF1 stimulates the dissemination of breast cancer and leads to the metastasis of cancer¹⁹. Using a FGF-1-specific single-chain antibody, the growth and metastasis tendency of breast cancer was significantly restrained²⁷. Lung cancer stem cells exhibited increased proliferative potential, enhanced tumorigenic activity, upregulated epithelial-to-mesenchymal transition under FGF1-based 3D culture¹⁷. Although the clinical relevance of FGF1 has been described in various cancers, its role in NPC development and progression has not been discussed. Combining these studies with our findings, genetic ablation of FGF1 significantly reversed the promoting effects of LHX2 on proliferation, migration, invasion, and EMT of NPC, we conclude that LHX2 increase the malignant behavior of NPC by modulating the FGF1.

FGFs bind to the receptor tyrosine kinase family FGFRs, and the subsequent downstream signaling occurs through the intracellular receptor substrates FGFR substrate 2 (FRS2) and phospholipase C γ (PLC γ), resulting in the phosphorylation and activation of MAPK/ERK, JAK2/STAT3, and PI3K/AKT signaling pathways, which has been identified as key mediators in tumor progression promotion²⁸. FGF1 promoted the amplification and enhanced stemness of LCSCs dependent on MAPK/ERK signaling pathway, leading to an increased proliferation and invasion¹⁷. FGFR activation induces accumulation of hyaluronan (HA) and increases the proliferation, migration and therapeutic resistance of breast cancer cells. Furthermore, FGFR-mediated HA accumulation requires activation of the STAT3 signaling pathway²⁹. Consistent with these observations, FGF1 promoted the growth of NPC cells primarily via activating phosphorylation of STAT3, ERK1/2 and AKT, which could be blocked by FGFR1/2 inhibitor AZD4547.

Epithelial-Mesenchymal transition is a physiological phenomenon, during this process, epithelial cells possess the characteristics of mesenchymal cells, including enhanced motility and invasive ability³⁰. LHX2 plays an important role in epithelial-mesenchymal interactions, identified in hair, liver and brain^{10,31,32}. Forced expression of LHX2 induced EMT and increased migration and invasion ability of breast cancer cells³³. Consistent with these observations, we figure out the results that LHX2 increased the EMT process, thus facilitated the migration and invasion of NPC cells. In mechanism, as the direct target of LHX2, FGF1 facilitates the EMT via activating the β -catenin signaling pathway. β -catenin, is an integral E-cadherin cell-cell adhesion adaptor protein and transcriptional co-regulator. As the key initial step in the EMT, the downregulation of E-cadherin expression was observed in metastatic tumors, the transcriptional level of E-cadherin is repressed by several factors including, ZEB1 and Twist1, which have been identified as the effectors of β -catenin/TCF4 signaling in tumor invasiveness³⁴⁻³⁶. In the absence of Wnt signal, APC/Axin/GSK-3 β could target β -catenin leading to its ubiquitination and proteasomal degradation³⁷. The activation of GSK3 β requires phosphorylation at Tyr216, on the contrary, many signaling pathways are reported to downregulate GSK3 β activity by phosphorylation at Ser9^{38,39}, including AKT. In our study, the FGF1 binds to the FGFR1 and FGFR2, resulting in the phosphorylation of Akt, which in turn phosphorylates GSK3 β at Ser9, rendering the kinase inactive and resulting in decreased phosphorylation of downstream substrates, including β -catenin.

Conclusions

In the current study, we identified LHX2 as a novel transcription factor related to NPC progress. LHX2 was highly expressed in NPC and correlated with tumor metastasis. In NPC cell lines we identified that LHX2 transcriptionally regulated FGF1 expression and promoted the proliferation, invasion, and metastasis of NPC in a FGF1/FGFR-dependent pathway. Elevated FGF1 by LHX2 could be secreted outside the cells and binds to FGFR1 and FGFR2, then the subsequent downstream signaling occurs through the intracellular receptor substrates FGFR substrate 2 (FRS2), resulting in the phosphorylation and activation of MAPK/ERK, JAK2/STAT3, and PI3K/AKT signaling pathways. Phosphorylated AKT could stimulate the phosphorylation of Ser9-GSK3 β , leading to the activation of β -catenin signaling and the promotion in the

transcription of its targeted ZEB1 and TWIST1 genes. Furthermore, FGF/FGFR trapping by AZD4547 could block the LHX2/FGF1-induced promotion on tumor growth and metastasis. Our study suggested A novel biomarker for NPC diagnosis and prognosis evaluation, as well as targets for therapeutic treatment.

