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Long non-coding RNA OR3A4 is associated with poor prognosis of human non-small cell lung cancer and regulates cell proliferation *via* up-regulating SOX4

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Abstract. – OBJECTIVE: **Recent studies have uncovered that long noncoding RNAs (lncRNAs) play a crucial role in the progression of malignant tumors. Non-small cell lung cancer (NSCLC) is a common type of fatal cancer worldwide. The aim of this study was to identify the specific function of lncRNA OR3A4 in the progression of NSCLC, and to explore the po[illegible] underlying mechanism.**

PATIENTS AND METHODS: **LncRNA O[illegible]A4 expression in 52-paired NSCLC tissues an[illegible]jacent normal tissues was detected by quan[illegible]tive real-time polymerase chai[illegible]tion (q[illegible]PCR). Cell proliferation assa[illegible]ptos[illegible] assay were used to inves[illegible]te the [illegible]ction o[illegible] OR3A4 in NSCLC. Fur[illegible]ore, t[illegible] underlying mechanism was [illegible] Western blot assay[illegible]**

RESULTS: **OR3[illegible] expressio[illegible] remarkably upregulated in [illegible] tissues w[illegible] compared with adjacent [illegible]rma[illegible]ues. The o[illegible]ll survival of NSCL[illegible] patients [illegible]h OR3A4 expression group w[illegible] significantly [illegible] than those in low OR3A[illegible]xpression group. [illegible]r the silence of OR[illegible] the p[illegible]oliferation of NSCLC cells was sig[illegible]tly [illegible]ibited. Besides, the apoptosis of NS[illegible]s was [illegible]markably promoted after [illegible] sile[illegible]f OR[illegible]. Meanwhile, knockdown [illegible]3A4 [illegible]fi[illegible]ly down-regulated the mRN[illegible]nd prot[illegible]vels of SOX4 in NSCLC cells. Fu[illegible]ermore, t[illegible] expression of SOX4 was found [illegible] in both NSCLC tissues and cells.**

CO[illegible]SIONS: **These above results suggest[illegible] that OR3A4 could promote cell proliferation [illegible] suppress cell apoptosis in NSCLC through u[illegible]egulating SOX4. Our findings demonstrated that OR3A4 might serve as a new therapeutic intervention for NSCLC patients.**

Key Words

KLong noncoding RNA, OR3A4, NSCLC, SOX4.

Intr[illegible]ction

[illegible]ung cancer is one of th[illegible] leading causes of cancer-related death[illegible] worldwide, remaining a public th[illegible] to the who[illegible]ociety[1]. As the main subtype of lun[illegible]cer, non[illegible]all cell lung cancer (NSCLC) accou[illegible] of all lung cancer cases[2]. During the past three decades, the mortality of NSCLC has [illegible]ed for more than four times. It is estimated [illegible]LC may remain the major health problem for the next 50 years at least[3,4]. Dramatic advances have been made in molecular tumorigenesis and therapeutic strategies for NSCLC. However, during the past decades, the 5-year survival rate of NSCLC patients is still less than 15%[5]. Therefore, there is an urgent need to realize the underlying mechanism and search for novel treatment strategy for NSCLC. Increasing evidence revealed that long noncoding RNAs (lncRNAs) are related to various cellular functions, including cell proliferation and apoptosis. For example, up-regulation of lncRNA LINC01510 is negatively associated with the prognosis of patients with colorectal cancer, which may offer a potential independent prognostic biomarker[6]. LncRNA MALAT1 promotes the progression of oral squamous cell carcinoma by inducing epithelial-mesenchymal transition (EMT)[7]. Through targeting miR-221/SOCS3, lncRNA GAS5 suppresses the proliferation and metastasis, whereas promotes gemcitabine resistance in pancreatic cancer[8]. Silence of lncRNA TUG1 inhibits the proliferation and migration of renal cell carcinoma (RCC) cells through regulating YAP expression by binding to miR-9[9]. Another study has indicated that lncRNA MEG3 inhibits proliferation and metastasis of osteosarcoma through depressing Notch

