IncRNA XIST regulates cell proliferation, migration and invasion via regulating miR-30b and RECK in nasopharyngeal carcinoma

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Abstract. Long non-coding RNA (IncRNA) X-inactive specific transcript (XIST) plays an essential role in the development and progress of nasopharyngeal carcinoma (NPC). MicroRNA-30b (miR-30b) has been confirmed to play an inhibitory role in various types of cancer. The molecular mechanisms underlying the IncRNA XIST-mediated regulation of the metastasis of NPC cells by miR-30b is not clear. qPCR and western blot analysis were used to detect the expression of XIST, miR-30b, and reversion inducing cysteine rich protein with kazal motifs (RECK) in NPC tissues and cell lines. The detection of luciferase reporter gene confirmed the relationship between lncRNA XIST, miR-30b and RECK. CCK-8 and Transwell assays were performed in order to detect the proliferation, migration and invasion of the NPC cells. The results of qPCR and western blotting indicated that the expression levels of lncRNA XIST and RECK were higher in the NPC tissues and cell lines than that of the control group, while the expression of miR-30b was lower. Knockdown of lncRNA XIST significantly inhibited cell proliferation, migration and invasion in the NPC cell lines. In addition, IncRNA XIST was found to negatively regulate the expression of miR-30b, resulting in the upregulation of RECK. Overexpression of RECK was found to reverse the inhibitory effect of IncRNA XIST knockdown or miR-30b on NPC cell metastasis. Our results showed that cell migration and invasion were inhibited by knockdown of IncRNA XIST, suggesting that the IncRNA XIST/miR-30b/RECK axis is involved in the development of NPC.

Introduction

Nasopharyngeal carcinoma (NPC), that originates from the epithelium of the nasopharynx, is a malignant head and neck tumor characterized by local invasion and early distant metastasis (1). Recently, more and more researchers have realized that non-coding RNAs (ncRNAs), including microRNAs (miRNAs/miRs) and long non-coding RNAs (lncRNAs), are new sets of clinical biomarkers and potential tumor treatment targets (2). A lncRNA is a transcript of more than 200 nucleotides in length, lacking the ability of protein coding. IncRNAs seem to participate in a variety of biological processes, such as cell proliferation, invasion, apoptosis and cancer progression (3). There is evidence that lncRNA disorders are involved in cell transformation and development of a variety of cancers, including NPC (4,5). A recent study demonstrated that silencing of IncRNA SRRM2-AS inhibited NPC cell proliferation, colony formation and angiogenesis, blocked cell cycle progression and enhanced apoptosis (6). Xue and Cao found that CASC15 enhanced NPC cell proliferation and metastasis via sponging miR-101-3p in vitro and in vivo (7). The inhibitory influence of XIST on miR-491-5p was found to suppress the growth of NPC tumors in vivo (8). miRNAs are a type of conserved endogenous ncRNAs, which can negatively regulate gene expression at the post transcriptional level (9). As confirmed, miRNAs can function as proto-oncogenes or tumor-suppressor genes to participate in various cell biological processes, such as cell proliferation, migration and autophagy (10). miR-30b is one member of the miR-30 family, which was found to play essential roles in proliferation, invasion, and autophagy in osteosarcoma cells (11). However, there is no direct evidence to support the involvement of miR-30b in NPC progression and processes.

X-inactive specific transcript (XIST) is an IncRNA derived from the XIST gene (12). XIST is highly expressed in a variety of tumors including ovarian cancer, non-small cell lung cancer, glioblastoma, breast cancer and liver cancer (13-17). Silencing of XIST was found to inhibit cell growth, metastasis and promote cell apoptosis, and knockdown of XIST can also inhibit tumor growth and promote high survival rate in nude mice, which indicates that XIST plays a pivotal role in the occurrence, development and progress of malignant tumors (17). However, the role of XIST in NPC and its potential biological mechanism remains to be explored.
Overexpression of miR-30b was confirmed to inhibit cell migration in NSCLC (18). Interestingly, although miR-30b has been reported to play an inhibitory role in certain types of cancer, it has also been shown to play a role as an oncogene in melanoma (19,20). The matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent proteolytic enzymes and play an important role in osteoelastic differentiation (20). Relevant experiments show that targeting cysteine rich protein with kazal motifs (RECK) may be an effective way to prevent the progression of oral cancer (21). The molecular mechanism of lncRNA XIST involved in the regulation of invasion and migration of NPC cells by miR-30b remains unclear.