Abbreviations

NPC: nasopharyngeal carcinoma; LHX2:LIM-homeodomain transcription factor 2; FBS: Fetal bovine serum; FGF1:Fibroblast Growth Factor 1; FGFR : Fibroblast Growth Factor Receptor; GSEA: Gene set enrichment analysis; IHC: immunohistochemistry; EMT: epithelial-mesenchymal transition; NPEC: nasopharyngeal epithelial cell; ChIP: Chromatin Immunoprecipitation; SDS-PAGE: SDS-polyacrylamide gel electrophoresis; shRNA: Short hairpin RNA; siRNA: Small interfering RNA.

Declarations

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Availability of supporting data

The obtained results of the research are available on reasonable request

Ethics approval and consent to participate

This study was performed in accordance with the ethical standards and the Declaration of Helsinki and according to national and international guidelines. Our study has been approved by the Institutional Ethical Review Board of Affiliated Cancer Hospital and Institute of Guangzhou Medical University.

Consent for publication

None

Author contributions

T.X performed the majority of the experiments; J.L and Y.B. provided clinical samples; T.X., Y.W. and H.J. performed the in vivo experiments; B.W., R.L., Y.T., X.H., and Z.X. provided technical support; T.X. and J.Z. contributed to study concept and design; T.X drafted the manuscript; J.Z and Y.Y contributed to study supervision.

Competing interests

The authors declare no conflicts of interest.

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Figures

Figure 1

LHX2 is highly expressed in NPC. (A) LHX2 mRNA expression level is higher in nasopharyngeal carcinoma tissues than in normal tissues. The data were analyzed in GSE12452, GSE53819 and GSE64634. (B) Western blot analysis of LHX2 in nasopharyngeal carcinoma tissues and in normal tissues. (C) RT-qPCR analysis of LHX2 expression levels in nasopharyngeal carcinoma tissues and in normal tissues. (D) Western blot analysis of LHX2 in the NP69 and NPC cell lines. (E)

Immunohistochemical staining and (F) statistical analysis of LHX2 in Normal tissues, primary NPC tissues with (LN+) or without (LN-) lymph node metastasis and primary NPC tissues with distant metastasis. Data are shown as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

.Effects of LHX2 on NPC cell growth in vitro and in vivo. (A)Western blot analysis of LHX2 protein expression in HONE1 and SUNE1 cells stably overexpressing LHX2. The CCK-8 assay (B), colony

formation assay (C) and EdU assay (D) of HONE1 and SUNE1 cells stably overexpressing LHX2. (E) Western blot assay revealed the p-STAT3, pERK1/2 and p-AKT were activated by LHX2. (F) Images of Xenograft tumors of nude mice 30 days after injecting with HONE1 and SUNE1 cells stably overexpressing LHX2 or Vector. (G) Tumor volume growth curves. (H) Average xenograft tumor weights. (I) Immunohistochemistry assay of LHX2, Ki67 and PCNA protein expression in xenograft tumors. Data are shown as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3

.LHX2 strengthens NPC cells migration and metastasis. (A) GSEA enrichment plots demonstrated that metastasis was associated with up-regulation of LHX2 in GSE12452 (left and middle) and GSE53819 (right). (B) Wound healing assay in HONE1 and SUNE1 cells stably overexpressing LHX2 or empty Vector. (C) Transwell migration and invasion assays of HONE1 and SUNE1 cells stably overexpressing LHX2 or empty Vector. (C) GSEA enrichment plots demonstrated that metastasis was associated with up-regulation of LHX2 in GSE12452 (left and middle) and GSE53819 (right). (D-F) Representative images of metastatic lung (D), Representative HE images (E) and numbers of metastatic foci per lung (F) in HONE1 and SUNE1 cells stably overexpressing LHX2 or empty Vector (n=5). Data are shown as means $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4

