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Long-chain non-coding RNA LOC554202 promotes proliferation, migration, and invasion of nasopharyngeal carcinoma cells by binding to microRNA-31 expression and regulating RhoA expression

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Abstract. – OBJECTIVE: Long-chain non-coding LOC554202, as a host gene for microRNA-31, has been shown to play a crucial role in a variety of diseases, especially tumors. However, its biological function in nasopharyngeal carcinoma (NPC) has not been reported.

PATIENTS AND METHODS: The expression levels of LOC554202 and microRNA-31 in NPC tumor tissue samples and cell lines were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The impacts of LOC554202 and microRNA-31 on the biological functions of NPC cells were examined by Cell Counting Kit-8 (CCK-8) and transwell assays. In addition, the modulation of LOC554202 on the expressions of microRNA-31 and RhoA was further confirmed by qRT-PCR and Western blot analysis.

RESULTS: The data of this study indicated that LOC554202 expression in NPC tissues and cell lines was remarkably upregulated, while microRNA-31 level showed an opposite tendency. Increasing LOC554202 expression remarkably enhanced the growth and metastasis of NPC cells, which was inhibited by overexpression of microRNA-31. Overexpression of LOC554202 downregulated microRNA-31 expression but upregulated that of RhoA, which may be a potential mechanism for the implication of LOC554202 in NPC.

CONCLUSIONS: As a host gene of microRNA-31, LOC554202 enhances RhoA expression and thus promotes the proliferative capacity and invasiveness of NPC cells.

Key Words:
NPC, LOC554202, MicroRNA-31, Cell proliferation, Cell migration, Cell invasion.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor that originates from the nasopharyngeal mucosa, with invasive squamous cell carcinoma as its most common pathological type[1]. Multiple factors, such as genetic susceptibility, epigenetic variation, environmental factors, ethnic background, and latent Epstein-Barr (EB) virus infection are engaged in the occurrence and development of NPC[2]. Common clinical manifestations of NPC include nasal congestion, nosebleeds, hemorrhage, hearing loss, tinnitus, headache, cervical lymph node metastasis or distant metastasis[3]. NPC can be divided into three subtypes, namely, keratinized squamous cell carcinoma, differentiated non-keratinized cancer, undifferentiated non-keratinized cancer, of which the last one is the most common subtype[4]. Currently, the treatments for NPC mainly include radiotherapy, combination of radiotherapy and chemotherapy, molecular targeted therapy and antiangiogenic drug treatment[5]. However, most NPC patients in advanced stage have distant metastasis or local recurrence, leading to poor prognosis. In addition, adverse effects of chemotherapy and radiotherapy include bone marrow suppression and upper gastrointestinal damage, which seriously reduce the life quality of patients with nasopharyngeal cancer[5]. Therefore, it is particularly important to find therapeutic targets for NPC diagnosis and treatment. The Human Genome Project shows that of the 3 billion base pairs that make up the human genome, less than 2% of the nucleic acid sequences encode proteins, and the remaining more than 98% are non-protein encoding sequences[6]. According to the length of the transcript, non-coding RNA can be divided into small non-coding RNA and long non-coding RNA (lncRNA), whose latter is a type of transcript with a length of more than 200 bp. LncRNA cannot encode stable polypeptides or

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protein for its low sequence conservation and the lack of functional open reading frame. However, lncRNA plays a pivotal role in modulating gene transcription, post-transcription, and translation through interaction with other molecules and thus participates in cell activities, such as cell proliferation, differentiation, and survival[7]. Compared with its regulation of normal physiological activities, its function in the diagnosis and treatment of diseases is more attractive. Some lncRNAs that are dysregulated in certain cancer tissues with a high degree of tissue specificity are expected to become new targets for cancer diagnosis and treatment[8]. LINC00511 affects the cell proliferation ability of papillary thyroid carcinoma through cyclin-dependent kinases[9]. LINC00483 prompts the progression of lung adenocarcinoma through sponge adsorption of microRNA-204-3p[10]. In addition, GATA3-AS1 enhances proliferation ability and metastasis of hepatocellular carcinoma cells *via* inhibiting PTEN, CDKN1A and TP53[11].

LncRNA LOC554202, as a host gene of microRNA-31, plays a cancer-promoting gene in non-small cell lung cancer[12], colorectal cancer[13], breast cancer[14] and other malignant tumors. It can regulate the expression of downstream genes by regulating microRNA-31 expression and participate in the progression of the diseases. However, LOC554202 expression in NPC and its biological function have not been reported. Therefore, the expression of LOC554202 in NPC and its possible molecular mechanism were preliminary explored.

