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Original Article

Long chain non coding TUG1 regulating lung cancer invasion and proliferation by targeting Mir-300 in A549 cell line

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Abstract: Objective: To investigate the expression of long-chain non-coding RNA TUG1 in lung cancer and the mechanism of action of TUG1 and miR-300 in the invasion and proliferation of lung cancer cells. Methods: The expression of TUG1 in different lung cancer tissues and paracancer tissues was detected by qPCR. Transwell invasion assay was used to detect the invasion ability of lung cancer cells after silencing TUG1, and the platelet clone formation assay was used to detect the proliferation of lung cancer cells after silencing TUG1. The interaction between TUG1 and miR-300 was detected by Dual-luciferase Reporter (DLR). The effect of overexpression miR-300 on recovering invasion ability of lung cancer after silencing TUG1 was detected through Transwell invasion assay, and the effect on recovering of proliferation ability was detected through cloning formating assays. In addition, western blot assay was used to investigate the expression of TLR signal pathway protein. The tumor size and volume of lung cancer was detected by subcutaneous tumorigenesis in nude mice. Results: Compared with paracancer tissues, the expression of TUG1 in lung cancer tissues was increased significantly, and with the progress of tumor stage the expression of TUG1 was gradually increased. That is to say, the expression of TUG1 was positively correlated with pathological stage of lung cancer and local lymphatic metastasis. The expression of A549 in lung cancer cell A549 was the highest. The invasion and proliferation of lung cancer cells can be inhibited by silencing TUG1. TUG1 can specially bind to 3'UTR of miR-300 TUG1. After silencing TUG1, overexpression of miR-300 could recover invasion and proliferation ability of lung cancer cell, and the down regulated expression of TLR pathway protein was also recovered. Compared with LV3-TUG1 group, the tumor volume and weight of LV3-TUG1+miR-300-mimic tumor-bearing mice is significantly increased. Conclusion: TUG1 plays an important role in the development and progression of lung cancer. TUG1 can regulate the invasion and proliferation ability of lung cancer cells by targeting miR-300 through TLR signaling pathway.

Keywords: TUG1, lung cancer, MiR-300, invasion, proliferation

Introduction

Lung cancer is a kind of tumor behaved highest incidence and mortality all over the world. Although the surgical treatment, chemotherapy, radiotherapy and targeted therapy have made great progress recently, the five-year survival rate of lung cancer patients is still only 15%, and improved little in the last two decades [1, 2]. The failure of treatment patient death is mainly caused by recurrence and distant metastasis of lung cancer [3]. Therefore, exploring new diagnostic and therapeutic molecular targets, finding new genes and targets related to lung cancer metastasis, and clarifying the mechanism of lung cancer metastasis becomes the focus of the majority of researchers.

Long-non-coding RNAs (lncRNAs) are RNA molecules with more than 200 nucleotides in transcribe length [4]. With development of research in recent years, the understanding of lncRNAs has transferred from the initial "transcriptional noise" to regulating gene expression in multi aspects, like the apparent genetic level, transcription level and post-transcriptional level, to effect the occurrence and progress of various diseases [5]. Yuan et al. [6] found that lncRNA MVIH in liver cancer patients behaves high expression, and the expression level is closely

related to tumor cell microvascular infiltration, stage and prognosis. In vivo experiments also confirmed that MVIH can activate angiogenesis to promote growth and migration of tumor, which suggests that MVIH can be used as an independent risk factor for hepatocellular carcinoma. In addition, lncRNA can interact with miRNA to affect the function of the body. Cai et al. [7] showed that the expression of lncRNA was up-regulated in vascular endothelial cells of glioma and affected the permeability of blood-brain barrier by interacting with miR-144, which affected the therapeutic effect of chemotherapeutic drugs. LncRNA TUG1 (taurine up-regulated 1) is located in the 22q12 region of chromosome. A lot of studies have shown that TUG1 is closely related to initiation and development of tumor [8]. However, researches about TUG1 in lung cancer are still little. Exploring the expression of TUG1 in lung cancer and its role in the initiation and progression of lung cancer may provide new methods and new ideas for early diagnosis and treatment of lung cancer.

Materials and methods

Samples collection

The tumor tissue and normal paracancer tissue of lung cancer patients was selected from 82 cases treated in our hospital from August 2015 to August 2016. Postoperative pathologic diagnosis, histological grade, lymph node metastasis, and clinic pathological staging were redefined by two sub-titles of pathology diagnostic physicians according to WHO guidelines and Frank's reports. Resected hilum, mediastinum and lymph nodes of lung during routine pathology surgical were used to determine the lymph node metastasis of lung cancer. In vitro tumor tissue was divided into three parts, one of which was quickly putted into the RNA preservation solution. The other one of the tissue was washed with cold phosphate buffer treated with coke diethyl carbonate ester (DEPC) to remove the blood and quickly putted into liquid nitrogen for cryopreservation.