LHX2 promotes the epithelial-mesenchymal transition of NPC via activating β -catenin signalling pathway. (A) GSEA enrichment plots demonstrated that β -catenin signaling was associated with up-regulation of LHX2 in GSE53819. (B) Western blot assay of β -catenin and Ser9-GSK3 β in HONE1 and SUNE1 cells stably overexpressing LHX2 or empty Vector. (C) RT-qPCR analysis of β -catenin response genes in HONE1 and SUNE1 cells stably overexpressing LHX2 or empty vector. (D) Representative images

of immunofluorescent staining of β -catenin in HONE1 and SUNE1 cells stably overexpressing LHX2 or empty Vector. (E) Western blot assay of EMT-related protein expression in HONE1 and SUNE1 cells stably overexpressing LHX2 or empty Vector. (F) Immunohistochemistry assay of E-cadherin and Vimentin protein expression in xenograft tumors. (G) Immunofluorescence staining was performed to detect the E-cadherin and Vimentin expression in HONE1 and SUNE1 cells stably overexpressing LHX2 or empty Vector. Data are shown as means $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5

.LHX2 promotes NPC growth and metastasis via transcriptionally activating FGF1 expression (A) LHX2 motif (B) Correlations between LHX2 and FGF1 mRNA expression in NPC tissues. (C) FGF1 mRNA expression level is higher in nasopharyngeal carcinoma tissues than in normal tissues. (D and E) Western blot analysis and RT-qPCR analysis of FGF1 expression in HONE1 and SUNE1 cells stably overexpressing LHX2 or Vector. (F and G) Western blot analysis and RT-qPCR analysis of FGF1 expression in HONE1 and SUNE1 cells transfected with shRNA or control. (H) Representative immunostaining of LHX2 and FGF1 in xenografted tumors. (I) Representative immunostaining of LHX2 and FGF1 in consecutive paraffin sections of NPC tissues. (J) Luciferase activity of the reporter gene driven by serially truncated/mutated FGF1 promoters. (K) ChIP assay confirmed the direct binding of LHX2 to the FGF1 promoters in HONE1 and SUNE1 cells.

Figure 6

.Autocrine secretion of FGF1 promotes the malignant behaviors of NPC. (A and B) HONE1 and SUNE1 cells were serum-starved, treated with AZD4547 (2μM) or DMSO, and stimulated with FGF1(20ng/ml), Whole cell lysates were subjected to Western blotting analysis. (C-F) HONE1 and SUNE1 cells were serum-starved, treated with AZD4547(2μM) or DMSO, and stimulated with FGF1 (20ng/ml), the cells were harvested and subjected to CCK-8 assay (C), colony formation assay (D), wound healing assay (E) and

Transwell migration and invasion assays (F). FGF1 overexpressed or Vector HONE1 cells (2×10^6) were injected into the dorsal flank of mice. One week after injection, the mice were treated with AZD4547 (12.5 mg/kg/d) or vehicle orally for 3 weeks. (G) Images of Xenograft tumors. (H) Tumor volume growth curves. (I) Average xenograft tumor weights. FGF1 overexpressed or Vector HONE1 cells (1×10^6) were injected into the tail vein of nude mice. One week after cell injection, the mice were administrated with AZD4547 (12.5 mg/kg) orally every other day for 3 weeks. Representative images of metastatic lung (J), Representative HE images (K) and numbers of metastatic foci per lung (L).

Figure 7

A working model of Lhx2-induced NPC progression. Lhx2 transcriptionally activates FGF1 expression. Tumor cells produced FGF1 binds to FGFR1 and FGFR2, and the subsequent downstream signaling occurs through the intracellular receptor substrates FGFR substrate 2 (FRS2), resulting in the phosphorylation and activation of MAPK/ERK, JAK2/STAT3, and PI3K/AKT signaling pathways. (i) These

pro-survival pathways are responsible for the promotion of tumor proliferation and growth.(ii)
Phosphorylated AKT stimulates the Ser9-GSK3 β / β -catenin signaling, leading to the EMT of NPC via the β -catenin targeted ZEB1 and TWIST1 genes and promotes tumor cell migration and invasion.(iii)
FGF/FGFR trapping by AZD4547 could block the .LHX2/FGF1-induced promotion on tumor growth and metastasis.

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