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and transforming growth factor (TGF)-beta signaling pathway[10]. In addition, silence of lncRNA OIP5-AS1 inhibits the proliferation, migration and promotes the apoptosis of glioma by blocking Wnt-7b/beta-catenin pathway and up-regulating miR-410[11]. In the present study, the results revealed that lncRNA OR3A4 expression in NSCLC samples was significantly up-regulated. Moreover, *in vitro* experiments demonstrated that OR3A4 significantly regulated the apoptosis and proliferation of NSCLC cells. Furthermore, our findings discovered that lncRNA OR3A4 played its function in NSCLC cells by regulating SOX4.

Patients and Methods

Patients and Cells

Human NSCLC tissues were collected from 52 NSCLC patients receiving surgery at China-Japan Union Hospital of Jilin University between 2009 and 2013. All collected tissues were stored at –80°C for subsequent use. Written informed consent was obtained from each subject before the surgery. This study was approved by the [illegible]ics Committee of China-Japan Union Hos[illegible] Jilin University. Four NSCLC cell lines (H[illegible]99, SPCA1, PC-9 and A549), as well as one no[illegible]l human bronchial epithelial cell line (16HBE) w[illegible] purchased from Chinese Aca[illegible] Scienc[illegible]. All cells were cultured in [illegible]becco[illegible] Modifie[illegible] Eagle's Medium (DMEM[illegible]ermo F[illegible]er Scientific, Inc., Waltham, M[illegible]A, [illegible] co[illegible] 10% fetal bovine serum ([illegible]S; The[illegible]sher S[illegible]ific, Inc., Waltham[illegible] USA), an[illegible]tained in a humidified in[illegible]h 5% CO_2 [illegible]C.

Cell Tra[illegible]ection

Le[illegible]rus expressing [illegible]t-hairpin RNA (sh[illegible]; Bios[illegible]ttiaInc., San Diego, CA, USA) aga[illegible] OR[illegible] was first synthesized. OR3A4 shRNA[illegible]R3A4) a[illegible] empty vector (EV) were [illegible] tran[illegible]d i[illegible] H1299 cells according to sp[illegible]ic inst[illegible]s. Transfection efficiency was ve[illegible]ed by q[illegible]antitative real-time polymerase [illegible]tion (qRT-PCR) 48 h after transfection.

[illegible]A Extraction and qRT-PCR

[illegible]tal RNA in NSCLC samples and cells wa[illegible] extracted in strict accordance with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNAs) using reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). QRT-PCR was conducted on ABI 7500 system (Applied Biosystems, Foster City, CA, USA), and SYBR Green real-time PCR was applied. Primers used in this study were as follows: OR3A4, forward 5′-CCTATCCCTTTCTCTAAGAA-3′ a[illegible] [illegible]verse 5′-ACTTCTGCAAAAACG[illegible]TG-3′; SOX4, forward 5′-CTTGACAT[illegible]TAGCT-GGCATGATT-3′ and reverse [illegible]CTGTG-CAATATGCCGTGTAGA-3′; GAPD[illegible]orward 5′-CCAAAATCAGATGG[illegible]CAATG[illegible]G-3′ and reverse 5′-TGATG[illegible]ATGGACTG[illegible]T-CATTCA-3′. The therm[illegible]ycle wa[illegible]s follow[illegible] s at 95°C, 5 s at 95°C [illegible]es, and 35 s at 60°C.

Cell Prolif[illegible] Assay

Prolifera[illegible]ion o[illegible]sfected ce[illegible]s in 96-well plates [illegible] monitored [illegible] 24 h according to the ins[illegible]s of cell coun[illegible]kit-8 (CCK-8) assay ([illegible]indo Molecular Technologies, Inc., Kuma-m[illegible]o, Japan). A[illegible]orbance at 450 nm was measu[illegible] by a specti[illegible]hotometer (Thermo Scientific, Wa[illegible]n, MA, [illegible]A).