Cell lines

Human lung cancer cells H1299 and A549 were purchased from Wuhan Cell Collection Centre, and were cryopreserved in liquid nitrogen. After the recovery, A549 cells were cultured in DMEM medium containing 10% fetal bovine serum, and H2199 were cultured in 1640 medium containing 10% fetal bovine serum, under 37°C and 5% CO_2 saturation humidity. Fetal bovine serum, RPMI 1640, and DMEM medium was purchased from Gibco Corporation. Transwell Chamber was purchased from Corning Corporation. Matrigel was purchased from Becton, Dickinson and Company (BD, US). Lipofectamine 2000 transfection reagent was purchased from Invitrogen Company. MiR-300-inhibitor and negative control was purchased from Shanghai Gemma gene. Trizol was purchased from Japan Takara Corporation. Reverse transcription kit (FSQ-101) was purchased from Takara Corporation. PCR kit was purchased from Japan Takara Corporation. The luciferase activity assay kit (Prl-TK) was purchased from Promega Corporation (Promega Biotech Co., Beijing, China). The luciferase reporter vector was synthesized by Promega Corporation (Promega Biotech Co., Beijing, China).

Quantitative real-time polymerase chain reaction

The tissue was removed from liquid nitrogen and grinded into powder, following precooling of the mortar. 2-3 mL of Trizol reagent was added and well mixed. The total RNA was extracted according to Trizol operating instructions. The cells of the different groups were inoculated to culture flask respectively with inoculation density of 1×10^6/L and culture cell fusion degree of about 80%. The total RNA cells were extracted following Trizol operation instructions. The concentration and purification of the nucleic acid was measured using an ultraviolet spectrophotometer. The upstream primer of TUG1 was 5'-TAAGCGCCCTCCACTCCAGAT-3', and the downstream primer was 5'-AGGCACCAGCTTCAAAACCC-3'. The upstream primer of MiR-300 was 5'-GGAGGTTAATGCTAATCGTGATAG-3', and downstream primer was 5'-GTGCAGGGTCCGAGGT-3'. All groups were diluted to the same concentration of total RNA with DEPC. The reagents were added in step-by-step, and the final cDNA was stored at -20°C after the reaction. The reaction system was prepared according to the operating instructions of Takara Corporation. The volume of each reaction system was 20 μL, and three wells were set up for each sample. Fluorescence quantitative PCR amplification of

the target RNA and GAPDH was carried out for each group of cDNAs. According to ΔΔCT method, the reaction conditions to calculate and detect gene were as follows: 37°C 15 min, 98°C 5 min. Then PCR reaction was carried out following the PCR kit instructions and the obtained data was used to calculate the amount of mRNA expression by $RQ=2^{-\Delta\Delta CT}$.

Cell transfection

Five days before the experiment, 5×10^3 A549 cells were inoculated into 96-well plates with cell fusion degree of 40%~60%. According to the Gemma Lentiviral Operation Manual, the suitable MOI (Multiply of Infection) of TUG1 lentivirus was measured with a gradient of 0, 10, and 100. The steps were as follows:

Two sterile EP tubes were prepared. 10 μl of 1×10^8 TU/ml virus was added to the first tube, and gently mixed avoiding forming foam. Then 10 ul of virus from the first tube was added into the second tube and mixed well. Get three virus groups in different gradient: stock solution, 10 times dilution, 100 times dilution.

10 ul of virus solution in three different gradients was added to three holes of each group. As the calculation shows, the MOI of the three holes were 100, 10, and 1 respectively. The optimum MOI is 100.

The experiment was divided into two groups: the silent group was transfected with TUG1 silencing lentivirus (LV3-TUG1), and the control group was transfected with negative control lentivirus (NC). 100 times diluted virus solution was added into LV3-TUG1 group, and 100 times the negative control virus was added into NC group. The fluorescent expression of GFP was observed after 24 h.

Transwell invasion assays

Lung cancer A549 cells in the logarithmic growth phase were taken. The digested cells were cultured in serum-free medium and made cell suspension in density of 8×10^7/L. 200 uL of cell suspension was added into the upper chamber of each group of the Transwell chambers, and 600 uL of culture medium containing 10% fetal bovine serum was added into the lower chamber. 3 repeated wells were set for each group and cultured at 37°C and 5% CO_2 for 24 h. Transwell chamber was removed and the matrix glue and the dis-perforating cells were wipe away with the cotton swab gently. The cells were fixed with methanol for 10 min, stained with 0.1% of crystal violet for 40 min. The number of perforated cells was counted under 100 times magnification and five visual fields were selected randomly to take the average. The experiment was repeated three times.