Patients and Methods

Sample Collection

A total of 30 cases of osteosarcoma tissue samples were collected from NPC patients diagnosed in Jinan Fifth People's Hospital from December 2017 to June 2019, and 12 normal tissues were used as control. All patients did not receive any treatment before surgery and were diagnosed as NPC pathologically. Tumor pathological classification and staging standards are implemented in accordance with the staging standards of the Union for International Cancer Control (UICC). The nasopharyngeal carcinoma tissues were confirmed by two pathologists in our hospital, respectively. All subjects volunteered to participate in the study and signed written informed consent. This investigation has been approved by the Ethics Committee of Jinan Fifth People's Hospital.

Cell Culture

Human nasopharyngeal epithelial cells NP69 and NPC cell lines (5-8F, C666-1, 6-10B, and HONE1) provided by American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin in a 37°C cell incubator with 5% CO_2.

Transfection

LOC554202 overexpression plasmid was constructed into the pSicoR lentiviral vector, and 293T cells were used for virus production. Lentivirus was used to infect NPC cells, and puromycin was used to select cells with LOC554202 overexpression. For transient transfection, Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was mixed with microRNA-31 mimics and microRNA NC (GenePharma, Shanghai, China) and then added into cells when cell density reached to 50%.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and underwent reverse transcription using PrimeScript™ RT kit (TaKaRa, Otsu, Shiga, Japan). Next, qRT-PCR was carried out with the SYBR Green kit (TaKaRa, Otsu, Shiga, Japan), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 as internal references. The primer sequences were shown below: LOC554202 Forward: 5'-TCTCTGGTGCTTCCCTCCTT-3', Reverse: 5'-TCTCTGGTGCTTCCCTCCTT-3'; miR-31 Forward: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTAT-3', Reverse: 5'-TAATACTGCCTGGTAATGATGA-3', RhoA Forward: 5'-AGCCTGTGGAAAGACATGCTT-3', Reverse: 5'-TCAAACACTGTGGGCACATAC-3', U6 Forward: 5'-GCTGAGGTGACGGTCTCAAA-3', Reverse: 5'-GCCTCCCAGTTTCATGGACA-3', Forward: 5'-TCCGATCGTGAAGCGTTC-3', Reverse: 5'-GTGCAGGGTCCGAGGT-3', and GAPDH Forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Cell Counting Kit-8 (CCK-8) Assay

Cells were plated in 96-well plates ($2'10^3$ cells/well) in 100 μL of culture medium. CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) was performed according to the manufacturer's protocol.

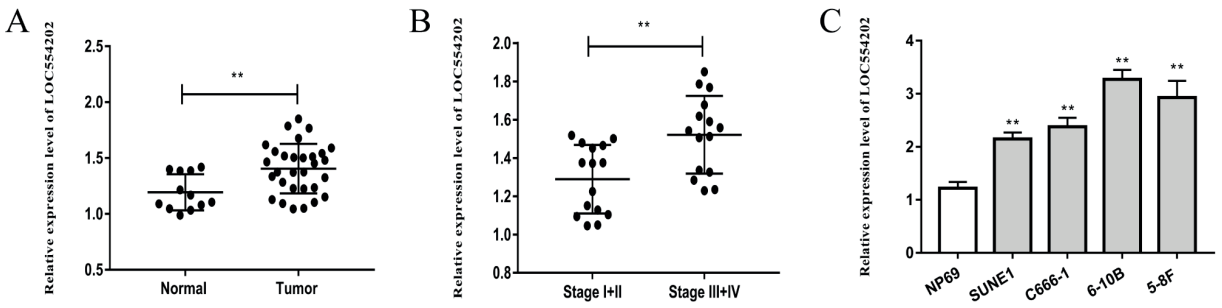

Figure 1. LOC554202 was highly expressed in nasopharyngeal carcinoma tissues and nasopharyngeal carcinoma cells. **A,** The expression of LOC554202 in nasopharyngeal carcinoma tissues and normal control tissues was detected by qRT-PCR. **B,** The expression level of LOC554202 in nasopharyngeal carcinoma patients with different TNM stages was analyzed by qRT-PCR. **C,** Detection of LOC554202 expression in normal control cell line NP69 and nasopharyngeal carcinoma cell lines (6-10B, 5-8F, SUNE1, and C666-1) by qRT-PCR. $^{**}p<0.01$

Transwell Assay

Cell migration or invasion ability was measured using a 24-well plate cell according to instructions. Cells were prepared into cell suspensions and seeded in the upper transwell chamber (10,000 cells/well) supplemented with serum-free 1640 medium, and then, the medium containing 10% FBS was added to the lower compartment. Lastly, the migrated cells were counted and observed after stained by crystal violet under a microscope, and 5 fields of view were randomly selected.