Materials and methods

Tissue specimen. Thirty-five pairs of matched tumor tissues and adjacent non-tumor tissues were collected from NPC patients (15 women and 20 men; age range, 44-70 years; median age, 61 years), who were surgically operated on at Liaocheng People's Hospital from February 2010 to October 2016. This study was approved by the Ethics Committee of Liaocheng People's Hospital. Prior to the study, all participants provided informed consent.

Cell culture. Human nasopharyngeal epithelial cell line NP69 and human NPC cell lines (SUNE1 and HK1) were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin in 5% CO\(_2\) in a 37˚C incubator. The primary normal human nasal epithelial cell line was cultured in epithelial cell growth medium (Promocell) at 37˚C in 5% CO\(_2\).

Cell transfection. siRNA of XIST (si-XIST), siRNA control (siNC), miR-30b mimic (miR-30b), miRNA control (MIR con) and miR-30b inhibitor were purchased from GenePharma Co., Ltd. NPC cells were cultured into a 6-well plate and cultured in a complete growth medium. Antibiotics were not used for at least 24 h before transfection. Then, the cells were transiently transfected with siRNA or co-transfected with si-XIST and miR-30b inhibitor or mimic using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The transfected cells were collected and analyzed. The transfected siRNA XIST, siNC, miR-30b mimic and inhibitor were as follows: si-XIST, 5'-GACCUUGUCAGUGGAUA UTT-3' (forward) and 5'-AUUACCACAGGUCACGU TT-3' (reverse); si-NC, 5'-UUCCUAAGCAUCGUGAC CAGUTT-3' (forward) and 5'-ACCGUACACGUCGGAGA ATT-3' (reverse); miR-30b mimic, 5'-UGUAAACAUCUCA CUCAGCU-3' (forward), and 5'-ACAUUUAGAAGUGU GAGUCA-5' (reverse); miR-30b inhibitor, 5'-AGCGGAGU UAGGAUGUUUACA-3'; miR-30b mimic control, 5'-UCA CACUCCUCAGAAGAGUGA-3'; miR-30b inhibitor control, 5'-UCACAACCUCUAGAAAGAGUGA-3'.

Quantitative real-time PCR (qPCR) assay. The total RNA from NPC cells was separated by TRIzol reagent. The synthesis of cDNA required the use of the M-MLV Reverse Transcriptase Kit (Toyobo). RT-qPCR was performed with the SYBR Green Real-Time PCR analysis Kit (Takara). U6 and GAPDH were respectively used for internal controls for RT-qPCR and western blot analysis. The qPCR cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 45 sec, and a final extension step of 95˚C for 15 sec, 60˚C for 1 min, 95˚C for 15 sec and 60˚C for 15 sec. Calculation of target gene expression and protein level was performed using the 2\(^{-ΔΔCT}\) method (22). Primer sequences were as follows: XIST forward (F), 5'-AATTGA ACAGGGCTGAGTTTAG-3' and reverse (R), 5'-TCATCC GCTTGGCTTCTAGATAG-3': miR-30b F and B, 5'-UGUAACAUCCU ACACUGACGU-3' and R, 5'-ACAUUUAGAAGUGAAGU GA-3': RECK F, 5'-TGTTGACCTGTTTACGGGATGTT-3' and R, 5'-GAAGAATGCTTGACGGCTTTGTGGT-3': GAPDH F, 5'-AGCGTGTGGGAAGATTCCT-3' and R, 5'-ACTGTGG GAAACTGGAATGGC-3'; U6 F, 5'-TGCGGGTGCCTGCTT GCGCAG-3' and R, 5'-CCAGTGAGGGTCGAGGAT-3'.

CCK-8 assay. Cell proliferation was analyzed and determined using the CCK-8 assay. In short, 1x10\(^4\) cells/well were laid in triplicate on a 96-well culture plate (Costar, Corning Inc.), cultured for 24 h, and then transfected with si-XIST, si-NC, or si-XIST + miR-30b inhibitor. At 24, 48 and 72 h after transfection, 10 µl CCK-8 solution was added to each well. The cells were incubated for an additional 1 h, and then the proliferative activity of the cells was measured at 450 nm by VersaMax (Molecular Devices, LLC).

Detection of double luciferase reporter assay. The putative binding site for miR-4301 in HOTTIP was predicted using bioinformatics tool starBase (http://starbase.sysu. edu.cn/index.php). The wild-type (WT) XIST 3'-UTR (untranslated region) and mutated (MUT) XIST 3'-UTR without miR-30b were designed and constructed. The mature miR-30b and its negative control (NC) sequences were cotransfected with XIST 3'-UTR-WT and XIST into cells. The fluorescent enzyme activity reagent (Promega Corp.) of the sample was detected by double fluorescent enzyme reporter gene assay. After 48 h, the cells were harvested and detected for luciferase activity. The double fluorescein reporter gene assay system (Promega Corp.) was used. Luciferase co-transfection was used as a standard control.