Plate colony assay

The cells in different logarithmic growth stage from each group were seeded in 6-well plates. 5 ml of sterile PBS solution was added, and the cells were gently washed and centrifuged. After repeated three times, the single cell suspension was prepared. The cell concentration was measured and adjusted to 1000 cells/ml. The single cell suspension was uniformly grown in a sterile 6-well plate and subjected to plate cloning experiments. Two weeks later, the formation of plate clonal cells was observed and the cell proliferation rate was calculated. The experiments were repeated three times.

Luciferase activity assays

The experiments were conducted following Lipofectamine 2000 instructions.

On the day before transfection, 5×10^6 293T cells in the logarithmic growth phase were picked and counted by routine trypsin digestion. The cells were inoculated into 6-well plates and the DMEM medium with 10% fetal bovine serum was changed 1 h before transfection, followed by culturing at 37°C and 5% CO_2. The luciferase reporter vector and LV3-TUG1 was co-transfected into 293T cells. After 293T cell fusion degree reached 60%, it was washed with PBS solution twice. And Lipofectamine 2000 was added and incubated at room temperature for 20 min. Then the recombinant plasmids and miRNAs were co-transfected into 293T cells, followed by changing culture medium and conducting double luciferase reporter system examination. The relative luciferase activity was calculated by the equation: Relative luciferase activity = firefly luciferase activities value/bloody luciferase activity value.

Western blot

The total protein was extracted from the cells using RIPA lysate, and the protein concentration was determined by BCA protein concentra-

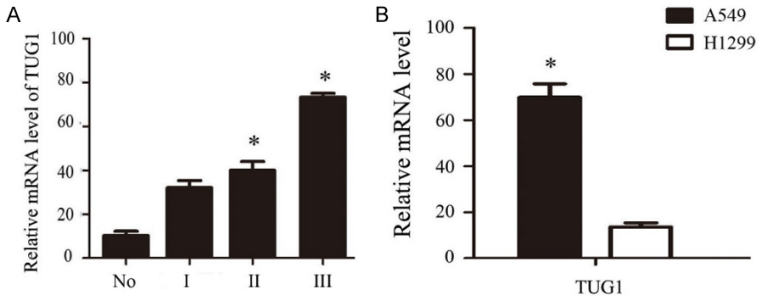

Figure 1. Increased Expression of TUG1 in lung, lung cancer tissues, and lung cancer cell lines. qPCR was employed to detect the expression of TUG1 in different stage of lung cancer (A) and para-cancerous tissue (B).

tion assay kit. Each group was added in buffer solution and boiled for 10 min. 20 ug of protein sample was taken for SDS-PAGE electrophoresis, using wet transfer method to transfer film. The film was closed by TBST containing 5% of skim milk powder for 2 h. After dilution and first resistance, the samples were incubated under 4°C overnight, and then washed by TBST. After washing, the secondary antibody was labeled by HRP, and incubated at 37°C for 2 h, followed by lighting under ECL luminescence system. The experiments were repeated three times.

Lung cancer xenografts

10 female nude mice in age 4-6 weeks and weighting 18-22 g were randomly chosen, and divided into two groups. The first group was inoculated with LV3-TUG1, and the second group was inoculated with LV3-TUG1+miR-300-mimic, with 5 mice in each group. The mice were fed in animal grade sterile feeding room without specific pathogen. Tumor cell suspension in concentration of 5×10^6~8×10^6/ml were inoculated in armpits. After the formation of subcutaneous tumor, the longest length (a) and shortest length (b) of tumor was measured with a vernier caliper measurement by the same experimental staff every three days. Nude mice were sacrificed 30 days later and the tumor tissue was removed under aseptic conditions. The tumor volume and weight was measured, and tumor volume was calculated by the followed equation: $V (mm^3) = 1/2 \times a \times b^2$.

Statistical analysis

The data was statistically treated using SPSS 21.0 software and expressed as mean standard deviation ±. The data were analyzed by one-way ANOVA. The data with heterogeneity of variance was treated by Karuskal-wallis method. The results with differences $P<0.05$ performed statistic meaning.

Results

Increased expression of TUG1 in lung, lung cancer tissues, and lung cancer cell lines

As shown in **Figure 1A**, the expression TUG1 in lung paracancer tissue was weaker. The expression of TUG1 in cancer tissue was significantly stronger than the paracancer tissue, and became stronger with the progress of the lung cancer level. The difference behaved statistically significant, indicating that TUG1 played an important role in promoting growth of lung cancer. The qPCR detection of TUG1 expression in different lung cancer cell lines (**Figure 1B**) showed that, compared with H1299 cells, A549 cells behaved significantly higher expression of TUG1. A549 cells were selected as the further experimental object.