Western Blot

NPC cells were lysed using PRO-PREPTM protein lysate (Pierce, Rockford, IL, USA), shaken on ice for 30 minutes, and centrifuged at 14,000 ´ g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA). Later, immunoblotting was carried out using specific antibodies, and the intensity of protein expression was finally determined using alpha SP image analysis software.

Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA). Measurement data were expressed as mean ± SD (standard deviation). Two-sample *t*-test was applied for comparison between two groups. $p<0.05$ represented statistically significant differences.

Results

LOC554202 Was Highly Expressed In NPC

First, LOC554202 expression was detected in 30 cases of NPC tissue samples and 12 cases normal control tissues by qRT-PCR. The results indicated that NPC tissues contained significant higher expression of LOC554202 than the control samples (Figure 1A). Based on TNM stage, NPC patients were divided into I + II group and III + IV group, and it was found that LOC554202 expression in the latter was higher than that in the former (Figure 1B). Consistently, *in vitro* cell experiments also showed the same tendency of LOC554202 expression (Figure 1C). The above observations indicated that LOC554202 played a critical part in the progression of NPC.

MicroRNA-31 Was Underexpressed In NPC

Figure 2A showed that microRNA-31 expression in above NPC tissue specimens was remarkably lower than that in normal control tissues. Through analysis, we found that microRNA-31 expression in tumor tissues of NPC patients in stage III + IV was markedly lower than that of those in stage I + II group (Figure 2B). Further, microRNA-31 expression was also remarkably reduced in NPC cell lines in comparison to the normal control cell line NP69 (Figure 2C). Correlation analysis revealed that the level of microRNA-31 in NPC tumor tissues was negatively correlated with that of LOC554202 (r=-0.5704, $p<0.001$) (Figure 2D).

Overexpression of MicroRNA-31 Partially Reversed the Biological Effects Induced by Overexpression of LOC554202

To verify the biological effects of LOC554202 and microRNA-31 on NPC cells, 6-10B and 5-8F cells were selected for *in vitro* cell experiments. First, a NPC cell line stably overexpressing LOC554202 was established by transfecting a lentiviral vector encapsulating