Colony Formation Assay

[illegible]299 cells at a concentration of 1.5×10^3 cells/[illegible]e first seeded into 6-well plates. 10 days later, formed colonies were fixed with 10% formaldehyde for 30 min and stained with 0.5% crystal violet for 5 min. Finally, Image-Pro Plus 6.0 (Silver Springs, MD, USA) was used for data analysis.

Flow Cytometry

Annexin-V-FITC (fluorescein isothiocyanate) apoptosis detection kit (BD, Franklin Lakes, NJ, USA) was used to detect the apoptosis of NSCLC cells. Briefly, harvested cells were first washed twice with ice-cold phosphate buffered saline (PBS). Subsequently, 100 µL flow cytometry binding buffer was added. After mixing 5 µL Annexin V/FITC and 5 µL Propidium Iodide (PI), the cells were stained at room temperature for 15 min in dark. 400 µL binding buffer was then added in each tube. FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyze apoptotic cells.

Western Blot Analysis

Total protein in cells was extracted using radio-immunoprecipitation assay (RIPA). Subsequently, the concentration of extracted protein was quantified by the bicinchoninic acid (BCA)

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protein assay kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the membranes were incubated the primary and secondary antibodies. Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-GAPDH and rabbit anti-SOX4, as well as goat anti-rabbit secondary antibody. Chemiluminescent film was applied to assess the expression of proteins with Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Student *t*-test and Kaplan-Meier method were chosen when appropriate. Experimental results were presented as mean ± SD (Standard Deviation). $p<0.05$ was considered statistically significant.

Results

Expression Level of OR3A4 in NSCLC Tissues and Cells

QRT-PCR was first conduct[illegible]ct OR3[illegible] expression in 52 patients' t[illegible]es a[illegible] NSCL[illegible] cell lines. As a result, O[illegible]4 was s[illegible]ificantly upregulated in NSCL[illegible] tis[illegible]am[illegible]hat of adjacent tissues [illegible]gure 1[illegible]mpare[illegible]ith 16HBE cells, the[illegible]ression le[illegible] OR3A4 in NSCLC cells [illegible]s[illegible]cantly up-r[illegible]ted (Figure 1B). Bas[illegible]on the [illegible]ge expression level of OR3A4, 52 NSCLC patients were divided into two groups, including high OR3A4 expression group and low OR3A4 expression group. Kaplan-Meier analysis showed that the overall survival of NSCLC patients in high OR3A4 expression group was significantly worse than those in low O[illegible] expression group (Figure 1C).

OR3A4 Knockdown Inhibite[illegible]he Proliferation of NSCLC Cells [illegible]itro

According to OR3A4 [illegible]pression [illegible] NSCLC cells, H1299 NSCL[illegible]ells were cho[illegible] OR3A4 knockdown. O[illegible]4 shR[illegible] (sh-OR[illegible]) and corresponding empt[illegible]to[illegible](EV) were synthetized and tran[illegible]cted in[illegible]299 cell[illegible]ransfection efficie[illegible] of OR3A4 [illegible]determined by qRT-PCR ([illegible]A). Subseq[illegible] cell proliferation assay foun[illegible]t knockdown of OR3A4 signif[illegible]ly inhibite[illegible]CLC cell proliferation (Fi[illegible]). Furthermo[illegible]sults of colony form[illegible]on assay showed that [illegible]e number of formed c[illegible]nies of NS[illegible]C cells was significantly redu[illegible] after OR3[illegible] knockdown (Figure 2C).

OR3[illegible]down Promoted the Apoptosis of NSCLC Cells

[illegible]w cytometry was then applied to explore [illegible]e[illegible]t of OR3A4 on NSCLC cell apoptosis. The results revealed that after OR3A4 knockdown in NSCLC cells, the apoptosis rate of H1299 NSCLC cells was remarkably increased (Figure 3).