Positive relationship between the expression of TGU1 and clinicopathological features of lung cancer

The relationship of the expression level of TUG1 with the age, sex, tumor size, tissue type, differentiation, lymph node metastasis and TNM staging in 82 patients with lung cancer was analyzed (**Table 1**). As the result showed, the high expression of TUG1 was positively correlated with TNM staging and local lymph node metastasis in lung cancer, and behaved no correlation with age, sex, and tumor type and differentiation level of lung cancer patients.

Decreased expression of miR-300 after silencing TUG1

The interaction between TUG1 and miR-300 has been predicted by bioinformatics tools. To investigate the possible interaction of miRNA with TUG1, we have processed further study through silencing TUG1 using lentivirus.

Table 1. Relationship between TGU1 expression and clinicopathological features of lung cancer

Clinicopathological data	Number	Low expression of TUG1	High expression of TUG1	χ^2	*P* value
Sex				16.25	0.96
Male	38	24 (63.16%)	14 (36.84%)		
Female	44	28 (63.67%)	16 (36.33%)		
Years				12.63	0.818
≤60	35	23 (65.74%)	12 (34.26%)		
>60	47	29 (61.70%)	18 (38.30%)		
Differentiation				10.06	0.174
High-middle	38	21 (55.26%)	17 (44.74%)		
Low	44	31 (70.45%)	13 (29.56%)		
Pathological staging				6.124	0.005
I	45	34 (75.56%)	11 (24.44%)		
II-III	37	21 (56.76%)	17 (43.24%)		
T1	35	26 (74.29%)	9 (25.71%)	4.98	0.105
T2-T4	47	26 (55.32%)	21 (44.68%)		
Lymph node metastasis				5.56	0.007
N0	51	38 (74.51%)	13 (25.49%)		
N1-N3	31	14 (45.16%)	17 (54.84%)		

As the results of Western blot showed, the expression of LV3-TUG1 was significantly lower than that of NC group (P<0.05). The difference behaved statistically significant, indicating that the expression of TUG1 can be silenced by LV3-TUG1.

The results from QPCR showed that the expression of miR-300 in LV3-TUG1 group was significantly lower than that in NC group (**Figure 2A**), which indicated that the expression of miR-300 was decreased after TUG1 was silenced.

The invasion and proliferation ability of lung cancer cells was inhibited after silencing TUG1

As the Transwell experiments showed (**Figure 2C**), the number of cancer cells through Matrigel in the LV3-TUG1 group was significantly lower than that of the NC group (P<0.01), which indicated that silencing TUG1 can inhibit the invasion of A549 cells of human lung cancer.

As the clone formation experiments showed (**Figure 2D**), the number of lung cancer cells in LV3-TUG1 group was significantly decreased compared with NC group (P<0.01), which indicating that silencing TUG1 can inhibit the proliferation of lung cancer A549 cells.

Targeting relationship of TUG1 and miR-300 through Luciferase reporter gene detection

As shown in previous experiment, TUG1 regulate expression of miR-300. To further verify whether TUG1 was bind to the 3'UTR of miR-300, LV3-TUG1 and miR-300 were co-transfected into lung cancer cells. Luciferase reporter gene results showed (**Figure 3**) that TUG1 can significantly inhibited luciferase activity in miR-300, and bind specifically to the 3'UTR of miR-300.

To further investigate the effect of miR-300 on the biological behavior of lung cancer, the miR-300-mimic was used to overexpress miR-300. The qPCR result showed that the expression of miR-300 in miR-300-mimic group was significantly higher than that in NC group (P<0.05), indicating that the efficiency of miR-300-mimic was in accordance with the experimental requirements.

Restoring effect of overexpress miR-300 on invasion and proliferation ability of lung cancer cells after silencing TUG1

The Transwell experiment showed that (**Figure 3C**), the number of lung cancer cell line A549 through Matrigel matrix in LV3-TUG1+miR-300-mimic group was significantly increased, compared with LV3-TUG1 group (P<0.01). So the invasion ability of human lung cancer A549 cells can be recovered through overexpress of miR-300 after silencing TUG1.

