LncRNA HOTAIR contributes to the tumorigenesis of nasopharyngeal carcinoma via up-regulating FASN

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Abstract. – OBJECTIVE: The therapeutic options for nasopharyngeal carcinoma (NPC) treatment have been restricted mainly to radiotherapy or chemotherapy, and the clinical treatment effect remains unsatisfactory. The primary purpose of this study was to investigate the molecular mechanisms of NPC and to find effective novel therapeutic targets for NPC.

PATIENTS AND METHODS: In order to analyze the expression level of lncRNA HOTAIR and FASN in human NPC clinical tissues or NPC cells, total RNA was extracted with TRIzol and the relative mRNA expression levels were detected by quantitative Real-time PCR. The endogenous expression of HOTAIR was modulated using lentivirus vectors transfection. The protein levels of Fatty acid synthase (FASN), p21 and MMP-9 in NPC cells were determined by Western blot when HOTAIR was knockdown. A free Fatty acid quantitation assay was performed to detect the intracellular free fatty acid in NPC cells. The CCK-8 and colony formation assays were performed for cell viability and proliferation determination. The cell cycle of NPC cells was also determined by flow cytometric analysis. A matrigel invasion assay was performed to analyze the invasive ability of NPC cells.

RESULTS: In this study, we observed a significant upregulation of lncRNA HOTAIR in NPC cells and clinical NPC specimens. The expression of Fatty acid synthase (FASN) was positively correlated to HOTAIR in NPC specimens. Knockdown of HOTAIR led to downregulation of FASN in NPC cells, thus suppressing cell proliferation and invasion. Additionally, de novo synthesis of cellular free fatty acid in NPC cells was inhibited when HOTAIR was knockdown. Furthermore, the protein levels of MMP-9 and p21 were downregulated when HOTAIR was knockdown.

CONCLUSIONS: We suggest that HOTAIR is important in the progression and recurrence of NPC, perhaps through upregulating FASN. Targeting HOTAIR may be an effective therapeutic strategy for NPC.

Key Words: Nasopharyngeal carcinoma, lncRNA HOTAIR, Fatty acid synthase, Oncogene, Tumorigenesis.
of gene transcription, post-transcriptional regulation, and epigenetic regulation. Additionally, some IncRNAs are associated with protein localization and fatty acid metabolic process in numerous human diseases. HOX antisense intergenic RNA (HOTAIR), an IncRNA that was first identified from a custom-tilling array of the HOXC locus, is encoded from the HOXC locus on chromosome 12q13.1. HOTAIR with 2,158 nucleotides plays an important role in gene regulation by modifying chromatin structure. As a potential oncogene, HOTAIR was initially identified in breast cancer. It was reported to have a role in tumorigenesis and metastasis in a variety of carcinomas, such as colorectal cancer, cervical cancer, bladder cancer, hepatocellular carcinoma, gastrointestinal stromal tumors, and pancreatic cancer. Its expression positively correlates with poor prognosis, tumor progression and recurrence in these cancers. Although HOTAIR has been reported as an independent prognostic marker for NPC progression and survival, as well as mediated angiogenesis, the underlying mechanism still needs to be further explored.

Fatty acid synthase (FASN) is a multi-functional enzyme that catalyzes the biosynthesis of palmitate in a NADPH dependent manner. It has been reported FASN ubiquitously expressed in normal cells in adult tissues, but the levels were from low to moderate. However, most of normal cells which primarily import lipids from the extracellular milieu, do not have a strict requirement for FASN activity. In contrast, an increased requirement for lipids in functions, such as membrane biosynthesis, protein modification, and signaling molecules, were observed in tumor cells. Therefore, tumor cells are more dependent on de novo palmitate synthesis catalyzed by FASN than normal cells. Accordingly, FASN is overexpressed in many solid and hematopoietic tumors, such as breast, ovarian, prostate, colon, lung, and pancreatic. Moreover, it was also found FASN inhibition has anti-tumor activities in biologically diverse preclinical tumor models and provides mechanistic and pharmacologic evidence that FASN inhibition presents a promising therapeutic strategy for treating a variety of cancers. All these reports suggested that FASN could be used as diagnostic biomarker and therapeutic target in treatment of NPC.