The Association Between OR3A4 and SOX4 in NSCLC

QRT-PCR results showed that the mRNA expression of SOX4 was significantly down-regulated in NSCLC cells transfected with OR3A4

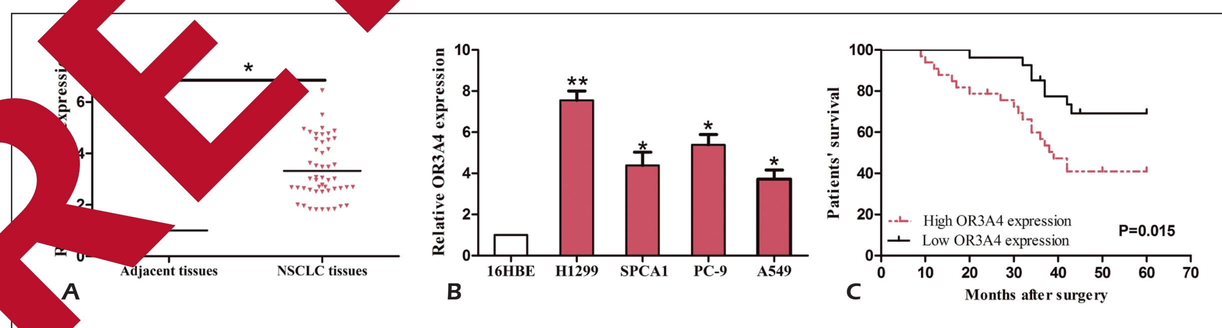

Figure 1. Expression level of OR3A4 was significantly increased in NSCLC tissues and cell lines. ***A***, OR3A4 expression was significantly increased in NSCLC tissues when compared with adjacent normal tissues. ***B***, Expression level of OR3A4 relative to GAPDH was determined in human NSCLC cell lines and normal human bronchial epithelial cell line (16HBE) by qRT-PCR as well. ***C***, High expression level of OR3A4 was associated with worse overall survival of NSCLC patients. Data were presented as mean ± standard error of the mean. $*p<0.05$. $**p<0.01$.

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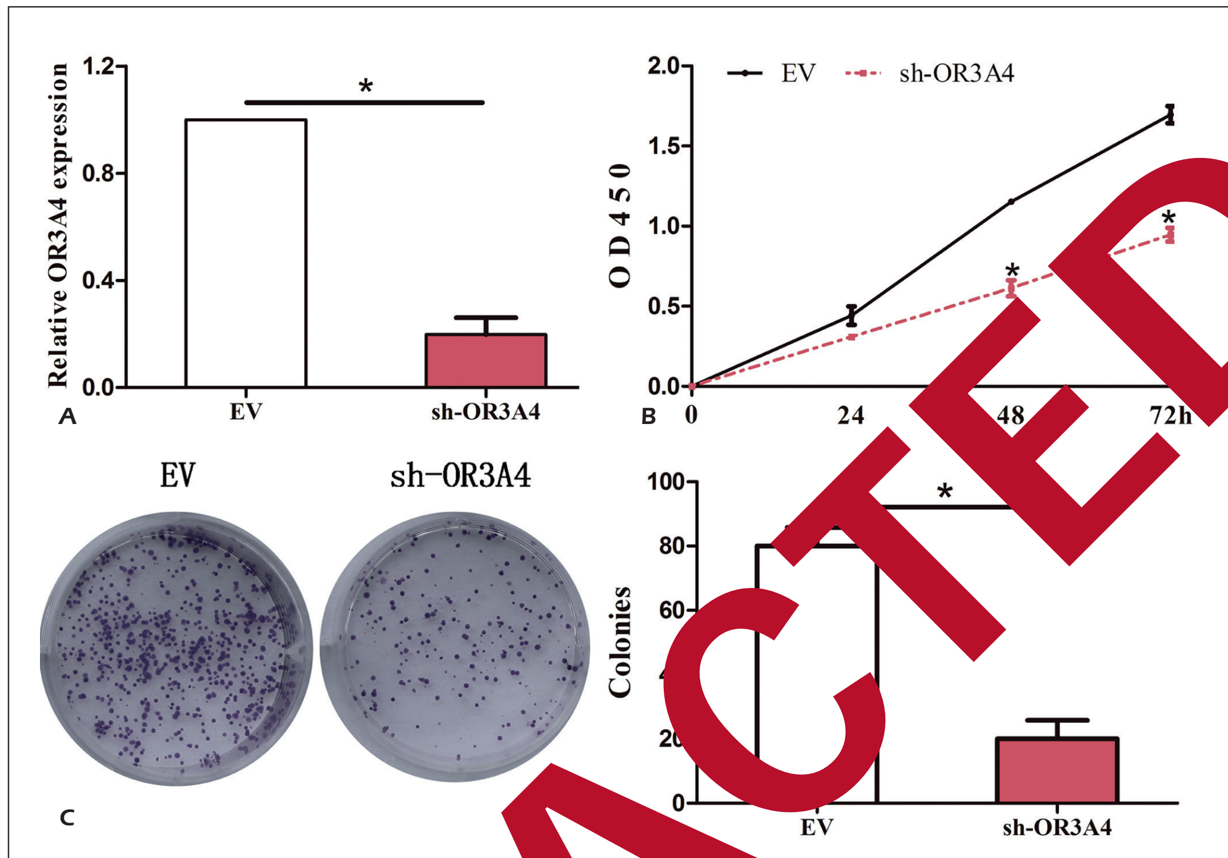