As the clone formation assay showed, the number of clone formed lung cancer cells in LV3-TUG1+miR-300-mimic group was significantly

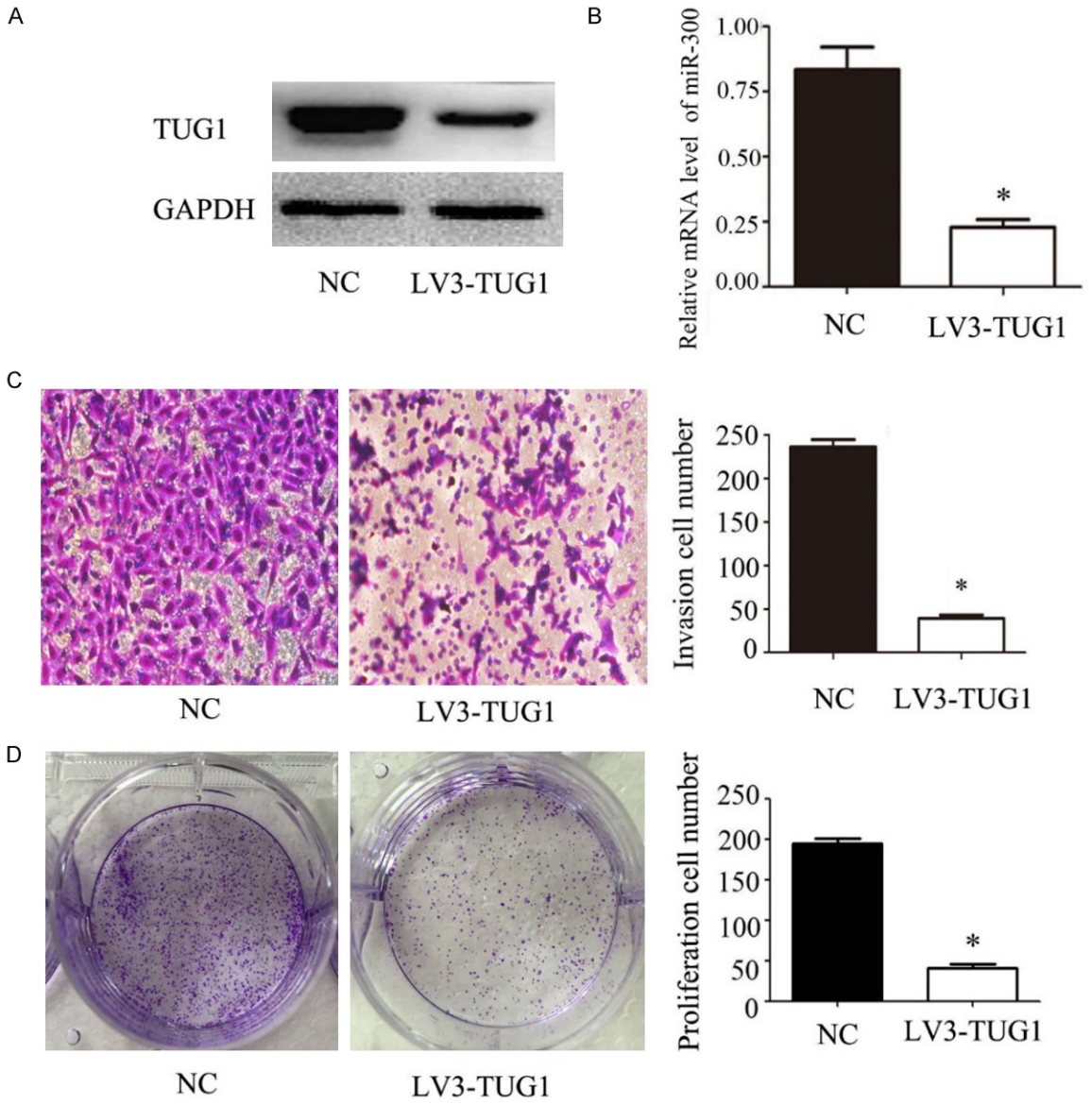

Figure 2. The invasion and proliferation ability of lung cancer cells was inhibited after silencing TUG1. A. Silencing efficiency of LV3-TUG1 was detected by Western blot; B. qPCR was used to detected the expression of miR-300 after silencing TUG1; C. Transwell invasion assays were used to detected the invasion ability of A549 after silencing TUG1; D. Cloning formating assays were used to detected the proliferation ability of A549 after silencing TUG1.

higher than that in LV3-TUG1 group ($P<0.01$), indicating that overexpression of miR-300 after silencing TUG1 can restore the proliferation ability of human lung cancer A549 cells.

Recovering effect of overexpressing miR-300 on TLR signaling pathway after silencing TUG1

Western blotting showed that the expression of genes related to TLR signaling pathway (like TLR4, IRAK4 and TRAF6) in LV3-TUG1 group was significantly lower than that in NC group ($P<0.01$) (**Figure 4**). The differences behaved statistic significant. However after added in miR-300-mimic, the expression of TLR4, IRAK4 and TRAF6 recovered obviously, suggesting that TUG1 could target miR-300 through TLR signaling pathway.