In the present study, our results confirmed the expressions of HOTAIR and FASN were markedly upregulated in NPC cells and specimens, and also revealed a positive correlation between FASN and HOTAIR expression in NPC specimens. Further studies showed that FASN as well as the synthesis of cellular free fatty acid in NPC cells were inhibited when HOTAIR was knockdown. The subsequent functional studies revealed that HOTAIR knockdown suppressed cell proliferation and invasion in vitro. Our study dissected a novel de novo palmitate synthesis function of HOTAIR in NPC and it might serve as a promising diagnostic and therapeutic target for NPC patients.

Patients and Methods

Cell Culture and Clinical Specimens

The normal human nasopharyngeal NP460 and the human NPC cell lines CNE1, CNE2, 6-10B, 5-8F and HONE1, were obtained from the Southern Medical University (Guangzhou, China). All the NPC cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (BI, Salt Lake City, UT, USA) and 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA). The normal human nasopharyngeal NP460 was maintained in a 1:1 mixture of refined KSFM medium (Invitrogen, Carlsbad, CA, USA) and Epilife (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured in a humidified 5% CO₂ atmosphere at 37°C.

A total of 23 clinical NPC specimens were obtained from the affiliated Hospital of Jining Medical College (Jining, Shandong, China). The study was approved by the Ethics Committee of our hospital (JN2015-015, approval date, 2015.3.1), and all the recruited patients signed informed consent before participating in this study. Specimens were obtained immediately after surgical resection and stored at -80°C for further analysis. Among them, 14 specimens were from male and 9 from female patients.

RNA Extraction and qRT-PCR

Total RNA was extracted from NPC tissues or NPC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. Complementary DNA (cDNA) was synthesized with Reverse Transcription M-MLV (TaKaRa Biotechnology, Dalian, Liaoning, China). Primers used for HOTAIR amplification were: forward 5′-GGTAGAAAAAGCAACCAGAAGC-3′ and reverse 5′-ACATAAACCTCTGTCTGTGAGTGCC-3′. Primers used for FASN
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amplification were: forward 5'-TGCTAGCT-GATCGATCGATCGTGC-3' and reverse 5'-CG-TAGCTGATGATCGTGCATAGC-3'. GAPDH (forward 5'-TGCTAGGCTAGGACGCTAGC-3' and reverse 5'-CTGGGCTAGATCGAGAGCT-3') was used as an internal control. The relative expression levels of HOTAIR and FASN were quantified by Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the supplier's protocol. The relative mRNA expression levels were analyzed and expressed relative to threshold cycle values (ΔCt), then converted to fold changes using the 2^-ΔΔCt method.32,33.

Construction of HOTAIR siRNA Lentiviral Expression Vector

The RNA interference sequences for human HOTAIR were obtained from previous articles.20,34 The sequences of HOTAIR siRNA and negative control were 5'-GAACGGGAGUACGAGAGA-3' (siHOTAIR-1), 5'-CCACAUGAACGCCAGAGAUU-3' (siHOTAIR-2), and 5'-CGGAUCAGCUCGCGCUAUCAUCGCA-3' (siNC). The oligonucleotides encoding short hairpin RNA (shRNA) were then constructed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and annealed into double strands by Annealing Buffer for RNA Oligos (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The double stranded DNA molecules were inserted into PLKO.1 lentiviral vector. All of the constructed plasmids were confirmed by DNA sequencing. The plasmids carrying shHOTAIR or the negative control shNC and packaging vectors were co-transfected into HEK293T to produce lentiviruses designated as Lv-ShHOTAIR-1, Lv-ShHOTAIR-2 and Lv-ShNC. The lentiviruses particles were further used for infection of NPC cells. For stable cell line, infected NPC cells were selected by puromycin (Sigma-Aldrich, St. Louis, MO, USA) in the concentration of 2 μg/mL. After antibiotics selection for about 7 days, the cultured cells were collected and the knockdown of HOTAIR was confirmed by qRT-PCR.