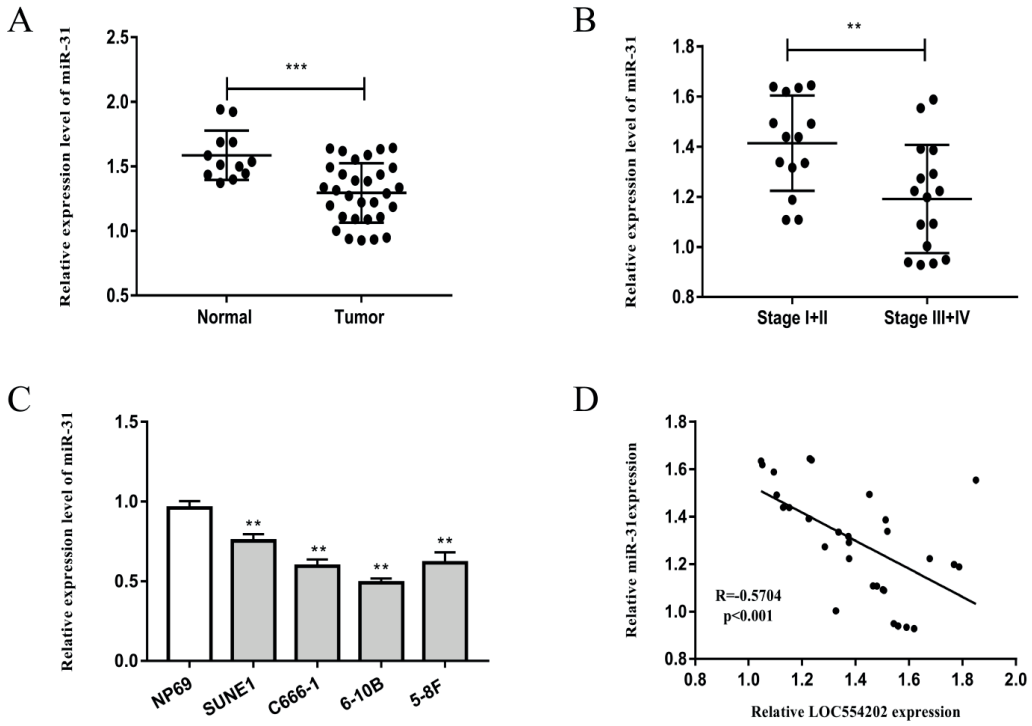

Figure 2. MiR-31 was underexpressed in nasopharyngeal carcinoma tissues and nasopharyngeal carcinoma cells. **A,** The expression of miR-31 in nasopharyngeal carcinoma tissues and normal control tissues was detected by qRT-PCR. **B,** The expression level of miR-31 in nasopharyngeal carcinoma patients with different TNM stages was analyzed by qRT-PCR. **C,** The expression of miR-31 in normal control cells NP69 and nasopharyngeal carcinoma cell lines (6-10B, 5-8F, SUNE1, and C666-1) was detected by qRT-PCR. **D,** Analysis of the correlation between the relative expression of LOC554202 and miR-31 in nasopharyngeal carcinoma tissues. $^{**}p<0.01$, $^{***}p<0.001$

the LOC554202 overexpression plasmid, which was observed by qRT-PCR assay (Figure 3A). Meanwhile, microRNA-31 mimics were applied to upregulate microRNA-31 expression in NPC cells (Figure 3B). Afterwards, CCK-8 and transwell experiments were carried out to reveal that upregulation of LOC554202 markedly enhanced the proliferation and metastasis of NPC cells, which were relatively weakened by upregulation of microRNA-31 (Figure 3C and 3D). Therefore, it was concluded that overexpression of microRNA-31 could partially reverse the biological effects caused by LOC554202 upregulation.

LOC554202 Could Inhibit MicroRNA-31 Expression and Promote RhoA Expression

QRT-PCR results showed that overexpression of LOC554202 in 6-10B and 5-8F cells remarkably inhibited microRNA-31 level, while it was partially enhanced by transfection with microRNA-31 mimics (Figure 4A). Previous studies have reported that microRNA-31 can target RhoA to inhibit its expression. Therefore, the mRNA and protein expression levels of RhoA were detected by qRT-PCR and Western blot. Overexpression of LOC554202 significantly increased RhoA expression, which was partially reduced by upregulation of microRNA-31 (Figure 4B, 4C). These results indicated that LOC554202, as a host gene of microRNA-31, elevated RhoA expression by inhibiting microRNA-31 expression, thus participating in the progression of NPC.

Discussion

The unconspicuous clinical symptoms of NPC in the early stage and the hidden site of NPC bring difficulties in the early diagnosis and treatment of NPC. Therefore, most NPC patients have been in the middle or advanced stage when diagnosed[15,16]. Due to the lack of specific early diagnosis indicators, the problems of early diagnosis and early treatment of NPC remain to be solved.

LncRNAs are involved in epigenetic modifications, through the control of upstream promoter gene transcription, inhibition of the activity of RNA polymerase, interface structure of chromatin reorganization and interference in mRNA shear, and thus participate in lots of physiological processs[17,18]. At present, they have also been shown to play an important biological role in the progression of NPC. So, long non-coding RNA ZFAS1 can promote the occurrence and metastasis of NPC by sponge adsorption of microRNA-892 and regulation of LPAR1 expression[19]. LncRNA DLX6-AS1 increased the expression of HIF-1 and promoted the malignant phenotype of NPC cells by targeting microRNA-199a-5p[20]. Silencing long non-coding RNA SRRM2-AS inhibits angiogenesis of NPC by activating the MYLK-mediated cGMP-PKG signaling pathway[21].