Restrained subcutaneous tumorigenesis in nude mice after silencing TUG1

The results indicated the effect of overexpressing miR-300 on tumor growth after silencing TUG1 (**Figure 5A**). The nude mice were killed through cutting the neck 30 days after the for-

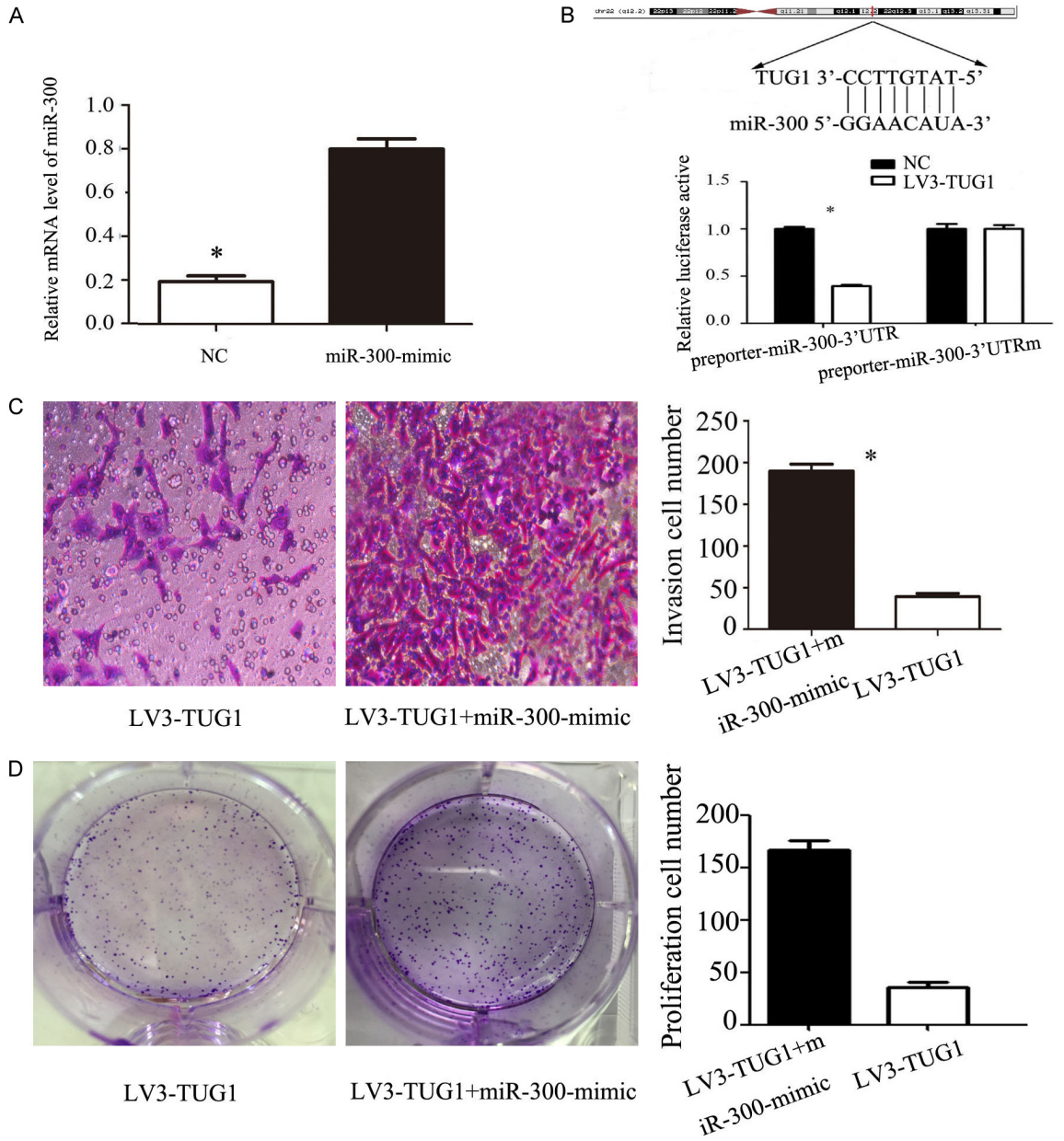

Figure 3. Restoring Effect effect of overexpress miR-300 on invasion and proliferation ability of lung cancer cells after silencing TUG1. A. qPCR was used to detect the overexpress efficiency of miR-300-mimic; B. Luciferase report gene detects miR-300 the direct target of TGU1; C. Transwell invasion assays were used to detected the invasion ability of A549 after silencing TUG1 and overexpression miR-300; D. Cloning formation assays were used to detected the proliferation ability of A549 after silencing TUG1 and overexpression miR-300. Error bars represent standard error. *p<0.05.

mation of subcutaneous tumor. The body was anatomized and tumors can be seen grown in left armpit. The tumor was in gray color, solid, and in round or oval shape, with nodular processes on the surface. The section of tumor was fish-like and tumor rate was 100%.