CCK-8 Assay and Colony Formation Assays

The CCK-8 and colony formation assays were performed for cell viability and proliferation determination. For CCK-8 assay, cells were plated in 96-well plates at a density of 5000 cells in 100 μL medium per well one day before the experiment. The cell viability was examined by CCK-8 kit (Keygentec, Nanjing, China) according to the manufacturer’s instruction. For colony formation assay, cells were seeded into 6-well plate at a density of 5 × 10^3 cells per well and cultured for another fortnight. The colonies were stained with Coomassie brilliant blue (Beyotime, Shanghai, China) and counted under a fluorescent microscope (IX70, Olympus, Tokyo, Japan). All the experiments were performed in triplicate.

Flow Cytometric Analysis

The cell cycle of NPC cells was also determined by flow cytometric analysis. HOTAIR knockdown stable cells or the shNC control cells (2 × 10^4/well) were seeded in 12-well plate. After 72 h, cells for cell cycle analysis were harvested by trypsinization and fixed in 70% ethanol overnight. The fixed cells were rehydrated in phosphate-buffered saline (PBS) and digested with RNase A and labeled with propidium iodide (PI), then analyzed by Flow cytometry. Data were analyzed using Flow Jo analysis software (Tree Star, Ashland, OR, USA).

Western Blotting

For Western blotting analysis, total protein was extracted and quantified using the Bradford method. About 60 g protein of each sample were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After the membranes were blocked with 5% milk in phosphate buffered saline (PBS)-0.05% Tween, they were incubated with primary antibody against β-actin were purchased from Santa Cruz Biotechnology (Beverly, MA, USA). FASN was purchased from Abcam (Cambridge, MA, USA), MMP-9 and p21 were purchased from Cell Signaling Technology (Beverly, MA, USA).

Invasion Assay

Transwell invasion assay was performed using Boyden’s chambers. Cells were planted in the upper chamber consisting of 8-m membrane filter inserts coated with Matrigel (BD Biosciences,
Franklin Lakes, NY, USA). The chemoattractant in the lower chamber was supplemented with medium containing 10% fetal bovine serum (FBS). Cells on the upper surface were removed by a wet cotton swab after 24 h, and those attached on the lower side of the membrane were fixed and stained with crystal violet before counting under a microscope in five randomly selected fields.

**Intracellular Free Fatty Acid Assay**

Intracellular free fatty acid in NPC cells was determined by a free fatty acid quantitation kit according to manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA).

**Statistical Analysis**

All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LDS). p values < 0.05 were considered statistically significant.

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### Results

**LncRNA HOTAIR and FASN Were Up-Regulated in Human NPC Clinical Tissues and NPC Cell Lines**

In order to analyze the expression level of lncRNA HOTAIR and FASN in human NPC clinical tissues, 23 NPC tissues and their adjacent normal ones were collected. Next, the total RNA of each specimen was extracted and the gene expression levels were detected by Real-time PCR. Our results revealed that both HOTAIR and FASN were considerably upregulated in NPC tissues compared to adjacent normal ones (Figure 1A-B). Moreover, as shown in Figure 1C, FASN mRNA levels were positively related to the elevation of HOTAIR in NPC tissues ($r = 0.4520$, $p = 0.0304$). Furthermore, we evaluated the expression levels of lncRNA HOTAIR and FASN in six NPC cell lines (CNE1, CNE2, 6-10B, 5-8F and HONE1) by Real-time PCR. The results showed HOTAIR and