Figure 2. Knockdown of OR3A4 inhibited NSCLC ce[illegible]li[illegible]tion. ***A***, OR3A4 expression in NSCLC cells transduced with empty vector (EV) or OR3A4 shRNA (sh-OR3A4) was [illegible]ed by qRT-PCR. GAPDH was used as an internal control. ***B***, CCK8 assay showed that the viabilit[illegible]NSCLC c[illegible] OR3A4 shRNA (sh-OR3A4) group was significantly inhibited when compared with empty vect[illegible]V) gro[illegible] ***C***, Colony [illegible]ation assay showed that knockdown of OR3A4 significantly decreased the growth ability o[illegible]99 NSCL[illegible]ells. The r[illegible]lts represented the average of three independent experiments (mean ± standard error of the me[illegible]*p*<0.0[illegible]<0.01.

shRNA (Figure 4[illegible] Western blo[illegible]lysis results further demon[illegible]at the prote[illegible]pression level of SOX4 was r[illegible]kably down-regulated in NSCLC cells transfected with OR3A4 shRNA (Figure 4B). To explore the interaction between OR3A4 and SOX4, the expression level of SOX4

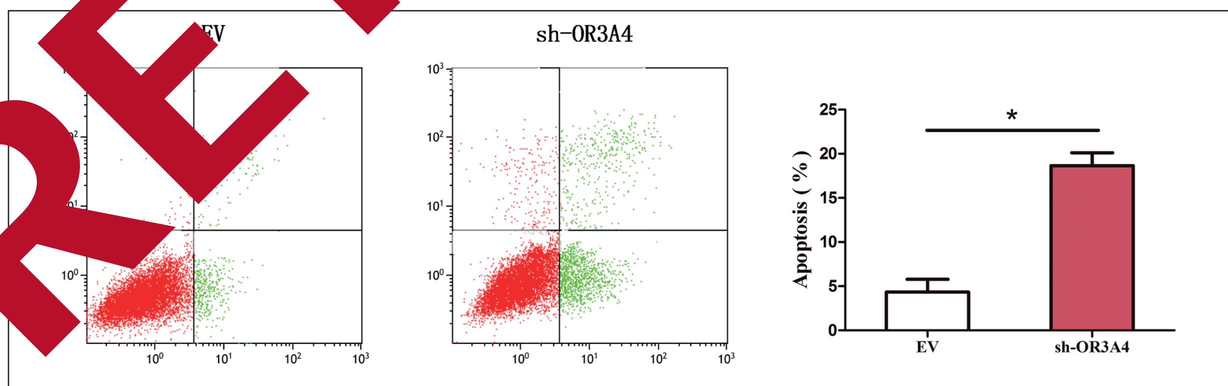

Figure 3. Knockdown of OR3A4 promoted NSCLC cell apoptosis. Apoptosis assay showed that the apoptosis rate of NSCLC cells was significantly promoted after knockdown of OR3A4 in NSCLC cells. The results represented the average of three independent experiments (mean ± standard error of the mean). **p*<0.05. ***p*<0.01.