The growth of tumor showed that, the tumor size of LV3-TUG1+miR-300-mimic group was significantly higher than that of LV3-TUG1 group (P<0.01). The tumor volume and weight of LV3-TUG1+miR-300-mimic group were also significantly increased (P<0.01), and the difference behaved statistically significant.

Discussion

lncRNA in abundant in human genome, and it has been proved to be functional. LncRNA can be used as a regulatory factor for gene expres-

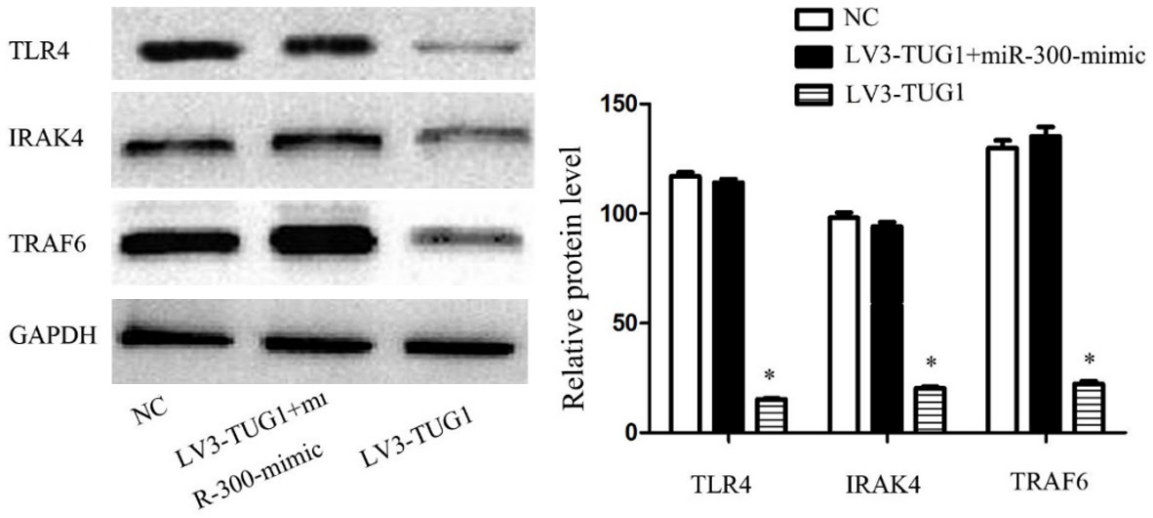

Figure 4. Recovering Effect effect of overexpressing miR-300 on TLR signaling pathway after silencing TUG1. Expression of TLR4, IRAK4 and TRAF6 were detected by Western blotting. Error bars represent standard error. *p<0.01.

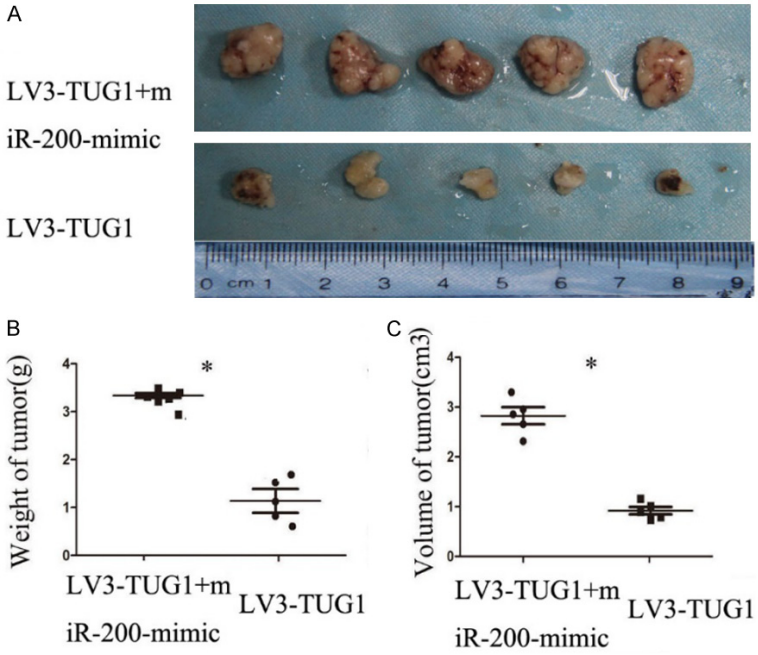

Figure 5. Restrained Subcutaneous subcutaneous tumorigenesis in nude mice after silencing TUG1. Effect of miR-300 on tumorigenesis after silencing TUG1 in vivo. A. Comparison of the tumor size of nude mice. B, C. Compare the volume and weight of the tumor in nude mice.

sion, to regulate gene expression before or after transcriptional through a variety of ways. The regulatory mechanism is very complex. The commonly theoretic is that, the interaction a variety of lncRNA, miRNA, and protein would induce the formation of regulatory network in charge of the accurate regulation of protein expression [9, 10].