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**Figure 1.** Relative lncRNA HOTAIR and FASN expression in NPC tissues and NPC cell lines assessed by Real-time PCR. A-B, lncRNA HOTAIR and FASN were upregulated in 23 NPC tissues compared with 23 normal nasopharyngeal epithelial samples. C, qRT-PCR assays examined the expression of both HOTAIR and FASN mRNA in each of 23 NPC tissues, and the relevance is listed in each blot ($r = 0.4520$, $p = 0.0304$). D-E, lncRNA HOTAIR and FASN were upregulated in a panel of NPC cells compared with normal nasopharyngeal epithelial NP460 cell line. The data were analyzed using 2-ΔΔCt method and presented as the mean ± standard deviation. n = 3, *$p < 0.05$, **$p < 0.01$.
FASN were also significantly increased in NPC cells compared with the immortalized nasopharyngeal cell line NP460 (Figure 1D-E). Among the five cell lines evaluated, CNE2 and 5-8F with higher HOTAIR and FASN levels, were more aggressive cell lines when compared with CNE1 and 6-10B, respectively. Therefore, high level of HOTAIR frequently occurs in NPC consistent with previous studies, suggesting HOTAIR may mediate NPC development and progression.

**Knockdown of LncRNA HOTAIR Inhibits FASN in NPC Cells**

To explore the relationship between HOTAIR and FASN in vitro, two NPC tumor cell lines including CNE2 and 5-8F were selected for the highest upregulation of HOTAIR among the five NPC tumor cell lines. SiRNAs and lentiviral vectors were used to limit HOTAIR expression in CNE2 and 5-8F cells, investigating whether knockdown of HOTAIR could inhibit FASN in NPC cells. The relative expression level of HOTAIR was detected by RT-qPCR at 48 h after transfection. Figure 2A shows that the relative level of HOTAIR in NPC cells was significantly decreased by Lv-shHOTAIR-1 or Lv-shHOTAIR-2, indicating that HOTAIR can be knocked down by Lv-shHOTAIR-1 or Lv-shHOTAIR-2 successfully. The relative expression level of HOTAIR transfected with Lv-shHOTAIR-1 was reduced by 66.15% in CNE2 cells and 69.28% in 5-8F cells; the reduced proportion was each with 53.23% and 73.48% when transfected with Lv-shHOTAIR-2. The relative expression level of FASN was also detected by RT-qPCR, the level of FASN in NPC cells was significantly decreased when HOTAIR was knocked down (Figure 2B). Next, Western blot assays were carried out to detect the protein levels of FASN in CNE2 and 5-8F when HOTAIR was silenced. Our results showed that the FASN protein levels were significantly reduced in Lv-shHOTAIR groups, compared with the control groups (Figure 2C).

![Graphs showing HOTAIR and FASN expression](image1)

**Figure 2.** HOTAIR was relevant to FASN expression in vitro. **A,** HOTAIR expression was suppressed by Lv-shHOTAIR in NPC cells. **B,** FASN expression was suppressed when HOTAIR knockdown. **C,** Protein levels of FASN were downregulated in the Lv-shHOTAIR infected CNE1 and 5-8F cells. Actin served as the internal control. **D,** HOTAIR knockdown inhibits the production of cellular free fatty acid in CNE1 and 5-8F cells. The data were presented as the mean ± standard deviation. *p < 0.05, **p < 0.01, n = 6.
Furthermore, the fatty acid synthase abilities of tumor cells were also detected by measuring cellular free fatty acid. HOTAIR knockdown significantly decreased the cellular free fatty acid from 25.3 μM to 14.2 μM or 17.9 μM in CNE2 when transfected with Lv-shHOTAIR-1 and Lv-shHOTAIR-2, respectively. In 5-8F, cellular free fatty acid was decreased from 39.8 μM to 26.5 μM or 24.7 μM when transfected with shHOTAIR-1 and Lv-shHOTAIR-2, respectively (Figure 2D). In summary, our results showed knockdown HOTAIR inhibits FASN in NPC cells, which makes HOTAIR a possible therapy target in treatment of NPC.