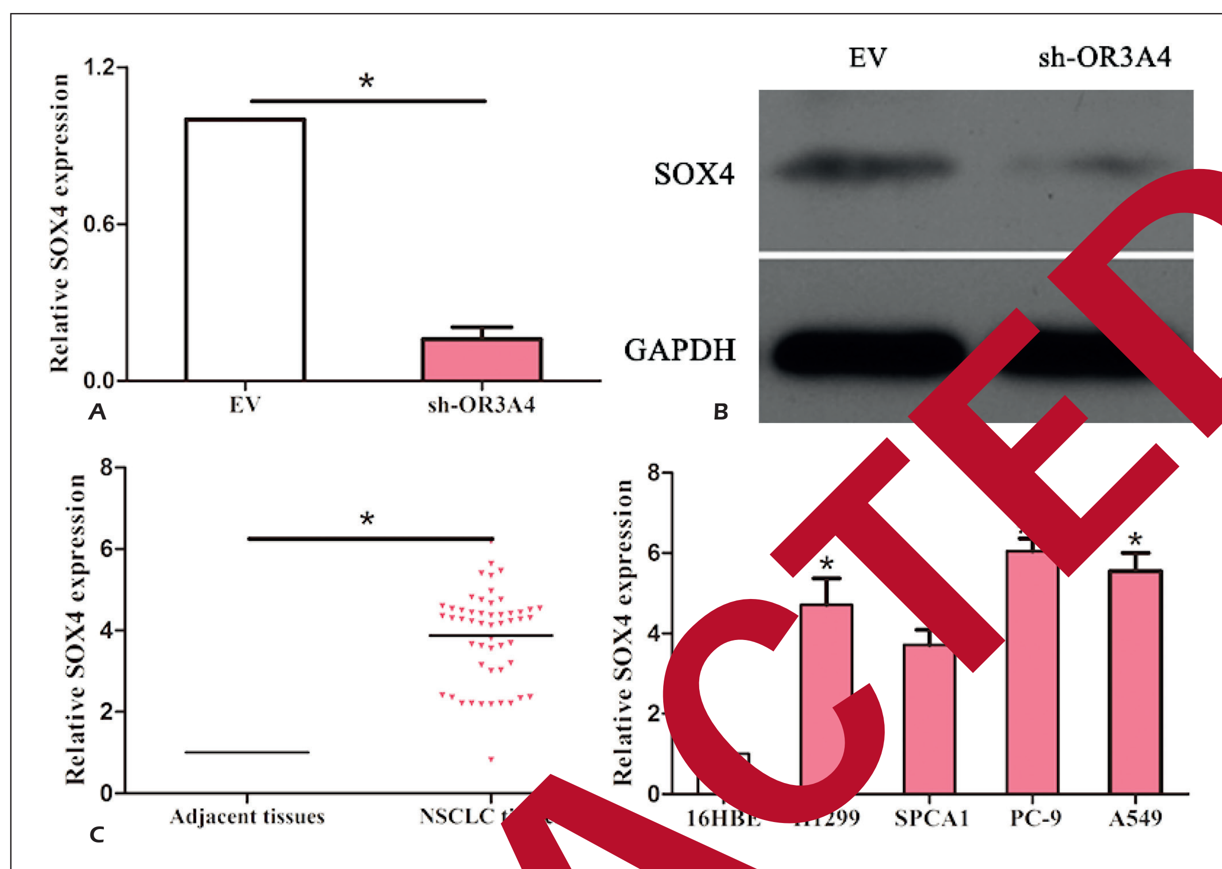

Figure 4. Interaction between SOX4 and OR3A4 in [illegible], The mRNA expression level of SOX4 in H1299 cells of OR3A4 shRNA (sh-OR3A4) group wa[illegible]ficantly d[illegible]d when compared with empty vector (EV) group. ***B***, Protein expression of SOX4 in H1299 cells [illegible]ly decre[illegible] after knockdown of OR3A4. ***C***, SOX4 was significantly up-regulated in NSCLC tissues when co[illegible]red wit[illegible]acent no[illegible]issues. ***D***, SOX4 was significantly up-regulated in human NSCLC cell lines when compare[illegible] normal h[illegible]an bronchi[illegible]pithelial cell line (16HBE). The results represented the average of three independent experimen[illegible] we[illegible] as mean ± standard error of the mean. *$p<0.05$. **$p<0.01$.