TUG1 behaved wide expression in human tissues and has the highest expression in retina and nerve tissue. Studies have shown that TUG1 is a necessary gene for regulating the normal development of the retina and nervous system [11]. Many factors are involved in the process of tumorigenesis, including proto-oncogene activation, expression disorders of tumor suppressor gene, abnormalities of DNA repair gene and abnormalities of apoptosis gene [12]. Hu et al. [13] found that the expression of proteases in Caspase family (a combination of pro-apoptotic molecules) can be inhibited through silencing TUG1, contributing to the reduction of cells apoptosis, which may promote the development and progression of tumor. Subsequent study found that the expression levels of TUG1 increased obviously in osteosarcoma, bladder cancer and esophageal cancer. The tumor proliferation and invasion ability was significantly inhibited after interfering expression of TUG1 [14, 15]. In this study, 82 cases of lung cancer were detected by PCR. The expression of TUG1 in the lung paracancer tissues was significantly lower, and

the expression of TUG1 increased gradually with the progress of tumor. Through analyzing the expression of TUG1 and the clinicopathological parameters, we can get a conclusion that the expression of TUG1 was positively correlated with TNM stage and metastasis of local lymph node, which indicated that TUG1 had a carcinogenic effect in lung cancer. Furthermore, the invasion and migration of lung cancer cells after silencing TUG1 were detected. As the result showed, the invasion and migration ability of lung cancer cells decreased obviously, which indicated that TUG1 also played an important role in the invasion and migration of lung cancer cells.

Presently, it has been shown that lncRNA-mediated post-transcriptional regulation is based on the complementary pairing of its own sequence and target sequence, which competes with miRNA to bind 3'UTR of target gene, thereby inhibiting the negative regulation of miRNA to target gene to achieve the purpose of regulating mRNA post-transcriptional processing [16, 17]. In this study, the possible interaction between TUG1 and miR-300 was predicted by bioinformatics tools. And the hypothesis was further verified by silencing TUG1. As a result, the expression of miR-300 was decreased after silencing TUG1. The enzyme reporter assay revealed that TUG1 specifically binds to the 3'UTR of miR-300, thereby affecting the biological function of lung cancer cells. In order to clarify whether miR-300 can restore the invasion and proliferation of lung cancer cells, miR-300-mimic was added on the basis of silencing TUG1. The results showed that the invasion and proliferation of lung cancer cells were significantly increased after adding miR-300-mimic restore.

TLRs plays an important role in innate immunity by recognizing pathogen components and inducing signaling pathways, leading to the generation and development of acquired immune responses [18]. TLRs positions are mainly associated with inflammatory cells. Monocyte-macrophages as important defensive cells play an important role in the anti-tumor immune function of the body. The characteristic marker C14 binds to LPS to act with TLR4. And then activate TLR signaling pathways of tumor cells, thus promote cell proliferation, inhibit apoptosis, and promote tumor cell migration, infiltration and angiogenesis [19]. Bohnhorst et al. [20] reported that TLR-TRAF6 plays an important role in verifying the role of cell in promoting tumor growth. It was found that TLR signaling pathway plays an important role in the process of lung cancer metastasis. In this study, the expression of relative cells in TLR signaling pathway was examined by WB test. It was found that TUG1 could target miR-300 through TLR signaling pathway.

In this Investigation, the expression of TUG1 and miR-300 in lung cancer and paracancer tissues was investigated by qPCR, and the interaction between TUG1 and miR-300, the function of TUG1 and miR-300 in lung cancer cell invasion and proliferation was further investigated. The results showed that the expression of TUG1 is upregulated in lung cancer, and TUG1 behaved direct interaction with miR-300. TUG1 can regulate the invasion and proliferation of lung cancer through target miR-300. It can also regulate the expression of TLR4, IRAK4 and TRAF6, which indirectly indicated that TLR signaling pathway plays a role in the process of regulating TUG1 regulating miR-300 to affect the biological function of lung cancer cells by. The results suggested that TUG1 and miR-300 may be involved in the invasion and proliferation of lung cancer cells, and may be a symbol for predicting the process of lung cancer, prognosis and monitoring of therapeutic effects.

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Disclosure of conflict of interest

None.

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