Western blot analysis. The total protein was extracted from NPC cells and tissues RIPA lysis buffer (Beyotime Biotechnology). Measurement of total protein concentration was performed using the BCA kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amount of protein (12 µg per lane) from the cell lysates was separated by 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp.). At room temperature, the PVDF membrane was blocked with 5% fat-free milk for 1 h. Subsequently, the membranes were rinsed with TBST twice and incubated with primary antibodies, including RECK (cat. no. ab238162, 1:1,000; Abcam); E-cadherin (cat. no. 3195, 1:2,000; Cell Signaling Technology, Inc.); N-cadherin (cat. no. 13116, 1:2,000; Cell Signaling Technology, Inc.); Vimentin (cat. no. 5741, 1:2,000; Cell Signaling Technology, Inc.) and glyceraldehyde-3-phosphate dehydrogenase
after knockdown of XIST.

Transwell assays. Transwell chambers (8-µm pore size; Costar, Inc.) were used to assess the migration and invasion of NPC cells. For the migration assays, 5x10^5 cells were added into the upper chamber. For the invasion assays, 1x10^5 cells were added into the upper chamber precoated with Matrigel (BD Bioscience). Matrigel was dissolved overnight at 4°C, diluted with serum-free medium at a ratio of 1:3, and added at 50 µl/hole to the top chamber of a Transwell chamber. Then the plate was air dried in an incubator for 4-5 h. An amount 500 µl medium with 15% FBS was then placed into the basolateral chamber. The chamber was maintained at 37°C in a 5% CO₂ incubator for 48 h. The cells on the lower surface of the membrane were then fixed with 4% paraformaldehyde (25°C for 10 min), stained with 0.5% crystal violet (25°C for 30 min). The images were captured in four randomly selected fields under a inverted microscope (CKX41; Olympus, Japan) and images were captured at x200 magnification.

Statistical analysis. Results are displayed as mean ± SD from experiments conducted in triplicate. Student's t-test and one-way analysis of variance (ANOVA) with Tukey's post hoc test were used to analyze differences between two groups and multiple groups, respectively. All statistical analyses were performed using SPSS 20.0 software (IBM Corp.) and GraphPad Prism 5.02 Software (GraphPad Software, Inc.). P-value <0.05 was considered to indicate a statistically significant result.

Results

Expression of XIST in NPC and adjacent tissues and cell lines. First, the expression of XIST in 35 NPC and adjacent tissues and 2 types of NPC cell lines (SUNE1 and HK1) was detected by qPCR. The results showed that the expression level of XIST in tumor tissue was significantly higher than that in the adjacent normal tissue (Fig. 1A). Similar to this result, the expression level of XIST in NPC cell lines (SUNE1 and HK1) was significantly higher than that in the NP69 cells (Fig. 1B).

Knockdown of IncRNA XIST inhibits the proliferation and metastasis of NPC cells. In our study, SUNE1 HK1 cell lines were selected to knockdown XIST in vitro. qPCR was used to detect the expression of XIST following knockdown of XIST (Fig. 2A) and confirm the knockdown efficiency. CCK-8 assay demonstrated that the growth ability of SUNE1 and HK1 cells were significantly inhibited by the knockdown of XIST (Fig. 2B). Transwell assay showed that the number of invasive and migratory cells in the SUNE1 (Fig. 2C and E) and HK1 (Fig. 2D and F) cell lines was significantly decreased after knockdown of XIST.

Expression level of miR-30b in NPC tissues and cell lines. First, the expression of miR-30b in 35 NPC tissues and adjacent tissues and NPC cell lines (SUNE1 and HK1) was detected by qPCR. The results stated clearly that the expression level of miR-30b in tumor tissues was significantly lower than that in the adjacent normal tissues (Fig. 3A). Similar to this result, the expression level of miR-30b in the NPC cell lines was significantly lower than that in the NP69 cells (Fig. 3B).