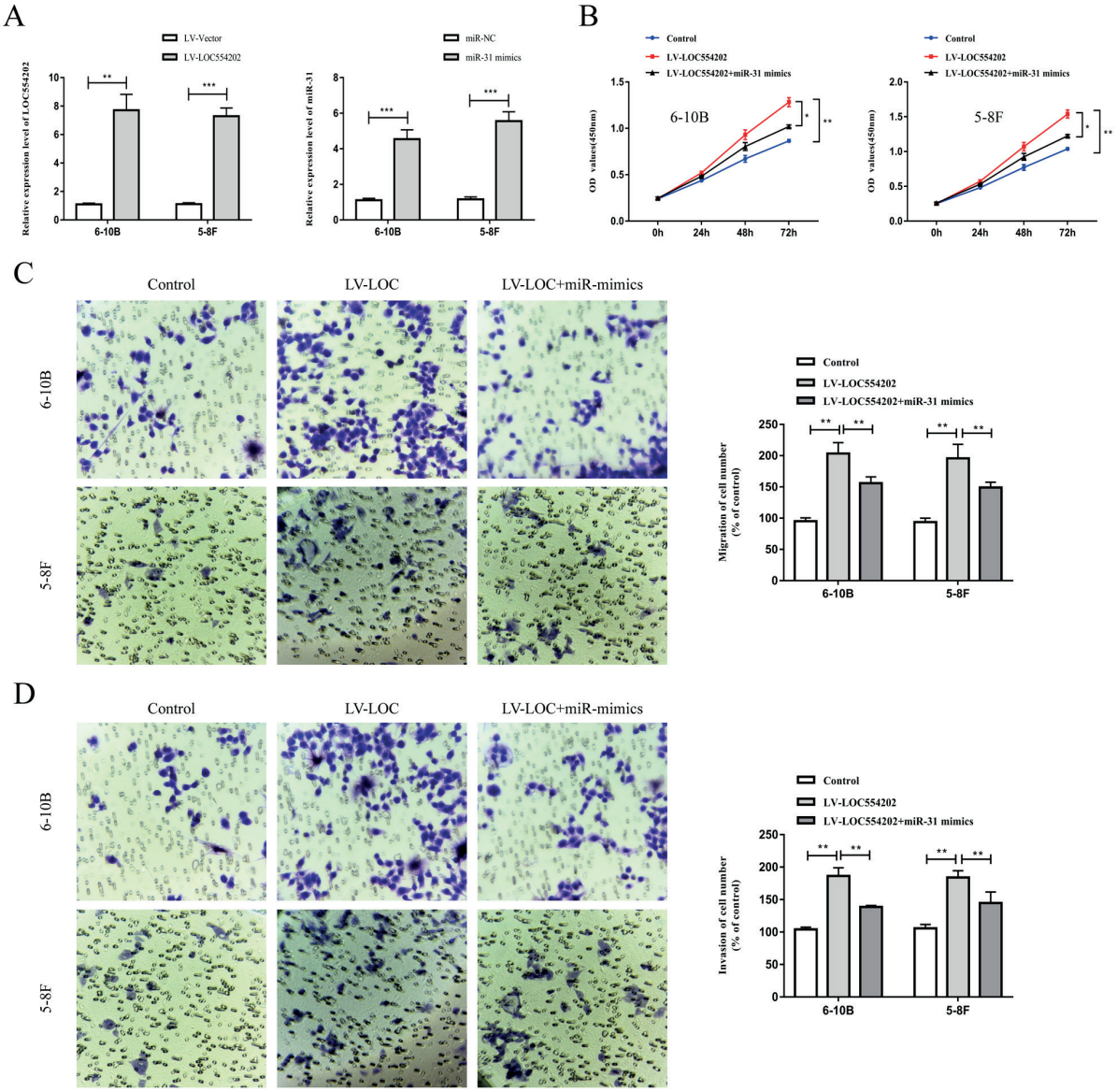

Figure 3. Overexpression of miR-31 partially reversed the biological effects caused by overexpression of LOC554202. **A,** The expression of LOC554202 in 6-10B and 5-8F cells transfected with LV-NC and LV-LOC554202 was detected by qRT-PCR. **B,** The expression of miR-31 in 6-10B and 5-8F cells transfected with miR-NC and miR-31 mimics was indicated by qRT-PCR. **C,** The effects of LOC554202 and miR-31 on the proliferation of 6-10B and 5-8F cells were tested by CCK8 experiment, (magnification: 20×). **D,** The effects of LOC554202 and miR-31 on the migration of 6-10B and 5-8F cells were tested by transwell migration experiments, (magnification: 20×). *p<0.05, **p<0.01, ***p<0.001