**Downregulation of IncRNA HOTAIR Inhibited the Proliferation of NPC Cells**

FASN is a vital enzyme in tumor cell biology, and inhibition of FASN prevents tumor cell growth, blocks tumor cell proliferation and survival. As our results indicated that the expression level of FASN positively correlated with HOTAIR expression, HOTAIR knockdown inhibits FASN in NPC cells. In this study, the effect of HOTAIR on the proliferation of NPC cells was investigated. After CNE1 and 5-8F cells were infected with Lv-shHOTAIR-1 or Lv-shHOTAIR-2 or Lv-shNC, CCK–8 assays were used to examine the effect of IncRNA HOTAIR on the proliferation of NPC cells. Although the cell proliferative ability among shHOTAIR-1, Lv-shHOTAIR-2 and Lv-shNC infected cells did not significantly change within 24 h, the cell proliferative ability of shHOTAIR-1 and Lv-shHOTAIR-2 groups markedly inhibited compared to Lv-shNC groups from 48 h (Figure 3A). Furthermore, the cell cycle of shHOTAIR-1, Lv-shHOTAIR-2 and Lv-shNC groups was also analyzed by flow cytometry when the cells were infected for 72 h. The results showed that the percentage of G1 phase cells was increased.

**Figure 3.** HOTAIR mediated cell growth in NPC cells. A, Growth of CNE2 and 6-10B cells were detected by Cell Counting Kit-8 assay every 24 h from 0 h to 72 h when HOTAIR expression was suppressed by Lv-shHOTAIR. B, The cell cycles of CNE2 and 6-10B cells were analyzed by Flow cytometry when the cells were infected for 72 h. C-D, Downregulation of IncRNA HOTAIR inhibited colony formation of CNE2 and 6-10B cells. n = 3, *p < 0.05, **p < 0.01.
about 15% ($p < 0.01$) after HOTAIR knockdown by shHOTAIR-1 and about 12% ($p < 0.05$) when knockdown by Lv-shHOTAIR-2 in CNE1 cells compared with Lv-shNC groups, and the percentage of G1 phase increased in 5-8F cells was about 11% and 14%, respectively. The results showed the cell cycle of NPC cells was arrested in G1 phase after downregulation of HOTAIR. There were no significant changes among the remains phases of cell cycle (Figure 3B). Moreover, Lv-ShHOTAIR infected NPC cells formed much fewer colonies compared with those obtained with Lv-ShNC infected cells (Figure 3C-D). All these data suggested that downregulation of HOTAIR inhibits proliferation of NPC cells.

**Knockdown of HOTAIR Suppresses Cell Invasion of NPC**

Our studies indicated that silence of HOTAIR inhibited the proliferation of CNE2 and 5-8F cells. We subsequently investigated the invasion of CNE2 and 5-8F cells after infected with LV-shHOTAIR. Matrigel invasion assays were performed to investigate whether lncRNA HOTAIR was involved in the invasion of NPC cells. The results showed that lncRNA HOTAIR knockdown cells exhibited impeded invasion abilities in both CNE2 and 5-8F cell lines (Figure 4A). Statistical analysis indicated that the difference between LV-shHOTAIR-1 or LV-shHOTAIR-2 group compared with LV-shNC group was significantly ($p < 0.01$); the cells that passed through the filters were reduced more than 60% when infected with LV-shHOTAIR (Figure 4B). Our results demonstrated that downregulation of HOTAIR inhibits invasiveness of NPC cells in vitro.

We further investigated the mechanism of HOTAIR in the progression of cell metastasis in NPC cells. Western blot showed that the expression of matrix metalloproteinase (MMP)-9 and p21...
were downregulated when HOTAIR knockdown (Figure 4C). It has been reported that MMP-9 and p21 positively correlated with migration and invasiveness of tumor cells. When MMP-9 and p21 were silenced, the invasion of tumor cells was suppressed. Although HOTAIR in regulating the protein levels of MMP-9 and p21 directly or through FASN remains to be investigated, our findings showed that silencing HOTAIR could inhibit cells invasion by downregulation of MMP-9 and p21 in NPC cells.