IncRNA XIST is targeted by miR-30b and negatively regulates its expression. To explore the regulatory mechanisms of IncRNA XIST, the prediction of target sites between IncRNA XIST and miR-30b was performed by StarBase v3.0 (Fig. 4A). miR-30b was selected from these miRNAs that interacted with IncRNA XIST. In addition, the miR-30b expression was explored by qPCR in SUNE1 and HK1 cell lines following IncRNA XIST knockdown. The results demonstrated that miR-30b expression was significantly increased in SUNE1 and HK1 cell lines transfected with si-XIST (Fig. 4B). As shown in Fig. 4C, miR-30b expression was higher when cells were transfected with miR-30b mimic in the SUNE1 and HK1 cell lines. The luciferase reporter gene assay revealed that co-transfection of XIST-WT and miR-30b mimic significantly decreased luciferase activity. However, no significant differences were observed in luciferase activity after co-transfection of XIST-MUT and miR-30b mimic (Fig. 4D and E). In addition, the outcome of the linear correlation analysis demonstrated that miR-30b expression was negatively correlated with IncRNA XIST expression in the NPC tissues (Fig. 4F). In a word, these results imply that IncRNA XIST may serve as a competitive endogenous (ce)RNA to directly bind to miR-30b and negatively regulate its expression.
Knockdown of lncRNA XIST or miR-30b suppresses cell proliferation, migration and invasion in SUNE1 and HK1 cells. As shown in Fig. 5A, we therefore transfected the si-NC, si-XIST and miR-30b inhibitor into the SUNE1 and HK1 cell lines. The expression of miR-30b was upregulated by si-XIST, and this tendency was reversed by the miR-30b
Figure 3. Expression of miR-30b was determined by qPCR in NPC and adjacent tissues and NPC SUNE1 and HK1 cells. (A) Expression of miR-30b in NPC and adjacent normal tissues. (B) Expression of miR-30b in NPC cells and NP69 normal cells. **P<0.01, compared to the adjacent tissues or NP69 cells. NPC, nasopharyngeal carcinoma.

Figure 4. IncRNA XIST targets miR-30b and negatively regulates its expression (A) Putative binding sites between IncRNA XIST and miR-30b. (B) Expression of miR-30b is significantly increased by knockdown of XIST. (C) Expression of miR-30b after transfection with miR-30b mimic. (D and E) The luciferase activity was determined by dual-luciferase reporter gene assay in NPC SUNE1 and HK1 cells. (F) Correlation between IncRNA XIST and miR-30b relative expression. **P<0.01, *P<0.05, compared to the NC. XIST, X-inactive specific transcript; NPC, nasopharyngeal carcinoma; NC, negative control; WT, wild-type; MUT, mutated.
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inhibitor. Meanwhile, we tested the expression of epithelial to mesenchymal transition (EMT) markers at the protein level in the SUNE1 and HK1 cell lines following transfection with si-XIST, si-NC, or si-XIST and miR-30b inhibitor. The outcome indicated that the E-cadherin (E) protein level was apparently increased in the SUNE1 and HK1 cells transfected with si-XIST, si-NC, or si-XIST and miR-30b inhibitor. E, E-cadherin; N, N-cadherin; V, vimentin. (C-F) SUNE1 and HK1 cell migration and invasion after transfection with si-XIST, si-NC, si-XIST and miR-30b inhibitor (x100 magnification). **P<0.01, *P<0.05, compared to the si-NC group. XIST, X-inactive specific transcript; NPC, nasopharyngeal carcinoma; NC, negative control; EMT, epithelial-to-mesenchymal transition.

Figure 5. lncRNA XIST knockdown suppresses NPC cell invasion and migration by targeting miR-30b. (A) Expression of miR-30b in NPC SUNE1 and HK1 cells transfected with si-XIST, si-NC, or si-XIST and miR-30b inhibitor. (B) The protein levels of EMT-related marker protein in SUNE1 and HK1 cells transfected with si-XIST, si-NC, or si-XIST and miR-30b inhibitor. E, E-cadherin; N, N-cadherin; V, vimentin. (C-F) SUNE1 and HK1 cell migration and invasion after transfection with si-XIST, si-NC, si-XIST and miR-30b inhibitor (x100 magnification). **P<0.01, *P<0.05, compared to the si-NC group. XIST, X-inactive specific transcript; NPC, nasopharyngeal carcinoma; NC, negative control; EMT, epithelial-to-mesenchymal transition.
RECK is a direct miR-30b target in NPC cells. In order to study the mechanism of the effect of miR-30b on NPC cells, we analyzed the target genes of miR-30b and predicted that RECK is a direct target of miR-30b by using TargetScan (http://www.targetscan.org/vert_72/) (Fig. 6A). The analysis of luciferase reporter gene displayed that the co-expression of miR-30b with RECK-3’-UTR reporter gene plasmid significantly inhibited luciferase activity in the SUNE1 and HK1 cells, but no significant change was observed in the mutant plasmid (Fig. 6B and C). The expression level of RECK was significantly decreased by the miR-30b mimic in SUNE1 and HK1 cells as shown in Fig. 6D. Protein level results showed that the expression of RECK was reduced by si-XIST, which were obviously counteracted when the miR-30b inhibitor was co-transfected into the SUNE1 and HK1 cells (Fig. 6E).