in NSCLC tiss[illegible] a[illegible]lls was det[illegible]. As a result, SOX4 expression [illegible]SCLC tissues was obviously hi[illegible]er than that o[illegible]cent normal tissues (Figur[illegible]C). Similarly, the e[illegible]ssion of SOX4 in NS[illegible] cells w[illegible]s significantly higher when compare[illegible]h t[illegible] of 16HBE cells (Figure 4D).

[illegible]iscussion

[illegible]NAs have been emerged as important [illegible]egulat[illegible]n the progression of NSCLC. For in[illegible]nce, by sponging miR-124-3p, lncRNA OG-[illegible] participates in regulating the proliferation of NSCLC cells[12]. LncRNA PRNCR1 functions as an oncogene in NSCLC and promotes tumor progression through regulating miR-488-HEY2 signal network[13]. Knockdown of lncRNA CRNDE inhibits the proliferation, migration and invasion of NSCLC cells by targeting the downstream target of microRNA-338-3p[14]. Other studies have shown that overexpression of lncRNA-p21 suppresses the apoptosis of NSCLC cells by directly down-regulating the expression level of PUMA[15]. Moreover, up-regulation of lncRNA FENDRR inhibits the proliferation and malignancy of NSCLC by serving as a sponge of miR-761[16]. LncRNA Olfactory receptor family 3 subfamily A member 4 (OR3A4) (Accession Number: NR_024128.1) has recently been explored to be exceptionally expressed. Previous studies have indicated that OR3A4 is correlated with the progression of several malignancies. For instance, OR3A4 promotes the growth, angiogenesis, migration and tumorigenesis of gastric cancer[17]. Overexpression of OR3A4 facilitates the proliferation and metastasis of breast cancer cells through inducing EMT[18]. In the present study, OR3A4 was found significantly up-regulated in

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both NSCLC tissues and cells. Besides, higher expression level of OR3A4 indicated worse prognosis of NSCLC patients. Furthermore, after OR3A4 knock-down, the growth ability of NSCLC cells was significantly inhibited, whereas cell apoptosis was promoted. These data suggested that OR3A4 functioned as an oncogene in NSCLC. Sex-determining region Y-related high-mobility group box 4 (SOX4), known as a transcription factor, has been identified as an oncogene in various malignancies. For example, SOX4 functions as an important molecular factor in leukemia. Up-regulation of SOX4 has been reported to be closely correlated with poor prognosis of acute myeloid leukemia[19]. By targeting EMT, SOX4 promotes the proliferation and migration of prostate cancer cells. This indicates that SOX4 may serve as a potential therapeutic target[20]. Moreover, SOX4 facilitates the invasion and metastasis of renal cell carcinoma by inducing EMT[21]. In addition, lncRNA TUG1 enhances the proliferation and inhibits the apoptosis of human osteosarcoma cells by downregulating SOX4[22]. In our study, the results of functional assays indicated that SOX4 was significantly down-regulated after OR3A4 knock-down *in vitro*. What's more, SOX4 expression was up-regulated in NSCLC tissues and cells. The above results all revealed that OR3A4 might exert its function in NSCLC *via* up-regulating SOX4.

Conclusions

Our research suggests that lncRNA OR3A4 may serve as a new biomarker in the development of NSCLC. The results [illegible] indicate that lncRNA OR3A4 plays an important role in the carcinogenesis of NSCLC, and [illegible] could be a promising marker for NSCLC.

Conflict of Interests

The authors declared no conflict of interest.

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