Discussion

As one of the most common tumors in Southern China and Southeast Asia, NPC is a specific regional genetic disease and its development might be associated with multiple factors. Among all of these factors, the activation of oncogenes or the inactivation of tumor suppressor genes that disrupt the homeostasis of cellular gene expression results in the occurrence of NPC as multiple other human cancers. Although encouraging developments in the mechanism of NPC have been achieved, the prognosis and treatment for patients with advanced NPC remain unfavorable. Hence, the molecular-targeted therapy will provide a more specific treatment for NPC and might provide new insight into its pathogenesis. LncRNA is considered as an important factor that influences the development of human tumors. With the development of technological methods, such as lncRNA microarray and RNA sequencing, more and more lncRNAs have been found to be dysregulated in tumors, which function as oncogenes or tumor suppressors. Previous studies have demonstrated that abnormal expression of lncRNAs was able to change the biological functions of tumor cells by affecting various cellular processes. A recent research revealed that LncRNA SLERT regulates the transcription of RNA polymerase I. Dysregulated rRNA synthesis by RNA polymerase I is associated with uncontrolled cell proliferation which may afford the development of tumor. These data suggest the important roles of lncRNAs in the pathogenesis and treatment of tumors. However, the specific functions and mechanisms of lncRNA in the occurrence of tumors remain to be elucidated.

As demonstrated in several previous researches, the expression of lncRNA HOTAIR was upregulated in many primary tumor tissues including NPC. Subsequent studies documented that HOTAIR promotes invasion and metastasis in a group of tumors. In the present work, the expression of IncRNA HOTAIR was examined in 23 primary NPC tissues and 23 normal nasopharyngeal epithelial tissues, and in five NPC and one normal nasopharyngeal epithelial cell line. The results revealed that HOTAIR expression was upregulated in NPC specimens compared with the normal tissues, and the extent of the increase in five NPC cells ranged from about 5- to 20-fold compared with the normal nasopharyngeal epithelial cell line. HOTAIR level was much higher in two aggressive cell lines: CNE2 and 5-8F. To explore the biological function of HOTAIR in NPC progression, a lentivirus-mediated shRNA expression system capable of interfering with HOTAIR expression in CNE2 and 5-8F cells was developed in the present study. The data demonstrated that knockdown of HOTAIR significantly suppressed the proliferation and invasion of CNE2 and 5-8F cells.

Previous reports suggested that inhibition of de novo palmitate synthesis via FASN inhibition provides an interesting approach to cancer therapy with a strong biological rationale. It has been found that lncRNAs are functionally associated with essential biological processes, including fatty acid metabolic process. The present investigation examined the expression of FASN in NPC tissues and in NPC cell lines. Our results revealed that the mRNA level of FASN positively correlated with HOTAIR expression in NPC specimens. Furthermore, the protein levels of FASN were downregulated when HOTAIR was silenced in NPC cell lines. In all, knockdown of HOTAIR inhibits fatty acid synthase in NPC cells. Furthermore, the progression promotion in NPC by HOTAIR was partially activated by the FASN, MMP-9 and p21. However, whether the effects of HOTAIR in regulating the protein levels of MMP-9 and p21 were directly or via targeting FASN, need to be further investigated. Based on this, we suggested that HOTAIR promotes nasopharyngeal carcinoma cell proliferation and invasion via targeting FASN. However, further research is necessary to clarify the exact relationship between HOTAIR and FASN.

Conclusions

We identified the role of HOTAIR in mediating tumorigenesis via upregulating FASN. These data demonstrated that HOTAIR might serve as
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a promising diagnostic biomarker and therapeutic target for NPC. Therefore, disruption of the HOTAIR mediated de novo palmitate synthesis is highly promising for developing therapeutic strategies for NPC patients.

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Author Contributions
DDM, LLY, and LQL conceived and designed the experiments. LLY and DDM conducted patient management and monitoring. DDM, LLY, and LQL collected and analyzed the data. DDM and LLY performed most of the experiments. All authors contributed toward data analysis, drafting, and revising the paper, and agree to be accountable for all aspects of the work.

Conflict of Interest
The Authors declare that they have no conflict of interests.

References