Discussion

Nasopharyngeal carcinoma (NPC), that originates from the epithelium of the nasopharynx, is a malignant head and neck tumor characterized by local invasion and early distant metastasis (1). Despite the possibility of initial radical treatment, approximately 30% of NPC patients present with metastasis or disease relapse (23). When the tumor undergoes metastasis after treatment, the prognosis of NPC patients is
One study found that lncRNA SRRM2-AS silencing prevented the angiogenesis of NPC cells by upregulating MYLK and activating NFR (6). It was also found that the high expression of lncRNA MALAT1 was related to the poor prognosis of pancreatic cancer patients (27). Knockdown of lncRNA TUG1 was found to significantly inhibit tumor proliferation and angiogenesis in vitro, and inhibit various phenomena of tumor cell metastasis (28). Wang et al demonstrated that the high expression of lncRNA XIST may play a significant role in the process of cancer cell lesions by promoting the proliferation of GBM cells (29). The abnormal expression of lncRNA XIST has been found in various cancers. Upregulation of the expression of XIST affecting the behavior of glioma cells (15). Similar to these results, our study showed that according to qPCR results, when compared with normal nasopharyngeal epithelial cells, lncRNA XIST was upregulated in NPC cells. The results of CCK-8 and Transwell assays demonstrated that inhibition of lncRNA XIST suppressed the proliferation, migration and invasion of NPC cells, which suggests that lncRNA XIST may affect the process of NPC metastasis. Our results suggest that lncRNA XIST is upregulated and enhances the development and progression of NPC. However, the specific mechanism of lncRNA XIST in NPC needs further study.

Zhuang et al found that lncRNA XIST can significantly inhibit the interaction of its target gene miR-92b. XIST inhibited the proliferation and metastasis of HCC cells by targeting miR-92b (17). Ma et al demonstrated that lncRNA XIST also controls the downstream target MACC1 through competitive endogenous (ce)RNA, so as to boost the proliferation and invasion of GC cells (30). miR-30b, which belongs to the miR-30 family, is located in the genomic region of chromosome 8q24. miRNA expression profiles have highlighted miR-30b downregulation as a common event in human malignancies. For example, overexpression of miR-30b inhibits cell migration and invasion in non-small cell lung cancer (31), and high levels of miR-30b inhibit the growth of gastric cancer cells and promote apoptosis (32). In our research, we detected the expression of lncRNA XIST and miR-30b, and found that they were negatively correlated in NPC cells. Furthermore, on the basis of the analysis of bioinformatics results, we discovered that lncRNA XIST includes a target combined site with miR-30b. Luciferase assay showed that lncRNA XIST interacted directly with miR-30b and regulated it. These results indicate that the competitive binding of lncRNA XIST to miR-30b and its expression are negatively regulated.

It has been confirmed that miRNAs can play a significant role in many types of cancer. Overexpression of miR-491-5p was found to significantly inhibit NPC cell proliferation, migration and invasion in vitro and tumor growth in vivo by targeting Notch3 (33). For example, miR-96 and miR-21 are highly expressed in non-small cell lung cancer (NSCLC) tumor tissues, and the regulation of target genes can significantly affect the migration and invasion of NSCLC cells (34,35). A previous studies has shown that miR-21 can alleviate osteoporosis by targeting the RECK gene, which suggests that RECK may be a new target for the treatment of osteoporosis (36). RECK has been proven to be a target of miR-30b by bioinformatics analysis and fluorescein reporter gene analysis. More importantly, miR-30b mimic suppressed RECK mRNA expression. Knockdown of lncRNA XIST significantly reduced RECK expression, which was reversed by the miR-30b inhibitor. We suggest that lncRNA XIST regulates RECK expression by negatively regulating miR-30b. In addition, our results showed that si-XIST or miR-30b inhibitor inhibited cell migration and invasion, suggesting that the lncRNA XIST/miR-30b/RECK1 axis participates in the progression of NPC (Fig. S1). The lack of using external data portal (GEO, ICGC and Arrayexpress) and online database (PROGeneV2, GEPIA, UCSC xena, SurvExpress, UALCAN, Linkedomics, cbioportal, OncomiR and Oncomine) may be a limitation of the present study. Results using these databases could help to confirm the findings of our study.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LS and HQ conceived and designed the study, and drafted the manuscript. LS, MZ and HQ collected, analyzed and interpreted the experimental data. LS revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Liaocheng People's Hospital (no. 201002008, Liaocheng, Shandong, China). Signed written informed consents were obtained from the patients and/or guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
References


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