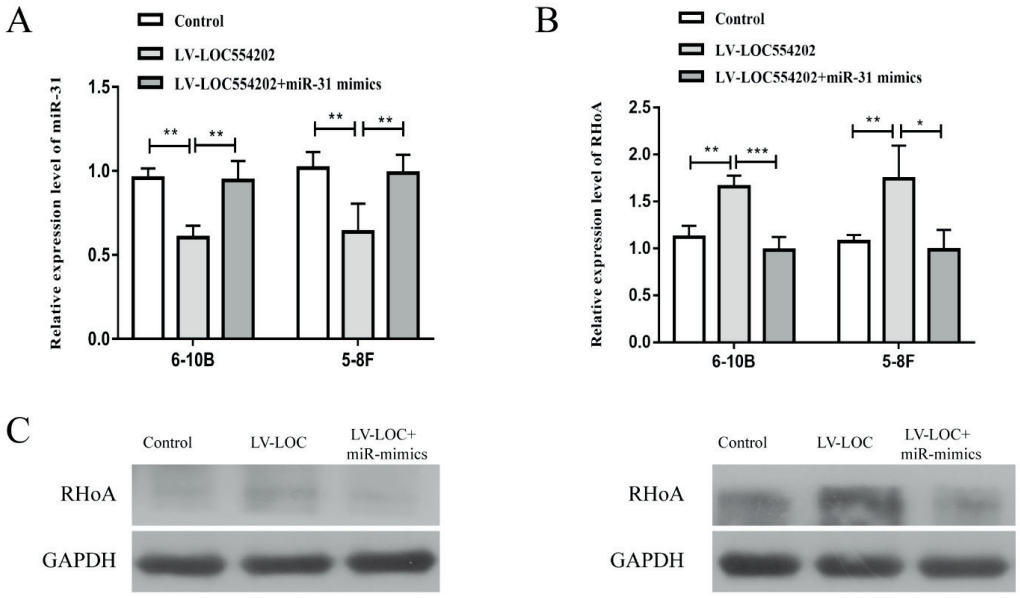

Figure 4. LOC554202 inhibited miR-31 expression and promoted RhoA expression. **A,** After LOC554202 and miR-31 were overexpressed simultaneously in 6-10B and 5-8F cells, miR-31 expression level was detected by qRT-PCR. **B,** After LOC554202 and miR-31 were overexpressed simultaneously in 6-10B and 5-8F cells, RhoA mRNA expression level was detected by qRT-PCR. **C,** After LOC554202 and miR-31 were overexpressed simultaneously in 6-10B and 5-8F cells, RhoA protein expression level was detected by Western blot. *p<0.05, **p<0.01, ***p<0.001

RhoA, as a small G protein with a molecular weight of 20-30 ldga in the Rho subfamily, is an important intracellular signaling molecule that can regulate cell growth, apoptosis and cell cycle by regulating cytoskeletal activity and cell morphological changes[22]. Intracellular RhoA mainly exists in two forms, namely, active type (RhoA bound to GTP) and inactivated type (RhoA bound to GDP)[23]. The active RhoA regulates actomiosin-dependent cell contraction and movement by regulating a variety of effectors, including ROCKl, mDia and PKN, and promotes tumor development by activating the STAT3 signaling pathway[24,25].

In this study, it was found that in comparison to that in control normal tissue samples, LOC554202 gene expression in NPC tissues was remarkably increased, while microRNA-31 expression was oppositely reduced. *In vitro* cell assays demonstrated that overexpression of LOC554202 significantly promoted migration and proliferation of NPC cells, while it was inhibited by overexpression of microRNA-31. LOC554202, as the host gene of microRNA-31, enhances RhoA expression, the target gene of microRNA-31, thus participating in the progression of NPC. It was confirmed that overexpression of RhoA can promote migration and proliferation of NPC cells. However, there are still many shortcomings in this study. It is still unknown whether LOC554202 can regulate RhoA expression through other mechanisms, such as the binding of other miRNAs through the ceRNA mechanism. Further, the function and mechanism of LOC554202 need to be further verified in animal experiments.

Conclusions

Taken together, the above data showed that LOC554202, abnormally highly expressed in NPC, could accelerate the proliferation rate and metastasis of NPC cells by suppressing microRNA-31 and promoting RhoA expression. Therefore, LOC554202/microRNA-31/RhoA axis may be a potential therapeutic target for NPC treatment. This study is expected to provide a new perspective for the occurrence and development mechanism of nasopharyngeal carcinoma, as well as new ideas for the clinical diagnosis and treatment of NPC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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